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# Effects of lncRNA BANCR on endometriosis through ERK/MAPK pathway

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**Abstract. – OBJECTIVE: To investigate the regulatory role of long non-coding ribonucleic acid (lncRNA) BRAF-activated non-coding RNA (BANCR) in rats with endometriosis (EMs) and its mechanism of action.**

**MATERIALS AND METHODS: A total of 30 healthy, unmated, female Sprague-Dawley (SD) rats were selected and divided into sham-operation group, model group and lncRNA BANCR intervention group, and a rat model of EMs was established by means of autotransplantation. The volume of eutopic endometrium in each group of rats was measured, and hematoxylin and eosin (HE) staining was applied to detect the impacts on the pathological morphology of ectopic endometrial tissues in each group. The levels of vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2) and MMP-9 in the rat serum were determined by virtue of enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure the messenger RNA (mRNA) levels of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) in the uterine tissues in each group of rats, and Western blotting assay was adopted to detect the levels of phosphorylated ERK and MAPK proteins in the rat uterine tissues in each group.**

**RESULTS: Compared with those in sham-operation group, the volume of eutopic endometrium in the rats was increased markedly, the pathological morphology was poorer, and the content of VEGF, MMP-2 and MMP-9 in the serum, the mRNA levels of ERK and MAPK in the uterine tissues, and the levels of phosphorylated ERK and MAPK proteins were elevated notably in model group. The rats in lncRNA BANCR intervention group had evidently decreased volume of eutopic endometrium, improved pathological morphology and significantly declined content of serum VEGF, MMP-2 and MMP-9, ERK and MAPK mRNA levels, and phosphorylated ERK and MAPK protein levels in the uterine tissues than those in model group.**

**CONCLUSIONS: LncRNA BANCR inhibitor can repress the development of ectopic endometrial tissues by inhibiting the generation of angiogenic factors in the EMs focus, and its mechanism may be related to the inhibition on the ERK/MAPK signaling pathway.**

*Key Words:*
Endometriosis, lncRNA BANCR, ERK, MAPK, VEGF.

## Introduction

Endometriosis (EMs) is a common sex hormone-dependent gynecologic disease with an incidence rate of 10-25%, which mainly occurs in women of childbearing age[1,2]. Although it is a benign disease, it is characterized by infiltration, invasion and metastasis, with such clinical manifestations as irregular menstruation, dysmenorrhea and infertility[3]. The pathogenesis of EMs is complex, and modern studies have manifested that EMs is closely associated with the abnormal process of angiogenesis. Currently, the treatment of EMs is dominated by surgeries, and administration of drugs inhibiting the ovarian function is used as the adjunctive therapy. However, long-term administration of the drugs will damage the reproductive health and quality of life of the women of childbearing age, and the prevalence rate of postoperative complications is fairly high, so it is urgent to find the medicines for EMs[4,5]. Long non-coding ribonucleic acids (lncRNAs), a category of non-coding RNAs[6,7] with a length of over 200 nucleotides, possess no function of encoding proteins, play important roles in molecular genetics and cellular process, participate in transcriptional regulation, cleavage and translation of gene as well as cell structure, and affect cell cycle, material exchange inside and outside cells, etc. Discovered by Lou et al[8] in melanoma cells, BRAF-activated non-coding RNA (BANCR), with a length of about 693 bp, is located in chromosome 9. In-depth studies have revealed that the BANCR expression is



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increased remarkably in cancers such as papillary thyroid cancer, retinoblastoma, lung cancer, colorectal cancer, gastric cancer and bladder cancer, suggesting that regulation of BANCR is possibly a new approach to treat cancers[9,10]. Extracellular signal-regulated kinase (ERK) is involved in cell growth, proliferation, differentiation, apoptosis and other processes, which can transmit exogenous signal stimulus into the cells after being stimulated and activated, further phosphorylating mitogen-activated protein kinase (MAPK), promoting MAPK to enter the nucleus and controlling the release and expression of downstream effectors. Among them, vascular endothelial growth factor (VEGF) is positively regulated by MAPK, which exerts crucial regulatory effects on the development of ectopic endometrial tissues. Therefore, this paper aims to study the regulatory role of lncRNA BANCR on angiogenic factors in EMs rats and to investigate its regulatory mechanism.

## Materials and Methods

### Animals

A total of 30 healthy, unmated female Sprague Dawley (SD) rats weighing (200±20) g were purchased from Guangdong Medical Laboratory Animal Center (license number: CNAS L3623). This study was approved by the Animal Ethics Committee of Ningbo Women and Children's Hospital Animal Center.

### Reagents

TRIzol lysis buffer (Invitrogen, Carlsbad, CA, USA), enzyme-linked immunosorbent assay (ELISA) kits for VEGF, matrix metalloproteinase-2 (MMP-2) and MMP-9 (R&D systems, Minneapolis, MN, USA), Bradford kit, primary antibody diluent for Western blotting, diethyl pyrocarbonate (DEPC)-treated water (Beyotime Institute of Biotechnology, Shanghai, China), lncRNA BANCR inhibitor (Guangzhou RiboBio Co., Ltd., Guangzhou, China), anti-rabbit horse reddish peroxidase (HRP)-labeled primary antibodies of phosphorylated ERK and MAPK (CST, Danvers, MA, USA) and goat anti-rabbit secondary antibody (CST, Danvers, MA, USA).

### Instruments

Super-clean bench (Thermo Fisher, Waltham, MA, USA), microplate reader, electrophoresis tank and gel imager (Bio-Rad, Hercules, CA, USA) and -80°C ultra-low-temperature refrigerator (Haier, Qingdao, China).

## Methods

### Preparation of a Rat Model of EMs

A rat model of EMs was prepared by means of autotransplantation. Before modeling, the rats received gavage-using diethylstilbestrol, so as to enter estrus. Next, the rats were anesthetized by chloral hydrate on a sterile operation platform, and the skin at about 1 cm above the median urethra was cut open to expose the uterus. Approximately 1 cm-long uterine fragment was excised, and the endometrium and muscularis were separated. Then, 2 fragments of endometrial tissue (about 5 mm × 5 mm) were fetched and sutured on the abdominal wall (one on the left, and the other on the right), followed by washing the abdominal cavity with gentamicin and skin suture. Anatomical observation showed that there was effusion in the uterus, enlarged volume of eutopic endometrium and angiogenesis in the rats, implying that the model was successfully established, available for subsequent experiments.

### Detection of Changes in Pathological Morphology of Ectopic Endometrial Tissues in Rats via Hematoxylin and Eosin (HE) Staining

The ectopic endometrial tissues in each group were embedded in paraffin, sliced into sections, stained by means of HE staining, deparaffinized and permeabilized using xylene, soaked in ethanol of high and low concentrations (100%, 90%, 80%, 70% and 50%) for 3 min, respectively, and rinsed with distilled water for 5 min, followed by staining of intercellular substance with hematoxylin staining solution for 5 min, staining of nucleus with eosin staining solution for 5 min, soaking in ethanol of low and high concentrations, fixation with xylene and observation of staining under a microscope.

### Detection of VEGF, MMP-2 and MMP-9 levels in Rat Serum Via ELISA

The blood in the abdominal aorta was drawn from each group of rats, followed by centrifugation, collection of serum and preservation at -80°C. The standard curves for different concentrations of standard substances were plotted

according to the instructions of ELISA kit, and every blank well was added with 100 μL sample diluent, sealed with parafilm and incubated at room temperature for 2 h. Then ,100 μL Biotin-Conjugate liquid were added, followed by film sealing and incubation for 1 h. After that, 100 μL streptavidin-HRP liquid were added and incubated for 1 h. Finally, TMB Substrate Solution was added, and the absorbance was measured (λ =620 nm).

### Detection of Messenger RNA (mRNA) Levels of ERK and MAPK in Ectopic Endometrial Tissues of Rats via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The rats' ectopic endometrial tissues were extracted, added with 1 mL TRIzol lysis buffer and 0.2 mL chloroform, shaken vigorously for 15 s and centrifuged. The supernatant was collected, added with 0.5 mL precooled isopropyl alcohol and inverted for several times, followed by centrifugation and collection of precipitate, which was dissolved in 0.01% DEPC-treated water, and the RNA concentration was determined. In accordance with the RT-PCR instructions, the tissues were added with the reagent to synthesize complementary deoxyribose nucleic acid (cDNA), followed by amplification reaction for 35 cycles, gel running and observation of optical density of band under the gel imager. Primer sequences used in this study were as follows: ERK, F: 5'-CGCTACACGCAGTTGCAGTACA-3', R: 5'-AACCGCAGCAGGATCTGCA-3'; MAPK, F:5'-GCTCGTACAGAACACGACCTG-3', R: 5'-AGTAGAGAGAATCGGGCGTCCG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC -3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

### Detection of ERK and MAPK Protein Levels in Ectopic Endometrial Tissues of Rats Via Western Blotting

The rats' ectopic endometrial tissues were lysed with the lysis buffer after extraction, and the protein concentration was detected through Bradford method, followed by metal bath for 15 min, loading and gel running. After the sample crossed the boundary between separation gel and spacer gel, the voltage was altered to 120 V, followed by membrane transfer at constant current for 2.5 h. Next, the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) where the objective band was located was clipped according to the marker, and the primary antibodies were incubated at 4°C overnight. On the next day, the secondary antibodies were added, diaminobenzidine (DAB) developer was utilized for color development, and ImageJ software was adopted for analysis of the optical density.

### Statistical Analysis

The experimental data were expressed by mean ± standard deviation, and GraphPad 5.0 software (La Jolla, CA, USA) was used for statistical analysis of data. The one-way analysis of variance was adopted for comparisons of data in line with normal distribution among groups. $p<0.05$ suggested that the difference was statistically significant.

## Results

### Analysis on Volume of Eutopic Endometrium in Rats

Compared with those in sham-operation group, there were apparent eminences in the eutopic endometrium of rats receiving autotransplantation in model group, which were hard in texture and difficult to be dissected, and adhered to surrounding tissues. The volume of graft in lncRNA BANCR inhibitor group was notably smaller than that in model group ($^{\#\#}p<0.01$), and the development of graft tissues in the endometrium was repressed prominently (Figure 1).

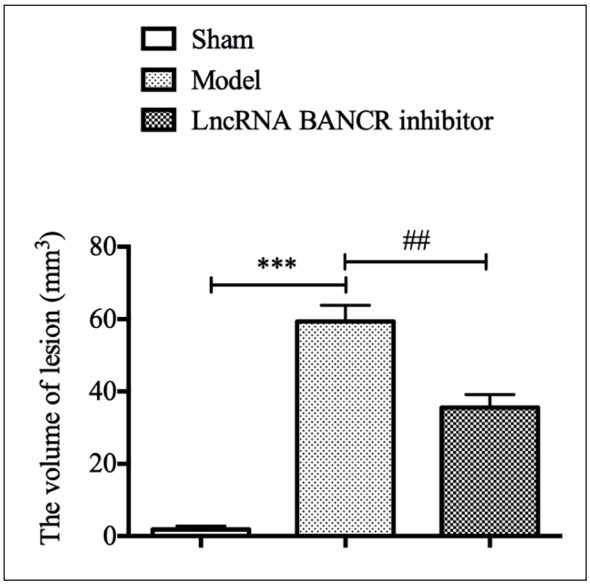


**Figure 1.** Volume of graft of eutopic endometrium in rats ($^{***}p<0.001$, $^{\#\#}p<0.01$).

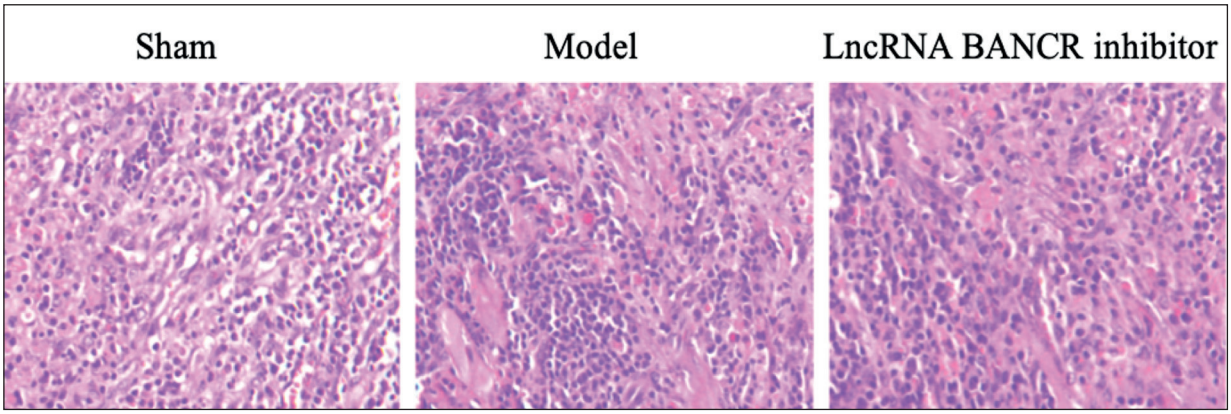


**Figure 2.** Change in pathological morphology of ectopic endometrial tissues in rats (20×).

### Analysis on Pathological Morphology of Ectopic Endometrial Tissues in Rats

According to the HE staining, the structure of the rats' endometrial epithelial cells was continuous and complete and arranged regularly, and the gland was full in sham-operation group (Figure 2). In model group, the endometrial epithelial cells were flat and irregular, with abundant blood vessels and shrunk glands. The rats in lncRNA BANCR inhibitor group had varying degrees of improved pathological changes in endometrial epithelial cells, markedly decreased number of interstitial cells and declined blood supply compared with those in model group, manifesting that the lncRNA BANCR inhibitor can significantly ameliorate the pathological morphology of ectopic endometrial tissues in rats.

### Content of VEGF, MMP-2 and MMP-9 in Rat Serum

In comparisons with those in sham-operation group, the content of serum VEGF, MMP-2 and MMP-9 was increased remarkably in model group ($^{**}p<0.01$, $^{*}p<0.05$, $^{*}p<0.05$). The content of serum VEGF, MMP-2 and MMP-9 in lncRNA BANCR inhibitor group was evidently lower than that in model group ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#}p<0.05$) (Table I), indicating that the lncRNA BANCR inhibitor can significantly suppress the production of angiogenic factors.

### Levels of ERK and MAPK mRNAs in Ectopic Endometrial Tissues of rats

As shown in the RT-PCR band patterns (Figure 3A), the rats in model group had remarkably higher levels of ERK and MAPK mRNAs in ectopic endometrial tissues than those in sham-operation group ($^{*}p<0.05$, $^{*}p<0.05$). Compared with those in model group, the levels of ERK and MAPK mRNAs in ectopic endometrial tissues were lowered obviously in lncRNA BANCR inhibitor group ($^{\#}p<0.05$, $^{\#}p<0.05$) (Figure 3B). Those results revealed that the lncRNA BANCR inhibitor is able to decrease the levels of ERK and MAPK mRNAs in ectopic endometrial tissues of rats to a great extent.

### Levels of ERK and MAPK Proteins in Ectopic Endometrial Tissues of Rats

According to Figure 4A, the levels of ERK and MAPK proteins in ectopic endometrial tissues in model group were notably higher than those

**Table I.** Content of VEGF, MMP-2 and MMP-9 in rat serum.

| Group | VEGF (pg/mL) | MMP-9 (pg/mL) | MMP-2 (pg/mL) |
|---|---|---|---|
| Sham-operation group | 6.25 ± 1.37 | 10.33 ± 2.16 | 7.82 ± 1.89 |
| Model group | 12.56 ± 2.14** | 21.56 ± 2.56* | 12.38 ± 2.23* |
| LncRNA BANCR inhibitor group | 9.34 ± 1.98$^{\#}$ | 15.19 ± 2.05$^{\#\#}$ | 10.59 ± 1.45$^{\#}$ |

*Note:* Model group vs. sham-operation group (**$p<0.01$, *$p<0.05$), lncRNA BANCR inhibitor group *vs*. model group ($^{\#\#}p<0.01$, $^{\#}p<0.05$).

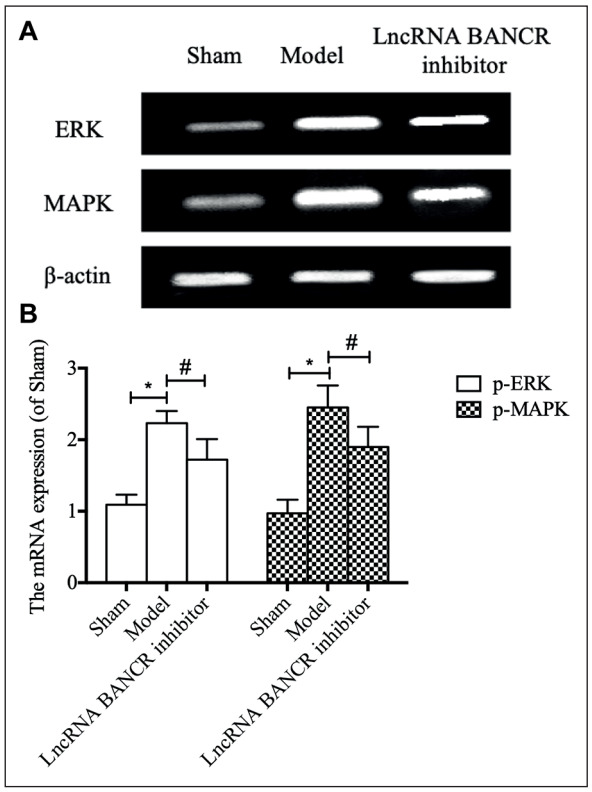


**Figure 3.** Levels of ERK and MAPK mRNAs in ectopic endometrial tissues of rats. Note: ***A,*** RT-PCR band patterns, ***B,*** RT-PCR statistical chart ($^{*}p<0.05$, $^{\#}p<0.05$).

in sham-operation group ($^{*}p<0.05$, $^{*}p<0.05$). The levels of ERK and MAPK proteins in ectopic endometrial tissues declined markedly in lncRNA BANCR inhibitor group in comparison with those in model group ($^{\#}p<0.05$, $^{\#}p<0.05$) (Figure 4B), further proving at protein level that the lncRNA BANCR inhibitor is capable of reducing the expressions of ERK and MAPK proteins in ectopic endometrial tissues of rats.

## Discussion

The first case of EMs developing into ovarian cancer was reported in 1925, and a growing number of researchers are paying attention to this gynecologic disease with malignant potential[11]. Modern studies have revealed that the volume of EMs tissues is enlarged significantly, and there are abundant blood vessels, implying that the occurrence or development of the disease is closely correlated with angiogenesis. If the expression of angiogenic factors is inhibited effectively, the enlargement of lesion volume will be repressed efficiently, thus controlling the occurrence and development of the disease[12]. Ma et al[13] discovered that the miR-34a-5p expression is decreased obviously in ectopic endometrial tissues of EMs patients, and its overexpression can target and inhibit the VEGF expression, providing new directions for the treatment of EMs. LncRNAs, as hotspots of cancer research in recent years and a category of long non-coding RNAs, do not participate in protein encoding but can regulate gene expressions from multiple aspects as functional RNA molecules, further inducing the occurrence, development and metastasis of tumors[14,15]. Sun et al[15] conducted wild-type genome sequencing for 25 EMs patients, and they discovered that a total of 948 lncRNAs and 4088 mRNAs are abnormally expressed in the patients. According to the experiment of Li et al[16], the expression of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is decreased remarkably in the EMs patients, especially in those with EMs-induced infertility. In addition, it is negatively correlated with P21 mRNA, and the proliferation of uterine granulosa cells can be inhibited by knocking down

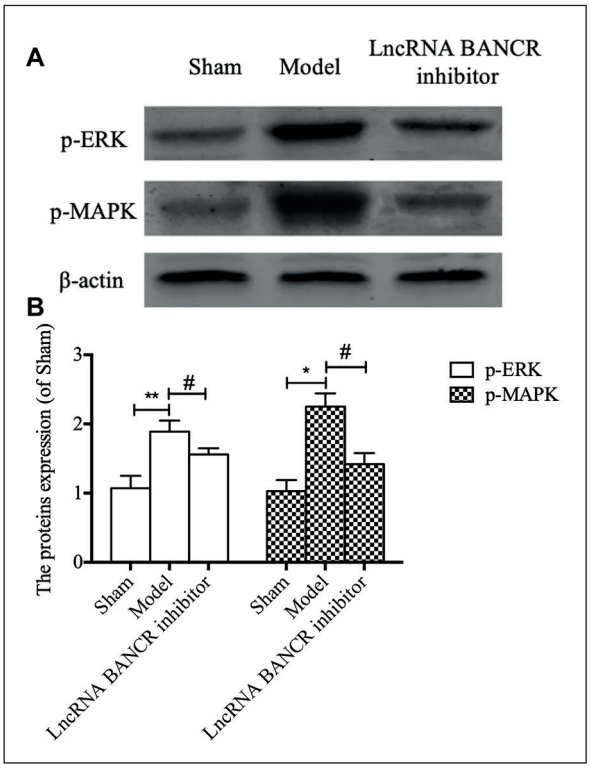


**Figure 4.** Levels of ERK and MAPK proteins in ectopic endometrial tissues of rats. Note: ***A,*** Western blotting band patterns, ***B,*** Western blotting statistical chart ($^{*}p<0.05$, $^{**}p<0.01$, $^{\#}p<0.05$).

the lncRNA MALAT1 expression, whose mechanism is possibly associated with the ERK/MAPK signaling pathway activated by the up-regulation of P21[17]. ERK, also known as P44/42, has two subtypes (ERK1 and ERK2), whose activation can serve as a vital node for cell signal transduction. The activated ERK can transmit extracellular signals into the cells and exert crucial effects on cell proliferation, differentiation and apoptosis. As an important signaling pathway in cells, the ERK/MAPK signaling pathway is involved in numerous physiological processes in the body[18,19]. Ding et al[19] revealed that in the case of EMs, P2X3 can trigger neuropathic pain through mediating the activation of the ERK signaling pathway. The mechanism may be related to the inhibition on the MEK/ERK signaling pathway, manifesting that ERK plays crucial roles in the occurrence, development and pathological change of EMs, and repressing the activation of this signaling pathway is a novel idea for treating the disease. Therefore, this research proposed to establish a rat model of EMs by means of autotransplantation. It was revealed that the effusion in the rat uterus and the volume of graft were increased significantly in model group. The HE staining indicated that there were engorged blood vessels, shrunk glands and inflammatory infiltration in the rat uterus in model group. However, after the administration of lncRNA BANCR inhibitor, the volume of graft was decreased significantly, and the pathological morphology was ameliorated markedly. Subsequently, the content of angiogenic factors (VEGF, MMP-9 and MMP-2) was detected, which was lowered notably in lncRNA BANCR inhibitor group, illustrating that the lncRNA BANCR inhibitor can prominently suppress the expression of angiogenic factors. In order to further explore the regulatory mechanism of lncRNA BANCR in EMs rats, the ERK and MAPK expressions in the ERK/MAPK signaling pathway at the gene and protein levels were measured separately. The results indicated that the lncRNA BANCR inhibitor can reduce the expressions notably, manifesting that the lncRNA BANCR inhibitor may exert its therapeutic effects on EMs rats by repressing the activation of the ERK/MAPK signaling pathway.

## Conclusions

We showed that the lncRNA BANCR inhibitor can suppress the expression of angiogenic factors in EMs rats and inhibit the development of graft, whose mechanism is possibly associated with the regulation of the ERK/MAPK signaling pathway.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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