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LncRNA ROR promotes proliferation of endometrial cancer cells *via* regulating Notch1 pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of long non-coding ribonucleic acid regulator of reprogramming (lncRNA ROR) on the proliferation and apoptosis of endometrial cancer (EC) cells, and to explore its possible underlying mechanism.

PATIENTS AND METHODS: The expression levels of lncRNA ROR and Notch1 in EC tissues were detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The changes in Notch1 protein were detected via Western blotting. Subsequently, the regulatory mechanism of lncRNA ROR on Notch1 was analyzed using Luciferase reporter gene assay. Moreover, the changes in cell proliferation and apoptosis were determined through cell counting kit-8 (CCK-8) assay and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, respectively.

RESULTS: Both lncRNA ROR and Notch1 were highly expressed in EC tissues ($p<0.05$). After overexpression of lncRNA ROR, HEC-1A cells had significantly enhanced proliferation ($p<0.05$) and weakened apoptosis ($p<0.05$). Meanwhile, the mRNA and protein levels of Notch1 rose remarkably compared with those in control group ($p<0.05$). Luciferase reporter gene assay revealed that lncRNA ROR could bind to the Notch1 regulatory factor miR-34a and inhibit its activity.

CONCLUSIONS: LncRNA ROR regulates the proliferation and apoptosis of EC cells via promoting the expression of Notch1 protein.

Key Words:
LncRNA ROR, Notch1, HEC-1A, Proliferation, Apoptosis.

Introduction

Currently, women are often diagnosed with uterine cancer in the world. Endometrial cancer (EC) is the most common type of uterine cancer, with about 382,069 new cases every year[1]. The mortality rate of EC is 4.3-4.5/100,000, 8.2-8.9/100,000 and 2.5/100,000 in the white people, black people and Chinese, respectively[2,3]. The 5-year survival rate of patients with early and local EC is around 95%, which is only 18% in patients with advanced EC[3,4]. Nearly 80% of EC is hormone-driven and hormone-dependent endometrioid adenocarcinoma[5]. Currently, the risk of EC in young women is increasing, and about 5% of EC patients are aged below 40 years old. These young patients often have a long history of abnormal uterine bleeding or infertility, with a strong desire to retain fertility. High-dose progesterone is a common method of fertility preservation in endometrioid adenocarcinoma[6]. Obesity remains the strongest risk factor for EC so far. Meanwhile, its risk factors include early menarche, late-onset menopause and infertility[7-9]. Diabetes mellitus and metabolic syndrome, exposure to tamoxifen, and family history are associated with increased risk of EC. Moreover, it has been confirmed that oral contraceptives and smoking can prevent EC development[10-13].

Long non-coding ribonucleic acids (lncRNAs) are a kind of RNA molecules with more than 200 nt in length, most of which are located in the nucleus. LncRNAs are transcribed by 4-9% of nucleotide sequences in mammalian genome sequences[14,15]. A large number of lncRNAs have been identified with the development of sequencing technique, most of which are involved in biological events[16,17]. They may enhance the expressions of target genes through ceRNA. In addition, some lncRNAs participate in gene expression *via* regulating DNA/RNA methylation and acetylation[18]. LncRNAs are potential molecular biological markers, such as lncRNA GAS5 and lncRNA

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HOTAIR[19,20]. In recent years, lncRNA regulator of reprogramming (ROR) has been determined as an important regulator of differentiation and reprogramming of induced pluripotent stem cells[21]. LncRNA ROR, also known as lincRNA-ST8SIA3, is located on chromosome 18. It plays an important regulatory role *via* pluripotent transcription factors[22] and can serve as a potent negative regulator of P53 and respond to DNA damage[23]. LncRNA ROR is associated with various biological parameters of tumors, such as growth, metastasis and invasion[24,25]. Besides, it serves as an important oncogene for such malignancies as gallbladder cancer, hepatocellular carcinoma and pancreatic ductal adenocarcinoma[26-29]. In addition, lncRNA ROR acts as the sponge absorbing miR-145 in EC and mediates the differentiation of EC stem cells[30]. Considering the universal extensive effects of lncRNA ROR, its role in the proliferation and differentiation of EC cells was explored in this study.

As an evolutionarily conserved signaling pathway, Notch signal transduction pathway regulates the differentiation of embryonic and postnatal tissues, and determines cell fate and proliferation[31]. In many malignant tumors, the activated Notch pathway may exert both pro- and anti-tumor functions[32]. Serrated and Delta-like ligands interact with the Notch receptor to induce its cleavage and nuclear translocation of the intracellular domain. After the activation of Notch, downstream target genes will be transcribed. In addition, the target gene Notch1 regulates key biological events, such as cell fate, proliferation and apoptosis[33]. Over the past few years, the potential role of lncRNA ROR in EC has been greatly elucidated in the literature. However, whether it regulates the proliferation and apoptosis of EC cells *via* affecting the Notch1 signaling pathway remains unknown.

Patients and Methods

Patients and Reagents

EC HEC-1A cells were donated by Sun Yat-sen University Laboratory. 10 pairs of EC tissues and normal tissues were provided by the Gynecology Department of The Fifth Affiliated Hospital, Sun Yat-sen University. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This investigation was approved by the Ethics Committee of the Hospital. MiR-34a mimics were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China), Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) from Nanjing BioChannel Biotechnology Co., Ltd. (Nanjing, China), TransFast transfection reagent from Promega (Madison, WI, USA), psiCHECK-2 Luciferase plasmids from Shanghai Kelei Biological Technology Co., Ltd. (Shanghai, China), and radioimmunoprecipitation assay (RIPA) lysis buffer, polyvinylidene difluoride (PVDF) membranes, enhanced chemiluminescence (ECL) Plus Western blotting reagent, Notch1 antibody and horseradish peroxidase (HRP)-coupled secondary antibody from ImmunoWay (Plano, TX, USA).

Cell Culture and Transfection

HEC-1A cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution in an incubator with 5% CO_2 at 37°C. For cell transfection, HEC-1A cells were first inoculated into 24-well plates. Subsequently, the cells were transfected with 100 nM pcDNA-ROR or pcDNA-control using TransFast according to the instructions of the miRNA transfection reagent (GenePharma, Shanghai, China). After 48 h, transfected cells were collected for the following experiments.

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

Total RNA was extracted from cells and quantified using NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using miScript II reverse transcriptase (Qiagen, Hilden, Germany). LncRNA ROR was quantified in strict accordance with the lncRNA SYBR Green PCR kit. After cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622, Waltham, MA, USA), Notch1 was quantified using the SYBR Green PCR kit. Detection was then performed using the 7500-Fast real-time PCR system. The expressions of miR-34a, lncRNA ROR and genes were detected *via* the $\Delta\Delta C_t$ method. Primer sequences used in this study were shown in Table I.

Cell Counting Kit-8 (CCK-8) Assay

HEC-1A cells were first inoculated into 96-well plates at a concentration of 70-80%. After the cells completely adhered to the wall, 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well, fol-

Table I. Primer sequences in qRT-PCR.

Index	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
MiR-34a	AGGCAGAGACAAAGCAGGAAGA	TGGTGTCGTGGAGTCG
Notch1	GAGGCGTGGCAGACTATG	CTTGTACTCCGTCAGCGTG
LncRNA ROR	CGAACGAGAGGACCGAAG	GCCAAGTTCTAGATAAGC

lowed by incubation for 3 h in the dark. Absorbance at 450 nm was detected using a micro-plate reader at 0, 24, 48 and 72 h, respectively.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

HEC-1A cells were fixed with formaldehyde and washed with phosphate-buffered saline (PBS) for 3 times. Next, the cells were permeabilized with 1% Triton X-100 and reacted with TdT solution at room temperature, followed by color development. Finally, the cells were observed under a microscope, and the number of $TUNEL^+$ cells was counted.

Western Blotting

Transfected cells were first washed with PBS, and an appropriate amount of lysis buffer was added. After fully mixing, the cells were taken and ultrasonically centrifuged to obtain protein samples. The concentration of extracted protein was measured using the bicinchoninic acid assay (BCA) method, and the volume of all protein samples was adjusted till the same concentration. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples in the gel were transferred onto PVDF membrane. Next, the membranes were incubated with the primary antibody of anti-Notch1 at 4°C overnight. On the next day, the membranes were incubated again with the horseradish peroxidase (HRP)-coupled secondary antibody at room temperature for 1 h. Immunoreactive bands were finally developed using ECL Plus Western blotting reagent.

Luciferase Reporter Gene Assay

Wild-type and mutant-type pisCHECK-lncRNA-3'UTR was transfected into HEC-1A cells (ROR wild-type sequences: 5'-UCUUGAGCA-3', and mutant-type sequences: 5'-GGGUCCCGG-3'). The cells were then co-transfected with miR-34a mimics or miR-NC, respectively. After 48 h, Luciferase activity was measured using a micro-plate reader (BioTek, Biotek Winooski, VT, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21 software (IBM, Armonk, NY, USA) was used for data analysis. The *t*-test was performed for two independent samples. $p<0.05$ was considered statistically significant.

Results

Expression of LncRNA ROR Was Up-Regulated In EC Tissues

QRT-PCR revealed that the expression of lncRNA ROR in EC tissues was about 3.3 times that in normal tissues, showing statistically significant differences ($p<0.01$; Figure 1).

Expression of Notch1 Was Up-Regulated In EC Tissues

According to quantitative results, both mRNA and protein expressions of Notch1 increased significantly in EC group compared with those in control group ($p<0.05$, Figures 2 and 3).

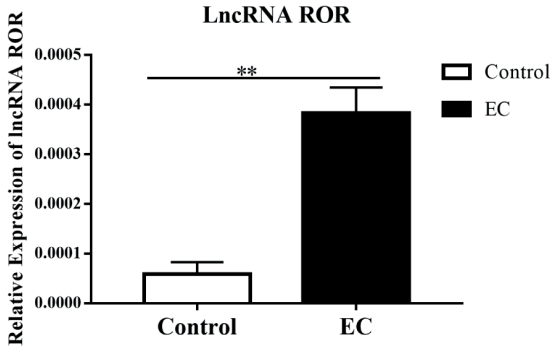

Figure 1. Relative expression of lncRNA ROR in EC tissues and normal tissues. Note: The expression of lncRNA ROR is higher in EC group than that in control group ($^{**}p<0.01$).

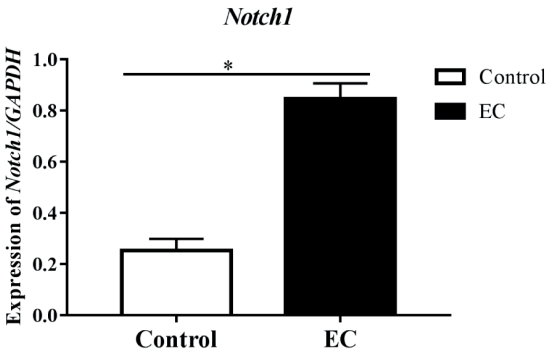

Figure 2. Relative mRNA expression of Notch1 in EC tissues and normal tissues. Note: The mRNA expression of Notch1 is higher in EC group than that in control group ($^{*}p<0.05$).

Effects of LncRNA ROR on Proliferation and Apoptosis of HEC-1A Cells

HEC-1A cells were transfected with pcDNA-control or pcDNA-lncRNA ROR, respectively. Quantitative results showed that the expression of lncRNA ROR in pcDNA-lncRNA ROR group rose remarkably ($p<0.05$, Figure 4A). Subsequent CCK-8 assay indicated that cell proliferation rate increased significantly at 48 h and 72 h after transfection in pcDNA-lncRNA ROR group ($p<0.05$, Figure 4B). TUNEL assay showed that the number of apoptotic cells declined markedly in pcDNA-lncRNA ROR group compared with control group ($p<0.05$, Figure 5).

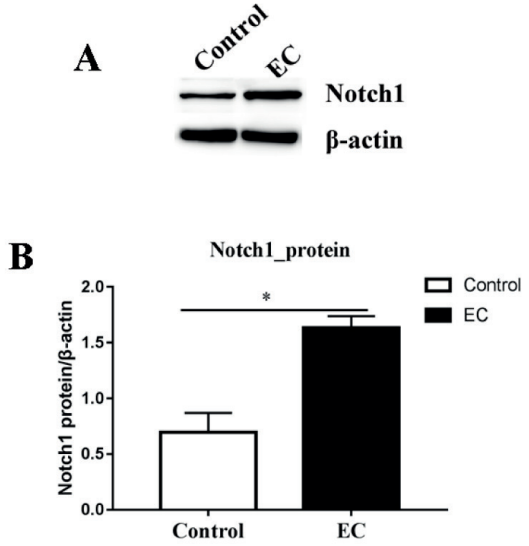

Figure 3. Relative protein expression of Notch1 in EC tissues and normal tissues. **A,** Protein electrophoresis bands. **B,** Protein quantitative results. Note: The protein expression of Notch1 is higher in EC group than that in control group ($^{*}p<0.05$).

Effect of LncRNA ROR Overexpression on Notch1

QRT-PCR was performed after HEC-1A cells were transfected with pcDNA-control or pcDNA-lncRNA ROR. It was found that the mRNA expression of Notch1 rose significantly in pcDNA-lncRNA ROR group ($p<0.01$, Figure 6A). The results of Western blotting manifested that the protein expression of Notch1 was significantly higher in pcDNA-lncRNA ROR group than control group ($p<0.01$, Figure 6B, C).

LncRNA ROR Could Inhibit Activity of MiR-34a

Currently, it has been confirmed that Notch1 is one of the target genes of miR-34a[34]. After HEC-1A cells were transfected with pcDNA-control or pcDNA-lncRNA ROR, quantitative results demonstrated that the expression of miR-34a was significantly in pcDNA-lncRNA ROR group than control group ($p<0.01$, Figure 7). According to the results of Luciferase reporter gene assay, Luciferase activity was markedly enhanced after co-transfection of wild-type lncRNA ROR and miR-34a ($p<0.05$). However, no evident changes were observed after co-transfection of mutant-type lncRNA ROR and miR-34a (Figure 8).

Discussion

EC is characterized by high global incidence, strong invasion and poor prognosis. Currently, biomarkers used for disease evaluation and diagnosis include diagnostic indexes in blood, urine or tissues, such as plasma markers[35]. Increasingly more marker genes, miRNAs and lncRNAs in EC have been confirmed to facilitate its diagnosis[36,37]. In recent years, there is still a lack of effective indexes for the diagnosis of EC. Meanwhile, the mechanisms of action of a great number of markers remain unclear. Therefore, the prognosis of EC patients is still far from satisfactory. LncRNA ROR has the potential to play a regulatory role in many cancers, including EC. It can serve as a novel biomarker or therapeutic target[38]. In this study, the role of lncRNA ROR in the progression of EC was explored from the perspective of effects of lncRNA ROR on the proliferation and apoptosis of EC cells.

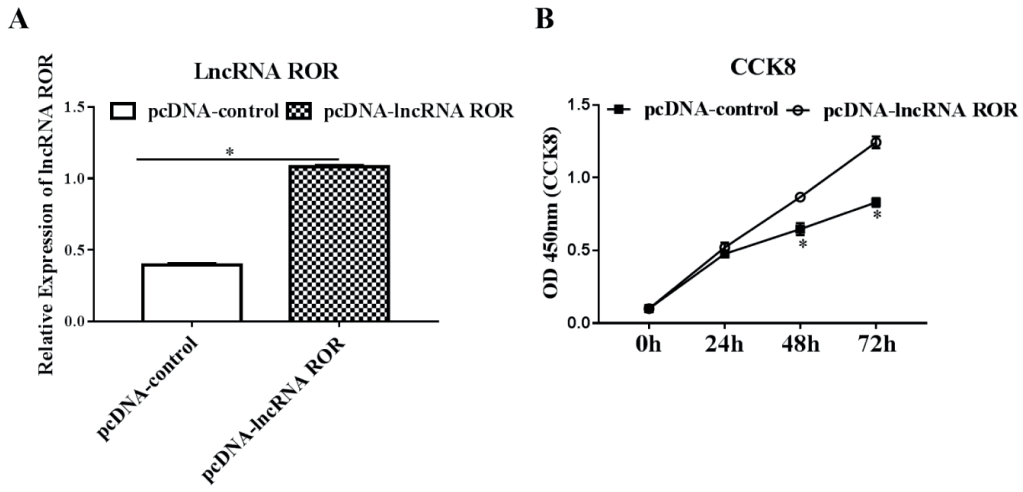

Figure 4. Effect of lncRNA ROR on proliferation of EC HEC-1A cells. **A,** Expression of lncRNA ROR. **B,** CCK-8 assay results. $^{*}p<0.05$.

LncRNAs may perform a wide variety of functions in gene regulation and other cellular processes. Some lncRNAs exhibit important functions, but the specific functions remain rather controversial[39]. Changes in lncRNA expression are associated with cancers and some neurological diseases[40,41]. Meanwhile, they can interact with a variety of proteins. LncRNAs play an important role in early embryonic development, keep the pluripotency of human embryonic stem cells and participate in somatic reprogramming[21,42]. They can also control the level and function of miRNAs in the pathogenesis of human diseases[43,44]. Current studies have demonstrated that the dynamic expression of lncRNAs is involved in human canceration. LncRNA ROR acts as an endogenous sponge that inhibits miR-145 and mediates EC stem cell differentiation[22]. However, its exact function in EC has not been reported yet. In this study, lncRNA ROR was found highly expressed in EC patients and could promote the proliferation of HEC-1A cells.

The Notch signal transduction pathway is activated mainly through the physical binding of 4 transmembrane receptors (Notch1, Notch2, Notch3 and Notch4) to adjacent surface ligands (Jagged-1, Jagged-2, Delta-1, Delta-3 and Delta-4)[45]. The abnormalities of Notch signal transduction pathway have been found to play an indispensable role in the occurrence of many cancers[46-48]. Located on chromosome 1, miR-34a is a member of the miR-34 family. The role of its expression has been confirmed in various cancers. Hermeking[49] indicates that p53-induced miR-34a is involved in regulating cell cycle *via* down-regulating the expression of Bcl-2 and promoting the expression of B-Myb. Previously, it has been proved that Notch1 is a downstream target gene of miR-34a, which also participates in many pathophysiological processes jointly with miR-34a[34,50]. Therefore, it was hypothesized in this study that lncRNA ROR changed in the Notch1 signaling pathway *via* regulating the expression of miR-34a, thereby participating in the proliferation and apoptosis of EC cells.

There are still some limitations in this study. First, the clinical mechanism was not investigated due to the lack of *in vitro* experiments. Therefore, more research is still needed prior to clinical application in the future. In conclusion, the differential expression of lncRNA ROR in EC tissues was clearly determined, which might lay a solid foundation for the subsequent exploration of its mechanism in the pathogenesis of EC. In terms of the mechanism, it was found that lncRNA ROR affected the expression of Notch1 through directly acting on miR-34a.

Conclusions

The novelty of this study was that all our findings provide a theoretical basis for the application of lncRNA ROR in the treatment of EC, and improve the possibility of lncRNA ROR as a potential therapeutic target for EC. Furthermore,

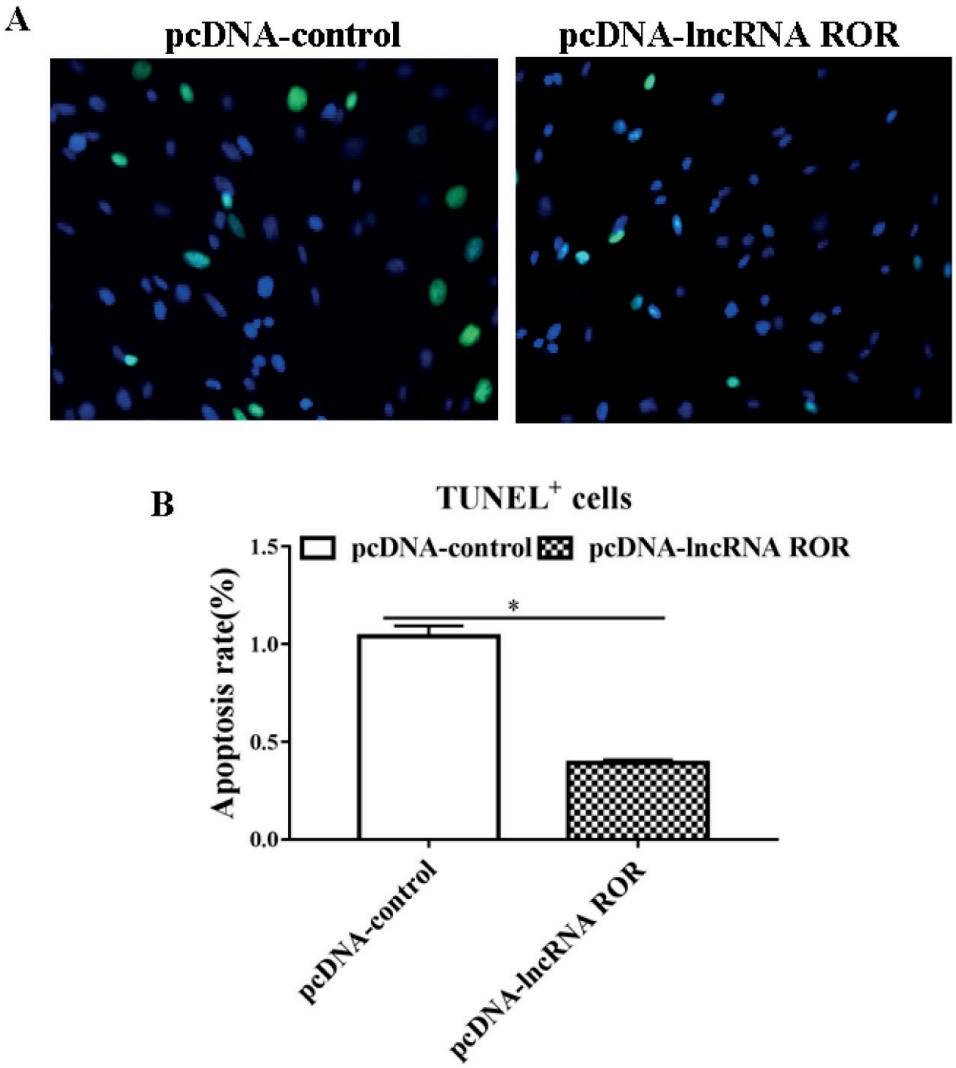

Figure 5. Effect of lncRNA ROR on apoptosis of EC HEC-1A cells. **A,** Fluorescence staining of apoptotic cells. **B,** Apoptotic cell count. (magnification: 400×) $^{*}p<0.05$.

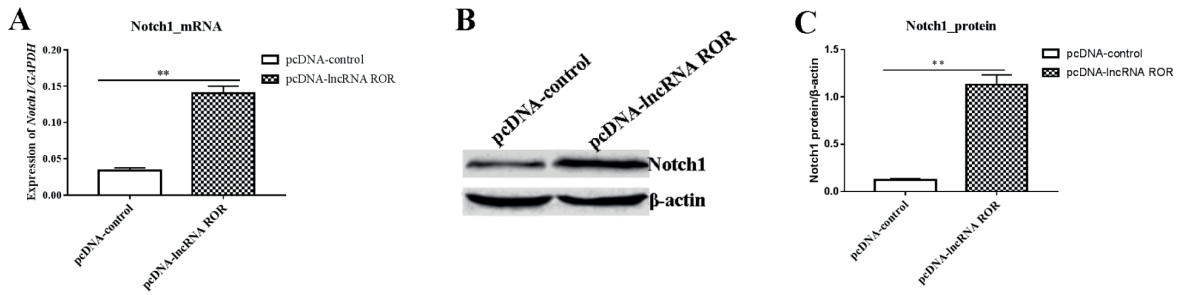

Figure 6. Effect of lncRNA ROR overexpression on Notch1. **A,** Notch1 mRNA expression. **B,** Notch1 protein bands, β-actin as the internal reference. **C,** Notch1 protein quantification. $^{**}p<0.01$.

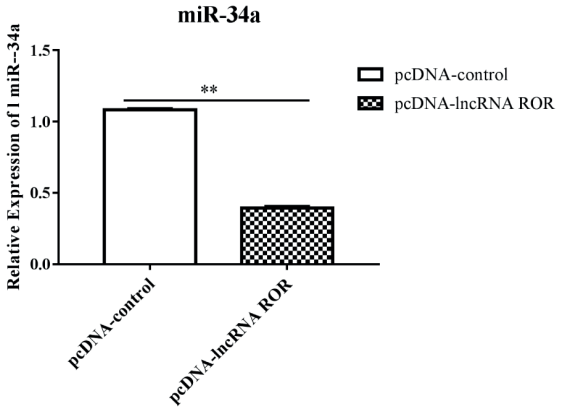

Figure 7. Changes in miR-34a expression after lncRNA ROR overexpression. Note: The expression of miR-34a declines in pcDNA-lncRNA ROR group compared with that in pcDNA-control group. $^{**}p<0.01$.

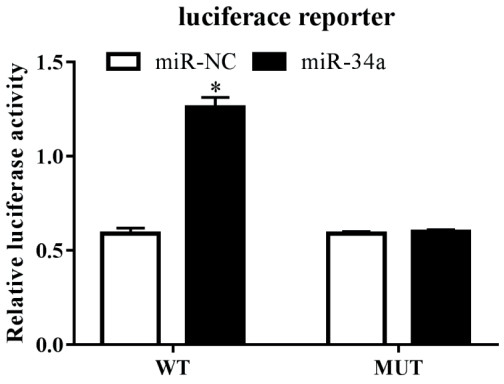

Figure 8. LncRNA ROR can bind to miR-34a. Note: WT/MUT: wild-type/mutant-type lncRNA ROR luciferase activity. $^{*}p<0.05$.

our study offers theoretical insights into lncRNA ROR as a potential therapeutic target for other related diseases, and enriches the role of lncRNA ROR in diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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