Activation of Rictor/mTORC2 signaling acts as a pivotal strategy to protect against sensorineural hearing loss

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The Food and Drug Administration–approved drug sirolimus, which inhibits mechanistic target of rapamycin (mTOR), is the leading candidate for targeting aging in rodents and humans. We previously demonstrated that sirolimus could treat ARHL in mice. In this study, we further demonstrate that sirolimus protects mice against cocaine-induced hearing loss. However, using efficacy and safety tests, we discovered that mice developed substantial hearing loss when administered high doses of sirolimus. Using pharmacological and genetic interventions in murine models, we demonstrate that the inactivation of mTORC2 is the major driver underlying hearing loss. Mechanistically, mTORC2 exerts its effects primarily through phosphorylating in the AKT/PKB signaling pathway, and ablation of P53 activity greatly attenuated the severity of the hearing phenotype in mTORC2-deficient mice. We also found that the selective activation of mTORC2 could protect mice from acoustic trauma and cisplatin-induced ototoxicity. Thus, in this study, we discover a function of mTORC2 and suggest that its therapeutic activation could represent a potentially effective and promising strategy to prevent sensorineural hearing loss. More importantly, we elucidate the side effects of sirolimus and provide an evaluation criterion for the rational use of this drug in a clinical setting.

mTORC2 | hearing | hair cells

With increasing progress and a rapid improvement in the living standards of individuals in society, considerable importance is being given to hearing problems. Therefore, it is of great significance to study the occurrence and understand the mechanisms underlying the development of hearing disorders to effectively prevent, detect, and treat deafness. Among the hearing-impaired population, sensorineural deafness accounts for the majority of cases of hearing loss. Studies have shown that almost 50% of hereditary sensorineural deafness is caused by mutations in hair cell (HC) enriched genes, although HCs constitute only approximately one-tenth of the cells in the sensory epithelium of the cochlea (1). These factors responsible for injury, including genetic defects, can lead to irreversible damage and the loss of cochlear HCs, which is the main cause of sensorineural hearing loss (2). Therefore, understanding the mechanism of the development and survival of auditory HCs and being able to restore the function of cochlear sensory epithelium is an ideal approach in auditory reconstruction and also the primary focus in the field of otology.

Mechanistic target of rapamycin (mTOR), now defined as the mechanistic target of sirolimus, is a highly conserved serine/threonine protein kinase, which controls the growth of cells and organisms induced by growth factors and nutrients. The mTOR is assembled into two multiprotein complexes, namely, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which can be distinguished based on their related proteins and their sensitivity to sirolimus (3). Evidence indicates that mTORC1 is mainly responsible for cell growth and proliferation in response to growth factors, nutrients, or stress, and that the two main downstream targets of mTORC1, namely, p70S6 kinase (S6K) and elongation factor 4E binding protein (4E-BP1), are key regulators of cap-dependent protein translation (4, 5). Although the mechanisms that regulate mTORC1 are well understood, the aspect of regulation of mTORC2 is relatively poorly characterized. The mTORC2 signaling is insensitive to nutrients; however, it responds to growth factors, such as insulin, through poorly defined mechanism(s) that require(s) PI3K (6). Experiments in yeast and cultured mammalian cells have indicated that mTORC2 plays a role in the regulation of the actin cytoskeleton (7, 8). The mTORC2 controls several members of the AGC kinase subfamily, including Akt, protein kinase C-α

Significance

The mechanistic target of rapamycin (mTOR) plays a central role in growth, metabolism, and aging. It is assembled into two multiprotein complexes, namely, mTORC1 and mTORC2. We previously demonstrated the efficacy of sirolimus in ARHL in mice by decreasing mTORC1. However, the aspect of mTORC2 regulation in the cochlea is poorly characterized. Herein, based on pharmacological and genetic interventions, we found that a high dose of sirolimus resulted in severe hearing loss by reducing the mTORC2/AKT signaling pathway in the cochlea. Furthermore, selective activation of mTORC2 could protect against hearing loss induced by acoustic trauma and cisplatin-induced ototoxicity. Hence, the therapeutic activation of mTORC2 in conjunction with decreasing mTORC1 might represent a promising and effective strategy in preventing hearing loss.

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(PKC-α), and serum and glucocorticoid-induced protein kinase 1 (SGK1) (9–11). Although mTORC2 is relatively insensitive to acute sirolimus treatment, recent studies have shown that prolonged exposure to sirolimus can inhibit the mTORC2 complex assembly (12–14).

As one of the central regulators of cellular activities, the mTOR signaling pathway has been attracting increasing attention in recent years (4, 15). Several studies now focus on the function of mTOR in metabolic tissues, largely because these tissues are particularly sensitive to the three inputs (nutrients, insulin, and energy status) that control mTOR (16, 17). Additionally, abnormalities in the mTOR signaling pathway can lead to conditions such as epilepsy (18, 19), diabetes mellitus (20, 21), tuberous sclerosis syndrome (22, 23), and tumors (24, 25). Nevertheless, existing knowledge regarding the functioning and regulation of the mTOR pathway in the maintenance of the auditory system and homeostasis is limited. To date, the available evidence suggests that auditory disorders result from changes in the mTOR signaling pathway (26–28). Recently, our study group provided evidence that inhibition of mTORC1 resulting from an intraperitoneal (i.p.) injection of 1 mg/kg sirolimus or from a genetic disorder leading to a decrease in mTORC1 activity via deficiency of *Raptor* attenuated age-related hearing loss (ARHL) in C57BL/6J mice, whereas overactivation of the mTORC1 signaling pathway in the neurosensory cells (NSE) caused premature HC death and progressive hearing loss (29). However, as a Food and Drug Administration (FDA)-approved drug, although an i.p. injection of sirolimus in wild-type (WT) C57BL/6J mice can prevent ARHL, some caveats must be considered when translating this finding to a clinical setting. Accordingly, the efficacy and safety of sirolimus should be comprehensively understood prior to its use in treating ARHL. While studying the effects of different doses of the drug, we serendipitously found that high doses of injected sirolimus could result in a significant loss of hearing in mice. Coincidentally, a recent study reports that the inhibition of mTOR by sirolimus results in dose-dependent damage of auditory HCs cultured ex vivo (30). Collectively, these findings suggest the complex functions of the mTOR signaling pathway in the auditory sensory epithelium, especially in auditory HCs. It is currently unknown whether mTOR acts through mTORC1, through mTORC2, or through the synergistic action of these two complexes in the development and survival of HCs. Neither the genetic nor the pharmacological manipulation of mTOR kinase is adequate to distinguish the role of these two complexes in cochlear HCs. In addition, the correlation between mTORC1 and mTORC2 in cochlear HCs and the downstream molecular regulatory signals remains to be elucidated.

Results

Sirolimus Protects Mice against Cocaine-Induced Hearing Loss. Cocaine abuse is a persistent health problem in the United States, with few available treatment options. Studies have reported that, in humans, bilateral sensorineural hearing loss occurs following cocaine abuse. It is reported that the inhibition of mTORC1 by sirolimus blocks cocaine-induced locomotor sensitization. In addition, our study group and others have shown that sirolimus is a potential therapeutic drug for protecting against ARHL and other hearing abnormalities in mice by decreasing mTORC1 (29, 31). We were keen on examining whether sirolimus played a role in treating hearing loss caused by cocaine abuse. To test this possibility, we first examined whether cocaine exposure involved the mTORC1 pathway in the cochleae and also assessed whether treatment with sirolimus affected cocaine-induced hearing sensitization. We found that injection of cocaine (20 mg/kg) markedly stimulated the phosphorylation levels of S6 (a reliable readout of mTORC1 activity) (*SI Appendix*, Fig. S1 *A* and *B*). After confirming that the mTORC1 signaling pathway was activated by cocaine, we further evaluated whether cocaine-induced hearing impairment was related to mTORC1. To this end, we coinjected mice with cocaine (20 mg/kg) and sirolimus (2 mg/kg) for three consecutive days. Cocaine-injected mice showed a significant elevation of the auditory brainstem response (ABR) threshold. However, the ABR threshold of mice coinjected with sirolimus and cocaine was significantly lower than that of mice injected with cocaine alone and was indistinguishable from that of the vehicle-treated mice (*SI Appendix*, Fig. S1*C*). In addition, we found that S6 phosphorylation was abolished by sirolimus (*SI Appendix*, Fig. S1 *D* and *E*).

High Concentration of Sirolimus Led to Severe Hearing Loss in C57BL/6J Mice. As an FDA-approved drug, the most effective dose with minimal side effects of sirolimus to the auditory system was determined, following which, we evaluated the efficacy of different concentrations of sirolimus on the hearing of C57BL/6J mice. Notably, consistent with our previous reports, low doses of sirolimus (i.p. to 6-mo-old C57BL/6J mice every other day at 1 mg/kg to 2 mg/kg) had no obvious effect on the hearing sensitivity of mice compared with the untreated WT mice (Fig. 1 *A* and *D*). In contrast, unexpectedly, high doses of sirolimus (10 mg/kg to 20 mg/kg) injection for 2 wk led to severe hearing loss in C57BL/6J mice (Fig. 1*A*). ABR thresholds to tune bursts between 4 and 32 kHz and click stimulation were significantly elevated by about 30 dB to 50 dB sound pressure level (SPL) in mice injected with high concentrations of sirolimus (Fig. 1*A*). The latency of the ABR wave I was increased (Fig. 1*B*), and the ABR peak 1 (P1) amplitudes were decreased concurrently (Fig. 1*C*), underscoring the auditory deficits in mice injected with high doses of sirolimus. In addition, distortion product otoacoustic emission (DPOAE) intensities were substantially reduced in mice that were injected with high doses of sirolimus (Fig. 1*D*). Besides changes in hearing, the mice receiving high doses of sirolimus showed a trend in weight loss (*SI Appendix*, Fig. S2*A*); the mice even died occasionally when the drug concentration reached 20 mg/kg, suggesting that high concentrations of sirolimus could likely lead to metabolism-related changes. Organs, including the kidney, heart, pancreas, and liver, did not appear to be pathologically or morphologically different compared to those of mice administered low-dose sirolimus (*SI Appendix*, Fig. S2*B*). In vivo electrophysiological data indicated that the hearing dysfunction in mice administered a high dose of sirolimus originated from impaired signal processing in the auditory periphery. To investigate the underlying causes of hearing, using histopathology, we found that high concentrations of sirolimus led to severe hair cell loss without obvious alterations in other key regions of the cochleae, such as the spiral ganglion neurons (SGNs) and the stria vascularis (StV) (Fig. 1 *E*–*G*), suggesting that HCs were the most vulnerable to damage from high-dose sirolimus injections in cochleae. Consistent with the cochlear phenotype in vivo, we observed a significant loss of inner hair cells (IHCs) and outer hair cells (OHCs) in cochlear explants with increasing concentrations of sirolimus ex vitro in a dose-dependent manner; almost all HCs were lost when the sirolimus concentration in the culture medium was 150 nm (Fig. 1 *H* and *I*). Collectively, our in vivo and ex vitro results demonstrated that different concentrations of sirolimus had different effects on auditory HCs, and high concentrations of sirolimus were detrimental to the hearing of C57BL/6J mice, suggesting the pivotal yet incompletely understood role of sirolimus in the auditory system.

High Dose of Sirolimus Did Not Alter mTORC1 but Decreased mTORC2 Signaling Compared to Low-Dose Sirolimus Treatment. Given the controversial data suggesting that down-regulation of mTORC1 prevents ARHL and cocaine-induced hearing loss, we

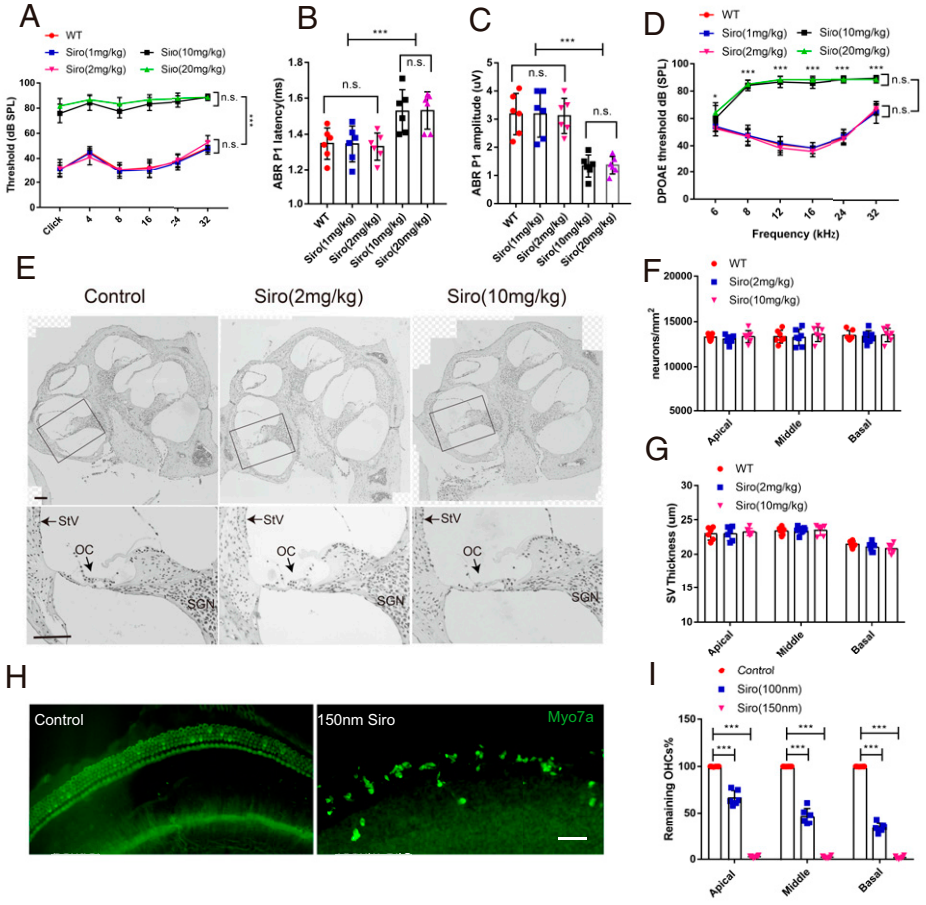

Fig. 1. High concentration of sirolimus leads to severe hearing loss in C57BL/6J mice. (*A–D*) Mice treated with different concentrations of sirolimus for 2 wk were tested based on ABR and DPOAE. (*A*) ABR thresholds, (*B*) ABR peak 1 (P1) latency, (*C*) ABR peak 1 (P1) amplitudes, and (*D*) DPOAE thresholds. ABR thresholds and DPOAE thresholds were greatly elevated in high-dose (10 and 20 mg/kg) treated groups compared to low-dose (1 and 2 mg/kg) treated groups; $n = 6$. (*E*) Transverse sections of mice cochleae treated with different concentrations of sirolimus were subjected to H&E staining. Severe degeneration of HCs in the OC (Organ of Corti) was observed in mice treated with high doses but not in controls and those treated with low doses. However, no significant differences in SGN and StV were observed between the three groups. (Scale bar: 100 μm.) Similar ganglion cell survival patterns (*F*) and StV thickness (*G*) were observed between the three groups. (*H*) Myo7a staining images of cultured cochlear sensory epithelia treated with sirolimus. (*I*) Quantitative analysis of surviving OHCs treated with sirolimus by analyzing cochlear turns at different concentrations; $n = 6$. (Scale bar: 20 μm.) Data are presented as the mean ± SD, "n.s." represents not significant, $*P < 0.5$, $***P < 0.001$, using two-tailed Student's *t* test.

were curious to evaluate the effects and the underlying mechanism when high doses of sirolimus injection were administered. For a comprehensive understanding and to address these dichotomous findings, we evaluated how high-dose sirolimus treatment affected mTOR signaling; first, we evaluated mTORC1 activity using different doses of sirolimus. As anticipated, we found that all doses of sirolimus reduced mTORC1 activity; however, notably, high sirolimus concentrations (10 mg/kg) did not further reduce the expression of P-S6 (S235/236) and P-S6K1 (T389), both of which are sensitive readouts downstream of mTORC1 (*SI Appendix*, Fig. S3 *A–C*). Accordingly, a high concentration of sirolimus (10 mg/kg) may lead to severe hearing loss in an mTORC1-independent manner. To further test this possibility directly, we examined the effects of high doses of sirolimus on the auditory function of mTORC1-deficient mice (29). Raptor is the mTORC1-specific component, and we established mice with conditional ablation (cKO) of Raptor. The *Atoh1-Cre* mouse line was selected because the Cre-mediated recombination was most expressed in cochlear HCs. Therefore, *Raptor-cKO* mice demonstrate hair cell–specific inactivation of the mTORC1 signaling pathway. Since sirolimus is a specific inhibitor of mTORC1, sirolimus injection should have no effect on *Raptor-cKO* mice. However, WT C57BL/6J mice and *Raptor-cKO* mice (6 mo old) that received 10 mg/kg i.p. injection of sirolimus for 2 wk showed a marked increase in ABR threshold (*SI Appendix*, Fig. S3*D*). Hence, these data support the notion that the adverse effects of sirolimus on the auditory system can be attributed to factors independent of mTORC1.

Apart from mTORC1, another functionally and structurally distinct mTOR-containing complex, mTORC2, has been recently identified. While little is known regarding its upstream regulator and downstream effectors, mTORC2 contains Rictor (sirolimus-insensitive companion of mTOR) (*SI Appendix*, Fig. S3*E*) as an essential component, which is largely insensitive to acute treatment with sirolimus (32). However, off-target effects, most notably the inhibition of mTORC2, have been described after the long-term and high doses of sirolimus administration (33, 34). Therefore, mTORC2 signaling was studied using the cochleae of mice that were injected with high doses of sirolimus and compared to those that were administered low doses of the drug. Using immunoblotting, the levels of pT1135-Rictor and Rictor of the whole cochleae were found to be similar in mice injected with different doses of sirolimus; however, phosphorylation of S473-Akt and PKCa-S657 (two reliable readouts of mTORC2 activity) decreased significantly in mice administered high doses of sirolimus compared to those that received a low dose (*SI Appendix*, Fig. S3 *F*, *G*, and *I*). Concurrently, we examined the phosphorylation of AKT at threonine 308 (pT308-AKT), which is not mTORC2-dependent, and did not detect obvious alterations in pT308-AKT levels (*SI Appendix*, Fig. S3*H*), indicating that phosphorylation of T308-AKT was not absolutely required. To further confirm that these effects were caused by the destruction of mTORC2, we immunoprecipitated mTOR from the cochleae of mice treated with different doses of sirolimus and observed the components of mTORC1 (Raptor) and mTORC2 (Rictor) using immunoblotting (*SI Appendix*, Fig. S3*J*). High levels of sirolimus disrupted the association of mTOR with Rictor in the cochlea without further reducing the binding ability of mTOR to Raptor (*SI Appendix*, Fig. S3*J*). Moreover, as opposed to mice that received a low drug dose, a high dose of sirolimus reduced mTORC2 signaling without significantly altering mTORC1 activity, leading us to infer that disruption of mTORC2 might have contributed to sirolimus-induced alterations in the hearing function of mice.

Local Pharmacological Inhibition of Pan-mTOR Signaling with Torin1 Resulted in Auditory Hair Cell Damage In Vitro and In Vivo. High doses of sirolimus could be fatal to C57BL/6J mice, indicating its negative influence on the other systems in vivo. Meanwhile, apart from inhibiting mTORC1 and mTORC2 in the cochlea, it cannot be conclusively stated that a high dose of sirolimus will not cause changes in other signaling pathways that are essential for auditory function. To rule out the detrimental effects of these factors to the auditory system, we injected the drug directly into the tympanic membrane, because this method can avoid potential toxicity and is also well documented in in vivo studies and clinical trials for the treatment of middle- and inner-ear diseases (35) (Fig. 2*A*). Torin1, a second-generation mTOR-specific inhibitor, was chosen for injection (36). A transtympanic injection of 5 mg/kg Torin1 was performed in one of the ears of adult C57BL/6J mice, whereas an equal volume of 0.5% dimethyl sulfoxide (DMSO) was injected in the other ear. Mice injected with 5 mg/kg Torin1 did not exhibit obvious differences in gross morphology and body weight compared to untreated mice. The mTORC1 and mTORC2 were inhibited by Torin1, as shown by decreased p-S6 (235/236) and P-Akt (S473) (Fig. 2 *B–D*). The ABR thresholds were measured for each ear at 2 wk after drug delivery. As anticipated, the mean ABR threshold was found to be significantly elevated in the Torin1-injected ear, with a mean elevation of click ABR thresholds of ∼35 dB and reduced amplitudes, compared to the other ear (Fig. 2*E*). In addition, the DPOAE intensities were substantially decreased in the Torin1-injected ear (Fig. 2*F*). In accordance with the results of the ABRs, the Torin1-injected ear showed significant hair cell loss (Fig. 2 *G* and *H*). However, the ribbon synapse of the IHCs, which were immunostained with the antibody against Ctbp2/Ribeye (presynaptic IHC ribbon marker), were present in a similar number in both ears (Fig. 2 *E* and *J*); In addition, there were no morphological differences in other parts of the cochlea based on the results of hematoxylin/eosin (H&E) staining of the cochlea sections of the Torin1-treated ear and the control ear. Consistent with our in vivo findings, we found a significantly decreased number of HCs in Torin1-treated explants ex vivo; however, there was no obvious decrease in the number of HCs in the control group (Fig. 2*K*). Taken together, these results indicated that the pharmacological transtympanic coinhibition activity of mTORC1 and mTORC2 with Torin1 reduced the survival of HCs both ex vitro and in vivo, which resembled the phenotype of the whole-body injection of a high dose of sirolimus.

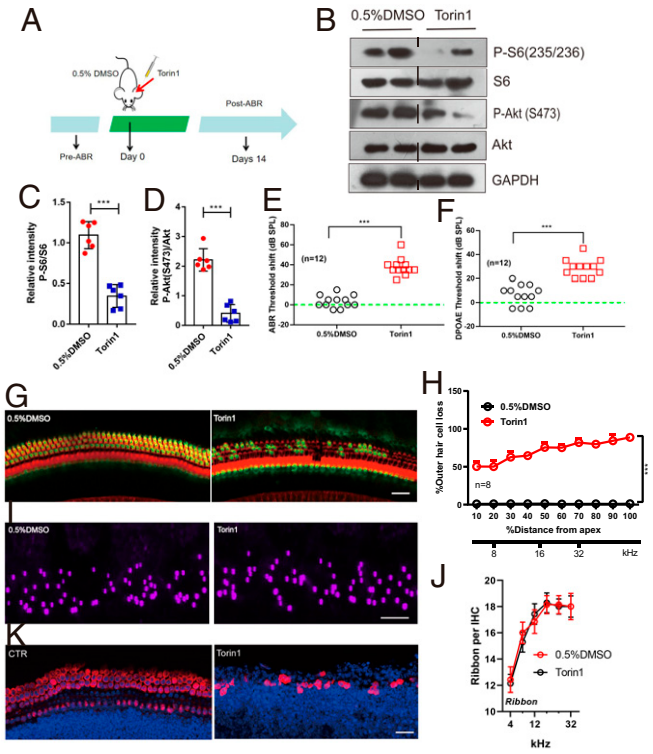

Fig. 2. Decreasing the mTORC1 and mTORC2 activity damages the cochlear HCs. (*A*) Schematic overview of the experimental design for transtympanic injection of Torin1 and ABR test. (*B*) Western blotting indicates mTORC1 and mTORC2 are coinhibited by Torin1 (5 mg/kg), as shown by decreased P-S6 (235/236) and P-Akt (S473), respectively. P-S6 (235/236) and P-Akt (S473) levels are quantified in *C* and *D*. Auditory function was damaged in Torin1-injected cochlea, as determined based on elevated threshold using either ABR (*E*) or DPOAE (*F*) assessments. (*G*) Confocal images of cochlear whole mounts labeled with Myo7A antibody (green) and counterstained with phalloidin to label filamentous actin (red) are shown. Only a few OHCs and partial IHCs remained in the middle and apical turns of Torin1-treated mouse cochleae. (Scale bar: 20 μm.) (*H*) Hair cell loss as a percentage for both Torin1-treated groups and 0.5% DMSO controls; $n = 6$. (*I*) Representative confocal images of IHC synapses from 32-kHz region of Torin1-treated and 0.5% DMSO-treated cochleae immunolabeled for presynaptic ribbons (CtBP2-violet). (Scale bar: 5 μm.) (*J*) Quantitative data show that Torin1 treatment does not alter the number of presynaptic ribbons compared to that in the controls. (*K*) Torin1 damages the hair cell ex vivo as determined using immunofluorescence staining with Parvalbumin (red) and DAPI (blue) in the cochlear explant cultures. (Scale bar: 20 μm.) Data are presented as the mean ± SD, $***P < 0.001$, using a paired *t* test.

mTOR Deficiency Was Nonessential for Early Hearing Development in Mice. Coinhibition of mTORC1 and mTORC2 by the systemic administration of high-dose sirolimus and transtympanic injection of Torin1 mainly affected the cochlear HCs in the auditory system. However, a study using these two methods is of limited value, since the systemic administration of both sirolimus and Torin1 affects the remnant cell types in the cochlea. Thus, the results obtained using both these drugs cannot be used to answer whether the observed phenotypes were the direct effects of a cell-autonomous function of mTOR signaling or whether they resulted from the additional impact of altered cell–cell communication. Thus, tissue/cell-specific analyses of mTOR signaling are better suited to determine the multifaceted role of mTOR. To address this question and for an in-depth understanding of the functional and intrinsic role of mTOR signaling in HCs, we used molecular genetics and the *Atoh1-Cre* mice line to generate mice expressing conditional ablation of *mTOR* (*SI Appendix*, Fig. S4*A*). At birth, the Cre-positive $mTOR^{flox/flox}$ pups (*mTOR-cKO*) were viable, born at Mendelian ratios, and indistinguishable from the control littermates. We further bred *Atoh1-Cre*/$mTOR^{fl/fl}$/*Rosa26-tdTomato* mice and isolated tdTomato-positive cells using flow cytometry (*SI Appendix*, Fig. S4*A*). Successful verification of the deletion of mTOR in *mTOR-cKO* was tested using PCR (*SI Appendix*, Fig. S4*B*). Western blot analysis of the sorted tdTomato-positive cells demonstrated an effective reduction of mTOR protein expression in *Atoh1-Cre*/$mTOR^{fl/fl}$/*Rosa26-tdTomato* mice (*SI Appendix*, Fig. S4 *C* and *D*). The phosphorylation of the mTORC1 target, S6, at S235/236 and 4E-BP1 at T37/46 was markedly reduced in *Atoh1-Cre*/$mTOR^{fl/fl}$/*Rosa26-tdTomato* mice (*SI Appendix*, Fig. S4 *C*, *E*, and *F*). In addition, the phosphorylation of the mTORC2 target, AKT, at S473 was also substantially attenuated (*SI Appendix*, Fig. S4 *C* and *G*). Immunolabeling for p-S6 (235/236) and pS473-AKT using the cochlear paraffin-embedded sections also showed a remarkable decrease in expression in *mTOR-cKO* mice (*SI Appendix*, Fig. S4 *I* and *J*). These findings demonstrated that the genetic loss of mTOR in cochlear HCs attenuated both the mTORC1 and mTORC2 signaling pathways.

The *mTOR-cKO* mice were indiscernible from the $mTOR^{flox/flox}$ littermates based on the gross morphology during the development period of the auditory system. H&E staining of *mTOR-cKO* mice cochleae showed no obvious structural abnormalities in the inner ear of *mTOR-cKO* mice compared to that of the littermate mice at P14 (*SI Appendix*, Fig. S4*H*). In *mTOR-cKO* mice, the IHCs and

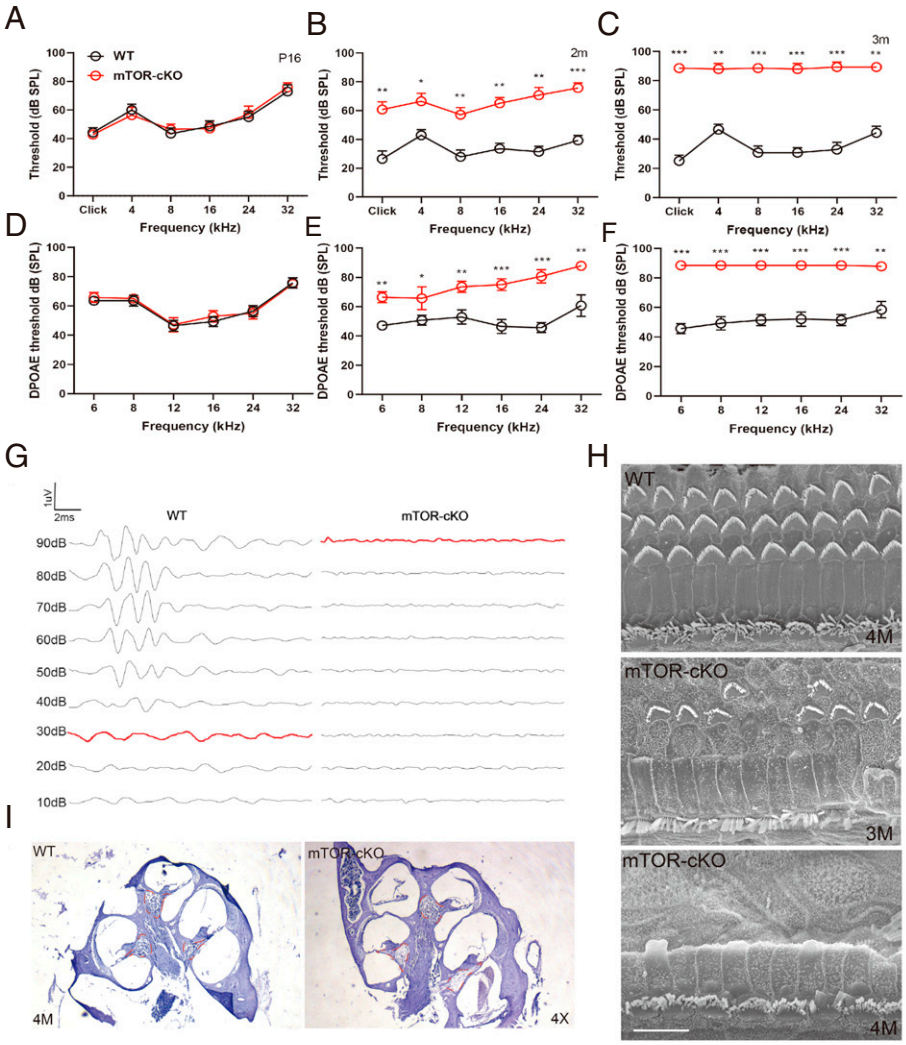

Fig. 3. Deletion of mTOR leads to rapid hair cell loss and progressive hearing loss. Increased age-related threshold shifts in *mTOR-cKO* mice, as measured using either ABR (*A–C*) or DPOAE (*D–F*). Threshold shifts were compared between *mTOR-cKO* and WT mice at similar time points (P16; 2 and 3 mo, respectively); $n = 6$ for each group. (*G*) Representative click ABR traces between 10 and 90 dB in control mice and *mTOR-cKO* mice at 3 mo. ABR thresholds of each genotype are highlighted in red; $n = 6$ for each group. (*H*) Representative SEM images of middle-base turn cochlear sensory epithelia from 4-mo-old control mice and 3- to 4-mo-old mTOR-cKO mice. Fused stereocilia (arrowheads) of IHCs are indicated in mTOR-cKO cochlea. (Scale bars: 20 μm.) (*I*) H&E staining shows severe degeneration of SGN in mTOR-cKO mice but not in WT mice at 4 mo. SGN is outlined in red. Data are presented as the mean ± SD, $*P < 0.5$, $**P < 0.01$, $***P < 0.001$, using a paired *t* test.

OHCs labeled with phalloidin were similar to those of the control mice, suggesting normal development of the HCs (*SI Appendix*, Fig. S5 *A* and *B*). Immunohistochemical analysis revealed that the expression levels and characteristic subcellular distribution of prestin (marker of OHCs), otoferin (marker of IHCs), Sox2 (supporting cell marker), and NF-20 (neuron filament marker) were indistinguishable between the WT and *mTOR-cKO* mice (*SI Appendix*, Fig. S5 *C–F*, *K*, and *L*). Moreover, confocal microscopy with FM1-43 staining showed normal functioning of the functional mechanotransduction of cochlear HCs in *mTOR-cKO* mice at postnatal day 7 (*SI Appendix*, Fig. S5 *G* and *H*). Next, we analyzed the afferent synapses of IHCs and found that GluR2/3 and Ctbp2 immunofluorescent spots were juxtaposed in both WT and *mTOR-cKO* mice, indicating identical counts of ribbon-occupied IHC synapses in both genotypes (*SI Appendix*, Fig. S5 *I* and *J*). Although *Atoh1* plays a role in the development of vestibular HCs, the shape of the stereocilia in vestibular HCs appeared normal in *mTOR-cKO* mice (*SI Appendix*, Fig. S5 *M–O*). Collectively, our findings indicated that decreasing both mTORC1 and mTORC2 is not necessary for the early development of hearing.

mTOR Deficiency Causes Rapid Hair Cell Loss and Progressive Hearing Loss. ABR and DPOAE were conducted over time to determine the auditory functions of *mTOR-cKO* mice in comparison to the WT littermates of the same age. ABR thresholds to tone bursts between 4 and 32 kHz and click stimulation were found to be similar at P16 (Fig. 3*A*). In contrast, at later stages, all ABR frequency thresholds were significantly elevated in *mTOR-cKO* mice (Fig. 3 *B, C,* and *G*), and the DPOAE intensities were substantially reduced in *mTOR-cKO* mice (Fig. 3 *D–F*). Consistent with results of the hearing test, scanning electron microscopy (SEM) findings revealed that the loss of OHCs was first detected in *mTOR-cKO* mice at nearly 4 wk of age, with no apparent change in IHCs during that time. Later, an extensive loss of OHCs occurred, and very few OHCs and only a marginal number of IHCs remained in the organ of Corti at 4 mo of age; however, the structure of HCs in WT mice was normal (Fig. 3*H*). After the degeneration of HCs in *mTOR-cKO* mice, SGN gradually degenerated after 4 mo (Fig. 3*I*). The *mTOR-cKO* mice exhibited no detectable impairment in balance and exhibited normal vestibular morphology throughout their lifespan. Thus, these findings provided convincing evidence that mTOR deletion in cochlear NSE could affect the long-term survival of HCs, leading to early-onset progressive hearing loss.

Dissecting Rictor/mTORC2 Deficiency Accounts for the Hearing Phenotype in mTOR-Deficient Mice. The mTOR, together with Raptor (encoded by *Rptor*) or Rictor (encoded by *Rictor*), acts through two distinct functional complexes, namely, mTORC1 and mTORC2. As mTORC1 deficiency in HCs alleviates ARHL, we hypothesized that mTORC2 inactivation might be the main cause of hearing abnormalities in *mTOR-cKO* mice. Specific pharmacological inhibitors of mTORC2 are not

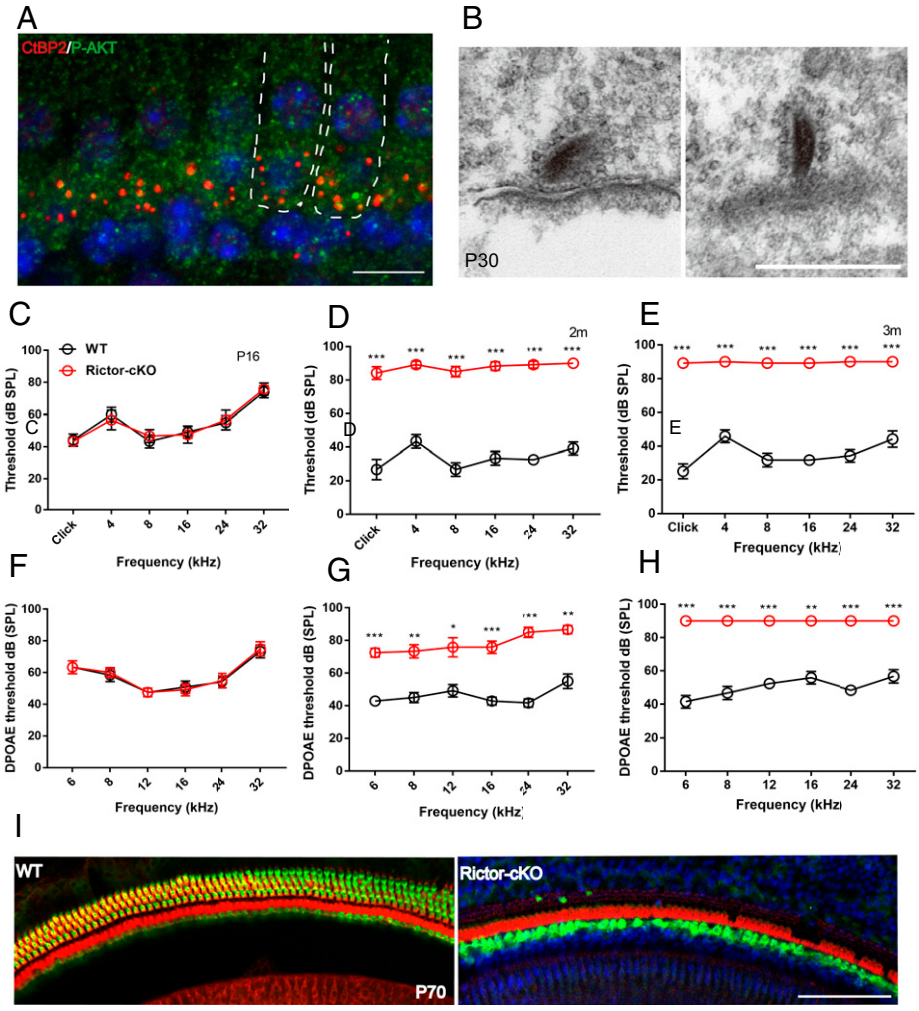

Fig. 4. *Rictor-cKO* mice undergo hearing degeneration with progress in age. (*A*) Immunofluorescence staining showing that P-Akt is not located in the synapses; cell nuclei are stained with DAPI (blue). (Scale bar: 10 μm.) (*B*) TEM images showing normal ribbon synapses (n = 14) in *Rictor-cKO* mice at P30. (Scale bar: 500 μm.) Increased age-related threshold shifts in *Rictor-cKO* mice, as measured using either ABR (*C–E*) or DPOAE (*F–H*). Threshold shifts were compared between *Rictor-cKO* and WT mice at similar time points (P16; 2 and 3 mo, respectively); n = 6 for each group. (*I*) Confocal microscopy images of cochlear whole mounts labeled with an Myo7A antibody (red) and counterstained with phalloidin to label F-actin (green). Representative images from the apical turns of the organ of Corti in WT mice at P70. (Scale bar: 20 μm.) Data are expressed as the mean ± SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, using two-tailed Student's t test.

available (37). To circumvent this problem and test our hypothesis, we generated NSE-specific *Rictor* knockout (*Rictor-cKO*) mice and investigated the selective involvement of mTORC2 and its functional relevance in cochlear pathophysiology compared to the loss of *mTOR*. *Rictor-cKO* mice were born at the expected Mendelian ratio and presented a body size that was comparable to the control littermates. Western blotting of the fluorescence-activated cell sorter (FACS)-isolated cochlear NSE suggested that Rictor protein was reduced in *Rictor-cKO* mice compared to that from the control littermates (*SI Appendix*, Fig. S6*A*). Results from the immunohistochemical analysis showed that Rictor protein was expressed in all segments of the cochlea in WT mice but was largely reduced in the HCs of *Rictor-cKO* mice cochleae (*SI Appendix*, Fig. S6*B*). Concurrently, mTORC2-mediated phosphorylation of Akt at Ser473 was greatly reduced in the NSE of *Rictor-cKO* mice but was normal in other parts of the basilar membrane, as determined using immunostaining (*SI Appendix*, Fig. S6*C*). The loss of Rictor abrogated the phosphorylation of mTOR at the mTORC2-specific residue, Ser2481, but did not affect mTOR levels (*SI Appendix*, Fig. S6*D*). Given that Akt is an upstream activator of mTORC1 (4), we investigated whether a reduction in Akt kinase activity resulting from mTORC2 disruption could affect the activation of mTORC1. As expected, in mTORC2-deficient mice, mTORC1-mediated phosphorylation of S6K1 at Thr389, of S6 at Ser235/236, and of 4EBP1 at T37/46 (three well-established readouts of mTORC1 activity) remained unchanged in NSE, supporting the results from previous findings that Ser473 phosphorylation is not necessary for Akt to disrupt mTORC1. Moreover, phosphorylation of the tuberous sclerosis complex 2 (TSC2), a downstream target of Akt and upstream inhibitor of mTORC1, was not altered (*SI Appendix*, Fig. S6 *D–K*). Thus, the conditional deletion of *Rictor* selectively reduced mTORC2 without affecting mTORC1 activity in the NSE of the cochlea.

Similar to that in *mTOR-cKO* mice, the auditory function developed normally in *Rictor-cKO* mice. No significant differences in hearing were found between *Rictor-cKO* mice and the age-matched WT mice at 2 wk of age when the hearing thresholds can be tested (*SI Appendix*, Fig. S7*A*). Additionally, no obvious changes in gross tissue morphology were detected in either the cochlea or the vestibule, using histological analysis. Similar to that in *mTOR-cKO* mice, the initial patterning of the cochlear NSE was found to be established normally in *Rictor-cKO* mice, based on the normal pattern and function of HCs and supporting cells determined using immunolabeling studies with Parvalbumin and Sox2, respectively (*SI Appendix*, Fig. S7*B*). Experiments using cultured mammalian cells and yeast reveal that mTORC2 plays an important role in regulating the actin cytoskeleton and that actin polymerization is reduced in the hippocampus of mTORC2-deficient mice (38). Based on these reports, we examined the stereocilia and the cuticular plates using phalloidin and spectrin, respectively. However, spectrin expression and actin content were not significantly different between the *Rictor-cKO* and WT mice (*SI Appendix*, Fig. S7*C*). Remarkably, the expression pattern of pS473-AKT

showed the form of point aggregation (*SI Appendix*, Fig. S6*C*), which led us to hypothesize that some pS473-AKT puncta were located in the ribbon synapse. However, immunofluorescence staining of pS473-AKT with Ctbp2/Ribeye revealed that pS473-AKT was not located in the ribbon synapse, indicating that mTORC2 did not directly affect the function of the ribbon synapse (Fig. 4*A*). Using transmission electron microscopy (TEM), no differences were found between the WT and *Rictor-cKO* mice in terms of the morphology of the ribbon synapse at P30 (Fig. 4*B*). Next, we assessed the hearing of *Rictor-cKO* mice by recording the ABR over time. The ABR thresholds to click stimulation were moderately elevated in *Rictor-cKO* mice directly after the development of hearing and progressively deteriorated with advancing age (Fig. 4 *C–E*). In addition, the DPOAEs were greatly reduced in *Rictor-cKO* mice across all frequencies (Fig. 4 *F–H*). When labeling the organ of Corti of WT and *Rictor-cKO* mice with Myo7a and Phalloidin, we first observed the loss of HCs at 3 wk of age in *Rictor-cKO* mice (*SI Appendix*, Fig. S7*D*) and this pattern of degeneration was similar in *mTOR-cKO* mice. However, contrary to that observed in *mTOR-cKO* mice, *Rictor-cKO* mice exhibited more serious and earlier hair cell loss compared to *mTOR-cKO* mice. By ∼P70, almost no outer HCs survived (Fig. 4*I*), accompanied by the collapse of the organ of Corti and subsequent loss of SGNs (*SI Appendix*, Fig. S7*E*). Since mTOR function is mediated by mTORC1 or mTORC2, it is reasonable to assume that mTORC1 deficiency alleviates ARHL and might, thus, compromise the hearing phenotype in *Rictor-cKO* mice. To test our hypothesis of whether HCs underwent apoptosis in *Rictor-cKO* mice, we conducted a terminal deoxynucleotidyl transferase dUTP nick-end labeling analysis of the hearing organ in P30 *Rictor-cKO* mice. Immunofluorescence analysis revealed apoptosis in some HCs (*SI Appendix*, Fig. S8*A*). Activation of apoptosis was confirmed based on the observation that chromatin condensation occurred in the nucleus of the HCs of *Rictor-cKO* mice (*SI Appendix*, Fig. S8*B*), which was significantly higher than that in the WT controls. Additionally, qPCR results indicated a significant increase in the expression of several proapoptotic genes, including Bax, Caspase 3, Caspase 8, and Caspase 9, and a significant decrease in the expression of the antiapoptotic gene, Bcl2 (*SI Appendix*, Fig. S8*C*). Moreover, we found that the transtympanic injection of Z-VAD-FMK, an apoptosis inhibitor, delayed the death of HCs in *Rictor-cKO* mice to a certain extent (*SI Appendix*, Fig. S8*D*). To further validate that mTORC2 deficiency was the primary cause of hair cell death and hearing loss in *mTOR-cKO* mice, we generated compound *mTOR-Rictor-DKO* mice (genetic deletion of Rictor in *mTOR-cKO* mice). The *mTOR-Rictor-DKO* mice were viable and developed normally. Of note is that the hearing phenotypes of compound-deleted mice largely resembled *Rictor-cKO* mice (*SI Appendix*, Fig. S9*A*). SEM results suggested that *mTOR-Rictor-DKO* mice displayed cochlear hair cell loss in a pattern that was not significantly different compared to that of the littermate *Rictor-cKO* mice (*SI Appendix*, Fig. S9*C*). Histological analysis of the cochleae of *mTOR-Rictor-DKO* mice revealed defects that resembled those in *Rictor-cKO* mice. As *Cre*-mediated recombination also occurred in cerebellar granule cells, we assessed the morphology of the cerebellum in both genetically modified mice. Compared to that of the WT control mice, the already small cerebellum in *Rictor-cKO* mice was further decreased in size compared to that of the *mTOR-Rictor-DKO* mice, which indicated a stark difference in the phenotype of the auditory system (*SI Appendix*, Fig. S9*B*). Thus, hearing impairment by *mTOR* was mainly attributed to Rictor/mTORC2 deficiency. To further test this possibility, we examined the effect of a high sirolimus dose (10 mg/kg) on auditory function in 4-wk-old mTORC2-deficient (*Rictor-cKO*) mice. As expected, results of ABR indicated no significant differences between the *Rictor-cKO* control mice and sirolimus-injected *Rictor-cKO* mice (*SI Appendix*, Fig. S9*D*). Collectively, these data strongly suggested that Rictor/mTORC2 deficiency was the main cause of hearing impairment in *mTOR-cKO* mice. Thus, we demonstrated that the benefits of lower mTORC1 signaling in *mTOR-cKO* mice were diminished, which is associated with a reduction in Rictor/mTORC2 signaling and hearing loss.

Rictor/mTORC2 Promoted Hair Cell Survival and Function Mainly through Phosphorylating AKT/PKB. Next, we attempted to delineate the mechanism through which Rictor/mTORC2 signaling could regulate hair cell survival. The mTORC2 plays an important role in the survival and development of many cell types, such as fat cells, pancreatic islets, T cells, skeletal muscle cells, hepatocytes, and myocardial cells, via the phosphorylation of several AGC family proteins, including Akt, PKC-α, and SGK1 (9, 11) (Fig. 5*A*). Thus, we were interested in determining the pathway downstream of mTORC2 that was affected. Accordingly, we determined the expression of the mTORC2 downstream target (phosphorylation of activation of Akt downstream targets, Foxo1 and GSK3β; phosphorylation of PKC-α and SGK1 downstream targets, and phosphorylation of NDRG1 at T346) at P5. Western blotting of FACS-sorted NSE showed that the levels of phosphorylation of Foxo1 at S256, GSK3β at S9, and PKC-α at S657 decreased obviously compared to that of the controls, whereas phosphorylation of NDRG1 at T346 was not altered (Fig. 5 *B–F*), suggesting that mTORC2 promoted hair cell survival independent of SGK1-NDRG1.

Next, we attempted to determine which downstream pathway of mTORC2 was responsible for the survival of HCs. Previously, our group, as well as some others, reported that the conditional deletion of *Pten* using the same *Cre* line did not lead to any hearing phenotype (29, 39); in addition, we have demonstrated, in a published study, that p-Akt (S473) levels in the cochlear NSE of *Pten-cKO* mice are higher than those in WT mice (29), suggesting that the overactivation of mTORC2 is not detrimental to cochlear HCs. However, it is noteworthy that, in the HCs of mice lacking *Pten,* mTORC1 is also up-regulated, although not promoted as effectively as observed in *Tsc1-cKO* mice. We have previously shown that mTORC1 overactivation in cochlear HCs (*Tsc1-cKO*) causes accelerated hearing loss. To further determine the effect of increased mTORC1 activity in *Pten-cKO* mice, we used conditional alleles to delete *Pten* in combination with *Tsc1* (*Tsc1-Pten-DKO*). Our results indicated that the *Tsc1-Pten-DKO* mice showed no significant changes in click ABR thresholds compared to that in the WT mice when assessed in the first 2 mo (*SI Appendix*, Fig. S10*A*), after which the *Tsc1-Pten-DKO* mice died prematurely in our study. In any case, these data provide strong evidence that mTORC2 and not mTORC1 plays a dominant role in the mTOR complex in cochlear HCs. We further observed that phosphorylation of PKC-α, and phosphorylation of the activation of the SGK1 downstream target, NDRG1, were unaltered in *Pten-cKO* mice compared to that in the WT mice (*SI Appendix*, Fig. S10 *B–D*). Subsequently, we genetically deleted *Pten* in combination with *Rictor* (*Rictor-Pten-DKO* mice) and found that the p-Akt (S473) level was increased compared with the *Rictor-cKO* mice, implying that the presence of an alternative kinase could increase the p-Akt (S473) levels in the cochlear HCs of *Rictor-Pten-DKO* mice. Then we checked whether the increase in p-Akt (S473) levels could restore the hearing ability in mTORC2-deficient mice. A comparison of the activation of mTORC2 downstream targets revealed only a selective increase of p-Akt (S473) in the NSE of *Rictor-Pten- DKO* mice compared to that in *Rictor-cKO* mice (Fig. 5 *G* and *H*). *Rictor-Pten-DKO* mice lived normally and showed no obvious gross morphological differences compared to *Rictor-cKO* mice. As expected, the *Rictor-cKO* mice developed both hearing defects and hair cell loss, whereas the

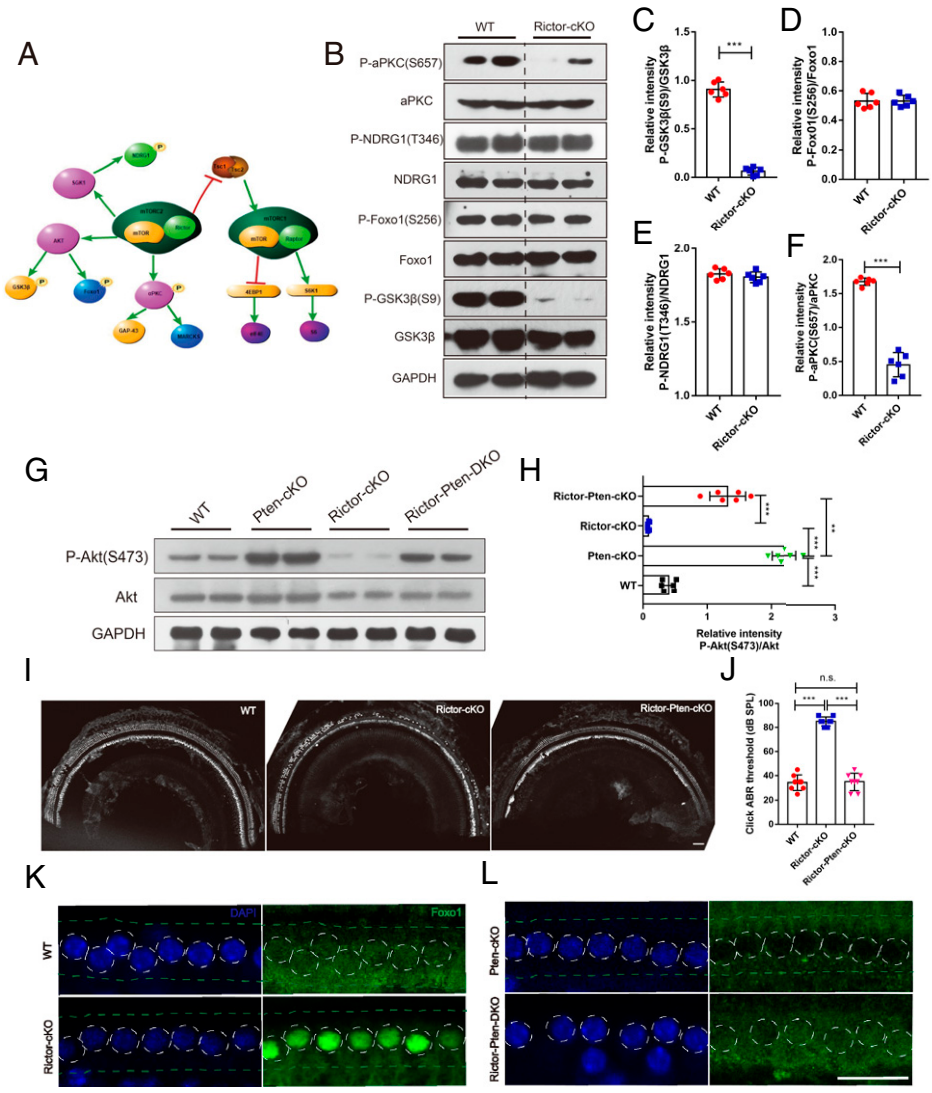

Fig. 5. The mTORC2 functions mainly through phosphorylating AKT/PKB. (*A*) Schematic representation of the signaling mechanisms upstream and downstream of mTORC1 and mTORC2. (*B*) Western blots of phosphorylation of mTORC2 targets aPKC, Akt, and SGK1 and the downstream targets. P-GSK3β, P-Foxo1, P-NDRG1, and P-PKC levels are quantified and shown in *C–F*, respectively. Representative Western blots (*G*) and quantifications (*H*) showing that, compared to that in WT mice, P-Akt (S473) is up-regulated in *Pten-cKO* mice. (*I*) Confocal microscopy images representing the loss of a large number of HCs in *Rictor-cKO* mice; however, this difference is not significant between WT and *Rictor-Pten-cKO* mice; (*J*) quantifications; $n = 8$. (Scale bar: 10 μm.) (*K*) Foxo1 immunolabeling of control HCs reveals that it is located in both the nucleus and cytoplasm. (Scale bar: 10 μm). In *Rictor-cKO* mice, Foxo1 is shifted almost entirely to the nuclei of HCs. (*L*) Foxo1 immunolabeling in *Pten-cKO* cochlear HCs showing a lack of nuclear Foxo1 accumulation and its concentration in the cytoplasm. In *Rictor-Pten-DKO* mice, the normal location of Foxo1 in HCs is restored to a pattern similar to that seen in WT mice. (Scale bar: 10 μm). Data are expressed as the mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$, using two-tailed Student's *t* test.

hearing ability and hair cell pattern observed in *Rictor-Pten-DKO* mice were similar to those of the WT mice aged 2 mo or younger (Fig. 5 *I* and *J*). The other evidence that supports mTORC2 functioning through AKT/PKB in HCs is the cellular distribution of Foxo1. As expected, on phosphorylation by AKT, Foxo1 was faintly detected in both the nucleus and cytoplasm of the HCs in WT mice (Fig. 5*K*). In *Pten-cKO* cochlear HCs, Foxo1 was not present in the nucleus but was concentrated in the cytoplasm (Fig. 5*L*). However, in *Rictor-cKO* mice, Foxo1 was shifted mostly entirely to the nuclei of HCs (Fig. 5*K*). In *Rictor-Pten-DKO* mice, the normal location of Foxo1 in HCs was restored to a pattern similar to that of the HCs in WT mice (Fig. 5*L*). Thus, the absence of Akt phosphorylation in *Rictor-cKO* HCs reduced Foxo1 phosphorylation and promoted the accumulation of Foxo1 in the nucleus. We evaluated the effects of Go6976, a selective inhibitor of PKC-α, in the hearing of WT mice by administering an i.p. injection to 2-mo-old C57BL/6J mice every other day at a dose of 2.5 mg/kg, and found that it effectively blocked the expression of PKC-α (*SI Appendix*, Fig. S10 *E* and *F*). However, the hearing level of Go6976-treated mice was comparable to that of WT mice (*SI Appendix*, Fig. S10*G*). Taken together, these data persuasively demonstrated that mTORC2 mainly exerted its effects via AKT/PKB in cochlear NSE.

mTORC2-Akt Is Involved in the Regulation of Hair Cell Survival in the Cisplatin Condition. Based on the findings that mTORC2 signaling was beneficial for hair cell survival, we aimed to further determine whether mTORC2 signaling was involved in cisplatin-induced toxicity. Cisplatin is a widely used chemotherapeutic agent; however, one of its significant side effects is irreversible sensorineural hearing loss, which occurs in 50 to 70% of patients receiving cisplatin treatment (40). Besides ototoxicity, nephrotoxicity is another adverse effect of cisplatin. Recently, mTORC2/Rictor signaling was shown to protect from cisplatin-induced nephrotoxicity (41). However, it remains unclear whether its mechanism of action is similar to that determined in the cochlea. Accordingly, we first investigated whether Akt activation was involved in the development of cisplatin-induced ototoxicity. We administered an i.p. injection of 40 mg/kg cisplatin to 2-mo-old WT mice for three consecutive days and measured the ABR in the control group and mice injected with cisplatin. The mean ABR threshold shift at all frequencies (4-, 8-, 16-, 24-, and 32-kHz tone burst and click) in mice administered cisplatin was significantly higher than that of mice in the control group (Fig. 6*A*). These results confirmed the damage caused by cisplatin on the hearing of mice. Notably, Western blot analysis of the whole cochleae revealed that neither Akt nor its downstream target, GSK3**β**, was phosphorylated or altered in the whole cochleae

after cisplatin injection (*SI Appendix*, Fig. S11 *A–C*). However, using formalin-fixed sensory epithelium tissues, we found that cisplatin was associated with higher levels of phosphorylation of Akt at S473 and phosphorylation of GSK3β at S9 (Fig. 6 *B–D*). Results of whole-mount immunofluorescence staining revealed that cisplatin considerably increased Akt phosphorylation (S473) in HCs, whereas no significant difference in activated Akt was observed in the remaining components of the basilar membrane (Fig. 6 *E* and *F*). In contrast, there were similarities in the activity of mTORC1 in HCs between the two groups, as indicated by the phosphorylation of S6 (*SI Appendix*, Fig. S11*D*). In our study, we could not detect the changes in activated Akt in the SGN after the administration of cisplatin (Fig. 6*E*). The increased activity of mTORC2 and unchanged activity of mTORC1 in the HCs of the cisplatin-treated group led us to infer that activated mTORC2 may resist the ototoxicity caused by cisplatin. To test our hypothesis, we used our experimental models of 2-wk-old WT, *Rictor-cKO* (mTORC2-deficient), *Rictor-Pten-DKO* (mTORC2-restored), and *Pten-cKO* (mTORC2-overactivated) mice to evaluate the effects of cisplatin treatment on the hearing levels among the four mice lines. No significant difference in hair cell pattern was found in these four mice lines before cisplatin treatment, and no hair cell loss was observed in any group (*SI Appendix*, Fig. S11*E*). After three consecutive days of injection, WT mice exhibited some extent of hair cell loss. Compared to that in the control groups, cisplatin injection resulted in a lower percentage of hair cell loss in *Pten-cKO* (mTORC2-overactivated) mice; in sharp contrast, a larger extent of hair cell loss was observed in *Rictor-cKO* mice (Fig. 6 *G* and *H*). Moreover, in *Rictor-cKO* mice, cisplatin administration significantly increased the ABR thresholds. Interestingly, *Pten-cKO* mice were more resistant to cisplatin intoxication. The average click ABR thresholds were determined to be 66.8 dB SPL and 48.7 dB SPL in *Rictor-cKO* and *Pten-cKO* mice, respectively (Fig. 6*I*). Collectively, these data suggested that mTORC2 was the crucial pathway that was activated in cochlear HCs to counteract the stress imposed by cisplatin exposure, and the defective activation of this pathway increased cisplatin ototoxicity, further confirming mTORC2 as the prosurvival signaling pathway in the cochlea.

Selective Activation of mTORC2 Enhanced Hair Cell Survival during Acoustic Trauma. We hypothesized that mTORC2 signaling was also implicated in noise-induced hearing loss (NIHL), because both cisplatin-induced hearing loss and NIHL likely involved similar mechanisms (40). To test our hypothesis, we developed a noise injury protocol using WT, mTORC2-deficient, and mTORC2-overactivated mice. As the complete deletion of mTORC2 in cochlear HCs resulted in premature hearing loss, we generated mice lacking only the *Rictor* allele in NSE (*Rictor-Het* mice). Western blot analysis from FACS-sorted NSE confirmed a reduction in the expression of Rictor protein and P-Akt (S473) (*SI Appendix*, Fig. S12 *A* and *B*), suggesting that *Rictor* is a haploinsufficient gene, and deleting one copy could reduce mTORC2 activity. Moreover, most of the *Pten-cKO* mice suddenly succumbed to epileptic symptoms caused by unexplained central nervous system effects upon exposure to noise. We used a low-molecular-weight compound, A-443654, which selectively increases P-Akt (S473) and found that an i.p. injection selectively promotes mTORC2 activity in the cochleae of WT mice (*SI Appendix*, Fig. S12 *C* and *D*). ABR thresholds in *Rictor-Het* mice and A-443654–injected mice were comparable to the WT controls in each test (*SI Appendix*, Fig. S12*E*). Exposure of mice at P30 to 105 dB SPL of broadband noise for 4 h resulted in elevations in threshold across all three groups that were comparable when evaluated 2 h later based on ABRs (*SI Appendix*, Fig. S12 *F* and *H*). ABR threshold results showed better hearing in the A-443654–injected group compared with that in the WT control mice, demonstrating almost complete recovery after acoustic stimuli. In contrast, *Rictor-Het* mice showed a significantly higher threshold compared with that of the other groups 2 wk after exposure (*SI Appendix*, Fig. S12*G*). Similarly, 2 wk after acoustic trauma, wave I amplitudes of ABR were higher in the A-443654–injected group than in the other groups (*SI Appendix*, Fig. S12*I*). However, the synaptic puncta densities of the IHCs were comparable among the three groups (*SI Appendix*, Fig. S12*G*). These results suggested that mTORC2 was involved in the regulation of hair cell survival during acoustic trauma, and that the overactivation of mTORC2 could protect against NIHL.

Deleting P53 Activity Rescued the Hearing Phenotype in *Rictor* Knockout Mice. Cisplatin increases the expression of P53, and the genetic or pharmacological ablation of P53 substantially attenuates cochlear hair cell loss resulting from cisplatin ototoxicity, thus preserving hearing (42). We subsequently determined P53 levels to test whether this was the case in the cisplatin-treated experimental conditions in our study. Concordantly, we found marked hyperactivity of P53 and the downstream effector, P21, as reflected by their accumulation and phosphorylation (*SI Appendix*, Fig. S13 *A* and *B*). The coenhanced activity of P53 and p-Akt (S473) after cisplatin injection was suggestive of the important role of P53 in the regulation of hair cell survival by mTORC2. In addition, enhanced P53 activity was also found in the NSE of *Rictor-cKO* compared with the WT controls (*SI Appendix*, Fig. S13 *C* and *D*). We hypothesized that hair cell loss caused by mTORC2 deficiency could be prevented by the inhibition of P53. To test this hypothesis, we first used PFT-a, a water-soluble small molecule, which is a powerful inhibitor of p53. Since our data clearly indicated that mTORC2 was the primary cause of hair cell death in organotypically cultured cochlea explants treated with Torin1, we attempted to determine whether PFT-a could protect HCs against Torin1 damage from P3-cultured explants. Cochlear explants treated with PFT-a alone did not exhibit any damage to hair cell layers, suggesting PFT-a to be nontoxic. Cultured cochlea explants treated with Torin1 exhibited disarray and a loss of sensory HCs at all turns. In contrast, pretreatment with PFT-a protected HCs from Torin1-mediated damage (*SI Appendix*, Fig. S13*E*). Next, we sought to determine whether the genetic lowering of P53 activity could prevent hearing loss in *Rictor-cKO* mice. We performed a genetic deletion of P53 in *Rictor-cKO* mice and established *Rictor-cKO*; $P53^{-/-}$ mice. Interestingly, *Rictor-cKO* mice showed nearly complete deafness threshold at 5 mo of age as evaluated using the mean ABR (*SI Appendix*, Fig. S13*F*), while the mean ABR threshold shifts at all frequencies in *Rictor-cKO*; $P53^{-/-}$ mice were significantly smaller than those in *Rictor-cKO* mice. In agreement with the ABR results, the *Rictor-cKO*; $P53^{-/-}$ mice exhibited more viable HCs than did the 5-mo-old *Rictor-cKO* mice (*SI Appendix*, Fig. S13*G*). However, both the rate of loss of HCs and the mean ABR threshold shifts in *Rictor-cKO*; $P53^{-/-}$ mice are still significantly higher than those in WT control mice, suggesting that the deficiency of P53 was not sufficient to completely rescue hearing defects in *Rictor-cKO* mice (*SI Appendix*, Fig. S13 *F* and *G*). These results indicated that, besides the P53 pathway, other pathways downstream of Akt also potentially contribute to hearing defects in *Rictor-cKO* mice. Together, these observations suggested that mTORC2 signaling in HCs functions partially through the P53 pathway and that deleting P53 activity attenuated the severity of the hearing phenotype in mTORC2-deficient mice.

Discussion

Sirolimus is clinically used to treat several disorders (43–46). To date, only sirolimus appears to delay the intrinsic aging in mammals, thus significantly prolonging their life span (47).

However, this drug is associated with certain side effects; for instance, sirolimus can induce impaired wound healing, edema, elevated circulating triglycerides, impaired glucose homeostasis, gastrointestinal discomfort, and mouth ulcers in patients with impaired renal function, who have undergone renal transplant (43). A study has provided the first physiopathological evidence for an uncoupled effect of sirolimus on mTOR complexes in vivo, with a predominant role of mTORC1 in extending life span and of mTORC2 in improving insulin resistance. In our previous study, we showed that sirolimus was effective in treating ARHL. Yet, in this study, instead of preventing ARHL, a high concentration of sirolimus was found to induce rapid deafness in mice. A possible reason for this occurrence could be that a high dose of sirolimus rapidly decreased the already low mTORC1 signaling in mice, which could have adversely affected the benefits of mTORC1 inhibition. However, it should be noted that mTORC1 signaling was not further reduced in mice administered high doses of sirolimus compared to those administered low doses of this drug. We cannot explain why high doses of sirolimus did not further decrease mTORC1 signaling in mice. In contrast, a significant reduction in mTORC2 was found in mice administered high doses of sirolimus. Moreover, we showed that the inhibition of mTORC2 mediated the deleterious effects of high doses of sirolimus. Two important pieces of evidence suggested that the down-regulation of mTORC2 was the cause of deafness in mice that were injected with a high dose of sirolimus. The first observation was a rapid decline of hearing in *Raptor* knockout mice that were injected with high levels of sirolimus. The second finding was that *Rictor* knockout mice did not exhibit further deterioration in hearing after being administered a high concentration of sirolimus. Because acute treatment with sirolimus does not affect mTORC2 signaling and FKBP12-sirolimus was not found to bind to the intact mTORC2, this complex was originally thought to be sirolimus insensitive (3, 8). However, it now appears that long-term treatment with sirolimus reduces mTORC2 signaling in some, but not all, cell types by suppressing mTORC2 assembly (12, 13). However, in the cochlear system, higher concentrations of sirolimus, but not prolonged sirolimus treatment, suppress mTORC2 activity. This is not unique to the cochlea. Ping Jun Zhu et al. (34) found that only high concentrations of sirolimus are required for mGluR-LTD in hippocampal slices. The reason for tissue specificity to sirolimus sensitivity during mTORC2 assembly is still unclear. Overall, our results suggested that a high concentration of sirolimus leads to hearing loss in mice; thus, the take-home message is to avoid sirolimus overdose in humans. Our findings unveil a molecular pathway revealing the adaptation of HCs to stress and show that mTORC2 acts as a survival factor and that its activation may prevent hair cell from being damaged. Moreover, our study findings indicated that sirolimus may not be suitable as an ideal pharmacological drug for treating ARHL, as high doses of sirolimus decreased mTORC2. Therefore, to prevent ARHL, it is necessary to develop specific and efficient inhibitors of mTORC1 that do not inhibit mTORC2.

In this study, we focused on highlighting the complexity of mTOR signaling in cochlear HCs. Based on the combined pharmacological and molecular approaches, the key findings of this study are as follows: 1) Sirolimus protected mice against cocaine-induced hearing loss; 2) a low dose of sirolimus inhibited only mTORC1, whereas mTORC1 and mTORC2 were concurrently inhibited when C57BL/6J mice were injected with high doses of sirolimus; 3) genetic mTOR deficiency resulted in rapid hair cell loss and progressive hearing loss; 4) mTORC2 deficiency, but not mTORC1, is the major driver underlying the hearing phenotype of *mTOR-CKO mice*; 5) mTORC2 sustained hair cell survival via PKB/Akt signaling, and lowering P53 activity substantially attenuated the severity of the hearing phenotype in *mTOR-CKO* mice; and 6) mTORC2 signaling was involved in the regulation of hair cell survival upon exposure to cisplatin and noise, and the overactivation of mTORC2 could protect from ototoxicity and NIHL. These findings collectively suggested that mTORC1 and mTORC2 signaling have distinct functions in cochlear HCs, indicating a dichotomy of mTOR complex function in the pathophysiology of cochlear HCs. Systemic mTORC2 deletion results in embryonic death, whereas tissue-specific mTORC2 deletion (such as that in skeletal muscle, adipose tissue, and kidney) usually leads to a weak phenotype (48–51). The weak phenotypes caused by mTORC2 deletion are in sharp contrast to the severe phenotypes caused by mTORC1 deletion in the same tissues (51, 52). Therefore, the deletion of mTOR largely resembles the phenotype of mice lacking raptor in some tissues, such as skeletal muscle and skin (53, 54). Thus, these results indicated that there are significant differences in the roles of mTORC1 and mTORC2 between tissues, and most of the known functions of mTOR in multiple tissues are mediated by mTORC1. Only recently, a study has highlighted the role of mTORC2 in regulating cardiac responses (55), M2 macrophage differentiation in response to helminth infections, adaptive thermogenesis (56), and T follicular helper cell differentiation (57). In the current study, we report the phenotype of mouse models where *Rictor* was conditionally and selectively deleted in cochlear HCs and, thus, provide strong evidence that mTORC2 deficiency results in severe hair cell damage.

Although many studies have attempted to elucidate the molecular mechanisms that lead to hair cell loss during cisplatin chemotherapy (42, 58), the signaling pathways that allow HCs to survive after the ototoxicity of cisplatin are still unknown. Surprisingly, little is known about the specific role of mTORC2 in cell survival. Moreover, to the best of our knowledge, evidence concerning the direct role of mTORC2 in regulating survival after cisplatin damage in cochlea has not been reported. Our study points to mTORC2 as a major determinant that prevents hair cell damage and death after cisplatin exposure. In fact, we found that mTORC2 was activated in HCs in response to cisplatin-induced stress, which is consistent with previous studies of the activation of Akt in kidney podocytes and tubular cells subjected to cisplatin stress (41). We also demonstrated that the preclusion of such activation by *Rictor* disruption led to severe hair cell damage in mice, and that overactivated P-Akt (S473) by *Pten* disruption could prevent cisplatin-induced ototoxicity. It is worth noting that P-Akt (S473) only increased in the cochlear OHCs but not in the supporting cells and SGNs or StV after cisplatin treatment. It is still unclear why mTORC2 increased specifically in cochlear HCs. In the cochlea, which is the sensory organ of hearing, cisplatin-induced ototoxicity is thought to be initiated after its uptake into the sensory HCs. Therefore, an increase in mTORC2 specifically in cochlear HCs could likely be attributed to the fact that HCs may be the first to respond to cisplatin treatment. Using Western blotting of the cochlear sensory epithelia, we found that total p-Akt (S473) levels in aged mice were enhanced possibly because HCs may have dominated the populations of cells responding to p-Akt (S473). However, further studies are required to elucidate how mTORC2 signaling is regulated in different parts of the cochleae in cisplatin-treated mice. Collectively, these results suggested that mTORC2 activation could protect the cochlea from cisplatin-induced ototoxicity. Targeting the mTORC2 signaling pathway can reduce cochlear hair cell death and salvage hearing during cisplatin treatment. Moreover, considering ototoxicity and NIHL share a similar mechanism, we also found that mTORC2 activation could prevent NIHL. A-443654 was used for activating mTORC2 in our study; however, it is also worth noting that Okuzumi et al. (59) did not find direct evidence that A-443654

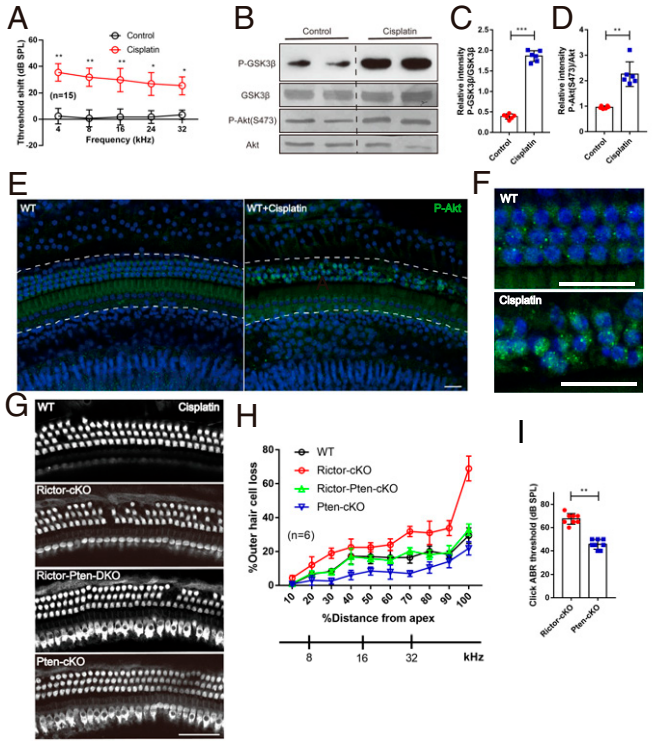

Fig. 6. Activation of mTORC2-Akt prevents hair cell loss after cisplatin exposure. (*A*) ABR-hearing thresholds are increased in cisplatin-treated mice compared to that in WT control mice; n =15. (*B*) Western blots of cochleae show increased P-GSK3β and P-Akt (S473) in cisplatin-treated mice; n = 3. Relative P-GSK3β and P-Akt (S473) levels are quantified and shown in *C* and *D*. (*E* and *F*) P-Akt (S473) immunolabeling (red) is stronger in outer HCs in the organ of Corti in cisplatin-treated mice than in WT control mice; however, no significant changes are seen in the SGN. (*G*) Representative images from the middle turns of the organ of Corti in mice after cisplatin administration at P14. Compared to that in the *Rictor-cKO* mice, cisplatin injection results in a lesser percent of lost HCs in *Pten-cKO* (mTORC2-overactivation) mice. (Scale bar: 20 μm). (*H*) Cochleograms were recorded in mice at P14. Graphs show the percentage loss of OHCs as a function of the percentage distance from the apex. (*I*) ABR-hearing threshold results in *Rictor-cKO* and *Pten-cKO* mice. Data are presented as the mean ± SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, using two-tailed Student's t test.

activated the mTORC2 signaling pathway in HEK 293 cells. Therefore, the specific mechanism of A-443654 activating the mTORC2 pathway in cochlear HCs deserves further investigation. There is a possibility that the environment in vivo is different from that of cultured cells in vitro, which led to the different effects of A-443654 in mouse models and the cultured cells. Anyway, based on our past and current findings, we propose the following concept: Appropriately timed inhibition of mTORC1 in conjunction with mTORC2 potentiation could be an effective approach in ameliorating the progression of degenerative changes of cochlear HCs caused by cisplatin ototoxicity and noise exposure.

The tumor suppressor protein p53 is a key regulator of several processes, including cell proliferation, senescence, and death (60). In our study and as shown previously, the p53 inhibitor, PFT-α, significantly protected HCs from cisplatin damage. Given the efficacy of PFT-α in preventing hair cell loss by aminoglycoside drugs (42, 60), we concluded that inhibiting p53 may be beneficial in preventing ototoxic drug-induced hair cell damage. Several studies have suggested a relationship between mTOR and the p53 pathway (61). Overexpression of P53 and decreased mTOR function have been confirmed in many genetic diseases in humans, such as Treacher Collins syndrome and Roberts syndrome. These diseases are characterized by facial dysplasia due to defective cell proliferation. Considering that mTORC2 potentiation could prevent cisplatin-induced hair cell loss, we attempted to examine the correlation between mTORC2 and P53 in cochlear HCs. We found that a decrease in P53 significantly prevented hair cell death caused by mTORC2 deficiency. TSC1/TSC2 is a negative regulator of mTOR. It has now well known that p53 inhibits the mTOR pathway by regulating TSC1/TSC2; however, the mechanism of mTOR feedback to p53 is still unclear. Results from several studies indicate that the mTOR and p53 pathways may converge during ribosomal biogenesis, wherein mTOR plays a key role, and its stress can lead to the activation of p53 (61). However, whether a similar mechanistic pattern applies to cochlear HCs remains to be determined. It is worth noting that lowering P53 activity failed to fully rescue the hearing defects in mTORC2 knockout mice. These results together suggested that the additional mTORC2 substrate might also play a role in the observed phenotype.

In summary, our findings indicate that mTORC2 plays an important role in maintenance the function of cochlear HCs. This finding will be helpful in the development of drugs that target the mTOR pathway, especially in the management of ototoxicity. Moreover, our findings support the concept that mTORC1 and mTORC2 signaling have distinctive functions in the control of ARHL. Therefore, combined with our previous findings and the results from the current study, the therapeutic activation of mTORC2 in conjunction with decreasing mTORC1 might represent a promising and effective strategy for the maintenance of hair cell function, to prevent cisplatin-induced and noise-induced hair cell loss, and to retard ARHL.

Materials and Methods

Animal models, in vivo auditory tests, SGN counting, immunolocalization studies, SEM and TEM studies, extraction of proteins from formalin-fixed sensory epithelia and Western blot, histological analysis, transtympanic administration of Torin1, cochlear explant cultures, isolation and collection of HCs, single-cell isolation and complementary DNA synthesis, quantitative real-time PCR, and statistical analysis are described in *SI Appendix, SI Materials and Methods*.

Data Availability. All study data related to this work are available in the main text and/or *SI Appendix*.

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