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LncRNA H19 inhibitor represses synovial cell proliferation and apoptosis in rats with rheumatoid arthritis *via* Notch signaling pathway

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Abstract. – OBJECTIVE: To study the roles and underlying mechanisms of long non-coding ribonucleic acid (lncRNA) H19 in the synovial cell proliferation and apoptosis in rats with rheumatoid arthritis (RA).

MATERIALS AND METHODS: A total of 30 Sprague-Dawley rats were randomly divided into Control group and Model group. The rat model of RA was induced by using type II collagen in Model group. The primary synovial cells were isolated from the synovial tissues of the rats and were assigned into Control group, Model group, and lncRNA H19 inhibitor intervention group. 5-Ethynyl-2'-deoxyuridine (EdU) staining was applied to detect cell proliferation in each group. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was employed to detect the cell apoptosis in each group. Western blotting assay was adopted to measure the expression levels of Notch1 and hairy/enhancer-of-split homolog-1 (Hes1) in each group of cells.

RESULTS: The RA incidence of the Model group was higher than that of the Control group. Compared to the Control group, the expression of lncRNA H19, Notch1 and Hes1 of the synovial cells in the Model group were significantly elevated. Besides, the cell proliferation rate of the Model was also increased, while the cell apoptosis rate was decreased compared with those in the Control group. Moreover, in comparison with Model group, lncRNA H19 inhibitor intervention group exhibited low lncRNA H19 level, remarkably reduced proliferation rate and protein levels of Notch1 and Hes1, as well as notably increased cell apoptosis rate.

CONCLUSIONS: Our results indicated that lncRNA H19 inhibitor could repress the proliferation and promote the apoptosis of synovial cells in RA rats, which might be attributed to the inhibition of the Notch signaling pathway.

Key Words:
Long non-coding RNA H19, Rheumatoid arthritis, Proliferation, Apoptosis, Notch signaling pathway.

Introduction

Rheumatoid arthritis (RA) is a kind of autoimmune disease with structural damage of synovial joint and long-term chronic inflammation in synovium as the major pathological characteristic. RA can occur at any age and is clinically mainly treated with non-steroidal anti-inflammatory drugs, glucocorticoid, antirheumatic drugs, etc. Most of these drugs can only relieve the symptoms rather than eradicate the disease[4,5]. Therefore, exploration of the pathogenesis of RA is necessary prerequisite in the search for therapeutic drugs and methods.

The imbalance between proliferation and apoptosis of synovial cells is one of the principal precipitating factors for RA[6]. Therefore, it is of great importance to effectively control the local proliferation and apoptosis of synovial cells for the treatment of RA. The Notch signaling pathway was proved to widely exist in vertebrates and invertebrates with high evolutionary conservation. The Notch signaling pathway is composed of Notch receptors and Notch ligands[7]. The activated Notch receptor will be cleaved and its intracellular domain will then enter the nucleus and bind to the transcription factor CSL, thereby activating downstream target genes and fulfilling corresponding biological functions[8,9]. Hence, regulation of the cell proliferation and apoptosis *via* Notch signaling pathway might have great potential in the treatment of RA in the future.

Long non-coding ribonucleic acids (lncRNAs) are a category of ncRNA molecules with a length of more than 200 nt and have been reported to directly or indirectly influence the gene expression[10]. LncRNA H19 is a type of ncRNA with a molecular weight of 2.3 kb, and its expression is up-regulated in

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multiple malignant tumors, including gastric cancer, lung cancer, and breast cancer[11-13]. The highly expressed lncRNA H19 could facilitate the proliferation, invasion, and migration of tumor cells, indicating that lncRNA H19 might be a potential therapeutic target of malignant tumors. However, the impacts of H19 on the proliferation and apoptosis of synovial cells in RA rats have not been clarified yet.

Therefore, the rat model of RA was induced using type II collagen was established in this study. The primary synovial cells were extracted from the rats and transfected with lncRNA H19 inhibitor to investigate the influences and underlying mechanisms of lncRNA H19 on the proliferation and apoptosis of synovial cells in RA rats.

Materials and Methods

Reagents

Type II collagen (Sigma Aldrich, Louis, MO, USA), Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA), fetal bovine serum (FBS; HyClone, South Logan, UT, USA), culture plate and culture flask (Corning, Corning, NY, USA), lncRNA H19 inhibitor (Shanghai GenePharma Co., Ltd., Shanghai, China), TRIzol solution (Shanghai Shiyi Biotech Inc., Shanghai, China), 5-Ethynyl-2'-deoxyuridine (EdU) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kits (Nanjing KeyGen BioTech Co., Ltd., Nanjing, China), Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), SYBR Premix Ex Taq fluorescence quantitative polymerase chain reaction (qPCR) kit (TaKaRa, Otsu, Shiga, Japan), lncRNA H19 and U6 primers (GenePharma), and anti-Notch1, anti-Hes1 and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit primary antibodies and horseradish peroxidase (HRP)-labeled secondary antibodies (Beijing Bioss Biological Technology Co., Ltd., Beijing, China).

Instruments

Inverted fluorescence microscope (Olympus, Tokyo, Japan), CO_2 incubator and cryogenic refrigerator (Thermo Forma, Waltham, MA, USA), fluorescence qPCR instrument (Thermo Fisher Scientific, Waltham, MA, USA), thermostatic water bath (Gilson, Middleton, WI, USA), high-speed low-temperature centrifuge Eppendorf (EP; Hamburg, Germany), and high-temperature sterilization pot (Beijing Ruibang Xingye Science & Technology Co., Ltd., Beijing, China).

Rats

A total of 30 Sprague-Dawley rats (8 weeks old, 180-200 g) were purchased from Shaanxi Institute for Food and Drug Control [permit number: SYXK (Shaanxi) 2018-002]. All rats were housed in a clean environment with constant temperature and humidity, given sufficient standard feed and water. All rats were free to move in the cage. The research was approved by the Animal Ethics Committee of Xi'an Jiaotong University Animal Center.

Establishment of the Model of RA

The type II collagen was fully mixed with an equal volume of Freund's complete adjuvant to prepare the type II collagen emulsion (1 mg/mL). Then, the emulsion was intracutaneously and subcutaneously injected into the footpad of left hind limb of the rats. The second induction was performed 7 d later. Subsequently, the RA score of each rat was recorded every 4 days from 14th d until the 30th d after the first injection. The rats without arthritis were scored 0 point, those with redness in 1-2 joints were scored 1 point, those with redness in 3-4 joints were scored 2 points, those with redness in 5 and more than 5 joints were scored 3 points, and those with severe arthritis in the hind paw were scored 4 points.

Culture and Transfection of Synovial Cells in RA Rats

The rats were sacrificed 30 days after inflammation induction. The synovial tissues were taken under sterile conditions, cut into pieces using surgical scissors, and cultured in complete DMEM containing 1 mg/mL type II collagen for 2 h. The medium containing tissue fragments was collected, centrifuged, and added new complete medium. The synovial cells harvested from Control group were cultured with complete medium without 1 mg/mL type II collagen. Next, lncRNA H19 inhibitor was transfected into the synovial cells using Lipofectamine 2000. The total RNA was extracted *via* TRIzol reagent. Finally, the lncRNA H19 level in each group was determined *via* quantitative reverse transcription-PCR (qRT-PCR). The primers were shown in Table I.

Table I. Information of primer sequences of lncRNA H19 and U6.

Gene name	Sequence
LncRNA H19	5'-TACAACCACTGCACTACCTG-3' 5'-TGGAATGCTTGAAGGCTGCT-3'
U6	5'-GCGCGTCGTGAAGCGTTC-3' 5'-GTGCAGGGTCCGAGGT-3'

Detection of Influence of LncRNA H19 on Proliferation of Synovial Cells in RA Rats Via EdU Staining

The synovial cells in each group were added with 50 μL of cytological fixing solution and incubated at room temperature for 30 min. Each well was added with 50 μL of glycine (2 mg/mL), incubated for 5 min, and then washed with phosphate-buffered saline (PBS) for 5 min. Then, every well was added with 100 μL of penetrant and incubated for 10 min following by the incubation with EdU staining solution in the dark for 30 min. After discarding the staining solution and addition of anti-fluorescence quencher, the fluorescence of each well was observed under the fluorescence microscope.

Detection of Influence of LncRNA H19 on Apoptosis of Synovial Cells in RA Rats Via TUNEL Staining

After fixed in 4% paraform for 30 min, the cells in each group e permeabilized with 0.3% Triton X-100 r for washed with phosphate buffe (PBS), ter, 50 μL of termina xynucl transferase-mediated dUT k end labe (TUNEL) staining solutio ded into eac well, and the cells we ce dark. After washed with PBS r 5 min an with anti-fluorescence c cher, cell's flu nce staining was obse und the microscope.

De of In nces of LncRNA H19 on L of Notch1 and Hes1 in novi ia Western Blotting

The cell each group were lysed using radio precipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein concentration was measured after the protein curve was plotted. Next, dodecyl sulfate sodium-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. The proteins were run on a space for 20 min and then on a separation gel at After that, proteins were transferred on poly-vinylidene difluoride (PVDF) membr he membrane was blocked in 5% ski milk p solution for 1 h followed by th ubation primary antibodies at 4°C night he ne day, the membrane was r i ated with HRP-labeled secondary an or 2 h e optical density of the s wa te he-miluminescence r d. The an sed in this study were vs: Notch1 (00), Hes1 (1:1000), and nce GAPDH (1:1000).

Statist nalysis

S Product and Service Solutions (S 19.0 (IBM Corp., Armonk, NY, USA) so re was em ed for data management. All th were r nted as mean ± standard deviati was adopted for comparison of the dif between the two samples. $p<0.05$ gested that the difference was statistically t.

Results

The Rat Model of RA Was Established Successfully

The rats in Control group had normal hind paws. In comparison with those in Control group, the RA score was remarkably increased in Model group at 14 d after inflammation induction (*$p<0.05$). Besides, the inflammation score was also raised as the induction days were increased (Table II).

LncRNA H19 Level in Synovial Cells of RA Rats Declined Markedly After Transfection with LncRNA H19 Inhibitor

According to the qRT-PCR results (Figure 1), the level of lncRNA H19 in the synovial cells was notably elevated in Model group compared

Table II. Polyarthritis index of rats (*$p < 0.05$).

Group	14 d	18 d	22 d	26 d	30 d
Control	0.056 ± 0.012	0.081 ± 0.022	0.103 ± 0.023	0.104 ± 0.034	0.112 ± 0.027
Model	0.154 ± 0.024*	0.198 ± 0.027*	0.231 ± 0.029*	0.341 ± 0.031*	0.428 ± 0.031*

Note: *$p<0.05$: Model group *vs*. Control group.

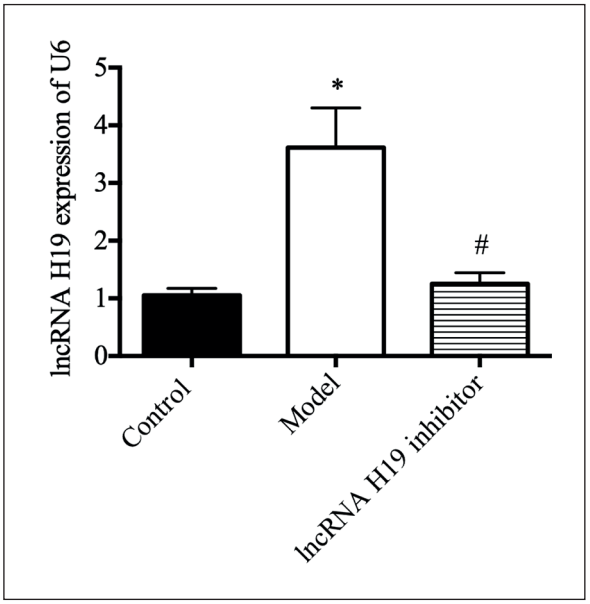

Figure 1. LncRNA H19 level in synovial cells in each group detected *via* qRT-PCR. (*p<0.05: Model group *vs.* Control group, #p<0.05: lncRNA H19 inhibitor group *vs.* Model group).

with that in Control group (*p<0.05). How... H19 was remarkably reduced in lncRNA ... inhibitor group in comparison with that in Mo... group (#p<0.05).

LncRNA H19 Inhibitor C... Evidently Repress the ... Synovial Cells in RA Rats

The statistical res... Edu... g (Figure 2A) indicated tha... synovial ... Model group had a distinctly higher proliferation rate than those of Control group (*p<0.05), while had a lower proliferation rate than those of lncRNA H19 inhibitor group (#p<0.05; Figure 2B). Our data suggested that lncRNA H19 inhibitor ... suppress the proliferation of synovial ... RA rats.

LncRNA H19 Inhibitor Could ... Prominently Promote the ...ptosis of Synovial Cells in RA ...

The TUNEL staining ... shown in Figure 3A. Compared wit... grou... synovial cells in M... grou... re-markable decreas... apoptosis ... <0.05). However, it w... sed notabl... lncRNA H19 inhibit... comparison with that in Model gro...p (#p<... Figure 3B). Above results ...rated that ...A H19 inhibitor cou... ate the apopto...s of synovial cells in ... rats.

Lnc... ...hibitor Could Signi... Repress the Protein ...els of Notch1 and Hes1 in ... Cells of RA Rats

...rding to the Western blotting bands (Figure 4A), the protein levels of Notch1 and Hes1 in synovial cells of Model group markedly enhanced compared with those in Control group (*p<0.05, *p<0.05), while they declined evidently in lncRNA H19 inhibitor group compared with those in Model group (#p<0.05, #p<0.05; Figure 4B).

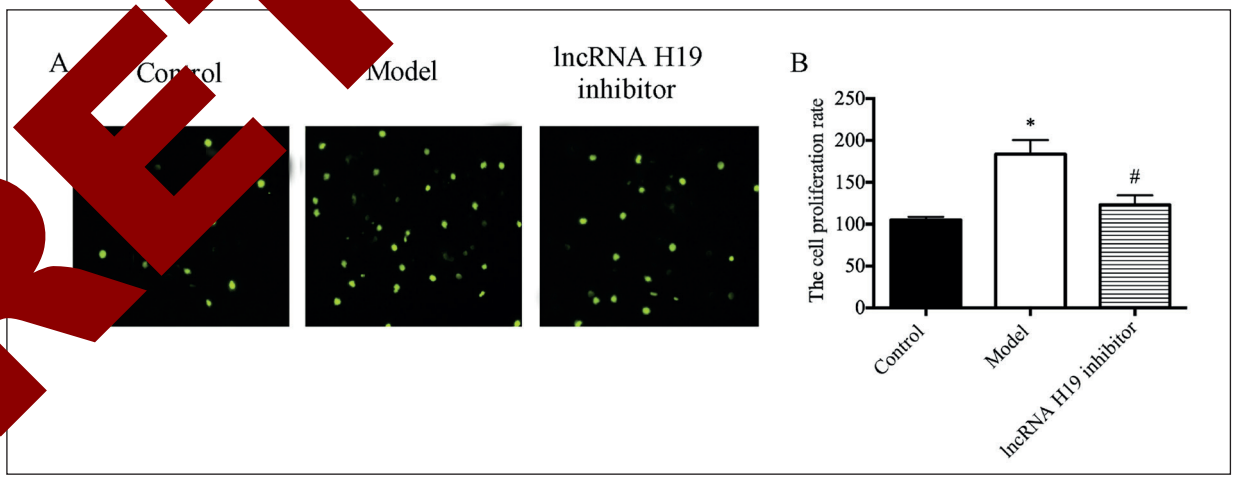

Figure 2. Cell proliferation rate in each group detected *via* Edu staining. **A,** Edu staining results (20×). **B,** Proliferation rate. (*p<0.05: Model group *vs.* Control group, #p<0.05: lncRNA H19 inhibitor group *vs.* Model group).

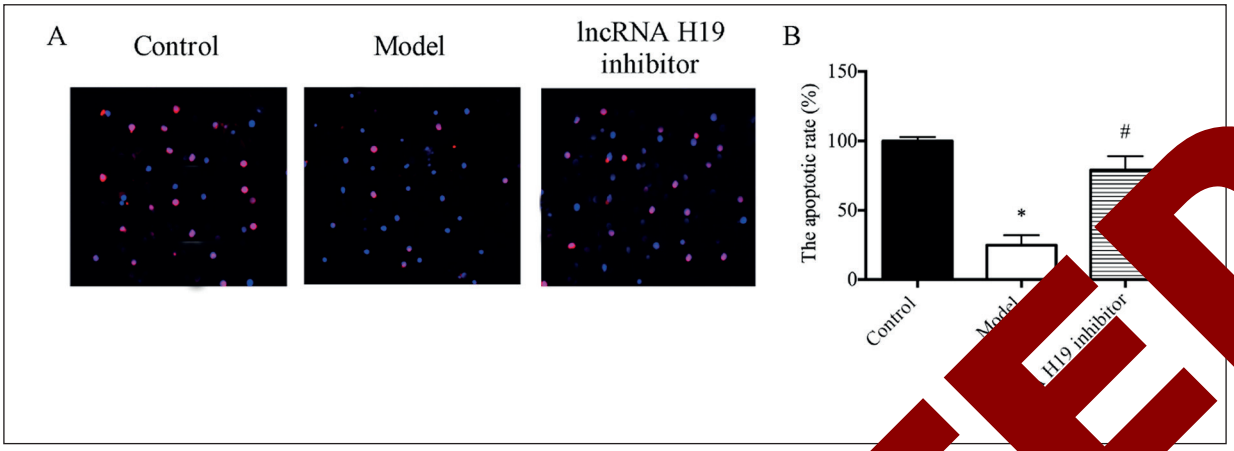

Figure 3. Cell apoptosis in each group detected *via* TUNEL staining. **A,** TUNEL stai ults (20×). **B,** l chart of apoptosis rate. (*p<0.05: Model group *vs.* Control group, #p<0.05: lncRNA H19 inh vs. Model grou

Discussion

Rheumatoid arthritis (RA) is an immune system disease characterized by chronic inflammatory lesions in the synovium[14]. The local inflammatory reaction will invade the b and joints if RA is not controlled efficientl thus leading to the deformity and function of the joints. It is worth noting that the viscera the body will also be affected b mmati during the pathological chang rves a one of the leading causes of of v capacity and disability of peop affecting the life qu ts. e, seeking for efficac herap rugs and protocols for RA ecome a s mission to be urgently

Although at is of RA is complicated, re rchers hav d in recent years that normal prolife ation and apoptosis of ovial cells ay pivotal roles in the onset o [16]. It als plies that searching for the m s regu g the proliferation and apoptosis ells will become a promising scheme treatment. With the development molecular biology, researchers have discov- lncRNAs are crucial players in tu- rther investigations also demonstrated hat lncRNAs occupied an important position in rheumatic diseases. Scientists have revealed that about 90% of gene expressions are controlled by lncRNAs, among which abnormally expressed lncRNA H19, located in the telomeric region of the human chromosome, can induce functional changes in organisms[17]. Nevertheless, the function of lncRNA H19 in RA remains unknown, so the rat model of RA was established first utilizing type II collagen induction in this research.

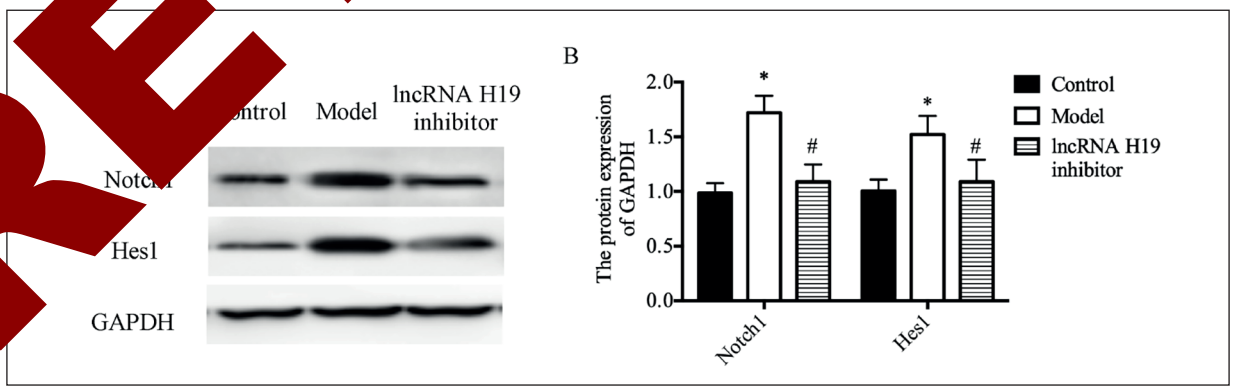

Figure 4. Protein expressions of Notch1 and Hes1 in each group of cells. **A,** Western blotting bands. **B,** Statistical charts of bands. (*p<0.05: Model group *vs.* Control group, #p<0.05: lncRNA H19 inhibitor group *vs.* Model group).

The RA score was increased distinctly in Model group compared with that in Control group, indicating that the rat model of RA was established successfully. The primary synovial cells were extracted from the synovial tissues, and those in Model group were stimulated by type II collagen continuously to prepare the model of RA synovial cells. Moreover, lncRNA H19 inhibitor was transfected into the synovial cells *via* Lipofectamine 2000 transfection technology, and its impact on the proliferation of synovial cells was determined through the Edu staining. According to Figure 3, lncRNA H19 inhibitor could evidently repress the proliferation of synovial cells in RA rats in comparison with Model group. Later, TUNEL staining was applied to examine the influence of lncRNA H19 inhibitor on apoptosis of synovial cells. The results manifested that the apoptosis rate of synovial cells in lncRNA H19 inhibitor group was remarkably higher than that in Model group. All those results indicated that the lncRNA H19 inhibitor could notably inhibit the proliferation and promote the apoptosis of synovial cells in RA rats.

It has been revealed that various cytok participate in some physiological and path cal processes of synovial cells, among whic Notch signaling pathway is one of the hotsp in the previous studies[18]. The N ignal organisms is involved in the r f cyto genesis, development, and m othe logical processes, dominated by c ife entiation, and apoptosi thr int n with adjacent cells. C d et a vered that BMI1 is capable o ressing the al proliferation of le lls, which m t be regulated by the ch g pathway. Matsuno et al[20] fou that modu he Notch signaling pathwa uld be a new t or the treatments of a s of d eases characterized by cell apopto dere measured the protein levels of No Hes1 Western blotting. LncRNA H19 r ably reduced the expression Notch 1 compared with Model group gure 4). an be seen that the lncRNA H19 r may affect the proliferation and apoptovial cells in RA rats by regulating the Notch signaling pathway.

Conclusions

In summary, the correlations of lncRNAs to the proliferation and apoptosis of synovial cells in RA rats were explored in this research. That is, H19 regulated the activation of synovial cells via the inhibition on the Notch signaling pathway. The results lay a solid foundation for scientific research on the application of cRNAs to the prevention, diagnosis, and ment of RA.

Conflict of Interest

The Authors declare that they h con interests.

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