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Modulation of Mcl-1 expression reduces age-related cochlear degeneration

Wei Ping Yang¹, Yang Xu², Wei Wei Guo¹, Hui Zhan Liu¹, and Bo Hua Hu^{3,*}

¹ Institute of Otolaryngology, Chinese PLA General Hospital, Beijing, PR China

² Department of Respiratory Medicine, Chinese PLA General Hospital, Beijing, P.R. China

³ Center for Hearing and Deafness, SUNY at Buffalo, Buffalo, New York, USA

Abstract

Mcl-1 is an anti-apoptotic member of the Bcl-2 family that modulates apoptosis-related signaling pathways and promotes cell survival. We have previously demonstrated a reduction of Mcl-1 expression in aging cochleae. To investigate whether restoring Mcl-1 expression would reduce aging-related cochlear degeneration, we developed a rat model of Mcl-1 overexpression. A plasmid encoding human Mcl-1/enhanced green fluorescent protein was applied to the round window of the cochlea. This *in vivo* treatment transfected both the sensory and supporting cells of the cochlear sensory epithelium and enhanced Mcl-1 expression at both the mRNA and the protein level. The upregulation of Mcl-1 expression reduced the progression of age-related cochlear dysfunction and sensory cell death. Furthermore, the transfection of Mcl-1 exerted its protective effect by suppressing cochlear apoptosis at the mitochondrial level. This study demonstrates that the genetic modulation of Mcl-1 expression reduces the progression of age-related cochlear degeneration.

Keywords

Mcl-1; gene delivery; cochlea; aging; intervention; hair cells; rat

1. Introduction

Age-related hearing loss (ARHL) is one of the most common impairments in the aging population (Cruickshanks et al., 1998; Roth et al., 2011; Seidman, et al., 2002). The degeneration of sensory cells in the cochlea is a major underlying pathology of ARHL (Kusunoki et al., 2004; Popelar et al., 2006; Sha et al., 2008; Tarnowski et al., 1991; Ter Haar et al., 2009). Emerging evidence suggests that hair cells degenerate via diverse death processes, such as apoptosis and necrosis (Hu et al., 2008; Sha et al., 2009), with apoptosis being the primary contributor in models of cell degeneration (Hu et al., 2008; Nevado et al., 2006; Op de Beeck et al., 2011; Park et al., 2010; Someya et al., 2009). Recently, interest

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*Corresponding author: Bo Hua Hu Center for Hearing and Deafness, State University of New York, 137 Cary Hall, 3435 Main Street, Buffalo, NY 14214, USA Phone: (716) 829-5316 Fax: (716) 829-2980 bhu@buffalo.edu. Wei Ping Yang : yangwp2002@yahoo.com Yang Xu : xuyang603@yahoo.com.cn Wei Wei Guo : gwent001666@yahoo.com.cn Hui Zhan Liu : liuhuizhan@xmsn.cn.

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has focused on suppressing age-related cell death through the modulation of signal transduction pathways that control the cell degeneration process (Wang et al., 2010).

Apoptosis is an active form of cell death that requires a cascade of signaling events. Several studies have documented the involvement of the mitochondrial signaling pathway in age-related cochlear apoptosis (Hu et al., 2008; Nevado et al., 2006; Sha et al., 2009). This cell death pathway includes the B-cell lymphoma-2 (Bcl-2) family, which contains both pro- and anti-apoptotic members (Someya et al., 2009). Genes in the Bcl-2 family are expressed in the rat cochlea (Alam et al., 2001; Hu et al., 2008; Hu et al., 2009; Hu and Cai, 2010), and a recent study using large-scale gene expression analysis implicated the Bcl-2 family in age-related cochlear degeneration (Tadros et al., 2008).

Mcl-1 is an anti-apoptotic member of the Bcl-2 family that was initially identified in differentiating myeloid cells (Kozopas et al., 1993). This gene is expressed in multiple cell types and acts as an upstream regulator of apoptosis by promoting cell survival (Michels et al., 2005). In aging cochleae, the expression of Mcl-1 is down-regulated at both the mRNA and protein levels (Yang et al., 2012). Importantly, this reduction in Mcl-1 expression is spatially related to sensory cell degeneration, suggesting a potential role for Mcl-1 in the aging process of the sensory cells. Given that Mcl-1 is required for the regulation of apoptosis, we hypothesized that enhancing Mcl-1 expression could promote sensory cell survival in aging cochleae and thus reduce ARHL. To test this hypothesis, we transfected rat cochleae *in vivo* with a plasmid encoding human Mcl-1/enhanced green fluorescent protein (pEGFP) and examined the effect on the progression of ARHL and sensory cell degeneration. We demonstrated that application of the plasmid to the round window of the cochlea resulted in transfection of both the sensory and supporting cells of the cochlear sensory epithelium, leading to enhanced Mcl-1 expression in the sensory cells. Importantly, the upregulation of Mcl-1 expression reduced the progression of ARHL and sensory cell death. Furthermore, the protective effect of Mcl-1 transfection was mediated by a reduction in cochlear apoptosis, which occurred at the mitochondrial level. This study demonstrates that the genetic modulation of Mcl-1 expression reduces the progression of age-related cochlear degeneration.

2. Materials and Methods

2.1. Animals

Fischer 344/DuCrIvR inbred rats (male and female, 24-25 months old, 300-400 g) were utilized to determine the effect of Mcl-1 transfection on the progression of the aging process in the cochlea. Initially, young Sprague Dawley rats (male and female, 2-3 months old, 200-250 g) were used to establish the protocol for transfecting cochleae with the recombinant Mcl-1/pEGFP plasmid. All animals were purchased from the Animal Center of Weitong Lihua in Beijing and were raised in the Animal Center of Chinese PLA General Hospital. The procedures involving the use and care of the animals were reviewed and approved by the Animal Center of Chinese PLA General Hospital.

2.2. Experimental protocol

All of the animals received a baseline hearing evaluation and were then randomly assigned to either the experimental or the control group. Subjects in the experimental group were transfected with the Lipofectamine-plasmid Mcl-1/pEGFP complex in one ear. Auditory function was reevaluated at defined time points following Mcl-1 transfection (see the following sections for details) and the cochleae collected after the final hearing evaluation. The collected cochleae were processed for either Mcl-1 expression analysis or morphological/biological analyses of the cochlear sensory epithelium.

Each experimental group was age-matched with a control group. During the development of the transfection protocol in young rats, the control group received a liposome treatment in one ear. The effect of this treatment was evaluated using the same protocols used in the experimental group. In the second phase of the study, aging rats were evaluated, and therefore the control group contained age-matched rats. Again, auditory function and cochlear morphology/biology were analyzed using the same paradigms used in the experimental group. The number of animals and cochleae included in each experimental condition or assay is described in the Results section.

2.3. Preparation of transfection complexes

The plasmid encoding human Mcl-1 and pEGFP (1 µg/µl) was constructed and verified using a previously described protocol (Guo et al., 2010). To obtain the Mcl-1 gene fragment, we extracted total RNA from human tonsil tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) and synthesized the first strand cDNA using a SuperScript™ III First Strand Synthesis System kit (Invitrogen). The Mcl-1 gene fragment was amplified by polymerase chain reaction (PCR). The following primers were utilized: Mcl-1-EcoR I: 5'-CGGAATTCATGTTTGGCCTCAAAGAAAC-3' Mcl-1-BamH I: 5'-CGGGATCCCGTCTTATTAGATATGCCAAACCAG-3' The reaction conditions were as follows: 95 °C for 5 min, 95 °C for 45 sec, 58 °C for 45 sec, 72 °C for 60 sec, and extension at 72 °C for 5 min, for a total of 40 cycles.

To construct the expression vector, the Mcl-1 PCR product and the expression vector pEGFP-N1 (Clontech BD Bioscience, Palo Alto, CA, USA) were digested with EcoRI and BamHI. The digestion products were incubated with T4 DNA Ligase at 22 °C for 3 h to ligate the Mcl-1 gene fragment into the pEGFP-N1 expression vector. The resulting recombinant plasmid was transformed into *E. coli* for replication and then extracted from the *E. coli* using an EndoFree Plasmid Maxi Kit (Cat. No 12362, Qiagen).

The quality of the construct was assessed using restriction endonuclease digestion and DNA sequencing. For the restriction endonuclease digestion, 15 U of the endonucleases EcoRI and BamHI (no more than 1/10 of the total reaction volume) were added to a reaction mixture containing 0.5 - 1 µg plasmid in a final volume of 20 µl. The solution was incubated in a 37 °C water bath for 2 h. Agarose gel electrophoresis was utilized to identify the positive recombinant in the digestion products. For DNA sequencing, a full-gene sequencing analysis of the expression vector was performed by Invitrogen.

Lipofectamine™2000 (Invitrogen) was used to deliver the Mcl-1/pEGFP plasmid to the rat cochlea. The Lipofectamine solution was mixed with the Mcl-1/pEGFP plasmid at a ratio of 1:3 (1 µl/µg plasmid to 3 µl lipofectamine) per the manufacturer's protocol. The mixture was incubated at room temperature under aseptic conditions for 20 min.

2.4. Surgical delivery of the Lipofectamine-Mcl-1/pEGFP plasmid complex to the cochlea

For *in vivo* gene delivery, the Lipofectamine-Mcl-1/pEGFP plasmid complex was surgically placed on the round window of the cochlea, which allowed the plasmid to pass through the round window membrane into the cochlea. Each animal was anesthetized with an injection of ketamine (30 mg/kg) and acepromazine (0.1 mg/kg) and the body temperature maintained at 38 ± 1°C with a heating pad. Under aseptic conditions, the right bulla was exposed using a ventrolateral approach, and a small hole (1.0 mm) was made in the wall of the bulla to access the round window of the cochlea. Four microliters of the Lipofectamine-Mcl-1/pEGFP plasmid complex solution (in the experimental animals) or the same volume of liposome solution (in the control animals) was placed on the bony groove of the round window niche using a micro-syringe. Subsequently, the hole in the bulla was sealed with

dental acrylic cement and the skin incision sutured. The animals were kept alive for a defined period of time and their auditory function reevaluated.

2.5. Cochlear tissue collection

At a defined time point after the surgery, the animals were anesthetized with an injection of ketamine (30 mg/kg) and acepromazine (0.1 mg/kg), sacrificed, and the cochleae collected for Mcl-1 expression analysis, morphological assessment of sensory cell degeneration, or biological analysis of apoptosis. The time schedule for cochlea collection is detailed in the Results section.

2.6. qRT-PCR

The expression of Mcl-1 mRNA was determined using quantitative real-time polymerase chain reaction (qRT-PCR) as described previously (Yang et al., 2012). Briefly, the cochleae were collected and dissected in an RNA stabilization reagent (RNAlater, Qiagen, USA). The cochlear tissue containing the sensory epithelia and the lateral wall was collected and the tissue from the two cochleae pooled to generate one sample. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized using a SuperScript™ III First Strand Synthesis System kit (Invitrogen).

The expression level of Mcl-1 was measured using a TaqMan® Gene Expression Assay Kit (TaqMan® MGB probes, FAM™ dye-labeled, Applied Biosystems, USA). qRT-PCR was performed using a Real-Time PCR System (7900 HT, Applied Biosystems, USA). Upon completion of the PCR run, the cycle threshold (Ct) values were calculated. To quantify the relative expression level of Mcl-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was processed and amplified along with the Mcl-1 mRNA in each sample.

2.7. Western blotting

The expression of Mcl-1 protein was assessed by Western blot according to a previously described method (Yang et al., 2012). Briefly, the cochlear tissue containing the sensory epithelia and the lateral wall was collected and the tissue from the two cochleae pooled to generate one sample. The tissue was homogenized and the homogenates centrifuged at 12,000 g at 4°C. The protein concentration was measured using a Microplate Reader (DNM-9602A, Perlong Medical Equipment Co., Ltd, China).

The proteins were separated and transferred to a polyvinylidene fluoride membrane via electrophoresis (DYY-7C, Beijing luiyi Instrument Factory, China). The membranes were blocked with blocking buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween containing 10% non-fat milk) and then probed with a mouse monoclonal antibody against Mcl-1 (1:100, sc-74437, Santa Cruz Biotechnology Inc., USA) at 4°C overnight. After rinsing, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG, Zhongshan Goldenbridre Biotechnology Co., Ltd, China). The stained membrane was visualized and imaged using a luminescence image analyzer (AX-II, Guangdong Yuehua Medical Instrument Factory Co., Ltd, China). To quantify the relative expression of Mcl-1 protein, β -actin was utilized as a loading control.

2.8. Immunolabeling of Mcl-1 and cytochrome c

Immunolabeling was performed to determine the spatial expression pattern of Mcl-1 protein in the cochlear sensory epithelium and the release of cytochrome c from mitochondria using previously described methods (Nicotera et al., 2003). The animals were sacrificed and the cochleae fixed with 10% buffered formalin overnight at 4 °C. The cochleae were dissected and the cochlear sensory epithelia collected. After membrane permeabilization with 0.2%

Triton X-100 in PBS, the tissues were incubated with either a mouse monoclonal antibody against Mcl-1 (1:50, sc-74437, Santa Cruz Biotechnology Inc., USA) or an anti-cytochrome c antibody (2.5 µg/ml, 6H2.B4; BD PharMingen, San Diego, CA) at 4 °C overnight. The tissue was then incubated with a secondary antibody (Mcl-1 staining: Alexa Fluor® 488 goat anti-mouse, 1:100 in 10 mM PBS, Invitrogen; cytochrome c staining: Cy2-conjugated goat anti-mouse IgG, 1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and counterstained with propidium iodide (see the subsequent section for more detail). To assess non-specific staining, a few specimens were incubated with only the secondary antibody.

2.9. F-actin staining

F-actin, an important structural protein in hair cells, was labeled to visualize the cuticular plates so that missing hair cells could be quantified. The procedure for staining F-actin has been previously described (Hu et al., 2002). Briefly, the animals were sacrificed and the cochleae fixed. After cochlear dissection, the sensory epithelia were incubated for 30 min in a staining solution that contained fluorescein isothiocyanate (FITC)-phalloidin (1:250, Sigma), 0.25% Triton-X-100 and 1% bovine serum albumin. After the F-actin staining, the specimens were counterstained with propidium iodide.

2.10. Nuclear staining

To identify sensory cell degeneration and to provide a structural marker for the assessment of cell-type-specific changes, we performed nuclear staining of the cells in the sensory epithelium using propidium iodide, a DNA-intercalating fluorescent probe, according to a previously described protocol (Yang et al., 2004). Briefly, the cochleae were collected and dissected. The organ of Corti was incubated in propidium iodide (5 µg/ml in 10 mM PBS, Invitrogen) in the dark for 10 min at room temperature. The specimens were rinsed in PBS and mounted on slides containing an anti-fade medium (ProLong™ Antifade kit, Invitrogen). When this labeling was performed as a counterstain for the immunolabeling described above, the tissue was always immunostained first and then counterstained with propidium iodide.

2.11. Assessment of sensory cell damage

Apoptotic, necrotic, and missing hair cells were quantified to determine the extent of sensory cell degeneration. The criteria for identifying these pathologies have been previously described (Yang et al., 2004). Within the hair cell area, missing hair cells were identified by a lack of nuclear staining, which was confirmed by examining the cuticular plates of the F-actin-stained cells. Hair cells exhibiting condensed nuclei and elevated propidium iodide fluorescence were classified as apoptotic. Hair cells with swollen nuclei and reduced propidium iodide fluorescence were categorized as necrotic. In accordance with our previous studies (Hu et al., 2008), the observed nuclear changes agreed with commonly used biological markers of apoptosis, such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 staining, indicating that these nuclear alterations are reliable indicators of the specific mode of cell death.

2.12. Measurement of auditory brainstem response thresholds

Auditory brainstem response (ABR) thresholds were measured before and at various time points after Mcl-1 transfection using a previously described protocol (Yang et al., 2012). The animals were anesthetized by an injection of ketamine (30 mg/kg) and acepromazine (0.1 mg/kg). The ABR was elicited with digitally generated tone bursts (4, 8, 16, 32, and 40 kHz; System III, TDT, USA) that were played through an electrostatic speaker (ES1, TDT, USA) placed 1 cm from the test ear at the level of the ear canal. The stimulus level was

calibrated using a sound level meter (BK 2231, BK Inc., Denmark) and stimuli delivered in descending 5-dB steps from 100 to 20 dB sound pressure level (SPL). The ABR was measured with subdermal needle electrodes over the vertex (non-inverting input), behind the test ear (inverting input), and behind the shoulder blade (ground) of the animal. The response signals were amplified, filtered (100-3000 Hz), and averaged (1024 sweeps). The ABR threshold was defined as the lowest stimulus intensity that reliably elicited a detectable response.

2.13. Data analyses

The relative quantification method (Livak and Schmittgen, 2001) was used to analyze the expression of Mcl-1 mRNA. The expression level of Mcl-1 was normalized to the expression level of GAPDH to generate the ΔCt . Then, the $\Delta\Delta\text{Ct}$ was calculated with the following formula: ΔCt of the experimental group – ΔCt of the control group. One-way Analysis of Variance (ANOVA) was used to evaluate significance. A fold-change equal to or greater than two was considered biologically significant.

In the Western blot analyses, the intensity of the Mcl-1 band was quantified using image processing software (IMAGE-Pro Plus, Media Cybernetics Co., USA). The intensity was normalized to that of β -actin on the same blot. The gray levels of the Mcl-1 bands in the control and test groups were compared using a two-way ANOVA (treatment $\times$ time).

To analyze Mcl-1 immunoreactivity, the tissue was photographed using confocal microscopy (Zeiss, LSM 510). The staining intensity was measured with image processing software (IMAGE-Pro Plus, Media Cybernetics Co. USA). Specifically, the gray level of the pixels within the cytoplasm of the selected cells (at least 15 representative cells for each sample) was quantified. Then, the gray levels of individual cells were averaged to generate a mean value for each sample. The average gray levels among the different experimental conditions were compared using a two-way ANOVA with treatment as one factor and cochlear site as the other.

To analyze the cytochrome c staining, we quantified the number of cytochrome c-positive cells; positive cells were defined as those cells exhibiting strong cytoplasmic immunolabeling when compared to the background staining in neighboring cells. The percentage of cytochrome c-positive cells was analyzed with a two-way ANOVA (treatment $\times$ cochlear sites).

To quantitatively analyze the number of damaged sensory cells, each organ of Corti was thoroughly examined from the apex to the base of the cochlea. The number of apoptotic, necrotic and missing hair cells was documented along the entire length of the organ of Corti, and the data were assembled into cochleograms. A two-way ANOVA (treatment $\times$ cochlear sites) was performed to compare the number of damaged sensory cells in the different groups.

A two-way ANOVA, with time/treatment as one factor and frequency as the other, was used to identify significant differences in the mean ABR thresholds among the different experimental conditions.

For all of the statistical analyses, the significance value was set at $p < 0.05$.

3. Results

3.1. Local application of Mcl-1/pEGFP to the round window transfected both sensory and supporting cells in the cochlear sensory epithelium

We first determined whether local application of the Lipofectamine-Mcl-1/pEGFP plasmid complex to the round window of the cochlea would transfect sensory and supporting cells in the sensory epithelium. Cochleae from young rats were examined with confocal microscopy at 5, 10, and 15 days after the treatment ($n = 6$ cochleae for each time point). Sporadic granular green fluorescence, an indication of the cellular expression of Mcl-1/pEGFP, was first observed in the supporting cell region 10 days after the treatment (Figure 1A). A strong fluorescence was then observed in sensory cells 15 days post-treatment (Figure 1B). At this time point, increased fluorescence was also detected in supporting cells, including pillar and Deiters cells. The cochleae of the control animals, which received liposomes alone, did not exhibit green fluorescence in the sensory epithelium (data not shown). Together, these observations suggest that, when applied locally, the Mcl-1/pEGFP plasmid can transfect both sensory and supporting cells in the sensory epithelium of the treated ear.

The contralateral ears of the animals, which did not receive the Mcl-1/pEGFP treatment, also exhibited Mcl-1/pEGFP fluorescence (data not shown), suggesting that Lipofectamine-Mcl-1/pEGFP plasmid complex applied to one ear can cross over to the contralateral ear, possibly through the cochlear aqueduct/the cerebrospinal fluid pathway. Because this type of crossover has been observed previously (Lalwani et al., 1997; Stover, et al., 2000), our subsequent analyses focused on changes occurring only in the treated ears.

3.2. Transfection of Mcl-1 did not affect sensory cell viability or cochlear function

Cochlear sensory cells are susceptible to changes in their intrinsic and extrinsic microenvironments. Therefore, we sought to determine whether the surgical procedure used to deliver the plasmid and the subsequent Mcl-1 transfection could affect sensory cell function. Sensory cell viability and cochlear function were examined at multiple time points following treatment with Mcl-1/pEGFP. This portion of the experiment was performed on young rats, which had normal hearing sensitivity and little sensory cell death in the cochlea, to preclude any difficulty distinguishing between age-related cochlear degeneration and Mcl-1/pEGFP-induced cochlear changes.

We examined nuclear morphology using propidium iodide staining at 7, 10, and 15 days as well as 1, 2 and 3 months after administration of the Mcl-1 plasmid ($n = 6$ cochleae at each time point). This time period covers both the short- and long-term effects of Mcl-1/pEGFP treatment. The sensory cells retained a normal nuclear shape and size at all of the time points. As expected, the control cochleae that received the liposome-only treatment displayed normal nuclear morphology (data not shown). These results indicate that the experimental paradigm used to transfect Mcl-1 did not affect sensory cell viability.

We next determined the ABR thresholds of the animals before and after Mcl-1 delivery (at 7 days and 1, 2 and 3 months, $n = 6$ cochleae at each time point) to evaluate cochlear function. No significant differences in threshold were found before and after Mcl-1 plasmid treatment at any of the tested frequencies (two-way repeated measures ANOVA with time and frequency as factors, main effect of time: $F = 1.478$, $df = 4, 125$, $p = 0.213$), indicating that the Mcl-1/pEGFP treatment did not compromise auditory function. Moreover, there were no significant changes in threshold in the control animals following the liposome treatment (two-way ANOVA, $F = 2.237$, $df = 4, 125$, $p = 0.069$). Together, these findings suggest that the cochlea tolerated the surgery for Mcl-1 plasmid delivery and subsequent Mcl-1 transfection.

3.3. Mcl-1 transfection increased the expression of Mcl-1 in aging cochleae

To determine whether the transfection of Mcl-1 using the Lipofectamine-Mcl-1/pEGFP plasmid complex increased the expression of Mcl-1 in the cochleae of aging rats (24-25 months), we examined the expression of Mcl-1 mRNA at 3, 6 and 9 days after the application of the Mcl-1 plasmid (n = 5 biological replicates for each time point). Compared to the controls, the treated ears exhibited a 3- to 3.5-fold increase in the expression of Mcl-1 (Figure 2), which was statistically significant (one-way ANOVA, $F = 93.9$, $df = 3, 11$, $p < 0.001$; Tukey test, $p < 0.001$ for control vs. each of the 3, 6 and 9 day groups). Therefore, the data indicate that transfection of the Mcl-1 plasmid increased the expression of Mcl-1 mRNA.

Next, we determined the spatial distribution and sites of altered Mcl-1 expression in the cochlear sensory epithelium following Mcl-1 transfection by examining Mcl-1 immunoreactivity in the cochleae of the experimental and the age-matched control animals one month after the Mcl-1/pEGFP and liposome-only treatment, respectively (n = 5 cochleae in each group). At this time, the pEGFP fluorescence could not be detected, therefore the green fluorescence observed in the tissue reflected the labeling of Mcl-1 immunoreactivity.

To better define the spatial distribution, we divided the sensory epithelium into three sections: apical, middle and basal, covering the region 0-30%, 35-70% and 75-100% from the apex of the cochlea, respectively. These three sections have differing degrees of sensory cell degeneration in aging cochleae (Hu et al., 2008).

The control cochleae displayed heterogeneous immunoreactivity in outer hair cells (Figure 3A and B). Intensely labeled outer hair cells were more prevalent in the apical and basal sections of the cochlea. Supporting cells and inner hair cells exhibited only weak fluorescence. Compared to control ears, Mcl-1/pEGFP-treated ears had increased Mcl-1 immunoreactivity in sensory cells (Figure 3C and D). To confirm the observed differences between treated and control ears, we measured the intensity of Mcl-1 immunoreactivity using an image analysis method. The data were analyzed with a two-way ANOVA with experimental condition (Mcl-1/pEGFP-treated and age-matched control ears) as one factor and cochlear site (the apical, middle and basal sections of the cochlear sensory epithelium) as the other factor. The effect of treatment was significant ($F = 5015.8$, $df = 1, 84$, $p < 0.001$, Figure 3E), indicating that the treated ears had increased Mcl-1 immunoreactivity. However, the interaction effect was not significant ($F = 2.2$, $df = 2, 84$, $p = 0.113$), indicating that the increased expression was not related to a particular cochlear section. Together, these expression analyses indicate that Mcl-1 transfection increased the expression of Mcl-1 in a cell-type specific manner.

To test the specificity of the Mcl-1 antibody, we used Western blot analysis to determine the molecular weight of the protein targeted by the antibody. A strong band appeared at 42 kDa and a weak band at 30 kDa; these bands are consistent with the reported molecular weight of Mcl-1 (Bae et al., 2000; Chao et al., 1998; Crossley et al., 2003). To evaluate non-specific staining, a few specimens were stained only with the secondary antibody; this tissue displayed weak background fluorescence (data not shown).

3.4. The up-regulation of Mcl-1 expression reduced the progression of sensory cell degeneration in aging cochleae

After successfully enhancing Mcl-1 expression in sensory cells of the aging cochlea, we determined whether this increase in expression affected the progression of age-related sensory cell degeneration. We examined ABR thresholds 3 months after the Mcl-1/pEGFP treatment (n = 18 cochleae) and compared the data with those from age-matched control animals (n = 18 cochleae). The treated ears had less hearing loss by an average of 12.7 ± 3.21

dB (two-way ANOVA with treatment and frequency as factors, ($F = 228.5$, $df = 2, 165$, $p < 0.001$, Figure 4), indicating that Mcl-1 transfection reduced the progression of age-related hearing loss.

To determine whether the hearing protection was accompanied by sensory cell protection, we examined the extent of sensory cell degeneration three months after Mcl-1 transfection ($n = 12$ cochleae) and compared the data with those collected from age-matched control animals ($n = 12$ cochleae). FITC-phalloidin labeling revealed the cuticular plates of the sensory cells, allowing us to identify the areas of missing cells, and propidium iodide, a DNA-intercalating fluorescent probe, highlighted the morphology of the sensory cell nuclei. The number of damaged outer hair cells (missing hair cells and hair cells with condensed or swollen nuclei) was quantified and the data assembled into a cochleogram.

In control cochleae, we observed site-dependent sensory cell degeneration in the sensory epithelium that was more prominent in the basal and apical sections (Figure 5A and B, Kruskal-Wallis one-way ANOVA, $p < 0.001$; Tukey test, both apex/middle and base/middle: $p < 0.05$). This pattern of sensory cell degeneration is consistent with our previous observations in the same rat species (Hu et al., 2008).

Both treated and control ears exhibited a similar spatial pattern of sensory cell degeneration. However, the magnitude of the sensory cell damage was reduced in the treated ears (Figure 5C and D). A two-way ANOVA (treatment $\times$ cochlear site) revealed a significant difference in the number of damaged cells between the Mcl-1-transfected and the control ears in all sections of the cochlear sensory epithelia ($F = 140.07$, $df = 1, 30$, $p < 0.001$; Tukey test, $p < 0.05$, Figure 5E). The difference was more prominent in the basal section than in the other two sections (one-way ANOVA, $F = 58.773$, $df = 1, 17$, $p < 0.001$; Tukey test, base vs. apex: $p < 0.001$, base vs. middle: $p < 0.001$). This base-dominated protection may be associated with the accelerated sensory cell degeneration that takes place in this region during this stage of aging (Hu et al., 2008). Together, the physiological and pathological analyses of Mcl-1/pEGFP-treated and control ears suggest that increased Mcl-1 expression reduced age-related cochlear degeneration.

3.5. The reduction of sensory cell degeneration was mediated by the suppression of apoptotic cell death

Our previous study revealed that the degeneration of cochlear sensory cells occurs via both apoptosis and necrosis (Hu et al., 2008). Given the potential role of Mcl-1 in the suppression of apoptosis, we determined whether Mcl-1 transfection reduced apoptosis in the sensory epithelium. To identify apoptotic cells, we observed the nuclear morphology illustrated with propidium iodide staining. Our previous study indicated that cells with nuclear condensation have increased caspase-3 activity, a biological hallmark of apoptosis (Hu et al., 2008). By contrast, cells with nuclear swelling lack the caspase activity, indicating that nuclear morphology is a reliable indicator of the mode of cell death.

Based on the nuclear morphology, we quantified the number of cells showing condensed or swollen nuclei ($n = 12$ cochleae in each group). In the aging cochleae (both the control and treated ears), more cells contained condensed nuclei than contained swollen nuclei (47.8 ± 31.0 for apoptotic cells vs. 5.6 ± 3.3 for necrotic cells, Mann-Whitney Rank Sum Test, $p < 0.001$), consistent with our previous observation that apoptosis dominates sensory cell degeneration in aging cochleae (Hu et al., 2008). Compared to the control ears, the Mcl-1-transfected ears had less sensory cell degeneration, stemming from a significant reduction in the number of apoptotic cells (two-way ANOVA with treatment and mode of cell death as factors, $F = 65.999$, $df = 1, 52$, $p < 0.001$; Tukey test, $p < 0.001$, Figure 6). However, the reduction in the number of necrotic cells was not statistically significant ($p = 0.458$). These

data suggest that the reduction of sensory cell degeneration was mediated primarily by the suppression of apoptotic cell death.

3.6. Suppression of apoptosis at the mitochondrial level

Mcl-1 interacts with mitochondrial proteins to regulate apoptotic events in the mitochondria (Cuconati et al., 2003; Thomas et al., 2010). Our previous research revealed that the release of cytochrome c from mitochondria is an early event in the age-related apoptosis of cochlear sensory cells (Hu et al., 2008). To determine whether the protection afforded by Mcl-1 is associated with inhibition of the mitochondrial apoptotic pathway, we investigated cytochrome c immunoreactivity, an indicator of cytochrome c release from the mitochondria, in cochleae collected 3 months post-transfection ($n = 6$ cochleae) and in age-matched control ears ($n = 6$ cochleae).

In the age-matched control cochleae, robust cytochrome c immunoreactivity was present in the cytoplasm of sensory cells. These cytochrome c-positive cells were most prevalent in the basal section and least prevalent in the middle section (Kruskal-Wallis one-way ANOVA on ranks, $p < 0.001$; Tukey test, $p < 0.05$ for all of the comparisons). This distribution pattern is consistent with the greater sensory cell damage observed in the apical and basal sections of the cochlea in both the present and previous studies (Hu et al., 2008).

There were significantly fewer cytochrome c-positive cells in the Mcl-1/pEGFP-treated cochleae than in the control ears (Figure 7) (two-way ANOVA, treatment $\times$ cochlear site, $F = 627.2$, $df = 1, 30$, $p < 0.001$; Tukey analysis: $p < 0.001$). The reduction was greatest in the basal section of the cochlear sensory epithelium (one-way ANOVA, $F = 110.705$, $df = 2, 15$, $p < 0.001$; Tukey test, base vs. apex: $p < 0.001$, base vs. middle: $p < 0.001$). These changes in cytochrome c release are consistent with the spatial pattern of the reduction in apoptotic cells observed after Mcl-1 transfection, suggesting that the reduction in apoptotic activity is mediated by the suppression of apoptosis at the mitochondrial level.

3.7. Mcl-1 transfection did not affect Mcl-1 expression in young cochleae

Our previous study demonstrated that Mcl-1 is robustly expressed in young cochleae (Hu et al., 2009). To determine whether the transfection of Mcl-1/pEGFP affected Mcl-1 expression in young cochleae, we examined Mcl-1 expression at both the mRNA and protein level in young rats both before and 1, 2, and 3 months after the Mcl-1/pEGFP treatment ($n = 6$ biological replicates at each time point). The qRT-PCR analysis revealed a small insignificant difference between the Mcl-1/pEGFP-treated and the control ears (Figure 8A) (one-way ANOVA, $F = 0.233$, $df = 3, 11$, $p = 0.871$). Moreover, there was no significant difference in protein expression at any of the time points, as assayed by Western blot (Figure 8B and C, two-way ANOVA, $p > 0.05$). These data indicate that Mcl-1 transfection did not enhance Mcl-1 expression in the young cochlea.

4. Discussion

The present study aimed to determine whether enhancing Mcl-1 expression would promote sensory cell survival and reduce the loss of functionality in aging rat cochleae. We demonstrated that local application of the Lipofectamine-Mcl-1/pEGFP plasmid complex to the round window of the aging rat cochlea effectively transfected both sensory and supporting cells in the organ of Corti and that the transfection increased Mcl-1 expression in the sensory epithelium in a cell type-dependent manner. Importantly, Mcl-1 overexpression reduced the progression of ARHL and sensory cell degeneration. This protective effect resulted from the suppression of sensory cell apoptosis and correlated with a reduction in mitochondrial signaling events that lead to apoptosis. The study clearly demonstrated that

genetic modulation of Mcl-1 expression reduced the progression of age-related sensory cell degeneration in the cochlea.

The primary pathology of ARHL is sensory cell degeneration in the cochlea (Li and Hultcrantz, 1994; Spongr et al., 1997). In previous studies, we have documented the progression pattern of hearing loss and sensory cell deterioration in aging animals (Hu et al., 2008; Yang, et al., 2010; Yang, et al., 2012). At 24-25 months old, rats experience significant hearing loss and a marked reduction in Mcl-1 expression in cochlear sensory cells (Hu et al., 2008; Popelar et al., 2006; Yang et al., 2010; Yang, et al., 2012). At this age, the cochlea demonstrates exacerbated sensory cell death, particularly in the basal section. This rapid progression provides an ideal time period to investigate the role of Mcl-1 expression in the aging process of the cochlea.

Age-related cochlear apoptosis involves the mitochondrial signaling pathway (Alam et al., 2001; Hu et al., 2008; Nevado et al., 2006). This pathway is regulated by the Bcl-2 family (Someya et al., 2009), which includes Bcl-2-associated protein X (Bax), a pro-apoptotic member that translocates from the cytosol to the mitochondria when cells receive an apoptotic stimulus (Green and Reed, 1998; Gross, et al., 1999; Wei et al., 2001). We previously identified an increase in Bax expression in the aging cochlea (Hu et al., 2008; Yang et al., 2012). Under physiological conditions, Bax is controlled by a set of anti-apoptotic genes that includes Mcl-1, an anti-apoptotic member of the Bcl-2 family that binds to and sequesters Bax. Mcl-1 expression increases in response to numerous signals that control cell growth, differentiation, and activation, but is rapidly turned over within the cell (Iglesias-Serret et al., 2003; Yang et al., 1996). The rapid down-regulation of Mcl-1 following the removal of survival factors or exposure to pro-apoptotic signals initiates apoptosis (Nijhawan et al., 2003). In the aging cochlea, the expression of Mcl-1 is significantly diminished. This down-regulation has been linked to sensory cell degeneration (Yang et al., 2012). Here, we demonstrated that enhancing the expression of Mcl-1 reduced the progression of age-related sensory cell apoptosis, suggesting a role for Mcl-1 in the suppression of age-related sensory cell degeneration. This finding is consistent with previous observations that Mcl-1 promotes cell survival under a variety of apoptosis-inducing conditions (Iglesias-Serret et al., 2003; Reynolds et al., 1994; Zhou et al., 1997).

The biological mechanism by which Mcl-1 inhibits apoptosis is not completely clear. Studies of non-cochlear cell systems have shown that the pro-apoptotic proteins Bax and Bcl-2 homologous antagonist killer (Bak) can trigger the mitochondrial events of apoptosis, including the formation of mitochondrial membrane pores that allow cytochrome c to be released into the cytoplasm (Cuconati et al., 2003; Thomas et al., 2010). Cytoplasmic cytochrome c activates caspases and initiates the apoptotic destruction of the cell. Studies from our lab and others have documented the release of cytochrome c in the aging cochlea (Hu et al., 2008; Riva et al., 2005). Here, we report that this apoptotic event is reduced following Mcl-1 transfection, indicating that the inhibition of cytochrome c release contributes to the observed suppression of apoptosis.

In the current study, we revealed that Mcl-1 transfection did not enhance Mcl-1 expression in young cochleae. This observation suggests that constitutive expression of the gene provides a feedback loop that controls the expression of the transfected gene. Because the young cochlea has a much higher expression level than the aging cochlea (Yang et al., 2012), the feedback control suppresses the expression of the transfected gene. Notably, we noticed a similarity in the levels of age-related reduction and transfection-induced increase in Mcl-1 expression. Specifically, we observed a 3.2-fold increase in Mcl-1 expression after transfection. This level of increase is comparable to the 3.1-fold reduction of Mcl-1 expression in aging cochleae compared to young cochleae (Yang et al., 2012). The absence

of a further enhancement of Mcl-1 expression following Mcl-1 transfection in the young cochleae provides additional evidence of the strict control of Mcl-1 in cochlear cells.

The modulation of cochlear gene expression *in vivo* is technically challenging due to difficulty accessing the cochlea and the complex structure of the inner ear. Here, we employed an established approach (Jero et al., 2001; Maeda et al., 2007; Wareing et al., 1999) to deliver the Lipofectamine-Mcl-1/pEGFP plasmid complex to the round window of the cochlea. This local application enabled us to alter cochlear Mcl-1 expression without systemically influencing gene expression. Our expression analysis revealed that transfection enhanced the expression of Mcl-1 at both the mRNA and the protein level in the aging cochlea. Using green fluorescent protein as a transfection reporter (Lalwani et al., 1997), we could observe the timing of the transfection. The fluorescence first appeared in supporting cells that directly contact the basilar membrane. This distribution pattern suggests that the route taken by the Lipofectamine-Mcl-1/pEGFP plasmid complex to the sensory cells passes from the canaliculi on the osseous spiral lamina to the basal domain of the supporting cells and then to the sensory cells, consistent with the route proposed by Shepherd and coworkers (Shepherd and Colreavy, 2004). Moreover, our functional and morphological data demonstrated that the transfection was relatively safe, with no significant influence on ABR thresholds or sensory cell viability. It should be noted that we did not examine the effects of Mcl-1 transfection on lateral wall cells or spiral ganglion neurons. Based on previous observations (Jero et al., 2001; Maeda et al., 2007), we suspect that cells in these regions could be transfected. Future investigations are needed to identify the contribution of lateral wall cells and spiral ganglion neurons to the effects of Mcl-1 transfection observed in the current study, so that the role of Mcl-1 in inhibiting apoptosis in these regions can be determined.

In summary

Using the Lipofectamine-Mcl-1/pEGFP plasmid complex, we successfully increased the expression of Mcl-1 in aging cochleae. Restoring Mcl-1 expression reduced the progression of ARHL and sensory cell degeneration. This protective effect was mediated by the suppression of apoptosis in sensory cells. Moreover, Mcl-1 transfection reduced the age-related release of cytochrome c, indicating that suppression of the apoptotic mitochondrial signaling cascade may contribute to the protection against age-related sensory cell apoptosis mediated by Mcl-1. Taken together, the results suggest that Mcl-1 may be a potential target for therapeutic interventions for age-related sensory cell degeneration in the cochlea.

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Figure 1. Mcl-1 transfection following local application of the Lipofectamine-Mcl-1/pEGFP plasmid complex to the round window of young rats

(A) Typical cochlear sensory epithelium examined 10 days after the treatment. The sporadic fluorescence (green) of Mcl-1/pEGFP is present in the supporting cell region (arrows). There is no detectable fluorescence in the outer hair cell region (marked by OHC). The sample was also stained with propidium iodide (red fluorescence) to demarcate nuclei. (B) Cochlear sensory epithelium examined 15 days after the treatment. Strong Mcl-1/pEGFP fluorescence is present in the region of outer hair cells, inner hair cells and pillar cells. OHC1, OHC2 and OHC3 indicate the first, second and third row of outer hair cells, respectively. PI indicates pillar cell. IHC indicates inner hair cell. The horizontal bar in (B) denotes 15 μ m.

Figure 2. Increase in the expression of Mcl-1 mRNA following Mcl-1 transfection in aging cochleae

The cochlear samples were collected 3, 6, and 9 days after application of the Lipofectamine-Mcl-1/pEGFP plasmid complex to the round window and expression levels measured using qRT-PCR. Compared to the expression levels in the untreated control cochleae, the transfected ears exhibited a 3- to 3.5-fold increase in Mcl-1 expression at the three time points examined. Asterisks indicate $p < 0.001$ (one-way ANOVA). Bars depict standard deviation.

Figure 3. Mcl-1 transfection increased Mcl-1 immunoreactivity in sensory cells of aging cochleae (A) Mcl-1 immunoreactivity in the apical, middle, and basal sections of the sensory epithelium of an aging control cochlea (25 months) without Mcl-1 transfection. Weak Mcl-1 immunoreactivity is visible in the cytoplasm, although some cells exhibit increased immunoreactivity (arrows). (B) Images in (A) superimposed with the propidium iodide staining to illustrate the orientation of the tissue. The cells with Mcl-1 immunoreactivity are sensory cells. (C) Mcl-1 immunoreactivity measured in the sensory epithelium of an aging cochlea collected one month after Mcl-1/pEGFP treatment. The three panels in (C) are of the apical, middle and basal sections of the sensory epithelium, respectively. Mcl-1

immunoreactivity is increased compared to the control ear (**A**) and immunoreactivity is evident in the cytoplasm (arrows). (**D**) Images in (**C**) superimposed with the propidium iodide staining to illustrate the structure of the tissue. Again, the cells with strong Mcl-1 immunoreactivity are sensory cells. OHC1, OHC2 and OHC3 indicate the first, second and third row of outer hair cells, respectively. IHC indicates inner hair cell. The horizontal bar in (**A**) represents 15 μm . All of the figures are presented at the same magnification level. (**E**) Comparison of the average optical density of Mcl-1 staining in hair cells of the control and treated cochleae in three cochlear sections (apical, middle and basal). Mcl-1-transfected ears have increased Mcl-1 immunoreactivity. Asterisks indicate $p < 0.001$ (two-way ANOVA).

Auditory Brainstem Responses

Figure 4. Reduction in the deterioration of ABR threshold in aging animals three months after Mcl-1 transfection

On average, treated ears exhibit 12.7 ± 3.21 dB less hearing loss compared to control ears.

Asterisks indicate that the difference is statistically significant ($p < 0.001$, two-way ANOVA). Bars depict standard deviation. N = 18 animals in each group.

Figure 5. Transfection of Mcl-1 reduces the progression of sensory cell degeneration

(A) The three panels depict the reticular lamina of the sensory epithelium stained with FITC-labeled phalloidin in the apical, middle and basal sections, respectively, of a control cochlea (27 months). Arrows point to sites of missing cells. (B) Propidium iodide staining in the same areas of sensory epithelium portrayed in (A). Arrows point to cells with condensed nuclei. (C) Treated cochlea collected 3 months after Mcl-1 transfection stained with FITC-labeled phalloidin. The three panels depict the reticular lamina of the cochlear sensory epithelium in apical, middle and basal sections, respectively. Arrows indicate sites of missing cells. (D) Propidium iodide staining of the same areas of the sensory epithelium depicted in (C). (E) Cochleograms illustrating the difference in the extent of sensory cell degeneration between control and Mcl-1-transfected ears. The data show the average number of damaged outer hair cells (the sum of the number of missing cells and cells with condensed or swollen nuclei) and are presented as a function of distance from the apex to the base of the cochlea. Both the treated and the control group had greater outer hair cell damage in the apical and basal sections (the shaded areas) than in the middle section of the sensory epithelium. Compared with control ears, treated ears had significantly fewer damaged cells in all cochlear sections. The asterisk indicates $p < 0.05$ (two-way ANOVA, treated vs. control). Bars depict standard deviation. $N = 12$ cochleae in each condition.

Figure 6. Average number of apoptotic and necrotic hair cells in control and Mcl-1-transfected cochleae three months after delivery of the Mcl-1 gene
Bars depict standard deviation. The asterisk indicates $p < 0.001$ (two-way ANOVA). N = 12 cochleae in each condition.

Figure 7. Mcl-1 transfection reduces cytochrome c immunoreactivity in sensory cells of the aging cochlea

(A) Cytochrome c immunoreactivity in the three sections (apical, middle and basal) of the sensory epithelium in an aging control cochlea (27 months). Arrows point to cells with strong cytochrome c immunoreactivity. These positive cells are more prevalent in the basal section. (B) Propidium iodide staining of the same regions illustrated in (A). All of the cytochrome c-positive cells are sensory cells (arrows). (C) Cytochrome c immunoreactivity in a cochlea examined three months after transfection. The cells with strong cytochrome c immunoreactivity are still present (arrows), but their number is markedly reduced compared to those observed in the control ear. (D) Propidium iodide staining of the same cochlear

regions shown in (C). Again, all of the cytochrome c-positive cells are sensory cells. OHC1, OHC2 and OHC3 represent the first, second and third row of outer hair cells, respectively. IHC indicates inner hair cell. The horizontal bar represents 15 μm . (E) Comparison of the percentage of cytochrome c-positive hair cells in the apical, middle and basal sections of the sensory epithelia in control and treated ears. Bars represent standard deviation. Asterisks indicate $p < 0.001$ (two-way ANOVA). N = 6 cochleae in each group.

Figure 8. Mcl-1 expression remains unchanged in the young rat cochlea following transfection of the Lipofectamine-Mcl-1/pEGFP plasmid complex

(A) Comparison of Mcl-1 mRNA expression levels in control and treated cochleae at 1, 2, and 3 months post-treatment. The small difference between the groups is statistically insignificant (one-way ANOVA, $p = 0.871$). (B) Representative Western blots of Mcl-1 protein expression in control and treated cochleae at 1, 2, and 3 months post-treatment. (C) Comparison of the expression of Mcl-1 protein in control and treated cochleae at 1, 2, and 3 months post-treatment. There is no significant difference in band intensity between the control and treated groups (two-way ANOVA, $p = 0.667$).