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UCHL1 regulated by Sp1 ameliorates cochlear hair cell senescence and oxidative damage

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Abstract. Age-related hearing loss (ARHL) is the most common cause of hearing loss in the elderly. Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is a deubiquitinating enzyme involved in several types of human disease. The present study aimed to investigate the effect of UCHL1 on a hydrogen peroxide (H_2O_2)-induced ARHL model in cochlear hair cells and uncover its underlying mechanism. Reverse transcription-quantitative (RT-q)PCR and western blot analysis were used to assess UCHL1 expression in HEI-OC1 cells exposed to H_2O_2. Following UCHL1 overexpression in H_2O_2-induced HEI-OC1 cells, cell activity was assessed by Cell Counting Kit-8 assay. The content of oxidative stress-associated markers including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and reactive oxygen species (ROS) was measured using corresponding commercial kits. Cell apoptosis was evaluated by TUNEL assay and western blot analysis. Cell senescence was assessed by senescence-associated β-galactosidase staining and western blot analysis. RT-qPCR and western blot analysis were applied to measure mRNA and protein expression levels, respectively, of specificity protein 1 (Sp1) in H_2O_2-treated HEI-OC1 cells. In addition, the association between UCHL1 and Sp1 was verified by luciferase reporter and chromatin immunoprecipitation (ChIP) assay. The mRNA and protein expression levels of UCHL1 were also determined in Sp1-overexpressing cells by RT-qPCR and western blot analysis, respectively. Following Sp1 overexpression in UCHL1-overexpressing H_2O_2-treated HEI-OC1 cells, cell activity, oxidative stress, apoptosis and senescence were assessed. Finally, the expression levels of NF-κB signaling-related proteins p-NF-κB p65 and NF-κB p65 were detected using western blot analysis. The results showed that UCHL1 was downregulated in H_2O_2-treated HEI-OC1 cells. In addition, UCHL1 overexpression enhanced cell viability and promoted oxidative damage, apoptosis and senescence in H_2O_2-induced HEI-OC1 cells. Furthermore, Sp1 was upregulated in H_2O_2-treated HEI-OC1 cells. Additionally, luciferase reporter and ChIP assays demonstrated that Sp1 interacted with the UCHL1 promoter to inhibit UCHL1 transcription. Sp1 overexpression reversed the effect of UCHL1 overexpression on cell viability, oxidative stress, apoptosis, senescence and activation of the NF-κB signaling pathway in H_2O_2-exposed HEI-OC1 cells. Collectively, the results suggested that UCHL1 transcriptional suppression by Sp1 protected cochlear hair cells from H_2O_2-triggered senescence and oxidative damage.

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Key words: age-related hearing loss, ubiquitin carboxyl-terminal hydrolase L1, specificity protein 1, senescence, oxidative stress

Introduction

Age-related hearing loss (ARHL) is characterized by bilaterally symmetric auditory dysfunction caused by aging and degeneration of the auditory system, the severity of which is associated with increasing age (1,2). ARHL is commonly characterized by decreased hearing ability for high frequencies that gradually include lower ones (2). It has been reported by the World Health Organization that >460 million individuals worldwide have hearing disorders, while by 2025 70-80% of individuals aged >65 years may have ARHL (3,4). Accumulating evidence has suggested that the development of ARHL is associated with social isolation, depression, anxiety and cognitive impairment (5,6). Therefore, early detection, intervention and delay of the occurrence and development of ARHL are of importance for the elderly population.

The ubiquitin-proteasome system is a key pathway involved in the degradation of cell protein and maintenance of normal deubiquitinating enzyme (DUB)-dependent cellular function (7-9). Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1, also called PARK5), belonging to the DUB family, is primarily involved in protein stability in cells via regulating the ubiquitin/proteasome pathway (10). Emerging evidence has indicated that UCHL1 is aberrantly expressed in age-associated diseases, including Parkinson's and Alzheimer's disease (11,12). More importantly, a recent study demonstrated that UCHL1 is downregulated in the cochlea of ARHL mice (13). Nonetheless, the effects of UCHL1 on ARHL remain unclear.

Specificity protein 1 (Sp1) is a member of the SP/Krüppel-like factor (KLF) transcription factor family, which is widely expressed in human cells under normal conditions and is involved in numerous cell processes, including cell proliferation, apoptosis, differentiation and transformation (14). Recent literature has elucidated that Sp1 mediates cochlear cell apoptosis in hearing loss models (15,16). Furthermore, it has been also reported that NF-κB participates in the pathogenesis of ARHL via interaction with immune-associated genes (17).

The present study aimed to evaluate the role of UCHL1 in ARHL and reveal the association between UCHL1, Sp1 and NF-κB signaling in ARHL.

Materials and methods

Bioinformatics analysis. The PROMO database (alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3/) was used to predict the association between UCHL1 promoter and Sp1.

Cell culture and treatment. Murine cochlea hair cells (HEI-OC1; cat. no. BFN60808695), obtained from BLUEFBIO, were cultured in DMEM supplemented with 10% FBS (both Shanghai ExCell Biology, Inc.) at 33˚C with 10% CO_2. To establish an *in vitro* ARHL model, HEI-OC1 cells were exposed to 1 mM hydrogen peroxide (H_2O_2) for 2 h at 33˚C, as previously described (18,19).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from HEI-OC1 cells (6-well plates at a density of $6x10^4$ cells per well) using TRIzol® reagent (Invitrogen) and cDNA was synthesized using the AffinityScript cDNA synthesis kit (Agilent Technologies) according to the manufacturer's instructions. qPCR was performed on a LightCycler 480 PCR instrument (Roche Diagnostics) using the AceQ® qPCR SYBR Green Master Mix (Vazyme Biotechnology Co. Ltd.). The following thermocycling conditions were used for qPCR: Pre-denaturation at 95˚C for 1 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 40 sec and extension at 72˚C for 15 sec. The changes in gene expression levels were assessed using the $2^{-\Delta\Delta Cq}$ method (20). The following primer pairs were used for qPCR: UCHL1, forward, 5'-AGGGACAGGAAGTTAGCCCTA-3' and reverse, 5'-AGCTTCTCCGTTTCAGACAGA-3'; Sp1 forward, 5'-CCTGGCATCCCACCAGAGTA-3' and reverse, 5'-GTGCAAGGAGCTGATCCCAA-3' and β-actin forward, 5'-GTTGGAGCAAACATCCCCCA-3' and reverse, 5'-CGCGACCATCCTCCTCTTAG-3'.

Western blot analysis. Total protein was extracted from HEI-OC1 cells ($1x10^6$ cells) using RIPA lysis buffer (Shanghai Yisheng Biotechnology Co., Ltd.) and the protein concentration was determined using a BCA protein assay kit (Beijing Solarbio Science & Technology Co., Ltd.). The proteins were transferred onto PVDF membranes (30 μg/lane) following separation by SDS-PAGE on a 10% gel. Following blocking with 5% skimmed milk at room temperature for 1 h, membranes were first incubated with primary antibodies at 4˚C overnight and then with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. ab205718; 1:2,000; Abcam) for 1 h at room temperature. The ECL Western Blot kit (Jiangsu CoWin Biotech Co., Ltd.) was used to develop the immunoreactive signals and protein band intensity was calculated using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.). The primary antibodies were as follows: Anti-UCHL1 (cat. no. ab108986; 1:1,000; Abcam), anti-B cell lymphoma-2 (Bcl-2; cat. no. ab182858; 1:2,000; Abcam), anti-Bcl-2 associated X (Bax; cat. no. ab32503; 1:1,000; Abcam), anti-p16 (cat. no. ab51243; 1:1,000; Abcam), anti-p21 (cat. no. ab109199; 1:1,000; Abcam), anti-Sp1 (cat. no. ab227383; 1:1,000; Abcam), anti-phosphorylated (p)-NF-κB p65 (cat. no. ab76302; 1:1,000; Abcam), anti-NF-κB p65 (cat. no. ab32536; 1:1,000; Abcam) and anti-β-actin (cat. no. ab8227; 1:1,000; Abcam).

Plasmid transfection. The recombinant plasmids pcDNA3.1-UCHL1 (Oe-UCHL1) and pcDNA3.1-Sp1 (Oe-Sp1), as well as the empty vector pcDNA3.1 negative control (Oe-NC), were obtained from Sangon Biotech Co., Ltd. HEI-OC1 cells seeded into six-well plates at a density of $5x10^5$ cells/well were transfected with 5 μg plasmids using Lipofectamine® 2000 (Life Technologies; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h, according to the manufacturer's recommendation. The subsequent experiments were performed at 48 h following cell transfection.

Cell Counting Kit-8 (CCK-8) assay. To assess cell viability, a CCK-8 kit (Abnova) was used according to the manufacturer's instructions. Briefly, HEI-OC1 cells were seeded into 96-well plates at a density of $5x10^3$ cells/well and treated with 10 μl CCK-8 solution at 37˚C for 2 h. Subsequently, optical density (OD) at a wavelength of 450 nm was measured using a microplate reader (Infinite M200; Tecan Group, Ltd.).

Evaluation of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and reactive oxygen species (ROS). HEI-OC1 cells were seeded into 96-well plates ($5x10^3$ cells/well). Following cell lysis in 300 μl lysis buffer (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 8.1), protein concentration was measured using a BCA protein assay kit (Bio Basic, Inc.). Assay kits for SOD (cat. no. A001-1-2) and GSH-Px (cat. no. A005-1-2) were obtained from Nanjing Jiancheng Bioengineering Institute and used according to the manufacturer's instructions. The OD values at a wavelength of 450 nm were determined using a microplate reader (Infinite M200; Tecan Group, Ltd.). To measure ROS accumulation, HEI-OC1 cells seeded into 24-well plates were treated with 10 μM dichloro-dihydro-fluorescein diacetate (MilliporeSigma) at 37˚C for 30 min in the dark followed by washing with serum-free DMEM three times. ROS generation was assessed by flow cytometry (Merck KGaA) using FACSAria (BD Biosciences) using the corresponding kit (cat. no. ab113851; Abcam) according to the manufacturer's instructions. The data were viewed in FlowJo software (version 10; FlowJo, LLC).

TUNEL assay. HEI-OC1 cells were fixed with 4% paraformaldehyde for 30 min at room temperature and were then treated with 0.1% Triton X-100 for 5 min at room temperature. TUNEL assay was performed using TUNEL reagent (cat. no. MK1013; Wuhan Boster Biological Technology, Ltd.) for 60 min at 37˚C according to the manufacturer's instructions.

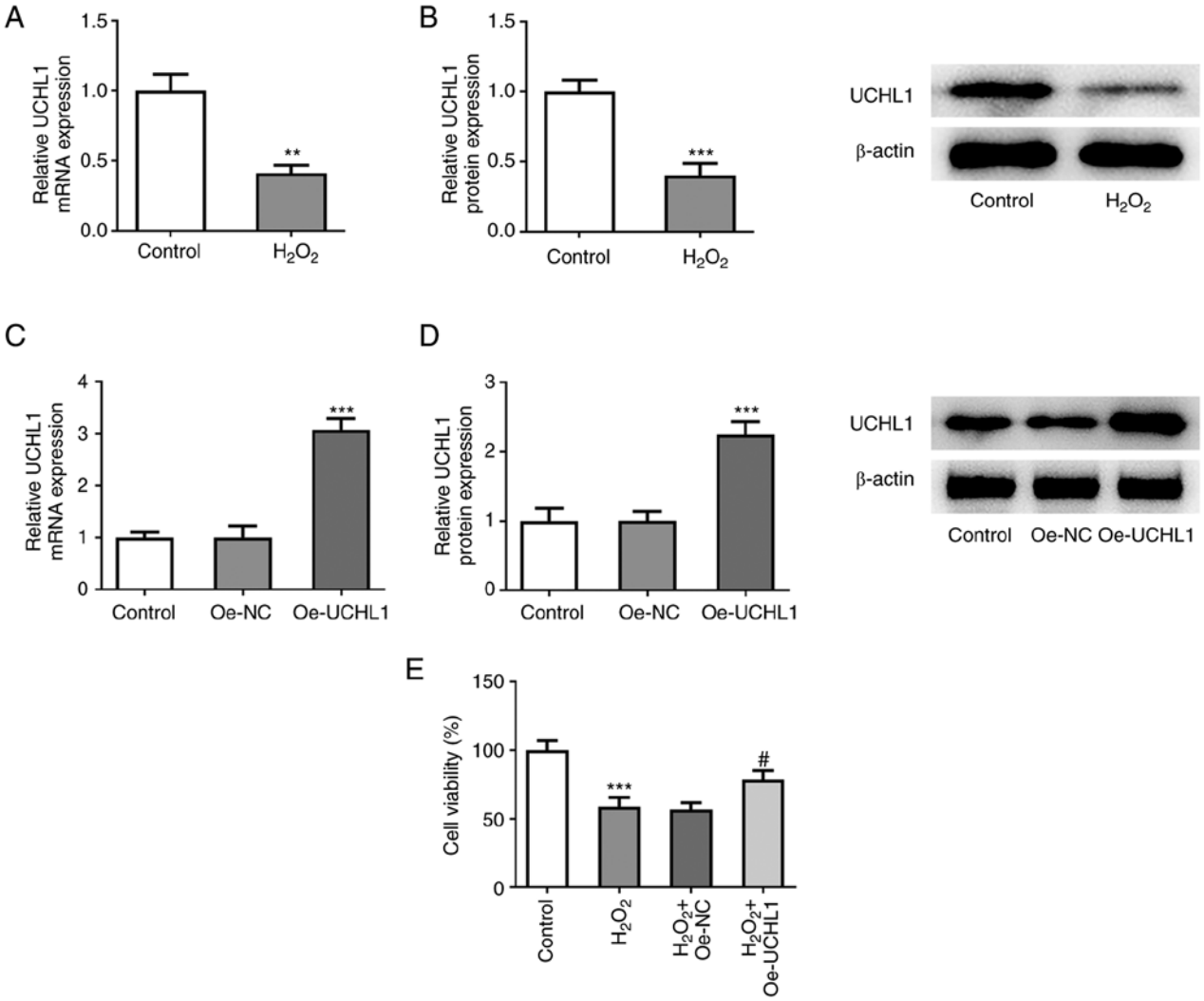

Figure 1. UCHL1 elevation improves the viability of H_2O_2-treated HEI-OC1 cells. (A) RT-qPCR and (B) western blot analysis of UCHL1 expression in H_2O_2-insulted HEI-OC1 cells. Analysis of overexpression efficacy of pcDNA3.1-UCHL1 recombinant plasmid via (C) RT-qPCR and (D) western blotting. (E) Viability of H_2O_2-exposed HEI-OC1 cells was evaluated by Cell Counting Kit-8 assay. **P<0.01 and ***P<0.001 vs. control. $^{\#}$P<0.05 vs. H_2O_2 + Oe-NC. UCHL1, ubiquitin carboxyl-terminal hydrolase L1; RT-qPCR, reverse transcription-quantitative PCR; H_2O_2, hydrogen peroxide; Oe-NC, overexpression negative control.

Cell nuclei were labeled with 10 mg/ml DAPI for 5 min at room temperature in the dark and images were captured from four random fields under a fluorescence microscope (LSM800; Carl Zeiss AG) after Antifade Mounting Medium (Beyotime Institute of Biotechnology) was added to the sections.

Senescence-associated-β-galactosidase (SA-β-gal) staining. Briefly, HEI-OC1 cells were seeded in six-well plates at a density of 5x104 cell/well. HEI-OC1 cells at ~80% confluency plated into 6-well plates were treated with 4% formaldehyde for 15 min at room temperature. Following washing with PBS, cells were incubated with SA-β-gal staining solution (cat. no. K320-250; BioVision, Inc.) overnight at 37˚C without CO_2. Finally, stained cells from 3 random fields of view were observed under a light microscope (Carl Zeiss AG).

Luciferase reporter assay. Wild-type (WT; 5'-CCCGCC CCG-3') or mutant (MUT; 5'-CAAAAAAAC-3') UCHL1 promoter were cloned into the pGL3 Basic vector (Promega Corporation). Cells ($5x10^5$) were seeded in 24-well plates for 24 h at 37˚C and were transfected with these plasmids as well as with Oe-Sp1 and Oe-NC using Lipofectamine® 2000 (Life Technologies; Thermo Fisher Scientific, Inc.) at 37˚C. After 48 h transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Gene Assay kit (Shanghai Qcbio Science & Technologies Co., Ltd.) according to the manufacturer's instructions. The relative luciferase activity was normalized to that of Renilla luciferase.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using the ChIP kit (Abcam). Briefly, 1% formaldehyde was added to HEI-OC1 cells for 10 min at room temperature. The fixed cells were washed twice with phosphate-buffered saline and were lysed using a lysis buffer (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 8.1) that contained 150 mM NaCl and a protease inhibitor, after which chromatin fragments were obtained using sonication using a 10 sec on and 10 sec off mode for 12 cycles at 4˚C. Following centrifugation at 13,000 x g for 10 min at 4˚C, the DNA fragments were incubated with antibodies against Sp1

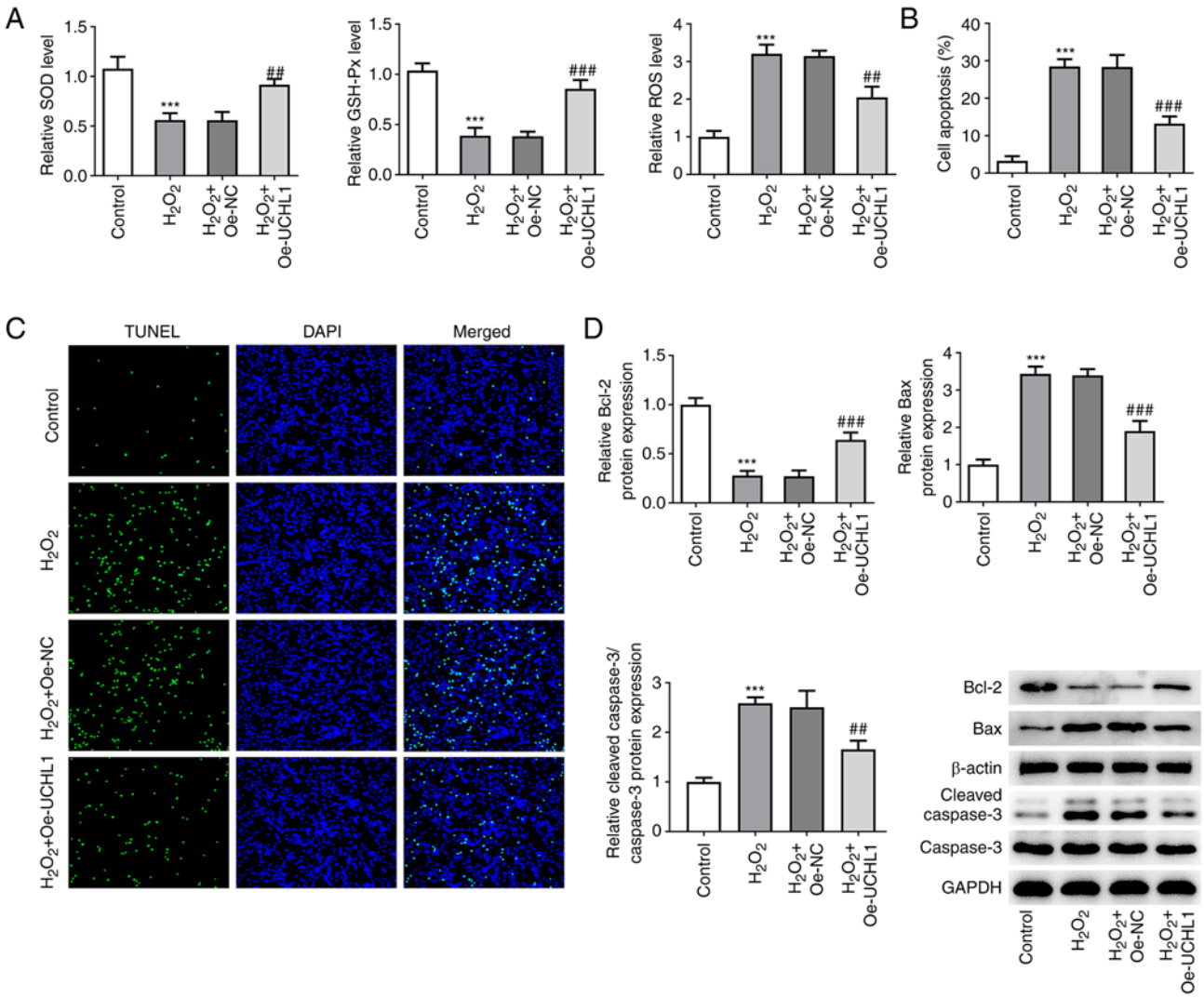

Figure 2. UCHL1 upregulation mitigates H_2O_2-mediated oxidative injury and apoptosis in HEI-OC1 cells. (A) Detection of levels of oxidative stress markers using the corresponding kits. (B) TUNEL assay estimated the apoptosis of H_2O_2-exposed HEI-OC1 cells. Magnification, x200. (C) Quantification of cell apoptotic rate. (D) Western blot analysis of expression of apoptosis-associated factors. ***P<0.001 vs. control. $^{##}$P<0.01 and $^{###}$P<0.001 vs. H_2O_2 + Oe-NC. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; Bcl-2, B cell lymphoma-2; H_2O_2, hydrogen peroxide; Oe-NC, overexpression negative control.

(cat. no. ab227383; 1:200: Abcam) or IgG (cat. no. ab6702; 1:40; Abcam) for 2 h at 4°C. The abundance of Sp1 on the UCHL1 promoter was measured by PCR as aforementioned. The sequence of oligonucleotides flagging the Sp1 binding site in the UCHL1 promoter was 5'-CCCGCCCCC-3'.

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 8 software (GraphPad Software, Inc.). Continuous variables are expressed as the mean ± standard deviation from three independent experiments. The differences between two groups were compared using unpaired Student's t test, while those between multiple groups were by one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

UCHL1 overexpression enhances the viability of H_2O_2-treated HEI-OC1 cells. To evaluate the role of UCHL1 in H_2O_2-induced HEI-OC1 cells, expression levels of UCHL1 were measured. RT-qPCR and western blot analysis showed that UCHL1 was downregulated in H_2O_2-treated HEI-OC1 cells (Fig. 1A and B). To determine the effect of UCHL1 on H_2O_2-treated HEI-OC1 cells, UCHL1 was overexpressed following cell transduction with Oe-UCHL1 plasmid. The overexpression efficiency was verified via RT-qPCR and western blot analysis (Fig. 1C and D). Furthermore, CCK-8 assay demonstrated that treatment with H_2O_2 diminished HEI-OC1 cell viability. However, UCHL1 overexpression increased the viability of HEI-OC1 cells exposed to H_2O_2. These findings indicated that UCHL1 protected HEI-OC1 cells from H_2O_2-triggered cell injury.

UCHL1 overexpression mitigates H_2O_2-mediated oxidative injury and apoptosis in HEI-OC1 cells. Contents of the oxidative stress-related markers SOD, GSH-Px and ROS were measured using the corresponding kits. The results revealed that exposure to H_2O_2 decreased SOD and GSH-Px

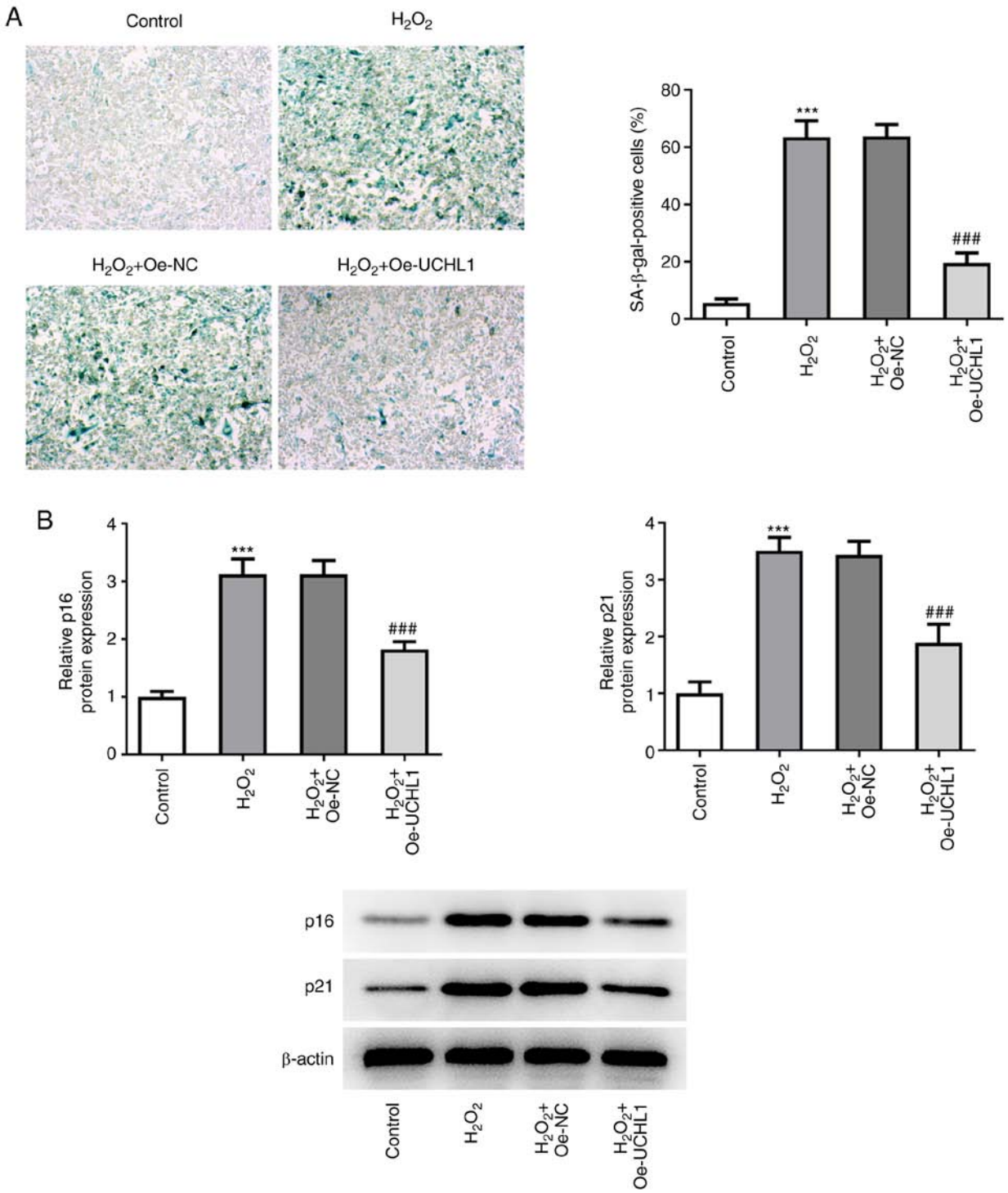

Figure 3. Overexpression of UCHL1 halts H_2O_2-triggered HEI-OC1 cell senescence. (A) SA-β-gal staining indicating cell senescence. Magnification, x100. (B) Western blot analysis of expression of senescence-associated factors. ***P<0.001 vs. control. $^{###}$P<0.001 vs. H_2O_2 + Oe-NC. UCHL1, ubiquitin carboxyl-terminal hydrolase L1; H_2O_2, hydrogen peroxide; Oe-NC, overexpression negative control; SA-β-gal, senescence-associated β-galactosidase.

levels but increased ROS activity. However, UCHL1 overexpression increased SOD and GSH-Px levels and attenuated ROS activity in H_2O_2-treated HEI-OC1 cells (Fig. 2A). Furthermore, TUNEL assay showed that UCHL1 ameliorated H_2O_2-induced HEI-OC1 cell apoptosis (Fig. 2B and C). In addition, western blot analysis revealed that cell exposure to H_2O_2 downregulated Bcl-2 and upregulated Bax, which were reversed by UCHL1 overexpression (Fig. 2D). Overall, these results suggested that UCHL1 exerted an inhibitory effect on H_2O_2-triggered oxidative stress and apoptosis in HEI-OC1 cells.

UCHL1 overexpression inhibits H_2O_2-induced HEI-OC1 cell senescence. SA-β-gal staining illustrated that the increased number of SA-β-gal-positive H_2O_2-treated HEI-OC1 cells was decreased following UCHL1 overexpression (Fig. 3A). In addition, UCHL1 overexpression suppressed the H_2O_2-mediated enhanced expression levels of p16 and p21 (Fig. 3B). These

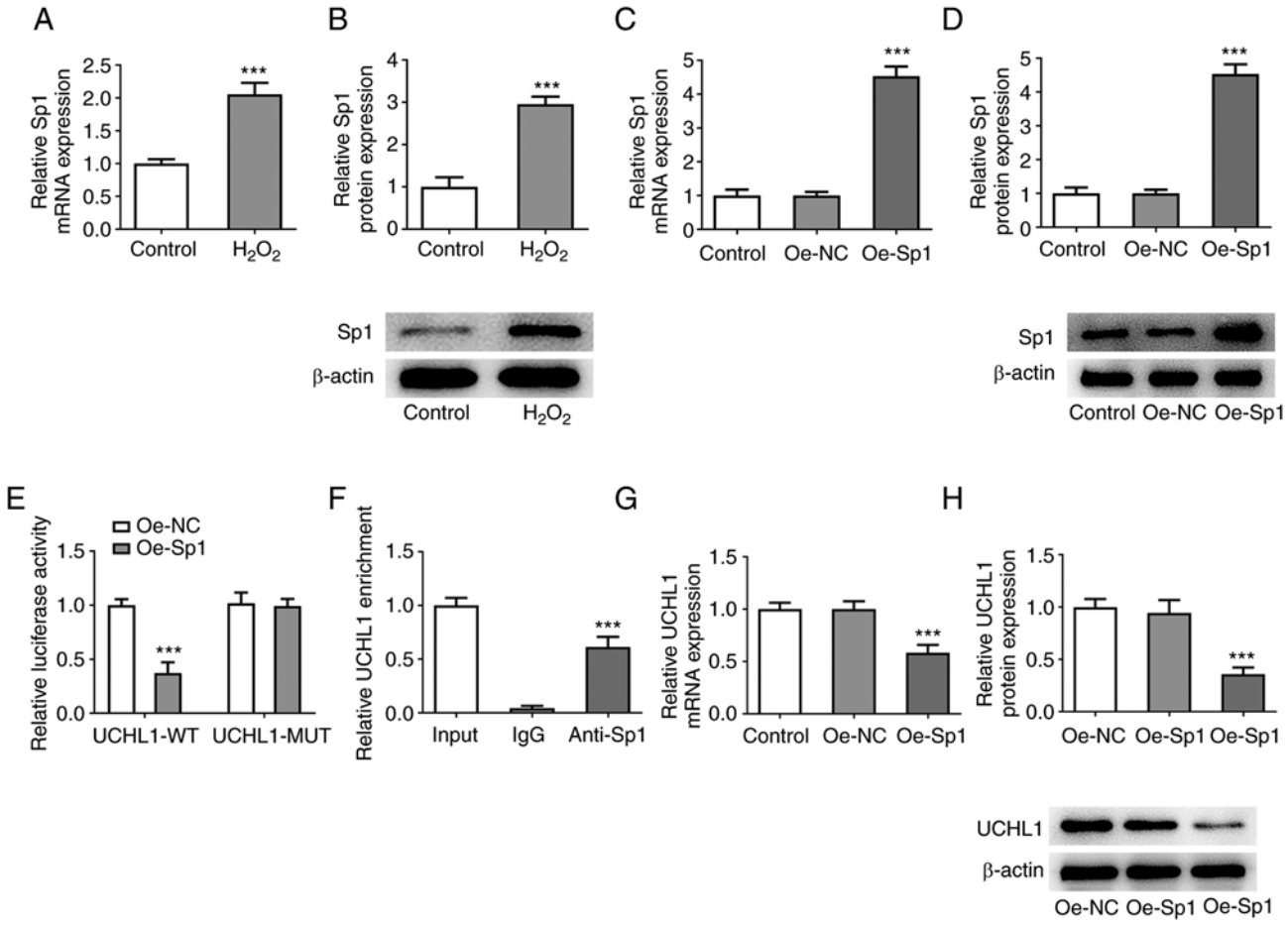

Figure 4. Sp1 suppresses transcription of UCHL1. (A) RT-qPCR and (B) western blot analysis Sp1 expression in H_2O_2-insulted HEI-OC1 cells. Analysis of overexpression efficacy of Oe-Sp1 plasmid by (C) RT-qPCR and (D) western blotting. $^{***}P<0.001$ vs. control. (E) Luciferase reporter assay verified the luciferase activity of UCHL1-WT and UCHL1-MUT. $^{***}P<0.001$ vs. Oe-NC. (F) Chromatin immunoprecipitation assay identified the accumulation of UCHL1 promoter in Sp1 antibody. $^{***}P<0.001$ vs. input. (G) RT-qPCR and (H) western blot analysis of UCHL1 expression after Sp1 was overexpressed. $^{***}P<0.001$ vs. control. UCHL1, ubiquitin carboxyl-terminal hydrolase L1; Sp1, specificity protein 1; RT-q, reverse transcription-quantitative; H_2O_2, hydrogen peroxide; Oe-NC, overexpression negative control; WT, wild-type; MUT, mutant.

results indicated that the H_2O_2-mediated HEI-OC1 cell senescence was attenuated by UCHL1 overexpression.

Sp1 suppresses transcription of UCHL1. The PROMO database revealed that the UCHL1 promoter interacted with the Sp1 transcription factor. RT-qPCR and western blot analysis revealed that Sp1 was upregulated in H_2O_2-induced HEI-OC1 cells (Fig. 4A and B). Following Sp1 overexpression by cell transduction with Oe-Sp1 plasmid (Fig. 4C and D), luciferase reporter assay showed that Sp1 overexpression diminished the luciferase activity of UCHL1-WT compared with UCHL1-MUT (Fig. 4E). ChIP assay showed that UCHL1 was precipitated following incubation of nuclear extracts with Sp1 antibody (Fig. 4F). Additionally, Sp1 overexpression decreased expression levels of UCHL1 (Fig. 4G and H). Collectively, the aforementioned findings demonstrated that Sp1 was a transcriptional inhibitor of UCHL1.

Sp1 overexpression abrogates the protective effect of UCHL1 on H_2O_2-induced HEI-OC1 cell injury. To uncover the association between Sp1 and UCHL1 in H_2O_2-treated HEI-OC1 cells, functional experiments were performed in H_2O_2-induced HEI-OC1 cells co-transduced with Oe-UCHL1 and Oe-Sp1 plasmids. CCK-8 assay revealed that UCHL1 restored the suppressed viability of H_2O_2-induced HEI-OC1 cells. However, this effect was reversed by Sp1 overexpression (Fig. 5A). The enhanced SOD and GSH-Px activity, as well as the reduced ROS levels mediated by UCHL1 overexpression in H_2O_2-induced HEI-OC1 cells, were restored following Sp1 overexpression (Fig. 5B). In addition, the attenuated H_2O_2-induced HEI-OC1 cell apoptosis mediated by UCHL1 overexpression was further increased in cells co-transfected with Oe-Sp1 plasmid (Fig. 5C). This was further verified by western blot analysis, showing that Sp1 overexpression abrogated the enhanced Bcl-2 and decreased Bax expression levels in H_2O_2-exposed HEI-OC1 cells co-transduced with Oe-UCHL1 plasmid (Fig. 5D). Additionally, SA-β-gal staining and western blot analysis demonstrated that the decreased number of SA-β-gal-positive cells, as well as p61 and p21 downregulation triggered by UCHL1 overexpression in H_2O_2-induced HEI-OC1 cells, were reversed by Sp1 overexpression (Fig. 5E and F). Notably, UCHL1 overexpression restored the H_2O_2-induced p-NF-κB p65 upregulation, which was further abolished by Sp1 elevation (Fig. 5G). Taken together, these findings indicated that UCHL1, negatively regulated by Sp1, promoted H_2O_2-mediated cell injury, oxidative stress, apoptosis and senescence, and modulated NF-κB signaling in HEI-OC1 cells.

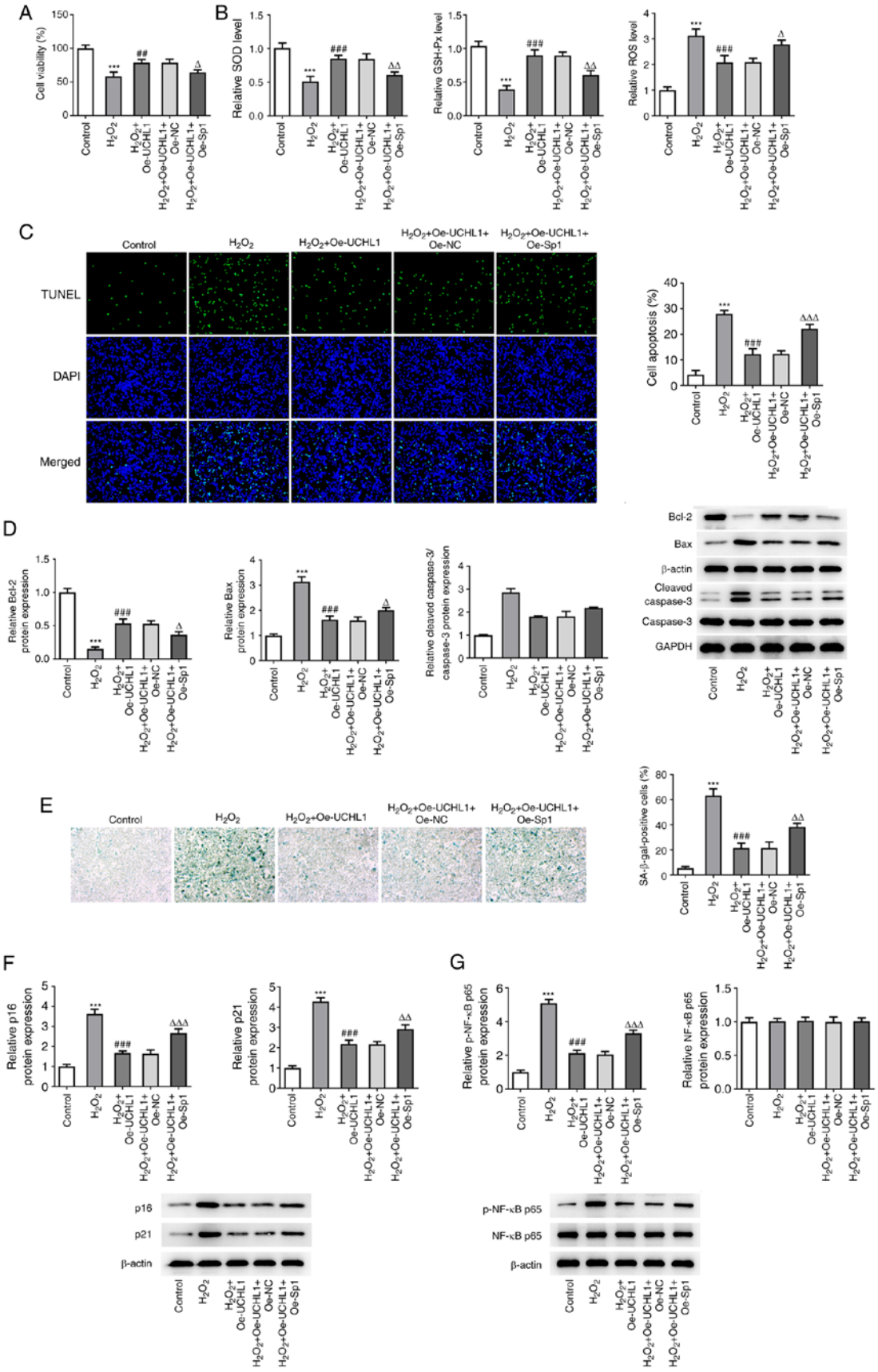

Figure 5. Overexpression of Sp1 abrogates the protective role of UCHL1 in H_2O_2-induced HEI-OC1 cell injury. (A) Viability of H_2O_2-exposed HEI-OC1 cells evaluated by CCK-8 assay. (B) Detection of the levels of oxidative stress markers using corresponding kits. (C) TUNEL assay of apoptosis of H_2O_2-exposed HEI-OC1 cells. Magnification, x200. (D) Western blot analysis of expression of apoptosis-associated factors. (E) SA-β-gal staining analysis of cell senescence. Western blot analysis of expression of (F) senescence- and (G) NF-κB signaling-associated factors. ***P<0.001 vs. control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. H_2O_2 + Oe-UCHL1 + Oe-NC. UCHL1, ubiquitin carboxyl-terminal hydrolase L1; Sp1, specificity protein 1; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; Bcl-2, B cell lymphoma-2; p-, phosphorylated; Oe-NC, negative control; SA-β-gal, senescence-associated β-galactosidase.

Discussion

ARHL is a common clinical condition with complicated pathogenesis (21). Globally, more than 500 million people have ARHL (18). Cochlear hair cells are mechanoreceptors of the auditory system and their loss is a predominant factor contributing to hearing loss (22). Apoptosis is a type of programmed cell death and accelerated cochlear hair cell apoptosis is considered a key factor leading to ARHL (23). Oxidative stress promotes cell or tissue damage caused by the imbalance between ROS production and elimination (24). A growing body of evidence has suggested that oxidative stress is associated with hearing loss and cochlear hair cell injury (23,25). A previous study suggested that cell senescence is a permanent and inevitable state of cell cycle arrest caused by ROS-mediated oxidative stress injury (26). H_2O_2 has been used to induce premature senescence in vascular endothelial cells (27) and keratinocytes (28). Therefore, in the present study, 1 mM H_2O_2 was utilized to induce cochlear hair cell senescence. Cell apoptosis, oxidative stress and senescence were investigated to uncover the underlying mechanism of ARHL.

UCHL1 is a key component of the ubiquitin-dependent protein degradation system, which is a highly conserved pathway involved in removal of damaged or misfolded proteins to prevent protein accumulation and maintain normal cell function (29). A recent study showed that UCHL1 is downregulated in the cochlea of ARHL mice (13). Additionally, UCHL1 regulates expression of ubiquitin proteasome system (UPS)-associated proteins to modify the aging process in the auditory cortex (30). Another study demonstrated that UCHL1 silencing facilitates autophagy-dependent auditory cell death following treatment with gentamicin (31). The results of the present study also revealed that UCHL1 was downregulated in H_2O_2-treated murine cochlea hair cells (HEI-OC1). Furthermore, UCHL1 overexpression effectively mitigated the loss of cell viability triggered by exposure of HEI-OC1 cells to H_2O_2. ROS comprise oxygen radicals, the level of which may reflect the degree of oxidative stress (32). It has been reported that the antioxidant enzymes SOD and GSH-Px eliminate excess ROS levels during the metabolic process to maintain balance (33). As expected, in the present study, cell exposure to H_2O_2 reduced SOD and GSH-Px levels but enhanced ROS activity. However, these effects were restored after UCHL1 overexpression, suggesting that UCHL1 could protect HEI-OC1 cells against H_2O_2-induced oxidative injury. Similarly, H_2O_2-induced HEI-OC1 cell apoptosis was also restored by UCHL1 overexpression. This was further supported since Bcl-2 downregulation and Bax upregulation in H_2O_2-treated HEI-OC1 cells were both reversed by UCHL1 overexpression. Furthermore, p16 and p21 serve a key role in regulating cellular senescence (34). Here, UCHL1 overexpression alleviated H_2O_2-induced cell senescence and decreased the H_2O_2-enhanced p16 and p21 expression levels in HEI-OC1 cells.

As a widely investigated transcription factor, Sp1 activates or inactivates the transcription of several genes encompassing putative CG-rich Sp-binding sites in their promoters (35-36). Bioinformatics analysis using the PROMO database predicted that Sp1 bound to the UCHL1 promoter. Additionally, Sp1 was upregulated in H_2O_2-induced HEI-OC1 cells. The strong affinity of Sp1 with the promoter region of UCHL1 was verified by luciferase reporter and ChIP assays. The results demonstrated that UCHL1 expression was negatively regulated by Sp1. Emerging evidence has suggested that Sp1 is involved in diverse biological events, including embryonic development, cell proliferation, death, senescence and angiogenesis (36). Consistent with the aforementioned findings, the experimental data of the current study also demonstrated that the effects of UCHL1 on viability, oxidative stress, apoptosis and senescence of H_2O_2-induced HEI-OC1 cells were counteracted by Sp1 overexpression.

NF-κB signaling is key in the development of ARHL (17). Furthermore, UCHL1 is considered a downstream protein of NF-κB signaling (37) and is involved in numerous human diseases by regulating NF-κB signaling (38,39). Xue *et al* (40) demonstrated that Sp1 is a regulator of NF-κB signaling in osteoarthritis. The results of the present study showed that the activation of NF-κB signaling mediated by exposure of HEI-OC1 cells to H_2O_2 was inhibited by UCHL1 overexpression. This effect of UCHL1 on NF-κB signaling was abrogated by Sp1 overexpression.

In conclusion, the present study suggested that UCHL1, negatively regulated by Sp1, could promote H_2O_2-mediated cell injury, oxidative stress, apoptosis and senescence and inhibit NF-κB signaling in an *in vitro* model of ARHL. To the best of our knowledge, this is the first study to report the role of UCHL1, as well as the association between UCHL1 and Sp1 in an H_2O_2-induced ARHL model in cochlear hair cells. Taken together, the results of the present study supported the efficacy of a novel targeted therapy for ARHL based on an UCHL1-mediated molecular mechanism. However, further studies in an *in vivo* animal model should be performed to verify the role of UCHL1 in ARHL and changes in the expression levels of UCHL1 and Sp1. In addition, how changes in expression of downstream factors of NF-kB signaling regulate UCHL1 expression should be further investigated.

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Availability of data and materials

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

Authors' contributions

XC and LL designed the study. LL, KX, XB and ZW performed the research. XT and XC analyzed the data. XC and LL drafted the manuscript. XC and LL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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