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Marginal Cell Damage in the Inner Ear of Mtdna4834-Deficient Rats is Associated with Methylation-Induced Downregulation of SOD2

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Abstract

Aging-related hearing deficiency is the most prevalent sensory disturbance in the aging population and is associated with the mitochondrial (mt)DNA4977 deletion (corresponding to mtDNA4834 deletion in rats). Superoxide dismutase 2 (SOD2), a key factor involved in aging, is located in the mitochondrial matrix and is related to DNA mutation. The present study investigated the relationship between SOD2 and aging-related mtDNA4834 deletion in rats. SOD2 expression was evaluated in marginal cells (MCs) isolated from the inner ear of rats subjected to D-galactose-induced aging-related mtDNA4834 deletion. SOD2 was downregulated in mtDNA-deficient MCs, and the process was associated with methylation of the SOD2-encoding gene. In addition, overexpression of SOD2 in mtDNA4834-deleted MCs resulted in increased cell viability and reduced apoptosis, as demonstrated by the upregulation of anti-apoptotic proteins and the downregulation of pro-apoptotic proteins. SOD2 overexpression suppressed mtDNA4834 deletion mutation and increased the copy number of mtDNA4834, while SOD2 silencing resulted in the opposite effect. The findings indicate that aging-related mtDNA4834 deletion is associated with SOD2 deficiency, implicating that SOD2 is a potential candidate for the treatment of mtDNA4977 deletion-related presbycusis.

Keywords: Presbycusis • SOD2 • Methylation • mtDNA4834 deletion • Apoptosis

Introduction

Aging is a natural and irreversible process generally defined as the accumulation of senescent cells and results in physiological dysfunction [1,2]. Aging-related hearing deficiency, or presbycusis, is the most prevalent sensory disturbance among the aging population [3,4]. More than 60% of people over 70 years of age suffer from some form of hearing disorder in the United States [5]. As a multifactorial disease, presbycusis is associated with a combination of medical history, environmental factors, and mitochondrial genetic variation [6,7]. From pathological observations, presbycusis has been classified into several categories based on variable amounts of degeneration of neurons, stria vascularis, and auditory receptors [8-10]. Among these, strial presbycusis has attracted much attention [11-13]. Damage to the stria vascularis has been correlated with marginal cell (MC) dysfunction, which is considered to be the origin of lesions in strial presbycusis. Thus, MCs have been highlighted in presbycusis research [2,9].

Mitochondria play a vital role in the development of presbycusis [14]. As the only extranuclear genetic material, mitochondrial DNA (mtDNA) is prone to mutations because of its unique genetic characteristics. Numerous clinical and experimental studies have revealed the strong relationship between presbycusis and mtDNA variation [15-18]. The 4977-bp deletion in human mtDNA (corresponding to the 4834-bp mtDNA deletion in rats), known as a common deletion, is widely found in many mitochondrial diseases and in normal aging tissues [2,19]. The mtDNA4977 deletion has been detected in presbycusis patients, suggesting the relationship between the common deletion and aging-related hearing conditions [6,20,21]. However, the specific mechanism through which the mtDNA4977 deletion participates in the pathogenesis of aging-related hearing disorders remains to be elucidated.

Superoxide dismutase 2 (SOD2) is a key factor of aging that is located only in the mitochondrial matrix [22,23]. Mitochondrial dysfunction leads to accelerated aging and associated diseases [24]. Cells exhibit functional mitochondrial defects in response to SOD2 deficiency [25,26], and SOD2 deficiency has been shown to increase the level of mutagenic DNA lesions [27,28]. However, whether there is a relationship between SOD2 and the mtDNA4834 deletion in aging-related hearing loss remains unknown. Based on our previous study, a rat model of inner ear senescence was established using D-galactose to mimic the aging-related mtDNA4834 deletion mutation [29]. The downregulation of SOD2 was confirmed in extracted MCs and was demonstrated to be related to the methylation of the SOD2-encoding gene. In addition, SOD2 was overexpressed or inhibited to investigate its effect on MC damage in mtDNA4834-deficient rats.

Experimental

Animal model construction

Eighteen one-month-old healthy Wistar rats (80-100 g) were purchased from the Laboratory Animal Centre of Huazhong Agricultural University. The animals were housed in a specific-pathogen-free environment under normal rat rearing conditions. The rats were randomly divided into control (CTRL, no treatment) and model (MOD, treated with D-galactose) groups (n = 9 per group). Rats in the CTRL group were injected every day with 0.9% saline (150 ml/kg) for 8 weeks, followed by intraperitoneal saline injection for 10 days. In the MOD group, the mtDNA4834 deletion was induced following previously reported protocols [29]. Briefly, rats in the MOD group were subcutaneously injected every day with 0.5% D-galactose (150 mg/kg; Sinopharm Group Co., Ltd., Beijing, China) for 8 weeks, followed by intraperitoneal injection of saline for 10 days. All animal experiments were approved by the institutional review board and performed under the Guidelines for Animal Care and Use of the

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Model Animal Research Institute at Wuhan Myhalic Biotechnology Co., Ltd. (approval number: HLK-20180620-01).

Polymerase chain reaction (PCR)

Rats in the CTRL and MOD groups were sacrificed under anesthesia using pentobarbital sodium at 110 mg/kg of body weight, and the bilateral auditory vesicles were removed and placed in pre-cooled (4°C) phosphate-buffered saline (PBS, pH = 7.4; Bioswamp, Myhalic Biotechnology Co., Ltd., Wuhan, China). Inner ear tissue containing spiral ligament, semicircular canals, basilar membrane, saccule, and utricle were collected under guidance of a microscope. Total DNA was extracted from the obtained tissue using TRIzol reagent (TaKaRa Bio, Dalian, China) following the manufacturer's instructions. Amplification was performed at 95°C for 3 min; 39 cycles of denaturation at 95°C for 5 s, annealing at 56°C for 10 s, and extension at 72°C for 25 s; and final extension at 65°C for 5 s and 95°C for 50 s using a GE48527 apparatus (Bio-Gener Technology Co., Ltd., Hangzhou, China). The following primers of the conserved 770-bp fragment of total mtDNA were used: forward, 5'-AGGACTTAACCAGACCCAAACACG-3' and reverse, 5'-CCTCTTTTCTGATAGGCGGG-3'. The mtDNA4834 deletion mutation was evaluated using nested PCR by forward (5'-GCGAAGCTTAGAGCGTTAAC-3') and reverse (5'-AGTGAGATAAGGAAGCCTGC-3') primers to amplify a 614-bp product, which was used as a template for subsequent PCR using forward (5'-TTTCTTCCCAAACCTTTCCT-3') and reverse (5'-AAGCCTGCTAGGATGCTTC-3') primers to obtain a 459-bp product corresponding to the 4834-bp deleted mtDNA [30,31]. The amplified 459-bp and 770-bp fragments were electrophoresed on 3% agarose gels for detection of the mtDNA4834 deletion mutation.

MC extraction and culture

MCs were extracted according to a previously reported method [32]. Briefly, rats in the CTRL and MOD groups were sacrificed under anesthesia using pentobarbital sodium at 110 mg/kg of body weight and the bilateral auditory vesicles were removed and placed into D-Hanks solution. The cochlear stria vascularis with or without spiral ligament was isolated with microscopy guidance and cut into small pieces of approximately 0.5 mm × 0.5 mm × 0.5 mm. The pieces were placed into a Petri dish and digested with 0.1% collagenase II for 40 min, followed by centrifugation for 5 min at 1000 × g. Then the cells were cultured with serum-free minimum essential medium-alpha (MEM-α Hyclone, Logan, UT, USA) containing 2 mmol of L-glutamine (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin-amphotericin B solution (Bioswamp, Wuhan, China) for 1 h in a polylysine-coated 6-well plate. Finally, the obtained cells were incubated in MEM-α containing 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. Dead and non-adherent cells were removed by replacing the culture medium after 12 h of culture and every three days thereafter. Cell morphology was observed under microscopy guidance (Nikon, Tokyo, Japan) and MCs were identified by cytokeratin-18, a characteristic marker of MCs [33,34], using flow cytometry and immunofluorescence.

Immunofluorescence

Immunofluorescence was performed to identify MCs and evaluate the expression of SOD2. The cells were washed twice with PBS for 2 min each, followed by fixation in 4% paraformaldehyde for 30 min at room temperature and three washes with PBS for 3 min each. The cells were immersed in 0.5% Triton X-100 (Bioswamp) for 20 min and blocked with 5% bovine serum albumin for 1 h at 37°C. The cells were then incubated with primary antibodies against cytokeratin 18 (ab133263, 1:100 dilution; Abcam, Cambridge, UK) and SOD2 (ab13533, 1:100 dilution; Abcam) overnight in a humidified chamber at 4°C and then incubated with Alexa Fluor 594-conjugated goat anti-rabbit antibodies (PAB160018, 1:200 dilution; Bioswamp) in a humidified chamber at 37°C for 30 min. 4',6-Diamidino-2-phenylindole (DAPI; Bioswamp) was added to stain the nuclei, after which the cells were observed using an inverted fluorescence microscope (Leica, Wetzlar, Germany).

SOD activity assay

Supernatants from inner ear tissue were collected to evaluate the activity of SOD using a Total Superoxide Dismutase assay kit (hydroxylamine method) according to the manufacturer's instructions (A001-1-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Methylation assay

The methylation status of SOD2 in MCs extracted from rats was analyzed by methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP). BSP was performed to determine the methylation condition of CpG island sequences of SODS. While MSP was performed to evaluate the methylation condition of SOD2 gene CpG island [35,36]. Briefly, Genomic DNA was extracted using the TIANamp Genomic DNA kit (DP304; Tiangen Biotech Co., Ltd., Beijing, China), followed by bisulfite conversion using a DNA Bisulfite Conversion Kit (DP215; Tiangen Biotech). The converted DNA (75 ng) was then subjected to amplification. For MSP, the unmethylated (U) and methylated (M) sequences were as follows: SOD2-U forward, 5'- AAGATGAATATATTTAAATTTGTGA-3' and reverse, 5'- AAACCCTACTACTAATCAAAACCATAACA-3'; SOD2-M forward, 5'- AAGATGAATATATTTAAATTTGCGA-3' and reverse, 5'-CCTACTACTAATCAAAACCATAACGTA-3'. For BSP, the SOD2 primers were as follows: forward, 5'-TAAGTGAGTTAGAAGGATTTTGA-3' and reverse, 5'-TATACTCCACCCTCAAACTAAACC-3'. The reaction procedure was performed at 95°C for 5 min; 30 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 10 s; extension at 72°C for 10 s; and final extension at 72°C for 5 min using a T100-Thermal Cycler apparatus (Bio-Rad, Hercules, CA, USA).

Quantitative reverse transcription PCR (qRT-PCR)

The mRNA expressions of SOD2 and DNA methyltransferase 1 (Dnmt1) were detected using qRT-PCR. TRIzol (Ambion, Austin, TX, USA) was used to extract total RNA and DNA was eliminated by DNase I (Fermentas, Waltham, MA, USA). The isolated RNA was reversed-transcribed into cDNA using the M-MuLV kit (TaKaRa Bio). The cDNA (25 ng) was amplified using a CFX-CONNECT 96 apparatus (Bio-Rad) with the following conditions: 95°C for 3 min; 39 cycles of denaturation at 95°C for 5 s, annealing at 56°C for 10 s; extension at 72°C for 25 s; and final extension at 65°C for 5 s and 95°C for 50 s. The primer sequences were as follows: SOD2 forward, 5'-ATTGCCGCCTGCTCTA-3' and reverse, 5'-CTCCCAGTTGATTACATTCC-3'; Dnmt1 forward: 5'-ACTGCGTCTCGGTCATT-3' and reverse, 5'-AGAGGGTCAGAGGTGGC-3'; GAPDH (internal control) forward, 5'-CAAGTTCAACGGCACAG-3' and reverse, 5'-CCAGTAGACTCCACGACAT-3'. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the relative mRNA expression levels [37].

Western blot

Proteins were extracted using radioimmunoprecipitation assay lysis buffer (Bioswamp) and quantified using a bicinchoninic acid assay kit (Bioswamp). Total protein (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk for 2 h at room temperature and incubated overnight at 4°C with primary antibodies, followed by incubation with secondary antibodies at room temperature for 1 h. The membranes were visualized using a model 5200 apparatus (Tanon, Shanghai, China) and the relevant band gray values were read using TANON GIS software (Tanon). Antibody information is provided in Table 1.

Cell transfection

Small-interfering RNA (siRNA) and negative control (si-NC, non-targeting) for SOD2 were designed and synthesized by Ribobio Co., Ltd. (Guangzhou, China) with the following sequences: siRNA1, 5'-GCCTGCACTGAAGTTCAAT-3'; siRNA2, 5'-GCAAGGTCGCTTACAGATT-3'; siRNA3, 5'-GGAGCACGCTTACTATCTT-3'; and si-NC, 5'-GGACGCACATTTATCGCTT-3'. To overexpress SOD2, the SOD2 cDNA fragment (forward: 5'-CTCTAGATGTTGTGTCGGGCG-3', reverse: 5'-GGAATTCTCACTTCTTGCAAACTAT-3') was cloned into pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vectors (Addgene, Watertown, MA, USA) to generate pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro-SOD2. Cells extracted from rats in the MOD group (1×10^6 cell/ml) were transfected with siSOD2, si-NC (siSOD2-

Table 1. Antibodies used in western blot.

Antibody	Species	Company	Product code	Dilution ratio	Protein size (kDa)
Primary antibodies					
Cleaved caspase 3	rabbit	Abcam	Ab2302	1:1000	17
Caspase 3	rabbit	Abcam	Ab13847	1:1000	17
Bcl-2	rabbit	Abcam	Ab196495	1:1000	26
Bax	rabbit	Abcam	Ab182733	1:2000	21
Cyt-c	rabbit	Abcam	Ab133504	1:5000	14
Dnmt1	rabbit	Abcam	Ab188453	1:1000	183
GAPDH	rabbit	CST	2118	1:1000	37
Secondary antibody					
Goat anti-rabbit IgG	goat	Bioswamp	SAB43658	1:10000	

NC), pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro-SOD2 (SOD2-OE), or empty pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro (termed SOD2-OE-NC) using Lipofectamine 2000 (Invitrogen, Valencia, CA, USA) following the manufacturer's instructions. Non-transfected MCs with (MOD) or without (CTRL) mtDNA4834 deletion served as controls for transfection. The transfection rate was detected by qRT-PCR after 48 h of transfection and the expression of apoptosis-related proteins was measured by western blot (Table 1). The mtDNA3843 copy number was evaluated by qRT-PCR as mentioned above with the following primer sequences: mtDNA forward, 5'-GCCCACATAGGATGAATAA-3' and reverse, 5'- ATTGGAACAGTAAGTAGGATG-3'; cytochrome c oxidase subunit 4 (internal control) forward, 5'-GCTTTCCCCACTTACGCTG-3' and reverse, 5'-TTGGTGCCCCTGTTCATCT-3'.

Viability assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was performed to detect the viability of transfected cells. Cells in the logarithmic growth phase (5×10^3 cells/well, 180 µl) were seeded into 96-well plates and cultured at constant humidity at 37°C in an atmosphere containing 5% CO_2 overnight. After 24, 48, and 72 h of transfection, the cells were treated with 20 µl of MTT reagent (5 mg/ml) for 4 h, followed by the addition of 150 µl of dimethyl sulfoxide. The absorbance was measured using a microplate reader (Thermo Fisher Scientific, Shanghai, China) at 490 nm after 10 min of shaking.

Flow cytometry

Flow cytometry was carried out to identify MCs, detect cell apoptosis, and assess the mitochondrial membrane potential of the transfected cells. For the identification of MCs, 100 µl of cells at a concentration of 1×10^6 cell/ml were incubated with 2 µl of antibody against cytokeratin 18 (ab133263; Abcam) for 45 min at 4°C. After centrifugation, the cells were resuspended in binding buffer (Bioswamp) and cultured with fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit (Bioswamp) in the dark for 30 min at 4°C and then subjected to flow cytometry (Beckman Coulter, Brea, CA, USA). To evaluate apoptosis, the Annexin V-FITC/propidium iodide (PI) assay (BD, Shanghai, China) was carried out according to the manufacturer's protocol. After 48 h of transfection, 1×10^5 harvested cells were resuspended in 200 µl of binding buffer (BD). The cells were incubated with 10 µl of Annexin V-FITC and 10 µl of PI in the dark for 30 min at 4°C and subjected to flow cytometry. The mitochondrial membrane potential assay was performed according to the instruction of the corresponding assay kit (PAB180068; Bioswamp). The harvested cells (5×10^5) were stained with 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1) solution for 20 min at 37°C, followed by centrifugation at 600 × g for 4 min at 4°C. The cells were resuspended in JC-1 staining buffer and centrifuged at 600 × g at 4°C for 4 min. This was repeated twice and the cells were resuspended in JC-1 staining buffer and subjected to flow cytometry.

Statistical analyses

The data are presented as the mean ± standard deviation. Differences between more than two groups were analyzed using one-way analysis of variance followed by least significant difference or Dunnett's test and those between two groups were analyzed using t-test. A p-value < 0.05 was considered to be statistically significant.

Results

Detection of mtDNA4834 deletion mutation in inner ear of rats

The PCR results in Figure 1 demonstrated that the 770-bp conserved mitochondrial DNA fragment, which represents total mtDNA, was amplified in the inner ear tissues isolated from both CTRL and MOD rats, indicating the successful extraction of the mtDNA template. Additionally, the 459-bp product corresponding to the 4834-bp mtDNA deletion was only amplified in inner ear tissues extracted from MOD rats, as demonstrated by the presence of bands on the MOD gel. These observations indicated that CTRL rats did not exhibit the mtDNA4834 deletion mutation, whereas the administration of D-galactose induced this mutation and marked the successful establishment of the aging rat model of inner ear senescence.

Isolation of MCs

Successful isolation of MCs was confirmed by conventional microscopy based on the typical morphology with pleomorphic growth patterns and clear boundaries (Figure 2A). As cytokeratin-18 is a characteristic marker of MCs [34,38], the purity of MCs was verified using flow cytometry by assessing the proportion of cells that were cytokeratin 18-positive, which exceeded 90% (Figure 2B). Immunofluorescence was also performed to detect the positive staining of cytokeratin 18. Isolated cells showed strong positive signal of cytokeratin 18, which was stained red (Figure 2C). The collective data revealed that MCs were successfully isolated from the inner ear of the CTRL and MOD rats.

Expression of SOD2 in mtDNA4834-deleted MCs

As shown in Figure 3, compared to control MCs, the expression of SOD2 in D-galactose-treated cells was downregulated, as demonstrated by the significant decreases in the mRNA expression of SOD2 ($p < 0.01$) (Figure 3A) in the supernatant. The SOD activity in the supernatant was also decreased ($p < 0.01$) (Figure 3B). In addition, immunofluorescence revealed weak positive staining of signals in the MOD cells compared to the signals in CTRL cells (Figure 3C).

Methylation of the SOD2-encoding gene in mtDNA4834-deleted MCs

As shown in Figure 4, MSP detection revealed the methylation of SOD2 in mtDNA4834-deleted MCs, as demonstrated by the amplification of the methylation primer (Figure 4A). BSP was then performed to detect the methylation of CpG sites within the SOD2 gene. MCs treated with D-galactose showed a higher proportion of methylated CpG sites than that in untreated MCs (Figure 4B). Furthermore, the expression of Dnmt1, a protein associated with DNA methylation, was measured using qRT-PCR and western blot. The mRNA ($p < 0.01$) and protein ($p < 0.01$) expression of Dnmt1 were significantly increased in the MOD group compared to those in the CTRL group (Figure 4C and 4D). These results indicated that the methylation level of the SOD2-encoding gene was increased in mtDNA4834-deleted MCs compared to that in untreated MCs.

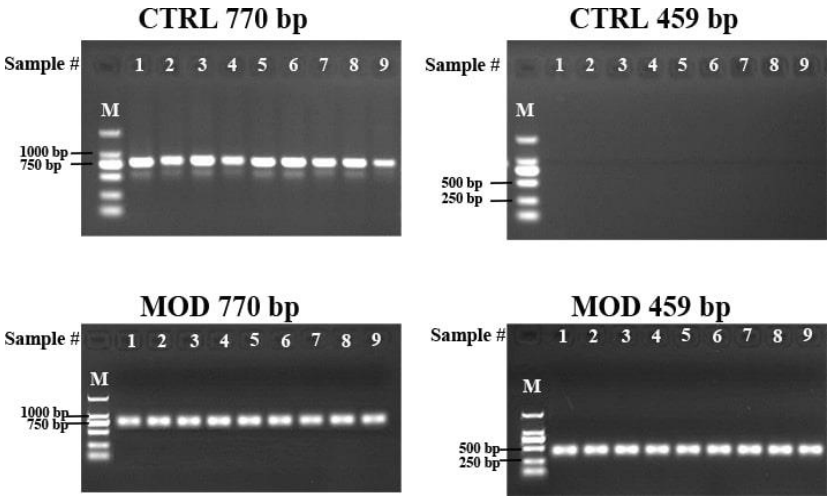

Figure 1. Graphical representation of comparison of Duncan Ely's Pre and Post Test.

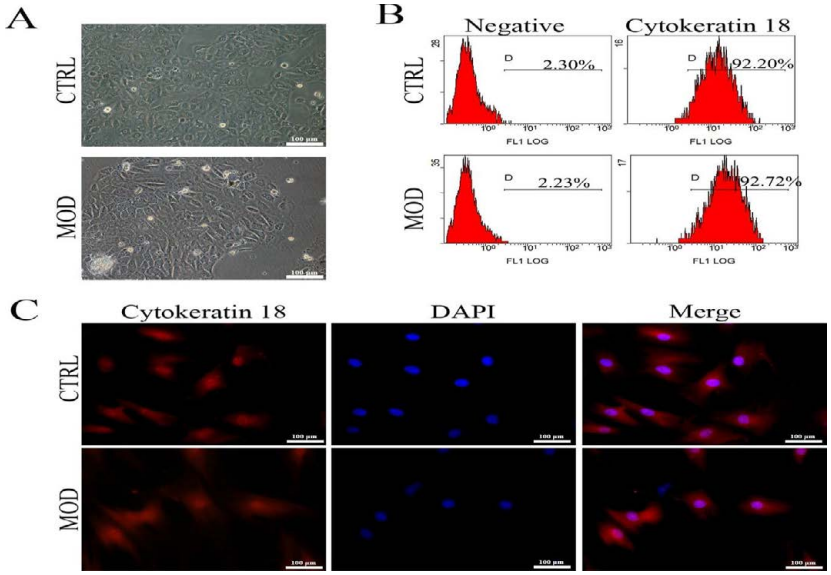

Figure 2. Characterization of MCs extracted from inner ear of rats with or without mtDNA4834 deletion mutation. (A) Morphology of MCs (scale bar = 100 μm). (B) Flow cytometry detection of the proportion of cytokeratin 18-positive cells in control and model MCs. (C) Immunofluorescence staining of cytokeratin 18 in control and model MCs (scale bar = 100 μm). CTRL represents control; MOD represents model.

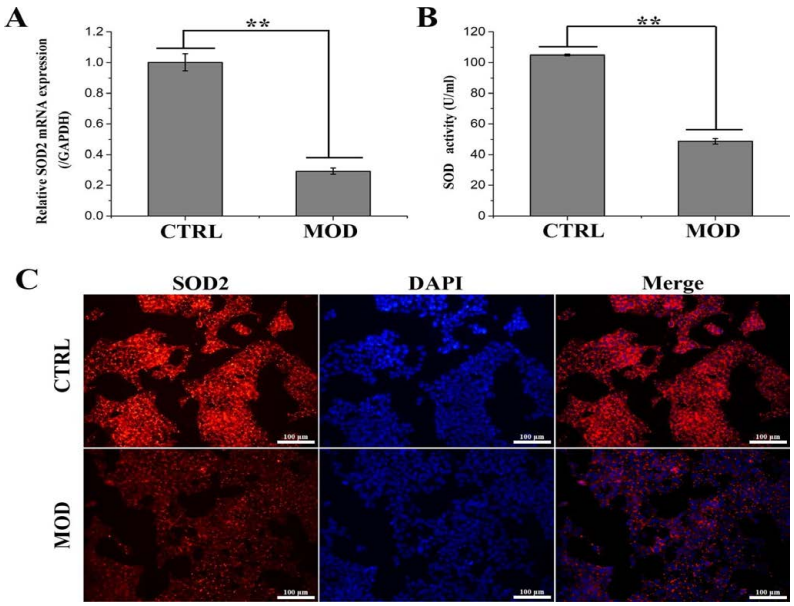

Figure 3. SOD2 expression in MCs. (A) qRT-PCR detection of relative mRNA expression of SOD2. (B) Detection of SOD activity in the supernatant of inner ear using corresponding assay kit. (C) Immunofluorescence staining of SOD2 in control and model MCs (scale bar = 100 μm). Data represent the mean ± SD (N = 3). ** denotes $p < 0.01$. CTRL represents control; MOD represents model.

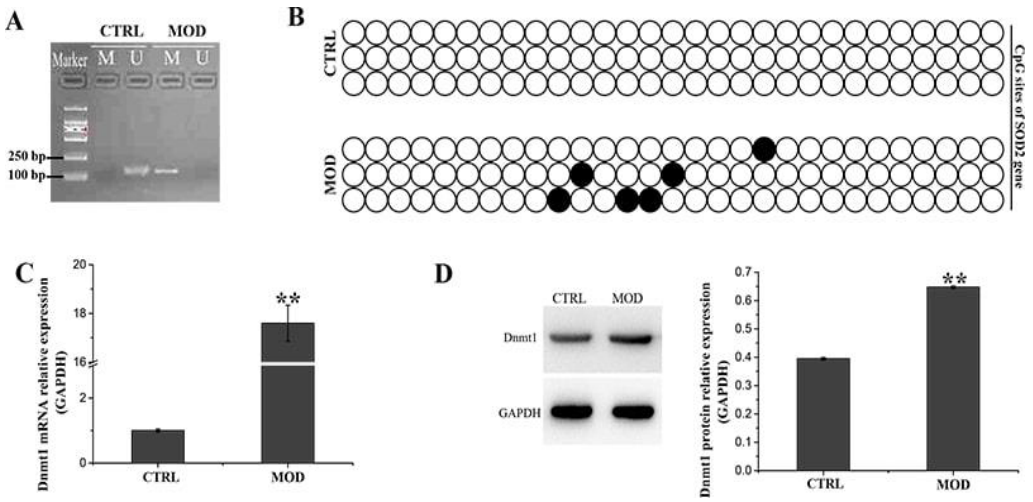

Figure 4. Methylation of the SOD2-encoding gene. (A) Methylation-specific PCR of the SOD2-encoding gene in control and model MCs. M represents methylated and U represents unmethylated. (B) Bisulfite sequencing PCR of the methylation level of the SOD2 gene promoter region in control and model MCs. Methylated and unmethylated CpG sites are shown as black and white dots, respectively. (C) qRT-PCR detection of the mRNA expression of Dnmt1 in control and model MCs. (D) Western blot detection of the protein expression of Dnmt1 in control and model MCs. Data represent the mean ± SD (N = 3). ** denotes $p < 0.01$. CTRL represents control; MOD represents model.

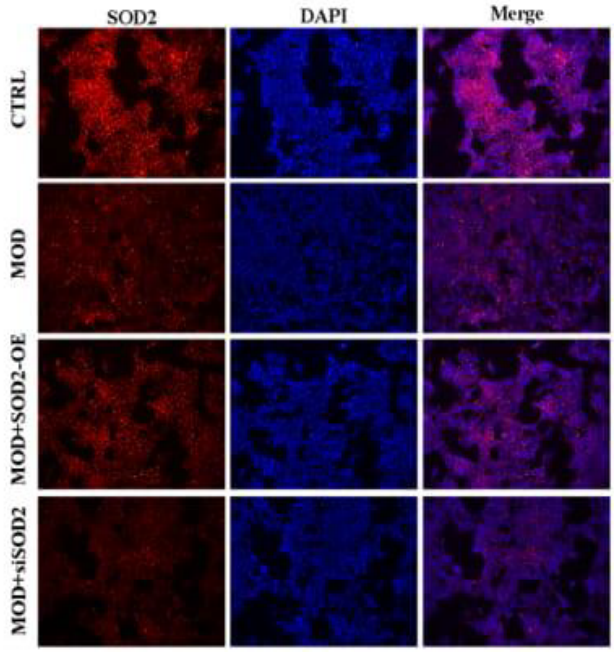

Figure 5. Immunofluorescence staining of SOD2 in mtDNA4834-deleted MCs after transfection. CTRL represents control; MOD represents model. OE represents overexpression.

Effect of SOD2 on apoptosis of mtDNA4834-deleted MCs

The positive expression of SOD2 in MCs after transfection was detected by immunofluorescence staining. As shown in Figure 5, MOD cells showed weak positive SOD2 staining compared to that in CTRL cells. However, SOD2-overexpressing cells showed strong positive SOD2 signals, whereas SOD2-silenced cells showed weak positive SOD2 signals compared those in MOD cells. Additionally, the mRNA expression of SOD2 was measured by qRT-PCR to evaluate the plasmid transfection efficiency. Compared to the SOD2 expression in the MOD group, SOD2 was significantly upregulated in the SOD2-OE group ($p < 0.01$) and significantly downregulated in the siSOD2 group ($p < 0.01$), confirming the successful transfection of plasmids (Figure 6A). Among the three siRNAs tested, siSOD2-2 was selected for subsequent experiments as it induced the lowest expression of SOD (Figure 7). In addition, the empty plasmid (NC groups) showed no effect on SOD2 expression (Figure 7 and Figure 8). Compared to the CTRL group, cell viability was decreased in the MOD group ($p < 0.01$). Compared to MOD cells, SOD2 overexpression increased the viability of mtDNA4834-deleted MCs ($p < 0.01$), while SOD2 inhibition decreased cell viability (Figure 6B). Flow cytometry demonstrated that compared to the CTRL group, apoptosis was promoted in the MOD group ($p < 0.01$); compared to MOD cells, SOD2 overexpression suppressed the apoptosis of mtDNA4834-deleted MCs, while SOD2 inhibition accentuated it (Figure 6C and 6D).

Next, the expression of apoptosis-related proteins was examined using western blot. Compared to the CTRL group, the pro-apoptotic proteins cleaved caspase 3, Bax, and cytochrome c (cyt-c) were significantly upregulated ($p < 0.01$) and the anti-apoptotic protein Bcl-2 was significantly downregulated in MOD group ($p < 0.01$). Compared to the MOD group, the protein levels of cleaved caspase 3, Bax, and cyt-c were significantly reduced ($p < 0.01$) and that of Bcl-2 was significantly elevated ($p < 0.01$) by SOD2-OE. On the contrary, cleaved caspase 3, Bax, and cyt-c were significantly upregulated ($p < 0.01$) and Bcl-2 was significantly downregulated ($p < 0.01$) by siSOD2 (Figure 9), which was consistent with the results of flow cytometry (Figure 6). These results demonstrated that SOD2 overexpression inhibited apoptosis of mtDNA4834-deleted MCs.

Effect of SOD2 on the mtDNA4834 deletion mutation in mtDNA4834-deficient MCs

After mtDNA4834 deletion, MCs were subjected to different transfections and PCR was performed to detect the mtDNA4834 deletion mutation in

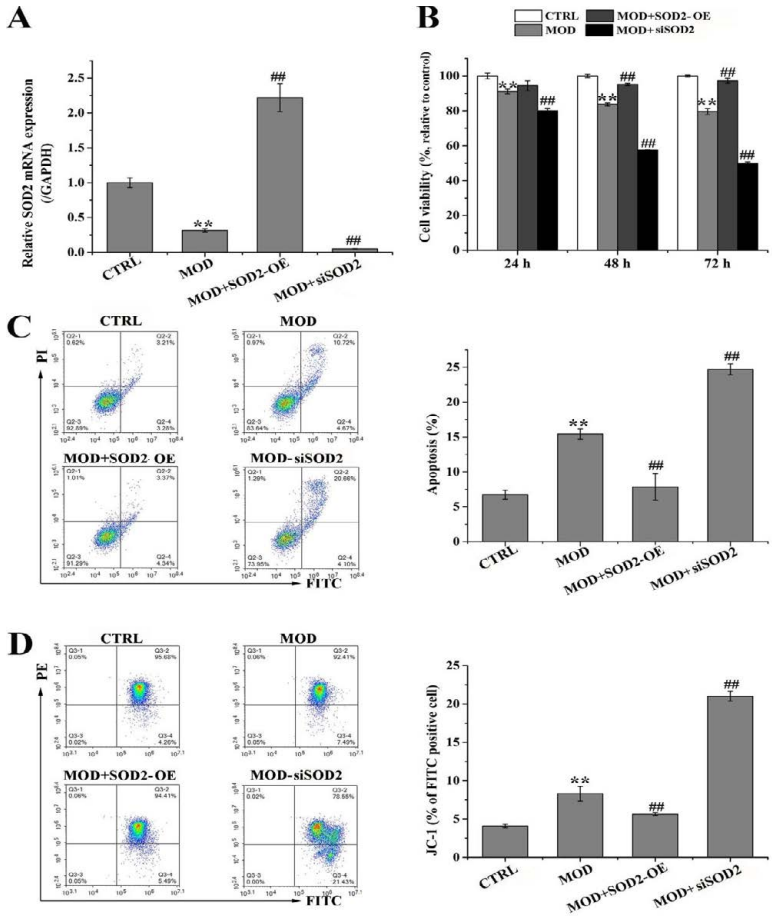

Figure 6. Effects of SOD2 on the proliferation and apoptosis of mtDNA4834-deleted MCs. (A) Relative mRNA expression of SOD2 after transfection. (B) Viability of mtDNA4834-deleted MCs after transfection for 24, 48, and 72 h. (C) Apoptosis and (D) mitochondrial membrane potential of mtDNA4834-deleted MCs after transfection for 48 h. Data represent the mean ± SD (N = 3). ** $p < 0.01$ vs. CTRL, ## $p < 0.01$ vs. MOD. CTRL represents control; MOD represents model. OE represents overexpression.

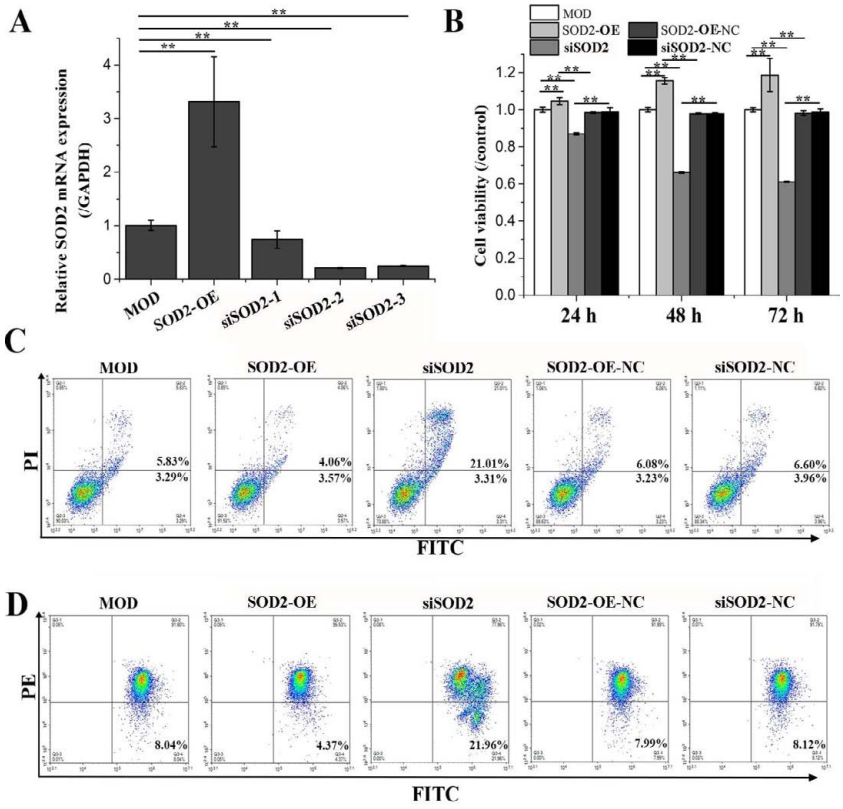

Figure 7. Effects of SOD2 on the proliferation and apoptosis of mtDNA4834-deleted MCs. (A) Relative mRNA expression of SOD2 after transfection. (B) Viability of mtDNA4834-deleted MCs after transfection for 24, 48, and 72 h. (C) Apoptosis and (D) mitochondrial membrane potential of mtDNA4834-deleted MCs after transfection for 48 h. Data represent the mean ± SD (N = 3). ** denotes $p < 0.01$. CTRL represents control; MOD represents model. OE represents overexpression.

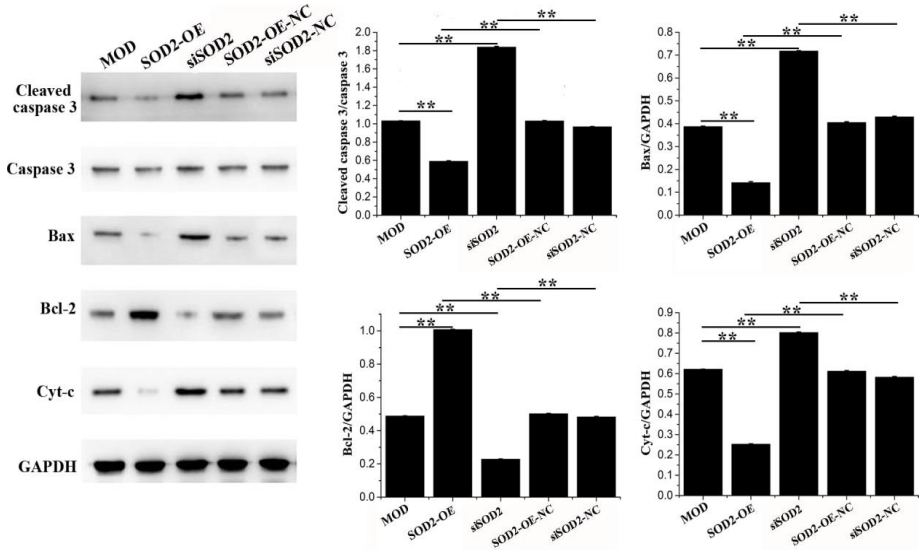

Figure 8. Western blot detection of the expression of apoptosis-related proteins in mtDNA4834-deleted MCs after transfection. Data represent the mean ± SD (N = 3). ** denotes p < 0.01. CTRL represents control; MOD represents model. OE represents overexpression.

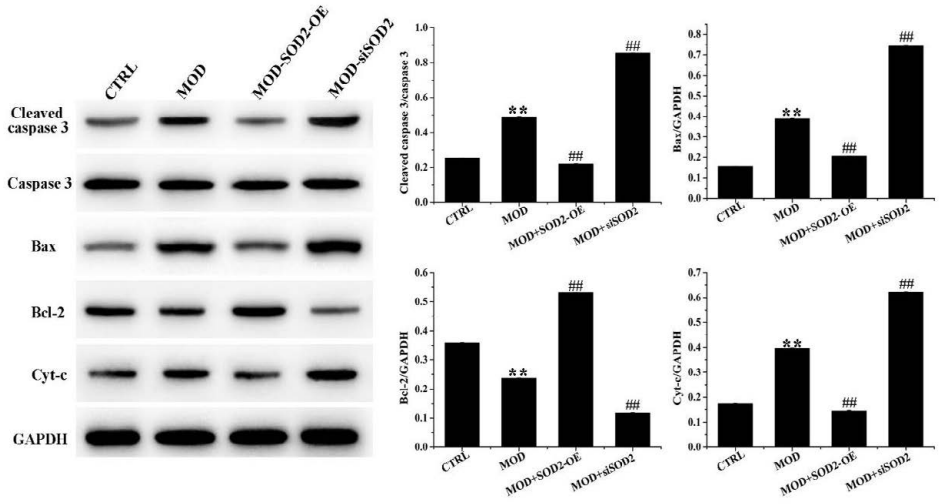

Figure 9. Western blot detection of the expression of apoptosis-related proteins in mtDNA4834-deleted MCs after transfection. Data represent the mean ± SD (N = 3). ** p < 0.01 VS CTRL group, ## p < 0.01 VS MOD group. CTRL represents control; MOD represents model. OE represents overexpression.

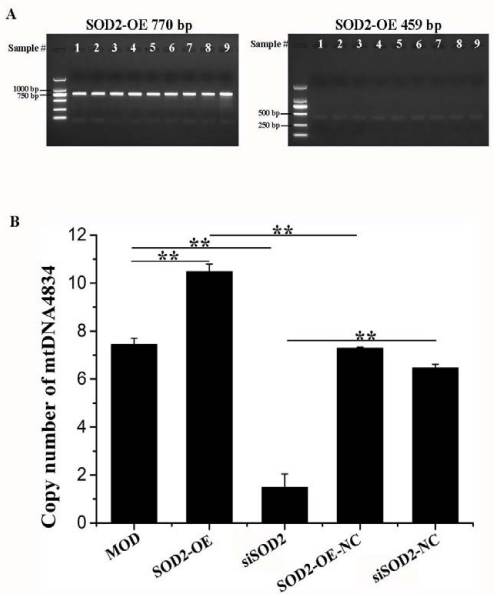

Figure 10. (A) PCR detection of mtDNA4834 deletion mutation in mtDNA4834-deleted MCs after SOD2 overexpression. (B) MtDNA4834 copy number after transfection of mtDNA4834-deficient MCs. Data represent the mean ± SD (N = 3). ** denotes p < 0.01. CTRL represents control; MOD represents model. OE represents overexpression.

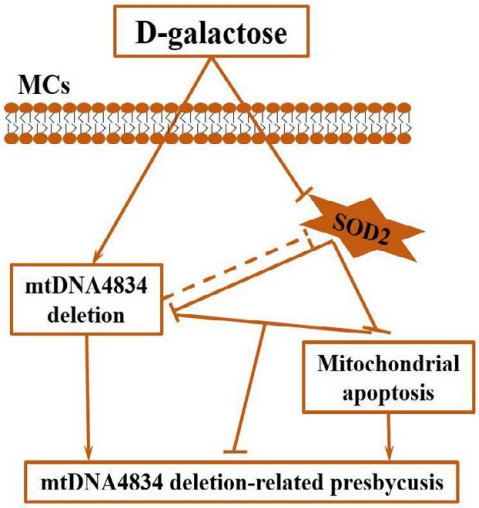

Figure 11. D-galactose-induced mtDNA4834 deletion-related presbycusis is associated with the down-regulation of SOD2. SOD2 overexpression suppressed the deletion of mtDNA4834 and mitochondrial apoptosis in MCs, thereby alleviating mtDNA4834 deletion-related presbycusis.

mtDNA4834-deficient MCs. As shown in Figure 10A, the 770-bp conserved mitochondrial DNA fragment, which represents total mtDNA, was amplified in MOD cells after SOD2 overexpression, indicating the successful extraction of the mtDNA template. The 459-bp product corresponding to the 4834-bp mtDNA deletion was barely amplified, as demonstrated by the weak bands in the SOD2-overexpressing MOD cells. Subsequently, the copy number of mtDNA4834 was evaluated using qRT-PCR. Compared to the MOD group, the mtDNA4834 copy number was significantly increased by SOD2-OE ($p < 0.01$) but significantly decreased by siSOD2 ($p < 0.01$) (Figure 10B). These findings indicated that SOD2 overexpression suppressed the deletion of mtDNA4834 in MCs.

Discussion and Conclusion

Mutations in mtDNA are related to the pathogenesis of a variety of diseases in humans, which include neurodegenerative diseases and cancer [39]. In addition, mutations in mtDNA are known to be associated with syndromic and non-syndromic forms of sensorineural deafness [29,40]. The mtDNA4977 deletion mutation (corresponding to the 4834-bp mtDNA deletion in rats), known as a common deletion, is reportedly associated with presbycusis [15,41,42]. Oxidative stress and mtDNA deletion are suggested to play crucial roles in the pathophysiology of age-related hearing loss [13], and oxidative stress leads to the accumulation of mtDNA deletions in cells [43]. SOD2, a key factor involved in aging, is located in the mitochondrial matrix and is associated with DNA mutation. Additionally, as an antioxidant enzyme, SOD2 is involved in the control of oxidative stress [44]. The relationship between SOD2 and mtDNA4834 deletion in the context of aging-related hearing loss has been unclear. In this study, MCs were extracted from the inner ear of rats treated with D-galactose to induce mtDNA4834 deletion, mimicking aging-related conditions. The expression of SOD2 was downregulated in mtDNA4834-deleted MCs compared to that in normal MCs.

Gene transcription is influenced by DNA methylation, which causes the chromatin structure to become dense and in turn impedes transcription [45]. DNA methylation is catalyzed by DNA methyltransferases including Dnmt1, and the upregulation of Dnmt1 leads to increased DNA methylation [45,46]. Presently, the degree of methylation in the SOD2-encoding gene was increased in mtDNA4834-deleted MCs compared to that in untreated MCs. Meanwhile, the expression of Dnmt1 was increased. The results presented here demonstrate that the downregulation of SOD2 in mtDNA4834-deleted MCs is associated with methylation of the SOD2-encoding gene and that Dnmt1 participates in its regulation. Although the methylation level of the SOD2-encoding gene is very low in this work, methylation did occur in the MOD group and inhibited the expression of SOD2 compared to that in the CTRL group. Changes in gene expression are not necessarily directly related to methylation levels. For example, Zhang et al. demonstrated that CRABP2 promoter methylation downregulated its expression, while the methylation frequency was also very low [47]. This phenomenon is similar to that in our present work.

To explore the relationship between SOD2 and aging-related mtDNA4834 deletion, SOD2 was overexpressed or silenced in mtDNA4834-deleted MCs through plasmid transfection. SOD2 overexpression increased the copy number of mtDNA4834, and the opposite effect was observed with SOD2 silencing. Additionally, SOD2 overexpression inhibited the apoptosis of mtDNA4834-deleted MCs. Apoptosis is closely related to hearing loss [41], and mitochondria are critically involved in the regulation of apoptosis. Aging-regulated mtDNA deletions induce mitochondrial dysfunction accompanied by inflammation and apoptosis, which are related to disease occurrence [48]. Previous research revealed that SOD2 knockout led to mitochondrial dysfunction and apoptosis, suggesting that SOD2 is involved in the induction of the mitochondrial apoptotic pathway [49]. Mitochondrial or endogenous apoptosis is characterized by the release of cyt-c in the cytoplasm, resulting in a series of cascade reactions [50,51]. Meanwhile, it is closely regulated by caspase-related cascades and members of the Bcl-2 family [52]. The findings presented here demonstrate that upregulation of SOD2 attenuated apoptosis of mtDNA4834-deleted MCs by modulating the expression of mitochondrial apoptosis-related proteins.

In conclusion, as shown in Figure 11, the findings of this study suggest that aging-regulated mtDNA4834 deletion in the inner ear is associated with methylation-induced downregulation of SOD2. The overexpression of SOD2 inhibits apoptosis of mtDNA4834-deleted MCs, which might be mediated by mitochondrial apoptosis-related pathways. The findings implicate SOD2 as a target for the treatment of mtDNA4977 deletion-related presbycusis. The limitation of the current study is that all experiments were performed in young rats and D-galactose treatment was used as the model of aging. The study would benefit from repeating the experiments in aged rats.

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Compliance With Ethical Standards

All animal experiments were approved by the institutional review board and performed under the Guidelines for Animal Care and Use of the Model Animal Research Institute at Wuhan Myhalic Biotechnology Co., Ltd. (approval number: HLK-20180620-01). *Conflicts of Interest.* The authors declare they have no conflict of interest.

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