# Expression of nestin, parvalbumin and otoferlin during cochlear development in the mouse: an immunofluorescence study

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ABSTRACT

To elucidate the proteins associated with cochlear development and auditory formation from a histomorphological point of view, this study examined the spatio-temporal expression pattern of nestin, parvalbumin, and otoferlin in the mouse cochlea from embryonic day 17 (E17) to postnatal day 28 (P28) using immunofluorescence. Our findings revealed that nestin was broadly expressed in developing otic mesenchyme cells beneath the basilar membrane, medial to the greater epithelial ridge, and adjacent to the developing stria vascularis during late embryonic stages (E17 and E18.5). From P1 to the onset of hearing (P14), nestin was primarily expressed in fibrocytes derived from otic mesenchyme cells in the spiral ligament and spiral limbus, as well as in tympanic border cells. Dual immunofluorescence staining of nestin with Isolectin B4 (IB4), a specific vascular endothelial marker, showed the location of nestin in the blood vessels within the cochlear lateral wall. Notably, in adults (P28), nestin expression was downregulated in the fibrocytes of the spiral ligament and spiral limbus but persisted in the tympanic border cells. Parvalbumin immunolabeling was consistently observed in spiral ganglion neurons (SGNs) and inner hair cells (IHCs) from E17 through adulthood. By P1, parvalbumin expression extended to all three rows of outer hair cells (OHCs) and persisted into adulthood. Transient parvalbumin expression was also noted in afferent nerve fibers innervating the IHCs during early postnatal stages. Otoferlin labeling was predominantly detected in the cytoplasm of IHCs, with limited temporal expression in OHCs from P6 to P10. Taken together, these results illustrated the dynamic expression of nestin, parvalbumin and otoferlin during cochlear development and suggested their important function in cochlear development.

**Key words:** nestin; parvalbumin; otoferlin; immunofluorescence; mouse; cochlea.

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## Introduction

The development of the mammalian cochlea is regulated by multiple proteins, including calcium-binding proteins,[1-3] high-mobility group box 1,[4] and connexins.[5] These proteins exhibited distinct spatio-temporal expression patterns in the cochlea in previous studies, with their distribution closely linked to their functional roles, suggesting diverse mechanisms involved in cochlear development. The molecular mechanisms underlying cochlear development in rodents, which are important for studies of congenital hearing loss and regenerative medicine,[6] have been elucidated over the past few decades,[7,8] to the comprehensive knock-out and mutagenesis screens,[9,10] and latterly, the scRNAseq studies which have given us deeper understanding of how the cochlea develops and functions.[11,12] Murine cochlear frozen sectioning, a backbone of inner ear research, preserves native cellular structures and antigen immunoreactivity, making it ideal for immunohistochemical studies.[13] Immunohistochemistry is one of the most powerful tools for direct visualization of distribution and localization of gene products. In this study, we employed cryosection immunofluorescence to analyze the immunolocalization of proteins closely associated with normal auditory function -though their precise roles remained incompletely understood- including nestin, otoferlin, and parvalbumin during mouse cochlear development.

Nestin, an intermediate filament protein, co-constituted the cytoskeleton with microtubules and microfilaments to maintain the cellular morphology and elasticity.[14] It was highly expressed in mammalian neural progenitor cells and widely used as a marker for these cells.[15] Recently, nestin-positive cells were also found in other tissues such as the pancreas,[16] muscle,[17] and teeth.[18] Additionally, nestin was expressed in various malignant tumor cells, with expression intensity positively correlating with tumor malignancy and prognosis.[19-21] Previous studies reported nestin expression during early cochlear development and proposed its use as a specific markers for identifying and selecting of stem and progenitor cells in the mammalian cochlea, suggesting that nestin played essential roles in cochlear development and maturation.[22,23] However, some inconsistencies existed in reported immunohistochemical studies of nestin in the cochlea. For example, Taniguchi *et al.* reported preferential nestin expressed in the tympanic border cells of the mouse cochlea during late embryonic and early postnatal stages,[24] which was not found in the cochleae of rats and dogs.[25,26] Variability in strain, differences in staining techniques and preparation of frozen sections may account for these discrepancies. The immunolocalization of nestin in the developing cochlea had not yet been fully clarified.

Parvalbumin, a calcium-binding protein of the EF-hand family, served multiple crucial functions in biological systems. As a muscle relaxant factor, parvalbumin facilitated muscle relaxation.[27] In the nervous system, parvalbumin-positive inhibitory interneurons played a pivotal role in neuronal development and synaptic plasticity.[28,29] In the central auditory system, parvalbumin demonstrated extensive expression patterns.[30,31] Notably, during the ontogenetic development of the central auditory system, discernible alterations in parvalbumin immunoreactivity were documented.[32,33] Three calcium-binding proteins of the EF-hand family, calretinin, calbindin D28K and parvalbumin, are reported to regulate $Ca^{2+}$ influx and exocytosis in inner and outer hair cells (IHCs and OHCs) by acting as $Ca^{2+}$ buffers,[34] similar to in the brain.[35] Our prior studies separately characterized the expression patterns of calretinin and calbindin D28K in both developing and mature mouse cochleae, demonstrating their cell type-specific distribution.[1,2] In this study, we focused on the expression and developmental dynamics of parvalbumin. Studies have underscored the pivotal role of parvalbumin in auditory function by demonstrating that targeted deletion of oncomodulin-a calcium-binding protein belonging to the parvalbumin family-triggered progressive hearing loss.[36] Although parvalbumin was reported to exhibit selective expression in the HCs of the guinea pig, rat, and mouse cochlea,[37-39] its spatiotemporal expression remained unclear.

Recently, gene therapy has emerged as a highly promising approach for treating congenital deafness, particularly in Autosomal Recessive Deafness Type 9 (DFNB9).[40,41] DFNB9 is a common form of non-syndromic hereditary deafness caused by mutations in the OTOF gene.[42] The Otoferlin protein, encoded by the OTOF gene, is a C2 domain-containing $Ca^{2+}$-binding protein that plays a crucial role in IHC synaptic transmission and vestibular function.[43] Otoferlin depletion in zebrafish larvae resulted in abnormal expression of the calcium binding hair cell genes S100 and parvalbumin,[44] and otoferlin deficiency in zebrafish resulted in defects in balance and hearing.[45] Reports on the dynamic expression of otoferlin during mammalian cochlear development were scarce, despite morphological evidence supporting its specific localization in IHCs of the mature cochlea.[46-48]

In this study, we reported the expression characteristics and significance of nestin, parvalbumin, and otoferlin in the mouse cochlea at different developmental stages using immunofluorescence, which would provide morphological evidence for further functional studies on these proteins in the peripheral auditory system.

## Materials and Methods

### Animals

All animal studies, including the euthanasia procedure, were conducted in compliance with the regulations and guidelines of Southeast University's institutional animal care, adhering to the standards set by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines (approval No. 20200402025).

### Immunofluorescence

Pregnant BALB/c mice (gestational days 17-18.5) and postnatal mice (P1-P28) were anesthetized *via* intraperitoneal injection of 10% chloral hydrate at a dose of 0.2 mL/100 g (at the time when our project was funded by the National Natural Science Foundation of China, the use of chloral hydrate as an anesthetic for mice was still accepted by the Southeast University; a formal notice banning this anesthetic was released by the university only in 2024). The immunofluorescence protocol followed our previous study,[49] prioritizing transcardial perfusion for optimal frozen-section quality. Under a microscope, the heart apex of postnatal mouse was lifted with microforceps, a needle inserted 2 mm into the left ventricle, the right auricle snipped, and saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PH 7.4). After careful isolation from surrounding tissues, the cochleae were perilymphatically perfused with fixative and post-fixed in the same solution for 35 min at room temperature. Following the same protocol established for rat cochleae, mouse specimens were decalcified in 10% EDTA (pH 7.4) beginning at P5.[5,50] Following decalcification, the cochleae were immersed in a sucrose gradient (15% for 3 h and then 30% overnight). The cochlear tissues were then embedded in optimum cutting temperature compound at 4°C for 2.5 h, rapidly frozen at -20°C, and the cryoembedded specimens were sectioned into serial sections (8 μm) using a cryostat and mounted on glass slides. Cochlear cryosections were treated with a solution contain-

ing 10% donkey serum and 0.3% Triton X-100 in PBS for 45 min at room temperature to increase cell membrane permeability to antibodies. Subsequently, the sections were incubated with primary antibodies diluted in 0.01M PBS overnight or longer at 4°C. The primary antibodies used included rabbit anti-nestin antibodies (1:100, ab105389; Abcam, Waltham, MA, USA), rabbit anti-otoferlin antibodies (1:50, ab309197; Abcam), rabbit anti-parvalbumin antibodies (1:200, ab181086; Abcam), rabbit anti-neurofilament 200 antibodies (1:400, ab207176; Abcam), mouse anti-synaptophysin antibodies (1:200, #9020, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-peripherin antibodies (1:50, ab246502; Abcam), mouse anti-parvalbumin antibodies (1:500, MA5-47410; Invitrogen, Waltham, MA, USA), Sox2 Monoclonal Antibody (Btjce), Alexa Fluor™ 488 (1:100, #53-9811-82; Invitrogen), biotinylated isolectin B4 antibody (1:250, Vector Laboratories, Newark, CA, USA). In double-stained experiments, coralite 594-conjugated phalloidin (1:250, #PF00003; Proteintech, Wuhan, China) and coralite 488-conjugated phalloidin (1:250, #PF00001; Proteintech, China) was applied to detect F-actin in the hair cells of the organ of Corti. After rinsing three times for 15 min in 0.01 M PBS, the slides were incubated for 1 h at 37°C with the following secondary antibodies: donkey anti-rabbit IgG conjugated with Alexa fluor 488 or 555 (1:250; Yeasen, Shanghai, China), Streptavidin conjugated with Alexa fluor 594 (1:250; Yeasen). donkey anti-mouse IgG conjugated with Alexa 555 or 597 (1:400; Beyotime, Haimen, China). Control sections were incubated with 0.01 M PBS, without primary antibodies. Additionally, rabbit (DA1E) monoclonal antibody IgG XP Isotype control (#3900; Cell Signaling Technology) was used as a negative control. The sections were then washed with 0.01 M PBS, and fluorescence was preserved by sealing the specimens with an antifade mounting medium containing 4′,6-diamidino-2- phenylindole (Biyuntian Biotechnology, Shanghai, China). Cryostat sections were examined using a Zeiss (LSM900) laser scanning confocal microscope with 10x (NA= 0.45), 20x (NA = 0.8), 40x (NA = 0.95) and 63x oil (NA = 1.4) objectives at 1024 x1024 pixels. Zen3.0 acquisition software was used. Immunostaining presented in the figures is representative of three individual experiments. Images were cropped and resized using Adobe Photoshop CC 2019.

## Results

### Expression patterns of nestin in the mouse cochlea during development

By optimizing the protocol for frozen sectioning of cochlear tissue through refined perfusion fixation, we could achieve frozen section images that enabled precise histological analysis of developmental features in mouse cochleae. This was exemplified by clear visualization of cochlear neural markers (Neurofilament 200 and synaptophysin) in the organ of Corti (Figure 1). In this study, we investigated nestin expression patterns in the mouse cochlea from embryonic day 17 (E17) to postnatal day 28 (P28) *via* immunofluorescent staining. Nestin expression appeared as fiber-like profiles, cochlear fibrocytes deriving from the otic mesenchyme were immunopositive for nestin,[51] a gradient of nestin expression in different turns of mouse cochlea was not evident. At E17, nestin expression was observed in spatially discrete subpopulations of otic mesenchyme cells surrounding the developing cochlear epithelium, specifically in regions medial to the greater epithelial ridge, above the developing stria vascularis, and beneath the basilar membrane. No labeling was detected in other cochlear

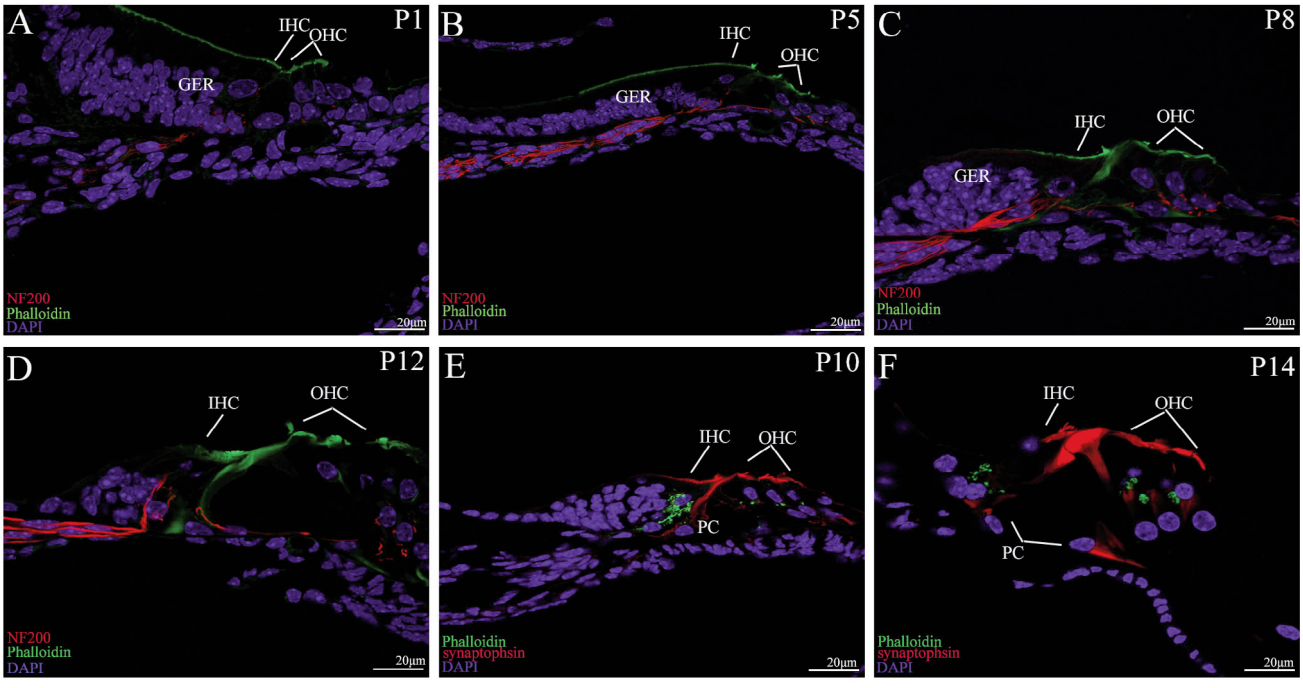


**Figure 1. A**) Detail of Neurofilament 200 (NF200) (red) immunolabeling in the middle turn of P1 mouse cochlea. **B-D**) Detail of NF200 (red) immunolabeling in the middle turn of P5, P8 and P12 mouse cochlea; NF200-positive cochlear afferent nerve fibers (red) innervated the IHC, NF200-positive cochlear afferent nerve fibers (red) projected beyond the floor of the tunnel of Corti to the OHCs. **E,F**) Detail of synaptophysin (green) immunolabeling in the middle turn of P10 and P14 mouse cochlea. presynaptic synaptophysin spots (green) were detected underneath the IHCs and OHCs. GER, greater epithelial ridge; IHC, inner hair cell; OHC, outer hair cell; PC, Pillar cells.

tissues (Figure 2 A-F). At E18.5, consistent with previous reports,[18] several-layered tympanic border cells derived from otic mesenchyme cells on the undersurface of the basilar membrane facing the scala tympani were nestin-positive. Double-labeling with nestin and isolectin B4 (IB4), a vascular endothelial marker,[52] showed that nestin-positive tympanic border cells were located near IB4-immunopositive vascular structures, such as the cochlear spiral modiolar artery. Nestin-expressing cells remained localized in the lateral strial regions and medial to the greater epithelial ridge which subsequently develops the spiral limbus (Figure 2 G-I). At P1, nestin expression emerged in Reissner's membrane. Additionally, nestin expression in fibrocytes of the spiral limbus and Reissner's membrane was prominent. Nestin was also expressed in basal cells of the nascent stria vascularis and in fibrocytes of the lateral cochlear wall adjacent to the stria vascularis. (Figure 3 A,B). Nestin remained expressed in the tympanic border cells (Figure 3C). Double-labeling with nestin and IB4 showed that partial co-expression in the cochlear spiral modiolar artery beneath the basilar membrane and vascular endothelial cells within the lateral cochlear wall (Figure 3 D-I). At P5, nestin was detected in the spiral limbus, Reissner's membrane, and tympanic border cells (Figure 4 A-C). In the cochlear lateral wall, nestin was localized to IB4-labeled capillaries within the stria vascularis. Additionally, nestin-positive fibrocytes of the spiral ligament extended beneath the spiral prominence (Figure 4 D-F). By P8, during the critical window of mouse cochlear development, the expression of nestin remained stable in both tympanic border cells and the spiral limbus. Nestin expression spreaded widely throughout most regions of

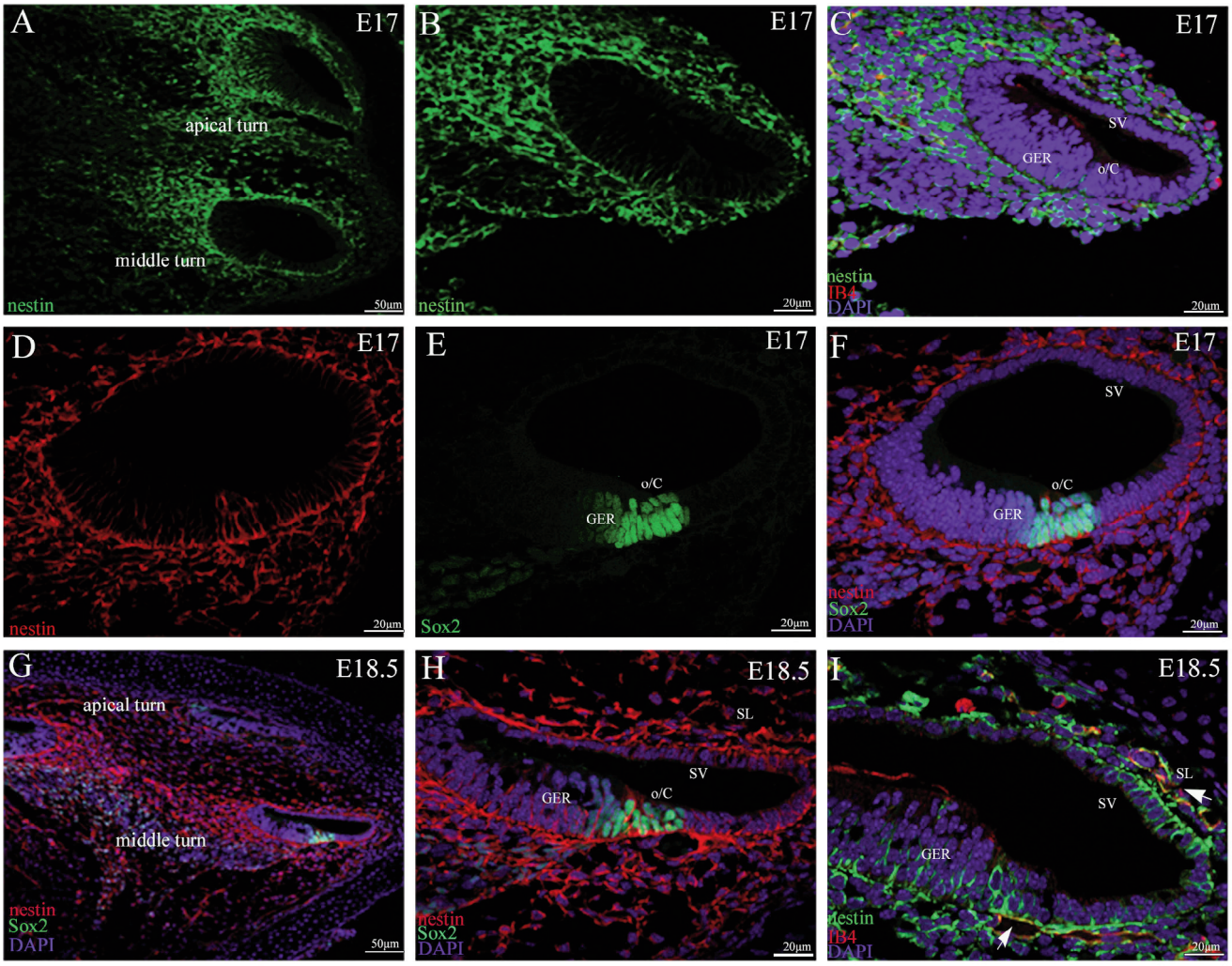


**Figure 2.** Nestin immunolabeling in the mouse cochlea at E17 and E18.5. **A**) An overview of cross-sections of the E17 mouse cochlea labeled with nestin (green), nestin-positive cells occurred in the apical turn of E17. **B,C**) Otic mesenchyme cells medial to the greater epithelial ridge and above the developing stria vascularis were positive for nestin; nestin-positive fibrocytes surrounded the E17 cochlear epithelium; double-labeling with nestin and IB4 revealed that nestin was rarely expressed in IB4-positive vascular endothelial cells. **D-F**) Double-labeling with nestin and Sox2 revealed that nestin-expressing cells were localized beneath the basal region of Sox2-immunoreactive the greater epithelial ridge. **G**) An overview of cross-sections of the E18.5 mouse cochlea labeled with nestin (red); nestin-positive fibrocytes (red) surrounded the E18.5 cochlear epithelium. **H**) Double-labeling with nestin and Sox2 demonstrated that nestin-expressing cells were localized not only beneath the Sox2-immunoreactive greater epithelial ridge but also extended into its basal region. **I**) Double-labeling with nestin and IB4 revealed that IB4-posiotive cochlear spiral modiolar artery was located on the undersurface of the basilar membrane, and nestin-positive cells were located near cochlear spiral modiolar artery (arrowheads) and IB4-posotive vascular endothelial cells (arrowheads) within the lateral wall. Fibrocytes in the spiral ligament was labelled for nestin. SV, stria vascularis; o/C, organ of Corti; SL, the spiral ligament; GER, greater epithelial ridge.

the spiral ligament. Nestin also labeled the endothelium of capillaries in the lateral wall (Figure 4 G-L). No discernible nestin immunolabeling was observed in spiral ganglion neurons (SGNs) (*Supplementary Figure S1)*. A similar pattern was observed at P14, with tympanic border cells labeled by nestin transforming from multilayered to single-cell layer structure. Meanwhile, other regions, including intrastrial capillaries, spiral ligament, and spiral limbus, continued to express nestin (Figure 5 A-F). In adult, nestin expression was downregulated in the fibrocytes of the lateral wall and spiral limbus. Only a few fibrocytes in the spiral ligament were labeled for nestin, In the spial limbus and the lateral wall blood vessels, staining for nestin was barely visible, but it remained evident in the tympanic border cells below the organ of Corti (Figure 5 G-K). In double-labeling controls, neither anti-rabbit nor anti-mouse IgG produced detectable signal (Figure 5L), and substitution of the primary antibody with rabbit IgG isotype control yielded no staining (*data not shown*).

## Expression patterns of parvalbumin and otoferlin in the mouse cochlea during development

We next investigated the expression patterns of two calcium-binding proteins, parvalbumin and otoferlin, in the developing and mature mouse cochlea using confocal immunofluorescence. In the apical turn of E17 mouse cochlea, parvalbumin was only expressed in the SGNs (Figure 6 A-C). In the middle turn of E17, the expression of parvalbumin occurred in both the IHCs and SGNs. Double-labeling with parvalbumin and Sox2, a marker for hair cell progenitors and supporting cells,[4] demonstrated that parvalbumin was absent from Sox2-stained IHC nuclei (Figure 5 D-F). By E18.5, the expression of parvalbumin emerged in the IHCs and one rows of OHCs (Figure 5 G-I). The cytoplasmic regions of parvalbumin-positive HCs did not overlap with Sox2-stained HC nuclei. At P1, three rows of OHCs were immunolabelled for parvalbumin, including the cytoplasm and the cuticular plates (Figure 6 J-L). Between P5 and P8, parvalbumin immunolabeling was observed in

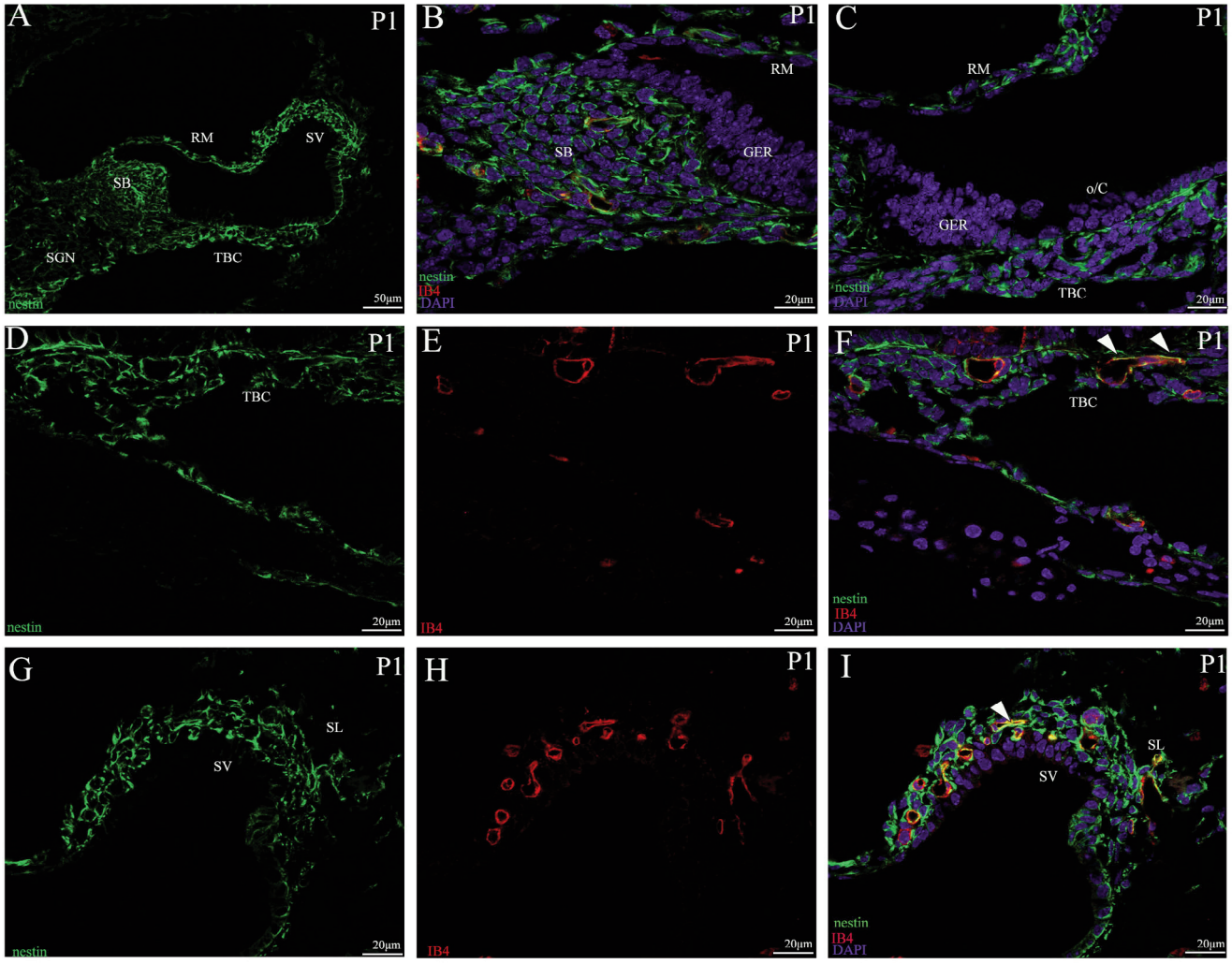


**Figure 3.** Nestin immunolabeling in the mouse cochlea at P1. **A**) An overview of cross-sections of the P1 mouse cochlea labeled with nestin (green) at P1. At P1, nestin began to be expressed in the Reissner's membrane. **B,C**) At P1, nestin expression was found in spiral limbus anchoring Reissner's membrane, as well as in the tympanic border cells beneath the basilar membrane. **D-I**) Double-labeling with nestin and IB4 showed that nestin was coexpressed partly (shown in yellow) with IB4 in the cochlear spiral modiolar artery (arrows) and vascular endothelial cells (arrows) within the lateral wall. Nestin was also expressed in the fibrocytes of the spiral ligament. SV, stria vascularis; o/C, organ of Corti; SL, the spiral ligament; GER, greater epithelial ridge; SB, the spiral limbus; RM, Reissner's membrane; TBC, tympanic border cells.

the afferent fibers innervating the IHCs. Parvalbumin continued to be expressed in the IHCs and OHCs, as well as the SGNs (Figure 7 A-E). From P14 through P28, parvalbumin was no longer detectable in the afferent fibers, and remained expressed in the HCs and SGNs (Figure 7F-H). Parvalbumin was not colocalized with peripherin in the adult type II SGNs (Figure 7I).

During the late embryonic stages, a lack of otoferlin expression in the mouse cochlea (*data not shown*). At P1, otoferlin was

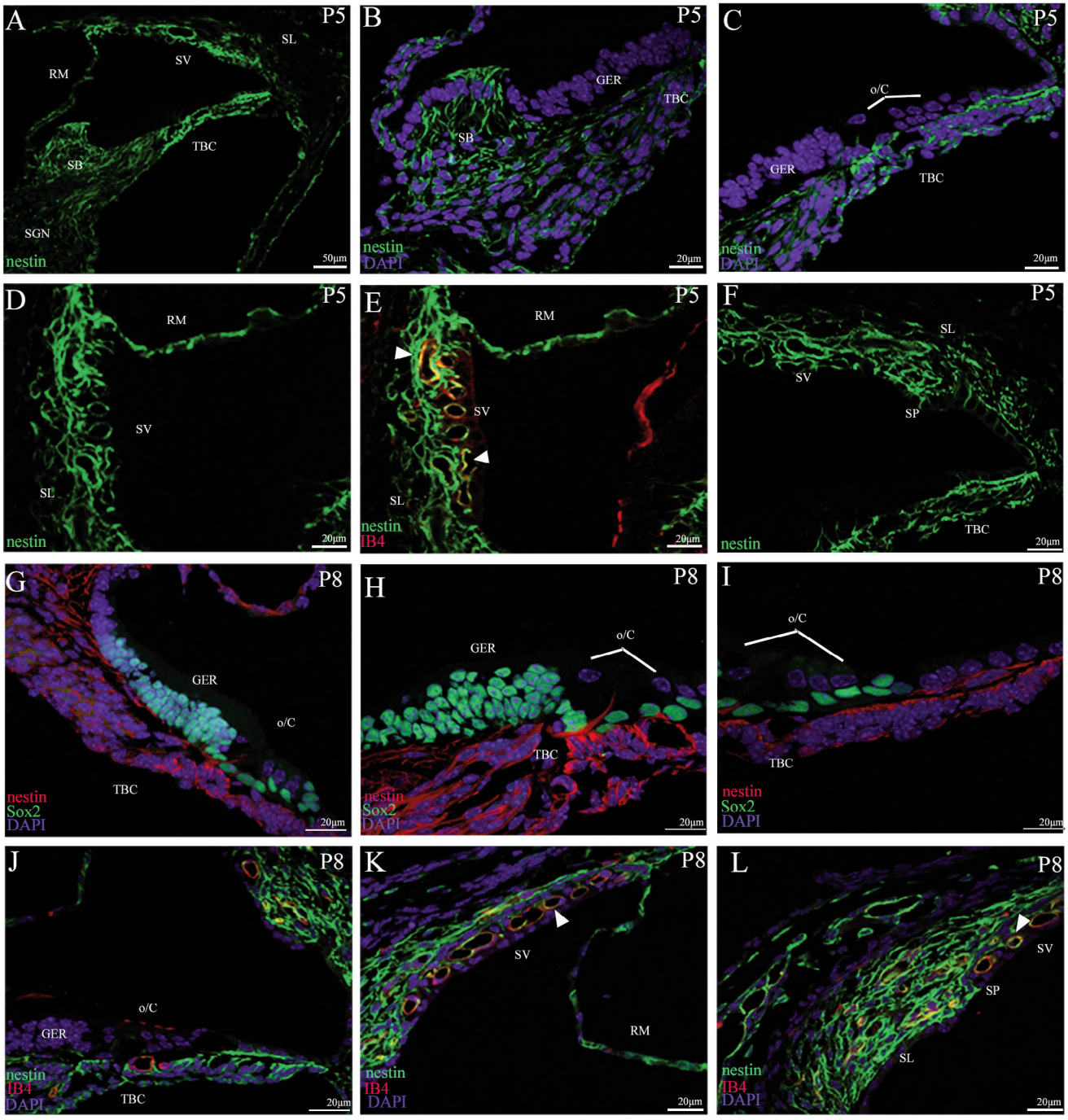


**Figure 4.** Nestin immunolabeling in the mouse cochlea at P5 and P8. **A**) A low-magnification view of cross-sections of the P5 mouse cochlea labeled with nestin (green) at P5, nestin was expressed in the Reissner's membrane. **B,C**) At P5, nestin was expressed in the fibrocytes of spiral limbus and the tympanic border cells. **D-F**) In the lateral wall of P5, nestin was expressed in type II fibrocytes in the spiral prominence; double-labeling with nestin and IB4 in the lateral wall of P5 showed that some vascular endothelial cells within the stria vascularis were double-labelled (shown in yellow) by nestin. **G-I**) Nestin immunolabeling in the mouse cochlea at P8; double-labeling with nestin and Sox2 revealed that nestin-labeled tympanic border cells included the undersurface of Sox2-labeled the greater epithelial ridge and supporting cells of the organ of Corti, and the undersurface of lateral region of supporting cells. **J**) At P8, double-labeling with nestin and IB4 showed nestin-labeled tympanic border cells were located near IB4-labeled the spiral vessel. **K-L**) At P8, nestin was broadly expressed in the fibrocytes of the spiral ligament; IB4 -immunopositive the endothelium of the capillaries (arrows), localized to the lateral wall, were coexpressed partly with nestin. SV, stria vascularis; o/C, organ of Corti; SL, the spiral ligament; GER, greater epithelial ridge; SB, the spiral limbus; RM, Reissner's membrane; TBC, tympanic border cells; SP, the spiral prominence.

weakly expressed in the HCs (Figure 8 A-C). At P6, otoferlin expression occurred in both IHCs and OHCs of the organ of Corti, otoferlin was detected throughout the cytoplasm of the IHCs, from apex (below the cuticular plate) to base. In the OHCs, otoferlin immunolabeling was largely distributed the lower end of the OHCs (Figure 8 D-F). At P10, both IHCs and three rows of OHCs retained otoferlin expression. Double-labeling with otoferlin and phalloidin revealed that otoferlin immunoreactivity was not pre-

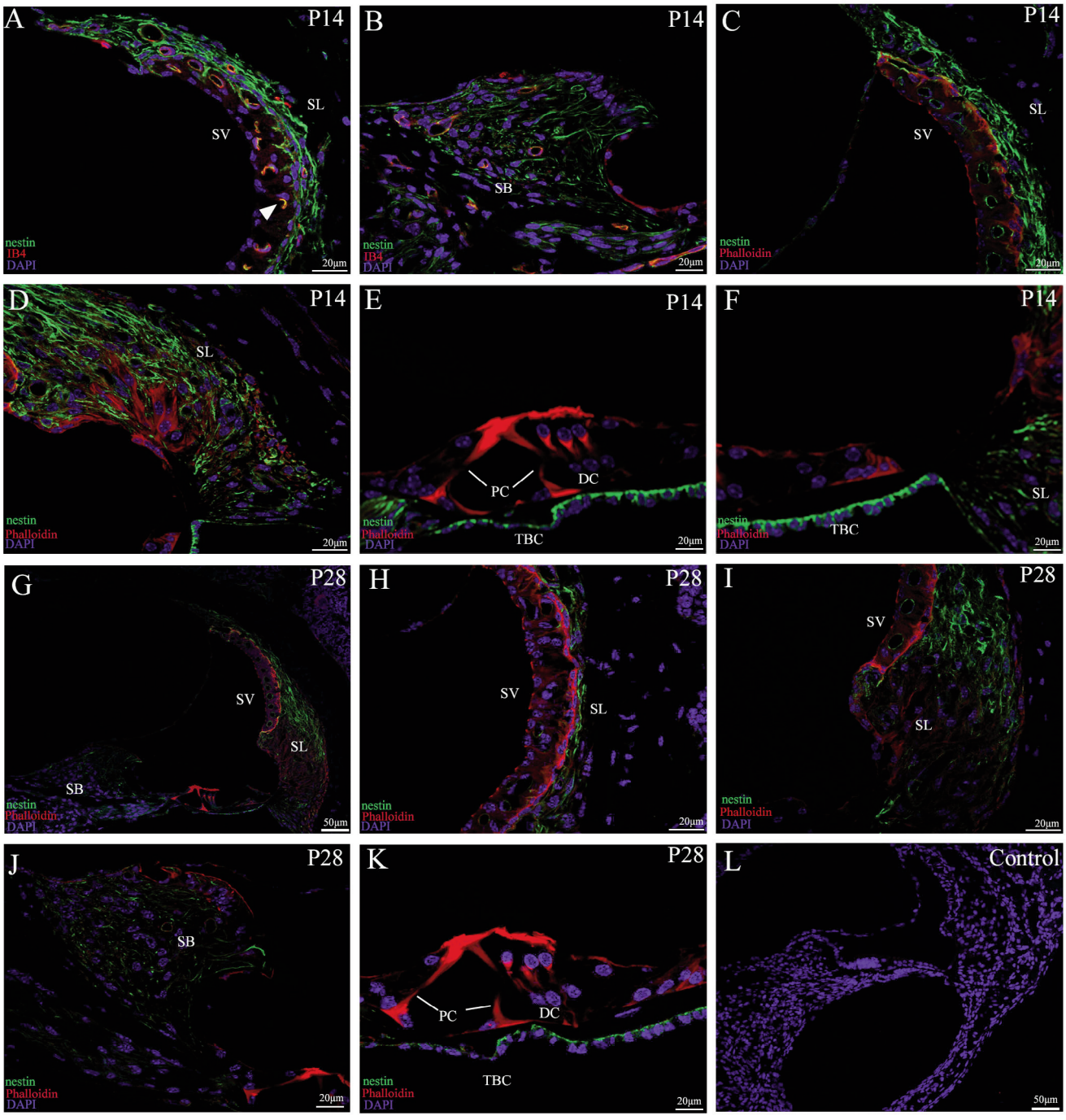


**Figure 5.** Nestin immunolabeling in the mouse cochlea at P14 and P28. **A,B**) At P14, nestin expression persisted in the IB4-positive intrastrial capillaries (arrows) and fibrocytes of the spiral limbus. **C,D**) Double staining with phalloidin (red) and nestin (green) revealed absence of nestin in phalloidin-labeled basal cells within the stria vascularis; nestin expression was concentrated in spiral ligament fibrocytes; note the root cell processes penetrated the spiral ligament, marked by phalloidin (red), are unlabeled for nestin. **E,F**) Nestin-positive, one-layered tympanic border cells were located beneath the footplates of the two pillar cells and beneath the lateral region of supporting cells. **G-J**) At P28, low-magnification images showed nestin labeling in a few fibrocytes of the spiral ligament, with weak expression in intrastrial capillaries and spiral limbus fibrocytes. **K**) Nestin-positive cells was observed in tympanic border cells beneath the footplates of the two pillar cells and the bases of three rows of Deiters' cells. **L**) No immunofluorescence labeling was detected in negative controls omitting the primary antibody. SV, stria vascularis; SL, the spiral ligament; SB, the spiral limbus; TBC, tympanic border cells; PC, pillar cells; DC, Deiters' cells.

sent in phalloidin-labeled the cuticular plates of HCs. Otoferlin immunolabeling was evenly distributed in most of the hair cell cytoplasm (Figure 8 G-I). However, no otoferlin labeling was observed in the OHCs from P14 to P28, otoferlin expression was constrained to the IHCs (Figure 8 J-O).

## Discussion

In the mammalian cochlea, HCs and supporting cells in the organ of Corti, as well as mesenchymal cells, contribute to proper auditory function through the expression of various functional pro-

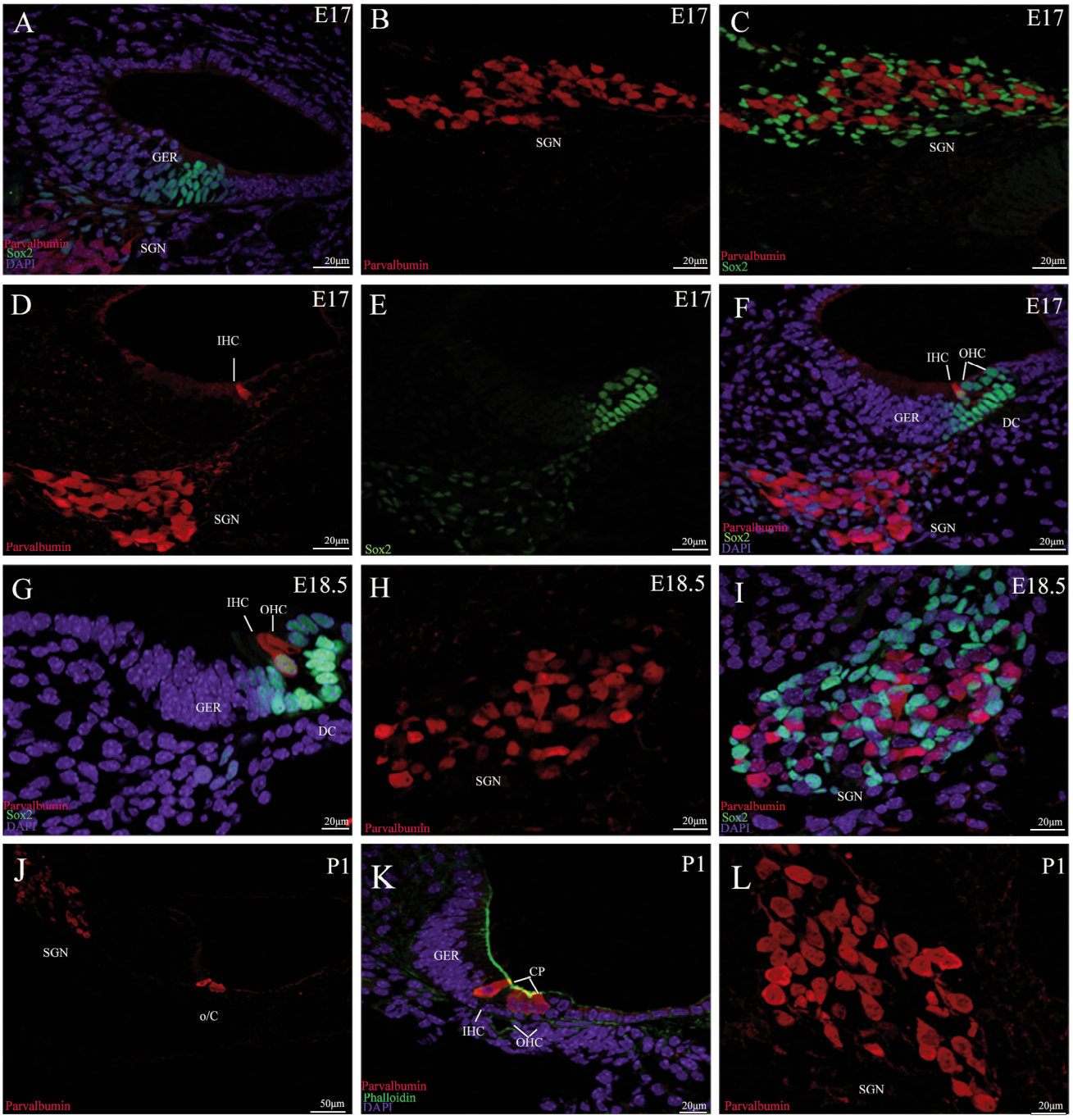


**Figure 6.** Parvalbumin immunolabeling in the mouse cochlea at E17, E18.5, and P1. **A**) In the apical turn of E17, parvalbumin labeling (red) was detected exclusively in the SGNs. **B,C**) Double labeling of parvalbumin with Sox2 showed parvalbumin-positive SGNs surrounded by Sox2-positive glial cells. **D-F)** In the middle turn at E17, Sox2 labeled HCs and Deiters'cell nuclei in the organ of Corti, while parvalbumin expression was observed in the IHCs and SGNs. **G-I**) At E18.5, double labeling with parvalbumin and Sox2 revealed parvalbumin expression in the IHCs, one row of OHCs, and SGNs. **J-L**) At P1, parvalbumin was expressed in the IHCs and three rows of OHCs in the organ of Corti; double-labeling with parvalbumin and phalloidin showed colocalization in the cuticular plates of both IHCs and OHCs; parvalbumin expression persisted in the SGNs. GER, greater epithelial ridge; SGNs, spiral ganglion neurons; o/C, organ of Corti; IHC, inner hair cell; OHC, outer hair cell; CP, cuticular plates; DC, Deiters' cells.

teins. To characterize proteins important for cochlear function, this morphological study examined the expression patterns of nestin, parvalbumin, and otoferlin in the mouse cochlea at different developmental stages. Using immunofluorescence, our results revealed widespread nestin immunoreactivity in structures derived from otic mesenchyme cells during the early stages of cochlear development, including tympanic border cells, the spiral limbus, and the lateral wall adjacent to the stria vascularis. These structures are known to be essential for normal cochlear development and hearing.[53] Additionally, we first demonstrated that the non-epithelial distribution of nestin was developmentally regulated, as evidenced by its downregulation in fibrocytes of the spiral ligament and spiral limbus in the adult. Consistent with previous studies, nestin was specifically expressed in tympanic border cells, which have been identified as slow-cycling cells in the developing cochlea.[24] In adults, nestin expression persisted in these cells. Some nestin-expressing cells in the adult cochlea have been proposed to indicate intrinsic repair potential, albeit to a more limited extent than during early postnatal stages.[54] It has been postulated that nestin-positive cells might serve as a source for newly formed HCs and supporting cells in the injured postnatal organ of Corti.[55] Several studies have reported changes in nestin expression in the mammalian cochlea following kanamycin ototoxicity and noise exposure.[56,57] These findings suggest that the presence and distribution of nestin may be essential for hearing. Notably, this study detected specific nestin expression in vascular endothelial cells of the mouse cochlea. Nestin is prominently expressed in vascular endothelial cells, where it plays a key role in blood vessels formation and maintenance and is recognized as a marker for neovascular endothelial cells.[58] It demonstrates robust expression across multiple tissues and is crucial for angiogenesis.[59,60] It is well known that blood vessels formation is closely linked to organ development. Suzuki *et al.* reported that nestin was expressed in vascular endothelial progenitor cells but not in mature endothelial cells, proposing it as a marker of neovascularization.[61] Moreover, nestin expression accompanies tumor angiogenesis, highlighting its potential as a marker for tumor neovascularization.[62,63] Our findings revealed extensive nestin expression in the microvasculature

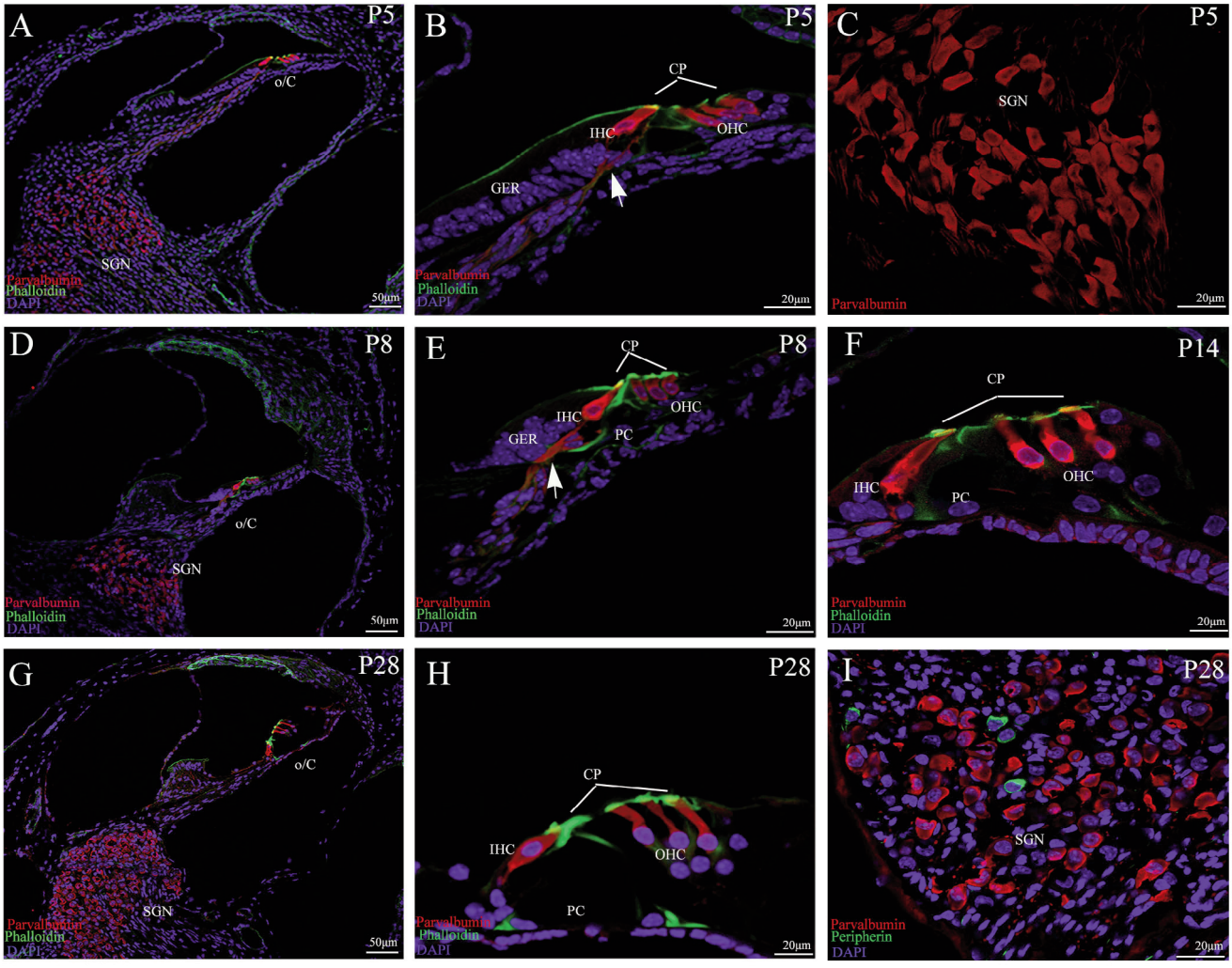


**Figure 7.** Parvalbumin immunolabeling in the mouse cochlea at P5, P8, P14 and P28. **A-C**) At P5, parvalbumin was expressed in the IHCs, three rows of OHCs, and SGNs; parvalbumin immunolabeling was also detected in afferent nerve fibers (arrows) innervating the base of the IHCs. **D,E**) At P8, parvalbumin expression persisted in the IHCs, OHCs, and SGNs, with immunoreactive nerve fibers (arrows) contacting the IHC base. **F-I**) At P14 and P28, parvalbumin labeling in afferent nerve fibers was absent, but expression remained in IHCs, OHCs, and SGNs. Double labeling of parvalbumin (red) and peripherin (green) in adult SGNs showed no colocalization of parvalbumin with peripherin-positive type II SGNs. GER, greater epithelial ridge; SGNs, spiral ganglion neurons; IHC, inner hair cell; OHC, outer hair cell; CP, cuticular plates; DC, Deiters' cells; PC, pillar cells; o/C, organ of Corti.

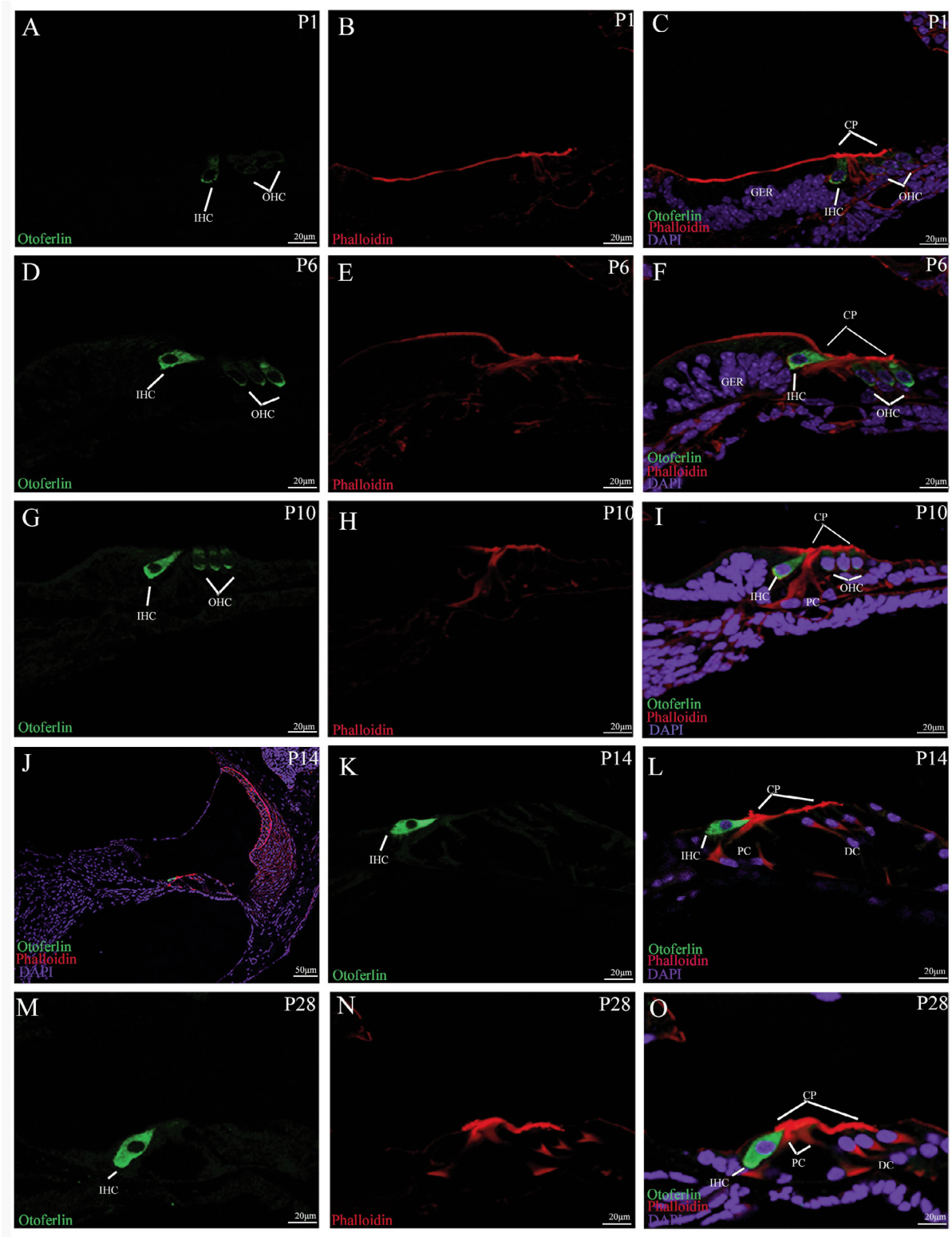


**Figure 8.** Otoferlin immunolabeling in the mouse cochlea at P1, P6, P10, P14, and P28. **A-C**) At P1, double-labeling with otoferlin (green) and phalloidin (red) revealed weak otoferlin expression in the IHCS and OHCs. **D-F**) At P6, otoferlin (green) was expressed in the IHCs and three rows of OHCs, with labeling observed throughout most of the IHCs cytoplasm and predominantly at the basal regions of the OHCs; no otoferlin immunoreactivity was observed in the pillar cells and Deiters' cells. **G-I**) At P10, otoferlin (green) expression persisted in the cytoplasm of both IHCs and OHCs; double-labeling showed no colocalization of otoferlin with phalloidin in the cuticular plates of HCs. **J**) An overview of cross-sections of the P14 mouse cochlea labeled with otoferlin (green); otoferlin expression was only detected in the IHCs. **K,L**) At P14, otoferlin immunolabeling in the organ of Corti was restricted to the cytoplasm of the IHCs. **M-O**) In the adult cochlea, otoferlin was distributed homogeneously throughout the IHC somata, while the cuticular plates remained unlabeled. GER, greater epithelial ridge; SGNs, spiral ganglion neurons; IHC, inner hair cell; OHC, outer hair cell; CP, cuticular plates; DC, Deiters'cells; PC, pillar cells.

of the cochlear lateral wall during the early postnatal period and around the onset of hearing, but its expression was virtually undetectable in the adult cochlea. These results suggested that nestin was associated with cochlear vascular development and thus played a role in cochlear maturation.

Calcium-binding proteins, widely distributed in various cells,[64] are involved in numerous functional roles in the cochlea.[3] Our findings on the distribution of the calcium-binding protein parvalbumin immunoreactivity showed that cells positive for parvalbumin were has the earliest onset at E17, first detectable in the IHC. Parvalbumin immunoreactivity in IHCs and three rows of OHCs cannot be clearly seen until P1. In the neonatal cochlea, the role of parvalbumin was proposed to regulate the spontaneous $Ca^{2+}$ signaling and the maturation of OHC afferent innervation in neonatal OHCs, possible through its inimitable $Ca^{2+}$ buffering capacity.[65,66] Parvalbumin exhibited distinct temporal expression pattern in HCs compared with calbindin-D28K and calretinin.[1,2] It was detected earlier in HCs than these two proteins, further supporting its role as an early marker for HCs. As the predominant calcium-binding proteins of the SGNs,[67] parvalbumin was also observed to be consistently expressed in the SGNs at all the ages examined. It has been postulated that the function of EF-hand calcium-binding proteins in type I SGNs may protect against deleterious consequences of $Ca^{2+}$ loading.[68] The expression distribution of parvalbumin in SGNs was similar with calbindin D28K and calretinin, it could thus be hypothesized that these three proteins may function in a synergistic manner. We observed stable parvalbumin expression in SGNs and HCs, suggesting that parvalbumin might play an essential role in auditory transmission. Additionally, transient parvalbumin expression in cochlear afferent fibers innervating the IHC indicated a possible role in the afferent innervation.

A variety of functional proteins were needed for maintaining proper $Ca^{2+}$ concentration in cochlear hair cells. Otoferlin, working as a $Ca^{2+}$-dependent mechanical sensor, has been implicated in $Ca^{2+}$-triggered synaptic-vesicle exocytosis in auditory hair cells and was known to interact with myosin VI.[69-71] At vestibular hair cell ribbon synapses, otoferlin was critical for a highly sensitive and linear calcium-dependent exocytosis.[72] Consistent with previous studies, otoferlin was selectively expressed in HCs of the organ of Corti during cochlear development and maturity.[35-37] By immunofluorescence staining of otoferlin on mouse cochlear cryosections, our results provided homogeneous labeling of otoferlin the cytoplasmic region of IHCs and its lack of expression in the cuticular plate and the stereocilia of IHCs. Age-related change in the expression of otoferlin was also found in IHCs during the onset of hearing-absent in late embryonic stages, weak at P1, obvious at P6, and persisting into adulthood, suggesting a possible role of otoferlin in postnatal development and maturation of the IHCs. It has been proposed that type I spiral ganglion neuron functional maturation depended on otoferlin-dependent IHC exocytosis during pre-hearing stages of development.[73] Similar to previous findings in the rat cochlea,[37] our results showed transient otoferlin expression in the developing OHCs, its expression vanished in parallel with the onset of hearing. In immature OHCs, otoferlin is also proposed to be important for synaptic exocytosis.[74] However, in hypothyroid mice, otoferlin expression persisted in the OHCs.[75]

This study examined the expression patterns of nestin, parvalbumin, and otoferlin in developing and adult mouse cochlear cryosections using immunofluorescence. Nestin was specifically expressed in fibrocytes of the spiral ligament and spiral limbus, blood vessels within the cochlear lateral wall, and tympanic border cells. Parvalbumin immunolabelling was observed in the SGNs from E17 to adult, and in IHCs and three rows of OHCs from birth to adult, with transient expression also observed in the cochlear nerve fibers. Otoferlin immunolabeling was preferentially detected in IHCs and transiently observed in OHCs during a limited developmental window from P6 to P10. By elucidating the spatiotemporal expression profiles of nestin, parvalbumin, and otoferlin in mouse cochlea across various developmental stages, this study provided theoretical insights into the mechanisms underlying auditory system development.

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*Supplementary Online Material*

*Figure S1. Nestin immunolabeling in the mouse cochlea at P8.*

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