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LncRNA MEG3 inhibits proliferation and promotes apoptosis of synovial cells in rats with knee osteoarthritis by regulating PTEN

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Abstract. – OBJECTIVE: To study the influences of long non-coding ribonucleic acid (lncRNA) maternally expressed gene 3 (MEG3) on the proliferation and apoptosis of synovial cells in rats with knee osteoarthritis by regulating phosphate and tensin homology deleted on chromosome ten (PTEN).

MATERIALS AND METHODS: In this experiment, rat synovial cell (RSC)-364 cells were cultured *in vitro*. Then, they were treated with PBS or lncRNA MEG3 overexpression lentiviruses and divided into normal control (NC) group and lncRNA MGE3 overexpression group (LncRNA MEG3 group). The messenger RNA (mRNA) expression levels of lncRNA MEG3 and PTEN in rat synovial cells were measured *via* qRT-PCR in each group, and Western blotting (WB) was performed to determine the protein levels of PTEN, cyclin D1, P21, B-cell lymphoma 2 (Bcl-2) and tubulin in rat synovial cells in both groups. The proliferation of rat synovial cells was detected *via* MTT assay, and the apoptosis was evaluated using FITC/PI double staining and flow cytometer.

RESULTS: Compared with NC group, LncRNA MEG3 group had notably overexpressed lncRNA MEG3 in RSC-364 cells ($p<0.01$), and an extremely substantially elevated mRNA level of PTEN ($p<0.05$). Besides, it was found through WB that the protein expression level of PTEN had a consistent trend with that of the mRNA level. The proliferation ability of cells was weakened ($p<0.05$), and the number of apoptotic cells was increased ($p<0.05$) in LncRNA MEG3 group compared with those in NC group. Finally, LncRNA MEG3 group had remarkably lower protein levels of cyclin D1 and Bcl-2, but a markedly higher protein level of P21 than NC group ($p<0.05$).

CONCLUSIONS: LncRNA MEG3 can raise the level of PTEN to weaken the proliferation ability but elevate the apoptosis level of RSC-364 cells.

Key Words:
LncRNA MEG3, PTEN, Cell proliferation, Cell apoptosis.

Introduction

Long non-coding ribonucleic acids (lncRNAs) are a class of non-coding long-stranded RNAs with more than 200 nt in length[1]. LncRNAs play a crucial role in multiple physiological and pathological processes, such as epigenetic regulation, and cell cycle, proliferation, differentiation and apoptosis, and they have become the hotspots in the research field of genetics in the 21st century[2,3]. Therefore, it is of great significance to explore the biological functions of lncRNAs for delving into the pathogenesis and pathogenic process.

LncRNA MEG3, a lncRNA measuring 1,600 nt in encoding length, is considered to possess the anti-tumor function[4]. LncRNA MEG3 can inhibit the proliferation but promote the apoptosis of gastric cancer cells, and its mechanism of action may be related to the regulation of the p53 signaling pathway[5]. However, the influences of lncRNAs on bone synovial cells have not yet been reported. Phosphate and tensin homology deleted on chromosome ten (PTEN) is the first discovered tumor suppressor gene with phosphatase activity[6,7], which is located on chromosome 10q23.3 and comprises 9 exons. PTEN protein can resist the activity of such phosphorylases as tyrosine kinases to repress the development and progression of tumors. PTEN, as the major negative regulatory factor of the phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (Akt) pathway, is able to decrease the level of phosphorylated Akt,

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thereby suppressing cell proliferation and promoting cell apoptosis[8].

The present study, therefore, aims to explore whether lncRNA MEG3 can affect the proliferation and apoptosis of rat synovial cells by regulating the PTEN protein level.

Materials and Methods

Cell Culture, Treatment and Grouping

Rat synovial cell (RSC)-364 cells purchased from China Cell Resource Seed Bank (Shanghai, China) were cultured using complete Dulbecco's Modified Eagle's Medium [DMEM, basic DMEM + 10% fetal bovine serum (FBS) + 0.1% P/S double antibodies + 0.1% glutamine] in an incubator with 5% CO_2 at 37°C (Gibco, Rockville, MD, USA). The cell culture was performed in accordance with the standard operation manual, and aseptic operation was required to avoid contamination. RSC-364 cells were cultured and subcultured into a 6-well plate, and when the density of cells reached 75%, phosphate-buffered saline (PBS) and lncRNA MEG3 overexpression lentiviruses (GenoMediTech Co., Ltd., Shanghai, China) were added into wells separately. The cells in normal control (NC) group and lncRNA MEG3 overexpression group (LncRNA MEG3 group) were harvested, and they were subjected to later proliferation and apoptosis assays.

Determination of Protein Expression Level Via Western Blotting (WB)

The cells in both NC group and LncRNA MEG3 group in the 6-well plate were added with RIPA cell lysis buffer (Beyotime Biotechnology, Shanghai, China), lysed on a shaking table at 4°C for 30 min, and centrifuged at 12,000 g and 4°C for 10 min. The supernatant was preserved. The concentration of total protein in rat synovial cells in each group was determined using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). A total of 10 μg of protein samples were taken from each group, added with loading buffer and subjected to metal bath at 100°C for 10 min, followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V and at 120 V when the protein samples passed through the boundaries of the spacer gel and separation gel. Subsequently, the gels were transferred onto a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and the membrane was washed using Tris-Buffered Saline with Tween-20 (TBST) for 10 min × 3 times, incubated with PTEN, cyclin D1, P21, B-cell lymphoma 2 (Bcl-2) and tubulin monocolonal primary antibodies diluted using 1% bovine serum albumin (BSA) at 1:1,000 (CST, Danvers, MA, USA) at 4°C overnight, washed with TBST for 10 min × 3 times, incubated again with horseradish peroxidase-conjugated secondary antibodies (CTS, Danvers, MA, USA) for 1 h, and washed again with TBST as above. Finally, enhanced chemiluminescence (ECL) developing solution (Thermo Fisher Scientific, Waltham, MA, USA) was added for color development, and the images of protein bands were acquired using a fluorescent developing instrument.

Measurement of Messenger RNA (mRNA) Expression Levels Via Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The cells in the 6-well plate in NC group and LncRNA MEG3 group were added with cell lysis buffer TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract mRNAs. Then, the concentration of mRNAs was determined. Subsequently, the mRNAs were reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to TaKaRa's specifications (Otsu, Shiga, Japan). First, 500 ng of RNAs were added into the mixture of 2 μL of 5 × PrimeScript RT Master Mix, 0.5 μL of OligodT, and 0.5 μL of reverse transcriptase, and the total volume of the overall reaction system was 10 μL. After that, PCR amplification was performed in accordance with the instructions of the PCR amplification kit as follows: 2 μL of cDNAs were with 10 μL of SYBR Premix Ex TaqII (2×), 0.8 μL of forward primers, 0.8 μL of reverse primers, and 0.4 μL of ROX reference dye II (50×).

Table I. List of primer sequences.

Gene	Forward	Reverse
LncRNA MEG3	5'-CTGCCCATCTACACCTCACG-3'	5'-CTCTCCGCCGTCTGCGCTAGGGGCT-3'
PTEN	5'-TTTGAAGACCATAACCCACCAC-3'	5'-ATTACACCAGTTCGTCCCTTTC-3'
GAPDH	5'-CAACGAATTTGGCTACAGCA-3'	5'-AGGGGTCTACATGGCAACTG-3'

Finally, ddH_2O was added to make the system constant at 20 μL, and the relative expression level of mRNAs was calculated based on the amplification cycle Ct value, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences are shown in Table I.

Detection of Cell Proliferation Via Methyl Thiazolyl Tetrazolium (MTT) Assay

The cells were first paved onto a 6-well plate at the density of 5×10^5 cells/well and divided into NC group and LncRNA MEG3 group. Then, they were digested using 0.25% trypsin, re-inoculated into a 96-well plate, and added with MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) at 12 h, 24 h, 48 h, and 72 h. After reaction for 3 h, the liquid in the wells was discarded, each well was added with 100 μL of dimethyl sulfoxide (DMSO) reagent (Sigma-Aldrich, St. Louis, MO, USA), and the mixture was shaken at room temperature for 10 min. Finally, the optical density (OD) of cells was read using a microplate reader at the wavelength of 490 nm. The larger OD indicates more cells, whereas the smaller OD suggests fewer cells. The proliferation ability of the cells in the two groups was determined using the above method.

Detection of Apoptosis Via Flow Cytometry

The cells in the 6-well plate in NC and LncRNA MEG3 groups were digested using trypsin without ethylenediaminetetraacetic acid (EDTA; Solarbio, Beijing, China), and 5 min later, the digestion was terminated using a complete medium. The resulting cells were rinsed using PBS twice, and the number of cells was re-counted. Then, $1\text{-}5\times10^5$ cells were added with binding buffer, and the cell deposits were re-suspended, mixed evenly with Annexin V Light 650 reagent, added with propidium iodide (PI) reagent and mixed evenly again, followed by reaction at room temperature in the dark for 15 min. Subsequently, the Annexin V Light 650 and PI fluorescence signals were detected through FL4 and FL2 channels, respectively, using a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA). Finally, the apoptosis of cells was determined according to the percentage of cells stained with fluorescein isothiocyanate (FITC) and PI.

Statistical Analysis

All data were subjected to analysis and statistics using GraphPad Prism 6.0 software (La Jolla, CA, USA), and expressed as $\bar{x}\pm s$. Two-tailed *t*-test was performed for data analysis. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ denoted statistically significant differences.

Results

LncRNA MEG3 Overexpression in RSC-364 Cells Detected

LncRNA MEG3 mimics were first transfected into RSC-364 cells using lentiviruses in this study, and it was found through qRT-PCR that the lncRNA MEG3 was considerably overexpressed in RSC-364 cells in LncRNA MEG3 group in comparison with that in NC group ($p<0.01$; Figure 1), suggesting a favorable expression effect of lncRNA MEG3 in lentiviruses and successful transfection and modeling.

Influence of LncRNA MEG3 on PTEN mRNA Level

After lncRNA MEG364 was successfully overexpressed in RSC-364 cells, the mRNA level of PTEN was further measured using qRT-PCR. According to the results, the mRNA level of PTEN in LncRNA MEG3 group was extremely substantially higher than that in NC group ($p<0.05$), implying that lncRNA MEG3 can notably raise the mRNA expression level of PTEN (Figure 2).

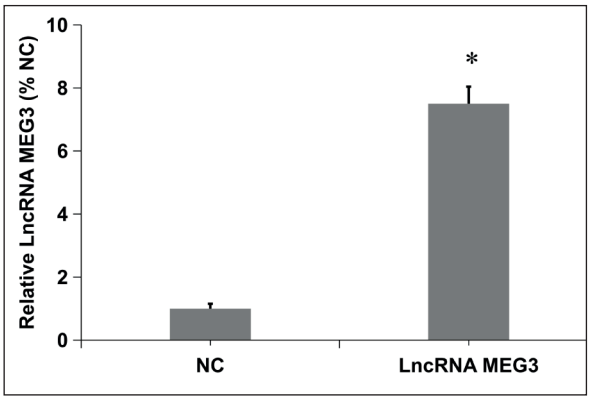

Figure 1. Expression of lncRNA MEG3 in RSC-364 cells detected *via* qRT-PCR. Expression of lncRNA MEG3 in cells in NC group and LncRNA MEG3 group detected *via* qRT-PCR. NC group: RSC-364 cells treated with PBS, LncRNA MEG3 group: RSC-364 cells treated with lncRNA MEG3 overexpression lentiviruses. *$p<0.01$: LncRNA MEG3 group *vs*. NC group.

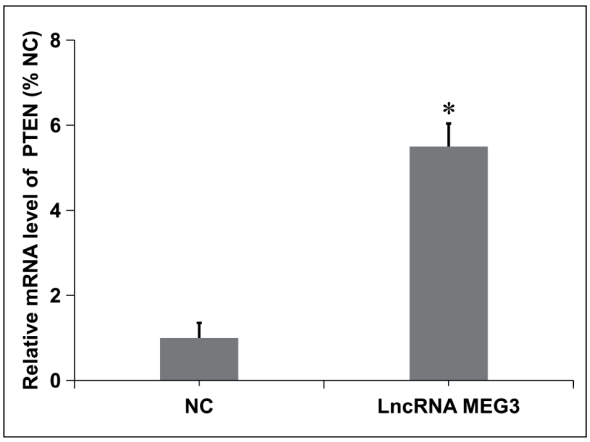

Figure 2. Influence of lncRNA MEG3 on PTEN mRNA level. MRNA expression of PTEN in cells in NC and LncRNA MEG3 groups detected *via* qRT-PCR *p<0.05: LncRNA MEG3 group *vs.* NC group.

Effect of LncRNA MEG3 on PTEN Protein Level

The effect of lncRNA MEG3 on the protein level of PTEN was detected *via* WB, and it was found that the protein expression level of PTEN exhibited a consistent trend with the mRNA level in rat synovial cells in LncRNA MEG3 group, suggesting that lncRNA MEG3 is able to increase the mRNA level of PTEN, thereby raising its protein level (Figure 3).

Influence of LncRNA MEG3 on Cell Proliferation

Subsequently, the number of RSC-364 cells was counted at 12 h, 24 h, 48 h, and 72 h *via* MTT assay, and it was discovered that the proliferation ability of cells in LncRNA MEG3 group declined compared with that in NC group (p<0.05; Figure 4), illustrating that lncRNA MEG3 can weaken the proliferation ability of cells.

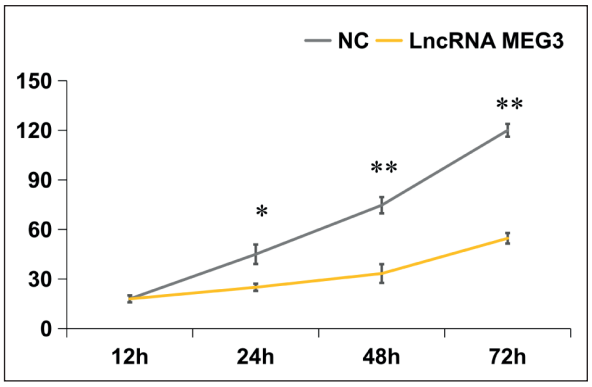

Figure 4. Influence of lncRNA MEG3 on cell proliferation. Number of RSC-364 cells at 12 h, 24 h, 48 h and 72 h in NC and LncRNA MEG3 groups detected *via* MTT assay. *p<0.05: LncRNA MEG3 group *vs.* NC group. **p<0.01: LncRNA MEG3 group *vs.* NC group.

Impact of LncRNA MEG3 on Cell Apoptosis

The impact of lncRNA MEG3 on the apoptosis of RSC-364 was detected *via* FITC/PI double staining and flow cytometer. The results revealed that LncRNA MEG3 group had remarkably more apoptotic cells than NC group (p<0.0; Figure 5), implying that lncRNA MEG3 can accelerate cell apoptosis.

Effects of LncRNA MEG3 on Proteins Related to Cell Proliferation and Apoptosis

Finally, it was found through WB that the protein levels of cyclin D1 and Bcl-2 notably declined, while the protein level of P21 rose substantially (p<0.05; Figure 6), suggesting that lncRNA MEG3 can considerably reduce cyclin D1 and increase P21 to inhibit cell proliferation, but lower Bcl-2 to promote cell apoptosis.

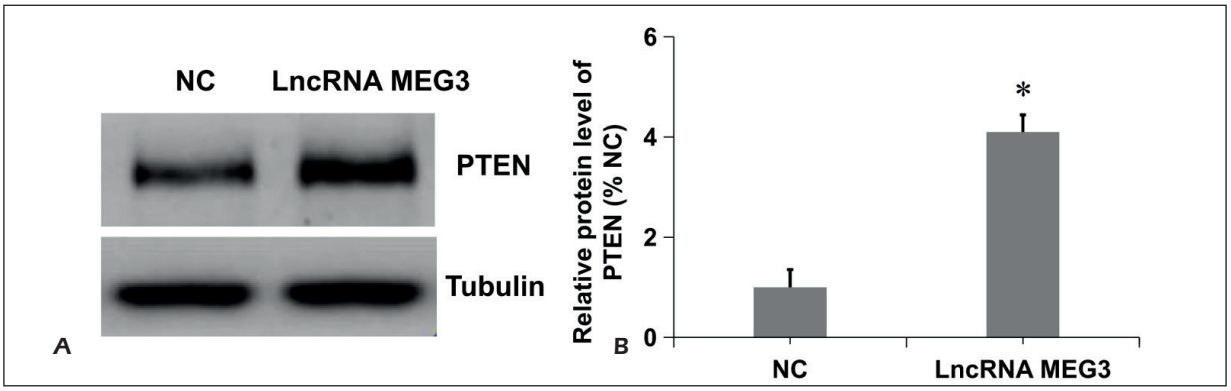

Figure 3. Effect of lncRNA MEG3 on PTEN protein level. **A**, Protein expression level of PTEN in NC group and LncRNA MEG3 group determined *via* WB. **B**, Quantification of the results in Figure A. *p<0.05: LncRNA MEG3 group *vs.* NC group.

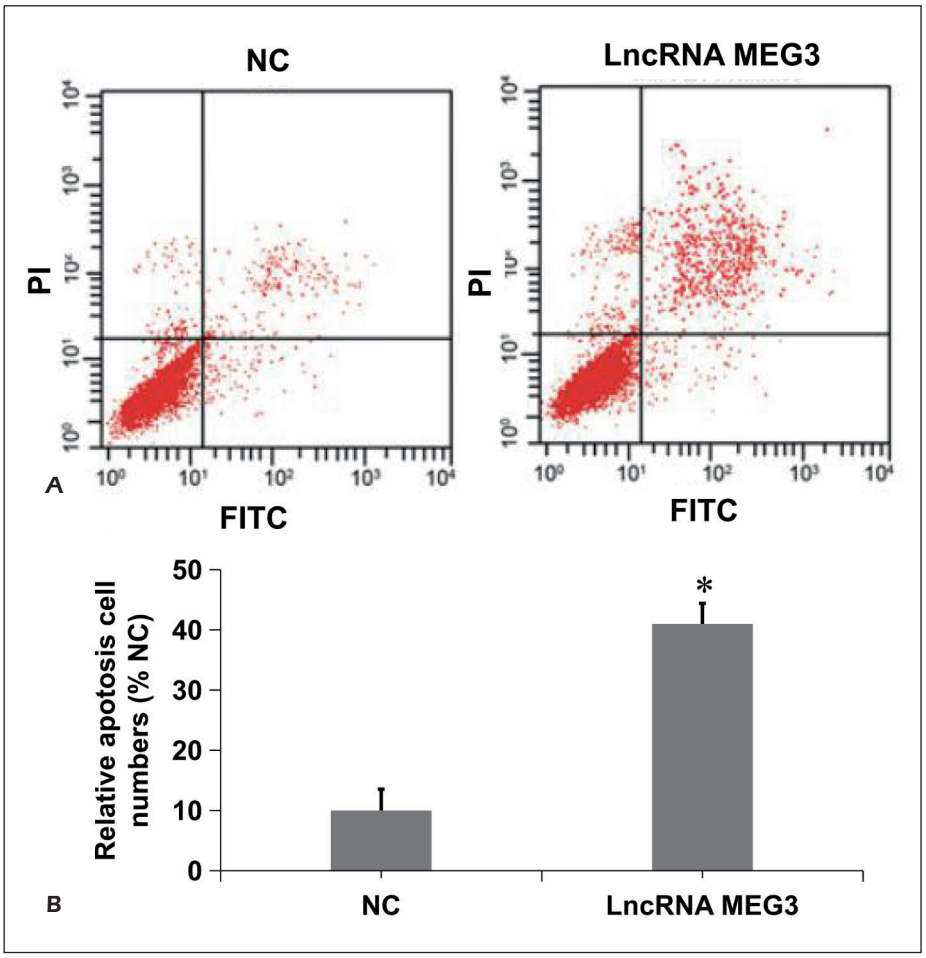

Figure 5. Impact of lncRNA MEG3 on cell apoptosis. **A**, Impact of lncRNA MEG3 on the apoptosis of RSC-364 cells detected *via* FITC/PI double staining and flow cytometer. **B**, Quantification of the results in Figure A. *$p<0.05$: LncRNA MEG3 group *vs.* NC group.

Discussion

RNAs can generally fall into mRNAs, tRNAs, rRNAs, microRNAs, lncRNAs, and antisense RNAs, among which lncRNAs are a kind of ncRNAs with the length beyond 200 nt[9] and without the ability to encode proteins. Studies have originally believed that lncRNAs do not pos-

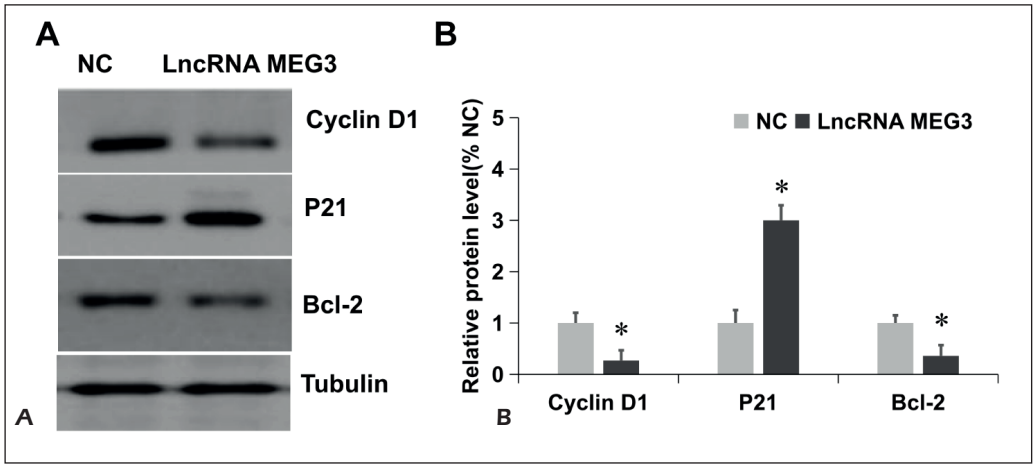

Figure 6. Effects of lncRNA MEG3 on proteins related to cell proliferation and apoptosis. **A**, Protein levels of cyclin D1, P21, and Bcl-2 in NC group and LncRNA MEG3 group determined *via* WB. **B**, Quantification of the results in Figure A. *$p<0.05$: LncRNA MEG3 group *vs.* NC group.

sess any biological function, but later it has been observed that they can regulate gene expression at epigenetics, transcriptional, and post-transcriptional levels. LncRNAs can induce chromatin remodeling and histone modification to regulate the epigenetic level[10]. A further study found that lncRNAs mainly adjust the selective slicing of genes or induce the formation of endogenous siRNAs, thereby functioning at the transcriptional level[11]. Therefore, lncRNAs bind to proteins to regulate the biological functions of genes at the post-transcriptional level[12]. However, the mechanism of action of lncRNAs is very complex, and it is still in the preliminary exploration and has not yet been fully clarified so far.

LncRNA MEG3 is a gene located on chromosome 14q32.3, with the encoding length of 1,600 nt. Previous studies have discovered that lncRNA MEG3 is lowly expressed in tumor tissues but highly expressed in normal tissues, which is considered as a lncRNA with the function of repressing tumors. LncRNA MEG3 is closely associated with meningioma, liver cancer, bladder cancer, and lung cancer[13-15]. LncRNA MEG3 can repress the proliferation and promote the apoptosis of tumor cells, but there are few research reports on its regulatory effects on the proliferation and apoptosis of bone synovial cells so far. Thus, this present study investigated the influences of lncRNA MEG3 on the proliferation and apoptosis of RSC-364 cells and its action of mechanism.

PTEN gene is the first tumor suppressor gene discovered to possess phosphatase activity[16,17]. As the major negative regulator of the PI3K/Akt pathway, PTEN can lower the phosphorylated Akt to inhibit cell proliferation and facilitate cell apoptosis. According to literature, the lack of PTEN is closely associated with many tumors[18-20]. Hence, whether lncRNA MEG3 affects the proliferation and apoptosis of rat synovial cells by regulating the protein level of PTEN was further explored in this study.

In the present research, RSC-364 cells were cultured *in vitro* and treated with PBS or lncRNA MEG3 overexpression lentiviruses in NC group and LncRNA MEG3 group. The qRT-PCR results showed that compared with that in NC group, lncRNA MEG3 was overexpressed in rat synovial cells in LncRNA MEG3 group, and that LncRNA MEG3 group had an extremely notably raised mRNA level of PTEN. The trend of PTEN protein expression level was found through WB to be consistent with that of the mRNA expression level. Besides, the MTT assay results revealed that compared with that in NC group, the proliferation ability of cells was weakened in LncRNA MEG3 group, and a substantial increase in the number of apoptotic cells was detected in LncRNA MEG3 group using flow cytometer compared with that in NC group. Finally, the results of WB revealed that LncRNA MEG3 group had substantially lower protein levels of cyclin D1 and Bcl-2, but a notably higher protein level of P21 than NC group. The main function of cyclin D1 is to promote cell proliferation. The P21 gene is an important member of the family of cyclin-dependent kinase inhibitors discovered in recent years and is involved in cell cycle regulation[21]. Although the present study found that lncRNA MEG3 can increase the mRNA and protein levels of PTEN, inhibit cell proliferation and facilitate cell apoptosis, whether the change in PTEN level induces the alteration of cell proliferation and apoptosis remains to be further researched. In subsequent research, PTEN needs to be knocked down using siRNAs to determine whether it influences the protein levels of cyclin D1, P21, and Bcl-2, thus affecting cell proliferation and apoptosis.

Conclusions

In summary, lncRNA MEG3 can inhibit the proliferation and promote the apoptosis of RSC-364 cells by increasing the PTEN level.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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