New Target of Oxidative Stress Regulation in Cochleae: Alternative Splicing of the p62/Sqstm1 Gene

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Abstract
We investigated oxidative stress and antioxidant response in the p62/Sqstm1-Keap1-Nrf2 pathway in C57BL/6 mice cochleae during age-related hearing loss (ARHL) and noise-induced hearing loss (NIHL), and the function of full-length and variant p62 in the regulation of Nrf2 activation. Groups of young (2 months), old (13–14 months), control, and acoustic trauma (AT) mice were examined for cochlear damage and oxidative stress as follows: auditory brainstem response and hair cell counts; malondialdehyde (MDA) levels measured by assay kit and 7,8-dihydro-8-oxoguanine (8-oxoG) detected by immunohistochemistry. Full-length and variant p62 were examined for expression in cochlea, hippocampus (HIP), and auditory cortex (AC) using immunoblotting. Keap1-Nrf2 pathway activation was based on immunoblotting of nuclear Nrf2 and quantitative real-time PCR of Nrf2 target genes HO-1/NQO-1. The oxidative function of full-length and variant p62 was examined in HEI-OC-1 cells by flow cytometry. The results showed hearing loss, and cochlear hair cell loss was associated with MDA accumulation and 8-oxoG expression during ARHL and NIHL. Nrf2 showed no obvious changes in nuclear protein. Expression levels mRNA for HO-1 and NQO1 were lower in old mice and mildly greater in AT Mice. The expression of p62 splicing variant lacking the Keap1-interacting region was greater than full-length p62 in cochleae. However, the expression of p62 splicing variant was lesser than full-length p62 in HIP and AC. For HEI-OC-1 cells, overexpression of full-length p62 decreased ROS levels induced by H2O2. Oxidative stress is closely related to ARHL and NIHL. Changing the ratio of full-length to variant p62 protein expression may be a new target to reduce the level of oxidative stress in cochleae.

Keywords Age-related hearing loss · Noise-induced hearing loss · Oxidative stress · Nrf2 · Alternative splicing of the p62 gene

Abbreviations
ARHL Age-related hearing loss
NIHL Noise-induced hearing loss
ROS Reactive oxygen species
ABR Auditory brainstem response
8-oxoG 7,8-Dihydro-8-oxoguanine
HIP Hippocampus
MDA Malondialdehyde
AC Auditory cortex

Introduction
Age-related hearing loss (ARHL) and noise-induced hearing loss (NIHL) are two major classes of sensorineural hearing loss. One of the primary pathological changes of ARHL and NIHL is irreversible loss of sensory cells (Frisina 2009; Li et al. 2019), which has been shown to be caused by an imbalance of redox regulation (Menardo et al. 2012; Honkura et al. 2016). It is plausible that regulation of redox reactions and inhibition of the production of reactive oxygen species (ROS) could help to prevent sensory cell damage.

The p62/Sqstm1-Keap1-Nrf2 pathway is a major mechanistic player in the regulation of cellular redox homeostasis (Chen et al. 2009; Harder et al. 2015; Pan et al. 2016). The stress-induced cellular protein p62/Sqstm1 (referred
to hereafter as p62) possesses several regions that mediate its interactions with other proteins to regulate cellular mechanisms such as autophagy, ubiquitination, and apoptosis (Bjorkoy et al. 2005; Katsuragi et al. 2015; Wang et al. 2017). In response to oxidative stress, p62 forms a homodimer via the K7-D69 hydrogen bond in its Phox1 and Bem1 domains (Wilson et al. 2003). This process facilitates p62 oligomerization and interaction with Keap1 via its Keap1-interacting region (KIR) (Komatsu et al. 2010; Lau et al. 2010), thereby causing Nrf2 stabilization and activation. In the nucleus, Nrf2 binds to the antioxidant response element (ARE) in DNA to activate antioxidant genes including HO-1, Nqo-1, Gclc, and Gclm (Kobayashi et al. 2004; Honkura et al. 2016; Fetoni et al. 2019). The p62 gene is also a target of Nrf2 binding ARE, and the nuclear translocation of Nrf2 promotes the expression of p62 protein (Jain et al. 2010; Kageyama et al. 2018). These findings suggest that there is a positive feedback loop in the p62-Keap1-Nrf2 axis.

In the human cochlea, expression of Nrf2 is predominantly observed in the organ of Corti, and rarely in the spiral ligament, stria vascularis, or Reissner’s membrane (Hosokawa et al. 2018). The neurons of the spiral ganglia are almost devoid of Nrf2. The organ of Corti, and its hair cells, in particular, are susceptible to the production of ROS. In animals, studies suggested that Nrf2 activation protects the inner ear from age- and ototoxic drug-related injuries (Kong et al. 2009; Hoshino et al. 2011; Kim et al. 2015). Similarly, drug-induced Nrf2 activation protects hair cells from ROS-related damage induced by overexposure to noise (Fetoni et al. 2015; Honkura et al. 2016). However, the expression of antioxidant genes regulated by Nrf2 barely increases following pure noise exposure (Honkura et al. 2016; Xiong et al. 2019). This indicates that ROS induced by noise exposure may not activate the Nrf2 pathway effectively under physiological conditions.

In the present study, we investigated whether noise- and age-related cochlear injuries would activate the p62-Keap1-Nrf2 pathway in C57BL/6 mice. We also measured the expression of a splicing variant and full-length p62 in the cochlea and other tissues then conducted experiments to elucidate the role of full-length and/or variant p62 in the regulation of Nrf2 activation in mouse HEI-OC1 cells.

Materials and Methods

Animals and Anesthesia

Male C57BL/6 mice were used for in vivo studies. The mice were divided into five groups, as follows: young group (51 mice, 2 months old), old group (51 mice, 13–14 months old), control group (56 mice, 2 months old), acoustic trauma (AT) group (58 mice, 2 months old), and tBHQ injection group (10 mice, 2 months old). The mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Care of the animals and experimental protocols were approved by the Animal Research and Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

Before conducting the hearing measurements and acoustic overexposures, the mice were anesthetized with chlorpromazine hydrochloride 20-mg/kg body weight (Harvest Pharmaceutical Co., Ltd., Shanghai, China) and ketamine hydrochloride 120-mg/kg body weight (Gutian Pharma Co., Ltd., Fujian, China).

Acoustic Overexposure

Mice were exposed to broadband noise (8–16 kHz) under anesthesia at 105 dB SPL for 2 h. The entire device was placed in a small, reverberant chamber. Noise was played over the horn. Noise calibration was performed before exposure where the noise level difference in each compartment was less than 1 dB.

Assessment of Auditory Function

Hearing threshold was assessed using click- and tone burst-auditory brainstem response (ABR) recordings. The TDT system III (Tucker-Davis Technologies, Alachua, FL, USA) was used to generate stimulus and record trigger signal. Generated tone burst stimuli were delivered at 8, 16, 24, and 32 kHz into the external auditory canal of the mice through an electrostatic speaker placed close to the head. Evoked potentials were filtered between 100 and 3000 Hz using the TDT system and the averages were noted 512 times. The highest stimulus intensity used was 90 dB with a sequential decrease by 10 dB until we identified the lowest sound level able to elicit a repeatable wave sufficient to be considered the threshold. The number of mice undergoing ABR testing included 13 mice in the young group, 13 in the old group, 8 in the control group, and 10 mice in the AT group. For the young and old groups, we performed ABR testing once. For the control and AT groups, we tested ABR thresholds at baseline (pre-AT) and 2 weeks after AT (post-2w): threshold shift = threshold (post-2w) – threshold (pre-AT).

tBHQ Administration

tBHQ injection group is a widely used Nrf2 activator that promotes Nrf2 for its nuclear translocation and its antioxidant activation. tBHQ was obtained from MedChemExpress (Monmouth Junction, NJ, USA). tBHQ solution was intraperitoneally injected at 50 mg/Kg. The injection was administered 12 h before sacrifice and tissue preparation. Ten mice were used for tBHQ injection.
Cell Cultures and \text{H}_2\text{O}_2\text{ Administration}

HEI-OC1 cells were cultured at 33 °C with 10% \text{CO}_2 in DMEM media containing 1.0 g/L glucose and 10% FBS. For plasmid construction, the CDS of full-length or variant p62 with 3 flag was separately cloned into a pcDNA3.1+ plasmid to create transients overexpressing various transcripts of the p62 gene. The control group was used pcDNA3.1+ vector. HEI-OC1 cells were treated with \text{H}_2\text{O}_2\text{ (200 mM)} for 24 h, after which cells were collected for ROS detection.

**Immunohistochemistry**

Immunohistochemistry was used to determine levels of 7,8-dihydro-8-oxoguanine (8-oxoG), a key biomarker of mitochondrial and nuclear DNA oxidative stress damage. Three mice per group underwent 8-oxoG testing. Mice were decapitated 6 h after acoustic overexposure. Cochleae were isolated, followed by immersion fixation in 4% paraformaldehyde overnight. Decalcification using 10% sodium EDTA was then performed for 2 days followed by overnight incubation in Hanks buffered saline with 25% sucrose. Cochleae were embedded in optimal cutting temperature compound, cryosectioned at 10-μm thickness, mounted on microscope slides, and stored at –20 °C. Slides with cochlear tissues were dried at room temperature for 30 min. Slides were then permeabilized for 10 min using 0.3% Triton X-100/PBS, washed with PBS, and blocked for 60 min using 10% goat serum/PBS. Finally, slides were incubated overnight with primary antibodies (mouse monoclonal anti-8oxoG-DNA lesion; 1:100; Santa Cruz, Santa Cruz, CA, USA) diluted in 0.5% BSA/PBS at 4 °C. On the following day, slides were washed with PBS and incubated for 1 h with secondary antibody (1:400; GAR4882, Multisciences, Hangzhou, China). After washing twice, the nuclei were dyed with DAPI. Slides were then carefully dissected, followed by permeation for 10 min with 0.3% Triton X-100/PBS and dyed using DAPI. Finally, they were observed under a fluorescence microscope (400×).

For outer hair cell counting, mice were decapitated 2 weeks after acoustic overexposure. Three mice from each group underwent outer hair cell counting. Cochleae were isolated, followed by fixation. Basal turn membranes were then carefully dissected, followed by permeation for 10 min with 0.3% Triton X-100/PBS and dyed using DAPI. Basal turn membranes were finally collected by centrifugation, and protein concentrations were measured using a bicinchoninic acid kit (G2026, Servicebio). Proteins were separated using SDS-PAGE gel and were then transferred to PVDF membranes. Membranes were blocked for 1 h using 3% BSA/TBST and then incubated overnight with primary antibodies (p62 (1:1000; ab91526, Abcam, Cambridge, UK), Nrf2 (1:1000; ab62352, Abcam), Lamin B1 (1:500; sc-374015, Santa Cruz), and beta-actin (1:1000, GB11001, Servicebio). On the following day, membranes were washed with TBST and incubated for 1 h with HRP-labeled goat anti-rabbit IgG (GB23033, Servicebio). Membranes were washed again and immersed in electrochemiluminescence solution (ECL, G2020, Servicebio) followed by exposure under an E-Gel Imager.

**MDA Assay**

Malondialdehyde (MDA) is a lipid peroxidation product. MDA levels in cochlea samples were measured using an MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Catalog No. A003-1), and the experimental methods were carried out according to the manufacturer’s instructions. The four groups (young, old, control, and AT group) used 15 mice respectively for cochlea MDA assay, and each experiment was repeated at least three times.

**qRT-PCR**

Dissections of cochlea were followed by immersion in cold PBS and then collection of whole cochlear tissue with the bone removed for qRT-PCR. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, USA), and 1 μg of the extracted total RNA was reverse-transcribed to cDNA following the protocol of ReverTra Ace (Toyobo, Osaka, Japan). Amplification of cDNA samples was performed on a LightCycler 480 II (Roche, Rotkreuz, Switzerland) using antibody (1:4000; GAR4882, Multisciences, Hangzhou, China). After washing twice, the nuclei were dyed with DAPI. Slides were then carefully dissected, followed by permeation for 10 min with 0.3% Triton X-100/PBS and dyed using DAPI. Basal turn membranes were finally collected by centrifugation, and protein concentrations were measured using a bicinchoninic acid kit (G2026, Servicebio). Proteins were separated using SDS-PAGE gel and were then transferred to PVDF membranes. Membranes were blocked for 1 h using 3% BSA/TBST and then incubated overnight with primary antibodies (p62 (1:1000; ab91526, Abcam, Cambridge, UK), Nrf2 (1:1000; ab62352, Abcam), Lamin B1 (1:500; sc-374015, Santa Cruz), and beta-actin (1:1000, GB11001, Servicebio). On the following day, membranes were washed with TBST and incubated for 1 h with HRP-labeled goat anti-rabbit IgG (GB23033, Servicebio). Membranes were washed again and immersed in electrochemiluminescence solution (ECL, G2020, Servicebio) followed by exposure under an E-Gel Imager.
SYBR Green Premix Ex Taq™ (Tli RNase H Plus; TaKaRa, Dalian, China). Cycling parameters were 3 min at 95 °C, followed by 40 cycles for 15 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C. Each sample was amplified three times followed by calculation of the mean value. Relative mRNA levels of NQO1 and HO-1 were normalized to beta-actin using the 2(− delta delta CT) method. The primers used in the present study are displayed in Table 1. The four groups (young, old, control, and AT groups) used 15 mice respectively for cochlea MDA assay and each experiment was repeated at least three times.

Agarose Gel Electrophoresis

We performed 2% agarose gel electrophoresis. The p62 qRT-PCR products from cDNA of cochleae and HEI-OC1 cells underwent gel electrophoresis to identify their sizes in the presence and absence of the last half of exon 7. After electrophoresis, the gels were exposed under an E-Gel Imager, and pictures were analyzed using ImageJ 1.52a.

Flow Cytometry

A reactive oxygen species (ROS) assay kit (Beyotime, S0033, Shanghai, China) was used for HEI-OC1 cell ROS analysis. Cell transfection and H₂O₂ treatment of HEI-OC1 cells were followed by trypsinization and collection by centrifugation at 2000 rpm for 5 min. Cells were washed in PBS twice. Cells obtained from one hole of a six-hole plate were resuspended in 500 μl DCFH-DA diluent. The cells were incubated for 20 min at 37 °C in the dark followed by analysis using flow cytometry.

Statistical Analysis

Data were expressed as means ± standard deviation (SD). N signified the number of animals per experimental group. Differences between groups with unpaired data were compared using the Student’s t-test. P-Values was presented as follows: *P < 0.05; **P < 0.01; ***P < 0.001; and ns = not significant.

Results

Cochlear Hair Cell Loss and ROS Accumulation in the ARHL Model

To determine the association between ROS and hearing loss, we examined hearing function, hair cells, and MDA levels in C57BL/6 mice. ABR, an objective electrophysiological test of hearing function, was used to monitor the progression of hearing loss. At all tested frequencies, the average hearing thresholds were significantly higher in old (13–14 months) mice than in young (2 months) mice (Fig. 1A). These results suggested that the mice had developed obvious ARHL.

To determine whether the functional deficits corresponded to the extent and localization of hair cell loss, cochleae were processed for surface preparations and quantitative hair cell counts following the ABR measurements. In the basal cochlear turn in young mice, surface preparations showed regular outlines, with one row of inner hair cells and three rows of outer hair cells (Fig. 1B, D); in the old mice, a nearly 68% loss of hair cells was observed in the same region. To determine whether oxidative stress might have contributed to the ARHL, cochlear MDA levels were measured in young and old mice. MDA levels were significantly higher in old cochleae (2.8 nmol/mg) than in young cochleae (1.4 nmol/mg) (Fig. 1C). This result was confirmed using fluorescence microscope analysis of 7,8-dihydro-8-oxoguanine (8-oxoG) staining. Compared with young cochleae, cochleae from old mice exhibited a higher density of dot-like 8-oxoG staining in the cytoplasm of sensory hair cells (Fig. 1E).

Cochlear Hair Cell Loss and Oxidative Stress in the NIHL Model

Next, we measured hearing function, hair cell counts, and ROS levels in the noise overexposure (AT) mice. At all tested frequencies, there were significant shifts in average hearing thresholds between AT mice and control mice (Fig. 2A). These results suggested that acoustic overexposure led to the development of severe hearing loss. Compared with the control group, AT mice showed a nearly 38% loss of hair cells in the basal cochlear turn (Fig. 2B, D), had significantly higher levels of cochlear MDA (4.5 nmol/mg) (Fig. 2C), and showed a higher 8-oxoG expression level in the cochlear basilar membrane (Fig. 2E).

Table 1 The primers used in the present study

| Primer       | Sequence            |
|--------------|---------------------|
| HO-1 forward | AAGCCGAGAATGCTAGTTC |
| HO-1 reverse | GCGCTGTAATATGTTACAAAGGA |
| NQO1 forward | TGGCGGAACACACAGAGG |
| NQO1 reverse | GAAATCGCCAGAGAATGAC |
| p62 forward  | GAAATGGAGGGAAGAAGAG |
| p62 reverse  | TCACAAATTTGAGGGTCCTTCG |
| Beta-actin forward | GGCTGTATTCCCCTCCATCG |
| Beta-actin reverse | CCAGTTGGTAACATGCGATGT |
Nrf2 Translocation and Target Gene Expression in Old Mice and AT Mice

Nrf2 nuclear translocation was examined using immunoblotting of nuclear proteins collected from the cochleae of young, old, control, AT, and positive control (PC) group mice. The PC group, which underwent tBHQ administration, showed marked nuclear translocation of Nrf2. By contrast, Nrf2 showed no obvious changes in nuclear protein during aging and noise overexposure (Fig. 3A). The mRNA expression levels of Nrf2 target genes HO-1 and NQO1 in the cochleae notably decreased with aging (Fig. 3B, C). In AT mice, there was only a slight increase in HO-1 expression levels, while those of NQO1 remained unchanged (Fig. 3D, E).

Overexpression of a p62 Splicing Variant Lacking the Keap1-Interacting Region

Two distinct p62 bands were observed in immunoblots of the cochlea. The predicted mRNA sequences of these two proteins were based on previous studies and the mouse Ensembl database (http://www.ensembl.org/Mus_musculus/Info/Index). Figure 4A illustrates a variant of p62 mRNA in which the last half of exon 7 of the full-length p62 mRNA is omitted; this variant protein would lack the final half of the KIR (Fig. 4B). Primers were designed to verify the existence of these two p62 transcripts in the cochleae and HEI-OC1 cells (Fig. 4C). The cDNA of control group cochleae and HEI-OC1 cells were used as templates for qRT-PCR, and the sizes of the PCR products were found to be consistent with the prediction (Fig. 4D).

In cochleae, expression levels of the variant p62 were higher than those of full-length p62, in the young, old, control, and AT mouse groups (Fig. 4E, F, H, and I). Furthermore, the relative ratios between variant and full-length p62 did not change with aging or noise overexposure (Fig. 4G, J). These findings suggested that there is a natural variant of p62 that lacks the KIR and whose expression is regulated identically to that of the full-length protein in the cochlea. In mice exposed to AT, cochlear expression...
levels of both the variant and the full-length p62 increased, with no significant differences between the young and old groups. In a comparison of variant and the full-length p62 expression in the HIP, AC, and the cochleae (Fig. 4K), we found there were substantial differences in the ratio between expression of the variant and the full-length p62. The ratio in cochleae was significantly higher than those of the HIP or AC (Fig. 4L).

**Overexpression of p62 and Oxidative Stress in HEI-OC1 Cells**

To confirm that HEI-OC1 auditory cells transfected with p62 variant and full-length overexpression plasmids transiently expressed high levels of both proteins, we used FLAG-tag immunoblotting (Fig. 5A). Expression levels of two Nrf2 targets, the antioxidant genes HO-1 and NQO-1, were first measured in HEI-OC1 cells incubated with \( \text{H}_2\text{O}_2 \) for 24 h to induce oxidative stress injury (Fig. 5B, C). We found that \( \text{H}_2\text{O}_2 \) at a concentration of 200 \( \mu \text{M} \) did not induce a significant increase in the expression of these antioxidant genes, nor did the presence of transiently overexpressed variant p62. By contrast, the gene expression levels of both HO-1 and NQO-1 were markedly increased under oxidative stress in cells overexpressing full-length p62.

Intracellular ROS levels were determined using flow cytometry in HEI-OC1 cells (Fig. 5D). We found substantial ROS accumulation under \( \text{H}_2\text{O}_2 \) treatment (red curve). Cells overexpressing the variant p62 combined with \( \text{H}_2\text{O}_2 \) treatment (blue curve) showed a similar accumulation of ROS. By contrast, the accumulation of ROS in cells overexpressing full-length p62 combined with \( \text{H}_2\text{O}_2 \) treatment (black curve) was less than that of cells treated with \( \text{H}_2\text{O}_2 \) alone.

**Discussion**

We found that the loss of hearing and hair cells was accompanied by increased ROS levels in aging and AT-exposed C57BL/6 mice. However, the Nrf2 pathway, an important
intracellular antioxidant response pathway, was not activated. A variant form of p62, a potential negative regulator of the Keap1-Nrf2 pathway, was highly expressed in cochleae. High expression levels of full-length p62, but not variant p62, promoted the activation of the Nrf2 pathway in HEI-OC1 cells, increasing the expression of two downstream antioxidant genes, and reducing the cellular levels of ROS.

The pathogenesis of age-related hearing loss involves many aspects, such as aging, oxidative stress, and genetic and environmental factors (Yamasoba et al. 2013). C57BL/6 model shows early age-related hearing loss, which is thought to be related to genetics (Yasuda et al. 2020; Johnson et al. 2017), but the model is also studied for other pathogenesis like oxidative stress (Brewton et al. 2016; Honkura et al. 2016). C57BL/6 has also been extensively used in noise studies (Lee et al. 2020). One limitation of this study was that we were unable to precisely isolate cochlear hair cells for in vitro experiments. Because Nrf2 is known to be mainly expressed in hair cells, with rarely expressed in other parts of the cochleae (Hosokawa et al. 2018), it would have been useful to investigate Nrf2 and p62 in hair cells directly. Instead, we used cultured HEI-OC1 cells to further elucidate our in vivo results. HEI-OC1 cells have been shown to express specific markers of cochlear hair cells and supporting cells (Kalinec et al. 2003). This cell line may represent a common progenitor for sensory and supporting cells of the cochlear organ of Corti and has been proposed as a useful in vitro system to investigate oxidative stress (Kalinec et al. 2016).

Oxidative damage is a factor in ARHL and NIHL (Fetoni et al. 2015; Honkura et al. 2016). In the cochleae of old mice, we found an imbalance of redox regulation that was manifested by the expression of 8-oxog and the accumulation of MDA. Expression levels of the Keap1-Nrf2 pathway target genes HO-1 and NQO1 were notably lower in old mice. It is well-understood that the reduction in antioxidant enzyme levels during aging causes oxidative stress (Menardo et al. 2012). Following noise exposure, the cochleae rapidly undergo significant oxidative damage (Wu et al. 2020) that is closely related to hair cell loss and permanent shifts in auditory thresholds. In this study, the Keap1-Nrf2 pathway in the cochlea was not significantly activated following acute oxidative damage. However, administration of tBHQ, an agonist of the Nrf2 pathway, enhanced the nuclear translocation of Nrf2. This suggests a mechanism in which the antioxidant pathway was difficult to be activated in the context of accumulation of ROS, rather than one in which...
it could not be activated in the mouse cochlea. We propose that there may be a negative regulator of the Nrf2 pathway in the cochleae under physiological conditions and that the Nrf2 pathway plays an important role in antioxidant reactions in the cochleae.

Under normal conditions, upon exposure to oxidative stress, specific cysteine residues of Keap1 are modified by oxidants, causing the Keap1 homodimer to lose its two-site binding affinity for Nrf2 (Bjorkoy et al. 2005; Rogov et al. 2014). As a result, Nrf2 escapes from the Keap1 interaction and translocates into the nucleus (Komatsu et al. 2010; Pan et al. 2016). Full-length p62 bearing the KIR region can also interact with Keap1, preventing it from trapping Nrf2 in the cytoplasm. However, the presence of variant p62 lacking the KIR region results in a negatively regulated Nrf2

Fig. 4 The exon of mRNA sequences and protein expression for full-length and splice variant p62. A The exon of mRNA sequences for full-length and splice variant p62. The variant p62 mRNA lacks the last half of exon 7. B The KIR region in the full-length and variant p62 proteins. C The qRT-PCR primers for identifying mRNA sequences of the full-length and splice variant p62. D Gel electrophoresis of RT-PCR products from cDNAs of control group cochleae and HEI-OC1 cells. E The immunoblot of p62 for young and old mice. F The analysis of E. The expression of the variant and the full-length p62 did not change during aging. G The analysis of E. The ratio between the variant and the full-length p62 did not change during aging. H The immunoblot of p62 for control and AT group. I The analysis of H. There was a marked increase of the expression of variant and the full-length p62 during noise overexposure. J The analysis of H. The ratio between the variant and the full-length p62 did not change during noise overexposure. K The immunoblot of p62 for cochlea, hippocampus (HIP), and auditory cortex (AC). L The analysis of Fig. 4 K. The ratio (variant/full-length p62) in cochleae was significantly higher than that in HIP or AC. Each experiment was repeated at least three times. The data are shown as mean±SD. Two-tailed, unpaired Student’s t-tests were used to determine statistical significance

Fig. 5 The HO-1/NQO-1 mRNA expression and the ROS levels in HEI-OC1 cells. A The immunoblot of flag for the overexpressing FLAG-tagged full-length p62, FLAG-tagged variant p62, and vector. The exogenous genes were significantly expressed. B, C HO-1/ NQO-1 mRNA expression in cells. No significant difference was observed between control group and H2O2 group. The HO-1/NQO-1 mRNA expression in H2O2+full-length p62 cells was higher than H2O2 cells. D ROS levels in HEI-OC1 cells. E The analysis of D. The accumulation of ROS in H2O2+full-length p62 cells was less than that in H2O2 cells. Each experiment was repeated at least three times. The data are shown as mean±SD. Two-tailed, unpaired Student’s t-tests were used to determine statistical significance
pathway (Kageyama et al. 2018). In this study, we observed high expression of variant p62 that was specific to the cochlea. This may explain why ROS was enhanced by exposure to AT, but the Nrf2 pathway was not activated. However, in the AC and HIP, the expression of full-length p62 was more than twice that of the splice variant. Unsurprisingly, the Nrf2 pathway was previously shown to be significantly activated following ROS stress in rat cerebral cortex and mouse hippocampal cells (Liu et al. 2014; Lee et al. 2016). Although the expression of both forms of p62 increased in mice exposed to AT, the ratio of splice variant to full-length protein remained unchanged. This suggested that the same regulatory mechanism controls the mRNA expression of both the full-length p62 and the variant lacking the KIR. In vitro experiments in HEI-OC1 cells under oxidative stress with H2O2 confirmed that the overexpression of full-length p62, but not variant p62, activated antioxidant genes in the Nrf2 pathway. There may have been high endogenous expression of the p62 splicing variant in these cells, such that its inhibition of the Nrf2 pathway reached a maximum. If so, the effect of exogenous overexpression of variant p62 on the Nrf2 pathway might not have been apparent.

The discovery of pre-mRNA splicing to generate multiple products from a single gene has greatly expanded genomic information (Lau et al. 2010). Exon 7 of mouse p62 carries a typical 5′ splicing site that is recognized by a component of the spliceosome, the U1 small nuclear ribonucleoprotein (snRNP; Matlin et al. 2005). If this site is recognized by U1 snRNP, the variant mRNA is generated; if not, full-length p62 mRNA is generated. As shown in Fig. 4E, H, the relative proportion of full-length and variant p62 remained constant with aging and noise exposure. This suggested that splicing occurred continuously, producing full-length and variant p62 at the same rate, irrespective of cellular conditions in mice. The regulation of p62 splicing should be further studied to identify targets for altering the ratio of full-length and variant p62 production in the cochlea.

**Conclusion**

ROS accumulation is known to be closely related to ARHL and NIHL. The inability of ROS accumulation to activate the Nrf2 antioxidant stress pathway under physiological conditions in this study may have been related to alternative splicing of the p62 mRNA in cochleae. Approaches to alter splicing for increased mRNA expression of full-length p62 relative to variant p62 may protect cochlear hair cells from oxidative stress-induced damage.

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