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ORIGINAL RESEARCH

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Low-dose rapamycin-induced autophagy in cochlear outer sulcus cells

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Abstract

Objectives: Autophagy is an intracellular housekeeping process that degrades cytoplasmic organelles, damaged molecules, and abnormal proteins or pathogens and is essential for normal hearing. Recent studies revealed the essential roles of autophagy in hearing and balance. The aim of this study was to evaluate the activation state of rapamycin-induced autophagy in cochlear outer sulcus cells (OSCs).

Methods: We used autophagy reporter transgenic mice expressing the green fluorescent protein-microtubule-associated protein light chain 3 (GFP-LC3) fusion protein and counted GFP-LC3 puncta in cochlear OSCs to evaluate the activation state of autophagy after oral administration of rapamycin.

Results: We observed basal level GFP-LC3 expression and an increase in the number of GFP-LC3 puncta in cochlear OSCs by oral administration of rapamycin. This increase was detected when the daily rapamycin intake was as low as 0.025 mg/kg, and it was dose dependent. The increased number of puncta was more at the basal turn than the apical turn.

Conclusion: Oral intake of low-dose rapamycin activates autophagy in cochlear OSCs.

Level of evidence: NA.

KEYWORDS

autophagy, hearing loss, inner ear, outer sulcus cell, rapamycin

1 | INTRODUCTION

Autophagy is an intracellular housekeeping process that degrades cytoplasmic organelles, damaged molecules, and abnormal proteins or pathogens,[1] thereby supporting normal cellular functions. When autophagy is induced, an isolation membrane encloses a portion of the cytoplasm, forming a characteristic double-membraned organelle termed the autophagosome. The autophagosome then fuses with the lysosome, which contains catabolic enzymes, forming the autolysosome. The contents of the autolysosome are then degraded via enzymes. A set of proteins encoded by autophagy-related genes (ATG) is required to induce autophagy and form the autophagosome.

Abbreviations: ATG, autophagy-related genes; DMSO, dimethyl sulfoxide; GFP-LC3, green fluorescent protein-microtubule-associated protein light chain 3; OSC, outer sulcus cells; PBS, phosphate-buffered saline.

Chika Saegusa and Makoto Hosoya contributed equally to this study.

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Microtubule-associated protein light chain 3 (LC3) is an ATG encoded by *ATG8*[2] that conjugates to phosphatidylethanolamine to form LC3-II, which is localized to isolation membranes and autophagosomes.[2] The level of LC3-II reflects the number of autophagosomes and autophagy-related structures and is therefore, widely used as an autophagosome marker. Green fluorescent protein (GFP)-LC3 mice expressing exogenous LC3 with an N-terminal GFP tag under the control of a constitutive CAG promoter have been used to monitor autophagy in vivo.[3,4] An increased number of GFP-LC3 puncta in tissue cryosections from these transgenic mice reflects autophagy activation.[3-5]

Autophagy reportedly contributes to the development of the inner ear, including remodeling of developmental tissue,[6] and maintaining a normal sense of balance[7] and homeostasis; furthermore, autophagy is involved in the response to ototoxic stress.[8] In addition, basal levels of autophagy are essential for hearing acuity and survival of hair cells in the inner ear.[9-11] Activation of autophagy is now considered a potential therapy or protective measure against hearing loss.[8,12-15]

Rapamycin is a drug often used to activate autophagy. Rapamycin binds to mammalian target of rapamycin complex 1 (mTORC1), which negatively regulates autophagy, and allosterically inhibits the kinase activity of mTORC1. The effectiveness of rapamycin in inducing autophagy in mammalian cells is dependent on cell type. In the inner ear, activation of autophagy by administration of rapamycin has been studied in detail in hair cells and spiral ganglion neurons. Rapamycin-induced activation of autophagy in hair cells reportedly alleviates ototoxicity induced by cisplatin,[16] gentamicin,[17] or neomycin.[14] Treatment with rapamycin also decreases cell death in hair cells caused by acoustic trauma.[8] Rapamycin also protects spiral ganglion neurons from gentamicin-induced degeneration.[18] However, the precise activation state of rapamycin-induced autophagy in other cells in the inner ear has not been sufficiently described, even though these cells are also important for normal hearing ability.

Many cases of cochlear hearing loss are thought to be caused by the dysfunction of lateral wall,[19-24] where endocochlear potential and ion homeostasis are generated and are thus essential for the maintenance of hearing. However, the role of autophagy in hearing loss related to the dysfunctions of lateral wall cells remains to be elucidated. Recently, we revealed that abnormal protein aggregations in outer sulcus cells (OSCs) in the lateral wall may be attributed to a cochlear pathophysiology of hearing loss, and rapamycin-induced autophagy ameliorated these aggregations and the susceptibility of diseased cells, by examining patient-derived OSCs of Pendred syndrome, a genetic disorder leading to hearing loss.[25] Therefore, we decided to elucidate the in vivo level of autophagy in OSCs in the lateral wall and its pharmacological activation.

Here, we report that oral administration of rapamycin activates autophagy in OSCs in the basal-to-mid cochlear turn of the mouse inner ear. Our results clearly show that rapamycin induces the activation of autophagy even at low doses.

2 | MATERIAL AND METHODS

2.1 | Animals

All animal care and treatment procedures were performed in accordance with institutional guidelines approved by the Experimental Animal Care Committee of the Keio University School of Medicine and the Guide for the Care and Use of Laboratory Animals (National Institute of Health, Bethesda, Maryland). GFP-LC3 transgenic mice that express GFP fused to the N-terminus of LC3 under the control of a CAG promoter were established as described previously.[3] This mouse strain (RBRC00806) was provided by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan, and maintained in a C57Bl6/J background. The mice were housed in a room with a 12-hours light/dark cycle and were fed ad libitum.

2.2 | Drug administration

Rapamycin was dissolved in dimethyl sulfoxide (DMSO; WAKO) at 0.0025 or 0.25 mg/mL concentration. GFP-LC3 mice were administered rapamycin once a day for 3 days (0.025 or 2.5 mg/kg) (Figure 1). Control GFP-LC3 mice were administered only DMSO once a day for 3 days. To avoid the effect of autophagy activation associated with aging, we used 12-13-week-old mice in this study.

2.3 | Immunohistochemistry

Animals were perfused with 4% paraformaldehyde (PFA; Nacalai Tesque). The temporal bones were dissected, fixed overnight in 4% PFA at 4°C to count the number of GFP-LC3 puncta, and decalcified with Decalcifying Solution B (WAKO) for 48-72 hours. For immunostaining with anti-Pendrin antibody, the temporal bones were fixed in 4% PFA at 4°C for 4 hours and decalcified for 24 hours. Samples were then embedded in Tissue-Tek OCT compound and then sliced into 7-μm sections. The sections were preblocked for 1 hour at room temperature in 10% normal serum in phosphate-buffered saline (PBS), incubated overnight with primary antibodies at 4°C, and then with Alexa Fluor-conjugated secondary antibodies for 1-2 hours at room

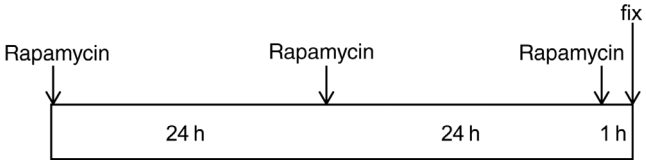

FIGURE 1 Schema of this study. To evaluate autophagy in the mouse inner ear, we used green fluorescent protein-microtubule-associated protein light chain 3 (*GFP-LC3*) transgenic mice expressing GFP fused to the N-terminus of LC3 under the control of a CAG promoter. *GFP-LC3* mice were administered rapamycin once per day for 3 days and then sacrificed to analyze autophagy

temperature. After washing with PBS, the cells were examined using a confocal laser-scanning microscope (LSM700; Carl Zeiss). Nuclei were counterstained with Hoechst33258. Four or six GFP-LC3 transgenic mice were used per group to count the number of GFP-LC3 puncta.

2.4 | Antibodies

The primary antibodies used in this study included anti-Pendrin (goat IgG, Santa Cruz Biotechnology, sc-16 894, 1:50), anti-LC3B antibody

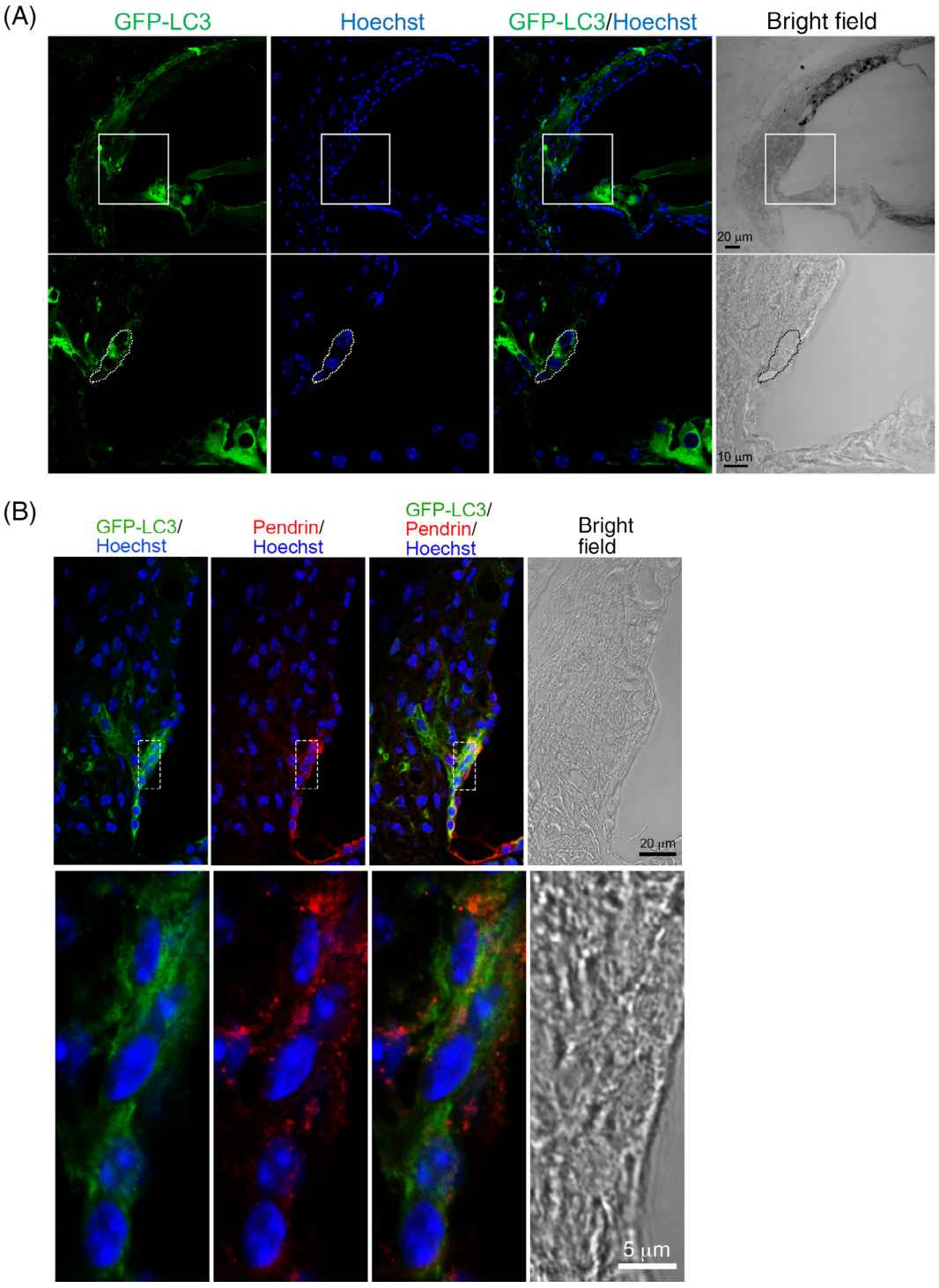

FIGURE 2 Expression pattern of green fluorescent protein-microtubule-associated protein light chain 3 (GFP-LC3) in the cochlea of control *GFP-LC3* transgenic mice. A, Cochlear sections of GFP-LC3 transgenic mice were co-immunostained with two GFP antibodies to identify GFP-LC3-expressing cells. The boxed areas in the upper panels are magnified in the lower panels. GFP-LC3 expression was observed in outer sulcus cells (OSCs, white broken line). B, OSCs were labeled with anti-Pendrin antibody (red). GFP-LC3 expression was observed in pendrin-positive cells in the cochlea. The boxed areas in the upper panels are magnified in the lower panels. Scale bars: 20 μm for upper panels and 10 μm for lower panels, A, 20 μm for upper panels and 5 μm for lower panels, B

(rabbit IgG, Novus Biologicals, NB100-2220, 1:100), and anti-p62 (guinea pig IgG, PROGEN GP62-C, 1:100). To count the number of GFP-LC3 puncta, we used a mixture of two anti-GFP antibodies (rabbit IgG, Medical & Biological Laboratory 598, 1:100; goat IgG, Rockland 600-101-215, 1:100). Immunoreactivity was visualized using Alexa Fluor-conjugated secondary antibodies (Thermo Fischer Scientific, 1:500).

2.5 | Intracellular GFP-LC3 puncta counting

Inner ear tissues were subjected to immunocytochemical analysis with anti-GFP antibodies. GFP-positive puncta were counted using a confocal laser-scanning microscope (LSM700; Carl Zeiss). We adjusted the laser power and PMT gains and offsets settings to capture images

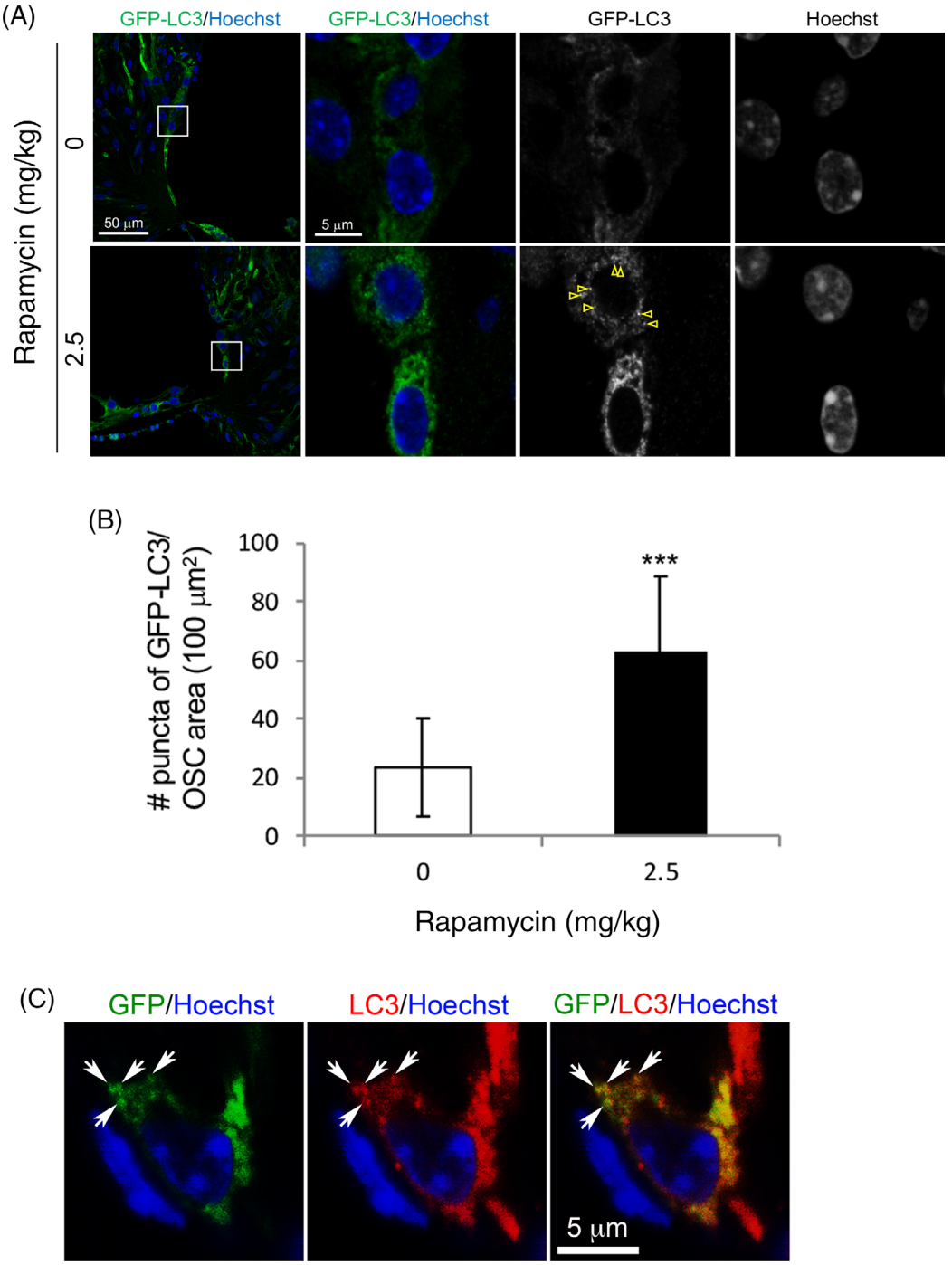

FIGURE 3 Oral administration of rapamycin-activated autophagy in outer sulcus cells (OSCs). Oral intake of rapamycin induced the formation of green fluorescent protein-microtubule-associated protein light chain 3 (GFP-LC3) puncta (arrowheads), A. Boxed areas in the left-most panels are magnified in the three right panels. Puncta formation was significantly increased in the rapamycin-treated group, B. GFP-positive puncta were also immunostained with anti-LC3B antibody (arrows), C. n = 72 and 48 OSC areas for the dimethyl sulfoxide (DMSO)-treated and rapamycin-treated groups, respectively. ***$P < .001$. Scale bars: 50 μm for the left-most panels, 5 μm for the right three panels in A, and 5 μm in C

of GFP-LC3 puncta. These settings were used to capture all images used in the quantification analysis of GFP-LC3 puncta. All images were captured using a ×63 objective (2048 × 2048 pixels, 16-bit data depth, and an average of four scans). The GFP-LC3 punctate structures in the cytoplasmic areas of OSCs were manually counted. Cytoplasmic areas of OSCs were calculated by subtracting the nuclear area from the whole-cell area using ImageJ. OSC areas at each cochlear turn (ie, the basal turn, midbasal turn, mid turn, and apical turn) were analyzed for each slice. Three slices were analyzed for each mouse. The analyses included six mice in the DMSO group, four mice in the rapamycin 0.025 mg/kg group, and four mice in the rapamycin 2.5 mg/kg group.

2.6 | Statistical analysis

Data are expressed as mean ± SD. A two-tailed, nonpaired Student's *t*-test was used to compare differences between two groups. Results with $P < .05$ were considered statistically significant.

3 | RESULTS

3.1 | Expression of GFP-LC3 puncta in inner ear OSCs

Although it was previously shown that a small number of GFP-LC3 puncta were observed in hair cells of cochlea explant culture established from postnatal day 5 GFP-LC3 transgenic mice,[9] we did not know that GFP-LC3 puncta can be observed in cochlear cells other than hair cells in adult GFP-LC3 mice. In this study, we first examined the expression profiles of GFP-LC3 in cochlea-frozen sections from adult GFP-LC3 mice. The expression of GFP-LC3 was broadly observed in the cochlea of GFP-LC3 mice at 12-13 weeks of age. Immunosignals of GFP-LC3 were clearly detected in the organ of Corti, OSCs, and lateral wall fibrocytes. In contrast, less GFP-LC3 expression was observed in the stria vascularis (Figure 2A). Based on the expression of GFP-LC3 in OSCs (Figure 2B), we suggest that GFP-LC3 mice can be used for the analysis of autophagy activation in cochlear OSCs. We also measured the auditory brainstem response of adult GFP-LC3 mice and

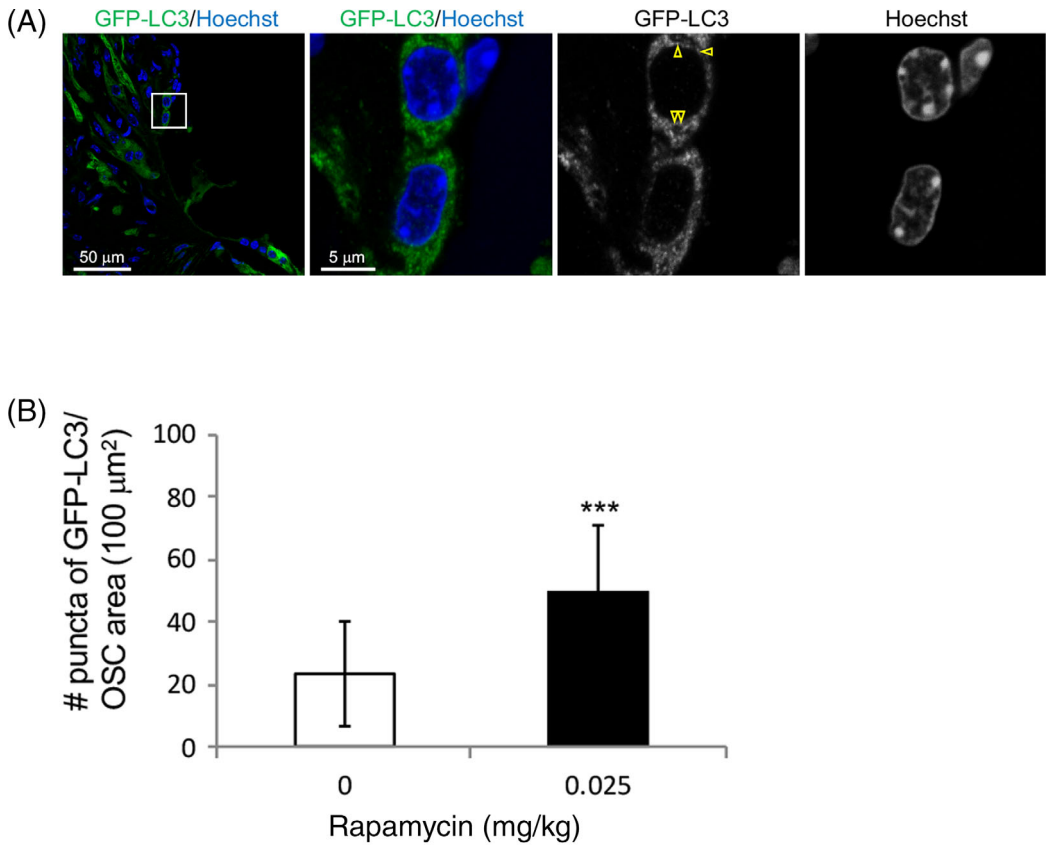

FIGURE 4 Oral administration of low-dose rapamycin activated autophagy in outer sulcus cells (OSCs). Green fluorescent protein-microtubule-associated protein light chain 3 (GFP-LC3) puncta (arrowheads) were induced in OSCs following oral administration of low-dose rapamycin, A. Boxed areas in the left-most panel are magnified in the right three panels. Puncta formation was significantly increased in the low-dose rapamycin-treated group, B. n = 72 and 48 OSC areas for the dimethyl sulfoxide (DMSO)-treated and rapamycin-treated groups, respectively. ***$P < .001$. Scale bars: 50 μm for the left-most panel and 5 μm for the right three panels

found that the auditory brainstem response threshold is not altered in GFP-LC3 mice compared to that in C57BL6 wild-type mice (data not shown), suggesting that the GFP-LC3 transgene insertion does not affect the hearing ability of mice, and GFP-LC3 mice can be used as a model to study autophagy related to auditory systems.

3.2 | Number of GFP-LC3 puncta is increased in inner ear OSCs by oral intake of rapamycin

Next, we orally administered rapamycin (2.5 mg/kg) to determine whether rapamycin mediates autophagy activation in OSCs. The

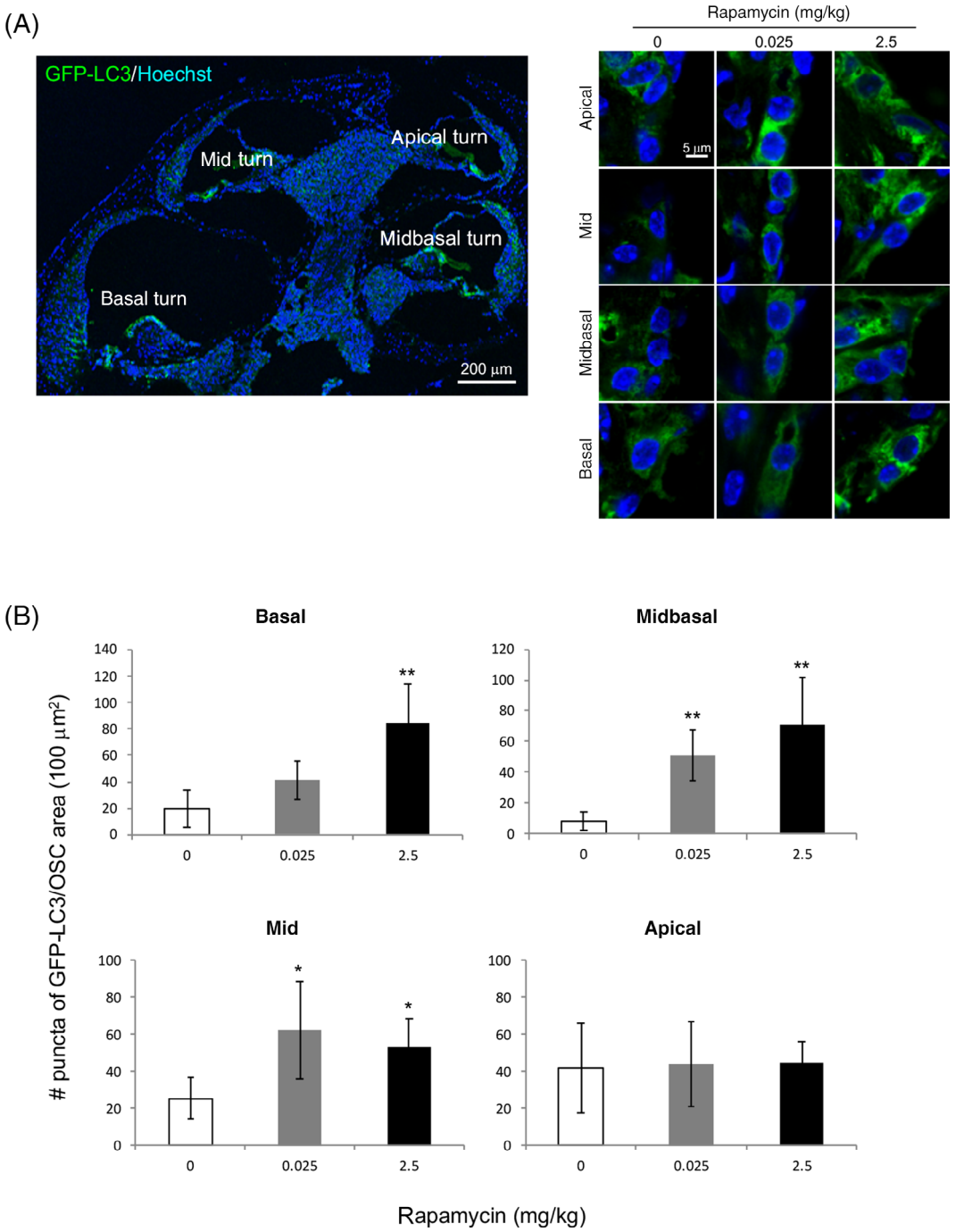

FIGURE 5 Regional differences in the effects of rapamycin on autophagy activation along the cochlear axis. A, Full view of the cochlear section (left) stained with anti-GFP antibody (green) and Hoechst (blue). Representatives of outer sulcus cells (OSCs) in each cochlea turn are shown on the right. To examine the effects of rapamycin for autophagy activation along the cochlear axis, GFP-LC3 puncta in OSC areas were quantified in each cochlear turn. Administration of rapamycin increased the number of GFP-LC3 puncta in the OSCs of basal-to-mid cochlear turns, whereas it did not cause any significant differences at the apical turn, B. n = 18, 12, and 12 OSC areas. *P < .05; **P < .01. Scale bars: 200 μm for the left panel and 5 μm for the right panels

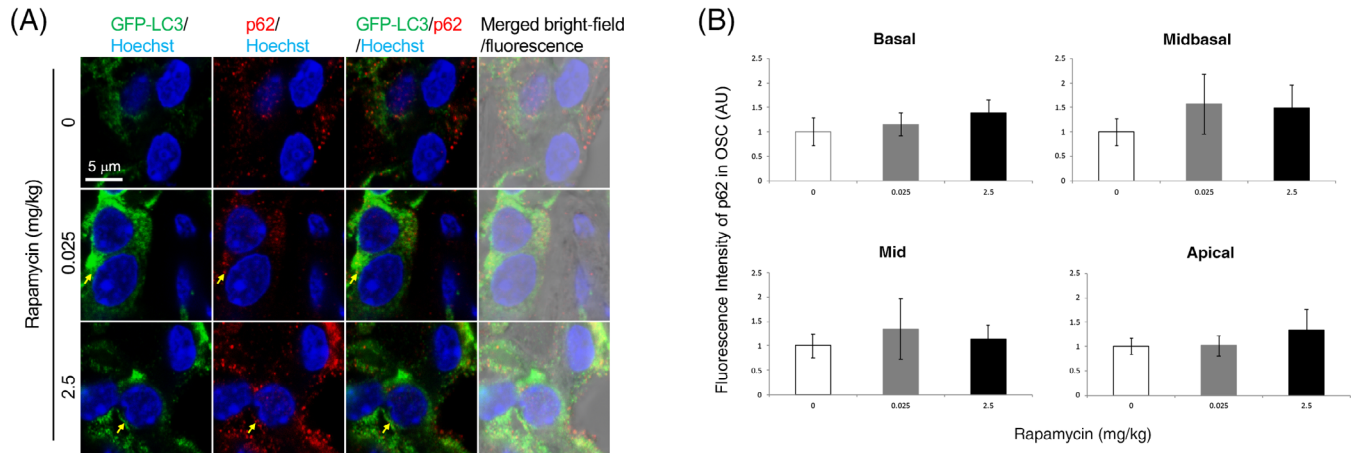

FIGURE 6 Effect of rapamycin administration on p62 accumulation in outer sulcus cells (OSCs). A, Confocal imaging of OSCs immunostained with anti-p62 (red) and anti-GFP antibodies (green). GFP-LC3 puncta were partially colocalized with p62 (arrows). B, Fluorescence intensity of p62-immunosignals in OSCs was calculated using ImageJ and compared between groups. There were no significant differences in the fluorescence intensity of p62-immunosignals between the groups. The mean fluorescence intensity of p62-immunosignals in OSC (AU, arbitrary units) of the dimethyl sulfoxide (DMSO)-treated mice was set to 1. Error bars indicate SD. n = 18, 12, and 12 OSC areas. Scale bar: 5 μm

administration was well tolerated by the animal. The formation of GFP-LC3 puncta, which was co-immunostained with LC3B antibody (Figure 3C), was significantly increased in OSCs following the administration of rapamycin ($P < .001$, Figure 3A,B), indicating that autophagy activation was achieved in OSCs after oral administration of rapamycin.

3.3 | Low-dose rapamycin activated autophagy in inner ear OSCs

To investigate the effect of oral low-dose rapamycin, we administered rapamycin at 0.025 mg/kg dose (a 100-fold lower dose than that used in Figure 3) to GFP-LC3 mice, in which the formation of GFP-LC3 puncta was significantly increased in OSCs (Figure 4). The administration of low-dose rapamycin activated autophagy in mouse OSCs.

3.4 | Regional differences in the effects of rapamycin on autophagy activation along the cochlear axis

We further examined regional differences associated with rapamycin administration in the cochlea by comparing the number of GFP-LC3 puncta in OSCs for each turn of the cochlea. An increased number of GFP-LC3 puncta were observed in OSCs in the basal-to-mid turn following the administration of rapamycin, whereas they were hardly observed in OSCs from the apical turn (Figure 5). The average number of GFP-LC3 puncta was higher in the apical turn than that in the basal-to-mid turn in the control mice.

3.5 | Effect of rapamycin administration on p62 accumulation in OSCs

We analyzed the effect of rapamycin on the accumulation of p62 protein in OSCs (Figure 6). p62/SQSTM1 is an autophagy-specific substrate and accumulates under autophagy-deficient conditions in hair cells of mouse inner ear.[9] We examined whether the expression level of p62/SQSTM1 in OSCs is altered by the oral administration of rapamycin. The expression of p62 was expected to be decreased by the activation of autophagy because aggregates containing p62 are degraded by the intracellular quality control system of autophagy. The results of immunostaining showed that the intensity of immunosignals of p62 in OSCs was not altered by the oral administration of rapamycin (Figure 6). Under our experimental conditions, the basal expression of p62 in OSCs of the control mice was low, and it may be difficult to detect further decrease in the expression of the p62 protein by the oral administration of rapamycin.

4 | DISCUSSION

Activation of autophagy is considered a potential therapeutic target for neurodegenerative diseases and cancers.[26-28] More than 10 autophagy activators, including rapamycin and its derivative compounds, are now under clinical trials for some diseases.[29-31] For instance, autophagy-mediated increase in the clearance of mutated and pathogenic proteins from neuronal cells is expected to become a new treatment option for neurodegenerative diseases associated with these proteins.[32] Recently, we reported that rapamycin is likely a feasible drug for the treatment of Pendred syndrome, which is thought to be caused by the accumulation of mutated proteins in inner ear cells.[25,33]

Clinically, rapamycin is used as an immunosuppressive drug for organ transplantations[34]; the immunosuppressive effects of rapamycin are observed at 16-24 ng/mL dose in the blood after renal transplantation and are attributable to the inhibition of mammalian target of rapamycin (mTOR). Rapamycin is also used as a treatment for lymphangioleiomyomatosis (LAM),[35] in which hyperactive mTOR signals associated with this disease are inhibited. LAM is treated with rapamycin at blood concentrations of 5-15 ng/mL; these concentrations are usually maintained via oral administration of 2 mg daily for adult patients. When rapamycin is clinically used at the described concentration, side effects such as stomatitis, diarrhea, nephrotoxicity, or thrombocytopenia are sometimes observed.[30,35] Therefore, we estimated the minimum effective dose and revealed the lowest effective concentration in vitro with Pendred syndrome patient-derived OSCs as 0.001 μM or approximately 0.9 ng/mL.[33] However, it was not clear whether oral administration of rapamycin at a low dose activates autophagy in the inner ear cells in vivo.

To address this issue, presenting results with the GFP-LC3 transgenic mice is helpful in monitoring the activation of autophagy. The accessibility of drugs to inner ear cells mainly depends on the distribution of the blood-labyrinth barrier. The tight junction is the main component of the blood-labyrinth barrier that creates a barrier between inner ear cells and systemic blood circulation. We have recently reported that the distribution of tight junctions around the lateral wall, including OSCs, in primates is similar to that in rodents by examining the cochleae of common marmosets using electron microscopy.[36] These previous observations suggest that the permeability and effectiveness of rapamycin in human inner ear cells can be predicted based on the results obtained from rodent models. Using GFP-LC3 mice, we found that autophagy is activated in cochlear OSCs at a low concentration of rapamycin (0.025 mg/kg) administered orally. Previously, it was reported that intraperitoneal injections of rapamycin (7.5 mg/kg) effectively activated autophagy in the inner ear hair cells of mice.[8] Our current study shows that a much lower concentration of rapamycin induces autophagy in inner ear cells when administered orally. Furthermore, we found regional differences in the effects of rapamycin along the axis of mouse cochleae. These results suggest that sensitivity to exogenous drugs or stimulation varies in inner ear cells depending on the region of the cochlear turn.

Previous studies have focused on the role of autophagy and/or the effect of rapamycin in the organ of Corti[8,14,16,17] or spiral ganglion neurons,[18] but activation of autophagy in the lateral wall has not been described in detail, although lateral wall cells, such as OSCs, are important for hearing function. Recently, we found that abnormal protein aggregation in OSCs in the lateral wall is a major pathophysiology[25] of the Pendred syndrome. Patients with the Pendred syndrome carrying *SLC26A4* mutations experience fluctuating and progressive hearing loss. The *SLC26A4* gene encodes the pendrin protein, which is abundantly expressed in OSC.[22,37,38] Interestingly, according to results from our previous study, abnormal protein aggregation in OSCs of these patients is a major pathophysiology, which can be decayed by activating autophagy using rapamycin.[25,33] Therefore, the activation of autophagy in OSCs in vivo by the oral intake of low-dose rapamycin is essential for the treatment of hearing impairment. In this study, we showed that oral administration of low-dose rapamycin activates autophagy in OSCs in mice. We are currently conducting a clinical trial involving oral intake of rapamycin at the aforementioned concentration for cochlear disease.[39] We believe this trial may clarify the effect of oral intake of low-dose rapamycin on hearing impairment at the clinical level.

To date, reports suggest that the normal activity of autophagy in the inner ear is essential for maintaining hearing and that autophagy activation in the inner ear protects against hearing loss induced by ototoxic stress.[8,9] Our results demonstrate that oral administration of low-dose rapamycin induces activation of autophagy in cochlear OSCs, suggesting that rapamycin could be a feasible drug to manipulate inner ear cells. Further studies are needed to confirm these possibilities.

5 | CONCLUSIONS

We showed that oral administration of low-dose rapamycin activates autophagy in the inner ear OSCs of mice. Considering the previous reports on the protective effect of low-dose rapamycin in diseased cells, our results suggest that low-dose rapamycin is a potential therapeutic strategy for treating inner ear disease caused by mutated proteins, including the Pendred syndrome.

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CONFLICT OF INTEREST

We declare no competing interests.

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