Article

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Protective Effect of ATP6V1B2 Against Kanamycin-Induced Cochlear Damage in Mice

Lianrong Wu[1], Wensi Huang[1,*]

[1]Department of Neurology, The People's Hospital of Pingyang, 325400 Pingyang, Zhejiang, China
*Correspondence: huangwensi1989@163.com (Wensi Huang)
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Background: Kanamycin (KM) is a commonly used antibacterial agent in clinical practice, but it can induce ototoxicity, leading to sensorineural hearing loss. Previous studies have reported that deletion of the vacuolar H^+-ATPase B2 subunit (ATP6V1B2) results in hearing impairment and that the mitogen-activated protein kinase (MAPK) pathway exerts a protective effect on cochlear cells in mice. Therefore, this study aimed to elucidate the role of ATP6V1B2 in KM-induced cochlear hair cell injury in mice and to explore the specific mechanisms involved.

Methods: KM and siRNA/overexpression constructs were used in combination *in vivo* and *in vitro* to evaluate the protective effect of ATP6V1B2 on cochlear hair cell injury. Auditory function in individual mice was assessed using auditory brainstem response (ABR) testing. HEI-OC1 cell viability was measured using the Cell Counting Kit-8 (CCK-8). Apoptosis in HEI-OC1 cells was detected with flow cytometry. Intracellular reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) through flow cytometry. The expression of vital regulatory factors, apoptotic markers, inflammatory mediators, and MAPK pathway proteins in HEI-OC1 cells was evaluated by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis.

Results: Following KM induction, the ABR threshold increased, the number of viable cells decreased, apoptosis was promoted, ROS accumulation was enhanced, and inflammatory factor expression in HEI-OC1 cells was elevated ($p < 0.05$). Compared with the KM group, Ad-ATP6V1B2 reversed the effects of KM on the ABR threshold, cell viability, apoptosis, ROS accumulation, and inflammatory factor expression ($p < 0.05$). In contrast, si-ATP6V1B2 exacerbated the effects of KM on the ABR threshold, viable cell number, apoptosis, ROS accumulation, and expression of inflammatory factors ($p < 0.05$). Ad-ATP6V1B2 exerted its protective effects on injured HEI-OC1 cells by inhibiting the p38 MAPK pathway and activating the ERK MAPK pathway ($p < 0.05$).

Conclusion: Ad-ATP6V1B2 protects against KM-induced HEI-OC1 cell injury by inhibiting the p38/MAPK axis and activating the ERK1/2/MAPK pathway. ATP6V1B2 effectively reverses KM-mediated cellular damage by modulating the MAPK signaling pathway. These findings suggest ATP6V1B2 may represent a novel therapeutic target for preventing and treating KM-mediated HEI-OC1 cell injury and hearing impairment.

Keywords: kanamycin; ATP6V1B2; hair cell; MAPK

Introduction

Despite the important role of aminoglycosides in combating Gram-negative bacterial infections, their side effects can cause irreversible ototoxic damage [1,2]. Aminoglycoside drugs such as kanamycin, administered intravenously, can induce nephrotoxicity and ototoxicity. A recent systematic review reported that the prevalence of hearing loss among treated patients ranges from 18% to 62% [3]. Aminoglycosides can also promote the generation of reactive oxygen species (ROS) [4]. Additionally, oxidative stress arising from this process triggers inflammation, contributing to the death of spiral ganglion neurons and cochlear hair cells [5,6]. Kanamycin (KM), an aminoglycoside antibiotic, is known to induce hearing loss as an adverse effect in clinical settings [7]. It primarily damages the hair cells in the inner ear. Risk factors for kanamycin-associated ototoxicity include dosage, duration of use, frequency of administration, renal function, age, and genetic susceptibility [8]. Frequent use of aminoglycosides carries serious side effects, including irreversible renal toxicity and ototoxicity [9,10]. Therefore, it is essential to identify and investigate potential target genes for the prevention and treatment of ototoxic injury.

The vacuolar H^+-ATPase B2 subunit (ATP6V1B2), located on chromosome 8p21.3, encodes the B2 subunit of the V1 domain of the vacuolar proton transporter ATPase (V-ATPase) [11]. V-ATPase is a multi-subunit enzyme responsible for mediating the acidification of intracellular organelles in eukaryotic cells. This acidification mechanism is essential for diverse processes, including protein sorting, zymogen activation, receptor-mediated endocyto-

sis, and the generation of synaptic vesicle proton gradients [12,13]. Pathogenic mutations in *ATP6V1B2* are associated with autosomal dominant hereditary deafness syndrome (MIM number 124480) and Zimmermann-Laband syndrome type 2 (ZLS2, MIM number 616455), which commonly present with severe congenital sensorineural hearing loss [14]. Yuan *et al*. [15] reported that a de novo mutation in ATP6V1B2 impairs lysosomal acidification and causes dominant deafness-onychodystrophy syndrome. Menendez *et al*. [16] further confirmed that a novel variant (c.1516C > T, p.Arg506) leads to DDOD in children, resulting in congenital deafness and nail dysplasia. Notably, Qiu *et al*. [17] observed that cochlea-specific *ATP6V1B2* knockout mice exhibit severe sensorineural hearing loss. However, studies investigating the role of ATP6V1B2 in kanamycin-induced cochlear damage in mice remain limited.

P38 and ERK are key members of the mitogen-activated protein kinase (MAPK) family. The ERK pathway regulates key processes such as cell differentiation and apoptosis [18]. The p38 pathway plays a central role in inflammatory responses and apoptosis, with sustained activation promoting the expression of inflammatory factor and cell death [19]. Previous studies have shown that the overexpression of superoxide dismutase 2 alleviates KM-induced hearing loss by suppressing the MAPK pathway [20]. Furthermore, research reported that radiation-induced mitochondrial damage and apoptosis in HEI-OC1 cells were attenuated by the p38 inhibitor SB203580, preventing stereocilia damage [21]. However, the impact of ATP6V1B2 on the MAPK pathway and its role in KM-induced cochlear hair cell damage have not yet been reported.

This study aimed to determine whether *ATP6V1B2* protects cochlear hair cells from KM-induced damage and to explore the possible underlying mechanisms. Our findings demonstrate that *ATP6V1B2* enhances cell viability, reduces ROS levels, and inhibits inflammation and apoptosis by suppressing the p38/MAPK pathway and activating the ERK/MAPK pathway, suggesting that ATP6V1B2 may represent a promising therapeutic target for the prevention and treatment of cochlear hair cell injury.

Materials and Methods

Animal

Male C57BL/6J mice (7–9 weeks, 15–25 g) were obtained from Shanghai Jie Si Jie Laboratory Animal Co., Ltd. A total of 120 experimental mice were used in this study. The animals were maintained under a 12-hour dark/light cycle, with a relative humidity of 50–55% and a temperature between 20–25 °C, and had free access to food and water.

Transfection

The siRNA (si-ATP6V1B2) and overexpression vector (Ad-ATP6V1B2) targeting ATP6V1B2 were designed and synthesized by HUAGENE (Shanghai, China), with si-NC and Ad-NC serving as controls. Mice were randomly assigned to four groups (n = 15): (1) Ad-NC group, (2) Ad-ATP6V1B2 group, (3) si-NC group, and (4) si-ATP6V1B2 group. Each mouse received 0.1 mL of a 1.0 nmol plasmid solution.

The si-ATP6V1B2 primer sequence was 5′-GGAUAUGCUUGGUCGAGUA-3′. The si-NC primer sequence was 5′-AGUGUACGGGUUCUAGUGA-3′. The Ad-ATP6V1B2 primers were forward 5′-ATGGCGTTGCGAGCGATGCGG-3′ and reverse 5′-GTGTTTTGCAGAGTCTCGAGGGT-3′. The Ad-NC primers were forward 5′-CAGTGCTGCAATGATACCGC-3′ and reverse 5′-TCCTTGAGAGTTTTCGCCCC-3′.

Plasmids were transfected into the epithelial region of the mouse ear according to previously described methods [22,23]. Forty-eight hours after transfection, euthanasia was performed via cervical dislocation. Cochlear tissues from the four groups were collected for reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis to confirm successful overexpression or knockdown of ATP6V1B2. Each experiment was repeated three times.

Animal Treatment

Mice were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). Kanamycin (K4000, Sigma-Aldrich, St. Louis, MO, USA) was administered through the round window membrane. Briefly, a small incision was made behind the right ear to expose the bulla. A hole was created in the bulla, and 7 µL of 1 mM KM saline was injected into the cochlea. After injection, a fascial graft was placed over the round window to prevent leakage of perilymph, and the incision was then sutured to allow recovery. The same procedure was applied to all groups.

Mice were randomly divided into four groups (n = 15): (1) Control group, (2) Kanamycin (KM) group, (3) Ad-ATP6V1B2 group, and (4) si-ATP6V1B2 group. The control group received 0.9% saline (0.6 mL/100 g) injected into the cochlea. The other three groups received 7 µL of 1 mM KM. Experiments were conducted 7 days post-surgery. Two days before KM injection, Ad-ATP6V1B2 and si-ATP6V1B2 were administered into the epithelial regions of mice in the third and fourth groups, respectively, to examine the effect of ATP6V1B2 on KM-induced ototoxicity. The mice will be reared normally in the laboratory after the experiment.

Auditory Brainstem Response (ABR)

ABR testing was performed on all groups 7 days after injection. Recordings were obtained using the RZ6 system and software (TDT, Alachua, FL, USA). Mice were

Table 1. RT-qPCR primer sequences.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
Caspase-3	GTCATCTCGCTCTGGTACGG	GTGGAAAGTGGAGTCCAGGG
Bax	AGGATGCGTCCACCAAGAAG	TGTCCAGCCCATGATGGTTC
Bcl-2	ACAGGGTACGATAACCGGGA	CAATCCTCCCCCAGTTCACC
Pou4f3	AGTCTCTCACTCTCTCGCACAA	GCTGTTCTTCTCTCGGTAGGC
Gfi1	GTGGAGTCGGAGCTGCTTTGCAC	GAGCAGATGTGTGGACAGCGTGG
β-actin	GGGAAATCGTGCCGTGACAT	GCGGCAGTGGCCATCTC

RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.

anesthetized with intraperitoneal pentobarbital sodium (30 mg/kg). After anesthesia, the recording electrode was inserted subcutaneously at the cranial vertex between the bilateral external auditory canals. A subdermal needle electrode was placed at the skull vertex. The ground electrode was positioned ventrolateral to the left external pinna, and the reference electrode ventrolateral to the right external pinna.

A speaker was placed 2 cm from the external auditory canal. The maximum output was a short pure tone at 90 dB, with a duration of 10 ms and a stimulation frequency of 21.1 per second. A total of 1024 averaged signals were collected. The low-pass filter was set to 100 Hz and the high-pass filter to 3000 Hz. The stimulus intensity began at 90 dB and was reduced in 5 dB increments until wave II disappeared, which was used to determine the hearing threshold. Detection frequencies included 4, 8, 16, 24, and 32 kHz.

Cell Culture and Treatment

HEI-OC1 cells were purchased from IMMOCELL (IM-M135, Xiamen, China). The HEI-OC1 cells used in this study were authenticated by STR analysis, and mycoplasma testing yielded negative results. Cell morphology showed no abnormal particles or signs of atrophy. HEI-OC1 cells were cultured in high-glucose DMEM (11965092, Gibco, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., USA) under conditions of 35 °C and 10% CO_2. The treatment groups were as follows: (1) Control: PBS added to wells containing 3×10^5 cells; (2) KM: 1 mM KM added; (3) Ad-ATP6V1B2: Ad-ATP6V1B2 plasmid transfected 2 days before KM treatment; (4) si-ATP6V1B2: si-ATP6V1B2 plasmid transfected 2 days before KM treatment; (5) Ad-ATP6V1B2 + DHCD: the p38 activator Dehydrocorydaline (DHCD, 20 µmol/L, HY-N0674, MCE) added to the Ad-ATP6V1B2 + KM group for 48 h; (6) Ad-ATP6V1B2 + PD: the ERK inhibitor PD98059 (20 µmol/L, HY-12028, MCE) added to the Ad-ATP6V1B2 + KM group for 48 h. The concentrations and durations of DHCD and PD98059 were determined based on previous studies [24,25]. The Ad-ATP6V1B2 + DHCD group and Ad-ATP6V1B2 + PD group were treated in the same manner. Subsequent experiments were performed 24 hours after treatment.

Cell Counting Kit-8 (CCK8) Assay

Cells were seeded at a density of 5×10^5, followed by the addition of 10 µL of CCK-8 solution (C0037, Beyotime, Shanghai, China) for 2 hours. Optical density (OD) values at 450 nm were measured using a MODEL680 microplate reader (Bio-Rad, Hercules, CA, USA).

mRNA Degradation Assay

Total RNA was extracted from HEI-OC1 cells, and cDNA was synthesized using a reverse transcription kit (D7180L, Beyotime, Shanghai, China). qRT-PCR was performed to detect *ATP6V1B2* mRNA levels using the TB Green® Premix Ex Taq™ Kit. β-actin served as an internal control, and relative mRNA expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers sequences are listed in Table 1.

Western Blot Analysis

HEI-OC1 cells were collected and lysed in PIPA buffer. Cell lysates were separated by SDS-PAGE, followed by immunoblotting. Proteins were transferred to PVDF membranes and blocked with non-fat milk for 1.5 hours. Membranes were incubated overnight at 4 °C with primary antibodies: anti-Pou4f3 (1:1000, ab272908, Cambridge, UK), anti-Gfi1 (1:1000, DF13541, Affinity, MI, USA), anti-Bcl-2 (1:500, ab194583, Cambridge, UK), anti-Bax (1:500, ab32503, Cambridge, UK), anti-Cleaved caspase-3 (1:500, AF7022, Affinity, MI, USA), anti-IL-6 (1:2000, ab290735, Cambridge, UK), anti-IL-1β (1:2000, ab254360, Cambridge, UK), and anti-β-actin (1:5000, ab8227, Cambridge, UK). After incubation with secondary antibodies (ab7090, Abcam, Cambridge, UK) for 2 hours, protein bands were visualized using a chemiluminescence reagent kit and imaged using a protein imaging system (1708265, Bio-Rad, California, USA). ImageJ software (Oracle Corporation, Redwood City, CA, USA; https://imagej.nih.gov/ij/) was used to quantify protein expression levels.

Flow Cytometry

According to the instructions provided in the apoptosis detection kit (559763, BD Biosciences, Inc. USA), cells were seeded at a density of 1.0×10^6 cells/mL in 6-well plates and cultured overnight. The plates were washed

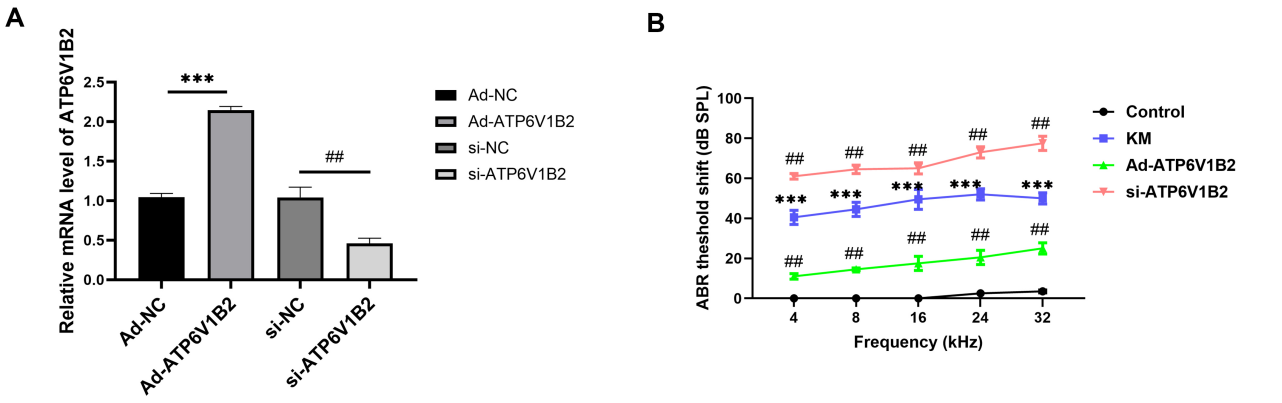

Fig. 1. Effect of *ATP6V1B2* on ABR thresholds in KM-treated mice. (A) RT-qPCR analysis of *ATP6V1B2* expression in overexpression and knockdown models. (B) ABR thresholds at different frequencies across groups. ***$p < 0.001$ vs. Ad-NC or control. $^{\#\#}p < 0.01$ vs. si-NC or KM. ABR, auditory brainstem response; KM, Kanamycin; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.

twice with PBS, followed by the addition of 1× Binding Buffer and 10 µL of 20 µg/mL FITC-labeled Annexin V, and incubated in the dark for 0.5 h. After centrifugation, the cell pellets were resuspended and mixed with 1× Binding Buffer and 5 µL of 50 µg/mL PI solution. Apoptosis was subsequently assessed using a flow cytometer (BD Biosciences, USA).

Intracellular Reactive Oxygen Species (ROS) Assay

Following the manufacturer's guidelines for ROS detection (E004, Nanjing Jiancheng, Nanjing, China), cells were incubated with 10 µM of DCFH-DA and 5 µM of dihydroethidium (DHE). They were then incubated for 30 minutes at 37 °C in an environment containing 5% CO_2. Intracellular ROS levels were evaluated by analyzing fluorescence intensity using a flow cytometer (Cytomics FC500 MCL, Beckman Coulter, USA) together with the FC500 imaging software.

Statistical Analyses

All experiments were performed in triplicate, and results are presented as the mean ± standard deviation (SD). Comparisons among multiple groups were analyzed using one-way or two-way ANOVA, followed by Tukey's post hoc test. ABR threshold data were evaluated using a two-way ANOVA. Differences between the two groups were assessed using Student's *t*-test, and $p < 0.05$ was considered statistically significant. GraphPad Prism 8 software was used for data analysis.

Results

Auditory Brainstem Response (ABR) Testing

The application of KM is well-recognized to induce severe hearing impairment in rodents [26]. In this study, an ABR test was conducted before drug administration to confirm that all mice exhibited normal auditory function. Furthermore, RT-qPCR was performed to determine whether overexpression or siRNA-mediated knockdown of *ATP6V1B2* was successfully achieved, and the results showed significant differences compared with the control group (Fig. 1A) ($p < 0.05$). Subsequently, ABR tests were performed in the mice treated with KM. Seven days after administration, ABR thresholds in the KM group were elevated compared with the control group (Fig. 1B) ($p < 0.05$), indicating that kanamycin impaired auditory function at all tested frequencies. Notably, ABR thresholds at all frequencies were significantly reduced in the Ad-ATP6V1B2 group ($p < 0.05$), while thresholds were increased in the si-ATP6V1B2 group, suggesting that *ATP6V1B2* exerts a protective effect against KM-induced ototoxicity (Fig. 1B) ($p < 0.05$).

ATP6V1B2 Enhanced the Viability of KM-Induced HEI-OC1 Cells

To investigate the effect of *ATP6V1B2* on the viability of KM-induced HEI-OC1 cells, we used the CCK-8 assay to evaluate the effects of *ATP6V1B2* overexpression and knockdown on KM-induced cellular damage. The results showed that, compared with the control group, KM significantly reduced the viability of HEI-OC1 cells ($p < 0.05$). Knockdown of *ATP6V1B2* further exacerbated the reduction in cell viability ($p < 0.05$), while overexpression of *ATP6V1B2* reversed this decline (Fig. 2A) ($p < 0.05$). Additionally, we examined the protein and mRNA levels of Pou4f3 and Gfi1, transcription factors essential for hair cell survival, using RT-qPCR and Western blot analysis. KM significantly reduced the expression of Pou4f3 and Gfi1 in HEI-OC1 cells ($p < 0.05$), while ATP6V1B2 overexpression increased both protein and mRNA levels (Fig. 2B,C) (p

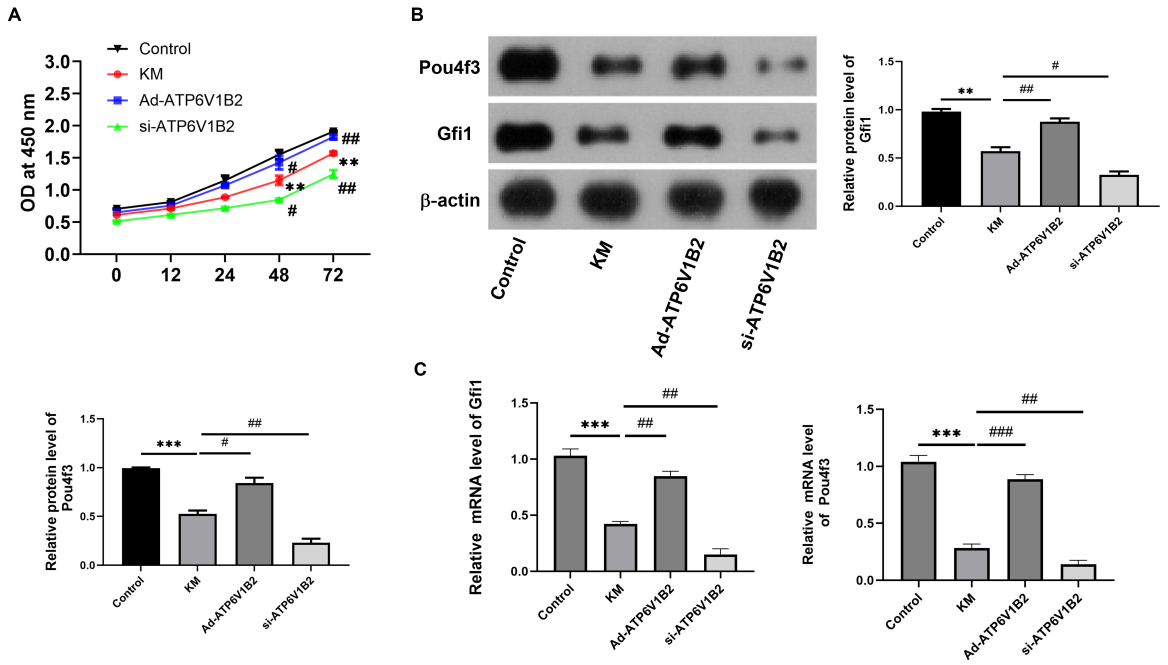

Fig. 2. The effect of *ATP6V1B2* on the viability of HEI-OC1 cells. (A) CCK-8 assay evaluating cochlear cell viability. (B,C) qPCR and Western blot analyses of mRNA and protein expression levels of cell viability markers Pou4f3 and Gfi1. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. KM. CCK-8, Cell Counting Kit-8.

< 0.05). These findings indicate that ATP6V1B2 enhances the viability of KM-induced HEI-OC1 cells.

ATP6V1B2 Inhibited the Apoptosis of KM-Induced HEI-OC1

To determine whether *ATP6V1B2* suppresses apoptosis in KM-induced HEI-OC1 cells, we first assessed apoptosis using flow cytometry. *ATP6V1B2* overexpression significantly inhibited apoptosis in HEI-OC1 cells ($p < 0.05$), while *ATP6V1B2* knockdown increased the apoptotic cell death (Fig. 3A) ($p < 0.05$). Furthermore, Ad-ATP6V1B2 intervention markedly increased the mRNA and protein levels of the anti-apoptotic marker Bcl-2 ($p < 0.05$), while decreasing the levels of Bax and cleaved caspase 3 ($p < 0.05$). si-ATP6V1B2 produced the opposite effects (Fig. 3B,C). Collectively, the results revealed that ATP6V1B2 inhibits apoptosis in KM-induced HEI-OC1 cells.

ATP6V1B2 Reduced the ROS Levels and Inflammatory Response in KM-Induced HEI-OC1 Cells

To analyze the role of *ATP6V1B2* in regulating ROS levels and inflammation in KM-induced HEI-OC1 cells, intracellular ROS levels were measured using DCFH-DA and flow cytometry. Knockdown of *ATP6V1B2* significantly increased the intracellular KM-induced ROS production ($p < 0.05$), while overexpression of Ad-ATP6V1B2 reduced the KM-induced intracellular ROS levels (Fig. 4A,B) ($p < 0.05$).

Western blot analysis further showed that ATP6V1B2 knockdown enhanced the KM-induced expression of pro-inflammatory cytokines ($p < 0.05$), while overexpression of ATP6V1B2 reduced the protein levels of IL-6 and IL-1β, thereby alleviating the inflammatory response in damaged cells (Fig. 4C) ($p < 0.05$). These findings indicate that ATP6V1B2 exerts a protective role by reducing inflammation, thereby enhancing hearing function.

ATP6V1B2 Exerts a Protective Effect on Damaged HEI-OC1 Cells by Regulating the MAPK Pathway

The MAPK signaling pathway plays a crucial role in cochlear hearing loss [19]. To determine whether the protective effect of *ATP6V1B2* on damaged cochlear cells is associated with MAPK signaling, we analyzed the phosphorylation status of p38 and ERK during ATP6V1B2-mediated protection. Compared with the KM group, phosphorylation of p38 was reduced in the Ad-ATP6V1B2 group and elevated in the si-ATP6V1B2 group (Fig. 5A) ($p < 0.05$). Conversely, ERK1/2 phosphorylation was elevated in the Ad-ATP6V1B2 group and reduced in the si-ATP6V1B2 group (Fig. 5A) ($p < 0.05$).

To further validate these observations, the p38 activator Dehydrocorydaline (DHCD) and the ERK inhibitor PD98059 were applied to examine the regulatory effects of Ad-ATP6V1B2 and si-ATP6V1B2 on p38/ERK1/2 activity. The p-p38/p38 ratio was significantly higher in the Ad-ATP6V1B2 + DHCD group than in the Ad-ATP6V1B2 group (Fig. 5B) ($p < 0.05$). Conversely, the p-ERK1/2/t-

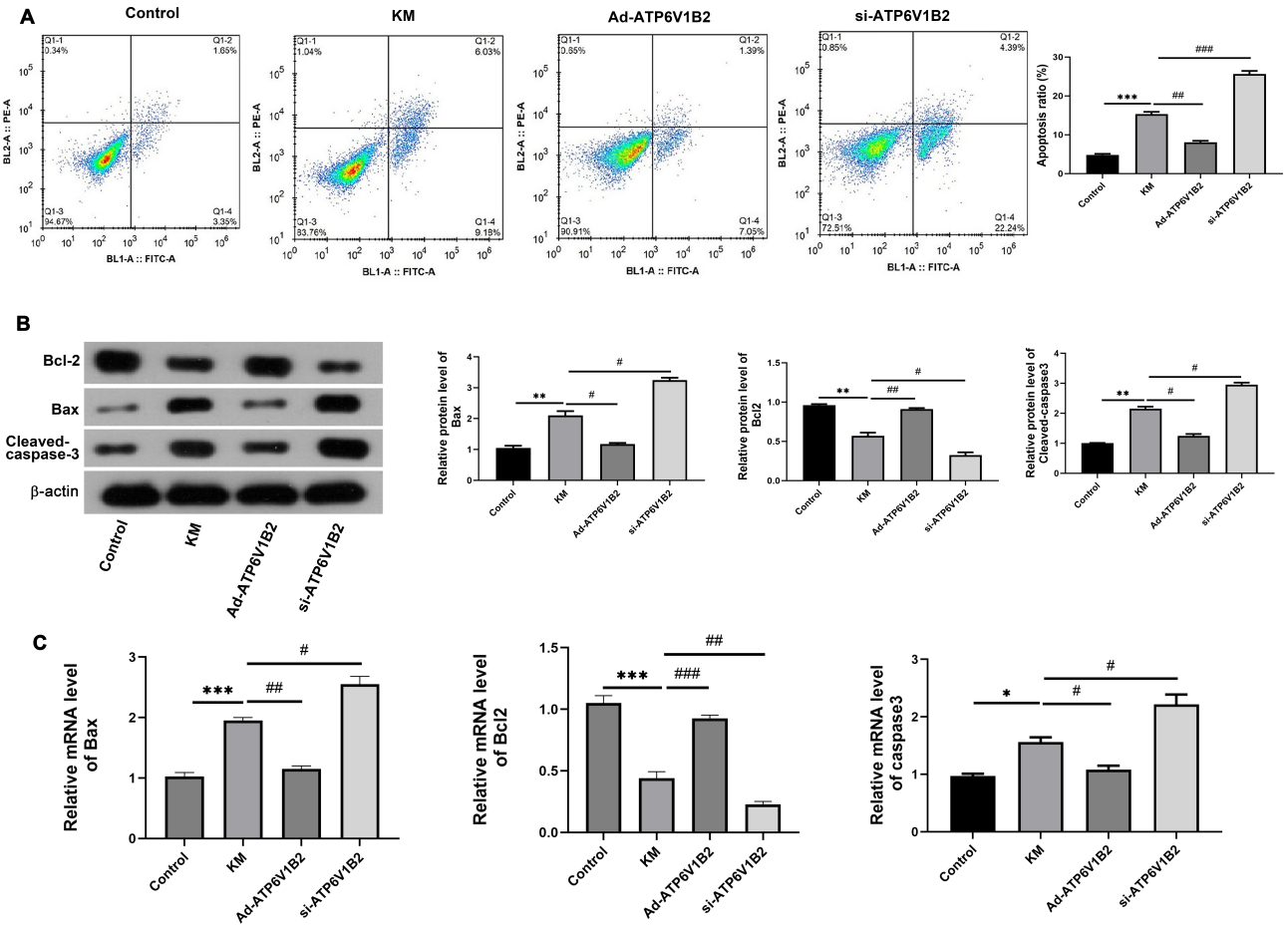

Fig. 3. Effect of *ATP6V1B2* on apoptosis in KM-induced HEI-OC1. (A) Flow cytometric analysis of apoptosis. (B) Western blot detection and quantification of apoptosis-related proteins. (C) qPCR analysis of mRNA expression of apoptosis-related proteins. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. KM.

ERK1/2 ratio was lower in the Ad-ATP6V1B2 + PD group compared with the Ad-ATP6V1B2 group (Fig. 5C) ($p < 0.05$). Taken together, the results suggest that *ATP6V1B2* plays a protective role in damaged cochlear hair cells by activating the ERK/MAPK pathway and inhibiting the p38/MAPK signaling.

Discussion

KM is an effective antibacterial agent. However, its ototoxic side effects severely limit its clinical application [7]. Therefore, identifying strategies that allow kanamycin to be used effectively against bacteria while preserving cochlear hearing function is of substantial clinical significance. Previous research has shown that knockdown of *ATP6V1B2* leads to severe sensorineural hearing loss [13], suggesting that *ATP6V1B2* may represent a potential target for alleviating the ototoxic side effects of KM. In this study, we investigated the role of *ATP6V1B2* in KM-induced hearing damage. We found that, following KM exposure, ABR thresholds were elevated in mice, the vitality of HEI-OC1 cells decreased, apoptosis increased, and both ROS levels and inflammatory responses were augmented. Overexpression of *ATP6V1B2* reversed KM-induced auditory damage in both mice and HEI-OC1 cells. The protective effect of *ATP6V1B2* overexpression against KM-induced damage appears to be primarily mediated through the MAPK signaling pathway.

ATP6V1B2 is widely expressed in mouse tissues, including the inner ear [26]. Therefore, we examined the effects of *ATP6V1B2* on cochlear function. In the *in vivo* experiments, ABR thresholds were significantly increased in the KM group compared with the control group, consistent with previous findings showing that KM induces cochlear hearing damage in mice [20]. Notably, overexpression of *ATP6V1B2* reduced ABR thresholds in mice across all tested frequencies. Yuan *et al.* [15] further demonstrated that *ATP6V1B2* knockdown (Atp6v1b2$^{Arg506*/Arg506*}$) resulted in abnormal lysosomal acidification, leading to hair cell damage and severe hearing loss in mice. These findings suggest that *ATP6V1B2* can improve hearing deficiencies in mice.

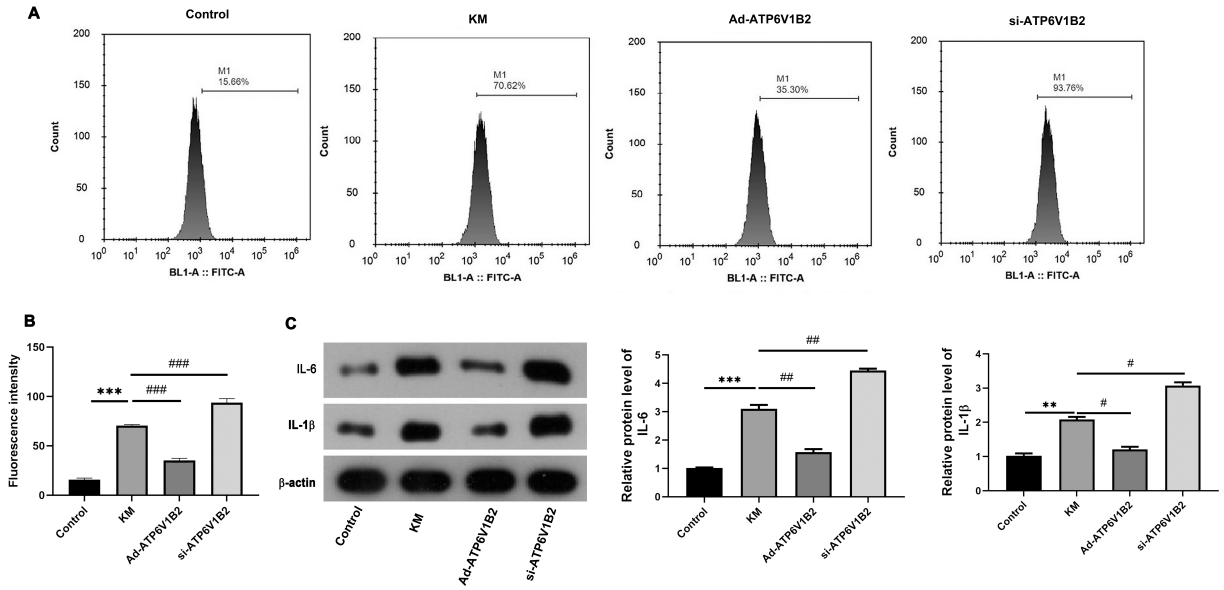

Fig. 4. Effect of ATP6V1B2 on ROS levels and inflammatory responses in KM-induced HEI-OC1 cells. (A) Intracellular ROS levels measured using DCFH-DA and flow cytometry. (B) Comparison of fluorescence intensity among groups. (C) Western blot analysis of pro-inflammatory cytokine protein expression. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. KM. ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescin diacetate.

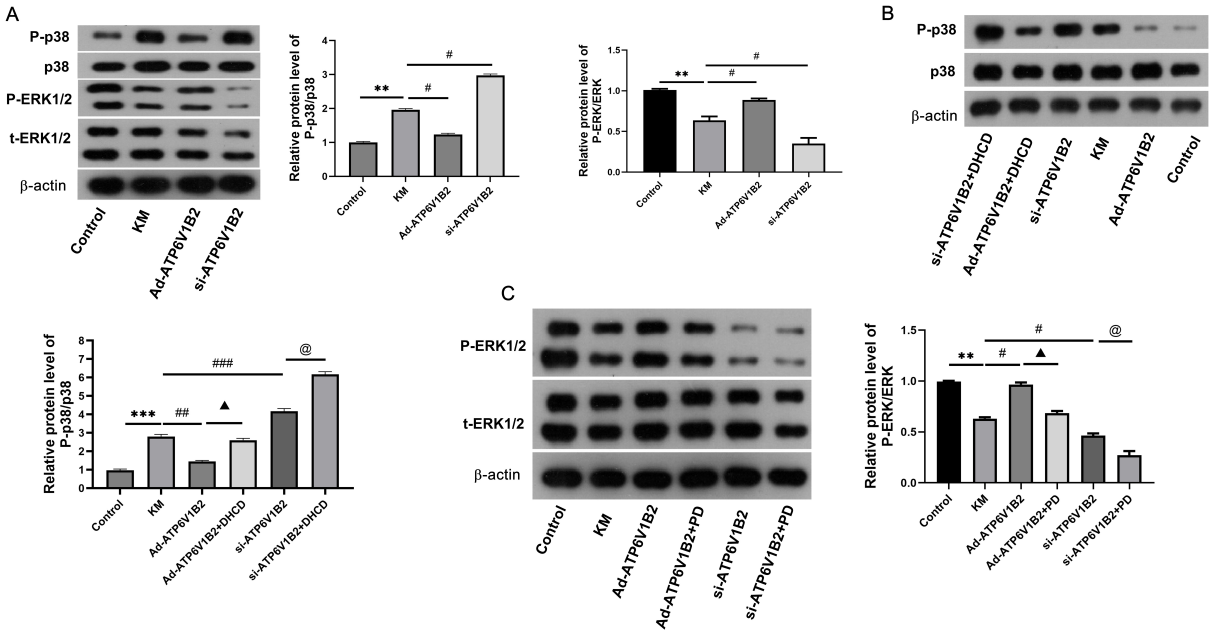

Fig. 5. Effects of the MAPK pathway on KM-induced HEI-OC1 cell damage. (A) Effect of ATP6V1B2 on ERK/p38 MAPK phosphorylation in cochlear HEI-OC1 cells. (B) Treatment with the P38 activator DHCD significantly increased p38 phosphorylation. (C) Treatment with the ERK1/2 inhibitor PD98059 significantly decreased ERK phosphorylation. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. KM; $^{\blacktriangle}p < 0.05$ vs. Ad-ATP6V1B2; $^{@}p < 0.05$ vs. si-ATP6V1B2. MAPK, mitogen-activated protein kinase; DHCD, Dehydrocorydaline.

Apoptosis is a primary mechanism underlying aminoglycoside-related ototoxicity [27], and mitochondria-mediated apoptosis has been extensively investigated [6]. Previous studies have reported increased levels of the terminal apoptotic effector caspase-3 in spiral ganglion neurons (SGNs) of Atp6v1b2$^{Arg506/Arg506}$ mice, indicating

that lysosomal dysfunction contributes to apoptosis [17]. In our study, flow cytometry and Western blot analysis indicated that Bax and cleaved caspase-3 levels were elevated in HEI-OC1 cells following KM treatment, accompanied by an increase in the apoptotic rate. Overexpression of ATP6V1B2 reversed these effects, suggesting that ATP6V1B2 inhibits apoptosis in KM-induced HEI-OC1 cell injury.

KM treatment may also induce endoplasmic reticulum stress, likely due to its antimicrobial mechanism, which disrupts microbial ribosomes and ultimately leads to the generation of ROS [25]. ROS-induced oxidative stress and inflammation play crucial roles in cochlear damage [28,29]. Previous research has reported that the overexpression of V-ATPase in tumor cells can inhibit apoptosis [30], while the inhibition of V-ATPase increases ROS production [31,32]. However, whether *ATP6V1B2* suppresses ROS accumulation and inflammatory responses in KM-induced cochlear injury has not been previously reported. Using the DCFH-DA probe to detect intracellular ROS levels and flow cytometry to measure pro-inflammatory factor expression, we observed that overexpression of *ATP6V1B2* alleviates KM-induced ototoxicity by reducing intracellular ROS accumulation and inhibiting the upregulation of IL-1β and IL-6.

Normal cochlear hearing function involves multiple signaling pathways, such as the PI3K/AKT pathway [33, 34], the MAPK pathway [19], and the Notch/Wnt/Atoh1 pathway [35,36]. Although previous studies have suggested a role for the MAPK signaling pathway in cochlear hearing damage, it remains unclear whether *ATP6V1B2* improves HEI-OC1 cell damage by modulating the MAPK signaling pathway. p38, ERK, and c-Jun N-terminal kinase (JNK) MAPKs play crucial roles in hearing impairment. Previous research has reported that inhibiting the activation of ERK1/2 in the cochlea enhances gentamicin-induced toxicity, leading to the loss of OHCs [37]. Interestingly, inhibition of p38 activation provides protection against aminoglycoside-, cisplatin-, and noise-induced cochleotoxicity [38]. Notably, aminoglycosides can induce ROS-mediated oxidative stress, activating the JNK pathway and regulating downstream transcription factors that promote apoptosis in damaged HCs [39]. This indicates that different MAPK kinases have varying effects on HCs.

The V-ATPase not only regulates neuroinflammatory diseases by inhibiting the MAPK signaling pathway [40], but can also activate the MAPK signaling pathway to influence overall physiological health [41]. Furthermore, previous studies have observed that inhibition of V-ATPase increases ROS production [32]. ROS is closely associated with inflammation [42]. Therefore, overexpression of ATP6V1B2 may enhance the vitality of cochlear hair cells, inhibit apoptosis, reduce ROS production, and alleviate inflammation by regulating the MAPK pathway. The present study suggests that overexpression of *ATP6V1B2* reverses the damaging effects of KM on HEI-OC1 cells by activating the MAPK/ERK1/2 pathway and inhibiting the MAPK/p38 pathway. This finding is consistent with previous studies [37,38].

To further confirm whether the MAPK/p38/ERK pathway participates in protecting against cochlear hearing damage, this study evaluated the effects of the ERK1/2 inhibitor PD98059 and the p38 activator DHCD on HEI-OC1 cell injury. The results indicated that treatment with PD98059 significantly reduced ERK1/2 activity in Ad-ATP6V1B2 cells, while treatment with the p38 activator DHCD significantly increased p-p38/p38 activity in Ad-ATP6V1B2 cells. These findings further suggest that ATP6V1B2 enhances cell viability, reduces reactive oxygen species levels, and inhibits inflammation and apoptosis by suppressing p38/MAPK and activating the ERK/MAPK pathway.

In summary, we established cochlear-specific *ATP6V1B2* knockdown and overexpression cell models. *ATP6V1B2* deficiency exacerbated KM-induced injury, leading to severe hearing loss, which was reversed by *ATP6V1B2* overexpression. These findings suggest that *ATP6V1B2* may serve as a novel target for hearing protection and provide a molecular basis for clinical diagnosis and future therapeutic interventions for cochlear hair cell damage and deafness. The results of this study deepen our understanding of the role of ATP6V1B2 in clinical cochlear injury and offer new theoretical support for the prevention and treatment of cochlear hearing impairment. However, the precise role and mechanisms of ATP6V1B2 in cochlear hearing impairment, as well as the underlying causes and potential therapeutic approaches for cochlear hearing impairment, require further investigation.

Conclusion

ATP6V1B2 reverses the damaging effects of KM on cochlear hair cells by modulating the MAPK pathway. *ATP6V1B2* may represent a novel molecular target for hearing protection and for the prevention and treatment of KM-mediated cochlear hair cell damage and hearing impairment.

Availability of Data and Materials

The data analyzed are available on the request from the corresponding author.

Author Contributions

Conceptualization: LW and WH. The initial draft: both authors. Critical revisions: both authors. Both authors have reviewed and approved the final manuscript and agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Ethics committee of The People's Hospital of Pingyang approval was obtained for all experimental protocols in this study (Approval No. 2023-013).

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Conflict of Interest

The authors declare there is no financial interest.

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