

RBPJ inhibits the movability of endometrial carcinoma cells by miR-155/NF- κ B/ROS pathway

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Background: Recombination signal-binding protein J (RBPJ) is a crucial downstream effector of Notch signaling, which is involved cell proliferation, differentiation, and apoptosis. It plays an important role in tumorigenesis although the further studies and concrete evidence are still needed. Especially for endometrial carcinoma, the functions and mechanism of RBPJ are still elusive.

Methods: The RNA expressions of RBPJ, miR-155, NF- κ B, TNF- α and κ B-Ras1 were examined by rt-PCR, and their protein levels were determined by Western Blot. Their expressions were inhibited by transient transfection of related siRNAs. Wound healing and transwell invasion assays were performed in ECC003 cells for measuring the migration and invasion ability, respectively. The ROS levels were detected by flow cytometry with H2DCFDA.

Purpose: This study was designed to investigate biological characteristics and molecular pathway of RBPJ in endometrial carcinoma cells, which may provide a potential therapeutic target for the treatments against endometrial carcinoma.

Results: It was shown in our study that the expression levels of RBPJ were significantly downregulated in different endometrial carcinoma cell lines. And a siRNA-mediated reduction of RBPJ enhanced the migration and invasion ability of ECC003 obviously. Besides, the results showed that the reactive oxygenspecies (ROS) levels increase when inhibiting RBPJ. To investigate the molecular pathway of RBPJ, we examined the expression of nuclear factor- κ B (NF- κ B), NF- κ B inhibitor interacting Ras-like protein 1 (κ B-Ras1), tumor necrosis factor- α (TNF- α) and miR-155. The results suggested that the expression of NF- κ B and TNF- α significantly was promoted, while κ B-Ras1 was inhibited. An upregulated expression was observed with miR-155 as well, which suggested the inhibition of NF- κ B signal pathway was mediated by miR-155. Our results of Notch intracellular domain (NICD) knockdown also demonstrated that NICD is required for the inhibition of RBPJ on miR-155. And knockdown of miR-155 could inhibit the mobility of endometrial carcinoma cells.

Conclusion: Our study suggested that RBPJ can inhibit the movability of endometrial carcinoma cells by miR-155/NF- κ B/ROS pathway.

Keywords: RBPJ, endometrial carcinoma, ROS, NF- κ B signal, miR-155

Introduction

As the transcription factor, recombination signal-binding protein J (RBPJ) belongs to the CSL family, which is essential for Notch signaling.^{1,2} RBPJ binding Notch intracellular domain (NICD) can modulate cell proliferation, differentiation, and apoptosis by regulating the expression of downstream target genes, which may be related to oncogenesis.^{3,4} It was reported that the expression of RBPJ was significantly downregulated in cancer cells, and depletion of RBPJ could obviously increase the survival rate of cancer cells and lead to a growth of tumor.⁵

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However, there is no study reported its functions and mechanism in endometrial carcinoma.

Previous study demonstrated that RBPJ repressed expression of miR-155 by binding to its promoter region. The absence of RBPJ led to an upregulation of miR-155.⁶ As a single-stranded non-coding RNA, miR-155 regulates expressions of many genes, and some of them play key roles in endometrial carcinoma.^{7,8} The expression of miR-155 also significantly upregulated in many other types of human cancers including thyroid adenoma,⁹ breast cancer,^{10–12} colon cancer,¹⁰ cervical cancer,¹³ pancreatic cancer^{14,15}, and lung cancer.¹⁶ miR-155 was proved to be involved in the invasion and metastasis of cancer cells. Inhibition of miR-155 expression can significantly reduce the proliferation of colon cancer cells and increase the cell apoptosis.

Nuclear factor- κ B (NF- κ B) is a major regulator that control cytokines expressions, such as tumor necrosis factor (TNF), by targeting promoter regions.^{17,18} Besides, NF- κ B has been shown to exhibit inhibitory effects on apoptosis by regulating the expression of anti-apoptotic genes.¹⁹ For tumorigenesis, NF- κ B can promote cancer cell growth and reduce cancer cell apoptosis in various cancers, like pancreatic cancer,²⁰ oral squamous cell carcinoma,²¹ colorectal cancer²², and breast cancer.²³ Reduced activity of NF- κ B can promote the apoptotic rate in cancer cells significantly.²⁴ It was reported that the NF- κ B inhibitor could inhibit the proliferation of prostate cancer cells and promoted cell apoptosis. Normally, NF- κ B remains in inactive bounding to the inhibitor, like κ B-Ras1.²⁵ κ B-Ras1 is the target gene of miR-155, and miR-155 can activate NF- κ B by targeting κ B-Ras1.⁶ The expression of κ B-Ras1 obviously downregulated in human tumors. And the deletion of κ B-Ras1 led to increased tumor growth.^{26,27}

TNF- α is a NF- κ B-regulated cytokine that has the ability to promote growth, invasion, and metastasis of cancer cell.^{6,17} As tumor-promoting factor, TNF- α was involved in the tumorigenesis. Its expression significantly increased in many cancers, including breast cancer, prostatic cancer, bladder cancer, colon cancer, liver cancer, and lymph cancer.²⁸ Previous study demonstrated that TNF- α promoted the tumorigenesis induced by chemical carcinogens, and TNF- α knock-out mice showed tolerance to chemical carcinogens.^{29,30} Besides, anti-TNF- α can be an effective treatment in colon cancer.^{31,32}

In this study, we examined the expression of RBPJ in different endometrial carcinoma cell lines. And its biological functions and molecular pathway was investigated in endometrial carcinoma cells with RBPJ knockdown,

which may provide a novel therapeutic target in the treatment against endometrial cancer.

Materials and methods

Cell culture

Cell lines RL95-2, ECC001, ECC003, Ishikawa, and ECC-E6/E7 were obtained from the ATCC (Manassas, USA). Cell lines were cultured at 5% CO₂ and 37 degrees centigrade with DMEM medium containing 1% FBS. Cells were used for experiment during the logarithmic growth phase.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the cells with TRIzol reagent. Cells were collected into disinfected tubes and washed with 1×PBS buffer. Cells lysis was added for cell fragmentation. Then, phenol/chloroform/isoamyl alcohol was added for removing impurities. After centrifugation, equal volumes isopropanol was added into the supernatant for RNA precipitation. RNA washed by 75% ethanol, and then dissolved by RNase-free ddH₂O. And first-strand cDNA synthesis reaction was referred to the manual of Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). The specific primer sequences were designed for RBPJ (5'-AAG GAG CCC CAC GAG AAA AAT-3'; 5'-ACC GAA CTT GCA TTG ATT CCA G-3') and NICD (5'-CGC TGA CGG AGT ACA AGT G-3'; 5'-GTA GGA GCC GAC CTC GTT G-3'). cDNA sample, primers, and qPCR Master Mix were used for real-time PCR, referring to the user manual provided with the SYBR® Green reagent (Roche, Indianapolis, IN, USA).

Transient transfection

Before transfection, cell density was reached up to 60–80%. Cells were transfected with 100 nm siRNA against RBPJ and NICD through Lipofectamine 2000. The specific siRNA sequences were designed against RBPJ (SiRBPJ-1: 5'-CCA GAU UUC UGC CUU AAU UGU -3'; SiRBPJ-2: 5'-GAU UUC UGC CUU AAU UGU UCU -3'; SiRBPJ-3: 5'-GAA GCU AUG CGA AAU UAU UUA-3') and NICD (SiNICD-1: 5'-CUA CUA UUU UUC GAU UUG AAU -3'; SiNICD-2: 5'-CUA UUU UUC GAU UUG AAU AGA -3'; SiNICD-3: 5'-ACC AAA ACC GAC UCU AUU CAA -3').

Wound healing assay

ECC003 cells, ECC003 cells transfected with SiRBPJ, and ECC-E6/E7 cells (5×10⁵ cells) were cultured at six-

well tissue culture plates and wounded with a sterile 10- μ L pipette tip. The loosely attached cells were removed by PBS buffer. The cells were cultured at serum-free medium and photographed.

Transwell invasion assay

ECC003 cells, ECC003 cells transfected with SiRBPJ and ECC-E6/E7 cells were suspended in serum-free medium containing BSA after washed by PBS buffer. Cell suspension was added to 24-transwell Boyden chamber with extracellular matrix-coated membrane inserts (8-mm pore size) (BD Falcon, Corning-Costar, New York, NY, USA). 24-transwell chamber was incubated overnight in incubator at 37 degrees centigrade and 5% CO₂. Invaded cells were fixed by formaldehyde for 10 mins and stained by crystal violet. The average number of cells per field was expressed as the percentage of the control after normalizing for cell number.

Detection of reactive oxygen species (ROS) level

Cell in logarithmic phase was cultured and seeded in six-well plate at a density of 4×10^5 /mL. Cell was washed by PBS buffer. After centrifugation, the cell was resuspended by PBS buffer. The probe H2DCFDA was added and the mixture was incubated at 37°C for 30 mins. ROS level was measured by flow cytometry.

Western blot

Total proteins were extracted with cell lysate. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane. The membrane was stained with 1×Ponceau S for 5 mins and then was closed by TBS solution for 1 hr. Primary antibody diluted with TBST was added for 2 hrs at RT. Then, the membrane was washed by TBST three times for 10 mins. Secondary antibody diluted with TBST and the membrane was incubated for 2 hrs. The result was analyzed by ECL detection system.

Statistical analysis

At least three repeated experiments have been performed. The data were processed by SPSS 25.0 statistical software. Two-tailed *t*-test and one-way ANOVA was applied in the analysis, in which **P*<0.05 and ***P*<0.01.

Results

The expression of RBPJ decrease in endometrial carcinoma cell lines

We detected the expression level of RBPJ in different endometrial carcinoma cell lines with real-time PCR. The results showed that the expressions of RBPJ were significantly downregulated in endometrial carcinoma cell lines compared to the normal cell line ECC-E6/E7 (Figure 1A and B). The mRNA and protein expression level of RBPJ varied as well among different endometrial carcinoma cell lines. According to this result, the expression of RBPJ was highest in ECC003, while the lowest expression of RBPJ was detected in Ishikawa.

RBPJ inhibits the proliferation, migration, and invasion capacities of endometrial carcinoma cell

To investigate the biological functions of RBPJ in endometrial carcinoma, the expression of RBPJ was knockeddown by siRNAs in ECC003 and ECC-E6/E7. Three different siRNAs were designed based on the sequence of RBPJ mRNA and tested for their interference abilities. Expressions of RBPJ mRNA were examined after transient transfections and incubation. As a result, all siRNAs can suppress RBPJ expression effectively, especially SiRBPJ-3 (Figure 1C). Thus, we selected SiRBPJ-3 for suppressing RBPJ expressions in further experiments.

The capacity of cell proliferation was investigated in ECC003 and ECC-E6/E7 with SiRBPJ-3. The results showed that the knockdown of RBPJ promoted the cell proliferation (Figure 2A). We also examined the migration and invasion of endometrial carcinoma cells by the wound healing and transwell invasion assays, respectively. It was suggested in the wound healing assays that the downregulation of RBPJ expression could promote the migration ability of ECC003 and ECC-E6/E7 cell line, in which the width of wound in SiRBPJ was significantly reduced compared to the control (Figure 2B). Similarly, in transwell invasion assays, the knockdown of RBPJ obviously improved the ability of ECC003 and ECC-E6/E7 cell line in invasion (Figure 2C). These results demonstrated that the RBPJ plays an important role in the proliferation and movability of endometrial carcinoma cells.

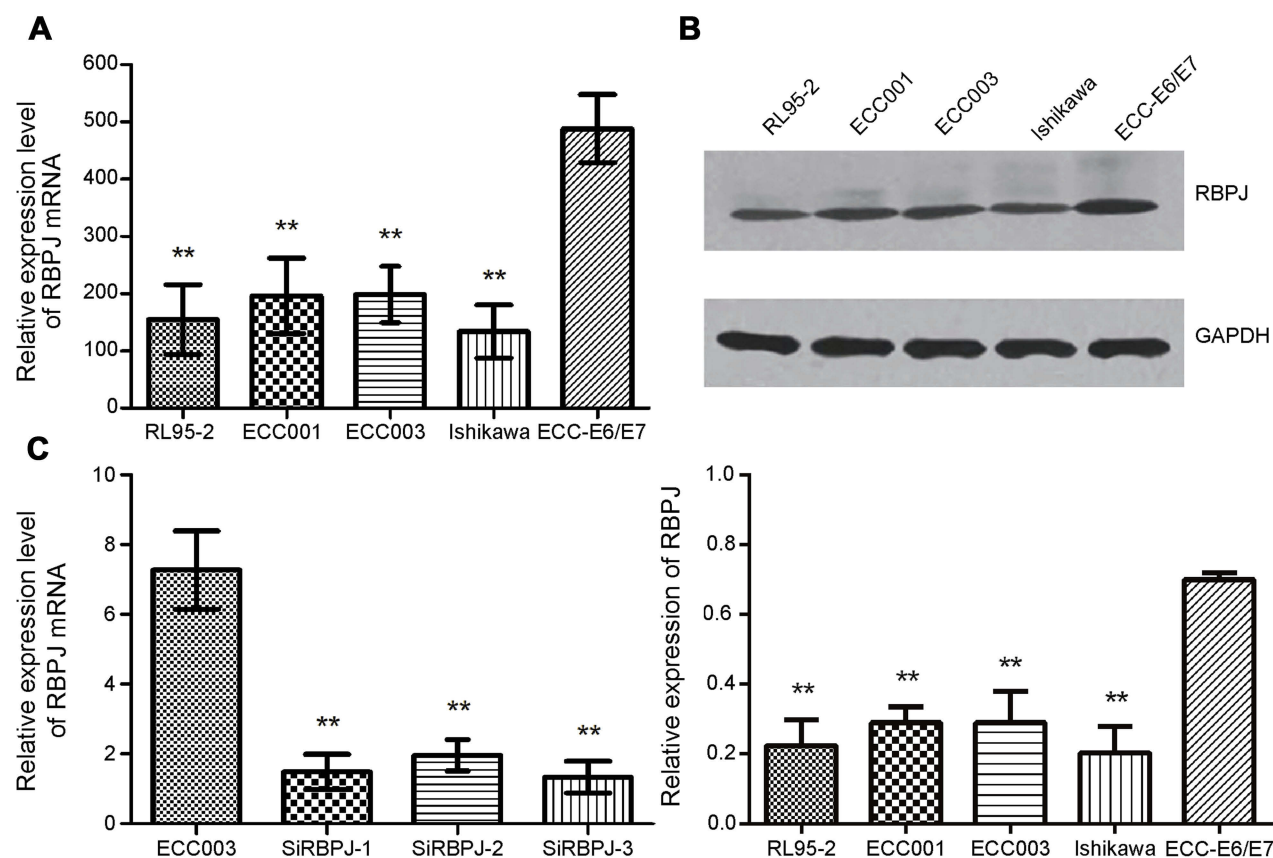


Figure 1 The relative expression of RBPJ. **(A)** The relative expression of RBPJ mRNA in endometrial carcinoma cell (RL95-2, ECC001, ECC003, and Ishikawa) and normal cell lines (ECC-E6/E7). **(B)** The relative expression of RBPJ protein. **(C)** The relative expression of RBPJ mRNA with three siRNAs in ECC003. At least three repeats were carried out. Data are presented as mean \pm SE. * $p < 0.05$; ** $p < 0.01$.

Abbreviation: RBPJ, recombination signal-binding protein J.

RBPJ suppresses endometrial carcinoma cells via reducing ROS level

It has been reported that ROS enhanced the ability of tumor movability. By measuring intracellular ROS levels, we found the ROS level in SiRBPJ-ECC003 was significantly higher than that in ECC003 and ECC-E6/E7 (Figure 3A). These results may suggest that the reduction of RBPJ could lead to an accumulation of ROS in endometrial carcinoma cell, which may enhance the movability of endometrial carcinoma cells.

RBPJ inhibits NF- κ B signal pathway in endometrial carcinoma

Since ROS was involved in NF- κ B signal pathway and inflammation via the release of TNF- α , we next detected the expression of NF- κ B, TNF- α , and κ B-Ras1, the upstream inhibitor of NF- κ B, by real-time PCR and Western blot. It was shown in our study that the expression of NF- κ B

and TNF- α significantly upregulated when RBPJ was suppressed (Figure 3B and C). Besides, the expression of κ B-Ras1 in SiRBPJ-ECC003 was inhibited simultaneously, which suggested RBPJ may inhibit NF- κ B signal pathway by regulating κ B-Ras1 in endometrial carcinoma cells (Figure 3B and C). These results above demonstrated that RBPJ can inhibit NF- κ B signal pathway, which may be involved in the growth of endometrial carcinoma.

RBPJ binding NICD reduces the expression of miR-155 in endometrial carcinoma

Previous study reported that κ B-Ras1 is a target gene of miR-155.⁶ Thus, we examined the expression of miR-155 by real-time PCR to investigate if RBPJ regulates the expression of miR-155 in endometrial carcinoma cells. The results showed that the expression of miR-155 was significantly raised in SiRBPJ-ECC003, and miR-155 expression was relatively

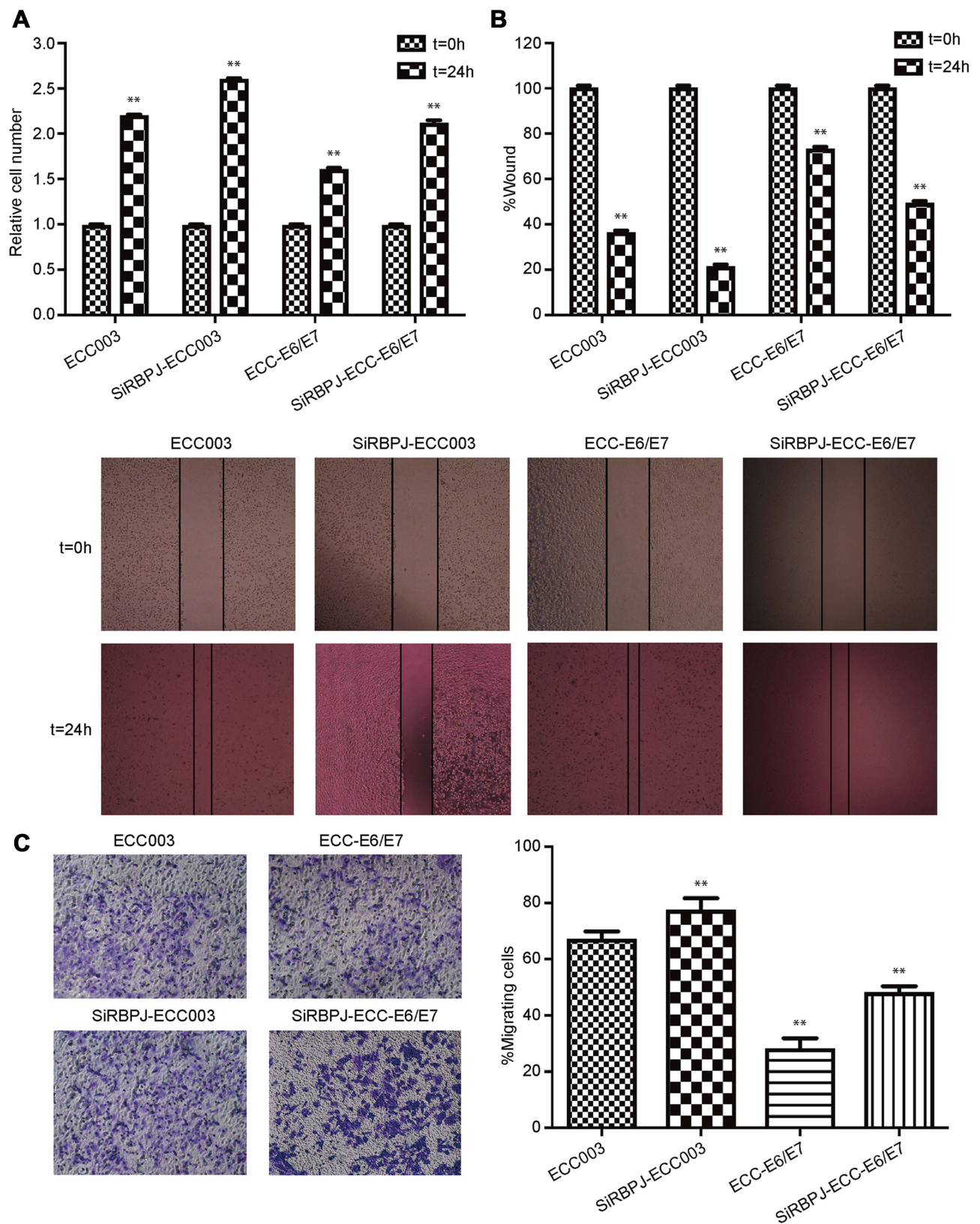


Figure 2 RBPJ knockdown promotes the proliferation, migration, and invasion of endometrial carcinoma cells. **(A)** The relative cell proliferation in ECC003 and ECC-E6/E7. Wound healing assays **(B)** and transwell invasion assays **(C)** were employed for determining the ability of cells in migration and invasion. At least three repeats were carried out, and the mean \pm SD was presented, * $p < 0.05$; ** $p < 0.01$.

Abbreviation: RBPJ, recombination signal-binding protein J.

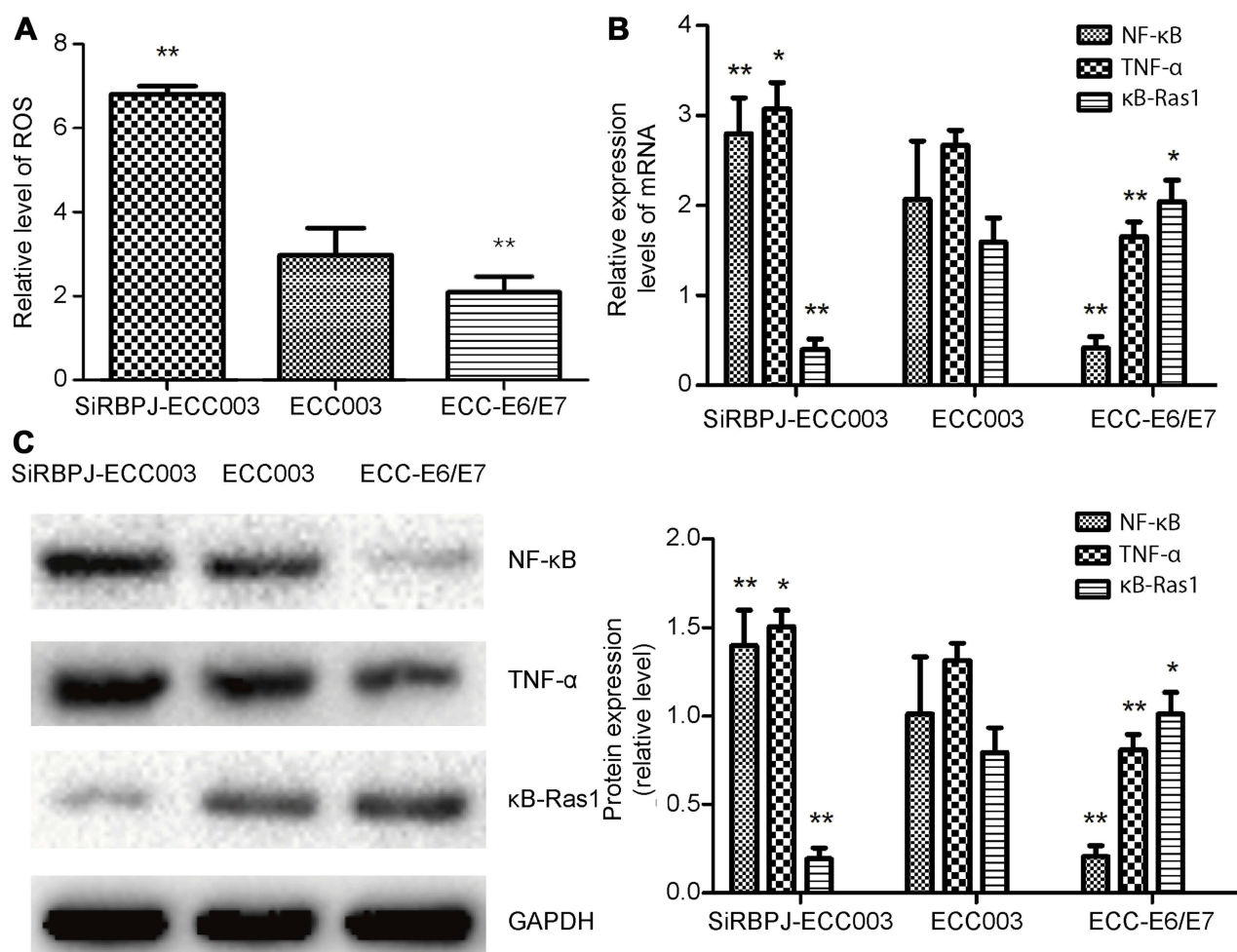


Figure 3 RBPJ regulates the NF-κB signaling pathway. (A) The ROS level in ECC-E6/E7, ECC003, and siRBPJ-3. (B) The relative expression of NF-κB, κB-Ras1, and TNF-α mRNA. (C) Western blot analysis for NF-κB, κB-Ras1, and TNF-α. All the above data are presented as mean ± SD, * $p < 0.05$; ** $p < 0.01$.

Abbreviations: RBPJ, recombination signal-binding protein J; NF-κB, nuclear factor κB; ROS, reactive oxygenspecies.

lower in ECC-E6/E7 (Figure 4A), which was consistent with our assuming.

To further confirm it, we interfered with the expression of NICD by siRNA, which can bind with RBPJ to activate its inhibitory ability. As we described before, three kinds of siRNA were designed to target NICD mRNA in ECC003, and we select SiNICD-1, the most efficient one, for next tests (Figure 4B). We detected the mRNA transcription level of κB-Ras1 and miR-155 in ECC003 and SiNICD-ECC003 (NICD knockdown in ECC003) by real-time PCR. The results showed that the mRNA level of miR-155 was markedly upregulated, while the mRNA level of κB-Ras1 was significantly downregulated in SiNICD-ECC003 (Figure 4C). Thus, we can conclude that the inhibitory effect of RBPJ on miR-155 was mediated by NICD in endometrial carcinoma cells. To further confirm it, the level of ROS was measured as

well. As expected, it significantly increased in SiNICD-ECC003 (Figure 4D). Moreover, we examined the migration and invasion of ECC003 treated with siRBPJ and miR-155 inhibitor, which suggested that the knockdown of miR-155 significantly inhibited the migration and invasion of SiRBPJ-ECC003 (Figure 4E and F). These results above indicated that the knockdown of RBPJ or NICD had a significant influence on the expression of miR-155 in endometrial carcinoma cells, and then abolished the inhibition on NF-κB signal pathway and intracellular ROS level.

Discussion

As a DNA-binding protein, RBPJ can recognize the promoter-specific sequence of target genes.² Particularly, RBPJ plays an important role in the Notch signaling pathway.^{33–35} As the downstream signal molecule of Notch, RBPJ binding NICD

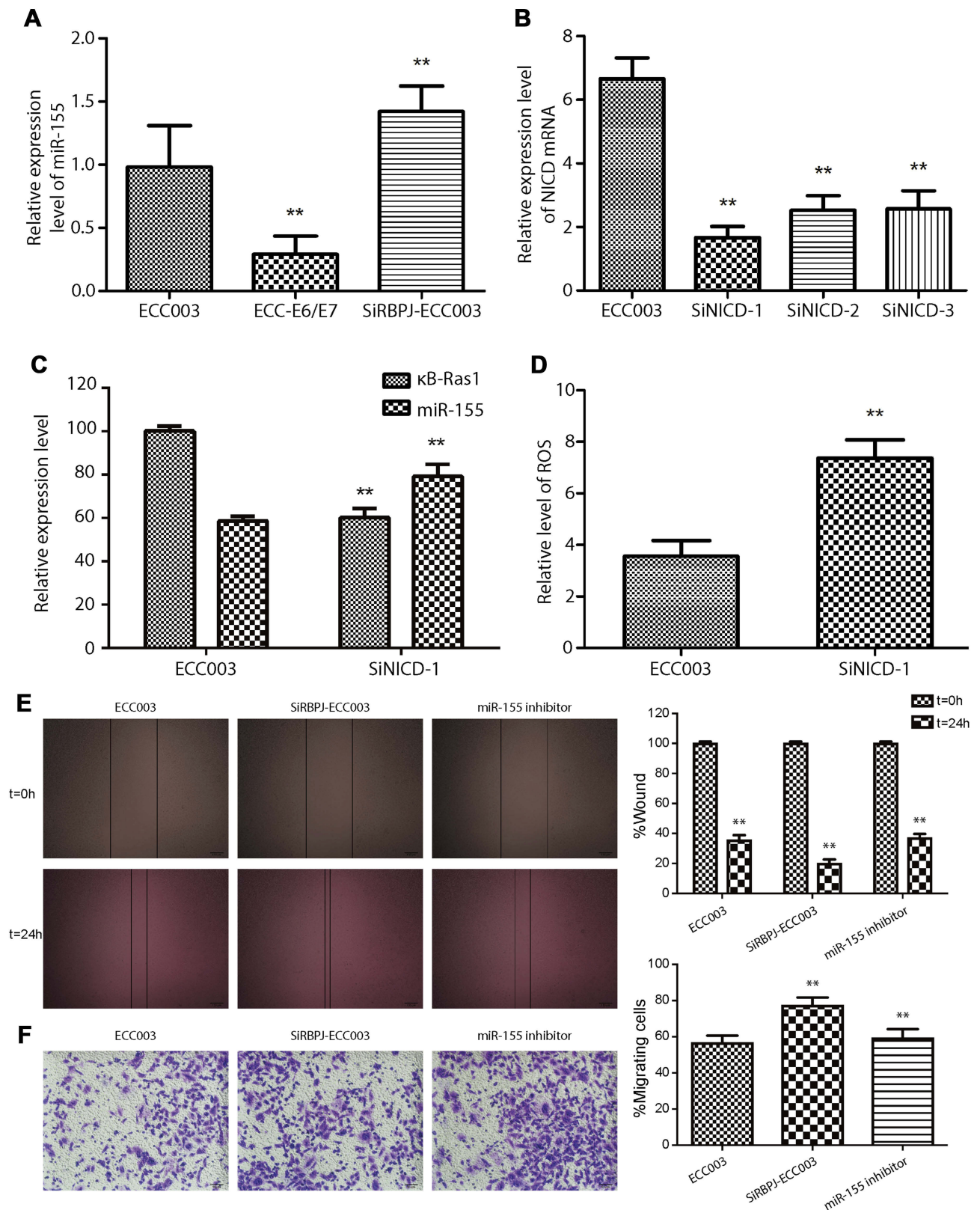


Figure 4 RBPJ binding with NICD inhibits miR-155. **(A)** The relative expression of miR-155 in ECC-E6/E7, ECC003, and siRBPJ-3. **(B)** The relative expression of NICD mRNA in siNICD-1, siNICD-2, and siNICD-3. **(C)** The relative expression of miR-155 and κ B-Ras1 in ECC003 and siNICD-1. **(D)** The ROS level in ECC003 and siNICD-1. Wound healing assays **(E)** and transwell invasion assays **(F)** were performed in ECC003 with siRBPJ and miR-155 inhibitor. At least three repeats were carried out, and the mean \pm SD was presented, * $p < 0.05$; ** $p < 0.01$.

Abbreviations: RBPJ, recombination signal-binding protein J; NICD, Notch intracellular domain; ROS, reactive oxygen species.

regulates the expression of target genes related to cell cycle or apoptosis, like Cyclin D1, Cyclin-dependent kinase 2, p21, and B-cell CLL/lymphoma 2 (Bcl-2).^{36,37} It has been proved that RBPJ is involved in tumorigenesis. Depletion of RBPJ in breast cancer and lung cancer could accelerate the growth of tumor.⁵ And RBPJ inhibition significantly reduced cell proliferation of small cell lung cancer.³⁸ However, there are no studies reported the functions of RBPJ in endometrial carcinoma, and its mechanism has not been clear.

In this study, we investigated the biological functions of RBPJ in endometrial cancer cells and revealed its molecular pathway. It was shown that RBPJ expression was reduced in endometrial carcinoma cell lines. In order to investigate the biological functions of RBPJ in endometrial carcinoma, the siRNAs targeting RBPJ were designed to reduce the cellular expression of RBPJ. The results of real-time PCR showed that the expression of RBPJ is reduced to about one fifth. Besides, we found knockdown of RBPJ significantly enhanced the cell proliferation and mobility. Considering the expression of RBPJ decreased gradually as well in these three lines, it suggested that RBPJ could effectively reduce the mobility of endometrial carcinoma cell, which may be involved in the metastasis of endometrial carcinoma.

ROS is the product of aerobic metabolism.^{39,40} ROS at a high level could cause DNA and protein damage and tumorigenesis. It was reported that, in various cancer cells, the level of ROS was elevated compared to normal cell.⁴¹ ROS is reported that it contributed to the metastasis of tumor cells and resistance to apoptosis.^{42–46} To investigate whether the inhibitory effect of RBPJ on endometrial carcinoma cell was mediated by ROS, we measured the ROS content. The results showed that the RBPJ gene expression and the ROS level were negatively related. This may because RBPJ directly inhibits the production of ROS or enhance the ability of scavenging ROS. Since ROS is associated with inflammation, the inflammation signal pathway was examined as well. The results showed that the expressions of inflammatory factors TNF- α and NF- κ B are significantly upregulated, which indicated the enhancement of inflammatory reaction. Moreover, the expression of κ B-Ras1, inhibitor of NF- κ B, was detected. The result showed that κ B-Ras1 expression was inhibited obviously, which suggested that RBPJ is involved in inflammation response by regulating the expression of κ B-Ras1.

Our study demonstrated that RBPJ inhibits the expression of miR-155 in endometrial carcinoma. Since previous

study on bone marrow niche indicated miR-155 could inhibit the expression of κ B-Ras1 by targeting the sequences at its 3'-UTR,^{6,47} we assumed that the expression of κ B-Ras1 might be promoted by RBPJ. It may be due to the miR-155's promoter targeting of RBPJ-NICD complex. To further investigate it, the expression of NICD was knocked down in endometrial carcinoma. The result indicated that NICD is required for the inhibition of RBPJ on miR-155. This may be due to the interaction between RBPJ and NICD, which activate the inhibitory activity of RBPJ. To investigate whether RBPJ inhibits the mobility of endometrial carcinoma cells by miR-155/NF- κ B/ROS pathway, we inhibited the expression of RBPJ and miR-155 in ECC003. By wound healing assay and transwell invasion assay, we found knockdown of miR-155 significantly decreased the mobility of SiRBPJ-ECC003. It suggested that RBPJ inhibits the mobility of endometrial carcinoma cells by inhibiting miR-155.

In summary, our study investigated the biological functions and molecular mechanism of RBPJ in endometrial carcinoma, which may provide a potential therapeutic target for endometrial carcinoma treatments.

Disclosure

The authors report no conflicts of interest in this work.

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