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Original Research

Therapeutic Effects of GDF6-Overexpressing Mesenchymal Stem Cells through Upregulation of the GDF15/SIRT1 Axis in Age-Related Hearing Loss

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Abstract

Background: It has been reported the therapeutic effects of mesenchymal stem cells (MSCs) on hearing loss. This study explored the therapeutic effects of growth differentiation factor 6 (GDF6) overexpression-induced MSCs (MSCs-GDF6) on age-related hearing loss (ARHL) and its underlying mechanisms. **Methods**: Reverse transcription-quantitative PCR and western blotting were used to evaluate gene expression. Flow cytometry and immunofluorescence assays were performed for the detection of apoptosis and autophagy, respectively. Hearing function and loss of outer hair cells (HCs) in ARHL rats were measured using the auditory brainstem response and cochlear silver nitrate staining, respectively. MSC proliferation was evaluated with the Cell Counting Kit-8 assay. **Results**: Growth differentiation factor 15 (GDF15) and sirtuin 1 (SIRT1) expression was significantly decreased in hydrogen peroxide (H_2O_2)-induced House Ear Institute-Organ of Corti 1 (HEI-OC1) cells and the cochlea of ARHL rats. Elevated apoptosis and blocked autophagic flux were uncovered in H_2O_2-induced HEI-OC1 cells and ARHL rats. GDF15 overexpression inhibited apoptosis and restored autophagic flux *in vitro* and *in vivo*. Meanwhile, GDF15 positively regulated SIRT1 protein expression. MSCs-GDF6 not only upregulated GDF15 and SIRT1 expression but also suppressed apoptosis and restored autophagic flux to reduce loss of HCs and hearing loss in ARHL rats. **Conclusions**: MSCs-GDF6 prevented loss of HCs to relieve ARHL by inhibiting apoptosis and restoring autophagic flux, likely in association with upregulation of the GDF15/SIRT1 axis.

Keywords: mesenchymal stem cells; GDF15; SIRT1; cochlear hair cell; age-related hearing loss

1. Introduction

Age-related hearing loss (ARHL), as known as presbycusis, is a type of sensorineural hearing loss that is bilateral, progressive, and symmetrical, and most noticeable in the higher frequencies; it is mainly caused by degeneration of the auditory system as people age [1]. According to World Health Organization estimates, about 2.5 billion adults aged >60 years will suffer from hearing loss by 2050 [2]. Elderly people with ARHL often have difficulties in hearing and speech communication, leading to psychological problems such as depression [3,4], which may be associated with dementia and brain cognitive dysfunction [5,6]. Hearing aids, osseointegrated auditory implants, and cochlear implantation are effective strategies for treating ARHL [7]. Due to the low use of hearing aids and high price of cochlear implantation, novel treatments are being developed. The major pathological changes in ARHL include the loss of hair cells (HCs), dysfunction of the stria vascularis, and degeneration of cochlear neurons [2,8]. Therefore, new technology research, including gene therapy, stem cell (SC) therapy, and pharmacotherapy, focuses on the regeneration and restoration of cochlear HCs during treatment of ARHL [7,9].

Mesenchymal SCs (MSCs) derived from human embryonic SCs (hESCs) are a type of pluripotent SC, which are present in almost all tissues and organs of the human body. An increasing number of studies has elucidated that MSCs can differentiate into HCs or auditory neurons, promote cochlea regeneration, and restore hearing function [10,11], suggesting the potential of MSC transplantation as a therapeutic strategy for treating deafness. Bettini *et al.* [12] indicated that intravenous injection of MSCs into mice with hearing loss can regenerate sensory structures damaged by kanamycin. Another study demonstrated that skin-derived MSCs prevented the loss of cochlear HCs and alleviated cisplatin-induced hearing loss in mice [13]. Nonetheless, the therapeutic potential of MSCs for ARHL treatment has not yet been explored.

Growth differentiation factor (GDF) family members, particularly GDF5 and GDF6, exhibit significant effects on the development of cartilaginous tissues, intervertebral discs, and joints [14]. Bademci *et al.* [15] demonstrated that GDF6 expression was higher in the cochlea than in the vestibule of developing and adult mice, and cochlear aplasia occurred in a $GDF6^{-/-}$ mouse model, revealing the crucial role of GDF6 in early cochlear development. *GDF6*

is the most upregulated gene associated with MSCs, playing a crucial role in the differentiation of hESCs into MSCs and contributing to the enrichment of MSC generation [16]. A previous study confirmed that GDF6 induces the differentiation of bone marrow- and adipose-derived MSCs [17]. Together, these findings identified an inducing factor that may play a major role in the future of regenerative medicine. Hence, the application of GDF6 overexpression-induced MSCs (MSCs-GDF6) may be a novel approach for ARHL treatment.

GDF15 is a member of the transforming growth factor beta superfamily and its primary biological roles include responses to inflammatory stimuli [18], oxidative stress [19], and the regulation of apoptosis [20]. Edderkaoui *et al*. [21] demonstrated that loss of the Duffy antigen/receptor for chemokines could alleviate noise-induced cochlear damage in mice, accompanied by inhibition of inflammation and an increase in GDF15 mRNA expression, indicating the important role of GDF15 in hearing loss. In addition, it has been reported that MSC treatment induces GDF15 upregulation in the myocardium [22]. However, it remains unknown whether the therapeutic effects of MSCs on ARHL are associated with GDF15.

This study explored the role of GDF15 and MSCs/MSCs-GDF6 in ARHL, and determined whether the effects of MSCs on cochlea are related to GDF15.

2. Materials and Methods

2.1 Cell Treatment

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line and rat bone marrow-derived MSCs were purchased from Yaji Biological Technology Co., Ltd. (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 33 °C, 10% CO_2. HEI-OC1 cells were induced with hydrogen peroxide (H_2O_2) to simulate ARHL *in vitro*, as previously described [23]. GDF15-overexpressing lentiviral vectors, and si-GDF15 and GDF6 overexpression plasmids were obtained from Obio Technology Co., Ltd. (Shanghai, China). The lentivirus was packaged and infected HEI-OC1 cells and MSCs for 48 h, after which the supernatants were harvested. All cell lines were validated by short tandem repeat profiling and tested negative for mycoplasma.

2.2 Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then RNA was reverse transcribed to synthesize cDNA. Using cDNA as a template, quantitative PCR (qPCR) was performed with 2×SYBR Green qPCR Mix (Aidlab, Beijing, China) on the ABI-7500 Real-Time PCR System (Thermo Scientific, Waltham, MA, USA). Finally, the $2^{-\Delta\Delta Ct}$ method was applied to analyze the relative mRNA expression. The list of primer sequences (5′-3′) are shown as below:

GDF15-F: CCTGGTCTGGGGATACTGAG;
GDF15-R: AGCAGGAACAGCAGGAACC;
GDF6-F: CTT TGT AGA CAG AGGACT GGA CGA T;
GDF6-R: GCT CTT CTT TGTCTG AGA GTG TGG;
β-actin-F: CCACTGCCGCATCCTCTTCC;
β-actin-R: CTCGTTGCCAATAGTGATGACCTG.

2.3 Western Blot Analysis

Proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer supplemented with PMSF (Servicebio Technology Co., Ltd., Wuhan, China) and quantified using the Bicinchoninic Acid Assay (BCA) Protein Quantification Kit (Vazyme, Nanjing, China). Then the proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene fluoride (PVDF) membrane. To block nonspecific binding, the membrane was incubated with 5% skim milk for 2 h at 25 °C. Next, the membrane was incubated at 4 °C overnight with the following primary antibodies: GDF15 (1:5000, 27455-1-AP; Proteintech, Chicago, IL, USA), sirtuin 1 (SIRT1) (1:5000, 13161-1-AP; Proteintech), B-cell lymphoma 2 (Bcl-2) (1:5000, 68103-1-Ig; Proteintech), Bcl-2-associated X (Bax) (1:10,000, 60267-1-Ig; Proteintech), cleaved caspase-3 (1:1000, #9664; Cell Signaling Technology, Danvers, MA, USA), light chain 3 (LC3)-I/II (1:2000, ab192890; Abcam, Cambridge, MA, USA), and p62 (1:1000, #23214; Cell Signaling Technology). After washing with phosphate-buffered saline (PBS), the membrane was incubated at 25 °C for 1–2 h with horseradish peroxidase-labeled IgG secondary antibodies. Finally, enhanced chemiluminescence was used to visualize the proteins. The results presented are from three independent biological replicates.

2.4 Flow Cytometry

Apoptosis of HEI-OC1 cells was evaluated by flow cytometry with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, CA, USA). After washing with PBS, HEI-OC1 cells (1×10^5) were resuspended in binding buffer. The cells were incubated with FITC Annexin V (5 µL) and propidium iodide (5 µL) for 15 min at 25 °C in the dark. After adding 400 µL binding buffer, the apoptosis rate was analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA) within 1 h.

2.5 Immunofluorescence

The analysis of autophagosome formation in HEI-OC1 cells was performed by immunofluorescence staining for LC3. In brief, HEI-OC1 cells in each group were successively fixed in 4% paraformaldehyde at room temperature for 30 min, and then blocked in 5% bovine serum albu-

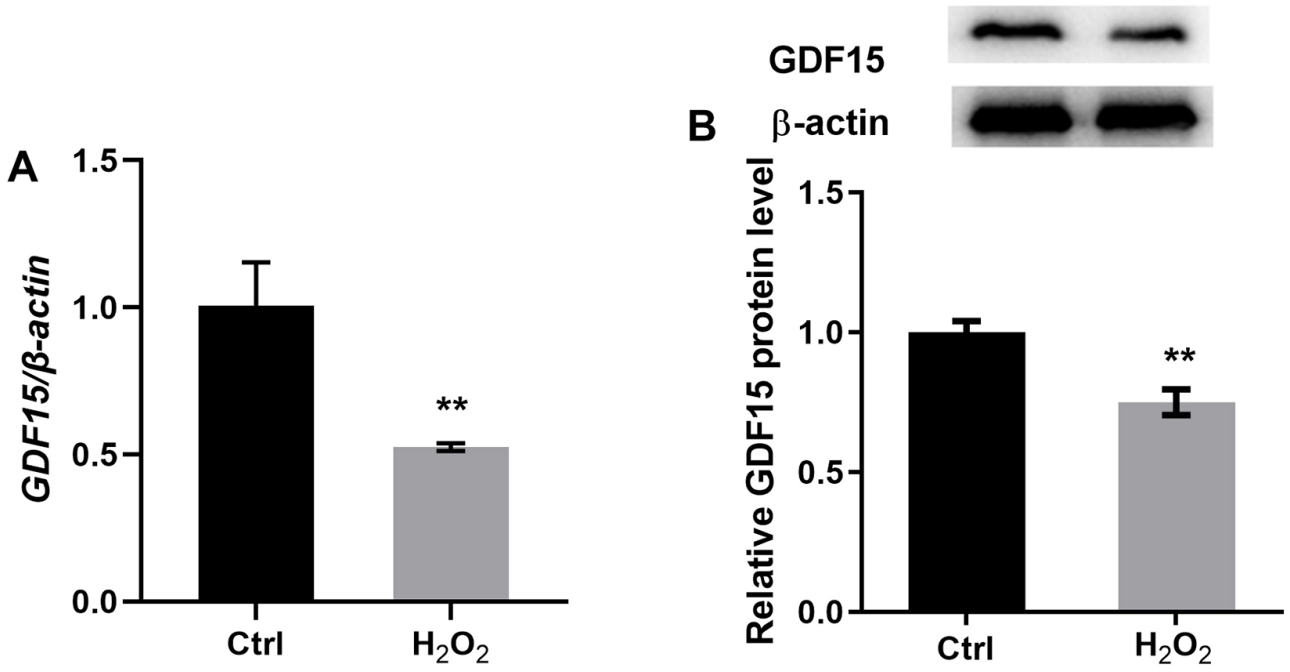

Fig. 1. GDF15 expression in H_2O_2-stimulated HEI-OC1 cells. HEI-OC1 cells were exposed to H_2O_2 to create an *in vitro* cell of ARHL. RT-qPCR (A) and western blot analysis (B) were used to analyze the mRNA and protein expression of GDF15 in H_2O_2-stimulated HEI-OC1 cells. $^{**}p < 0.01$ vs. the Ctrl group. GDF15, growth differentiation factor 15; ARHL, age-related hearing loss; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

min at 37 °C for 2 h. Next, HEI-OC1 cells were incubated overnight with LC3B antibody (Abcam) at 4 °C, followed by incubation with Cy3-labeled IgG secondary antibody at 37 °C for 1 h in the dark. After staining with Hoechst for 10 min, the photographs were taken by confocal microscopy (IX83; Olympus, Tokyo, Japan).

2.6 A Rat Model of ARHL

All procedures involving animals were performed in compliance with our institutional animal care guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanchang University (Approval No. 20221031014). A total of 36 male Sprague Dawley rats were purchased from Charles River (Beijing, China). Mice were randomly divided into six groups (n = 6/group): Normal, Model, Model + adeno-associated virus-negative control (AAV-NC), Model + AAV-GDF15, Model + MSCs-NC, and Model + MSCs-GDF6. Rats in the Model groups were intraperitoneally injected with D-galactose (150 mg/kg/d) for 60 days. AAV-GDF15/NC (Obio Technology) or MSCs-NC/MSCs-GDF6 (1×10^5) were retroauricularly injected into the scala media or tympani of the cochlea on Days 0 and 30, respectively. At the end of the experiments, all rats were euthanized with 100% compressed CO_2 gas.

2.7 Auditory Brainstem Response

Rats in each group were anesthetized with pentobarbital sodium (2%, 40 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection. A metal recording electrode was placed under the skin at the top of the skull, the reference electrode was placed under the earlobe on the acoustic side, and the ground electrode was placed under the skin behind the auricle on the opposite side. Different frequencies of tone burst (8, 12, 24 kHz) were given from the recording system. The stimulus intensity started from 100 dB sound pressure level (SPL) and decreased successively with 5 dB; the lowest stimulus intensity that could distinguish wave shape I or II was determined as the auditory brainstem response (ABR) threshold.

2.8 Cochlear Silver Nitrate Staining

Cochlear tissues were isolated from rats and placed under a dissecting microscope after repeated rinsing with pure water. The round window membranes were opened and a small hole was drilled in the tip of the cochlea with the needle of a 1 mL syringe. Silver nitrate solution (0.5%) was immediately perfused until outflowing from the oval foramen at the bottom of the cochlea. After fixing in 10% formaldehyde, the specimens were exposed to sunlight for about 2 h. Finally, morphological changes of the outer HCs (OHCs) in the basement membrane were observed under an optical microscope.

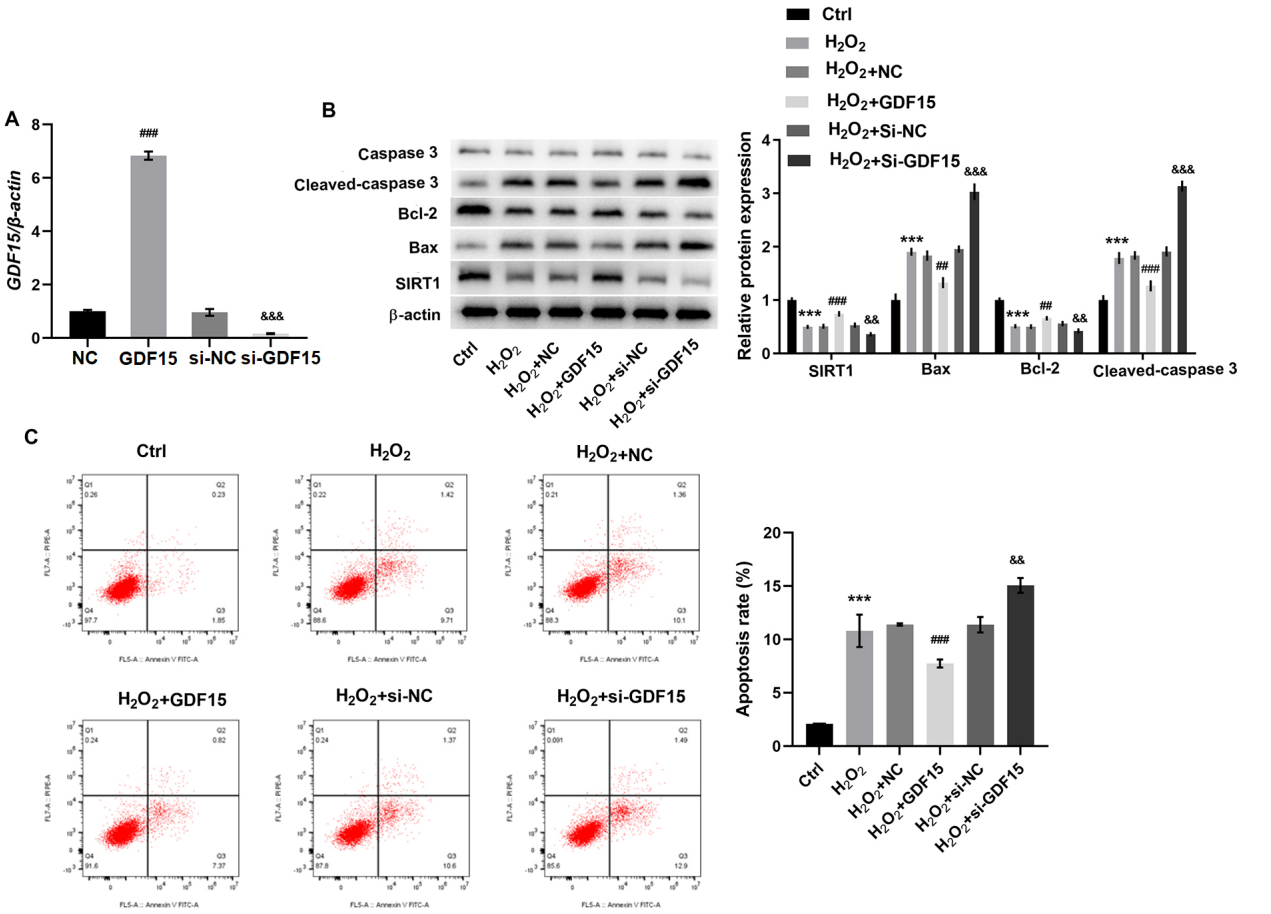

Fig. 2. Effects of GDF15 on the apoptosis of H_2O_2-stimulated HEI-OC1 cells. GDF15 overexpression or knockdown plasmid was transfected into H_2O_2-stimulated HEI-OC1 cells. (A) RT-qPCR was used to detect the transfection efficiencies of GDF15 overexpression and GDF15 silencing. (B) Detection of SIRT1, Bax, Bcl-2 and cleaved caspase-3 protein expression by western blot analysis. (C) Analysis of apoptosis rate using flow cytometry. $^{***}p < 0.001$ vs. the Ctrl group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 + NC group; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. the H_2O_2 + si-NC group. GDF15, growth differentiation factor 15; SIRT1, sirtuin 1; NC, negative control; si-NC, small interfering RNA- negative control; Bax, Bcl-2-associated X; Bcl-2, B-cell lymphoma 2.

2.9 Cell Counting Kit-8 Assay

The Cell Counting Kit-8 (CCK-8) assay was performed to evaluate MSC proliferation. MSCs were inoculated in 96-well plates. After the cell density reached 60–70%, MSCs were co-cultured with 10 µL CCK-8 (Beyotime Bio., Shanghai, China) in the dark for 1–4 h. The optical density value was obtained at 450 nm using a microplate reader (SpectraMax i3x, Molecular Devices, San Jose, CA, USA).

2.10 Statistical Analyses

Three replicates were set up for each experiment, and data from each experiment are expressed as the mean ± standard deviation. GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was applied for statistical analyses. The Student's *t*-test and one-way analysis of variance was used for comparisons between two groups and multiple groups, respectively. $p < 0.05$ was considered statistically significant.

3. Results

3.1 GDF15 Expression in H_2O_2-Stimulated HEI-OC1 Cells

HEI-OC1 cells were exposed to H_2O_2 to create an *in vitro* model of ARHL. The preliminary results of RT-qPCR and western blot analysis showed that the mRNA and protein expression of GDF15 was significantly decreased after exposure to H_2O_2 in HEI-OC1 cells (Fig. 1), indicating the potential role of GDF15 in ARHL. For all original western blot figures of Fig. 1B see **Supplementary Material**.

3.2 Regulation of Apoptosis and Autophagic Flux by GDF15 in H_2O_2-Induced HEI-OC1 Cells

To assess the effects of GDF15 on H_2O_2-stimulated HEI-OC1 cells, GDF15 overexpression or knockdown plasmid was transfected into H_2O_2-stimulated HEI-OC1 cells. Fig. 2A shows the remarkable transfection efficiency. Western blot analysis (Fig. 2B) and flow cytometry (Fig. 2C) showed that H_2O_2 stimulation induced apoptosis

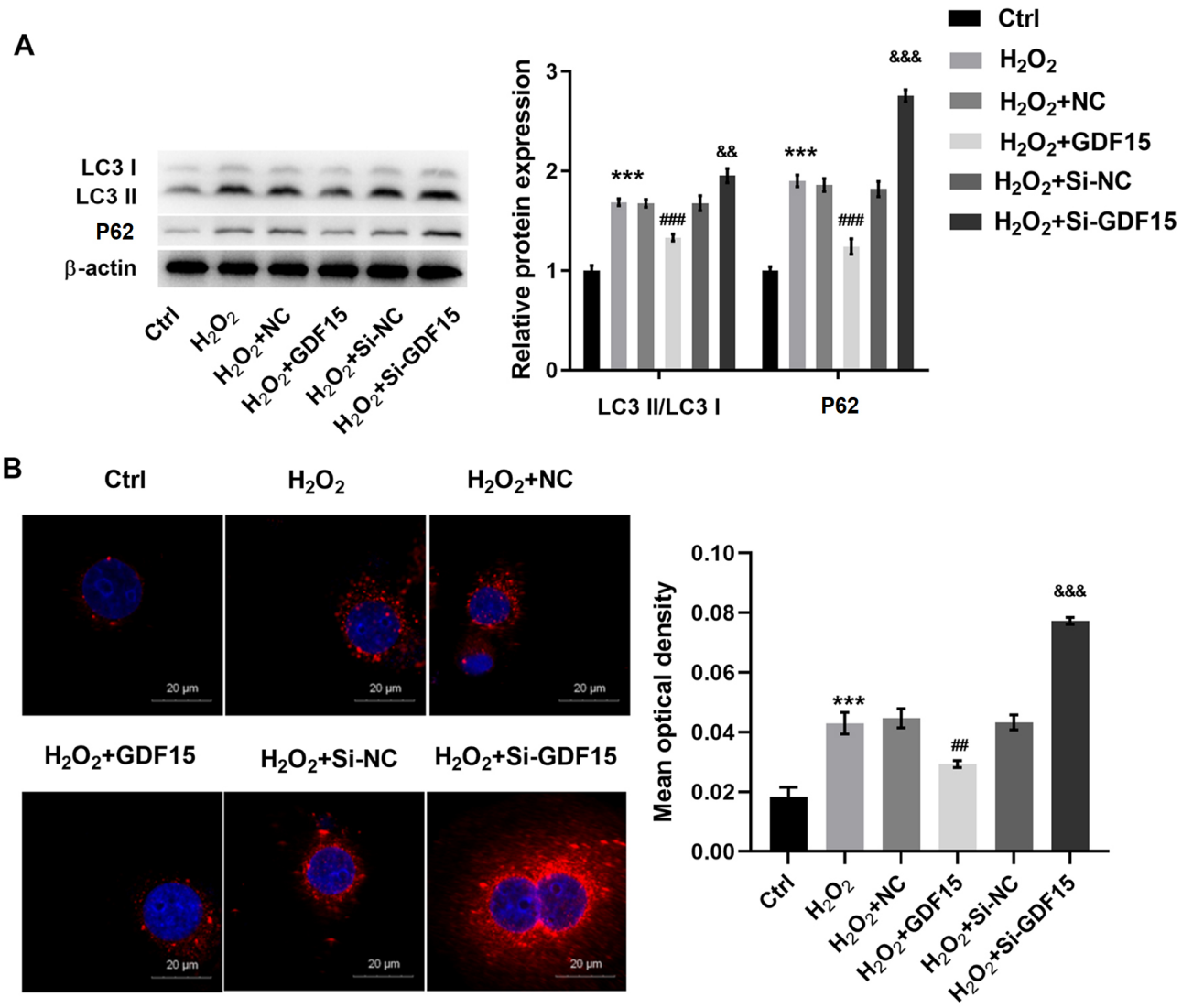

Fig. 3. Effects of GDF15 on the autophagic flux of H_2O_2-stimulated HEI-OC1 cells. GDF15 overexpression or knockdown plasmid was transfected into H_2O_2-stimulated HEI-OC1 cells. (A) Expression of autophagy-related proteins (LC3 I, LC3 II, p62) was detected by western blot analysis. (B) Immunofluorescence of LC3-positive autophagosomes (red fluorescence). Scale bar: 20 μm. $^{***}p < 0.001$ vs. the Ctrl group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 + NC group; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. the H_2O_2 + si-NC group. GDF15, growth differentiation factor 15; LC3, light chain 3; NC, negative control; si-NC, small interfering RNA- negative control.

in HEI-OC1 cells, which was evidenced by decreased anti-apoptotic protein (Bcl-2) expression, and increased pro-apoptotic protein (Bax and cleaved caspase-3) expression and apoptotic rate. For all original western blot figures of Fig. 2B see **Supplementary Material**. Furthermore, the results of GDF15 gain/loss-of-function studies demonstrated that GDF15 negatively regulated apoptosis in H_2O_2-induced HEI-OC1 cells. We also found that H_2O_2 treatment led to a decrease in SIRT1 expression, which was positively regulated by GDF15.

Next, the relationship between GDF15 and autophagic flux was further explored. As shown in Fig. 3A,B, H_2O_2 induced an increase in the LC3 II/LC3 I ratio and number of LC3-positive autophagosomes (Red fluorescence). For all original western blot figures of Fig. 3A see **Supplementary Material**. Furthermore, GDF15 overexpression decreased the LC3 II/LC3 I ratio and number of LC3-positive autophagosomes, whereas GDF15 silencing had the opposite effects on H_2O_2-induced HEI-OC1 cells. These data only showed that H_2O_2 treatment and GDF15 silencing promoted the formation of autophagosomes. To monitor autophagic flux, the level of p62 protein was also detected. Fig. 3A illustrates that H_2O_2 treatment and GDF15 silencing led to p62 accumulation in HEI-OC1 cells, corresponding with inhibition of autophagy activity, which is contradictory to the finding of increased LC3 II/LC3 I ratio and autophagosomes, suggesting autophagy disruption. The above findings indicate that H_2O_2 treat-

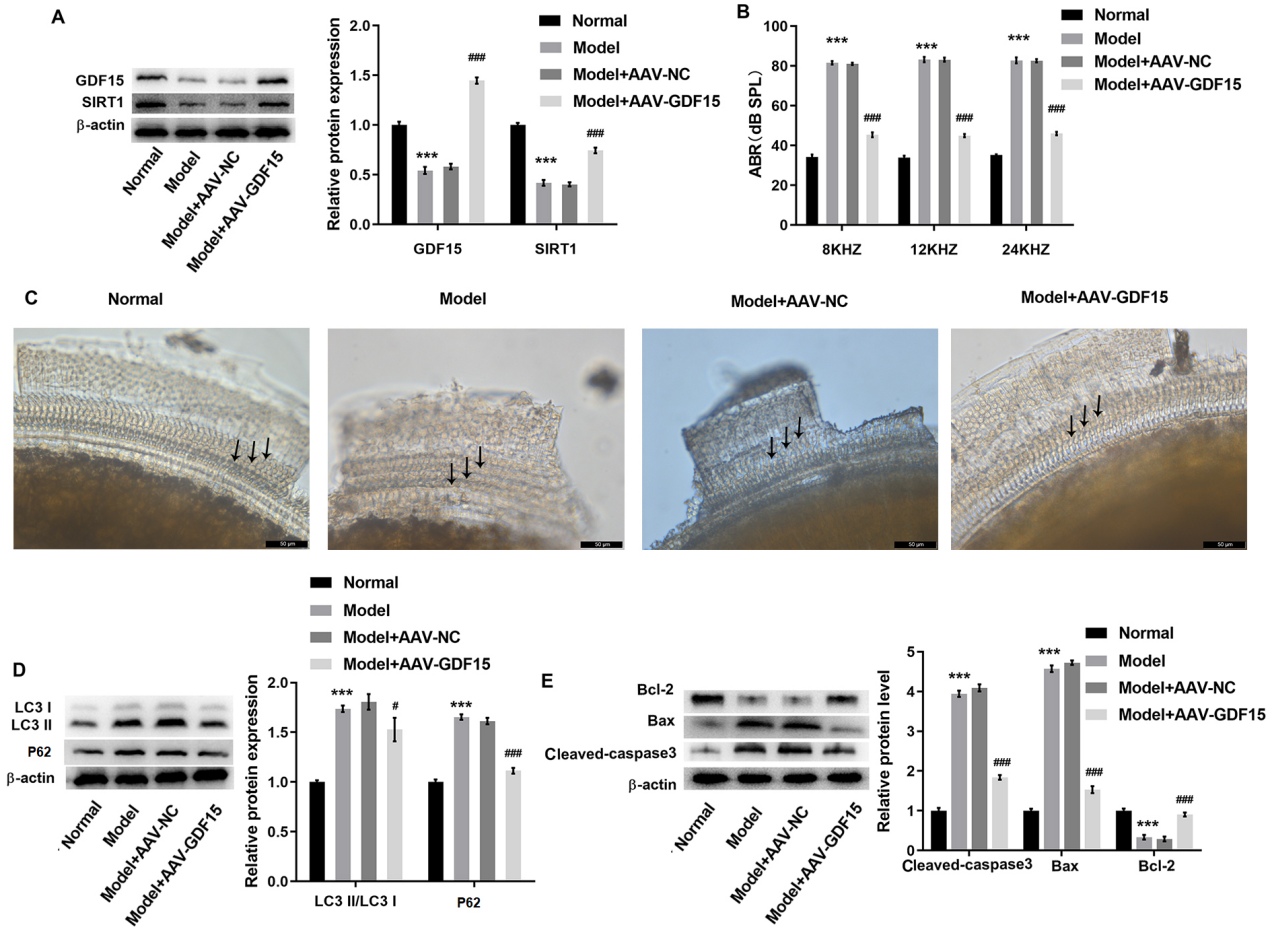

Fig. 4. Effects of GDF15 on a rat model of ARHL. A rat model of ARHL was established and treated with AAV-GDF15. (A) Western blotting was used to detect the protein expression of GDF15 and SIRT1. (B) ABR test. (C) Cochlear silver nitrate staining (Scale bar: 50 µm). The arrows show three rows of outer hair cells. Western blot analysis was used to detect the expression of autophagy (D) and apoptotic-related (E) proteins. $^{***}p < 0.001$ vs. the Normal group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. the Model + AAV-GDF15 group. GDF15, growth differentiation factor 15; ARHL, age-related hearing loss; AAV-GDF15, adeno-associated virus-growth differentiation factor 15; SIRT1, sirtuin 1; ABR, auditory brainstem response.

ment induced autophagic flux blockade in HEI-OC1 cells. Further gain/loss-of-function studies clarified that GDF15 overexpression restored autophagic flux, while GDF15 knockdown exacerbated autophagic flux blockade in H_2O_2-induced HEI-OC1 cells.

3.3 Effects of GDF15 on a Rat Model of ARHL

In view of the regulation of apoptosis and autophagic flux by GDF15, we established a rat model of ARHL to evaluate the protective effects of AAV-GDF15 on ARHL. Consistent with the *in vitro* experiments, GDF15 and SIRT1 expression was also downregulated in the cochlear tissues of ARHL rats (Fig. 4A), but upregulated after treatment with AAV-GDF15. For all original western blot figures of Fig. 4A,D,E see **Supplementary Material**. Next, we evaluated the hearing function of rats through ABR thresholds and observed OHCs using cochlear silver nitrate staining. Compared with the Normal group, ABR thresholds (Fig. 4B) were increased and there was great loss of OHCs in ARHL rats (Fig. 4C), indicating their weakened hearing function. Notably, treatment with AAV-GDF15 reduced the ABR thresholds and the loss of OHCs in ARHL rats. In addition, we used western blotting to measure the apoptosis and autophagy-related proteins. In ARHL rats, expression of the LC3 II/LC3I ratio, p62, and pro-apoptotic-related proteins (Bax and cleaved caspase-3) was increased (Fig. 4D), but expression of the Bcl-2 anti-apoptotic-related protein was decreased (Fig. 4E). These findings demonstrated that autophagic flux was blocked and apoptosis was upregulated in the cochlear cells of ARHL rats. However, these alterations were reversed after treatment with AAV-GDF15. Overall, these data suggest the protective effects of GDF15 overexpression on ARHL.

3.4 Effects of MSCs-GDF6 on a Rat Model of ARHL

Finally, MSCs were infected with lentivirus-containing GDF6 overexpression vectors, and the significant efficiency of GDF6 overexpression is illustrated in

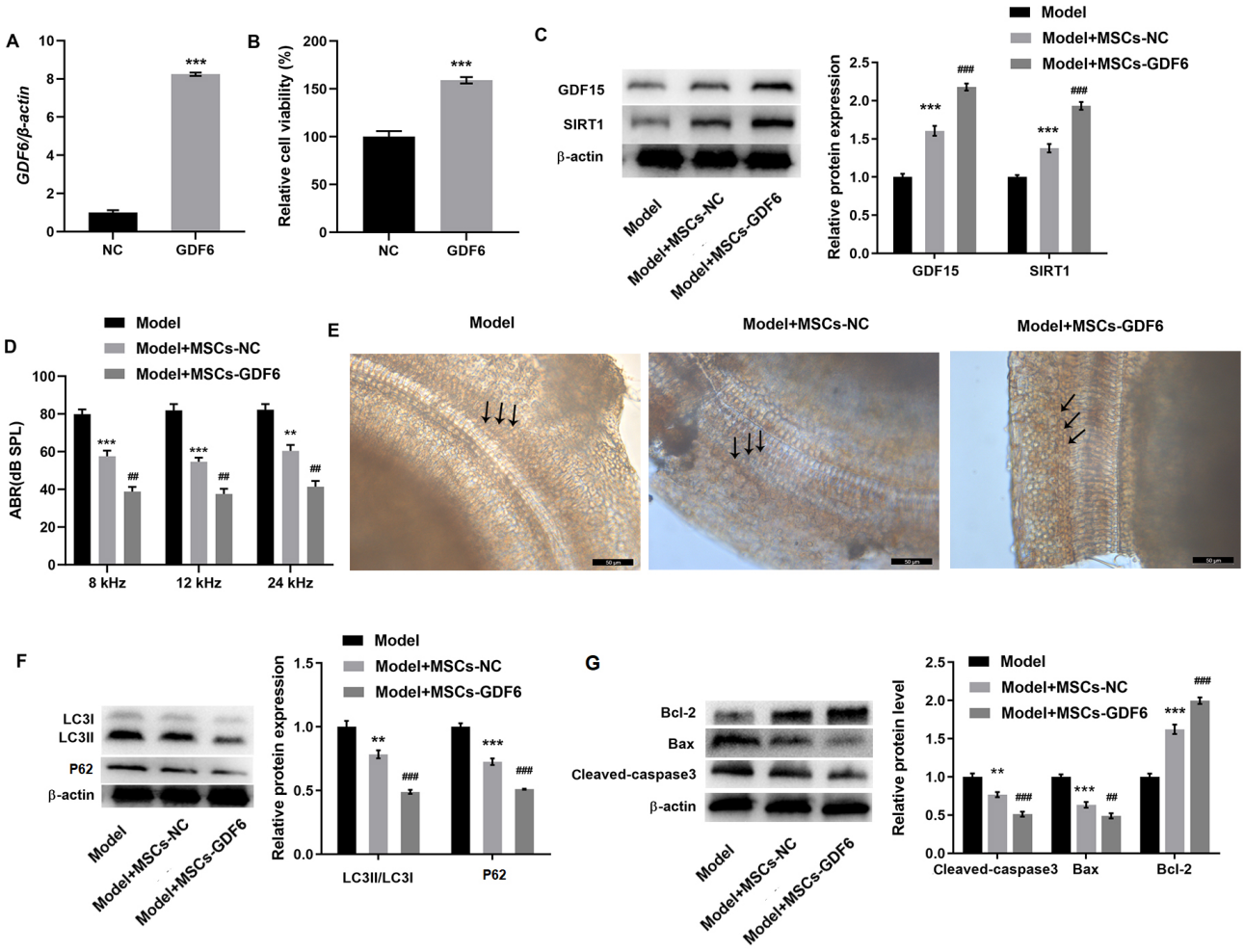

Fig. 5. Effects of MSCs-GDF6 on a rat model of ARHL. ARHL rats were treated with MSCs modified with GDF6 overexpression. (A) RT-qPCR was used to detect GDF6 overexpression efficiency. (B) The CCK-8 assay was used to analyze MSC proliferation. (C) Western blot analysis was used to detect the protein expression of GDF15 and SIRT1. (D) ABR test. (E) Cochlear silver nitrate staining (Scale bar: 50 μm). The arrows show three rows of outer hair cells. Western blotting was used to detect autophagy (F) and apoptotic-related (G) proteins. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the NC or Model group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the Model + MSCs-NC group. GDF6, growth differentiation factor; MSCs, mesenchymal stem cells; MSCs-GDF6, GDF6 overexpression-induced mesenchymal stem cells; ARHL, age-related hearing loss; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, cell counting Kit-8; SIRT1, sirtuin 1; ABR, auditory brainstem response; NC, negative control; MSCs-NC, mesenchymal stem cells-negative control.

Fig. 5A. The CCK-8 assay showed that GDF6 overexpression promoted MSC proliferation (Fig. 5B). Subsequently, ARHL rats were treated with MSCs-GDF6. Western blot analysis showed that MSC treatment increased GDF15 and SIRT1 expression in ARHL rats (Fig. 5C). As illustrated in Fig. 5D,E, MSC treatment reduced ABR thresholds, recovered the neatly arranged OHCs, and decreased the loss of OHCs in ARHL rats. In addition, autophagy-related proteins (LC3 II/LC3 I, p62) and pro-apoptotic-related proteins (Bax and cleaved caspase-3) were downregulated, while the Bcl-2 anti-apoptotic-related protein was significantly upregulated in ARHL rats treated with MSCs (Fig. 5F,G). For all original western blot figures of Fig. 5C,F,G see **Supplementary Material**. Interestingly, these outcomes were further enhanced after treatment with MSCs-GDF6. In general, MSCs-GDF6 prevented apoptosis and restored autophagic flux of cochlear HCs to alleviate ARHL.

4. Discussion

The pathological processes of ARHL include DNA damage, oxidative stress, inflammation, mitochondrial dysfunction, and mitochondrial DNA (mtDNA) mutations [9, 24]. Apoptosis is the main cause of loss of cochlear HCs, which can be induced by mtDNA mutations and oxidative stress-induced by reactive oxygen species (ROS) accumulation [2,25]. Liu *et al.* [26] showed by RNA sequencing that the upregulated pathways included apoptosis in the cochleae of aged mice. Another study revealed that H_2O_2-

induced oxidative stress led to the apoptosis of HEI-OC1 cells [27]. In line with our study, the elevated level of apoptosis was revealed in H_2O_2-induced HEI-OC1 cells and ARHL rats.

Activated autophagy helps remove damaged mitochondria, proteins, and other cellular components in response to oxidative stress and inflammation. Once autophagy flux is blocked, accumulation of mitochondria and proteins causes apoptosis, autophagy, or both [28]. Autophagy is a significant pathway in ARHL and may be regulated by ROS and inflammation [29]. Lv *et al*. [30] found activated autophagy in the cochlear HCs of aged C57BL/6 mice. Consistent with our results, the upregulated level of autophagy was observed in both H_2O_2-induced HEI-OC1 cells and the cochlea of ARHL rats, along with increased levels of autophagy-related proteins (LC3 II/LC3I) and LC3-positive autophagosomes. On the other hand, p62 acts as another marker to monitor autophagic activity as its degradation depends on the autophagic pathway. The upregulated p62 indicated that autophagic flux was blocked in H_2O_2-induced HEI-OC1 cells and the cochlea of ARHL rats. Given these findings, targeting apoptosis or autophagy is considered an effective approach to prevent the loss of cochlear HCs.

GDF15 is a stress response gene, and its aberrant expression is correlated with cellular stress and mitochondrial dysfunction in age-associated diseases [19,31]. Our findings showed that GDF15 expression was markedly downregulated in H_2O_2-induced HEI-OC1 cells and the cochlea of ARHL rats. Increasing evidence indicated that GDF15 has a regulatory role in apoptosis and autophagy. A study by Zou *et al*. [32] demonstrated that engineered exosomes with GDF15 inhibited apoptosis and promoted autophagy via activation of the AMP-activated protein kinase (AMPK) signaling pathway to prevent myocardial injury. Chen *et al*. [33] suggested that the absence of GDF15 could enhance autophagy and inhibit inflammation to alleviate chronic glomerulonephritis. In our study, the gain/loss-of function studies of GDF15 were conducted *in vitro* to determine the effects of GDF15 on the apoptosis/autophagy of cochlear HCs. The results showed that GDF15 overexpression inhibited apoptosis and restored autophagic flux in H_2O_2-induced HEI-OC1 cells, which was further evidenced by *in vivo* experiments with AAV-GDF15 treatment. It is worth noting that GDF15 had a positive regulatory effect on SIRT1 expression *in vitro*.

SIRT1 is a class III histone deacetylase that depends on NAD (+). It has been considered a potential therapeutic target for ARHL due to its anti-aging, anti-inflammatory, and antioxidant effects [34]. Previous studies have shown the lower expression of SIRT1 in aged mice compared to young mice [35,36]. In accordance with our results, a decrease in SIRT1 protein expression was observed in H_2O_2-induced HEI-OC1 cells and the cochlea of ARHL rats, which was upregulated by GDF15 overexpression. Previous studies have indicated the inhibitory effects of SIRT1 overexpression on apoptosis in HEI-OC1 cells [37,38]. On the other hand, activation of SIRT1 can enhance AMPK activity [34], which is an important effector in cell autophagy. Pang *et al*. [35] showed that SIRT1 upregulation restored autophagy in HEI-OC1 cells. Together these findings led us to conclude that GDF15 overexpression inhibits apoptosis and restores autophagic flux via upregulation of SIRT1 to relieve ARHL.

In light of the therapeutic effects of MSCs by inducing HC regeneration, differentiation, and proliferation in hearing loss [39], the therapeutic potential of MSCs in ARHL was investigated. First, GDF6 overexpression was found to promote MSC viability, suggesting that MSCs overexpressing GDF6 may have better efficacy. Subsequently, ARHL rats were treated with MSCs and MSCs-GDF6, with the results indicating that both MSCs and MSCs-GDF6 could reduce apoptosis and restore autophagic flux to ameliorate loss of HCs. Moreover, more significant effects were observed in ARHL rats treated with MSCs-GDF6. Notably, GDF15 and SIRT1 protein expression was markedly increased in ARHL rats treated with MSCs or MSCs-GDF6. As MSCs can be induced to secrete GDF15 [40,41], we propose that MSCs-GDF6 can exert their therapeutic effects by inhibiting apoptosis and restoring autophagic flux to prevent loss of HCs, thereby relieving ARHL, likely in association with upregulation of the GDF15/SIRT1 axis.

5. Conclusions

To sum up, the results of this study demonstrated that GDF15 upregulation could relieve ARHL by inhibiting apoptosis and restoring autophagic flux. In addition, MSCs-GDF6 treatment promoted the expression of GDF15 in ARHL rats. Importantly, MSCs-GDF6 treatment prevented loss of HCs to relieve ARHL by inhibiting apoptosis and restoring autophagic flux, likely in association with upregulation of the GDF15/SIRT1 axis (**Supplementary Fig. 1**). This study highlights the therapeutic effects of MSCs-GDF6 on loss of HCs, providing a novel strategy for ARHL treatment.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

JL, HP designed the research study and analyzed the data. JL HP, YL, CL, WX performed the research. WX provided help and advice on experimental methods, wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal protocol in this study was approved by the Institutional Animal Care and Use Committee in Nanchang University (No. 20221031014).

Acknowledgment

Not applicable.

Funding

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL26179.

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