

Original Article

The Spatiotemporal Expression of Ring1B in the Postnatal Mouse Cochlea

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BACKGROUND: Epigenetic regulation plays significant role in the development of the inner ear. It is known that Ring1B can monoubiquitinate H2AK119 to compact nucleosomes and block gene transcription. Ring1B plays crucial role in embryonic development, heterochromatin function, stem cell maintenance and so on. However, it is still unknown whether Ring1B plays a role in the development of inner ear.

METHODS: Here, we used postnatal C56BL/6J mice to examine the expression of Ring1B in the cochlea using real-time quantitative polymerase chain reaction, Western blot, and immunofluorescence staining.

RESULTS: Ring1B mRNA expression was observed at postnatal day 0 and postnatal day 14 cochlea. Ring1B protein was expressed in the cochlea on postnatal days 0, 14, and 30. The Ring1B was observed to be expressed in spiral ganglion cells at postnatal days 0, 14, and 30. Additionally, Ring1B was expressed in hair cells at postnatal days 0, 14, and 30.

CONCLUSION: Our results provide the basic expression pattern of Ring1B and might be helpful for future investigations of the detailed role of Ring1B in the cochlea.

KEYWORDS: Polycomb protein, Ring1B, postnatal, inner ear, cochlea

INTRODUCTION

The mammalian cochlea is a complex sensory organ that is responsible for sound detection, and the main structure includes Corti's organ, spiral ganglion, and lateral wall containing the stria vascularis. The development of the mouse cochlea begins with a thickening of the ectoderm adjacent to the hindbrain, invagination of the thickening ectoderm, formation of the otocyst, and gradual formation of Corti's and Kölliker's primordial.¹ At birth, cochlear base forms the scala tympani and scala vestibuli. The apical turn of the cochlea shows only Corti's primordial and perilymphatic space but no scala tympani and scala vestibuli. On postnatal (P) day 14, each turn of the cochlea is mature and stria vascularis is mature. The postnatal development of Corti's organ and stria vascularis is from basal turn to apical turn. The development of spiral ganglion neurons is similar to that of Corti's organ and stria vascularis.² There is no detectable hearing function at birth for mice, and the hearing begins to appear nearly at P10-14 and is basically mature at P30.³ Many genes are involved in the postnatal development of the cochlea, such as atonal bHLH transcription factor 1 (Atoh1), enhancer of zeste 2 polycomb repressive complex 2 subunit (Ezh2), BMI1 proto-oncogene polycomb ring finger (Bmi1), P27 Kip2, Myosin VIIa, calretinin, parvalbumin, adenosine triphosphatase Ca⁺⁺-transporting plasma membrane 2 (PMCA2), S100 calcium-binding protein (S100), and so on. We can find any epigenetic genes, such as Atoh1, Ezh2, Bmi1, and so on, involved in the development of cochlea.⁴⁻⁹

Epigenetic regulation plays important role in the development of the inner ear,⁵ and a recent report has revealed that the epigenetic mechanism behind Atoh1 regulation underlies hair cell differentiation and subsequent maturation.⁶ Dynamic changes in the histone modifications H3K4me3/H3K27me3, H3K9ac, and H3K9me3 correlate with the onset of Atoh1 expression during hair

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cell differentiation. Ring1B is a member of the polycomb protein family. Polycomb protein family includes 2 core protein complexes: polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2). Polycomb repressive complex 1 mainly comprises Ring1B and Bmi1. Polycomb repressive complex 2 trimethylates histone H3K27, and H3K27me3 can attract, bind, and immobilize PRC1. Polycomb repressive complex 1 can stabilize H3K27me3 to inhibit the expression of downstream genes, of which Ring1B monoubiquitinates H2AK119 to compact nucleosomes and block gene transcription.¹⁰

Ring1B plays an important role in embryonic development, stem cell maintenance, heterochromatin function, and cancer.^{11–15} However, the function of Ring1B in the inner ear is unclear so far.

In this study, we characterize the detailed spatiotemporal expression of Ring1B in the postnatal mammalian cochlear at mRNA and protein levels.

MATERIALS AND METHODS

Animals

Timed pregnancies were established for C57BL/6J wild-type mice. Postnatal day 0 was defined as the day of birth. Mice were housed with open access to food and water at Shanghai University of Medicine & Health Sciences. All experiments were approved by the Shanghai University of Medicine & Health Sciences, and all efforts were made to minimize suffering and reduce the number of animals used. Ethical committee approval was received from the Ethics Committee of Shanghai University of Medicine & Health Sciences (Approval no: 2021-SSF-16-002).

Quantitative real-time polymerase chain reaction

Total RNA was extracted by Trizol and then was reversely transcribed into cDNA by M-MLV kit (M1705, Promega, Wisconsin, America). Real-time polymerase chain reaction was performed on Roche LightCycler480 real-time PCR system (Roche, Basel city, Switzerland) using SYBR premix ex taq qPCR Master Mix (DRR041B, TAKARA, Tokyo, Japan). All primers were designed to flank individual exons and were tested by PCR. The optimized conditions were held constant for each sample to assure a valid comparison of the results. Primers sets used were as follows: GAPDH(F) 5'- TGGTGAAGGTCGGTGTGAAC -3'; (R) 5'- GCTCCTGGAAGATGGTGATGG-3', Ring1B(F) 5'- TGGCAACAAA GAGTGTCTTA-3'; (R) 5'- CACCATTATCTTCTGCTCCA-3'. Each group includes 3 mice, that is 6 cochleae.

Western Blot

Total protein was isolated using radio Immunoprecipitation Assay (RIPA) lysis solution. Proteins were separated by 10% SDS-PAGE. After electrophoresis, samples were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% non-fat dried milk in tbs+tween (TBST) for 1 hour at room temperature. The primary antibodies, including anti-Ring1B antibody (Abcam, ab181140, dilution 1:100) and anti- β -actin antibody (Abcam, ab115777), were added to the blocking buffer and kept overnight at 4°. After rinsing 4 times (10 minutes each) with TBST, the membranes were incubated with horseradish Peroxidase (HRP)-conjugated secondary antibody at a concentration of 1:5000 (Cell Signaling Technology, Boston, Mass, USA) for 1 hour at room temperature. The immunoreactive bands were visualized using an ECL-PLUS kit (Thermo).

Tissue and Frozen Section Preparation

Mix four ketamine (0.1g/dose) with 100mg methylthiazide, and then inject intraperitoneally at the rate of 3 μ l/g to anesthetize the mice. After anesthetizing the mice, postnatal days 0, 14, and 30 mice were decapitated to dissect the inner ear, and the cochleae were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.2. Postnatal days 0, 14, and 30 cochleae were decalcified in 10% ethylenediaminetetraacetic acid (EDTA). Specimens were embedded in 4% sucrose and cut into 10- μ m-thick sections and mounted onto glass slides.

Immunofluorescence

Epitopes were unmasked by microwave heating (1000 W) in retrieval buffer (Maixin Biotech, Fuzhou, China) for 5 minutes. Sections were permeated with 0.1% Triton X-100, blocked with 10% donkey serum in 10 mM PBS (pH 7.4) for 30 minutes at room temperature, and then incubated with the primary anti-Ring1B (Abcam, ab181140, 1:100), anti-Myosin7a (Proteus BioScience, 25-6790, 1:200), and anti- β -tubulin (Abcam, ab18207, 1:200) antibody overnight at 4° in a humidified chamber. The following day, the sections were rinsed with PBS and then incubated with secondary antibody (Alexa Fluor 568 donkey anti-rabbit IgG, A10042; Alexa Fluor 555 goat anti-mouse IgG, A21422, 1:1000, Life Science, Chestertown, America) for 30 minutes at room temperature. After washing with PBS, the sections were mounted in Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) (F6057, Sigma, Saint Louis, Mo, America). Images were taken using a confocal laser-scanning microscope (LSM 710 META, Zeiss, Oberkochen, Germany) with a 20 $\times$ objective.

Statistics

All data are expressed as means $\pm$ standard error of the mean. The Student's *t*-test was performed using SigmaPlot (ver.14; Systat Software Inc., San Jose, Calif, USA). In all analyses, *P* < .05 was taken to indicate statistical significance.

RESULTS

Ring1B mRNA Was Expressed in P0 and P14 Cochlea

We first observed the expression of Ring1B at the mRNA level. We used quantitative real-time polymerase chain reaction (qRT-PCR) technology in order to more accurately observe the expression abundance of Ring1B. Ring1B mRNA was expressed in P0 and P14 cochlea (Figure 1). The average $2^{-\Delta\Delta Ct}$ of P0 and P14 was 1.363 and 1.090, respectively.

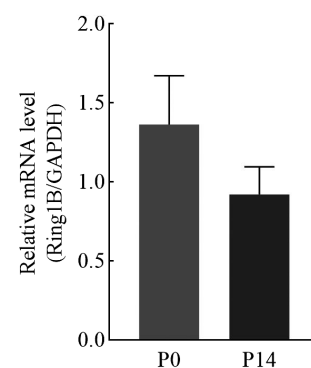


Figure 1. Real-time PCR data showed the mRNA levels of Ring1B at P0 and P14. *n* = 3 for each group. PCR, polymerase chain reaction.

Ring1B Protein Was Expressed in the P0, P14, and P30 Cochlea

To detect the Ring1B protein expression in the cochlea at P0, P14, and P30, Western blot was applied. We found that Ring1B protein was highly expressed in the P0 cochlea and gradually decreased during mice maturation in P14 and P30 cochlea (Figure 2).

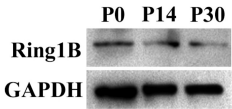


Figure 2. Ring1B protein was expressed in P0, P14, and P30 cochlea. P, postnatal. Ring1B protein was highly expressed in P0 cochlea and low expressed in P14 and P30 cochlea. P, postnatal.

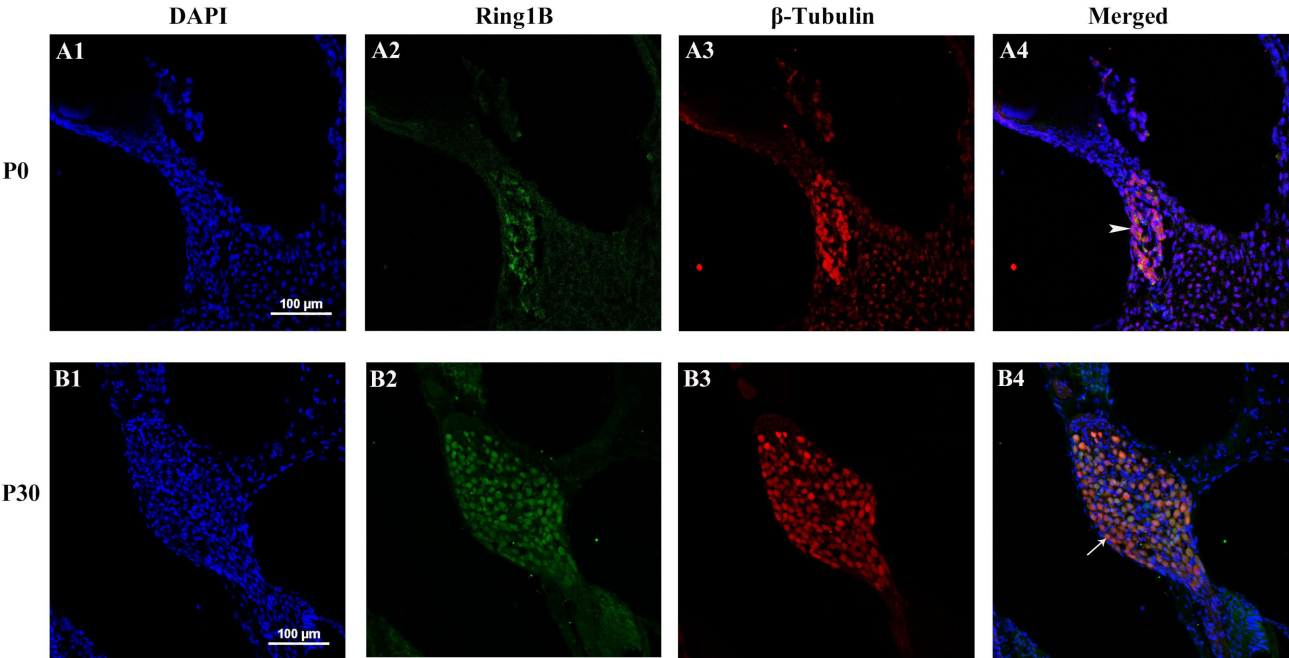


Figure 3. Ring1B protein was expressed in the spiral ganglion cytoplasm at P0 and P30. White arrowhead in image A4 and white arrow in image B4 indicate ganglion cell. P, postnatal.

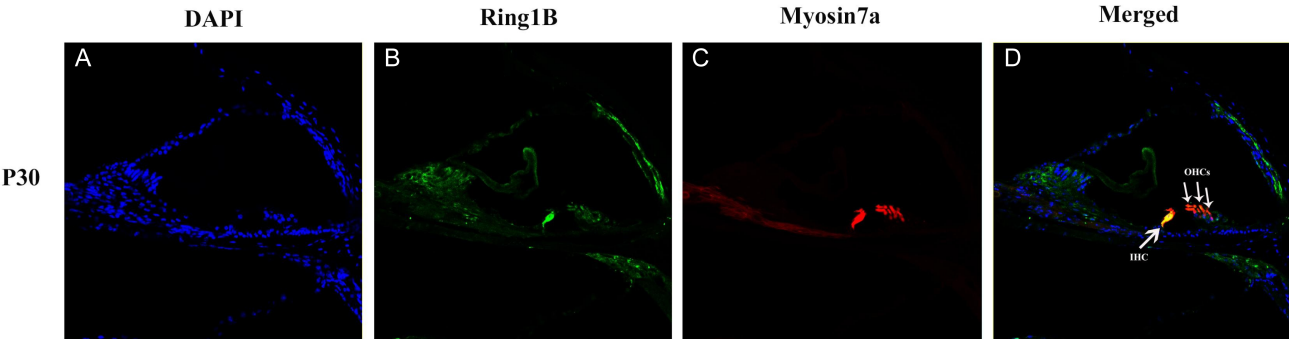


Figure 4. Ring1B was expressed highly in the inner hair cell cytoplasm and low in the outer hair cell cytoplasm at P30. IHC, inner hair cell; OHC, outer hair cells; P, postnatal.

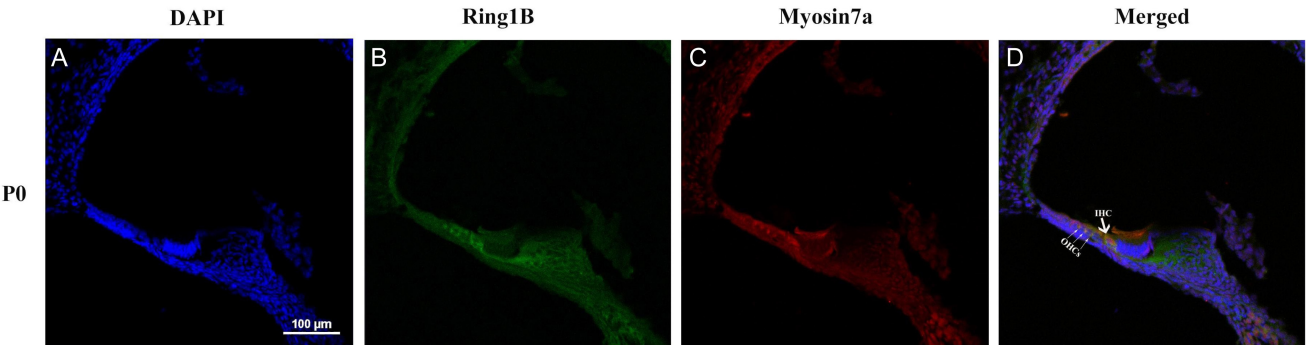


Figure 5. Ring1B was expressed in the inner Corti's organ (inner hair cell cytoplasm and outer hair cell cytoplasm) at P0. OHCs, outer hair cells; IHC, inner hair cell; P, postnatal.

Ring1B Protein Was Located in Hair Cells and Spiral Ganglion Neurons at P0 and P30

To investigate the spatial expression pattern of Ring1B protein, Ring1B protein immunofluorescence was used on cochlear cross-sections. We discovered that Ring1B protein was highly expressed in the spiral ganglion cytoplasm at P0 and P30 and used β -tubulin as the marker for spiral ganglion cells (Figure 3). Ring1B protein was expressed highly in the inner hair cell cytoplasm and low in the outer hair cell cytoplasm at P30, and we used Myosin7a as the marker for hair cells (Figure 4). Ring1B protein was observed in the outer hair cell cytoplasm and inner hair cell cytoplasm at P0 (Figure 5). At P14, we found that Ring1B protein was expressed in the spiral ganglion cytoplasm and the hair cell cytoplasm (data were not shown).

DISCUSSION

The cochlea is an anatomical structure of the inner ear, and it conducts and senses sound as part of the peripheral auditory system. The organ of Corti is the core part of the cochlea and is the auditory transduction organ that physically transforms mechanical signals from the middle ear into nerve impulses. The organ of Corti is located throughout the full length of the basilar membrane and contains inner hair cells and outer hair cells and at least 4 types of supporting cells.¹

Ring1B is a core component of the mammalian polycomb system. Ring1B is one of the epigenetic regulators of production of cell diversity during embryonic development and adult tissue maintenance.¹⁶ In addition, Ring1B protein is involved in multisystem development, such as an early proximal-distal specification of the forelimb bud, gastrulation development, mouse neocortical development, pectoral fin development, and mouse growing incisors.^{17–19} To further explore the function of Ring1B in inner ear during postnatal development, we need to understand the expression pattern of Ring1B in the cochlea.

In this study, we observed that Ring1B protein was highly expressed in P0 cochlea and gradually decreased with mice maturation in P14 and P30 cochlea, which indicates that the role of Ring1B in early cochlear development is very critical. In order to further clarify the cell type of Ring1B protein expression, we carried out immunofluorescence staining of cochlear sections. Ring1B was expressed in the cytoplasm of spiral ganglion cells at P0, P14, and P30 (Figure 3), which suggests that Ring1B may be involved in the postnatal development and maturation of spiral ganglion cells. We also demonstrated that Ring1B was expressed in the hair cell cytoplasm at P0, P14, and P30 (Figure 4–5). It indicates that Ring1B may participate in the postnatal maturation of hair cells. However, the Ring1B expression decreases in the outer hair cells compared to that of the inner hair cells at P30 (Figure 4).

We are preparing to block the protein in the next experiment to observe whether the cochlear development is affected.

CONCLUSION

In summary, our data show that Ring1B is expressed in spiral ganglion cells' cytoplasm and hair cells' cytoplasm during postnatal development. Our results provide the basic Ring1B expression

pattern, which might be helpful for studying the Ring1B function in the cochlea.

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Declaration of Interests: The authors have no conflict of interest to declare.

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