Conditional Deletion of Pten Leads to Defects in Nerve Innervation and Neuronal Survival in Inner Ear Development

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Abstract

All cellular phenomena and developmental events, including inner ear development, are modulated through harmonized signaling networks. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor, is a major signaling component involved in cross talk with key regulators of development; i.e., Wnt, Notch, and bone morphogenetic proteins. Although Pten function has been studied in various systems, its role in inner ear development is poorly understood. Here, we used inner ear-specific Pten conditional knockout mice and examined the characteristics of the inner ear. In a detailed analysis of the phenotype, reduced cochlear turning and widened epithelia were observed. Phalloidin staining of sensory epithelium revealed that hair cell patterns were disturbed; i.e., additional rows of hair cells were discovered. The neural abnormality revealed a reduction in and disorganization of nerve fibers, including apoptosis at the neural precursor stage. Pten deficiency induced increased phosphorylation of Akt at Ser473. The elevation of inhibitory glycogen synthase kinase 3β (GSK3β) was sustained until the neuronal differentiation stage at embryonic day 14.5, instead of pGSK3β downregulation. This is the first report on the influence of Pten/Akt/GSK3β signaling on the development of spiral ganglia. These results suggest that Pten is required for the maintenance of neuroblast number, neural precursors, and differentiation in the inner ear.

Introduction

Early in auditory system development, the otic placode undergoes invagination to form otocysts, which generate a complex membranous labyrinth and ganglia through a series of morphological events via harmonized coordination of various signaling molecules such as fibroblast growth factors, Notch, bone morphogenetic proteins, and Wnt [1, 2, 3, 4, 5]. To form the cochleovestibular ganglion (CVG) complex, neuroblasts delaminate from otic epithelium into the adjacent mesoderm between embryonic day E9.5 and E11.5 [6, 7]. After delamination, the CVG complexes are separated into the spiral and the vestibular ganglia through a series of morphogenetic proteins, and Wnt [1, 2, 3, 4, 5]. To form the cochleovestibular ganglion (CVG) complex, neuroblasts delaminate from otic epithelium into the adjacent mesoderm between embryonic day E9.5 and E11.5 [6, 7]. After delamination, the CVG complexes are separated into the spiral and the vestibular ganglia. Spiral ganglia undergo maturation processes around embryonic day 14.5, instead of pGSK3β downregulation. This is the first report on the influence of Pten/Akt/GSK3β signaling on the development of spiral ganglia. These results suggest that Pten is required for the maintenance of neuroblast number, neural precursors, and differentiation in the inner ear.

PTEN is a key modulator of phosphatidylinositol 3-OH kinase (PI3K) signaling that involves the regulation of diverse cellular events such as growth, differentiation, and migration, and specialized developmental functions [9, 10, 11]. PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) to produce phosphatidylinositol-4,5-biphosphate, thereby directly affecting the PI3K pathway [12]. The loss of PTEN leads to PI3K-hyperactivated accumulation of PIP3, which promotes Akt phosphorylation and induces phosphorylation of downstream effectors such as the mammalian target of rapamycin, forkhead box 1, and glycogen synthase kinase 3 (GSK3) α/β [13, 14, 15]. Recently, GSK3 has been reported to be a regulator of neurogenesis that coordinates multiple signaling pathways involving cell proliferation and differentiation of progenitors [16, 17]. During inner ear development, PI3K/Akt signaling has also been associated with insulin-like growth factor 1 (IGF-1). Mutation of the gene encoding its receptor (Igfr) is associated with insulin-like growth factor 1 (IGF-1). Mutation of the gene encoding its receptor (Igfr) is associated with severe cochlear defects and deafness [18, 19].

The function of Pten has been studied using transgenic animals. The conventional Pten knockout model exhibits early embryonic lethality, and hemizygous Pten mice undergo tumorigenesis in several organs [20, 21]. Conditional Pten deletion in neural
precursors leads to a significantly enlarged brain, increased proliferative capacity, and neuronal hypertrophy [22,23]. Haploinsufficiency of PTEN in subventricular zone progenitor cells results in extensive migration and resistance to hydrogen peroxide-induced apoptosis [24]. Marinho et al. [25] reported that loss of Pen disrupts cell layers in the developing cerebellum, suggesting a disturbance of migration and positioning. Treatment with PTEN-antisense oligonucleotide produces short neurites with apoptosis in the PC12 cell line [26]. These results indicate that Pen may participate in regulating neural organization, connection, and outgrowth, including neurodevelopment. In the inner ear, it has been reported that Pen expression is observed in the developing cochlea and that phosphorylated Pen accumulates in hair cell and supporting cell nuclei of the aging cochlea [27,28]. Despite extensive studies of Pen-null phenotypes in various systems, little is known about the neuronal roles and mechanisms of Pen during inner ear development. In this study, we characterized the inner ear of a Pen conditional-knockout model, and showed that Pen is one of the key molecules in neuronal maintenance of the developing CVG population.

Materials and Methods

Ethics Statement

All animal procedures were conducted according to the guidelines for the use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at the Korea Food and Drug Administration or Korea Centers for Disease Control and Prevention (10Sikyak30 and KCDC-017-11).

Mouse Lines

Pen<sup>loxp/loxp</sup> (stock number 006440), Tg(Neurog1-cre/ESR1)<sup>1</sup>-Good (stock number 00529), and Tg(Atoh1-cre/Esr1)<sup>1</sup>Fsh (stock number 007684) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and Tg(Pax2-Cre)<sup>1</sup>Akg (stock number 010569-UNC) mice were purchased from MMRRC (UNC, Chapel Hill, NC, USA). Tg(Neurog1-cre/ESR1)<sup>1</sup>-Good, Tg(Atoh1-cre/ESR1)<sup>1</sup>Fsh, and Tg(Pax2-Cre)<sup>1</sup>Akg mice were crossbred with Pten<sup>loxP/loxP</sup> mice to generate Pten<sup>+/loxP/+;Pen<sup>loxp/loxp</sup></sup>, Neurog1<sup>cre/cre</sup>;Pen<sup>loxp/+</sup>, and Atoh1<sup>cre/cre</sup>;Pen<sup>loxp/+</sup> double-transgenic animals. These animals were crossed with Pten<sup>loxP/loxp</sup> mice to generate conditional knockout mice of four genotypes: Cre<sup>+/cre</sup>;Pen<sup>loxP/+</sup>, Cre<sup>+/cre</sup>;Pen<sup>loxp/+</sup>, and Cre<sup>+/cre</sup>;Pen<sup>loxp/loxp</sup>. Cre<sup>+/cre</sup>;Pen<sup>loxP/loxp</sup> mice were selected as cKO mice. Mice of the other two phenotypes (Pen<sup>loxP/+</sup> and Pen<sup>loxP/loxp</sup>) are referred to as “wild type” because they have no abnormal phenotype. The morning of the day of the vaginal plug was referred to as “wild type” because they have no abnormal phenotype. The morning of the day of the vaginal plug was referred to as “wild type” because they have no abnormal phenotype. The morning of the day of the vaginal plug was referred to as “wild type” because they have no abnormal phenotype.

Immunofluorescence

Embryos were harvested and fixed in 4% paraformaldehyde (PFA) at 4°C overnight, cryo-protected in 30% sucrose, embedded in OCT compound, and sectioned to a thickness of 10 μm using a cryostat. The following primary antibodies were used: anti-PTEN mouse monoclonal (560002, 1:20; BD Biosciences, Sparks, MD, USA), anti-NeuroD goat polyclonal (sc-1084, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Isl1 mouse monoclonal (40.2D6, 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-Tuj1 mouse monoclonal (MMS-435P, 1:400; Covance, Emeryville, CA, USA), anti-Tuj1 rabbit monoclonal (MRB-435P, 1:400; Covance), anti-TrkC goat monoclonal (AF1404, 1:25; R&D Systems, Minneapolis, MN, USA), anti-phospho-Akt rabbit monoclonal (4060, 1:50; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-GSK3β rabbit monoclonal (9233, 1:50; Cell Signaling Technology), anti-cleaved caspase-3 rabbit monoclonal (9664, 1:200; Cell Signaling Technology), anti-PCNA mouse monoclonal (ab29, 1:100, Millipore, Billerica, MA, USA), anti-acetylated tubulin mouse monoclonal (T6793, 1:500; Sigma-Aldrich), anti-Prox1 rabbit polyclonal (PB-R238C, 1:200; Covance), and anti-neurollament rabbit monoclonal (N4142, 1:100; Sigma-Aldrich) antibodies. After antigen retrieval with 10 mM citrate buffer for 20 min at 95°C, sections were incubated in blocking solution and normal donkey serum as whole mounts using an MZ16FA rhodamine filter set (Leica Microsystems, Wetzlar, Germany).

Neural Tracer Application

Nerve fibers were traced using NeuroVue® Red Plus (Poly-sciences, Warrington, PA, USA) to reveal the general pattern of innervation, as described previously [31,32]. Briefly, a dye-coated filter triangle was used to cut the eighth cranial (vestibulo cochlear) nerve in E17.5 or E18.5 Pax2<sup>Cre/cre</sup>;Pen<sup>loxP/+</sup>, Neurog1<sup>Cre/Cre</sup>;Pen<sup>loxP/+</sup>, and Atoh1<sup>Cre/Cre</sup>;Pen<sup>loxP/+</sup> mice and their wild-type littermates (n>6 for each genotype). During diffusion, brains were incubated continuously in 4% PFA in 0.1 M phosphate buffer. After diffusion for 7 days at 37°C, the ears and otic ganglia were dissected away from the adjacent mesenchyme and photographed as whole mounts using an M216FA rhodamine filter set (Leica Microsystems, Wetzlar, Germany).
Western Blotting Analysis

E14.5 inner ears from four wild-type and Pax2<sup>Cre/+;Pten<sup>loxP/loxP</sup> embryos were dissected, pooled, and homogenized in lysis buffer (Cell Signaling Technology). Approximately 10 μg of protein was separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore). After blockade of nonspecific binding, the membranes were incubated with primary antibody and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The following primary antibodies were used: anti-PTEN rabbit monoclonal (9188, 1:500, Cell Signaling Technology), anti-phospho-Akt (Ser473) rabbit monoclonal (4060, 1:500, Cell Signaling Technology), anti-Akt rabbit monoclonal (4961, 1:500, Cell Signaling Technology), anti-phospho-<sup>α</sup>Serine (Ser9) rabbit monoclonal (9315, 1:500, Cell Signaling Technology), anti-<sup>α</sup>tubulin rabbit monoclonal (sc-47778, 1:1000; Santa Cruz Biotechnology) antibodies. The signals in the Western blots were detected with an ECL kit (Thermo Scientific, Rockford, IL, USA). Band intensity ratios were calculated with ImageJ (NIH).

Quantification of Cells

Neuroblasts were counted in 10-μm frozen sections. Consecutive sections through the entire CVG complex and epithelium were stained with anti-NeuroD and anti-Iles1 antibodies, and signal-positive cells with a clear nucleus were counted in every other section (every 20 μm) at 200× magnification using an Axio Imager A1 microscope (Carl Zeiss). Five to eight cochleae from four embryos of each genotype at E10.5 were analyzed. At E11.5, cleaved caspase-3-positive cells in the CVG were counted in every section (every 20 μm) from three cochleae from three embryos. At E16.5, vestibular ganglia were enumerated in every fourth section (every 40 μm) from seven vestibules from four embryos. At E18.5, spiral ganglia were enumerated in every third section (every 30 μm) from three cochleae from three embryos.

Statistical Analysis

Data are presented as means ± SEM. The statistical significance of differences was determined by unpaired Student’s t-test. Asterisks indicate level of significance (*P<0.05, **P<0.01, and ***P<0.001).

Results

Pten Expression Patterns during Inner Ear Development

To evaluate the role of Pten during inner ear development, we examined the expression pattern of Pten from embryonic day (E)10.5 to E16.5 in the developing inner ear (Fig. 1). At E10.5, Pten was expressed in the CVG area but hardly detected in the inner ear epithelium (Fig. 1A, a–f). We compared Pten expression in the CVG with that of NeuroD, TrkC, and Tuj1. NeuroD is a marker of neuroblasts, and TrkC, a neurotrophin-3 receptor, is a marker for early differentiated neurons [33,34]. At E10.5 to E11.5, most Pten expression in the CVG overlapped with TrkC signals rather than being co-expressed with NeuroD (arrows in Fig. 1A, f and l). From E12.5 to E14.5, during neuronal differentiation, Pten was clearly expressed in TrkC cochlear ganglia and pan-neuronal marker Tuj1-positive ganglion neurons (Fig. 1A, m–x). Pten immunoreactivity changed slightly over the course of inner ear development. During the early stages of development, Pten is expressed in the neuronal cell body since the outgrowth of neurites is not fully completed. From E16.5 to E18.5, Pten immunoreactivity remains in the neuronal cell body but seems to be stronger in the ends of neurites than at earlier stages (arrows in Fig. S1A). At E16.5, Pten-immunopositive signals were observed in the hair cells of the cochlear epithelium (arrowheads in Fig. 1B, b). In addition, Pten signals were detected in the area of Hensen’s and Claudius’ cells (arrows in Fig. 1B, b), which has not been reported previously. Tuj1-positive spiral ganglia showed Pten immunopositivity (Fig. 1B, j–l). Expression of Pten was also observed in the vestibular sensory epithelia, non-sensory epithelia, and ganglion (Fig. S1B).

Characterization of the Inner ear in Pax2<sup>Cre/+;Pten<sup>loxP/loxP</sup> Embryos

To determine the gross morphology of the inner ear in Pax2<sup>Cre/+;Pten<sup>loxP/loxP</sup> (Pten conditional knockout (cKO)) embryos, we used the paint-fill technique with a total of four embryos at E14.5 and five embryos at E17.5 (Fig. 2). At E14.5, every component of the inner ear was identifiable but the membrane surface was coarse compared to that of the wild-type. In the vestibular part, the ears of Pten cKO embryos had thickened semicircular canals and canal pouches. The endolympathic duct and common crus were also wider. The Pten-deleted cochlea exhibited a grossly wide morphology with a coarse pattern but with slightly reduced turning at E14.5 (Fig. 2A, b). This widened morphology became more evident at E17.5 (Fig. 2B, b and d). Next, we measured the cochlea length at E17.5. The inner length of the Pten-deleted cochlea was decreased by 30% (69.6±6.2 in 4–6 cochleae, P<0.01) compared with that of wild-type cochlea, although no significant difference in outer length was observed (4–6 cochleae, P>0.95; data not shown). The coiling of the cochlea also changed slightly (Fig. 2B, d). In the wild type, the coiling of the cochlea reached 1.75 turns, but 9 of 10 cochleae in the affected ears had only 1.5 turns (Fig. 2B, e and d).

Next, we investigated cochlear sensory epithelium development and the organization of hair cells using fluorescent staining in whole-mount dissected sensory epithelium (Fig. 2C). In Pten-deleted embryos, rows of outer hair cells were staggered with an extra row of hair cells in the basal and mid turn, and the array of inner hair cells was sparsely disrupted by intruding extra inner hair cells (arrowheads in Fig. 2C, b), as assessed by phalloidin staining with p75<sup>NTR</sup>, a marker of inner pillar cells at E18.5. In contrast, numbers of hair cells in the vestibular sensory epithelium (i.e., utricle, saccule, and ampulla) did not show statistically significant deficits in Pten cKO embryos (data not shown). Although some irregular patterning was evident in the cochlear hair cell array, the general orientations of the hair cell bundles in Pten cKO embryos were different from those in wild-type mice (Fig. S2). The p75<sup>NTR</sup>-positive pillar cells were widened and occasionally missing (data not shown) compared to those of the wild type (arrow in Fig. 2C, b). These results indicated that Pten plays a role in the structural development of the organ of Corti.

Neuronal Inner Ear Defects in the Pten cKO Embryos

We labeled the nerve innervation patterns by inserting a NeuroVue filter strip into the eighth nerve including both afferent and efferent nerves of inner ears at E18.5 to investigate neuronal phenotype (Fig. 3). The radial bundles of wild-type cochlea were evenly distributed (Fig. 3A, a and c). In contrast, Pten cKO mice had much thicker fascicles and showed possible pathfinding defects (arrowheads in Fig. 3A, b and d). Additionally, a shortened distance between the modiolus and spiral ganglion was observed in the basal turn, indicating an increased length between the spiral ganglion and hair cells from thirteen cochleae.
(201% increase, P<0.05; Fig. 3A, d). Ganglia in Pax2Cre/+;PtenloxP/loxP mice showed statistically significant percentage losses: 87% for spiral ganglion neurons at E18.5 (3 cochleae, P<0.01; arrows in Fig. 3C, D), and 58% for the vestibular ganglion at E16.5 (7 cochleae, P<0.01; Fig. S3).

Sensory neurons share a common progenitor with hair cells in the developing inner ear. Neural cell fate specification is decided by the expression of selector genes such as Neurog1 [1,6]. A ganglion defect can be caused by alteration of neurotrophic factors (i.e., NT-3 in the cochlea) secreted from the sensory domain of the epithelium [35]. Thus, sensory neuron and hair cell development may be influenced through cross-regulation. To verify the cause of the spiral ganglion defects, we performed selective deletion of Pten in either the sensory epithelium or the ganglia using Atoh1Cre+;PtenloxP/loxP and Neurog1Cre+;PtenloxP/loxP mice, respectively (Figs. S4 and 3B). When ganglion-selective deletion of Pten in the Neurog1Cre+;PtenloxP/loxP mice was performed using tamoxifen injections from E8.5 to E11.5, badly disorganized ganglion innervation patterns resulted (arrowheads in Fig. 3B, b and d), whereas a normal innervation pattern was observed in the Atoh1Cre+;PtenloxP/loxP mice (data not shown). Indeed, Pten depletion in Neurog1Cre+;PtenloxP/loxP mice did not influence the hair cell pattern in the cochlear epithelia (Fig. S4A). Our data imply that the Pten cKO neuronal phenotype results from defects in progenitors of the Neurog1-positive neural lineage and did not influence sensory epithelial alteration.
Loss of Neurons in the Pten cKO Spiral Ganglia

We examined the neuronal markers NeuroD and Islet1 at E10.5 to understand the effect of Pten deletion on the CVG (Fig. 4). Although the Pten cKO NeuroD-positive area was not reduced compared to that of the control, the number of NeuroD-positive cells was decreased by 40% in the CVG (5 cochleae, \( P < 0.001 \); Fig. 4B). This reduction was already evident in the epithelium before delamination of neuroblasts. The number of NeuroD-positive cells within the epithelium was reduced by 23% in Pten cKO mice compared to wild-type mice (8 cochleae, \( P < 0.05 \); Fig. 4C). Although NeuroD-positive CVG cells were disorganized and the neuroblasts became flattened in Pten cKO mice, they were still co-localized with PCNA-positive cells, as observed in the wild-type (data not shown). At neuronal maturation after exit from the cell cycle, neurons appeared to be expressed by both Islet1-positive and NeuroD-negative cells in the CVG at E10.5 (arrows in Fig. 4A, e and f). Islet1-positive and NeuroD-negative neurons also decreased significantly compared to those in the wild-type (6 cochleae, \( P < 0.05 \); Fig. 4D). Furthermore, apoptosis in the spiral ganglion increased markedly from E11.5 in Pten-deficient mice compared to that in the wild-type (Figs. 5 and 6). To identify whether the cleaved caspase-3-positive cells were neurons or Schwann cells, we determined the co-localization of apoptotic cells using immunoreactivity to Tuj1, a neuronal marker, and Sox10, a Schwann cell marker (Fig. S5). Immunofluorescence results showed that cleaved caspase-3 signals were co-localized in neurons expressed with Tuj1-positive cells but not with Sox10-positive Schwann cells (arrows in Fig. S5, B).

We performed PCNA immunofluorescence at E11.5 to examine whether the apoptotic neuronal cells were in a differentiation stage (Fig. 5B). Apoptotic neurons were distributed in the core of the non-proliferative area (arrows in Fig. 5B, f) and expressed Islet1, a neuronal marker, and Sox10, a Schwann cell marker (Fig. S5). Immunofluorescence results showed that cleaved caspase-3 signals were co-localized in neurons expressed with Tuj1-positive cells but not with Sox10-positive Schwann cells (arrows in Fig. S5, B).

We investigated Akt activity to determine the mechanisms of the Pten loss-induced spiral ganglion neuronal phenotype, as it is well known that PTEN loss promotes Akt signaling pathway activity. Akt activation was assessed by detecting Akt phosphorylation at the Ser473 residue. As shown in Figure 6, Akt phosphorylation increased in the spiral ganglion of Pten-deficient mice compared to that of wild-type mice (Fig. 6A, a–f). The relative intensity of the phospho-Akt signal was increased by 162% in Pten cKO mice compared to wild-type mice (4 cochleae, \( P < 0.05 \); Fig. 6B and C, a). We observed Ser9-phosphorylation of the GSK3β protein in the Pten-deleted inner ear at E13.5, the neuronal differentiation stage, which was co-expressed in the phospho-Akt-positive cells of
the spiral ganglion (Fig. 6A, d and j). The level of GSK3β phosphorylation at E13.5 was sustained at E14.5 (data not shown). However, at an earlier stage, such as E11.5, GSK3β phosphorylation was detected in both wild-type and Pten-deleted mice (data not shown). The relative intensity of the phospho-GSK3β/GSK signal was increased by ∼50% in Pten-deleted mice compared to wild-type mice (4 cochleae, *P* < 0.05; Fig. 6B and C, b). The cleaved caspase-3-positive cells no longer expressed phospho-Akt, phospho-GSK3β, or Islet1.

**Discussion**

In this study, we explored whether Pten plays a neuronal role in the regulation of development in the mouse inner ear. We found that inner ear-specific Pten deficiency resulted in severe neuronal abnormalities such as apoptosis of spiral ganglion with disorganization of nerve innervations. This is the first report of on the novel functions of Pten in regulating neuronal survival, differentiation, and axon pathfinding of neurons in the developing inner ear.

**Pten is Required for the Morphogenesis of the Developing Inner Ear**

Our results provide an initial characterization of the Pten-depleted inner ear. Notably, Pten-deficient mice have enlargement of the entire membranous labyrinth, which is dramatic in the endolymphatic sac (Fig. 2A, B). This phenotype is similar to that in the inner ear of anion-exchanger Pendrin-knockout mice [36]. This dysmorphogenesis may be associated with changes in ion channels, including transporters and exchangers. Recent studies have shown that Pten regulates ion channels through PI3K/Akt signaling [37,38,39]. The molecular mechanism behind the morphological defects in the Pten-depleted inner ear, however, requires further study.

Specifically, we observed neuronal defects in the Pten-deficient inner ear (Fig. 3), leading to aberrant axon pathfinding of spiral ganglion and disruptions of the accurate connections to the hair cells. This abnormality was influenced by the substantial reduction of neuronal cell number after neural fate commitment of progenitors (Figs. 3 and 4). The disorganized radial fibers and neuronal loss in the inner ear of Pten cKO mice is similar to the
phenotypes of several mutants (e.g., Brn3a<sup>−/−</sup>, TrkC<sup>−/−</sup>, or Ezh2<sup>−/−</sup>) required for the differentiation of the spiral ganglia [40,41]) and suggests that Pten may play a role in these gene-mediated signaling pathway. Thus, our results suggest that Pten is a functional regulator involved in axon pathfinding of the spiral ganglia during inner ear development.

**Pten Deficiency Induced Apoptosis of Differentiated Spiral Ganglia**

Several studies on Pten-deficient mice have reported hypertrophic characteristics such as an enlarged brain and tumorogenesis in various organs [20,21,22,23]. In contrast, we found that Pten deficiency induced apoptosis of pre-differentiated cells but not in proliferating CVG in the developing inner ear (Fig. 5). We observed neuronal loss of spiral ganglia in Neurog<sup>1<sup>cKO</sup> mice (Fig. 3B), similar to Pax2<sup>2<sup>cKO</sup> mice (Fig. 3A). This contradiction suggests that Pten has a particular function in the development of cochlear ganglia. The role of Pten in the inner ear agrees with a study in which PTEN-antisense in a neuronal cell line induced cell death with a loss of differentiating cells [26]. These results indicate that Pten deficiency promotes apoptosis in differentiating neurons in the delaminated CVG. A previous

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**Figure 4. Reduction in neuronal cell number in the cochleovestibular ganglia (CVG) of Pten conditional knockout (cKO) mice.** (A) NeuroD-positive cells are shown in green, and Islet1-positive cells are in red. Early matured neurons appeared to be expressed by both Islet1-positive and NeuroD-negative cells in the CVG at E10.5 (arrow in e, f). CVG, cochleovestibular ganglion; GG, geniculate ganglion; OV, otic vesicle. Scale bar: 100 μm. (B–D) Cell counts in the CVG (white outlines in A) and epithelium at E10.5. (B) The number of NeuroD-positive cells in Pten-deficient mice was reduced markedly to about half of that in wild-type mice (5 cochlea, \( P < 0.001 \)). (C) A significant reduction in NeuroD-positive cells in the inner ear epithelium was observed in Pten cKO mice (8 cochlea, \( P < 0.05 \)). (D) Early differentiated neurons expressed by Islet1-positive/NeuroD-negative in Pten-deficient mice showed a significant loss compared to those in wild-type mice (6 cochlea, \( P < 0.05 \)).

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study reported that prolonged PI3K activation could trigger apoptosis via chronic induction of a cell-cycle component [42]. Thus, further experiments are required to determine the mechanism by which \textit{Pten} deficiency induces apoptosis in spiral ganglia.

**Pten** Deficiency Causes GSK3β Phosphorylation in Spiral Ganglion Differentiation

To verify the proposed signaling mechanisms for spiral ganglion defects in \textit{Pax2Cre/+;} \textit{Pten}\textsuperscript{loxP/loxP} mice, we examined whether \textit{Pten} deficiency (Fig. S6) upregulates Ser1473-phosphorylation of Akt and triggers Ser9-phosphorylation of GSK3β (pGSK3β) in the PI3K signaling cascade. As shown in Figure 6, we found hyperactive Akt and hypoactive GSK3β by immunofluorescence in the spiral ganglion of Pten-deleted inner ears at E13.5. At E11.5, the level of pGSK3β in Pten cKO mice was similar to that of wild-type mice (data not shown). Although wild-type mice exhibited a decreased level of pGSK3β, indicating increased activation at E13.5, Pten cKO mice did not decrease the GSK3β phosphorylation level at the same developmental stage (Fig. 6). It was recently established that precise regulation of GSK3 activity is required to control neuronal differentiation. Inhibiting GSK3 facilitates or prevents axon formation and extension by regulating phosphorylation of its substrates; \textit{i.e.}, microtubule-associated protein 1B, collapsin response mediator protein 2, or adenoma-

**Figure 5. Neuronal apoptosis in the cochleovestibular ganglion (CVG) complex of \textit{Pten}-deficient mice.** (A–D) Apoptotic cells in the CVG were stained with anti-cleaved caspase-3 antibody at E11.5. Cleaved caspase-3 immunoreactivity (red) increased substantially in \textit{Pten}-deficient mice compared to that in wild-type mice. (A) Apoptotic neurons were occasionally co-localized with TrkC-positive (green) (arrows in f) or negative cells. Higher magnification images of d, e, and f are shown in the insets in d, e, and f, respectively. Scale bars: 100 μm. (B) Proliferating PCNA-positive cells (green) were not seen in apoptotic neurons (arrows in f). Higher magnification images of d, e, and f are shown in the insets in d, e, and f, respectively. Scale bars: 100 μm. (C) Cleaved caspase-3-positive cells (red) were also Islet1-negative cells (arrows in f). Apoptotic neurons (red) were distributed in the core of the non-proliferative area that expressed Islet1 (green). Higher magnification images of d, e, and f are shown in the insets in d, e, and f, respectively. Scale bars: 100 μm. (D) Numbers of cleaved caspase-3-positive apoptotic cells in the CVG were significantly increased in \textit{Pten}-deficient mice at E11.5 (3 cochleae, \(P<0.001\)).

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Figure 6. Akt and GSK3β phosphorylation in Pten-deficient mice. (A) Phosphorylation of Akt and GSK3β was measured by immunofluorescence staining using anti-phospho-Akt (Ser473) (pAkt) and anti-phospho-GSK3β (Ser9) (pGSK3β) antibodies at E13.5. Compared to wild-type spiral ganglia, Akt phosphorylation (green) increased substantially in Pten-deficient mice (a–f). GSK3β phosphorylation (green) was also highly expressed in the spiral ganglia of Pten-deficient mice compared to that in wild-type mice (g–l). In the absence of Pten, all pAkt and pGSK3β cells were maintained in Islet-1 positive cells (red) (a–l). Wild-type mice showed well-organized patterns of Islet-1-positive cells (a–c), whereas Pten-deficient mice showed a slightly scattered form of spiral ganglia (d–f). Cleaved caspase-3-positive apoptotic neurons (red) were sometimes co-
localized with TrkC-positive cells (green) (arrowheads in r). DAPI-stained nuclei (blue) are seen in all images. Scale bar: 100 μm. (B) Western blotting analysis of Pten, pAkt, Akt, pGSK3β, GSK3β, and β-actin expression in the inner ear at E14.5. Proteins were extracted from four pooled inner ears at E14.5. (C) The relative intensity of each phospho-protein was normalized to the total level of the same protein. Levels of both pAkt (a) and pGSK3β (b) were significantly increased in Pten cKO mice (4 cochleae, P<0.05).

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Figure S3 Neuronal loss of vestibular ganglia in Pten-deficient mice at E16.5. (A) Tuj1 immunoreactivity (green) were reduced in the vestibular ganglion of Pax2Cre/+;PtenloxP/loxP mice. U, utricle; VG, vestibular ganglion. Scale bar: 100 μm. (B) Numbers of vestibular ganglia were significantly reduced compared to wild-type mice (7 cochleae, P<0.01).

(TIF)

Figure S4 Epithelial phenotype in Neurog1Cre+;PtenloxP/loxP and Atoh1Cre+;PtenloxP/loxP Pten-deficient mice. (A) Whole-mount in situ hybridization for Pax2 expression (red) compared to that in wild-type mice (a, b). Scale bar: 20 μm. (B) Tamoxifen-inducible Pten deletion from E13.5 to E14.5 in Atoh1Cre+;PtenloxP/loxP mice included three rows of outer and one row of inner hair cells compared to that in wild-type mice (a, b). Scale bar: 20 μm.

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Figure S5 Apoptotic neurons in the cochleovestibular ganglion (CVG) complex of Pax2Cre/+;PtenloxP/loxP embryos. (A–D) Cleaved caspase-3-positive apoptotic cells (red) in the CVG were stained with Tuj1 (green), a neuronal marker, or Sox10 (green), a Schwann cell marker, at E12.5. (B) Apoptotic cells were co-localized with Tuj1-positive neurons in the CVG (arrows in B). U, utricle; SG, spiral ganglion. Scale bar: 100 μm.

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Figure S6 Reduction in Pten-positive immunoreactivity in Pax2Cre/+;PtenloxP/loxP mice. Pten immunopositivity (red) in the spiral ganglion at E16.5 was significantly decreased in Pax2Cre/+;PtenloxP/loxP mice compared to wild-type mice. DAPI-stained nuclei (blue) are seen in all images. SG, spiral ganglion. Scale bar: 100 μm.

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Author Contributions

Conceived and designed the experiments: HJK SKK. Performed the experiments: HJK HMW JR JB SBC. Analyzed the data: HJK SKK. Contributed reagents/materials/analysis tools: HJK JB JWK MHP HYP. Wrote the paper: HJK SKK.

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