

# ***PTEN* regulation of the proliferation and differentiation of auditory progenitors through the *PTEN/PI3K/Akt*-signaling pathway in mice**

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The organ of Corti, which is the sensory organ of hearing, consists of a single row of inner hair cells and three rows of outer hair cells in mice. The auditory hair cells develop from auditory progenitors. Hair cell development is related to several genes, including *PTEN*. Homozygous null mutant (*PTEN*<sup>-/-</sup>) mice die at around embryonic day 9, when hair cells are extremely immature. Moreover, in heterozygous *PTEN* knockout mice, it was found that *PTEN* regulates the proliferation of auditory progenitors. However, little is known about the molecular mechanism underlying this regulation. In the present study, we generated *PTEN* conditional knockout in the inner ear of mice and studied the aforementioned molecular mechanisms. Our results showed that *PTEN* knockout resulted in supernumerary hair cells, increased p-Akt level, and decreased p27<sup>kip1</sup> level. Furthermore, the presence of supernumerary hair cells could be explained by the delayed withdrawal of auditory progenitors from the cell cycle. The increased p-Akt level correlates with p27<sup>kip1</sup> downregulation in the cochlea in the *Pax2-PTEN*<sup>-/-</sup> mice. The reduced p27<sup>kip1</sup> could not maintain the auditory progenitors in the nonproliferative state and some progenitors continued to divide. Consequently, additional

progenitors differentiated into supernumerary hair cells. We suggest that *PTEN* regulates p27<sup>kip1</sup> through p-Akt, thereby regulating the proliferation and differentiation of auditory progenitors. *NeuroReport* 25:177–183 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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## **Introduction**

The inner ear is the innermost part of the vertebrate ear, and it is mainly responsible for balance and sound detection. The inner ear develops from the otic placode, which is a thickening of the surface ectoderm next to the hindbrain at embryonic day 8 (E8). Over time, the otic placode invaginates to form the otocyst at E9. By E12, the otocyst begins to develop morphologically into the cochlea and the vestibule in mice [1]. The cochlea is the auditory portion of the mammalian inner ear [2]. Its core component is the organ of Corti, which is the sensory organ of hearing. The organ of Corti in mice consists of a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) [3]. The auditory hair cells (HCs) are the sensory receptors that transform sound vibrations into electrical

signals. The electrical signals then travel through auditory nerve fibers to the brain. HCs develop from auditory progenitors and several genes coordinate to regulate this process [4,5]. All otocyst cells in mice continue to divide from E9 to E12 [2,6]. The auditory progenitors withdraw from the cell cycle and establish a zone of nonproliferating cells from E12.5 to E14.5. P27<sup>kip1</sup> activity maintains zone of nonproliferating cells in a nonmitotic state. At E15, p27<sup>kip1</sup> expression is selectively downregulated, and auditory progenitors differentiate into HCs and supporting cells [7]. The upstream regulator of p27<sup>kip1</sup> in the inner ear remains unknown. Using cell culture systems of 293T and COS-7 cells, p27<sup>kip1</sup> was found to be the substrate of Akt. Akt was converted into its activated form (p-Akt) through phosphorylation at specific serine and threonine residues. Subsequently, p-Akt promoted p27<sup>kip1</sup> degradation [8–10].

Mammalian HCs cannot regenerate spontaneously [11]. The HC loss caused by noise trauma and ototoxic drugs is permanent and often leads to hearing impairment. Thus, study of HC regeneration in mammals is a hot topic in hearing research. Several genes, such as *retinoblastoma (Rb)*, p27<sup>kip1</sup>, and *phosphatase and tensin homolog deleted on*

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*chromosome 10 (PTEN)*, are associated with HC regeneration [7,12–14].

*PTEN* is a tumor suppressor gene frequently mutated in tumors such as endometrial carcinomas and glioblastomas [15]. *PTEN* expression in the HCs and cochlear–vestibular ganglion in the inner ear of mice shows a transient, specific pattern [16,17]. *PTEN* null mice (*PTEN*<sup>-/-</sup>) die at around E9.5, long before the HCs mature [4,18]. By contrast, the number of HCs is increased in heterozygous *PTEN* knockout mice. Withdrawal of auditory progenitors from the cell cycle is delayed [14]. These results indicate that *PTEN* is involved in the proliferation of auditory progenitors. However, little is known about the molecular mechanism of *PTEN* in regulating the proliferation and differentiation of auditory progenitors. Thus, we conditionally knocked out *PTEN* in the inner ear of mice. Using this mouse model, we studied the role of *PTEN* in regulating the proliferation and differentiation of auditory progenitors.

## Materials and methods

### Animals

Mice homozygous for floxed *PTEN* exon 5 (*PTEN*<sup>LoxP/LoxP</sup>) [19] were crossed with Pax2-Cre mice [20]. Pax2-Cre recombinase was expressed in the developing otocyst, kidneys, and the midbrain–hindbrain boundary [20]. Mice for timed mating were placed together at 6:00 p.m. The day the vaginal plugs were established was considered embryonic day 0.5 (E0.5).

All experiments were conducted in accordance with the standards of the Shandong University Ethics Committee.

### Genotyping

The following PCR primers were designed to detect the genotypes: the *PTEN*<sup>LoxP/LoxP</sup> left primer, located upstream of exon 5, was 5'-gacctgaactcaatgtttgac-3', whereas the *PTEN*<sup>LoxP/LoxP</sup> right primer, located within exon 5, was 5'-gccccgatgaataaatatg-3'. The PCR cycling conditions were as follows: 33 cycles of 94°C for 10 min, 94°C for 30 s, 57°C for 1 min, 72°C for 30 s, and 72°C for 7 min. The Pax2-Cre left primer was 5'-tgcaacgagtgtgagggttc-3', whereas the Pax2-Cre right primer was 5'-acgaacctggtcgaaatcag-3'. The PCR cycling conditions were 30 cycles of 94°C for 10 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 7 min.

### Immunostaining of cryosections

Inner ears dissected from the heads of mice were fixed overnight in 4% paraformaldehyde at 4°C. Subsequently, the samples were placed in 15% sucrose for 8 h at room temperature for the first dehydration and in 30% sucrose at 4°C overnight for the second dehydration. Finally, the samples were embedded in OCT compound (Sakura Finetek, Torrance, California, USA) and frozen at -20°C until sectioning.

The sections were washed with 10 mM PBS and blocked (10% goat serum) for 1 h at room temperature. Primary antibodies in 5% goat serum were then introduced and the samples were incubated overnight at 4°C. The primary antibodies included anti-myosin VIIa (myo 7a) rabbit polyclonal antibodies (Proteus-Bioscience, Ramona, California, USA; 1:200), anti-p27<sup>kip</sup> rabbit polyclonal antibody (Abcam, Cambridge, Massachusetts, USA; 1:200), and anti-p-Akt rabbit monoclonal antibodies (Millipore, Billerica, Massachusetts, USA; 1:200). The samples were washed with PBS and incubated at room temperature for 2 h in secondary goat anti-rabbit IgG (H + L) Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA; 1:300) diluted in the same solution as the primary antibodies. A laser scanning confocal microscope (LSM700; Carl Zeiss AG, Pentacon, Germany) was used to analyze the samples.

### Whole-mount immunostaining

Inner ears dissected from the heads of mice were fixed overnight in 4% paraformaldehyde at 4°C. The cochlear were dissected into basal, middle, and apical sections. The resulting whole mounts were washed with 10 mM PBS and stained with rhodamine phalloidin (Sigma, Santa Clara, California, USA; 1:500) for 30 min at room temperature, followed by a final washing in PBS. The samples were analyzed under a laser scanning confocal microscope (LSM700). We counted the rhodamine-phalloidin-stained HCs.

### Western blot analysis

Cochlea proteins were incubated in cell lysis buffer (10 mM Tris, pH = 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF) and extracted using a homogenizer. The proteins from the samples (50 µg) were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. The primary antibodies used were anti-p-Akt rabbit monoclonal antibodies (Millipore; 1:200), anti-p27<sup>kip</sup> rabbit polyclonal antibodies (Abcam; 1:200), and anti-GAPDH mouse monoclonal antibodies (Millipore; 1:3333). Subsequently, the membrane was incubated for 1 h at room temperature with the appropriate secondary antibodies coupled with horseradish peroxidase. Finally, the immunoreactive bands were visualized using ECL detection reagents.

### 5-Bromodeoxyuridine labeling

Timed pregnant female mice at E13.5, E14.5, or E15.5 were injected three times a day with 5 µg/µl 5-bromodeoxyuridine (BrdU; Sigma) in PBS. At E18.5, the pregnant female mice were killed by cervical dislocation and their embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C.

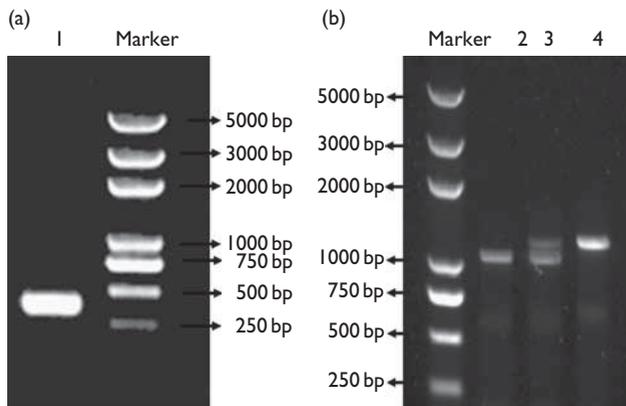
## Results

### PTEN knockout in the inner ear of mice

*PTEN*<sup>LoxP/LoxP</sup> mice, with the *PTEN* exon 5 flanked by *LoxP* sites [19], were crossed with Pax2-Cre mice to

generate *Pax2-PTEN*<sup>-/-</sup> mice [20]. The *PTEN* exon 5 flanked by *LoxP* sites were removed by Cre upon Cre expression. The genotypes of the pups were determined by PCR analysis (Fig. 1). The *Pax2-PTEN*<sup>-/-</sup> mice died perinatally. Although some of the *Pax2-PTEN*<sup>-/-</sup> mice remained alive after birth, others were stillborn. We dissected 10 pregnant female mice at E18.5 and all the *Pax2-PTEN*<sup>-/-</sup> embryos survived. The *Pax2-PTEN*<sup>-/-</sup> mice at P0.5 were physically similar to wild-type mice. No statistically significant differences in weight and length were observed between the *Pax2-PTEN*<sup>-/-</sup> and the wild-type mice.

Fig. 1

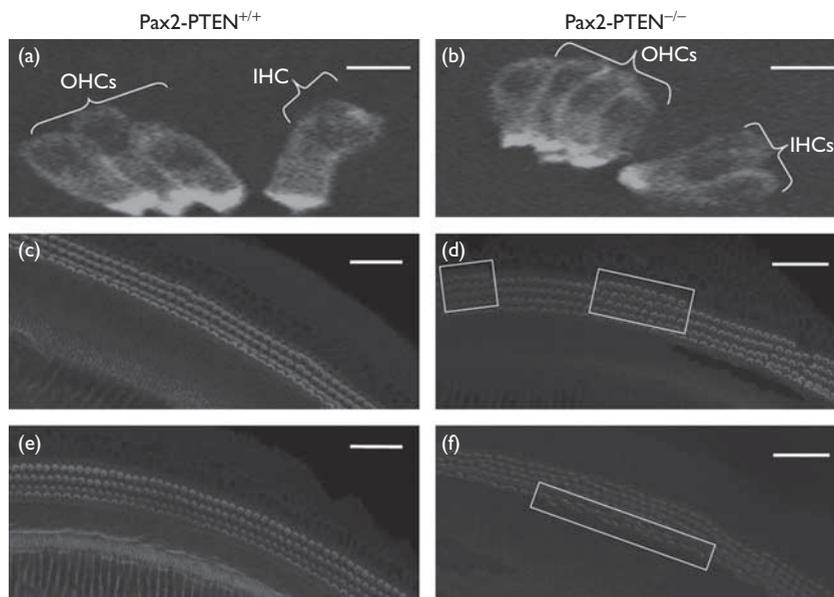


Genotyping through PCR analysis. (a) The PCR product of Pax2-Cre recombinase gene was 319 bp (1). (b) The product of the wild-type allele was 1200 bp and that of the *LoxP* allele was 1300 bp. A 1200 bp band was detected in the wild-type mice (2). A 1200 bp band and a 1300 bp band were detected in the heterozygous mice (3). Only the 1300 bp band was observed in the homozygous mice (4).

### Supernumerary auditory HCs in the cochlea of the *Pax2-PTEN*<sup>-/-</sup> mice

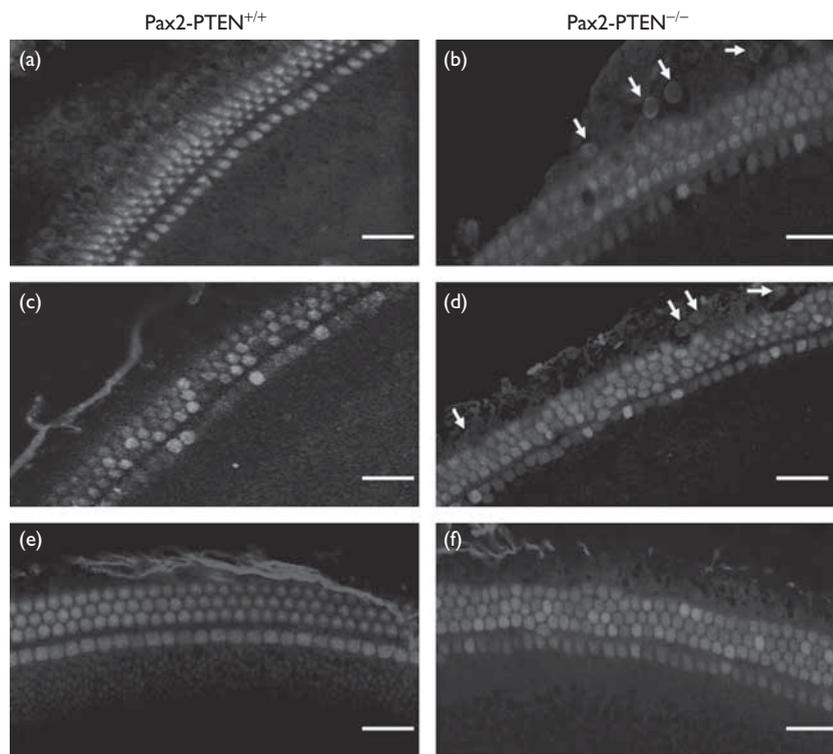
We labeled cryosections of wild type and knockout mice with MyoVIIa to identify HCs. In the wild-type mice, we observed the usual one row of IHCs and three rows of OHCs at P0.5 (Fig. 2a). However, supernumerary IHCs and OHCs were noted in sections of the *Pax2-PTEN*<sup>-/-</sup> mice at the same age (Fig. 2b). The supernumerary HCs were cylindrical and were similar to the HCs in wild-type mice (Fig. 2a and b). Whole-mount staining using rhodamine phalloidin was performed to determine the distribution of supernumerary HCs in the cochlea of the *Pax2-PTEN*<sup>-/-</sup> mice at P0.5. Most of the supernumerary

Fig. 2



MyoVIIa immunostaining of the cryosections of the cochlea and confocal images of cochlear whole mounts stained with rhodamine phalloidin at P0.5. (a) Three rows of OHCs and a single row of IHCs were found in *Pax2-PTEN*<sup>+/+</sup> mice. The HCs were cylindrical and were stained by MyoVIIa. Scale bar = 50 μm. (b) Four rows of OHCs and two rows of IHCs were observed in the *Pax2-PTEN*<sup>-/-</sup> mice. The supernumerary HCs were also cylindrical and were stained by MyoVIIa. Scale bar = 50 μm. (c) Three rows of OHCs were found in the mid-basal region of the cochlea of the *Pax2-PTEN*<sup>+/+</sup> mice. Scale bar = 50 μm. (d) Four rows of OHCs (demarcated by frames) were observed in the mid-basal region of the cochlea of the *Pax2-PTEN*<sup>-/-</sup> mice. Scale bar = 50 μm. (e) A single row of IHCs was found in the mid-basal region of the cochlea of the *Pax2-PTEN*<sup>+/+</sup> mice. Scale bar = 50 μm. (f) Two rows of IHCs (demarcated by frame) were found in the mid-basal region of the cochlea of the *Pax2-PTEN*<sup>-/-</sup> mice. Scale bar = 50 μm. HC, hair cells; IHCs, inner hair cells; OHCs, outer hair cells.

Fig. 3



Cochlear whole mounts stained with BrdU at E18.5. Pregnant female mice were injected with BrdU at E13.5, E14.5, and E15.5. BrdU stained the proliferating cells. The HCs were stained with rhodamine phalloidin. (a) In the *Pax2-PTEN*<sup>+/+</sup> mice that were injected with BrdU at E13.5, no BrdU staining was detected at E18.5. Scale bar=30 μm. (b) In the *Pax2-PTEN*<sup>-/-</sup> mice that were injected with BrdU at E13.5, BrdU staining (arrows) was detected at E18.5. Scale bar=30 μm. (c) In the *Pax2-PTEN*<sup>+/+</sup> mice that were injected with BrdU at E14.5, no BrdU staining was detected at E18.5. Scale bar=30 μm. (d) In the *Pax2-PTEN*<sup>-/-</sup> mice that were injected with BrdU at E14.5, BrdU staining (arrows) was detected at E18.5. Scale bar=30 μm. (e) In the *Pax2-PTEN*<sup>+/+</sup> mice that were injected with BrdU at E15.5, no BrdU staining was detected at E18.5. Scale bar=30 μm. (f) In the *Pax2-PTEN*<sup>-/-</sup> mice that were injected with BrdU at E15.5, no BrdU staining was detected at E18.5. Scale bar=30 μm. BrdU, 5-bromodeoxyuridine.

HCs were distributed in the middle and basal regions of the cochlea of the *Pax2-PTEN*<sup>-/-</sup> mice (Fig. 3d and f). The *Pax2-PTEN*<sup>-/-</sup> mice had 67.8% more OHCs than the wild-type mice. Furthermore, the IHCs were 22.6% greater in *Pax2-PTEN*<sup>-/-</sup> mice than those in wild-type mice ( $n = 10$ ;  $*P < 0.05$ ).

#### **PTEN knockout delayed the withdrawal of auditory progenitors from the cell cycle**

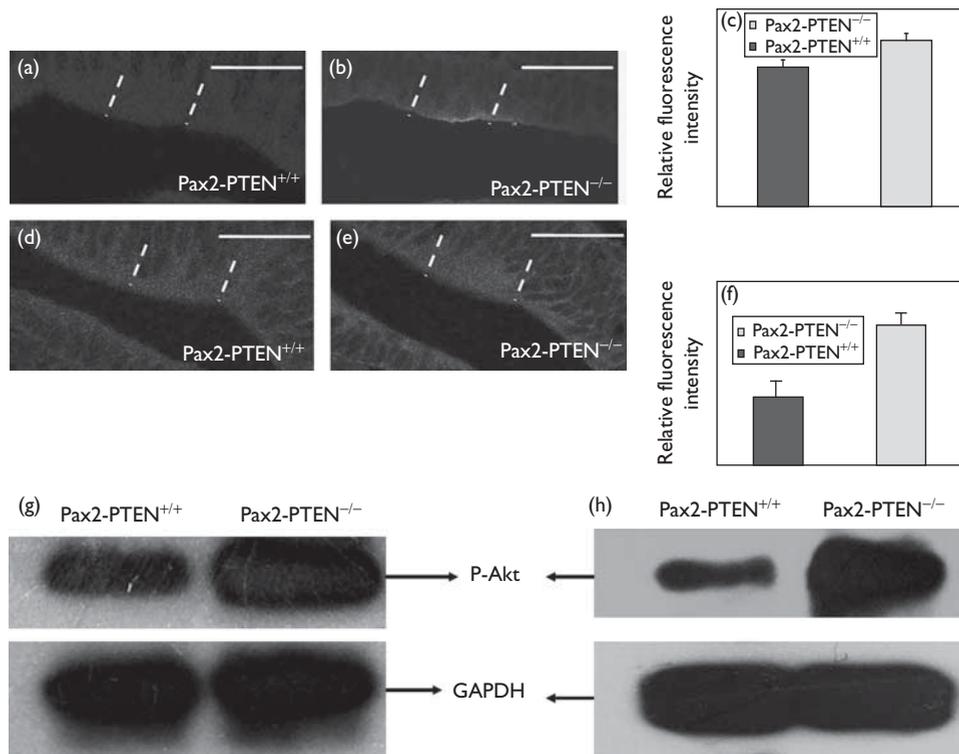
Pregnant female mice were injected with BrdU at E13.5, E14.5, or E15.5. The BrdU labeling was checked among the embryos at E18.5. BrdU labeling was detected in the *Pax2-PTEN*<sup>-/-</sup> mice injected with BrdU at E13.5 or E14.5 (Fig. 3b and d). However, no BrdU labeling was observed in the *Pax2-PTEN*<sup>-/-</sup> mice injected with BrdU at E15.5 or wild-type mice injected with BrdU at E13.5, E14.5, or E15.5 (Fig. 3a, c, e, and f). Thirty-two BrdU-labeled nuclei were observed in one cochlea of *Pax2-PTEN*<sup>-/-</sup> mice injected with BrdU at E13.5. Furthermore, 40 BrdU-labeled nuclei were observed in one cochlea of *Pax2-PTEN*<sup>-/-</sup> mice injected with BrdU at E14.5.

These results indicate that the auditory progenitors were still dividing at E13.5 and E14.5. They withdrew from the cell cycle in the *Pax2-PTEN*<sup>-/-</sup> mice late. The presence of supernumerary HCs can be attributed to the delayed withdrawal of auditory progenitors from the cell cycle in the *Pax2-PTEN*<sup>-/-</sup> mice.

#### **PTEN knockout increased the p-Akt level**

We checked the levels of Akt and p-Akt in the *Pax2-PTEN*<sup>-/-</sup> mice through immunofluorescence and western blot analysis. Immunofluorescence analysis was used under identical imaging conditions at the same cochlear location. The results showed that the Akt level remained unchanged (data not shown), but the p-Akt level increased in the *Pax2-PTEN*<sup>-/-</sup> mice compared with wild-type mice at E13.5 and E14.5 (Fig. 4a, b, d and e). Quantitative analysis of relative fluorescence intensity further confirmed these results (Fig. 4c and f). Western blot analysis also confirmed that the Akt level remained unchanged (data not shown) and the p-Akt level increased in the *Pax2-PTEN*<sup>-/-</sup> mice compared with wild-type mice (Fig. 4g and h).

Fig. 4



*PTEN* knockout increased p-Akt level. (a, b) Immunostaining for p-Akt in otocyst at E13.5 (demarcated by dashed lines). The p-Akt staining was more intensive in the *Pax2-PTEN*<sup>-/-</sup> mice. Scale bar=50  $\mu$ m. (c) Quantitative analysis of the relative fluorescence intensity showed that the *Pax2-PTEN*<sup>-/-</sup> mice had a higher p-Akt level compared with the *Pax2-PTEN*<sup>+/+</sup> mice at E13.5 ( $n=10$ ;  $P<0.05$ ). (d, e) Immunostaining for p-Akt in otocyst at E14.5 (demarcated by dashed lines). The p-Akt staining was more intensive in the *Pax2-PTEN*<sup>-/-</sup> mice. Scale bar=50  $\mu$ m. (f) Quantitative analysis of the relative fluorescence intensity showed that the *Pax2-PTEN*<sup>-/-</sup> mice had a higher p-Akt level compared with the *Pax2-PTEN*<sup>+/+</sup> mice at E14.5 ( $n=10$ ;  $P<0.05$ ). (g, h) Western blot analysis of the cochlea protein at E13.5 (g) and E14.5 (h) showed that the *Pax2-PTEN*<sup>-/-</sup> mice had a higher p-Akt level than the *Pax2-PTEN*<sup>+/+</sup> mice.

### *PTEN* knockout decreased the p27<sup>kip1</sup> level because of the loss of *PTEN*

p27<sup>kip1</sup> is one of the substrates of Akt and is phosphorylated by p-Akt [8]. We used immunofluorescence and western blot analysis to determine p27<sup>kip1</sup> levels. Immunofluorescence analysis was used under identical imaging conditions at the same cochlear location. p27<sup>kip1</sup> immunostaining was lower in the inner ear of the *Pax2-PTEN*<sup>-/-</sup> mice compared with wild-type mice at E13.5 and E14.5 (Fig. 5a, b, d and e). Quantitative analysis of relative fluorescence intensity further confirmed these results (Fig. 5c and f). Western blot analysis also showed that the p27<sup>kip1</sup> level was lower in the *Pax2-PTEN*<sup>-/-</sup> mice compared with wild-type mice (Fig. 5g and h).

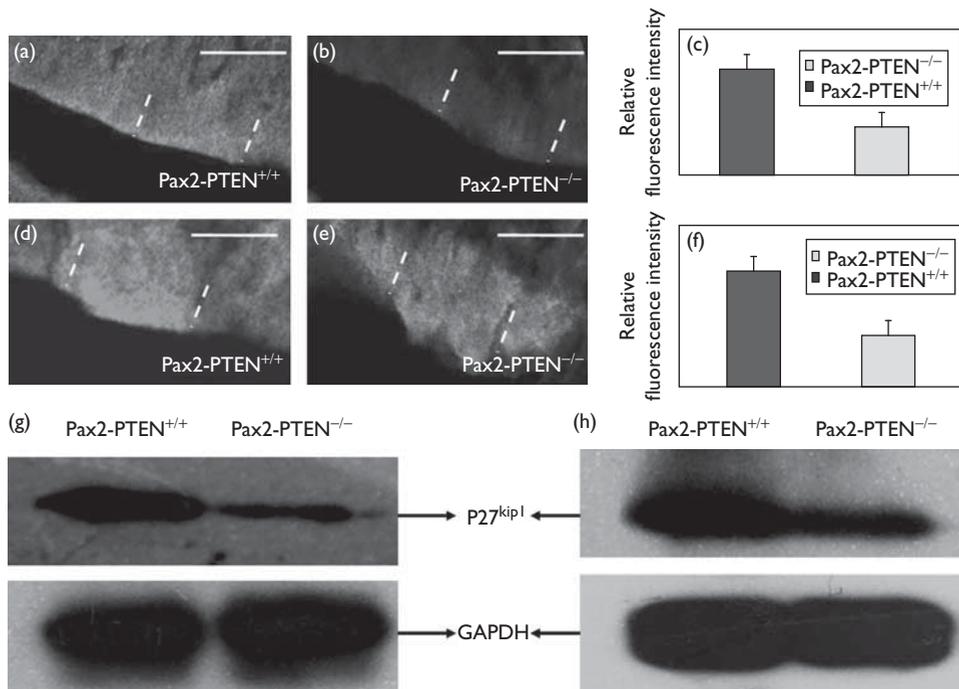
### Discussion

*PTEN* plays a crucial role during embryonic development [21]. The heterozygous *PTEN* knockout mice showed that *PTEN* regulates the proliferation of auditory progenitors. However, the molecular mechanism by which *PTEN* regulates the proliferation and differentiation of auditory progenitors remains unknown. Thus, to investi-

gate the molecular mechanism underlying *PTEN* regulation, we created *PTEN* conditional knockout in the inner ear of mice. Our results show supernumerary HCs in the *Pax2-PTEN*<sup>-/-</sup> mice. We evaluated the expression of genes that may be related to the proliferation and differentiation of auditory progenitors. The results show that the *Pax2-PTEN*<sup>-/-</sup> mice have higher p-Akt level and lower p27<sup>kip1</sup> level compared with wild-type mice at around E13.5 and E14.5.

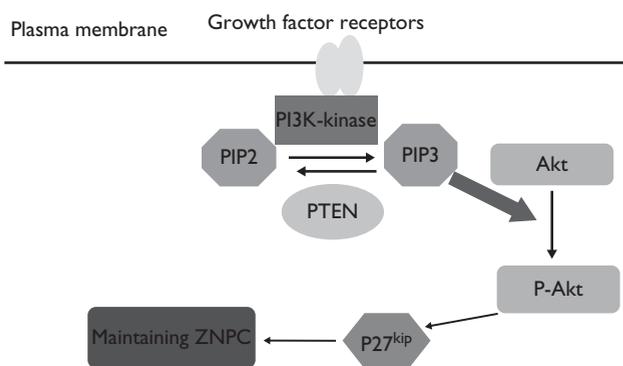
The p27<sup>kip1</sup> level is correlated with the proliferation and differentiation of auditory progenitors. From E9 to E12, little p27<sup>kip1</sup> expression occurs and all cells in the otocyst proliferate in mice [2,6]. From E12 to E14, with p27<sup>kip1</sup> expression, the auditory progenitors in the otocyst withdraw from the cell cycle, and the persistence of p27<sup>kip1</sup> maintains the progenitors in a nonproliferative state. Subsequently, the p27<sup>kip1</sup> level is downregulated during HC differentiation [7]. Nevertheless, little is known about p27<sup>kip1</sup> upstream regulators during inner ear development. Our results suggest that p27<sup>kip1</sup> is a downstream target of the *PTEN*/PI3K/Akt-signaling pathway, and it is regulated by Akt in the cochlea.

Fig. 5



*PTEN* knockout decreased the p27<sup>kip1</sup> level. (a, b) P27<sup>kip1</sup> immunolabeling in the otocyst at E13.5 (demarcated by dashed lines) showed that the p27<sup>kip1</sup> level was lower in the Pax2-*PTEN*<sup>-/-</sup> mice compared with the Pax2-*PTEN*<sup>+/+</sup> mice. Scale bar=50 μm. (c) Quantitative analysis of the relative fluorescence intensity showed that the p27<sup>kip1</sup> level was lower in the Pax2-*PTEN*<sup>-/-</sup> mice compared with the Pax2-*PTEN*<sup>+/+</sup> mice at E13.5 ( $n=10$ ;  $P<0.05$ ). (d, e) P27<sup>kip1</sup> immunolabeling in the otocyst at E14.5 (demarcated by dashed lines) showed that the p27<sup>kip1</sup> level was lower in the Pax2-*PTEN*<sup>-/-</sup> mice compared with the Pax2-*PTEN*<sup>+/+</sup> mice. Scale bar=50 μm. (f) Quantitative analysis of the relative fluorescence intensity showed that the p27<sup>kip1</sup> level was lower in the Pax2-*PTEN*<sup>-/-</sup> mice compared with the Pax2-*PTEN*<sup>+/+</sup> mice at E14.5 ( $n=10$ ;  $P<0.05$ ). (g, h) Western blot analysis of the cochlea protein at E13.5 (g) and E14.5 (h) showed a lower p27<sup>kip1</sup> level in the Pax2-*PTEN*<sup>-/-</sup> mice compared with the Pax2-*PTEN*<sup>+/+</sup> mice.

Fig. 6



Model of the PTEN/PI3K/Akt-signaling pathway for regulating the proliferation and differentiation of auditory progenitors. PI3K phosphorylates PIP2, converting PIP2 into PIP3. Conversely, PTEN dephosphorylates PIP3, converting PIP3 back into PIP2. PIP3 directly phosphorylates Akt to generate p-Akt. P-Akt phosphorylates p27<sup>kip1</sup> and triggers its degradation. The p27<sup>kip1</sup> level maintains ZNPCs in the nonproliferative state. Downregulation of p27<sup>kip1</sup> induces the differentiation of progenitors at ZNPC into HCs. Thus, *PTEN* may regulate the proliferation and differentiation of auditory progenitors through p-Akt and p27<sup>kip1</sup>. HCs, hair cells; ZNPCs, zone of nonproliferating cells.

In the *PTEN*/PI3K/Akt-signaling pathway, PIP2 is converted into PIP3 through phosphorylation by phosphatidylinositol 3-kinase (PI3K). Subsequently, Akt is converted into its activated form (p-Akt) through phosphorylation by PIP3. PIP3 can be dephosphorylated back into PIP2 by *PTEN* [9]. The *PTEN* spatiotemporal expression pattern is similar to that of p27<sup>kip1</sup> in the inner ear [16]. Loss of *PTEN* in the inner ear prevented the dephosphorylation of PIP3, which then phosphorylated more Akt and caused p-Akt accumulation. P-Akt was found to phosphorylate p27<sup>kip1</sup> and promote p27<sup>kip1</sup> degradation in cell culture [8]. Thus, p-Akt may function in a similar manner and downregulate p27<sup>kip1</sup> in the cochlea. Consequently, the p27<sup>kip1</sup> level was lower in the *PTEN* knockout mice (Fig. 6). The decreased p27<sup>kip1</sup> delayed the withdrawal of the auditory progenitors from the cell cycle. Some progenitors continued to divide. Therefore, more progenitors differentiated into supernumerary HCs.

**Conclusion**

P27<sup>kip1</sup> is expressed specifically and transiently in the inner ear of mice. p27<sup>kip1</sup> plays an important role in the

proliferation and differentiation of auditory progenitors. This study suggests that *PTEN* regulates p27<sup>Kip1</sup> through p-Akt, thereby regulating the proliferation and differentiation of auditory progenitors. *PTEN* knockout in the inner ear resulted in supernumerary HCs. Therefore, *PTEN* manipulation provides a potential strategy for regenerating HCs and treating hearing loss in mammals. (Supplementary video, Supplemental digital content 1, <http://links.lww.com/WNR/A267>).

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### Conflicts of interest

There are no conflicts of interest.

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