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LncRNA MEG3 inhibits proliferation and promotes apoptosis of osteosarcoma cells through regulating Notch signaling pathway

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Abstract. – OBJECTIVE: To explore the effect of long non-coding ribonucleic acid (lncRNA)-maternally expressed gene 3 (MEG3) on the Notch signaling pathway, and its influences on the proliferation and apoptosis of osteosarcoma MG-63 cells.

MATERIALS AND METHODS: LncRNA MEG3 was overexpressed in osteosarcoma MG-63 cells, and the cells were divided into Blank group, Len-con group, and Len-MEG3 group. The expression level of MEG3 in each group was detected *via* quantitative Polymerase Chain Reaction (qPCR), the cell proliferation level in each group was detected *via* Cell Counting Kit-8 (CCK-8) assay, and the apoptosis in each group was detected *via* Hoechst 33258 staining. Moreover, the content of the inflammatory factors in each group was determined using the Enzyme-Linked Immunosorbent Assay (ELISA), and the expression levels of apoptosis-related proteins and Notch signaling pathway-related proteins were determined through Western blotting.

RESULTS: The expression level of lncRNA MEG3 in Len-MEG3 group was significantly higher than that in the Blank group and Len-con group ($p<0.01$). The overexpression of lncRNA MEG3 could significantly weaken the proliferation ($p<0.01$) and enhance the apoptosis of osteosarcoma cells ($p<0.01$). The overexpression of lncRNA MEG3 could significantly increase the content of the inflammatory factor interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) ($p<0.01$), and remarkably decrease the content of the anti-inflammatory factor IL-10 in osteosarcoma cells ($p<0.01$). Besides, the overexpression of lncRNA MEG3 could evidently raise the expression of Caspase3 ($p<0.01$) and reduce the Bcl-2/Bax expression in osteosarcoma cells ($p<0.01$). Finally, the overexpression of lncRNA MEG3 could remarkably reduce the protein expressions of Jagged1, Notch1, and NICD1 in osteosarcoma cells ($p<0.01$).

CONCLUSIONS: The overexpression of lncRNA MEG3 can inhibit the proliferation and promote the apoptosis of osteosarcoma MG-63 cells by suppressing the Notch signaling pathway.

Key Words:
Osteosarcoma, LncRNA MEG3, Notch signaling pathway, Apoptosis.

Introduction

Osteosarcoma is a kind of highly malignant solid tumor derived from mesenchymal cells, also known as osteogenic sarcoma, which often occurs in the metaphysis of long bone[1,2]. Osteosarcoma accounts for more than half of all bone tissue-derived malignant tumors, characterized by a high degree of malignancy and proneness to metastasis, and it frequently occurs in children and adolescents at the age of 15-25 years old[3]. The pathogenesis of osteosarcoma remains unclear, and the viral factors, reflex factors, chemical factors, and genetic factors are all the precipitating factors[4]. Chemotherapy is one of the most common treatment means for osteosarcoma currently, and its cure rate can be up to more than 60%, but the curative effect of chemotherapy on patients with metastatic osteosarcoma is poor, and the drug resistance and further metastasis of tumor cells easily occur during treatment[5]. Therefore, it is of great significance to deeply understand the pathogenesis of osteosarcoma and to search for the influencing factors for the treatment and prognosis of osteosarcoma.

According to the epidemiological survey, osteosarcoma is associated with genetic fac-

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tors. The research evidence of cytogenetics suggests that chromosome recombination can occur in patients with osteosarcoma, and the gene mutation is an important influencing factor for the formation and prognosis of osteosarcoma[6]. The long non-coding ribonucleic acids (lncRNAs) can regulate various physiological and pathological processes, such as stress response, cell apoptosis, and differentiation[7]. Through genomic hybridization analysis, Liu et al[8] found that lncRNA maternally expressed gene 3 (MEG3) is lowly expressed or even not expressed in various tumor cells or tissues. Researchers argue that MEG3 has a certain inhibitory effect on various tumors. Huang et al[9] studied and found that the abnormally methylated CpG island can reduce the expression level of MEG3, thereby leading to massive proliferation and significantly reduced apoptosis of colon cancer cells, ultimately causing colon cancer. Currently, there are few reports on the effects of lncRNA MEG3 on the proliferation and apoptosis of osteosarcoma cells. The Notch signaling pathway is closely related to the proliferation and differentiation of various cells, which is involved in the regulation of the formation and maturation of osteoblasts. Dai et al[10] found that the occurrence of osteoarthritis and osteosarcoma may be closely related to the inhibition on the Notch signaling pathway in the cells. In the present work, the effect of lncRNA MEG3 on the Notch signaling pathway and its influences on the proliferation and apoptosis of osteosarcoma MG-63 cells were evaluated through *in vitro* cell researches.

Materials and Methods

Materials and Instruments

Human osteosarcoma MG-63 cell lines (Shanghai Cell Bank, Chinese Academy of Sciences, Shanghai, China), Cell Counting Kit-8 (CCK-8) assay kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA), Hoechst 33258 kit, interleukin-6 (IL-6) kit, tumor necrosis factor-α (TNF-α) kit and IL-10 kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), SYBR Premix Ex Taq kit and PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan), RIPA lysis buffer (Guge Servicebio, Wuhan, China), protease inhibitor (Guge Servicebio, Wuhan, China), antibodies (Abcam, Cambridge, MA, USA), microplate reader (Bio-Rad, Hercules, CA, USA), ultraviolet spectrophotometer Corning Incorporated (Corning, NY, USA), quantitative Polymerase Chain Reaction (qPCR) instrument (Illumina-Eco, Heidelberg, Germany), thermostatic water bath kettle (Shanghai Yiheng Technology Co., Ltd., Shanghai, China), 5% CO_2 cell incubator (Thermo Fisher Scientific, Waltham, MA, USA), electrophoresis apparatus (Corning, Corning, NY, USA), fluorescence microscope (Nikon, Tokyo, Japan), and pipettor (Eppendorf, Hamburg, Germany). Other unsourced reagents were described in the paper.

Construction of LncRNA MEG3-Overexpressing Cell Lines

The lncRNA MEG3-overexpressing cell lines were purchased from GenePharma (Shanghai, China), and they were constructed as follows: after the plasmids were successfully constructed and treated with 1a-25$(OH)_2$D for 48 h, they were transiently transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. With the blank plasmids as the control, the cells were divided into the Blank group (blank control group), Len-con group (transfected with blank plasmids), and Len-MEG3 group (transfected with lncRNA MEG3-overexpressing plasmids). Before transfection, the cells were paved into a 12-well plate at the density adjusted to 1×10^5/mL and cultured for 24 h until they covered 80% of the well. Then, the original medium was replaced with the serum-free medium, and the lncRNA MEG3-overexpressing plasmids and blank plasmids were mixed evenly with Lipofectamine 3000, respectively, and incubated at room temperature for 5 min. 100 μL of the mixed solution was added into each well for incubation under 5% CO_2 and 30°C for 36 h, and the cells were collected to detect the expression level of MEG3 *via* qPCR.

Detection of Expression Level of LncRNA MEG3 in Cells Via qPCR

The cells successfully transfected were collected and cultured until they covered 80% of the culture flask. Then, the cells in the three groups were collected and added with TRIzol (Invitrogen, Carlsbad, CA, USA) to extract the total RNA. The concentration and purity of RNA in each group were measured using the agarose gel and nucleic acid-protein quantometer (Nan-

oDrop2000, Thermo Fisher Scientific, Waltham, MA, USA), and the total RNA was reversely transcribed into complementary deoxyribonucleic acids (cDNAs) (37°C for 15 min and 85°C for 5 s). The qPCR primers were designed using Primer6.0 according to the target gene sequences in the GenBank, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference, and synthesized by Jiangsu Synbio Technologies Co., Ltd. (Shanghai, China) (Table I). Then, the qPCR system was prepared: 10 μL of SYBR® Premix Ex Taq™ II (2×), 1.0 μL of primer mix and 1 μL of cDNA, as well as diethyl pyrocarbonate (DEPC)-treated water till the total volume of 20 μL. The reaction conditions were adjusted as follows: 95°C for 30 s, 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, for a total of 35 cycles. The relative expression levels of the genes in each group were calculated using $2^{-\Delta\Delta Ct}$, and the expression level of lncRNA MEG3 was expressed as MEG3/GAPDH.

Detection of Effect of LncRNA MEG3 on Cell Proliferation Using CCK-8 Assay

After successful transfection, the cells were collected and cultured, and the cell density was adjusted to 1×10^5/mL. The cells were cultured in a 96-well plate with complete medium under 5% CO_2 and 37°C for 48 h, and the cell proliferation in each group was detected using the CCK-8 kit. The CCK-8 reagent was mixed evenly with the medium at a ratio of 1:10, and then 100 μL of the mixture was added into the plate, followed by culture in an incubator with 5% CO_2 at 37°C for 1 h. Then, the optical density (OD) value was measured at a wavelength of 450 nm using a microplate reader, based on which the cell proliferation level in each group was calculated.

Detection of Effect of LncRNA MEG3 on Apoptosis Using Hoechst 33258 Staining

The cells successfully transfected were collected and cultured, and the cell density was adjusted to 1×10^5/mL. The cells were inoculated into the 6-well plate, added with the complete medium and cultured in the incubator with 5% CO_2 at 37°C for 48 h. The supernatant was discarded, and the cells were fixed with freshly prepared 4% paraformaldehyde for 30 min, washed with PBS for 3 times, and added with 50 μL of Hoechst 33258 dye, followed by incubation at room temperature in a dark place for 10 min. The cell conditions in each group were recorded, photographed, and observed under a fluorescence microscope. The normal cells showed the dark blue color, while the bright blue color in the nucleus indicated apoptosis, namely apoptotic cells. The apoptosis rate in each group was calculated.

Detection of Content of Inflammatory Factors in Cells Using ELISA kit

The cells successfully transfected were collected and cultured, with the cell density adjusted to 1×10^5/mL. The cells were inoculated into the 6-well plate and added with the complete medium, followed by incubation in the incubator under 5% CO_2 and 37°C for 48 h. The supernatant was discarded, and the cells in each group were collected for later use. The standard curves of IL-6, TNF-a, and IL-10 were plotted. 100 μL of standard solution or cell solution in each group was added into the antibody-coated 96-well plate, sealed and incubated at 37°C for 1 h. After the liquid was patted dry, the cells were added with 100 μL of biotin-labeled antibodies of IL-6, TNF-a, or IL-10, sealed and incubated at 37°C for 1 h. Then, the plate was washed with the diluted washing liquid (250 μL/well) for 3 times. The cells were added with 100 μL of ABC working solution, sealed, and incubated at 37°C for 30 min. The plate was washed again with 250 μL of washing liquid for 3 times. The cells were added with 90 μL of TMB developing solution, sealed, and incubated at 37°C for 30 min. Finally, the 3,3',5,5'-Tetramethylbenzidine (TMB) stop buffer was added and mixed evenly, and the absorbance in each group was measured at a wavelength of 450 nm using the microplate reader. After the standard curves were plotted, the content of IL-6, TNF-a, and IL-10 in each group was calculated.

Table I. PCR primers.

	Sequence
LncRNA MEG3	Forward primer: 5'-ATCATCCGTCCACCTCCTTGTCTTC-3' Reverse primer: 5'-GTATGAGCATAGCAAAGGTCAGGGC-3'
GAPDH	Forward primer: 5'-AATGCCTCCTGCACCACCAAC-3' Reverse primer: 5'-AAGGCCATGCCAGTGAGCTTC-3'

Detection of Effects of LncRNA MEG3 on Apoptosis-Related Proteins and Notch Signaling Pathway Through Western Blotting

The cells successfully transfected were collected and cultured. After the cell density was adjusted to 1×10^5/mL, the cells were inoculated into the 6-well plate and added with the complete medium, followed by incubation in the incubator under 5% CO_2 at 37°C for 48 h. The supernatant was discarded, and the cells in each group were collected for later use. After 1 mL of RIPA lysis buffer was added, the total protein was extracted, and the protein concentration in each group was determined using the BCA protein quantification kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). After the loading buffer at an equal concentration was prepared, the protein was boiled in the thermostat water bath kettle for 15 min, loaded with dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to electrophoresis under 80 V till the blue band reached the end of the gel. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane using the wet method under 90V for 100 min. Then, the protein band was cut according to the molecular weight of the protein, and the PVDF membrane was sealed with freshly-prepared 5% skim milk powder for 1 h, followed by incubation with the Rabbit-Caspase3, Rabbit-Bcl-2, Rabbit-Bax, Rabbit-Jagged1, Rabbit-Notch1, Rabbit-NICD1, and Rabbit-GAPDH antibodies at 4°C overnight. After the membrane was washed with freshly prepared TBST for 3 times (5 min/time), the protein was incubated again with the horseradish peroxidase-labeled secondary antibodies at room temperature for 1 h, and the membrane was washed again with TBST for 3 times. The developing solution was prepared for color development in a dark room. The proteins in each group were quantified, and the relative expression levels of the corresponding proteins were calculated based on Caspase3/GAPDH, Bcl-2/Bax, Jagged1/GAPDH, Notch1/GAPDH, and NICD1/GAPDH, respectively.

Statistical Analysis

The research results were expressed as mean ± standard deviation. SPSS 22.0 (SPSS Corp., Armonk, NY, USA) software was used for the data analysis. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). The homogeneity test of variance was performed. The Bonferroni's method was adopted in the case of homogeneity of variance, while Welch's method was adopted in the case of heterogeneity of variance. $p<0.05$ suggested that the difference was statistically significant.

Results

Construction of LncRNA MEG3-Overexpressing Cell Lines

The lncRNA MEG3-overexpressing cell lines were constructed, and the expression level of lncRNA MEG3 in each group was detected *via* qPCR. As shown in Figure 1, the expression level of lncRNA MEG3 in Len-MEG3 group was significantly higher than that in the Blank group and Len-con group ($p<0.01$).

Overexpression of LncRNA MEG3 Could Significantly Reduce the Proliferation of Osteosarcoma Cells

The effect of the overexpression of lncRNA MEG3 on the proliferation of osteosarcoma cells was detected using the CCK-8 assay. As shown in Figure 2, the cell proliferation level in Len-MEG3 group was significantly lower than that

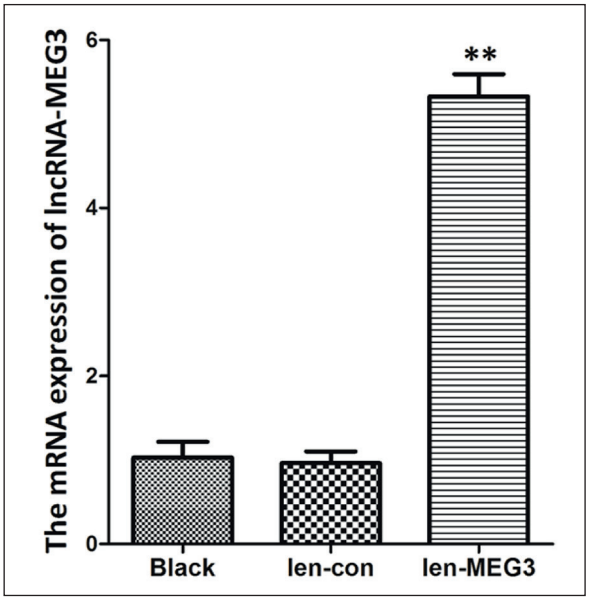

Figure 1. Expression level of lncRNA MEG3 in each group detected *via* qPCR. The expression level of lncRNA MEG3 in Len-MEG3 group is significantly higher than that in the Blank group. $^{**}p<0.01$ *vs*. Blank group.

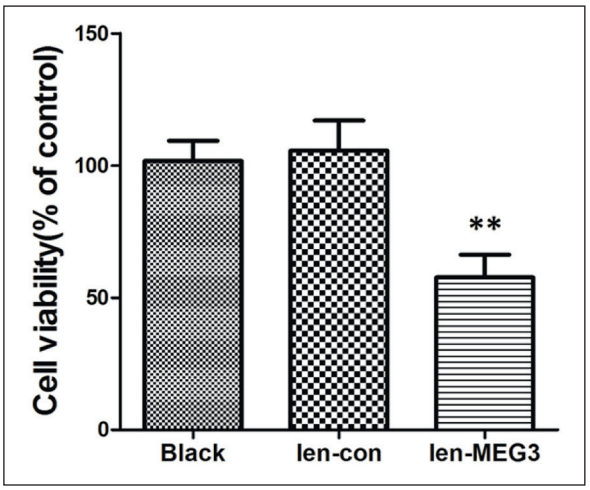

Figure 2. Cell proliferation level in each group detected using CCK-8 assay. Compared with that in the Blank group, the cell proliferation level in Len-MEG3 group is significantly decreased. **p<0.01 *vs*. Blank group.

in the Blank group and Len-con group (p<0.01), while it was similar between the Len-con group and Blank group (p>0.05).

Overexpression of LncRNA MEG3 Could remarkably Enhance the Apoptosis of Osteosarcoma Cells

The effect of the overexpression of lncRNA MEG3 on the apoptosis of osteosarcoma cells was detected using the Hoechst 33258 staining. As shown in Figure 3, the cell apoptosis level in Len-MEG3 group was remarkably higher than that in the Blank group (p<0.01), while it was similar between the Len-con group and Blank group (p>0.05).

Overexpression of LncRNA MEG3 Could Evidently Increase the Content of Inflammatory Factors in Osteosarcoma Cells

The content of the inflammatory factors in each group was determined *via* ELISA. The results showed that the overexpression of lncRNA MEG3 could evidently increase the content of IL-6 (p<0.01) and TNF-a in osteosarcoma cells (p<0.01), and evidently decrease the content of IL-10 (p<0.01). The levels of IL-6, TNF-a, and IL-10 were similar between the Len-con group and Blank group (p>0.05) (Figure 4).

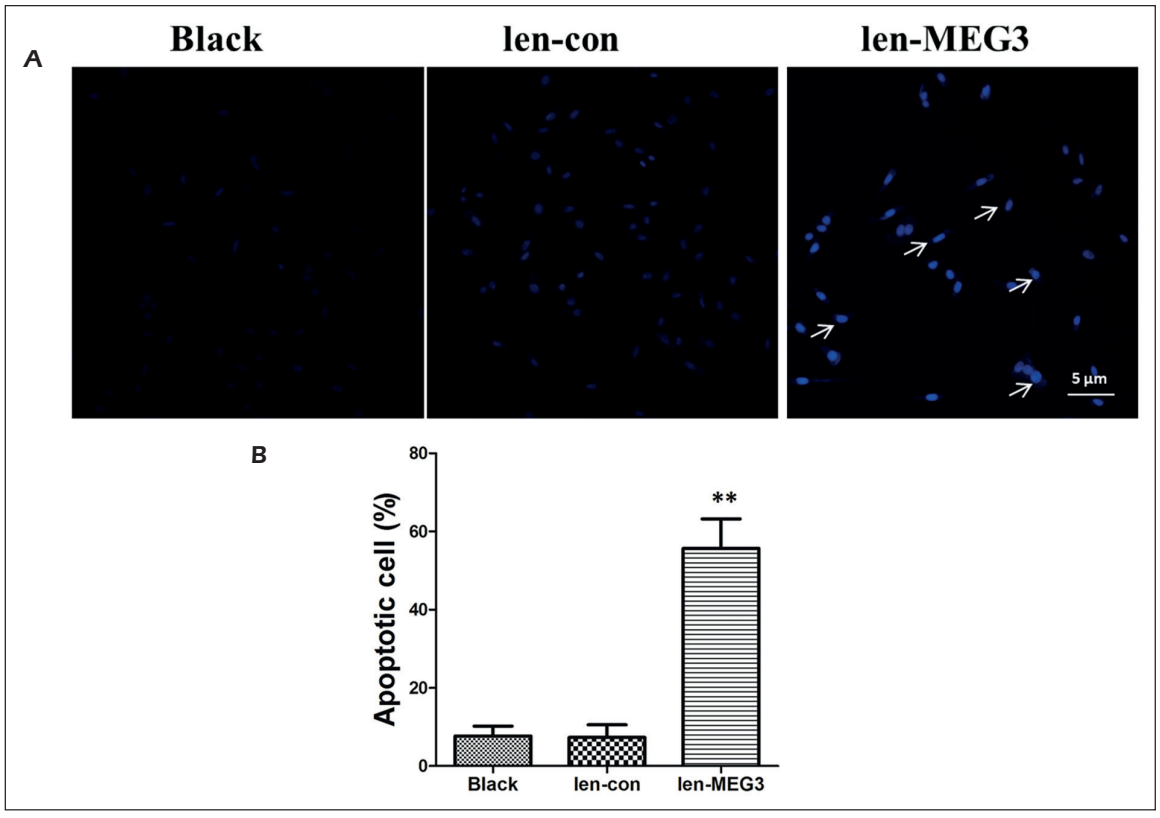

Figure 3. Apoptosis level detected through Hoechst 33258 staining. **A,** Micrograph (magnification: 40×). **B,** Statistical graph. The apoptosis level in Len-MEG3 group is remarkably higher than that in the Blank group (scale bar = 50 μm). **p<0.01 *vs*. Blank group.

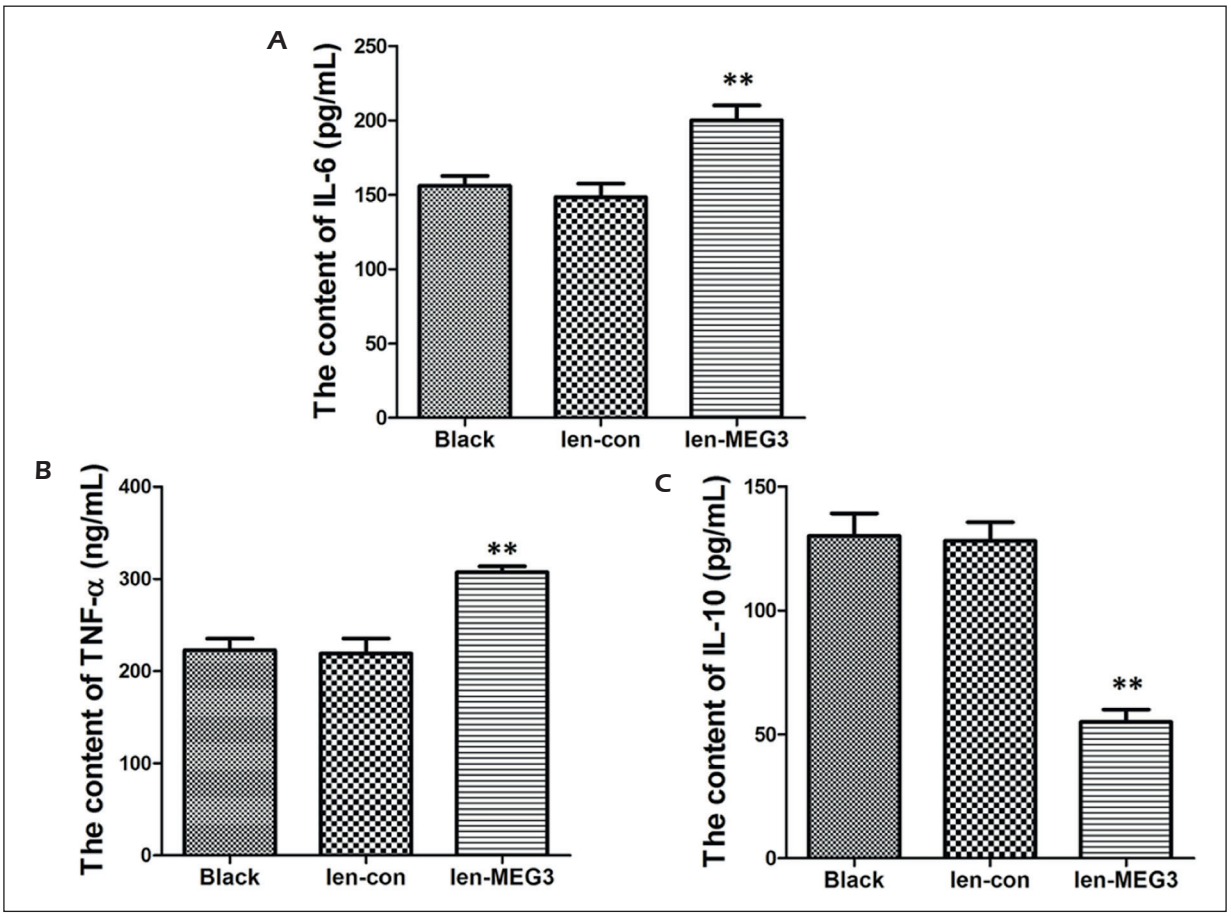

Figure 4. Content of TNF-a, IL-6, and IL-10 in cells detected using ELISA. **A,** Content of IL-6, **B,** content of TNF-a, and **C,** content of IL-10. Compared with the Blank group, Len-MEG3 group has evidently higher content of IL-6 and TNF-a, and evidently lower content of IL-10. ***p*<0.01 *vs.* Blank group.

Overexpression of LncRNA MEG3 Could Remarkably Increase the Expressions of Apoptosis-Related Proteins in Osteosarcoma Cells

The effect of lncRNA MEG3 on the expressions of the apoptosis-related proteins was determined using Western blotting. The results revealed that the overexpression of lncRNA MEG3 could remarkably raise the expression of Caspase3 ($p<0.01$) and reduce the Bcl-2/Bax expression in osteosarcoma cells ($p<0.01$). There were no differences in the expression levels of Caspase3 and Bcl-2/Bax between the Len-con group and Blank group ($p>0.05$) (Figure 5).

Effect of Overexpression of LncRNA MEG3 on Notch Signaling Pathway in Osteosarcoma Cells

The effect of the overexpression of lncRNA MEG3 on the Notch signaling pathway in osteosarcoma cells was determined through Western blotting. As shown in Figure 6, the overexpression of lncRNA MEG3 could remarkably reduce the protein expressions of Jagged1, Notch1, and NICD1 in osteosarcoma cells ($p<0.01$).

Discussion

LncRNAs are found in many species and involved in such biological processes as gene transcription, translation and silencing, and RNA splicing and modification *in vivo*, with tissue-specific expression[11]. Ma et al[12] studied and found that the expression of lncRNA MEG3 is low in a variety of tumor cells. It was found through the detection of the expression level of MEG3 in 23 cases of colon cancer and para-carcinoma tissues that the expression level of MEG3 is lower in cancer tissues, and it is even

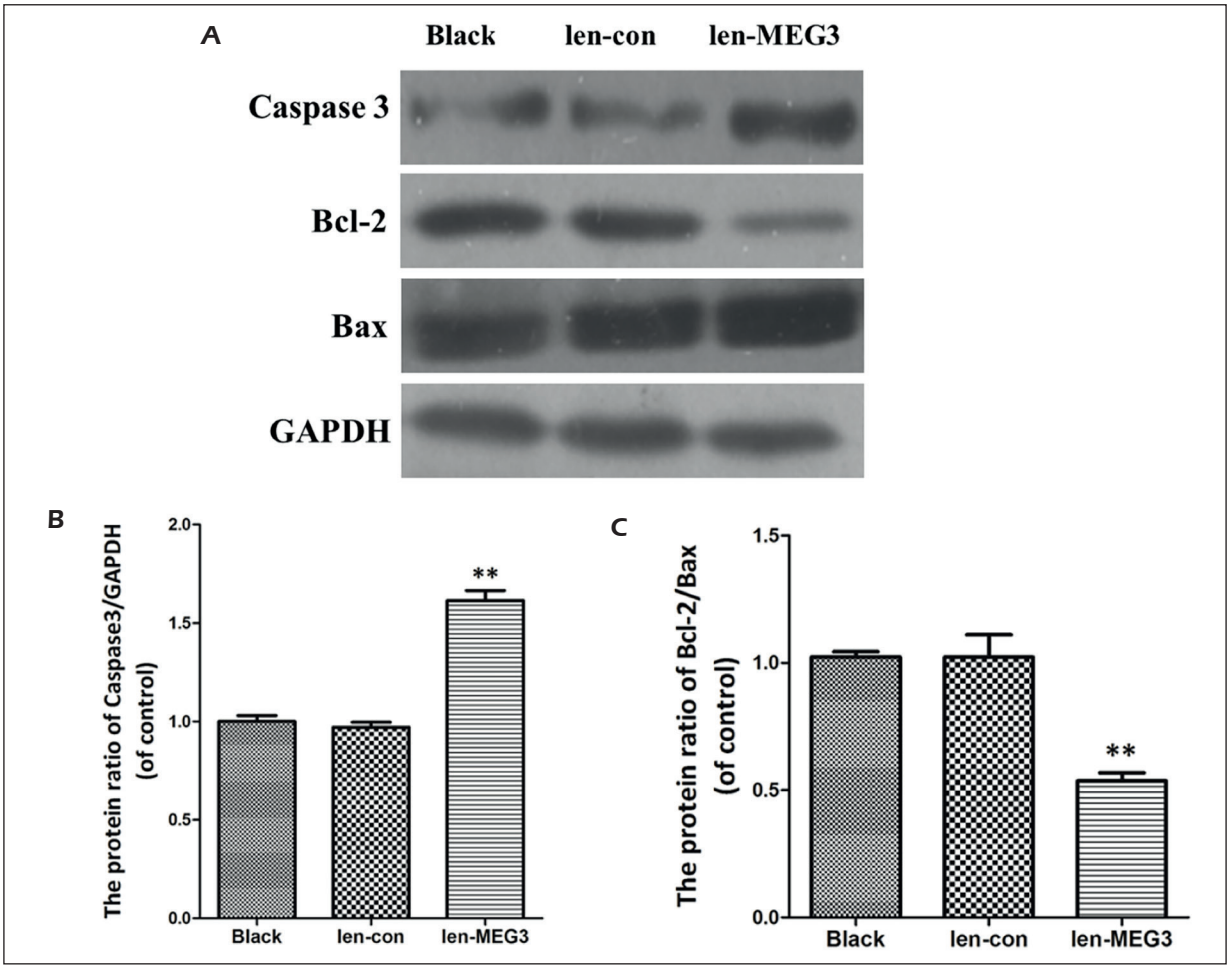

Figure 5. Expression levels of apoptosis-related proteins detected using Western blotting. **A,** Protein bands, **B,** Caspase3 protein, and **C,** Bcl-2/Bax. Compared with the Blank group, the Len-MEG3 group has remarkably increased the content of Caspase3 and remarkably decreased Bcl-2/Bax. $^{**}p<0.01$ *vs.* Blank group.

not detected in several samples. As a tumor suppressor gene, MEG3 has been identified in a variety of tumors. Through the bioinformatics analysis of MEG3, Liu et al[13] found that MEG3 reduces the p53 transcription by affecting the binding between the transcription factor p53 and DNA, thereby inhibiting cell proliferation and promoting apoptosis. In the present work, lncRNA MEG3 was overexpressed in osteosarcoma cells, and it was found that the apoptosis of osteosarcoma cells was significantly enhanced, the cell proliferation was significantly inhibited, and the expression of apoptosis-related protein Caspase3 was also significantly increased.

The Notch signaling pathway is mainly composed of Notch ligand, Notch receptor, and intracellular effector binding protein. Notch is a monomeric transmembrane protein on the cell surface, which participates in vesicular transport, and mediates the protein interaction and chemical modification. The activation of the Notch signaling pathway mainly depends on the binding between Jagged1 ligand and Notch1 receptor, playing a crucial role in cell development and differentiation[14,15]. Jin et al[16] studied and found that the Notch signaling pathway is involved in the regulation of physiological activities of osteoblasts, such as proliferation, invasion, and apoptosis. The regulation of the Notch signaling pathway has no second messenger involved, and it mainly depends on the intracellular NICD concentration. The low-dose NICD can activate the Notch signaling pathway, and mediate the vesicular transport, cell proliferation, and other physiological activities[17]. Moreover, Liu et al[18] found that leukemia in mice can be effectively treated

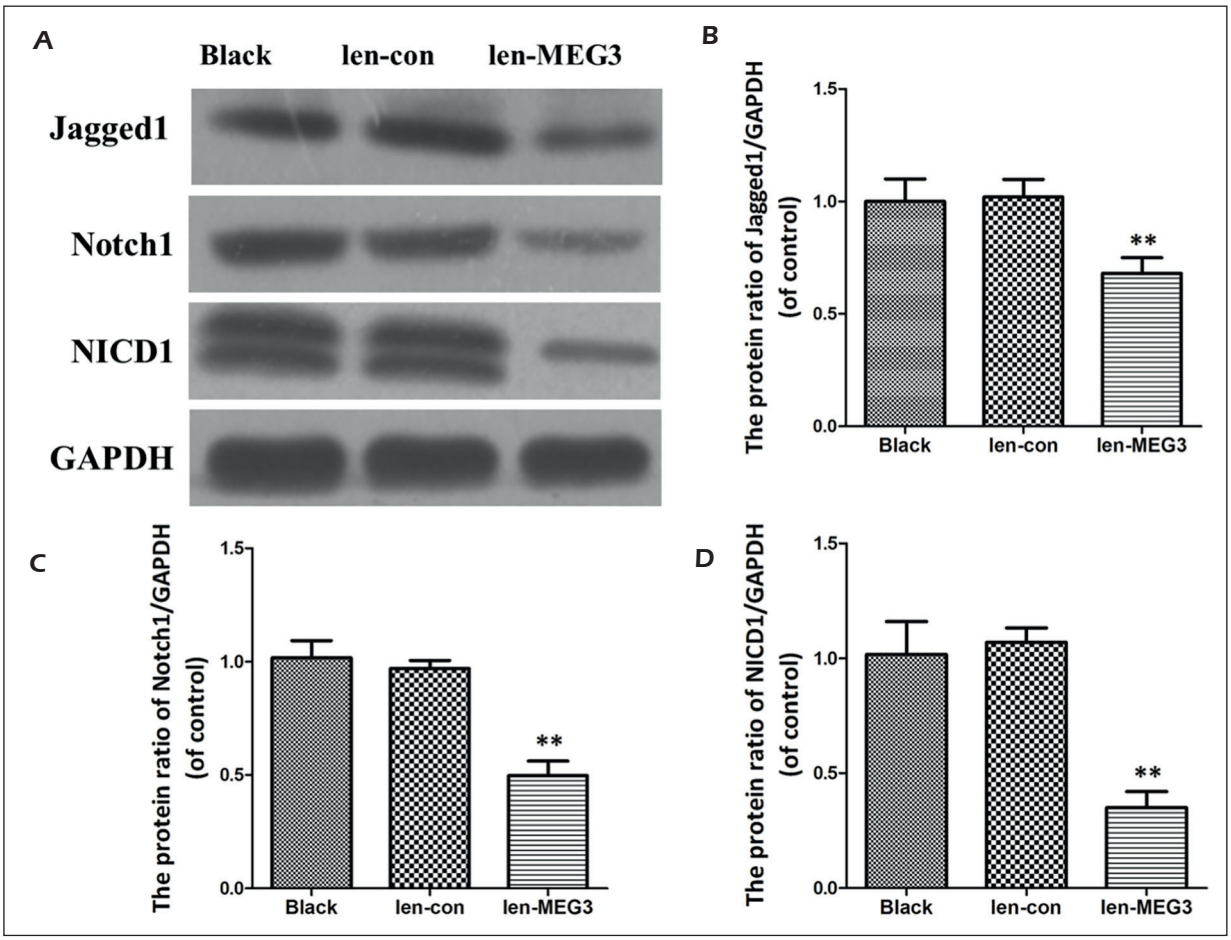

Figure 6. Expression levels of Notch signaling pathway-related proteins detected using Western blotting. **A,** Protein bands, **B,** Jagged1 protein, **C,** Notch1 protein, and **D,** NICD1 protein. The overexpression of lncRNA MEG3 can remarkably reduce the protein expressions of Jagged1, Notch1, and NICD1 in osteosarcoma cells. $^{**}p<0.01$ *vs.* Blank group.

by reducing the expression of Hes1 gene in stem cells to inhibit the Notch signaling pathway. Rho et al[19] showed that both metastasis and invasion of osteosarcoma cells can be significantly weakened by inhibiting the activity of intracellular deacetylase from reducing the expression of Notch1. The above results highly suggest that the Notch signaling pathway is an effective therapeutic target for tumors.

In this investigations, lncRNA MEG3-overexpressing osteosarcoma cells were constructed *in vitro*. It was found that the increased expression of lncRNA MEG3 in osteosarcoma cells could effectively inhibit the expression of NICD1 in cells. The expression levels of Jagged1 and Notch1 were also significantly reduced, indicating that the overexpression of MEG3 can effectively inhibit the activation of the Notch signaling pathway, thereby inhibiting proliferation and promoting apoptosis of osteosarcoma cells. Wang et al[20] found that by upregulating the expression of Notch1 in the Notch signaling pathway and activating the Notch signaling pathway can remarkably promote the growth and proliferation of osteosarcoma cells. Besides, the activation of the Notch signaling pathway can also act on target proteins, thereby regulating the tumor microenvironment, lowering the expressions of inflammatory factors in tumor cells, and facilitating the proliferation of tumor cells. In this study, it was found that in lncRNA MEG3-overexpressing osteosarcoma cells, the Notch signaling pathway was suppressed, and the content of the inflammatory factors IL-6 and TNF-a was evidently increased, while that of the anti-inflammatory factor IL-10 was evidently decreased. Xu et al[21] manifested that the Notch signaling pathway is also closely related to the sensitivity of tumor

cells to chemotherapeutic drugs, which may be an important theoretical basis for the ability of Notch signaling pathway to regulate proliferation and apoptosis of various tumor cells.

Conclusions

In summary, this study revealed through *in vitro* experiments that the overexpression of lncRNA MEG3 can effectively inhibit proliferation, promote apoptosis of osteosarcoma cells, and increase the expressions of intracellular inflammatory factors and apoptosis-related proteins, which may be related to the inhibition on the activation of the Notch signaling pathway in cells. The results of this report can provide a theoretical basis for the targeted therapy for osteosarcoma and bring new hope for the treatment of osteosarcoma patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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