Developmental expression of high-mobility group box 1 (HMGB1) in the mouse cochlea

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ABSTRACT

The expression changes of high-mobility group box 1 (HMGB1) in the mouse cochlea have recently been implicated in noise-induced hearing loss, suggesting that HMGB1 participates in regulating cochlear function. However, the precise role of HMGB1 in the auditory system remains largely unclear. This study aimed to investigate its function in the developing mouse cochlea by examining the expression pattern of HMGB1 in the mouse cochlea from embryonic day (E) 18.5 to postnatal day (P) 28 using double immunofluorescence on frozen sections. Our findings revealed that HMGB1 was extensively expressed in the cell nucleus across various regions of the mouse cochlea, including the organ of Corti. Furthermore, its expression underwent developmental regulation during mouse cochlear development. Specifically, HMGB1 was found to be localized in the tympanic border cells at each developmental stage, coinciding with the gradual anatomical transition in this region during development. In addition, HMGB1 was expressed in the greater epithelial ridge (GER) and supporting cells of the organ of Corti, as validated by the supporting cell marker Sox2 at P1 and P8. However, at P14, the expression of HMGB1 disappeared from the GER, coinciding with the degeneration of the GER into the inner sulcus cells. Moreover, we observed that HMGB1 co-localized with Ki-67-positive proliferating cells in several cochlear regions during late embryonic and early postnatal stages, including the GER, the tympanic border cells, cochlear lateral wall, and cochlear nerves. Furthermore, by dual-staining Ki-67 with neuronal marker TUJ1 or glial marker Sox10, we determined the expression of Ki-67 in the neonatal glial cells. Our spatial-temporal analysis demonstrated that HMGB1 exhibited distinct expression patterns during mouse cochlear development. The co-localization of HMGB1 with Ki-67-positive proliferating cells suggested that HMGB1 may play a role in cochlear development.

Key words: HMGB1; immunofluorescence; expression; mouse; cochlea; development.

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Introduction

High-mobility group (HMG) proteins are non-histone nuclear DNA-binding proteins, classified into three families: HMGA, HMGB, and HMGN.[1,2] Among these families, the HMGB protein family is the most abundant, consisting of three members (HMGB1-3), each containing two "box" domains (A and B box) in the N-terminus.[3] HMGB1, the most extensively studied and abundant HMG protein, is a 25-kDa protein composed of 215 amino acids. HMGB1 can be actively secreted or passively released from necrotic cells in response to infection, injury and cellular stress. When passively released, HMGB1 acts as a danger-associated molecular pattern (DAMP) molecule protein by binding to receptors for advanced glycation end-products (RAGE) and Toll-like family of receptors (TLRs).[4-7] It has also been found that the release of HMGB1 from supporting cells of the organ of Corti is involved in an inflammatory process in the amikacin-poisoned rat cochlea.[8] Besides its pathological role, HMGB1 has been proposed to have many potential roles, acting as a transcription factor and growth factor involved in various biological processes, including transcription,[9] replication, DNA repair, and recombination.[10,11] Studies have suggested that HMGB1 has a critical role in the development of the central nervous system.[12] Loss of HMGB1 *in vivo* impairs neural progenitor cell survival and proliferation.[13,14] HMGB1 is also involved in other developmental processes, such as spinal cord regeneration, endothelial cell proliferation, osteogenic differentiation, and myogenesis in other cell types.[15-18]

HMGB1 displays a complex temporal and spatial distribution pattern during tooth development and in the mouse brain.[19,20] Besides HMGB1, another member of the high mobility group family, HMGA2, has been proposed to possibly participate in inner ear development, based on its extended and overlapping expression with the stem cell and cochlear supporting-cell marker Sox2.[21] Previous data have indicated changes in HMGB1 expression during the development of the mouse cochlea, suggesting that HMGB1 may participate in the development of the mouse cochlea.[22] Two recent studies have shown that abnormal HMGB1 expression in the mouse cochlea is associated with noise-induced hearing loss and contributes to the ototoxicity of cisplatin to the inner ear.[23-25] In a recent study, Xiao *et al*. reported on the cytoplasmic accumulation of HMGB1 in cochlear hair cells, which mediated noise-induced cochlear damage.[26]

While the crucial role of HMGB1 in the cochlea was confirmed, its exact function of HMGB1 in the mammalian auditory systems remained ambiguous. In the present study, we conducted further investigations into the immunolocalization of HMGB1 in the mouse cochlea from embryonic day (E)18.5 to postnatal day (P) 28, utilizing double immunofluorescence histochemistry. Before the onset of hearing (approximately P14),[27] our findings revealed co-localization of HMGB1 with Sox2 in the supporting cells of the greater epithelial ridge (GER), a temporary structure during cochlea development.[28] As the hearing commenced, both HMGB1 and Sox2 expression ceased in the GER, aligning spatially and temporally with the degeneration of the GER into the inner sulcus cells by the second postnatal week. Furthermore, we observed specific and stable expression of HMGB1 in the tympanic border cells, located on the lower side of the basilar membrane, gradually transforming from a three- to four-cell layered structure at P1 into a one-cell layer structure in adulthood.[29] Notably, this study first reported that HMGB1 co-localizes with the proliferation markers Ki-67 in several regions of the late embryonic and early postnatal mouse cochlea, including the GER, tympanic border cells, cochlear lateral wall, and auditory nerves, where cochlear stem cells were identified. We confirmed the presence of nerve cells expressing Ki-67 within the osseous spiral lamina and spiral ganglion through dual-immunostaining for Ki-67 with the glial marker Sox10.[30] Overall, these results suggest that HMGB1 might have a vital role in cochlear development.

Materials and Methods

Animals

The husbandry and management of animals were approved by the Animal Use and Care Committee of Southeast University (approval No. 20200402025). All animal procedures conform to international bioethical guidelines. After mating, gestational age was counted as E1 when a vaginal plug was detected. Individual embryos were staged according to standard mouse development.

Immunofluorescence

BALB/c mice aged from E18.5 P28 were used in the present study. Pregnant (gestational day 18.5) and P1-P28 BALB/c mice were anesthetized using 10% chloral hydrate (0.2 mL/100 g mouse weight). Then, postnatal mice were intracardially perfused using normal saline and ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). Embryonic mice were immediately decapitated, and the cochleae were dissected. The detailed methods and procedures of immunofluorescence staining were described in previous publications.[31,32] Briefly, the cochleae were flushed with the fixative solution through the oval window and then fixed in 4% paraformaldehyde for 35 min at room temperature. The cochlea of mice older than P5 was decalcified in 10% EDTA at 4°C. In our protocol, P14 mouse cochlea was decalcified in 10% EDTA solution at 4°C for 6 h. The cochleae were washed with 0.01M phosphate-buffered saline (PBS; pH 7.4) for 5 min. The cochleae were processed in 15% sucrose for 3 h and then 30% sucrose for 12 h. After air-drying for 3 min, the cochleae were mounted in an optimum cutting temperature compound (OCT) at 4°C 3 h, and frozen at -80°C.

Cochlear tissue was cryosectioned at 6 μm thickness using a Leica (Wetzlar, Germany) cryostat microtome. The sections were incubated with 0.01M PBS containing 10% donkey serum and 0.3% Triton X-100 for 35 min at room temperature. Next, sections were incubated with primary antibodies in 0.01M PBS overnight or longer at 4°C. Primary antibodies used were as follows: rabbit anti-HMGB1 polyclonal antibody (1:200, Abcam, Cambridge, MA, USA), goat anti-Sox2 polyclonal antibody (1:200, Santa Cruz Biochemicals, Dallas, TX, USA), rat anti-Ki-67 monoclonal antibody (1:100; Invitrogen, Waltham, MA, USA), biotinylated-isolectin B4 antibody (1:250, Vector Laboratories, Newark, CA, USA), mouse anti-TUJ1 monoclonal antibody (1:200, Millipore, Burlington, MA, USA), rabbit anti-Sox10 monoclonal antibody (1:50, Abcam). After rinsing with 0.01M PBS three times for 15 min, the cryosections were incubated for 1 h at room temperature with the corresponding secondary antibodies: Alexa fluor 555 donkey anti-rabbit IgG (1:250, Yeasen Biotechnology, Shanghai, China), Alexa fluor 488 donkey anti-rabbit IgG (1:250, Beyotime, Haimen, China), Alexa fluor 488 donkey anti-goat IgG (1:250, Beyotime), Alexa fluor 488 donkey anti-rat IgG (1:250, Beyotime), Streptavidin conjugated with Alexa fluor 488 (1:250, Yeasen). Coralite488-conjugated Phalloidin was also used (1:250, Proteintech, Wuhan, China). Rabbit IgG control polyclonal antibody (Proteintech) and omission of the primary antibodies assessing non-specific secondary antibody binding was used as a negative control. After washing with 0.01M PBS three times for 5 min, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1:600, Biyuntian, Beijing, China) for 5 min to visualize cell nuclei. Cryostat sections were observed and photographed with a LeicaSP8 laser scanning confocal microscope with 63X

(NA=1.4) oil-immersion objectives or a Zeiss (LSW900) laser scanning confocal microscope with 20X (NA=0.8), 40X (NA=0.95) and 63X (NA=1.4) oil-objectives at 1024 by 1024 pixels, LAS AF Version 3.2.1.9702 acquisition software and Zen3.0 acquisition software was used. Immunostaining presented in the figures was representative of three individual experiments. Images were cropped and resized using Adobe Photoshop CC 2019.

Results

Co-localization of HMGB1 and Sox2 in the mouse organ of Corti at P1, P8, P14 and P28

This study employed double-labeling immunofluorescence to examine the distribution and expression of HMGB1 on cryosections of the mouse cochlea at various developmental stages. In the P1 auditory epithelium, immunofluorescence staining for HMGB1 was observed in the nucleus, and nuclear labeling for HMGB1 in the GER was identified by double-labeling with supporting-cell marker Sox2. Almost a complete co-localization of HMGB1 and Sox2 was observed. HMGB1 also co-expressed with Sox2 in the Deiters' cells and pillar cells of the organ of Corti. Positive expression of HMGB1 was observed in three-to-four-cell layered tympanic border cells located on the lower side of the basilar membrane (Figure 1 A-C). At P8, co-localization of HMGB1 and Sox2 continued to be detected in the GER and supporting cells of the organ of Corti. HMGB1 immunostaining was maintained in the inner and outer hair cells (IHCs and OHCs), and tympanic border cells (Figure 1 D-F). At P14, the organ of Corti was mature, the expression of HMGB1 and Sox2 disappeared from the GER that degenerated into the inner sulcus cells, and HMGB1 immunolabeling retained its expression in the tympanic border cells and the organ of Corti (Figure 1 G-I). In the adult (P28) mouse cochlea, the general expression pattern of HMGB1 was similar to that seen in P14. HMGB1 continued to be expressed in a one-cell layer of tympanic border cells and the organ of Corti (Figure 1 J-L). The location of HMGB1-positive cells in the IHCs and OHCs was demonstrated by double staining with phalloidin, a specific marker for F-actin (Figure 2 A-F). Co-immunostaining with HMGB1 and isolectin B4 (IB4), a specific vascular endothelial marker,[33] showed nuclear expression of HMGB1 around and inside IB4-positive blood vessels within the cochlear lateral wall, and HMGB1-labelled nuclei did not colocalize with isolectin B4 (IB4) (Figure 2 G-L).

Co-localization of HMGB1 and Ki-67 in E18.5, P1, P5, and P8 mouse cochlea

Ki-67, a nuclear protein marker for proliferating cells, is widely used to identify cells acting as stem cells in developing and adult tissues.[34,35] In the mammalian inner ear, Ki-67 is a reliable marker of proliferation.[36-38] Several developmental inner ear genes were identified by co-labeling with Ki-67.[39,40] In the current study, many Ki-67-positive proliferating cell nuclei were co-expressed with HMGB1-positive nuclei at later embryonic stages (Figure 3 A-C). Consistent with previous reports of expression of Ki-67 in the GER and tympanic border cells at embryonic developmental stages,[39,41,42] our results showed that Ki-67 immunostaining was limited to the apical surface of the GER in the apical turn of E18.5; almost all Ki-67-positive GER cells were co-localized with HMGB1-positive GER cells (Figure 3D), whereas no Ki-67 immunostaining was observed in the GER in the middle turns of the E18.5 cochlea (Figure 3G). Ki-67 and HMGB1 co-localized in the Reissner's membrane, cochlear lateral wall, spiral limbus and cochlear nerve cells within the spiral ganglion region (Figure 3 E-F, I-K). We also found that Ki-67-positive cells among the tympanic border cells were co-localized with HMGB1-positive cells. It is worth noting that HMGB1 and Ki-67 were co-localized in the tympanic border cells below the hair cells where the cochlear spiral modiolar artery exists (Figure 3H). No immunofluorescence labeling of HMGB1 and Ki-67 was detected in any parts of the E18.5 cochlea in negative controls omitting the primary antibody (Figure 3L). Also, no antibody binding was observed in negative controls stained with normal rabbit IgG (*data not shown*). At P1, the distribution of Ki-67 in the GER also differed between the apical, middle, and basal cochlear turns. Only at the apical turn of P1, immunostaining for Ki-67 was detectable in the GER (Figure 4 A-C). HMGB1-Ki-67 double-positive cells were abundantly present in the cochlear modiolus (Figure 4 D-F). Several tympanic border cells surrounding the cochlear spiral modiolar artery and located below the outer sulcus cells showed immunostaining for Ki-67 and HMGB1 (Figure 4 G-I), but Ki-67 expression was largely absent from the spiral limbus at P1 (Figure 4J). HMGB1-Ki-67 double-positive cells were also seen in the stria vascularis, the spiral ligament and the Reissner's membrane (Figure 4K). In the basal turn of the P1 mouse cochlea, HMGB1-Ki-67 double-positive cells could be detected in the tympanic border cells (Figure 4L). Previous studies suggested that the expression of Ki-67 is only detected in spiral ganglion neurons (SGNs) at E16 and E20 in rat.[43] In addition, we have previously shown that most Sox2-positive small and spindle-shaped glial cells were co-labeled with HMGB1 at this stage.[22] To investigate this further, dual immunostaining was performed using SGN markers for TUJ1 and either HMGB1 or Ki-67. Nuclear expression of HMGB1 in large and spherical SGN was identified by co-staining of TUJ1 with HMGB1, and spindle-shaped glial cells wrapping SGNs were immunolabeled for HMGB1 (Figure 5 A-C). At the same time, Ki-67 immunostaining was seen only in the nuclei of the non-neuronal (TUJ1-negative) cells in the spiral ganglion (Figure 5 D-F). Cochlear nerve cells within the spiral ganglion of the P1 Rosenthal canals were double-labeled with HMGB1 and Ki-67 (Figure 5 G-I). Given that the Sox10 transcription factor is a marker for glial cells in the neonatal and postnatal cochlea and other regions of the nervous system,[44-46] which regulates Wnt/β-catenin signaling in diverse developmental processes in normal tissues, dual-immunostaining for Sox10 and Ki-67 was performed to determine further the identification of the nerve cells expressing Ki-67 in the spiral ganglion of the Rosenthal canals. Consistent with previous observations, Sox10-positive nuclei were detectable in the stria vascularis, mainly in the strial marginal cells. Reissner's membrane, the interdental cells and supporting cells were also positive for Sox10[47] (Figure 6 A-C). Interestingly, Ki-67 was co-localized with Sox10 in the GER (Figure 6 D-F). Additionally, to the best of our knowledge, this is the first time that many Sox10-positive neonatal cochlear glial cells within the osseous spiral lamina and the spiral ganglion were found to co-label with Ki-67 (Figure 6 G-L). At P3, no Ki-67-positive cells were seen in the GER throughout the cochlear duct. Ki-67 continued to be expressed in the tympanic border cells, cochlear lateral wall and nerve cells within the spiral ganglion region, and colocalized with HMGB1 (Figure 7 A-F). From P5 onwards, Ki-67-positive cells were scarcely detectable in the Rosenthal canals. The distribution of HMGB1-Ki-67 double-positive cells was constrained to the tympanic border cells and cochlear lateral wall (Figure 7 G-I). As previously reported, at P8, the distribution of Ki-67-positive proliferating cells changed with the advancement of development,[48] and only a few Ki-67-HMGB1 double-labeled cells were scattered in the cochlear lateral wall and the osseous spiral lamina, but there is no significant colocalization between Ki-67 and HMGB1 in the Rosenthal canals and tympanic border cells at this time point (Figure 7 J-L).

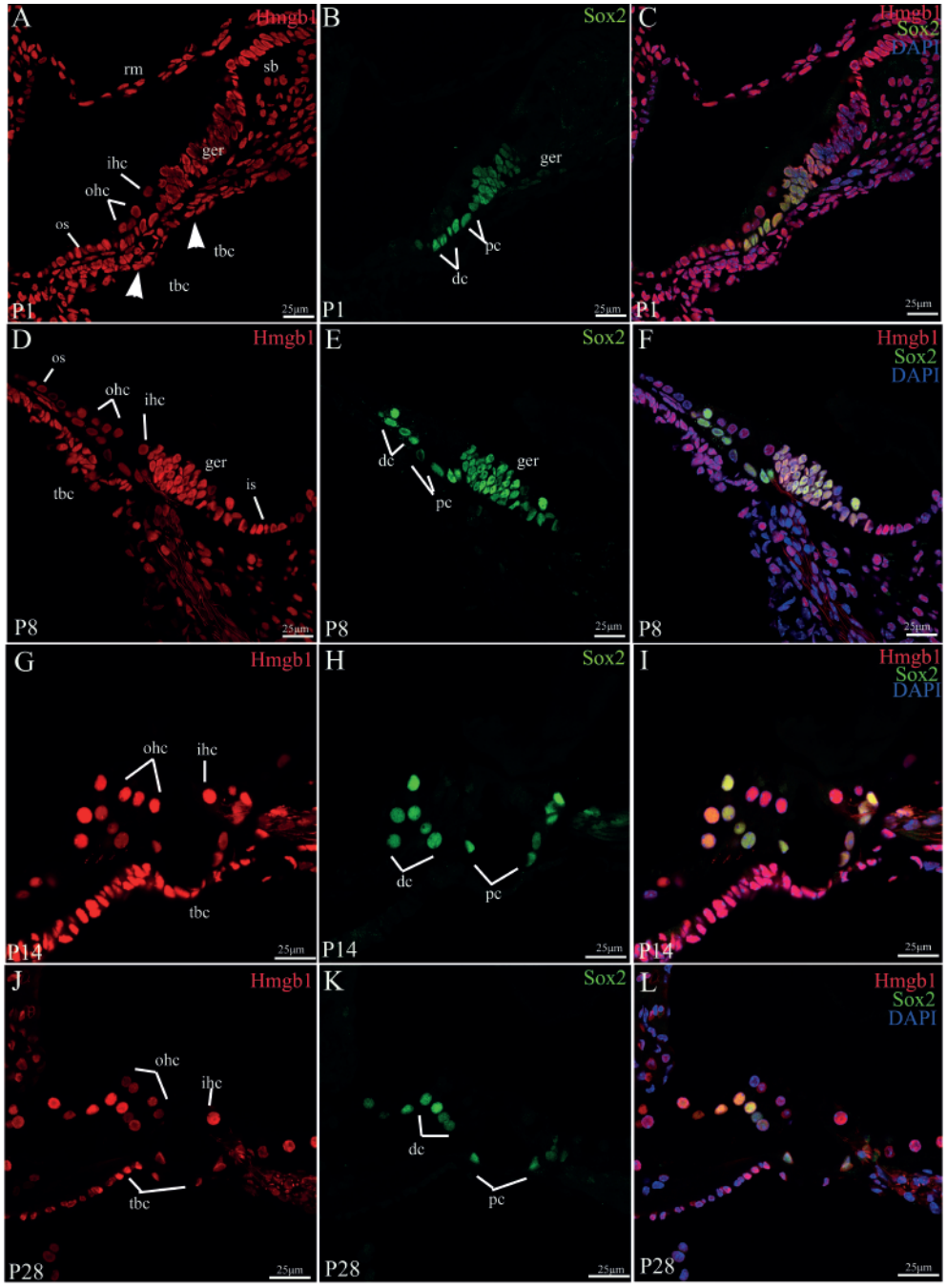

Figure 1. High magnification view of P1, P8, P14 and P28 mouse organ of Corti double labeled by HMGB1 (red), Sox2 (green) under confocal microscopy. **A-C**) Detail of HMGB1 labeling (red) and Sox2 (green) in the auditory epithelium in the middle turn of the P1 mouse cochlea and the merged image+DAPI (blue); HMGB1 labeled the nuclei of the greater epithelial ridge located medial to the inner hair cells, and HMGB1 (red) was colocalized (shown in yellow) with Sox2 (green) in the greater epithelial ridge; co-localization of HMGB1 and Sox2 was detected in the Deiters' cells and pillar cells separating the inner hair cells from the outer hair cells; in addition, nuclear expression of HMGB1 was detected in the inner and outer hair cells. HMGB1 was also detected in the spiral limbus, Reissner's membrane and outer sulcus cells; HMGB1 immunoreactivity was detected in the tympanic border cells (arrowheads) lining the underside of the basilar membrane, particularly in cells beneath Sox2-labeled supporting cells. **D-F**) Detail of HMGB1 labeling (red) and Sox2 (green) in the auditory epithelium in the middle turn of the P8 mouse cochlea and the merged image+DAPI (blue); HMGB1 immunolabeling (red) was detected in the inner and outer hair cells, and tympanic border cells; HMGB1 immunoreactivity was present in the inner sulcus cells, HMGB1 (red) was colocalized (shown in yellow) with Sox2 (green) in the greater epithelial ridge and the supporting cells of the organ of Corti. **G-I**) Detail of HMGB1 (red) labeling and Sox2 (green) in the P14 organ of Corti and the merged image+DAPI (blue); the expression of both HMGB1 and Sox2 disappeared from the greater epithelial ridge; HMGB1 colocalized with Sox2 in the supporting cells of the organ of Corti, HMGB1 labeling was retained in the inner and outer hair cells, tympanic border cells. **J-L**) Detail of HMGB1 (red) labeling and Sox2 (green) in the adult organ of Corti and the merged image+DAPI (blue); HMGB1 (red) immunolabeling was still present in the Sox2-labeled supporting cells of the organ of Corti, HMGB1 immunoreactivity was maintained in the nuclei of the hair cells and one-layered tympanic border cells. ihc, inner hair cells; ohc, outer hair cells; pc, pillar cells; dc, Deiters' cells; os, outer sulcus; is, inner sulcus; tbc, tympanic border cells; sb, spiral limbus; rm, Reissner's membrane; ger, the greater epithelial ridge.

Discussion

In this study, we investigated the expression and distribution of HMGB1 in the mouse organ of Corti by dual-staining for HMGB1 and Sox2. We observed age-related changes in HMGB1 expression in the GER, a crucial cell population essential for developing the auditory sensory epithelium. This suggests that HMGB1 expression may be associated with cochlear development. As the failure of the GER cell proliferation leads to hypoplasia of the organ of Corti,[49] the location of stem cells has been identified in the embryonic and neonatal mouse cochlea using the proliferation marker Ki-67.[41-44] Therefore, we performed dual-immunostaining for HMGB1 and Ki-67 on cryosections of the mouse cochlea to fur-

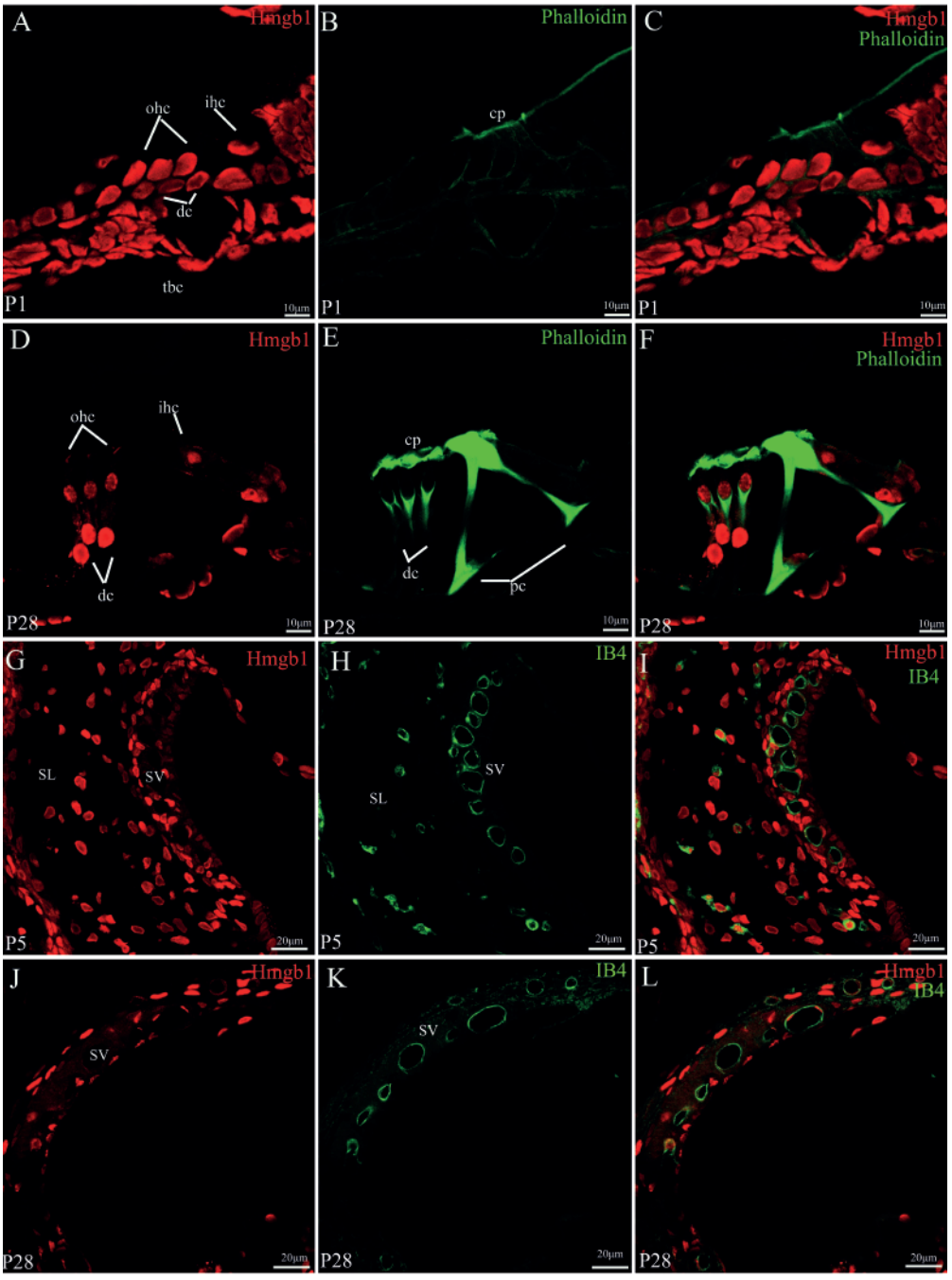

Figure 2. A-C) Double staining of phalloidin (green) and HMGB1 (red) in the mouse cochlea at P1; phalloidin labeled the cuticular plates of P1 inner and outer hair cells, HMGB1 was expressed in the cell nuclei of inner and outer hair cells. **D-F**) Double staining of phalloidin (green) and HMGB1 (red) in the mouse cochlea at P28; phalloidin staining was noted in Deiters' cups underlying HMGB1-positive outer hair cells. **G-I**) IB4-stained blood vessel (green) located in the stria vascularis and spiral ligament of P5 mouse cochlea appeared largely HMGB1 immuno-negative. **J-L**) Double immunofluorescence staining of IB-4 (green) and HMGB1 (red) in the mouse cochlea at P28; few IB4-HMGB1 double-labeled strial capillaries were detectable. ihc, inner hair cells; ohc, outer hair cells; pc, pillar cells; dc, Deiters' cells; tbc, tympanic border cells; SV, strial vascularis; SL, spiral ligament; cp, the cuticular plates.

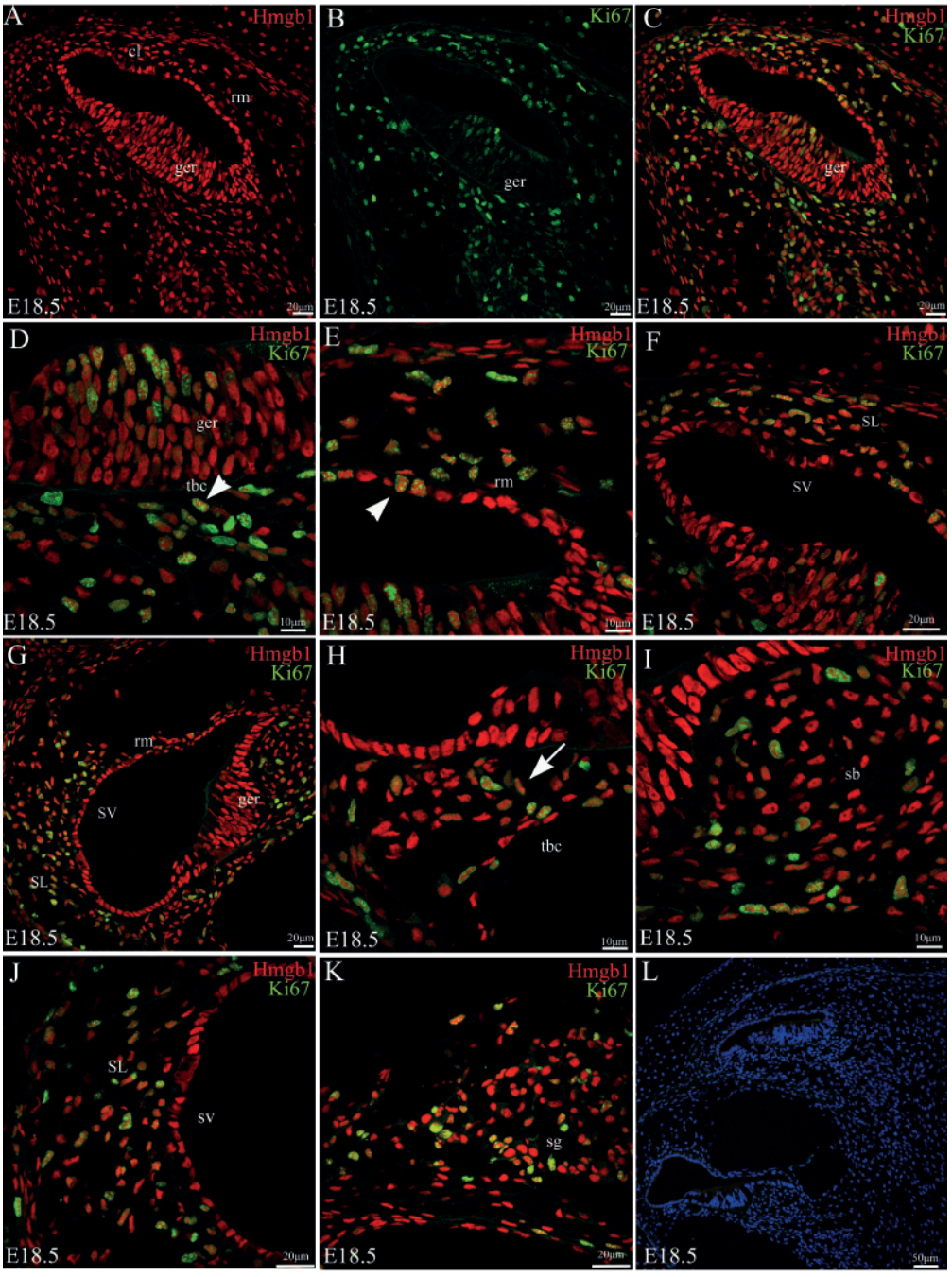

Figure 3. Double immunofluorescence staining of Ki-67 (green) and HMGB1 (red) in the mouse cochlea at E18.5. **A-C**) Low-magnification view of the expression of HMGB1 (red) and Ki-67 (green) in the apical turn of E18.5 and the merged image; the expression of Ki-67 was located at the apical portion of the greater epithelial ridge. **D,E**) HMGB1-Ki-67 double-positive cell nuclei (yellow) were restricted to the apical part of the greater epithelial ridge in the apical turn of E18.5; HMGB1-Ki-67 double-positive cell nuclei (yellow) were detected in the tympanic border cells (arrowheads) and the Reissner's membrane (arrowheads). **F**) In the lateral wall of the E18.5 cochlea, HMGB1-Ki-67 double-positive cell nuclei were mainly present in the spiral ligament. **G**) Double immunofluorescence staining of Ki-67 (green) and HMGB1 (red) in the middle turn of E18.5. Ki-67 was not expressed in the greater epithelial ridge in the middle turn of E18.5. **H**) In the middle turn of E18.5, HMGB1-Ki-67 double-positive cell nuclei (yellow) were found in the tympanic border cells located below the hair cells where a cochlear spiral modiolar artery (arrows) exists. **I,J**) Ki-67-positive nuclei in the spiral limbus and cochlear lateral wall were colocalized with HMGB1. **K**) HMGB1-Ki-67 double-positive nerve cells were observed in the spiral ganglion in the middle turn of E18.5. **L**) No immunofluorescence labeling of HMGB1 and Ki-67 was detected in negative controls. ger, greater epithelial ridge; tbc, tympanic border cells; SL, spiral ligament; SV, stria vascularis; rm, Reissner's membrane; sb, spiral limbus; sg, spiral ganglion.

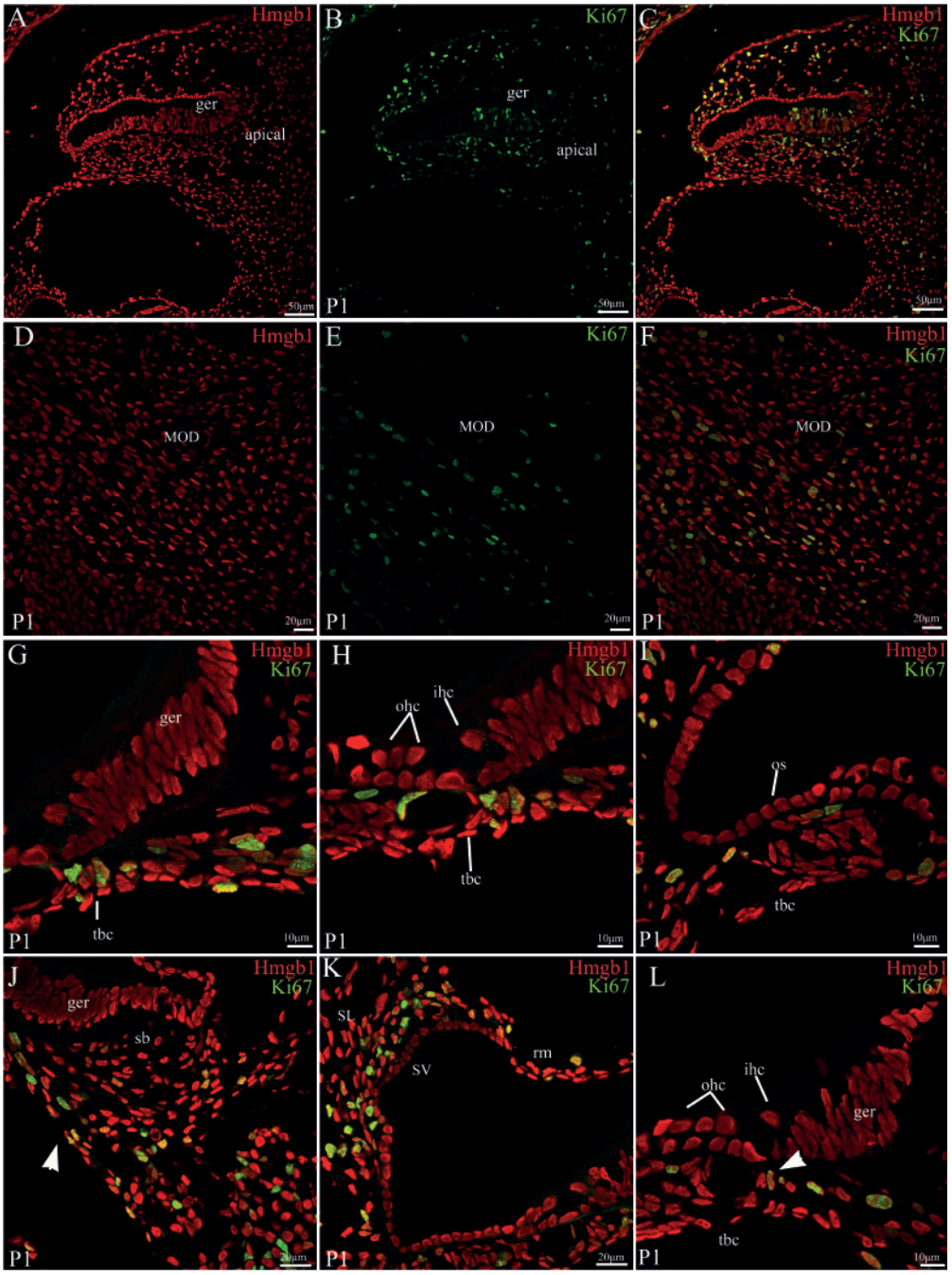

Figure 4. Double immunofluorescence staining of Ki-67 (green) and HMGB1 (red) in the mouse cochlea at P1. **A-C**) Low-magnification view of the expression of HMGB1 (red) and Ki-67 (green) in the apical turn of P1 and the merged image; the expression of Ki-67 was located at the apical portion of the greater epithelial ridge of P1. **D-F**) HMGB1-Ki-67 double-positive nerve cells were observed in the modiolus of P1 mouse cochlea. **G-I**) Double immunofluorescence staining of HMGB1 (red) and Ki-67 (green) in the auditory epithelium in the P1 middle turn; in the greater epithelial ridge, there is no colocalization between the two immunoreactivities. HMGB1-Ki-67 double-positive cells were detected in the tympanic border cells below the hair cells and the outer sulcus cells. **J**) Cochlear nerve cells (arrowheads) within the osseous spiral lamina were double immunostained for HMGB1 (red) and Ki-67 (green). **K**) Ki-67-HMGB1 double-positive cell nuclei (yellow) were detected in the spiral ligament, stria vascularis, and Reissner's membrane. **L**) Ki-67-HMGB1 double-positive cell nuclei (yellow) were also found in the tympanic border cells (arrowheads) in the basal turn of the P1 mouse cochlea. ger, greater epithelial ridge; tbc, tympanic border cells; SL, spiral ligament; SV, stria vascularis; rm, Reissner's membrane; sb, spiral limbus; sg, spiral ganglion; ihc, inner hair cells; ohc, outer hair cells; tbc, tympanic border cells; os, outer sulcus; MOD, the modiolus..

ther examine the potential roles of HMGB1 in cochlear development. Our results demonstrated that HMGB1 co-express with Ki-67 in several cochlear regions at later embryonic and early postnatal stages, including the GER, tympanic border cells, cochlear lateral wall and cochlear nerve cells. These findings suggest that HMGB1 might participate in cell proliferation during cochlear development, which aligns with previous studies showing the essential role of HMGB1 in cell proliferation of various cell types.[50-52] Knockdown of HMGB1 can significantly reduce the Ki-67 expression and inhibit the proliferative activities.[53] Previous studies reported that HMGB1 affects cell proliferation *via* modulation of multiple signaling pathways such as NF-κB, PI3K/Akt, JNK and RAGE/TLR4.[54-56] Given the expression of the RAGE, one of the main receptors for HMGB1 that contribute to cochlear maturation,[57] we hypothesized that HMGB1 could participate in cochlear development *via* signaling to RAGE. To the best of our knowledge, this is the first study reporting the expression of HMGB1 in the tympanic border cells and its co-localization with Ki-67. Tympanic border cells are Wnt-responsive and act as progenitors for postnatal mouse cochlear cells.[42] These cells express Ki-67 and exhibit active proliferation until the second week of postnatal development. Our data showed that HMGB1-Ki-67 double-positive tympanic border cells were located near the cochlear spiral modiolar artery, which is consistent with the specific localization of the slow-cycling cells within the tympanic border zone near the vascular structure. Previous studies reported that acoustic or drug-induced injury to the mammalian and chick cochlea induces the proliferation of tympanic border cells,[58,59] thus, we assumed that changes in HMGB1 expression in the tympanic border cells following cochlear damage may correlate with cochlear pathology, as demonstrated in the spiral limbus and spiral ligament.

Additionally, we found the presence of HMGB1 in Reissner's

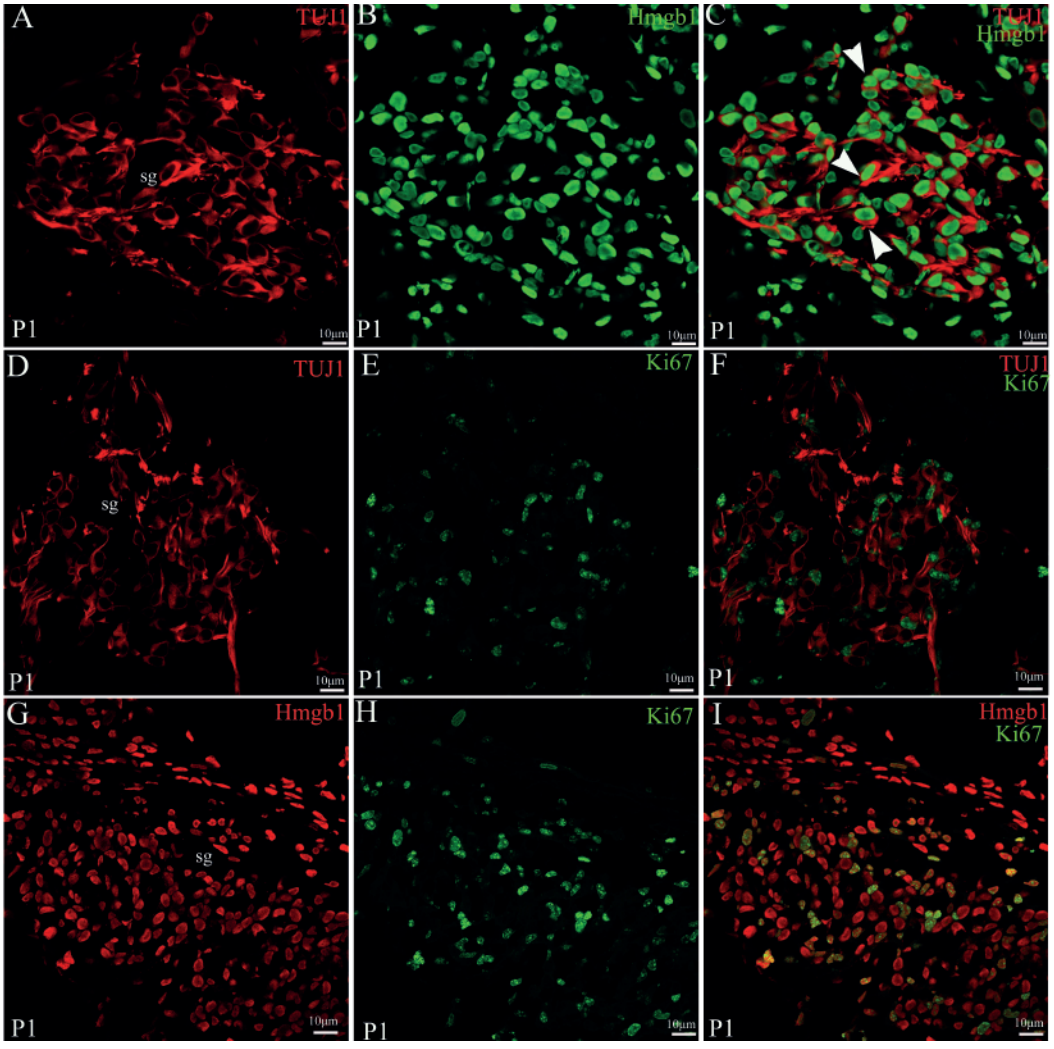

Figure 5. Double immunofluorescence staining of TUJ1(red) with both Ki-67 (green) and HMGB1 (green) in the spiral ganglion region of P1 mouse cochlea. **A-C**) Double immunofluorescence staining of TUJ1 (red) with HMGB1 (green) in the spiral ganglion region of P1; HMGB1 was expressed in the nuclei of TUJ1-positive spiral ganglion neurons and its surrounding glial cells (arrowheads). **D-F**) Double immunofluorescence staining of TUJ1 (red) with Ki-67 (green) in the spiral ganglion region of P1; TUJ1-positive the cytoplasm of spiral ganglion neurons was surrounded by Ki-67-positive cochlear nerve cells. **G-I**) HMGB1-Ki-67 double-positive nerve cells were observed in the spiral ganglion in Rosenthal's canal. sg, spiral ganglion.

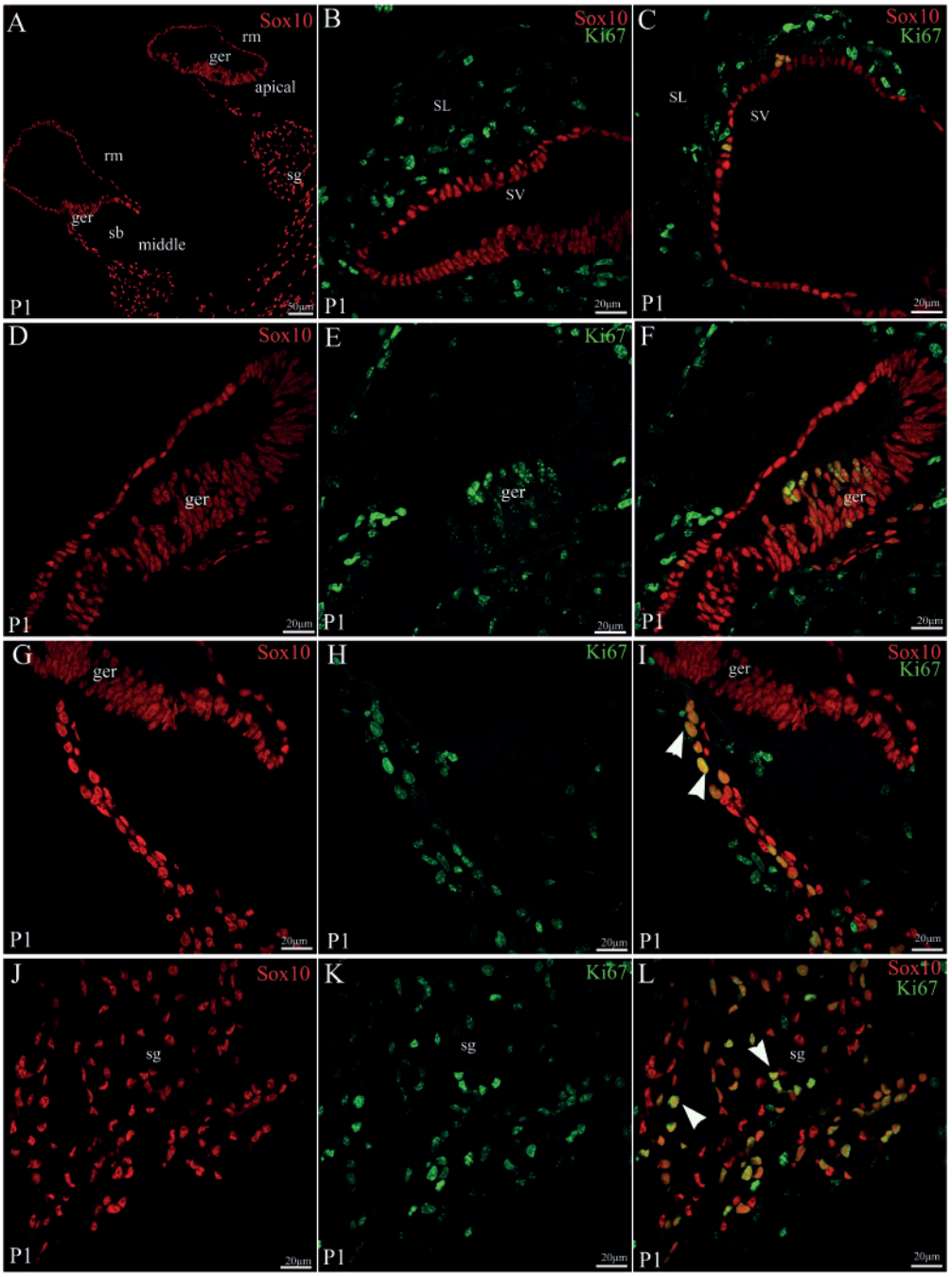

Figure 6. Double immunofluorescence staining of Ki-67 (green) and Sox10 (red) in the mouse cochlea at P1. **A**) Low-magnification view of the frozen sections of the P1 mouse cochlea labeled with Sox10 (red); Sox10 was mainly expressed in the greater epithelial ridge, Reissner's membrane, spiral limbus, the stria vascularis and glial cells within the spiral ganglion region of P1. **B,C**) Sox10 immunoreactivity was mainly found in the strial marginal cells in the apical turn of P1; most of Sox10-positive strial marginal cells did not colocalize with Ki-67. **D-F**) Sox10 partially colocalized with Ki-67 in the greater epithelial ridge. **G-I**) Sox10 partially colocalized with Ki-67 in the glial cells within the osseous spiral lamina. **J-L**) Many, not all of Sox10-positive glial cells colocalized with Ki-67. ger, greater epithelial ridge; tbc, tympanic border cells; SL, spiral ligament; SV, stria vascularis; sb, spiral limbus; rm, Reissner's membrane; sg, spiral ganglion.

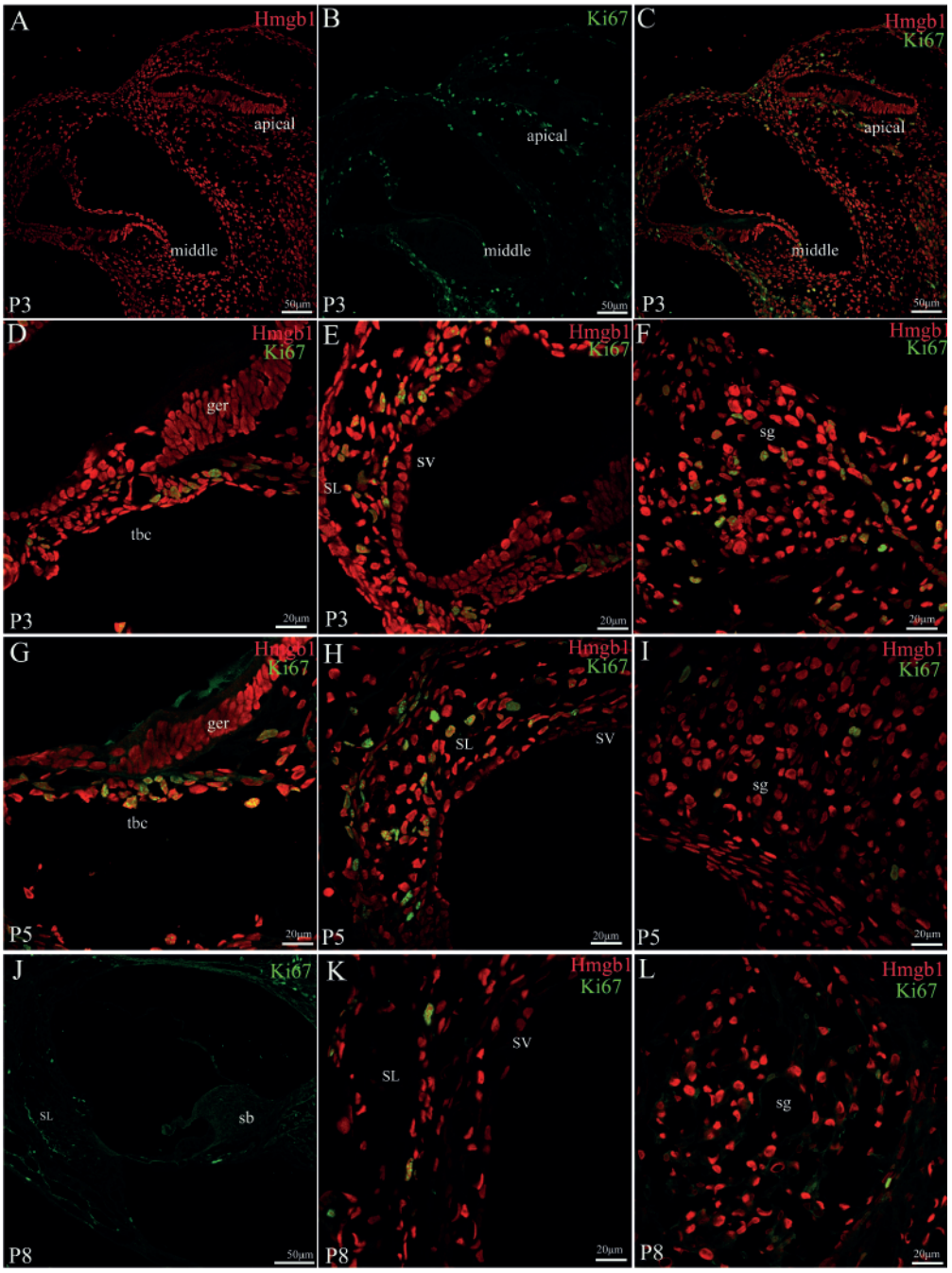

Figure 7. Double labeling for Ki-67 (green) and HMGB1 (red) in the mouse cochlea at P3, P5 and P8. **A-C**) Low-magnification view of the expression of HMGB1 (red) and Ki-67 (green) in the apical and middle turn of P3 and the merged image; note that immunostaining for Ki-67 disappeared from the greater epithelial ridge of P3. **D-F**) Ki-67-HMGB1 double-positive cell nuclei were detected in the tympanic border cells, the cochlear lateral wall and spiral ganglion region of P3. **G**) In the middle turn of the P5 mouse cochlea, Ki-67-positive tympanic border cells beneath the basilar membrane were co-labeled with HMGB1. **H**) Ki-67-HMGB1double-positive cells (yellow) were confined to spiral ligament fibrocytes. **I**) In the middle turn of P5 mouse cochlea, only a few scattered Ki-67-positive cells were seen in the Rosenthal canal. **J**) An overview of Ki-67 (green) labeling in the middle turn of P8. **K,L**) Only a few Ki-67-HMGB1 double-positive cells were scattered in the spiral ligament and the osseous spiral lamina, almost no Ki-67-HMGB1 double-positive cell nuclei were found in the tympanic border cells and the Rosenthal canal of P8 mouse cochlea. ger, greater epithelial ridge; tbc, tympanic border cells; SL, spiral ligament; SV, stria vascularis; sb, spiral limbus; sg, spiral ganglion; ihc, inner hair cells; ohc, outer hair cells; tbc, tympanic border cells.

membrane and the co-localization of HMGB1 and Ki-67 in Reissner's membrane at E18.5 and P1. Several proteins contributing to cochlear development, such as LaminB1 and Pax2,[60,61] are expressed similarly in Reissner's membrane, which acts as a barrier between endolymph and perilymph, maintaining their unique fluid compositions.[62,63] The cell nucleus of Reissner's membrane is resistant to degeneration resulting from acute atoxyl intoxication and is the last cell component to disintegrate.[64] The number of cells and cellular proliferation in Reissner's membrane has also been implicated in endolymphatic hydrops.[65,66] Therefore, the expression of HMGB1 in the Reissner's membrane observed in this study may be essential in cochlear function.

In our previous study, we found that HMGB1 was mainly expressed in the nuclei of the SGNs and Sox2-positive glial cells in the spiral ganglion regions at various developmental stages. Moreover, upregulation of HMGB1 expression in glial cells of the spiral ganglion regions was linked to cochlear pathogenesis after acoustic trauma.[24] Similar changes in HMGB1 expression were also observed in the SGNs of amikacin-treated rats.[67] Previous studies have shown that HMGB1 can promote neural stem cell proliferation.[68,69] In this study, we further extended our previous investigation by demonstrating the co-localization of HMGB1 and Ki-67 in the non-neuronal cells within the spiral ganglion regions during prenatal and early postnatal stages. Additionally, Ki-67 was co-expressed with Sox10 in cochlear neonatal glial cells. Sox10 is known to be essential for the determination, differentiation and maintenance of peripheral glial cells,[70] suggesting the potential involvement of HMGB1 in cochlear nerve cell proliferation during cochlear development.

In conclusion, we identified a specific expression pattern of HMGB1 in the tympanic border cells throughout the developmental period. Using double immunofluorescence histochemistry, we revealed that the changes in HMGB1 and Sox2 expression in the GER correlate with the morphological degeneration of the GER during postnatal development. Additionally, we found that Ki-67-positive proliferating cells co-localize with HMGB1 during late embryonic and early postnatal cochlear development. These findings offer some insights into the potential physiological functions of HMGB1 in the cochlea.

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