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Mechanism of NLRX1 mediating JNK apoptosis pathway to participate in functional impairment of cochlear hair cells in mice with senile deafness

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ABSTRACT

To explore the mechanism of cochlear hair cell damage and study the prevention and treatment of sensorineural hearing loss, the effect of NLRX1 gene expression on the functional damage of cochlear hair cells in presbycusis was comprehensively analyzed. In the in vivo detection, C57BL/6 mice of different ages were used as experimental subjects. Cochlear tissues were taken after the hearing test of mice, and the number of cells and protein changes in NLRX1 immunofluorescence staining were detected. In the in vitro detection, the cochlear hair cell HEI-OE1 was used as the experimental object, and the cell proliferation activity was detected after overexpression or silencing of NLRX1.In the in vivo and in vitro experiments, the expression of JNK pathway-related proteins was simultaneously detected. The results of in vivo experiments showed that the hearing threshold of 270d -old mice was substantially greater than that of 15d-, 30d-, and 90d-old mice (P <0.05). I addition, with increasing age, the expression of p-JNK, Bcl-2, Bax, and Caspase-3 in the mouse cochlea gradually increased (P<0.05). *In vitro* experimental results showed that cell proliferation activity decreased after overexpression of NLRX1, and the expression of p-JNK, Bcl-2, Bax, and Caspase-3 was substantially decreased (P<0.05). Silencing NLRX1 can inhibit the above phenomenon, indicating that NLRX1 can inhibit the proliferation of hair cells in old mice through the activation of the JNK apoptosis pathway, thereby promoting the occurrence of sensorineural hearing loss.

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Introduction

Sensorineural hearing loss is very common and is mainly characterized by progressive development and a long course of disease (1,2). Sensorineural hearing loss is caused by impairment of cochlear hearing cells, auditory nerves, or neurons at all levels in the auditory conduction pathway, resulting in impairment of sound perception or nerve conduction, and eventually deafness (3). Studies suggested that excessive accumulation of reactive oxygen species (ROS) in cochlear cells and cell injury apoptosis are closely related to deafness (4). The reduction of ROS generation can effectively protect against cochlear hair cell damage (5). ROS can activate the release of mitogen-activated protein kinase and its stress-activated protein kinase c-Jun N-terminal kinase (JNK), thus functioning in various cell activities (6,7). Studies confirmed that ROS/JNK signaling is involved in mediating auditory cell apoptosis, so it can be used as a therapeutic target for deafness (8).

The protein family containing nucleotide binding domains and leucine-rich repeats (NLRs) are a class of evolutionarily conserved proteins with multiple biological functions (9). NLRX1, one of the NLRs, is widely detected in vertebrate muscles and heart and other tissues, and it belongs to mitochondrial function regulatory proteins (10). NLRX1 can promote the generation of ROS by acting on the complex of the mitochondrial respiratory chain, thereby activating signal pathways such as JNK (11). In addition, studies confirmed that NLRX1 participates in early multi-tumor diseases, mainly by regulating signal pathways related to tumor progression (12,13). NLRX1 can activate Caspase-8 and induce apoptosis (14). In summary, NLRX1 can participate in physiological processes such as ROS response, impaired mitochondrial function, and apoptosis. However, it is rarely reported whether it is involved in the regulation of deafness caused by impaired cochlear hair cell function.

This study hypothesized that NLRX1 regulated apoptosis-related JNK pathways, thereby affecting cochlear hair cell survival. To verify it, C57BL/6 mice were used as research subjects to explore the expression of NLRX1 in the cochlea of mice of different ages, and the mechanism of NLRX1 expression on JNK pathway activation was analyzed with cochlear hair cells as subjects. It was hoped to provide the experimental basis for understanding the mechanism of hair cell damage in senile deafness.

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Materials and Methods

Experimental subjects

Newborn 15d-, 30d-, 90d-, and 270d-old wild-type C57BL/6 mice from Shanghai Jiesijie Laboratory Animal Co., LTD., were used. All mice were fed for one week with a free diet, the room temperature was 22-25 °C, the relative humidity was 55-60%, and the light was altered day and night for 12h/12h. Animal tests were carried out in accordance with the standards of practice established by the Animal Ethics Committee.

Cochlear hair cells (HEI-OC1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose containing 10% fetal bovine serum at 37°C and 5% CO_2. The cell culture medium was replaced regularly, and the cell growth state was observed. Cell passage was carried out when the convergence degree reached about 80%.

Auditory brainstem response (ABR) test

Before the test, the mice were weighed and anesthetized with 1% pentobarbital sodium (45 mg/kg) intraperitoneally injected. The mice were placed in a closed audiometry chamber with good sound insulation, and the electrodes of the audiometry instrument were placed under the skin of the cranial top, right mastoid process, and thigh of the mice. The mice were given auditory stimulation (initial intensity was 90 dB, then decreased step by step with 5 dB) to detect the lowest stimulus intensity, which was set as the auditory threshold.

Immunofluorescence staining

Anesthetized with 1% pentobarbital sodium (45 mg/kg) intraperitoneally injected, the mice were killed by neck dislocation, the cochlea tissue was harvested, and the oval window membrane, oval window membrane, and excess soft tissue were removed. Cochlear tissue was immersed in a 4% paraformaldehyde (PFA) solution and treated overnight at 4°C for tissue fixation. After being washed with phosphoric acid buffer, the cochlear tissues were immersed in a 10% ethylenediamine tetraacetic acid (EDTA) solution and treated with decalcification for three days until the tissues were transparent. The hair cells were then exposed completely under an anatomical microscope, and the basement membrane was taken as specimens. After that, phosphoric acid buffer with 0.5% Triton X-100 was applied to penetrate the tissues for 15 min, and the phosphoric acid buffer was washed. Then, poly (butylene terephthalate) (PBT) -1 solution was added, and tissue was sealed at 25°C for 1h, then added with 1:1,000 diluted NLRX1 primary antibody, then incubated overnight at 4°C. After the primary antibody was discarded, the tissues were rinsed with poly (butylene succinate-co-terephthalate) (PBST) solution, and fluorescently labeled secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) dye were added, which were incubated together at room temperature for 30min in the dark. Then, the tissues were rinsed with PBST, and Dako anti-quenching agent was added. The luminescence was examined by a fluorescence microscope in the dark.

Transfection of cells overexpressing/silencing NLRX1

After counting the HEI-OC1 cells, they were inoculated into 6-well plates at 5×10^5 cells/well. After adherence, cells were washed with a serum-free cell medium. The Opti-MEM-diluted Lipofectamine 2000 reagent was added for transfection of NLRX1 siRNA and overexpression of NLRX1 plasmid. The sequence of NLRX1 siRNA is 5'-UCAAGAAGGAGAUAUGCUCAU-3'. The NLRX1 expression sequence was amplified and connected to the expression vector pcDNA3.1 by T4 ligase. The NLRX1 expression sequence was transformed into DH5α, and the plasmid was extracted after propagation. Subsequent cell tests were performed 48h after transfection.

RT fluorescence qPCR

Total RNA was extracted from mouse cochlea basal membrane tissues and HEI-OC1 cells according to the traditional Trizol reagent extraction method. Ultraviolet spectrophotometry and agarose gel electrophoresis were adopted for the determination of RNA concentration and purity. The RNA concentration was normalized to 300 ng/μL, and the cDNA was reversely transcribed, referring to the cDNA reverse transcription kit (Taraka, Japan). Then, mRNA levels of target genes NLRX1 and β-actin were detected with cDNA as the template in accordance with the RT fluorescent qPCR kit instructions (Taraka, Japan). Primers for quantitative detection of target genes are shown in Table 1. With β-actin gene was set as the internal reference, and the relative level of NLRX1 was detected via $2^{-\triangle\triangle ct}$.

Cell proliferation viability detected by methyl tetrazolium (MTT) assay

HEI-OC1 cells were inoculated into 96-well plates at 5×10^3 cells/well. After cell adhesion, NLRX1 siRNA and overexpressed plasmid were transfected. After 24 hours of culture, cells were cleaned with phosphoric acid buffer, 100 μL 0.5mg/ mL MTT reagent was used, and the cells were incubated in the dark for 4 hours. Then, the supernatant was discarded, and 100 μL dimethyl sulfoxide (DMSO) reagent was applied to the cells, which were incubated in an incubator for 15min. The absorbance of all wells of cells was measured at 570 nm via a microplate reader. The blank group (normal cells without any treatment) and the negative control group (only transfection reagent was added) were set to calculate the cell proliferation activity.

Western blot

The basal membrane tissues of mouse cochlea and HEI-OC1 cells were taken, and RIPA protein lysate was added for tissue homogenization. Quantitative detection

Table 1. Primers for quantitative detection of target genes.

Gene	Primer sequence (5'→3')	Length (bp)
NLRX1	F: TAGGGCCTTTATCCGTTACCA	134
	R: TAAACCACTCGGTGAGGTTCC	
β-actin	F: ATGACCCAAGCCGAGAAGG	228
	R: TGCAATGACGTGAGGAACACT	

was implemented to detect the concentration of extracted protein by referring to the bicinchoninic acid (BCA) kit, and a standard curve was drawn. The 10% separated glue and 6% concentrated glue were prepared, and the proteins were heated with boiling water for 10min for denature. Then, proteins were electrophoretized at 80V-120V for 30 min and 60 min, respectively. polyvinylidene fluoride (PVDF) membrane was used for protein transmembrane processing, and then the proteins were soaked in a blocking solution containing 5% skim milk powder, and sealed at 25°C for 1h. TBST solution was added for cleaning. Rabbit anti-NLRX1 (1:1,000), rabbit anti-JNK (1:1,000), rabbit anti-p-JNK (1:1,000), mouse anti-Bcl-2 (1:1,000), rabbit anti-Bax (1:1,000), rabbit anti-Caspase-3 (1:1,000), and mouse anti-β -actin primary antibody (1:1,000) were added, and overnight incubation was at 4°C. TBST solution was used for cleaning, horseradish peroxidase labeled secondary antibody was applied, and the cells were incubated at room temperature under dark conditions for 2h. ECL luminescence detection kit was used to detect the luminescence reaction from light, and then developed in a gel imager. With β-actin gene as the internal reference, the gray value of target band was detected by ImageJ.

Statistical analysis

SPSS 19.0 was employed, and all experimental data were represented by means ± SD. Differences between groups were compared using a single-factor ANOVA procedure. $P<0.05$ was considered statistically considerable.

Results

In vivo animal test results

Mouse ABR audiometry results

The differences between the ABR audiometry results of 15d, 30d, 90d, and 270d newborn mice were illustrated in Figure 1. As the audiometry frequency (8 kHz - 32 kHz) increased gradually, the hearing threshold of mice of different ages showed a gradually increasing trend. Under 24 kHz and 32 kHz frequency stimulation, the hearing threshold of 90d-old mice was dramatically superior to that of 15d- and 30d-old mice ($P<0.05$). Under the stimulation of different listening frequencies, the hearing threshold of 270d-old mice was dramatically superior to that of other-day-old mice ($P<0.05$).

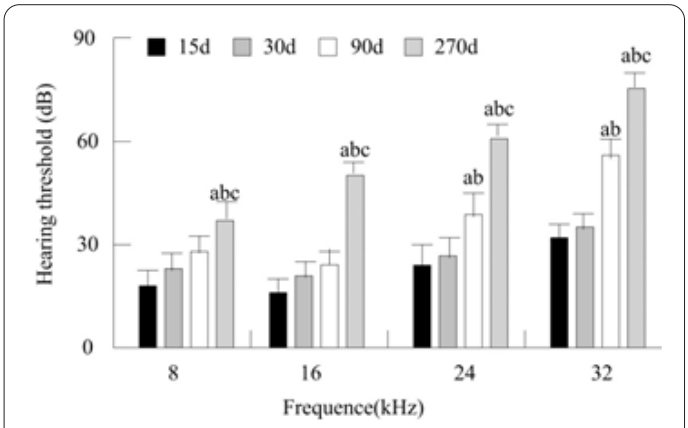

Figure 1. Comparison of ABR hearing test results in mice of different ages. Note: [a]$P<0.05$ vs. 15d- age; [b]$P<0.05$ vs. 30d- age; [c]$P<0.05$ vs. 90d- age.

NLRX1 immunofluorescence staining of the mouse cochlea

Immunofluorescence staining was used to examine the difference in NLRX1 expression in cochlear basement membrane tissues of mice of different ages, and the results were shown in Figure 2. There was no positive expression of NLXR1 in the cochlear hair cells of 15d-old mice. A small amount of positive expression of NLRX1 was seen in the cochlear hair cells of 30d-old mice, mainly with strong red fluorescent staining. The expression of NLRX1 in 90d-old mice increased slightly, and hair cell loss appeared. The positive expression of NLRX1 in 270d-old mice further increased, the fluorescence signal was strong, and they showed a neat and dense arrangement trend.

Changes in NLRX1 level in the mouse cochlea

The expression level of NLRX1 in cochlea tissues of mice of different ages was discussed, and the number of NLRX1 positive cells is shown in Figure 3. As the age of mice increased, the expression level of NLRX1 in the cochlea tended to increase gradually. After comparison, NLRX1 mRNA and protein levels of 30d-, 90d-, and 270d-old mice were substantially greater than those of 15d-old mice ($P<0.05$). NLRX1 mRNA and protein levels and the number of positively stained cells in 90d-old mice were substantially greater than those of 30d- and 270d-old mice ($P<0.05$). The NLRX1 mRNA and protein levels and

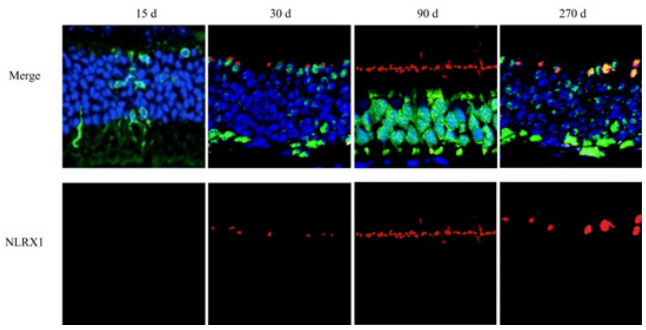

Figure 2. Immunofluorescence staining of NLRX1 in mice of different ages. Note: the microscope magnification was ×400; the red fluorescence was stained by NLRX1; the blue fluorescence was stained by DAPI; the green fluorescence was stained by Parvalbumin.

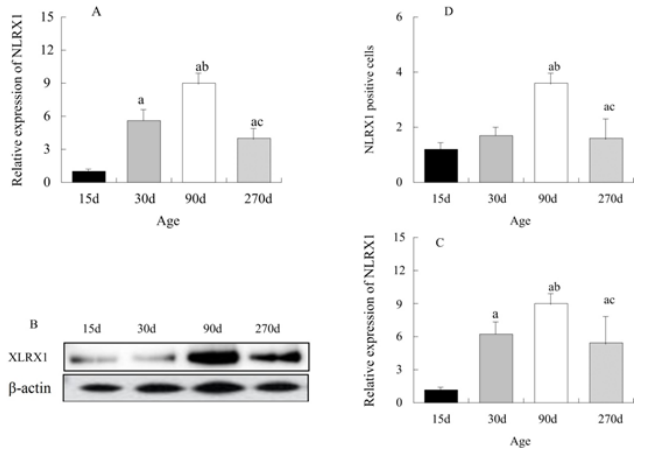

Figure 3. Comparison of NLRX1 expression in cochlea tissues of mice of different ages. Note: A. Difference in NLRX1 mRNA expression; B. The average number of NLRX1 positive staining cells; C. Western blot strips; D. Difference in NLRX1 protein expression. [a]$P<0.05$ vs. 15d- age; [b]$P<0.05$ vs. 30d- age; [c]$P<0.05$ vs. 90d- age.

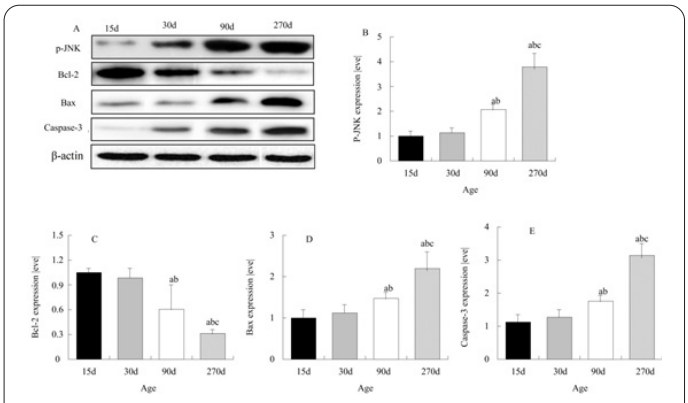

Figure 4. Comparison of the JNK pathway and apoptosis-related protein expression in cochlea tissues of mice of different ages. Note: A. Western blot strips; B. Differences of phosphorylated proteins in JNK pathway; C. Bcl-2 protein expression difference; D. Bax protein expression difference; E. Caspase-3 protein expression difference. [a]*P*<0.05 vs. 15d- age; [b]*P*<0.05 vs. 30d- age; [c]*P*<0.05 vs. 90-day age.

the number of positive staining cells were the largest in 90-day-old mice.

Detection of JNK pathway activation and apoptosis in mouse cochlear basement membrane

In Figure 4, as the age of the mice increased, the expression levels of p-JNK, Bax, and Caspase-3 proteins in the cochlear basement membrane showed a gradual increasing trend, and the expression of Bcl-2 showed a gradual decrease trend. Among them, the target gene protein level in 270d-old mice was the highest in p-JNK, Bax, and Caspase-3 and the lowest in Bcl-2. After comparison, the protein level of the target gene suggested no drastic differences between 15d- and 30d-old mice (P >0.05). The p-JNK, Bax, and Caspase-3 protein levels in mice aged 90d and 270d were dramatically higher versus that of 15d and 30d (P <0.05), while the Bcl-2 level was lower. Protein levels of p-JNK, Bax, and Caspase-3 in 270d-old mice were dramatically superior to that of 90d-old (P <0.05), while the Bcl-2 was lower.

In vitro cell test results

Detection of NLRX1 expression in HEI-OC1 cells

Real-time fluorescent quantitative PCR and western blot detected the differences of NLRX1 in HEI-OC1 cells after different treatments. Figure 5 shows no considerable difference in NLRX1 level in the cells of the control group and negative control group (P >0.05). NLRX1 in the cells of the overexpressed group was dramatically superior to that of the other groups (P<0.05). NLRX1 in the silenced group was substantially inferior to other groups (P <0.05).

Effect of NLRX1 on HEI-OC1 cell proliferation

The differences in the proliferation activity of HEI-OC1 cells in each group were shown in Figure 6. The cell proliferation activity after overexpression of NLRX1 was dramatically superior to the Ctrl group, negative control group, and silenced NLRX1 group (P<0.05). After the silencing of NLRX1, the cell proliferation activity increased substantially and was dramatically superior to the overexpressed NLRX1 group (P<0.05). Moreover, it was slightly inferior to the control group and the negative control group, involving no considerable differences (P>0.05).

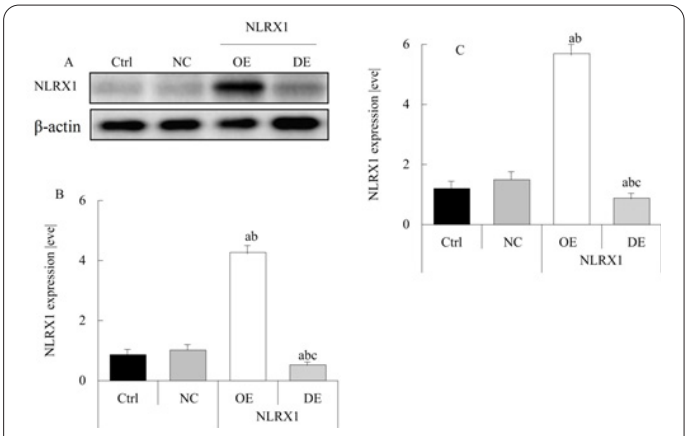

Figure 5. Comparison of NLRX1 expression in HEI-OE1 cells with different treatments.Note: Ctrl means the control group; NC means the negative control group; OE means the overexpression NLRX1 group; DE means the silenced NLRX1 group. A. Western blot strips; B. NLRX1 protein expression difference; C. NLRX1 mRNA expression difference. [a]*P*<0.05 vs. Ctrl group; [b]*P*<0.05 vs. NC group; [c]*P*<0.05 vs. OE group.

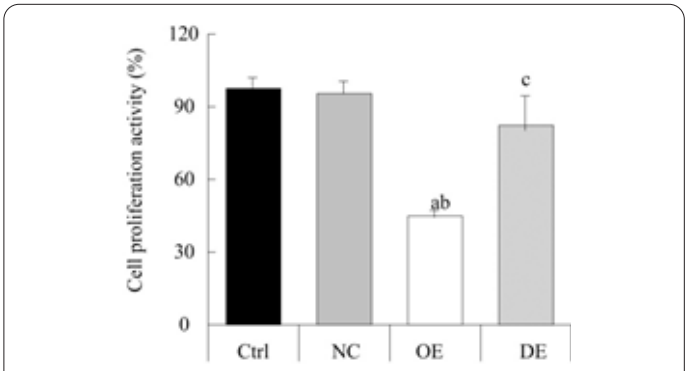

Figure 6. Comparison of the proliferation activity of HEI-OE1 cells with different treatments. Note: Ctrl means the control group; NC means the negative control group; OE means the overexpression NLRX1 group; DE means the silenced NLRX1 group. [a]*P*<0.05 vs. 15d- age; [b]*P*<0.05 vs. 30d- age; [c]*P*<0.05 vs. 90d- age.

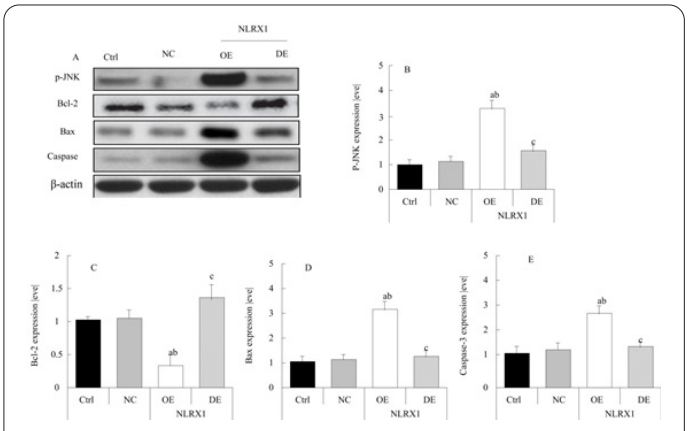

Figure 7. Comparison of the proliferation activity of HEI-OE1 cells with different treatments. Note: Ctrl means the control group; NC means the negative control group; OE means the overexpression NLRX1 group; DE means the silenced NLRX1 group. A. Western blot strips; B. Differences of phosphorylated proteins in JNK pathway; C. Bcl-2 protein expression difference; D. Bax protein expression difference; E. Caspase-3 protein expression difference. [a]*P*<0.05 vs. 15d- age; [b]*P*<0.05 vs. 30d- age; [c]*P*<0.05 vs. 90d- age.

Effect of NLRX1 on JNK pathway activation and apoptosis in HEI-OC1 cells

Figure 7 shows that p-JNK, Bax, and Caspase-3 protein levels in the overexpression NLRX1 group were the

highest, the Bcl-2 was the lowest, and the levels were substantially different from other groups (P<0.05). However, no great differences were indicated among the control group, the negative control group, and the silenced NLRX1 group (P>0.05).

Discussion

Due to the natural upward decay, the cochlear hair cells also decay and disappear, which is in line with the phenomenon of human presbycusis (15). Without manual intervention, C57BL/6 mice can also develop spontaneous presbycusis. It is close to the naturally occurring human presbycusis disease, so it is one of the ideal animal models for studying presbycusis (16,17). The ABR held that as the age of C57BL/6 mice increased, the hearing threshold also showed a gradually increasing trend, indicating that the elderly mice had a hearing impairment.

Furthermore, NLRX1 in mouse cochlear hair cells was explored. It turned out that NLRX1 was mainly expressed in mouse cochlear hair cells, and the expression level increased and then decreased with the growth and development of mice. Among them, 90-day-old mice had the highest expression. This may be due to the loss of cochlear hair cells in 270-day-old mice, so the expression level of NLRX1 decreased. Studies confirmed that NLRX1 can regulate cell apoptosis by activating the JNK signaling pathway (18,19). Bax and caspase-3 are pro-apoptotic factors, while Bcl-2 is an anti-apoptotic factor, which jointly regulates the process of cell apoptosis (20-22). As the age of mice increased, the phosphorylation level of JNK and protein levels of Bax and Caspase-3 in the cochlea tissue showed a gradually increasing trend. However, the expression of Bcl-2 showed a trend of down-regulation, indicating that as the age of the mice increased, the mouse cochlear hair cells underwent obvious apoptosis (23).

The effects of overexpression and silencing of NLRX1 on the proliferation of cochlear hair cell HEI-OE1 and activation of the JNK pathway were analyzed. It was revealed that NLRX1 can inhibit cell proliferation activity, and increase p-JNK, Bax, and Caspase-3 protein in the cell while inhibiting the Bcl-2 protein. When NLRX1 was silenced, the expression level of the target gene protein in HEI-OE1 cells changed substantially. The increase of p-JNK expression level indicated that NLRX1 caused the phosphorylation level of JNK, which in turn activated the signal pathway. The elevated levels of Bax and caspase-3 indicated that NLRX1 can promote dependent apoptosis (24). Therefore, NLRX1 can inhibit proliferation and promote apoptosis by activating the JNK apoptosis signaling pathway (25).

This study revealed that NLRX1 can enhance the activation of the JNK signaling, thereby promoting cochlear basement membrane hair cell apoptosis, inhibiting proliferation, and promoting the process of deafness. The phased role of NLRX1 in regulating the function of mouse cochlear hair cells was also explored via in vivo and in vitro experiments. However, whether NLRX1 plays a role in hair cell damage caused by other common deafness factors requires further preparation of NLRX1 knockout mice for verification. In conclusion, this work provides experimental evidence for exploring the therapeutic targets of sensorineural hearing loss.

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