Methionine sulfoxide reductase B3 deficiency causes hearing loss due to stereocilia degeneration and apoptotic cell death in cochlear hair cells

Tae-Jun Kwon1,*, Hyun-Ju Cho1,*, Un-Kyung Kim1,*, Eujin Lee6, Se-Kyung Oh1, Jinwoong Bok7,8, Yong Chul Bae2, Jun-Koo Yi3,9, Jang Woo Lee4, Zae-Young Ryoo3, Sang Heun Lee5, Kyu-Yup Lee5,* and Hwa-Young Kim6,*

1Department of Biology, College of Natural Sciences, 2Department of Oral Anatomy and Neurobiology, School of Dentistry, 3School of Life Science and Biotechnology, College of Natural Sciences, 4Graduate School of Electrical Engineering and Computer Science and 5Department of Otorhinolaryngology-Head and Neck Surgery, School of Medicine, Kyungpook National University, Daegu, Republic of Korea 6Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, Daegu, Republic of Korea, 7Department of Anatomy and 8BK21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea and 9Gyeongsangbuk-do Livestock Research Institute, Yeoungju, Republic of Korea

Received July 23, 2013; Revised and Accepted October 28, 2013

INTRODUCTION

The cochlea (the auditory portion of the inner ear) plays a vital role in the conversion of physical vibrations generated by sound waves into chemical signals in the process of hearing. The mammalian cochlea contains ~16,000 sensory hair cells that are arranged as three rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs) (1,2). Because these hair cells cannot be regenerated during the lifetime of an animal, damage to or loss of hair cells due to various factors, such as genetic defects, noise and drugs, is irreversible and leads to permanent hearing loss (3).

Methionine is one of the most sensitive amino acids to oxidation by reactive oxygen species (ROS). The oxidation of
methionine generates a diastereomeric mixture of methionine-
R-sulfoxide and methionine-S-sulfoxide (4), which may cause
significant changes to protein structure and function (5,6).
However, oxidized methionine can be repaired using the
enzyme methionine sulfoxide reductase (Msr). Two families of
MsrSs, MrSA and MrSB, have evolved to catalyze the stereospe-
cific reduction of methionine sulfoxide to methionine in proteins
(7–9). MrSA is specific for the S-form of methionine sulfoxide,
whereas MrSB only reduces the R-form (10,11). The functions of
MsrSs are to repair oxidatively damaged proteins, to protect
against oxidative stress and to regulate protein function.

In mammals, three genes encode MrSB proteins (MrSB1, 2 and
3, which have different subcellular locations), whereas a single
gene encodes MrSA (12–14). MrSB1 is a cytosolic and nuclear
selenoenzyme, MrSB2 is targeted to the mitochondria and me-
thonine sulfoxide reductase B3 (MrSB3) exists in two forms in
humans, MrSB3A and MrSB3B, which contain different
N-terminal regions generated via alternative first exon splicing.
MrSB3A is targeted to the endoplasmic reticulum (ER),
whereas MrSB3B resides in the mitochondria (13). However,
no evidence for the existence of alternatively spliced MrSB3
forms in mice has been reported. Instead, mouse MrSB3 contains
consecutive ER and mitochondrial signal peptides in the
N-terminal region and is targeted to the ER (15).

Recently, Ahmed et al. (16) reported that the MrSB3 gene
is associated with human DFNB74, a locus for autosomal reces-
sive hearing loss. Two mutations in MrSB3, one missense
and one nonsense mutation, were identified in families with
the DFNB74 locus. The missense mutation (p.Cys89Gly) is a
loss-of-function allele that results in a complete lack of MrSB3
enzymatic activity. The nonsense mutation (p.Arg19X) occurs
in the mitochondrial targeting sequence of MrSB3B. These data
suggest that functional MrSB3 is essential for hearing in
humans (16). However, the lack of animal models for MrSB3 defi-
ciency has hindered our understanding of the function of
MrSB3 and the pathogenesis of DFNB74 hearing loss.

Here, we present the first report of the generation and evalua-
tion of MrSB3 knockout mice as an animal model for DFNB74 and
propose a possible pathological mechanism leading to hearing
loss caused by MrSB3 deficiency in DFNB74 patients.

RESULTS

Generation of MrSB3 knockout mice

In mice, the MrSB3 gene is located on chromosome 10 and com-
prises ~120 kb of genomic DNA. MrSB3 knockout mice were
generated using homologous recombination. The knockout con-
struct was designed to replace exon 7 encoding the catalytic Cys
residue with aNeo cassette (Fig. 1A); correct gene targeting was
verified with the Southern blot analysis (Supplementary mater-
ial, Fig. S1).

In homozygous MrSB3/−/− mice, the MrSB3 protein was not
detected in the heart, skeletal muscle or testis, tissues in which
MrSB3 proteins are highly expressed in wild-type mice (Fig. 1B).
In addition, heterozygous MrSB3+/− mice exhibited lower
MrSB3 levels than wild-type MrSB3+/+ mice. We also examined
MrSA, MrSB1 and MrSB2 proteins expression levels in MrSB3
knockout mice. MrSB1 levels were higher in the heart and skeletal
muscle in MrSB3−/− mice than in MrSB3+/+ mice (Fig. 1B).
MrSB2 was expressed at similar levels in the heart but at slightly
decreased levels in the skeletal muscle and testis. The levels of
MrSA did not differ in any of the tissues examined (Fig. 1B).

Figure 1. Generation of MrSB3 knockout mice. (A) The mouse MrSB3 gene and its flanking region. The MrSB3 gene in mice consists of seven exons. The parentheses
represent the size of each exon. Exon 7, which contains the catalytic residue, was replaced with a Neo cassette. The lower panel illustrates the flanking DNA regions
used for homologous recombination. LA, 5′ left arm; RA, 3′ right arm. (B) Western blot analysis of MrSs in the heart, skeletal muscle and testis. Gapdh was used as a
loading control. +/+, wild-type; +/-, heterozygous MrSB3 knockout; −/−, homozygous MrSB3 knockout mice.
MsrB3 knockout mice are deaf

Because the MSRB3 gene is associated with DFNB74 deafness in humans (16), we sought to ascertain whether the MsrB3 knockout mice also exhibited a hearing impairment phenotype. We measured the hearing thresholds using an auditory brainstem response (ABR) test in MsrB3+/− and MsrB3−/− littermates at postnatal day (P) 20, an age when the hearing system is considered to have largely matured. Heterozygous MsrB3+/− mice exhibited an ABR threshold and waveform with an average hearing threshold of 29.5 decibels (dB) using click stimuli (Fig. 2, left panel) and average hearing thresholds of 27.5, 26.3 and 30.3 dB at 8, 16 and 32 kHz (n = 15), respectively, using tone bursts (Supplementary Material, Fig. S2, left panel). Homozygous MsrB3−/− mice did not respond to click stimuli or tone bursts (n = 15), indicating profound hearing impairment (Fig. 2, right panel, Supplementary Material, Fig. S2, right panel). However, we did not observe any abnormal vestibular behaviors such as circling, head tossing or walking disability in MsrB3−/− mice. These data clearly demonstrate that the deletion of MsrB3 leads to deafness in mice.

MsrB3 is expressed in the sensory epithelia of cochlear and vestibular tissues

MsrB3 expression was analyzed immunohistochemically in mouse inner ears from embryonic day (E) 12.5 to P18. The inner ear sections contained both the cochlea and vestibular end organs including the cochlear duct, utricle, saccule and crista ampullaris. No detectable MsrB3 signal was observed at E12.5 in any inner ear sections (Fig. 3A and I). MsrB3 expression began to be detected at E13.5 in the sensory epithelia of vestibular organs, such as the maculae of the utricle, saccule and all three cristae (Fig. 3J and data not shown). MsrB3 expression in the cochlea was not detected until E15.5, at which point MsrB3 signals were found primarily in the basal and middle turns of the cochlea, and later in all cochlear turns (Fig. 3D). These temporal and spatial expression patterns of MsrB3 follow the sequence of inner ear hair cell differentiation. The MsrB3 signals in the cochlea and vestibule were continuously detected until at least P18, when the mouse hearing ability matures (Fig. 3D–G and J–O). Magnified views of P18 specimens showed that the MsrB3 signals were present in the cochlear epithelium from the inner to the outer sulcus cells, encompassing the auditory hair cells (Fig. 3G and H) and the sensory epithelia of the vestibular organs (Supplementary Material, Fig. S3).

Cellular localization of MsrB3 at the base of the stereocilia and in the hair cell body

To assess the intracellular localization of MsrB3 in the hair cells, we performed whole-mount immunostaining of the organ of Corti from wild-type mice. In a top view of the organ of Corti, MsrB3 immunoreactivity was viewed as dot-like structures at the base of the stereocilia of both IHCs and OHCs (Fig. 4A and D). However, side views of the IHCs revealed that MsrB3 proteins were expressed in more rod-like patterns at the base of each stereocilium (Fig. 4B and C). Similar localization of MsrB3 proteins at the base of stereocilia was also observed in vestibular hair cells (data not shown). MsrB3 immunoreactivity was completely absent in the MsrB3−/− organ of Corti, whereas heterozygote MsrB3+−/− cochlea exhibited expression patterns similar to those of wild-type mice (Supplementary Material, Fig. S4). In addition, faint and scattered MsrB3 staining was also detected in the cell bodies of hair cells.

Figure 2. MsrB3−/− mice are profoundly deaf. A hearing test was performed using ABR in MsrB3+/− and MsrB3−/− mice at P20. Waveforms of ABR at the click are represented for MsrB3+/− (left panel) and MsrB3−/− (right panel) mice. Similar results were obtained from 15 MsrB3+/− and MsrB3−/− mice.
Msr level and activity in the inner ear

We analyzed the expression levels and activities of MsrBs and MsrA in the inner ears of MsrB3+/+, MsrB3+/− and MsrB3−/− mice. The expression of MsrB3, which was examined using western blot experiments, was reduced in the inner ears of MsrB3+/− mice and completely abolished in those of MsrB3−/− mice (Fig. 5A). The expression levels of MsrA and MsrB1 in the inner ears of MsrB3+/− and MsrB3−/− mice appeared slightly higher than those in the inner ears of MsrB3+/+ mice.

Surprisingly, inner ear MsrB activities were not reduced in heterozygote MsrB3+/− or homozygote MsrB3−/− mice compared with wild-type mice (Fig. 5B). Similarly, no significant reductions in MsrB activities were observed in the heart, skeletal muscle or testis in MsrB3−/− mice (data not shown). These results suggest that MsrB3 enzymatic function is either compensated for by increased MsrB1 isozyme activity in MsrB3+/− and MsrB3−/− mice or that this enzymatic function does not play a major role in MsrB activity.

The inner ears of MsrB3 knockout mice exhibit no significant changes in protein oxidation

To determine whether the observed MsrB3 deficiency increases cellular protein oxidation in the inner ear, we measured the levels of methionine sulfoxide and carbonyl proteins in MsrB3+/+, MsrB3+/− and MsrB3−/− littermates at P10, P20 and P30 using western blot analysis. H2O2-treated P10 wild-type cochlear tissues exhibited some increased band intensities and size shifts compared with non-treated cochlear tissues, validating our methodology for measuring methionine sulfoxide levels (Supplementary Material, Fig. S5). However, when we compared the inner ear proteins of MsrB3+/− and MsrB3−/− mice, no significant differences were observed in either the band intensity or size shift at all three stages tested (Supplementary Material, Fig. S5).

Furthermore, no increase in the carbonyl level of proteins was detected in the inner ears of MsrB3−/− mice compared with MsrB3+/+ and MsrB3+/− mice at all stages tested (Supplementary Material, Fig. S5).

Figure 3. Analysis of inner ear MsrB3 expression. Immunohistochemical analysis was performed in serial sections of inner ears containing the cochlear duct (A–H) and vestibular organs (I–O) from E12.5 to P18. MsrB3 was labeled with Alexa 555 fluorescein (red), and nuclei were labeled with DAPI (blue). The earliest expression of MsrB3 in the end of each sensory organ is indicated by an arrowhead. The magnification of the boxed region in (G) shows sensory epithelial cells expressing MsrB3 specifically (H); hair cells are indicated by an arrowhead. cd, cochlear duct; us, utriculosaccular space; ed, endolymphatic duct; mo, modiolus; sg, spiral ganglion; sv, scala vestibuli; sm, scala media; st, scala tympani; cr, crista amullaris; ut, utricle; and sc, saccule. Scale bars: 200 μm (A–G and I–O); 50 μm (H).
the integrity of the stereociliary bundles of inner ear hair cells progressively degenerate in MsrB3−/− mice

Our observations that MsrB3 proteins are strongly expressed in the basilar part of stereociliary bundles and that MsrB enzymatic activity is largely normal in the inner ear of MsrB3+/+ mice led us to examine the possible structural role of MsrB3 in stereociliary bundles. Using scanning electron microscopy, we examined the integrity of the stereociliary bundles of both OHCs and IHCs. (A) Side view of IHCs. MsrB3 is densely localized at the base of the stereocilia and scattered as spots throughout the entire cell body, a distribution that might correspond with ER localization. (C) Magnified stereocilia of OHCs. Scale bars: 5 μm (A, B, and D); 2 μm (C).

Deletion of MsrB3 leads to apoptotic cell death in the organ of Corti

We next examined whether stereociliary degeneration is associated with hair cell loss in MsrB3−/− mice using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and by immunolabeling active caspase 3 on the cochlear sections of MsrB3+/+ and MsrB3−/− mice at P20. Although no TUNEL-positive cells or active caspase 3 signals were observed in the MsrB3+/+ cochlea (Fig. 7A and C), the MsrB3−/− cochlea exhibited TUNEL-positive cells in the organ of Corti (including the hair and supporting cells) (Fig. 7B and B*). Active caspase 3 was also detected in the organ of Corti of MsrB3+/− mice (Fig. 7D and D*). We quantified the increase in apoptotic cells by counting TUNEL(+) cells in the organ of Corti of MsrB3+/− and MsrB3−/− mice. The number of TUNEL(+) cells was significantly increased in the cochlea of MsrB3−/− mice, with an average of 61.5 cells, compared with that in the cochlea of MsrB3+/− mice, with an average of 5 cells (n = 4, P < 0.008, Supplementary Material, Fig. S9). These data suggest that MsrB3 plays a protective role against apoptotic cell death in the cochlea.

DISCUSSION

Reduction and oxidation (redox) homeostasis is essential in almost all living organisms to delicately control the balance between the beneficial and harmful effects of free radicals such as ROS and reactive nitrogen species. The deleterious actions of these free radicals have been associated with the pathogenesis of a variety of diseases including cancer, diabetes mellitus, inflammatory diseases, neurodegenerative disorders and aging (17). A recent report demonstrating genetic mutations in the MsrB3 gene as a cause of prelingual hearing loss in DFNB74 patients also strongly suggested a crucial role of redox homeostasis in the development or functioning of the inner ear (16).

To explore the pathophysiological mechanisms of deafness caused by the null alleles of MsrB3, we generated MsrB3−/− mice. Similar to the phenotypes observed in the DFNB74 patients (16), the homozygote MsrB3−/− mice displayed profound hearing loss with normal vestibular function (Supplementary Material, Fig. S10), suggesting that our MsrB3−/− mice were excellent animal models for human DFNB74 hearing loss.
loss. Thus far, knockout models for some other Msr genes have been reported. MsrA \(^{-/-}\) mice exhibited various neurological abnormalities, such as tiptoe walking, abnormal behavior and lower locomotor activity, as well as a dramatically shortened life span; however, the hearing ability of MsrA \(^{-/-}\) mice has not been described (18–20). MsrB1 \(^{-/-}\) mice appeared generally normal, although the liver and kidney were slightly affected (21). These results suggest that MsrB3 is an important Msr protein involved in hearing function in mammals.

Consistent with the hearing loss phenotype of MsrB3 \(^{-/-}\) mice, we observed that MsrB3 proteins are densely localized at the base of the stereocilia on the apical surface of the hair cells in the organ of Corti. Recently, TRIOBP, an actin-bundling protein, has been reported to be localized at the base of stereocilia and is essential for hearing due to its role in the formation of stereocilia rootlets (22). Because Triobp \(^{-/-}\) mice showed stereociliary degeneration phenotypes similar to those observed in MsrB3 \(^{-/-}\) mice, we considered whether MsrB3 could also be associated with the formation of rootlets. However, rootlet structures were clearly observed in the hair cells of MsrB3 \(^{-/-}\) mice at P8 using transmission electron microscopy (Supplementary Material, Fig. S11). In addition, quantitative real-time PCR analysis showed no significant differences in the mRNA levels of TRIOBP between the inner ears of MsrB3 \(^{-/-}\) and MsrB3 \(^{+/+}\) mice at P5, P10 or P20 (data not shown). Therefore, it is unlikely that MsrB3 is functionally associated with TRIOBP in regulating the formation of stereociliary bundle rootlets. Instead, MsrB3 appears to control the maturation and/or maintenance of stereociliary bundles because the stereociliary bundles of MsrB3 \(^{-/-}\) mice exhibited morphological alterations starting at P8 and progressively degenerated during time points spanning the completion of hearing development in mice. Mechanotransduction activity was evaluated with FM1-43 dye in the hair cells of MsrB3 \(^{+/+}\) and MsrB3 \(^{-/-}\) mice at P3. We observed that FM1-43 uptake was normal in both MsrB3 \(^{+/+}\) and MsrB3 \(^{-/-}\) mice but completely abolished by adding 5 mM EGTA, which has been shown to disrupt tip links at the stereocilia (data not shown). These results indicate that the hair cells of the MsrB3 \(^{-/-}\) mice develop normally and have functional mechanotransduction channels until at least P3 and further suggest that degeneration rather than maldevelopment of the hair cells is the primary cause of hearing impairments in the MsrB3 \(^{-/-}\) mice.

However, the molecular mechanisms by which MsrB3 deficiency leads to degeneration of the stereocilia remain to be resolved. Recently, Hung et al. (23) showed that Mical, a mono-oxygenase, can selectively oxidize two methionines (46th and 49th Met) of the 16 Met residues in the actin polypeptide, thereby inducing F-actin to disassemble. Moreover, Lee et al. (24) reported that both MsrB1 and MsrB2 have been shown to reduce the oxidized Met46 and Met49 in Mical-treated F-actin. Based on these reports, we hypothesize that MsrB3 may play a crucial role in the redox regulation of F-actin in hair cells and that the deficiency of MsrB3 may result in increased oxidation of F-actin by Mical, which accelerates the disassembly of F-actin in stereocilia, causing their degeneration.

MsrB3 plays a protective role against oxidative stress in MsrB3 transgenic flies and yeast cells (25,26). However, no significant accumulation of carbonyl derivatives was observed in the inner ears of MsrB3 \(^{-/-}\) mice compared with MsrB3 \(^{+/+}\) and MsrB3 \(^{+/+}\) mice. These data suggest that oxidative stress may not be a major cause of hair cell degeneration induced by MsrB3 deficiency. Notably, no significant differences in MsrB enzyme activity were observed between the inner ears of MsrB3 \(^{-/-}\) and MsrB3 \(^{+/+}\) (or MsrB3 \(^{+/+}\)) mice. We were not able to examine the enzymatic activities of MsrB1, MsrB2 and MsrB3 individually because all three MsrB enzymes act on the same substrates, and their catalytic activities are similar (27). Interestingly, the expression levels of MsrB1 proteins in the inner ear were slightly higher in MsrB3 \(^{-/-}\) and MsrB3 \(^{+/+}\) mice than in the wild-type. Thus, it is possible that the increased activity of the MsrB1 isozyme compensated for the loss of MsrB3 activity in MsrB3 \(^{-/-}\) and MsrB3 \(^{+/+}\) mice. Taken together, these results suggest that the loss of MsrB3 activity controlling redox homeostasis is not a major pathological cause leading to hearing loss in MsrB3 \(^{-/-}\) mice.

Our TUNEL and anti-active caspase immunoassays demonstrated that MsrB3 deficiency induces apoptotic cell death in inner ear tissues. We consistently observed that knocking down MsrB3 expression in mammalian cell lines resulted in apoptotic cell death, confirming the anti-apoptotic role of MsrB3 (data not shown). The apoptosis of sensory hair cells is an important factor contributing to many types of hearing impairments including age-related, noise-induced, ototoxic and monogenic forms of hearing loss (28–30). These results
Figure 6. Ultrastructural morphology of hair cell stereocilia. The stereociliary bundles from MsrB3+/+, MsrB3+/− and MsrB3−/− mice were analyzed using scanning electron microscopy. (A) The images show all hair cell bundles from the middle turn in the organ of Corti. The upper three rows correspond with OHCs, and the single bottom row corresponds with IHCs. (B) Views at higher magnification. The left image presents a higher magnification view of OHCs, and the right image shows a representative IHC. The images represent five developmental stages (P5, P8, P13, P20 and P50). Scale bars: 10 μm (A); 1 μm (B).
human deafness caused by MSRB3 mutations. Our results provide novel insights into the pathogenesis of hair cells, which ultimately resulted in profound hearing loss.

We found that MsrB3 deficiency led to the progressive degeneration of stereociliary bundles, followed by the apoptotic loss of death was found in the inner ear sections of MsrB3−/− mice (A and C). In contrast, MsrB3+/− mice show TUNEL (B and B′) and active caspase 3 (D and D′) signals in the sensory epithelia, including hair cells, supporting cells and basilar membrane cells. Scale bars: 50 μm.

suggest that the role of MsrB3 in protecting cochlear hair cells might not be directly mediated by its well-known protein reduction activities.

In summary, we report the generation and characterization of an MsrB3−/− mouse model, which is useful for studying the pathological mechanisms of DFNB74 hearing loss in humans and understanding the function of MsrB3 in auditory hair cells. We found that MsrB3 deficiency led to the progressive degeneration of stereociliary bundles, followed by the apoptotic loss of hair cells, which ultimately resulted in profound hearing loss. Our results provide novel insights into the pathogenesis of human deafness caused by MSRB3 mutations.

**MATERIALS AND METHODS**

**Generation of MsrB3 knockout mice**

The MsrB3 knockout-targeting construct was designed to abolish exon 7 within the gene by replacing this exon with a neomycin resistance gene. Exon 7 harbors the active site of the enzyme, including the catalytic Cys residue. To clone the 5′ left arm, a first 1509-bp (LA1) fragment, a second 2778-bp (LA2) fragment downstream of LA1 and a third 3003-bp (LA3) fragment downstream of LA2 were amplified using PCR from 129/SvJ mouse genomic DNA and inserted into the NotI/NheI, NheIXhoI and XhoISalI sites, respectively, in the pDrive cloning vector. A 2681-bp fragment (RA) coding for the 3′ right arm was amplified using PCR and inserted into the SalI/NheI sites in the pDrive cloning vector. All cloned fragments were verified using DNA sequencing. The LA1 fragment containing the NotI/NheI sites was then ligated to the same sites of pDrive containing LA2. The MsrB3 knockout construct was prepared as follows: the RA fragment containing the SalI/NheI sites was cloned into the XhoIXhoI sites of the pOSDupDel Neo vector. The LA3 fragment containing the XbaISalI sites was then cloned into the same sites of pOSDupDel containing the RA fragment. The LA1/2 fragment containing the NotIXhoI sites was then cloned into the same sites of pOSDupDel containing the RA and LA3 fragments. The targeting construct contained a 7.69-kb 5′ left arm, a 1.3-kb neomycin cassette and a 2.68-kb 3′ right arm. We electroporated 129/SvJ embryonic stem (ES) cells with the targeting construct after it had been linearized using NotI. Positive recombinant ES cells were identified using PCR and then microinjected into C57BL/6 blastocysts. The resulting chimeric mice were then mated with C57BL/6 mice. Germ line transmission was confirmed using Southern blot analysis (Supplementary Material, Fig. S1). Genomic DNA was digested with SpeI and then probed with a 651-bp probe. The knockout allele yielded a 5.1-kb band, whereas the wild-type allele generated a 6.9-kb band.

**Animal preparation and experiments**

All mice used in the animal experiments were obtained by breeding heterozygous MsrB3 knockout mice of a 129/SvJ and C57BL/6 mixed background. The mouse genotypes were confirmed using PCR analysis. The animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University and Yeungnam University.

**Mice genotyping**

Genomic DNA was isolated from mouse tails. PCR genotyping was performed using the following primers: one forward primer (5′-CAAAATGCTGTCGAGCCATC-3′) and two reverse primers for the wild-type (5′-GAAAGTCGCATCTAGACGA GAGAGC-3′) and knockout (5′-GATGATCTCGTCGTGACCC ATG3′) alleles. The PCR products were 538 bp for the wild-type allele and 393 bp for the knockout allele.

**Auditory brainstem response**

The animals were intramuscularly anesthetized using a mixture of tiletamine–zolazepam (1.8 mg/100 g) and xylazine hydrochloride (0.7 mg/100 g). Body temperature was monitored using a rectal thermometer, and the animals were maintained on a heating pad at 37°C. All experiments were performed in a soundproof room. Sound-evoked ABRs were measured using a Tucker Davis Technology (TDT) system as previously described (31). Briefly, needle electrodes from the head stage (RA4LI, TDT) were connected to a pre-amplifier (RA4PA, TDT) to record ABRs and inserted into the vertex (+ charge), mastoid (− charge), and hind leg (ground). Acoustic stimuli were calibrated at 90 dB SPL (sound pressure level) with 8, 16 and 32 kHz tone bursts using calibration software (SigCalRP)
in TDT System 3 and the probe microphone system. Tone burst stimuli with a 1-ms rise/fall and a 5-ms plateau or transient click stimuli at frequencies of 8, 16 and 32 kHz were generated using signal design software (SigGenRP) and applied. The signals needed for the stimuli were generated using SigGenRP and an RP2.1 real-time processor and then transmitted in sequence through a programmable attenuator (PA5, TDT), speaker driver (ED1, TDT) and electrostatic speaker (EC1, TDT). Stimuli were generated every 36.1 ms for 500 repetitions from 90 dB SPL to the acoustic threshold in 5-dB decrements at every frequency. The phase of the stimuli was reversed after every stimulus to reduce noise caused by repetitive stimuli.

MsrB3 antibody

Four different polyclonal anti-MSRB3 antibodies were tested and their specificities were compared: two new antisera prepared by immunizing rabbits with a recombinant human MSRB3A, a rabbit polyclonal antiserum against human MSRB3 [as previously described (15)] and a rabbit polyclonal anti-human MsrB3 obtained from Sigma-Aldrich. To validate specificity against mouse MsrB3, we performed western blotting and immunohistochemistry analyses using MsrB3+/+ and MsrB3−/− mouse tissues and cells transfected with a mouse MsrB3–GFP fusion construct (15). Among the evaluated antibodies, the previously described anti-human MsrB3 antibody (15) exhibited the highest specificity for mouse MsrB3 (Supplementary Material, Figs S4 and S12). Thus, this antibody was used for further study.

Frozen-section immunohistochemistry

Inner ears were isolated from E12.5, E13.5, E14.5, E15.5 and E18.5, as well as P0 and P18 mice and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 day at 4°C. The inner ears from P18 mice were then decalcified in 10% ethylene-diaminetetraacetic acid in PBS for 2 days at 4°C. All tissues were dehydrated with 30% sucrose in PBS and a 1:1:30% sucrose:OCT compound (Optimal Cutting Temperature compound, Surgipath FSC22, Leica Microsystems) for 1 day each at 4°C. The inner ears were then embedded in OCT compound. Serial sections were prepared by cutting the tissues into 10 μm sections using a −27°C cryostat and dried for 30 min at room temperature (25°C). After permeabilization with 0.5% Triton X-100 in PBS for 5 min, the tissue sections were blocked with a blocking solution (5% normal goat serum and 2% bovine serum albumin in PBS) for 1 h and incubated overnight at 4°C with the rabbit anti-MsrB3 (1:50) and mouse anti-Myo7a antibody (1:100, DSHB 138-1) diluted in the blocking solution. Goat anti-rabbit IgG antibody conjugated with Alexa 555 fluorescein (Molecular Probes/Invitrogen) and goat anti-mouse IgG antibody conjugated with Alexa 488 fluorescein (Molecular Probes/Invitrogen) were used as secondary antibodies and diluted in the blocking solution (1:1000); the diluted antibody was incubated with the tissue sections for 1 h at room temperature. The tissue sections were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and visualized using a Zeiss DE/AX10 Imager A1 fluorescence microscope system (Carl Zeiss).

Whole-mount immunostaining

Inner ears were isolated from P6 and P15 MsrB3+/+ mice and P5 and P13 MsrB3−/− and MsrB3−/− mice; the ears were rapidly fixed by infusion with 4% paraformaldehyde in PBS via the round window and immersed in the same fixative solution at 4°C for 1 h. After fixation, the organ of Corti and vestibule were dissected using a dissecting microscope. The dissected samples were immunostained with an anti-MsrB3 antibody following the immunostaining procedure described above. Next, F-actin was labeled by incubation with Alexa Fluor 488 phal-loidin (Molecular Probes/Invitrogen). After mounting in Fluoromount (Sigma-Aldrich), the samples were visualized using a confocal laser scanning microscope (LSM700, Carl Zeiss) and the ZEN 2009 program.

Scanning electron microscopy

Cochleas were immediately isolated from the euthanized mice and perfused carefully through the round window using 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) after a hole was made at the top of the cochlea. The perfused cochleas were immersed in the same fixation mixture for 1 h at room temperature. The lateral wall, Reissner’s membrane and the tectorial membrane were removed via dissection under a dissecting microscope and fixed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde, 2 mM calcium chloride and 3.5% sucrose. After fixation, the dissected specimens were rinsed three times for 20 min at 4°C with 0.1 M sodium cacodylate buffer containing 2 mM calcium chloride. The specimens were treated post-fixation using the osmium tetroxide (OsO4)-thiocarbohydrazide method developed by Hunter-Duvar (32). Briefly, specimens were immersed in 1% OsO4 for 1 h at 4°C and then placed in saturated thiocarbohydrazide for 20 min at room temperature. This specimen treatment was repeated three times at room temperature. The specimens were then dehydrated using a graded series of ethanol solutions, dried using a critical point drier (HCP-2, Hitachi), affixed on a stub and coated with platinum using a sputter coater (E1030, Hitachi). The coated specimens were mounted on a stub holder and viewed using a cold-field emission scanning electron microscope (S-4300, Hitachi) operated at 15 kV.

Transmission electron microscopy

For ultra-thin sectioning, the temporal bones were rapidly removed and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 1× PBS (pH 7.4) for 24 h. After fixation, the samples were decalcified in 0.2 M EDTA (pH 7.4) with 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C for 2 weeks and then post-fixed using osmium tetroxide (OsO4) for 30 min. The specimens were rinsed three times in 0.1 M cacodylate buffer at 4°C and then dehydrated using a graded series of ethanol solutions. To remove residual ethanol, the dehydrated specimens were treated twice with propylene oxide at room temperature. Then, the specimens were placed in capsule caps filled with fresh Epon resin and incubated overnight at 65°C. Ultra-thin sections (60 nm) were obtained with a Leica Ultracut UCT ultramicrotome (Leica Microsystems) using a diamond
knife (Diatome) and collected on 15 nickel grids (400 mesh). Grids were examined on a transmission electron microscope (H-7500, Hitachi).

Histological analysis
Inner ears were isolated from P20 mice and fixed with 4% paraformaldehyde in PBS for 1 day at 4°C. After fixation, the inner ears were decalcified in 10% ethylenediaminotetraacetic acid in PBS for 2 days at 4°C. The samples were then dehydrated using a series of ethanol solutions, cleared in xylene and embedded in paraffin. Five-micrometer sections were cut on a RM2125 RT microtome (Leica Microsystems) and stained with hematoxylin and eosin (H&E).

TUNEL assay and active caspase 3 detection
Cochlear sections obtained from P20 MsrB3+/− and MsrB3−/− mice were prepared as described above. The TUNEL assay was performed using an In Situ Cell Death Detection Kit with Fluorescein (Roche Diagnostics) according to the manufacturer’s instructions. Activated caspase 3 was detected using an anti-cleaved caspase 3 antibody (Cell Signaling Technology) using the immunostaining procedure described above. The number of apoptotic cells was quantified by counting TUNEL (+) cells in the organ of Corti of MsrB3+/− and MsrB3−/− mice, and the difference was calculated by a t-test (SPSS for Windows, version 18, SPSS Inc.).

Msr enzyme assay
The Msr enzyme assay used protein from the whole otic capsule including both whole membranous and bony labyrinth of MsrB3+/+, MsrB3+/− and MsrB3−/− mice. The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol, 200 μM dabsylated methionine-R-sulfoxide (for MsrB) or methionine-S-sulfoxide (for MsrA) and 200 μg of crude protein. The reaction was conducted at 37°C for 30 min, and the reaction product dabsyl-Met was quantified using high performance liquid chromatography.

Analysis of methionine sulfoxide levels
The tissues used in the Msr enzyme assay were homogenized in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol and 1% 2-mercaptoethanol including Protease Inhibitor Cocktail Set 1 (Calbiochem). Supernatants were obtained via centrifugation. The hydrogen peroxide (H2O2) concentrations were determined using a BCA protein assay kit. Protein lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol and 1% 2-mercaptoethanol in-}

Measurement of carbonylated proteins
Carbonylated protein levels in the tissues used in the Msr enzyme assay were measured using the Oxyblot kit (Chemicon) according to the manufacturer’s instructions. The blots were quantitatively analyzed using ImageJ software.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

Conflict of Interest statement
None declared.

FUNDING
This work was supported by the National Research Foundation of Korea Grant (211-0006178 to H.Y.K.) and the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111774 to K.Y.L.).

REFERENCES


