

Original Article

Differential Expression of LaminB1 in the Developing Rat Cochlea

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OBJECTIVES: To explore the temporal expression pattern of LaminB1 in the cochlea of postnatal rat, and whether LaminB1 is associated with cochlear development.**MATERIALS AND METHODS:** Sprague–Dawley rats ranging from postnatal day 0 (p0) to 21 (p21) were used. The tissues of stria vascularis (STV) including spiral ligament, spiral ganglion cell (SGC), and basilar membrane (BM), including the organ of Corti, were dissected, respectively. Immunofluorescence, quantitative real-time polymerase chain reaction, and western blot were applied to detect the expression of LaminB1 in individual cochlear tissues at both mRNA and protein levels.**RESULTS:** Immunofluorescence revealed that LaminB1 was localized in the outer hair cells, inner hair cells, Kolliker's organ, Reissner's membrane, SGC, STV, and spiral ligament. The intensity of staining surrounding the scala media decreased during cochlear development. The expression of LaminB1 mRNA and protein in STV, SGC, and BM was at a maximum level at p0 but gradually declined to a minimum level at p21.**CONCLUSION:** Our research provided direct evidence that LaminB1 was expressed in the developing cochlea and developmentally regulated in cochlear tissues, suggesting a possible role of LaminB1 in cochlear development. Our result provided a theoretical basis for further study about the physiological function of LaminB1 in the peripheral auditory system.**KEYWORDS:** LaminB1, rat, cochlea, development

INTRODUCTION

LaminB1, a major component of the nuclear lamina ^[1], plays a vital role in chromatin organization, DNA replication, and transcription ^[2]. In addition, LaminB1 appears to play a developmental role in tissue differentiation and organ development ^[3]. Homozygous *Lmnb1* mutant mice survived embryonic development but died at birth with defective organogenesis of the lung and bone ^[4]. In LaminB1 null mice, defects in migration of neurons and spindle orientation in neural progenitor cells were also evident ^[5]. In addition, levels of LaminB1 finely modulated the differentiation of neural stem cells into neurons ^[6]. Thus, the expression patterns of LaminB1 during development have been the subject of many studies ^[7]. In mouse brain, Lin et al. ^[8] examined that LaminB1 protein and mRNA peak at birth or postnatal day 1, followed by a gradual decrease with age. In human tissues, LaminB1 was preferentially detected in proliferating epithelial cells, whereas muscle and connective tissues were negative ^[9]. However, the physiological expression patterns of LaminB1 in the developing cochlea were less known.

The mammalian cochlea, an excellent sensory organ, is sensitive to sound and can discriminate frequencies exquisitely ^[10]. In rats, the auditory nerve-brainstem evoked responses were observed only from postnatal day 12 to 14 with highly elevated threshold ^[11, 12], then reached adult level at approximately postnatal day 22. The ontogeny of auditory system function has been correlated with many events, for example, the differentiation of the outer hair cells (OHCs), maturation of the synapses, development of tissue space in the organ of Corti (OC), and degradation of Kolliker's organ ^[13].

As mentioned above, LaminB1 plays a key role in development and organogenesis. However, whether LaminB1 is associated with cochlear development still remains unknown. The aim of the present study was to investigate the localization and the temporal ex-

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pression pattern of LaminB1 in the developing rat cochlea by immunofluorescence, western blot, and quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Animals

A total of 120 Sprague-Dawley rats were provided by the Experimental Animal Centre. They were from postnatal day 0 (p0) to 21 (p21). The rats were randomly divided into four groups: p0, p7, p14, and p21. The study was approved by the local ethics committee.

Cochlea Sections Preparation

The cochleae were isolated immediately after euthanasia. They were gently fixed by perfusing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) through the round and oval window and soaked in this solution overnight at 4 °C. Then, the cochleae were washed and transferred to 10% sodium ethylenediaminetetraacetic acid (pH 7.3-7.4) for 7 days, followed by dehydration in 30% sucrose solution overnight at 4 °C. The cochleae were embedded in optimum cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA), cryosectioned at 8 µm thickness, and collected onto glass slides.

Immunofluorescence

Cochlear sections were permeated with 0.5% Triton X-100 for 15 min at a room temperature of about 25 °C and then blocked with blocking solution (10% goat serum in PBS with 0.01% Triton X-100) for 40 min. Diluted in blocking solution, the primary antibody against LaminB1 (1:400; Abcam, Cambridge, UK) was applied to the cochlea sections overnight at 4 °C. After rinsing three times in PBS, specimens were incubated with the DyLight™ 488-conjugated goat anti-rabbit IgG (1:500; Multi-Sciences, Hangzhou, China) at a room temperature of about 25 °C for 1 h, then counterstained with 10 µg/mL 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37 °C. Finally, the sections were washed, coverslipped, and observed with a laser scanning confocal microscope (Olympus Fluoview 500 IX 71; Olympus, Tokyo, Japan). The images were recorded digitally at the same magnification and time of exposure^[14].

qRT-PCR

The tissues of stria vascularis (STV) including spiral ligament, spiral ganglion cell (SGC), and basilar membrane (BM), including the OC, were dissected, respectively, in RNase-free D-Hank's solution by microdissection. Total RNAs were isolated using TRIzol (Invitrogen, Carlsbad, USA), and 1 µg extracted total RNA was reverse-transcribed to cDNA following the protocol of ReverTra Ace (Toyobo, Osaka, Japan).

The primers used in the present study were as follows:

GAPDH

Forward: 5'-GTCGGTGTGAACGGATTTGG-3'

Reverse: 5'-GACTGTGCCGTTGAACTTGC-3'

LaminB1

Forward: 5'-GTCCTTCTCCCGAGTGACC-3'

Reverse: 5'-CGCCTCTGATTCTCCACAT-3'

The amplification of cDNA samples occurred on a LightCycler 480 II (Roche, Rotkreuz, Switzerland) using SYBR Green Premix Ex Taq™ (Tli RNase H Plus; TaKaRa, Dalian, China). The cycling parameters were 3 min at 95 °C, followed by 40 cycles for 15 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C. Each sample was run three times, and the mean value was calculated. Relative mRNA levels of LaminB1 were normalized to GAPDH using the 2^(-Delta Delta CT) method^[15].

Western Blot

The tissues of STV, SGC, and BM were prepared and homogenized in RIPA buffer containing protease inhibitors. Bicinchoninic acid protein assay reagent (Sigma-Aldrich) was used to determine the concentration of protein in the supernatant of total tissue lysates. Equalized protein content (20 µg) was electrophoresed on a polyacrylamide gel at 80 V and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, USA). Membranes were blocked with 5% bovine serum albumin (Boster, Wuhan, China) in Tris-buffered saline containing 0.2% Tween-20 for 1 h at a room temperature of about 25 °C. Primary antibody against LaminB1 (1:1000; Abcam) and β-actin (1:2000; Sigma-Aldrich) were applied to probe with proteins overnight at 4 °C. After rinsing three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000; Sigma-Aldrich) for 1 h at a room temperature of about 25 °C. Then, protein bands were visualized using a chemiluminescence system (PTC-200; Bio-Rad Laboratories, Hercules, CA, USA) with enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA). All the tests were performed more than three times.

Statistical Analysis

All data were presented as mean±SEM. One-way analysis of variance (one-way ANOVA) with Student-Newman-Kuels (SNK) correction was used for statistical analysis. A *p*<0.05 was considered statistically significant.

RESULTS

Localization and Developmental Alteration of LaminB1 in the Cochlea

LaminB1 was expressed in the nucleus of the SGCs, hair cells (including OHCs and IHCs), Kolliker's organ, Reissner's membrane, and STV (including spiral ligament and spiral limbus) (Figure 1). The overall distribution of LaminB1 remained unchanged in the whole cochlea at all these stages of postnatal development. On the other hand, at high magnification, the intensity of staining was increased at p0 in the SGC, BM, and STV and gradually decreased at p21 (Figure 1).

Expression of LaminB1 mRNA in the Developing Cochlea

The mRNA expression of LaminB1 displayed difference at developmental stages in STV, SGC, and BM, respectively (one-way ANOVA, *F*=21.122, 38.190, and 23.193, *d.f.*=3, *p*<0.001) (Figure 2). The expression of LaminB1 in BM (Figure 2) was remarkably lower in the p14 and p21 groups than in the p0 group (SNK test, *q*=8.989 and 9.171, *p*<0.001). LaminB1 mRNA at p14 and p21 (SNK test, *q*=7.348 and 7.530, *p*<0.001) was also expressed less than that at p7. However, there was no significant difference between the p0 and p7 groups (SNK test, *q*=1.641, *p*>0.05) or between the p14 and p21 groups (SNK test, *q*=0.1816, *p*>0.05). Collectively, LaminB1 mRNA in BM reached a maximum level after birth but sharply decreased to the lowest level at p21. The expression patterns of LaminB1 in STV and SGC were similar to those in BM.

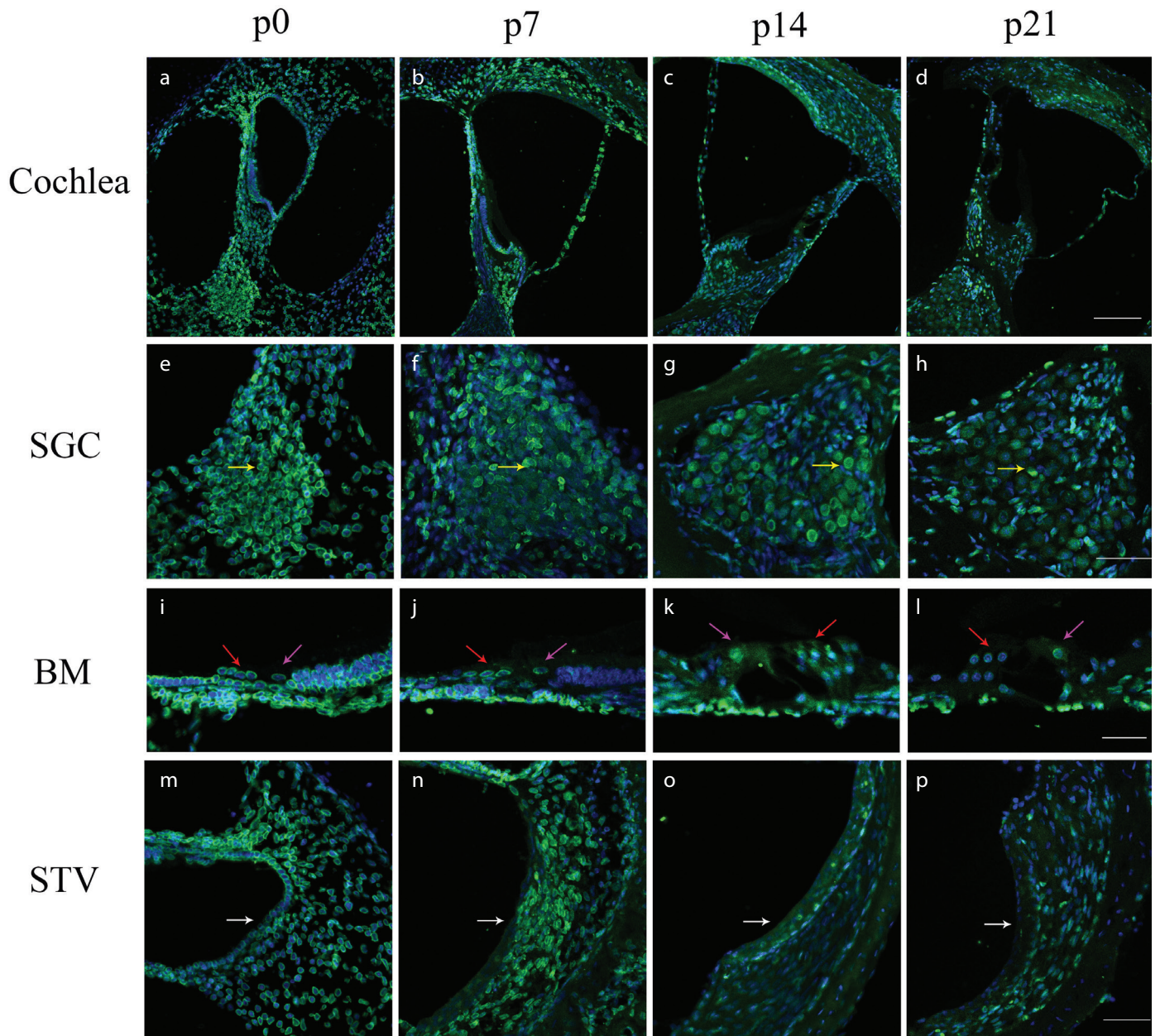


Figure 1. a-p. Localization and developmental changes of LaminB1 in the developing cochlea. (a, e, i, m) p0 group, (b, f, j, n) p7 group, (c, g, k, o), p14 group, (d, h, l, p) p21 group. The LaminB1 protein was labeled by fluorescein isothiocyanate (green fluorescence), and the nuclei were stained with DAPI (blue fluorescence). LaminB1 was mainly expressed in the SGC (a-h), BM (a-d, i-l), STV (a-d, m-p), Reissner's membrane (a-d), and limbus laminae spiralis (a-d). OHCs, red arrow; IHCs, pink arrow; SGC, yellow arrow; STV, white arrow. Scale bar: a-d, 100 μ m; e-h, m-p, 50 μ m; i-l, 25 μ m.

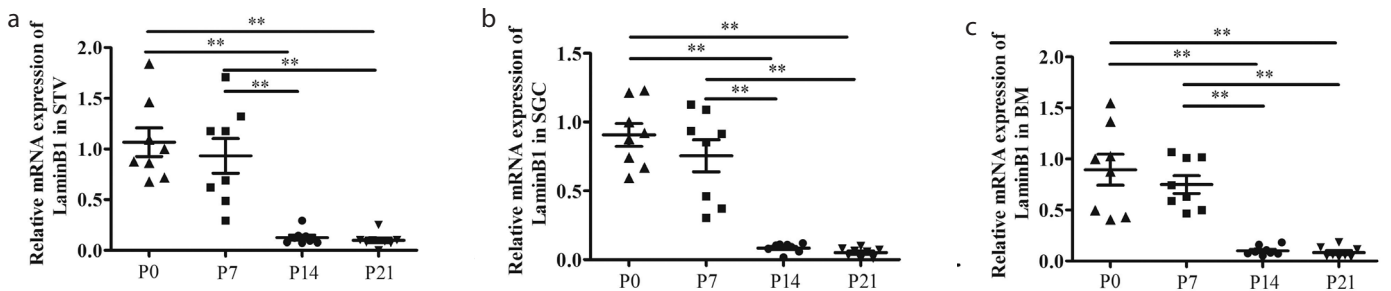


Figure 2. a-c. Expression of LaminB1 at the mRNA level in the developing cochlea. LaminB1 was down-regulated in the separated tissues of the cochlea during postnatal development. GAPDH was the reference gene. a) Relative mRNA expression of LaminB1 in STV. b) Relative mRNA expression of LaminB1 in SGC. c) Relative mRNA expression of LaminB1 in BM. ** $p < 0.001$.

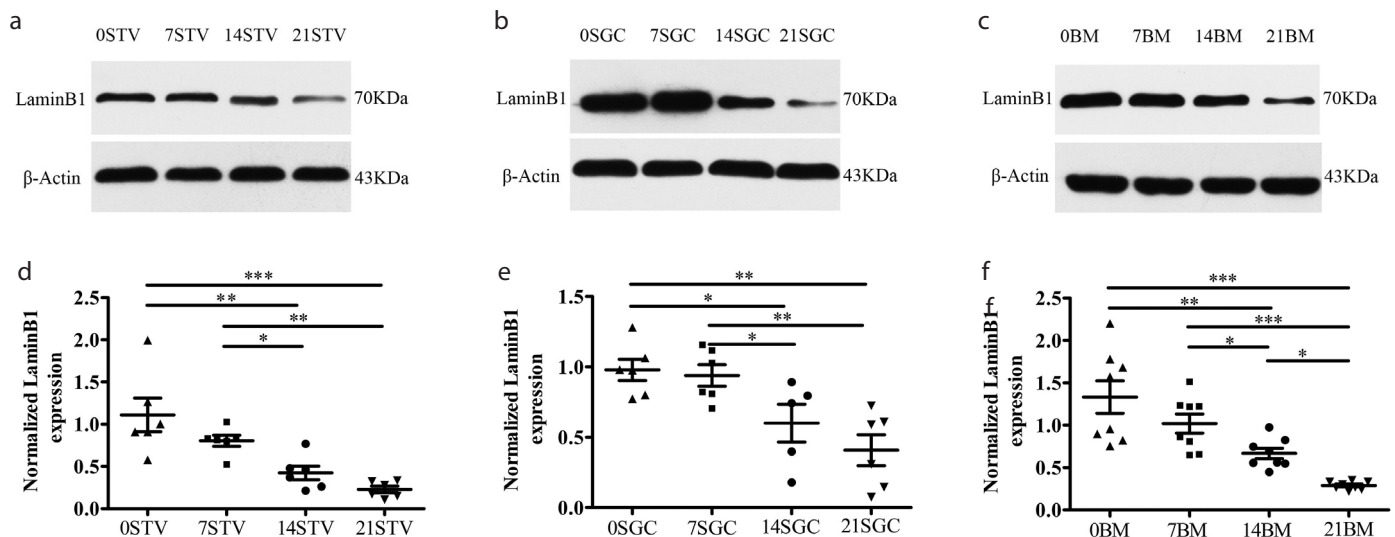


Figure 3. a-f. Expression of LaminB1 at the protein level in the developing cochlea. The 70 kDa and 43 kDa bands corresponding to LaminB1 and β -actin, respectively, were detected in STV, SGC, and BM (a-c). Relative quantification of the developmental expression of LaminB1 in different tissues of the rat's cochlea was shown (d-f). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 0STV, STV of p0 group; 0SGC, SGC of p0 group; 0BM, BM of p0 group.

Expression of LaminB1 Protein in the Developing Cochlea

Immunoreactive bands of 70 kDa and 43 kDa corresponding to LaminB1 and β -actin, respectively, were detected in STV, SGC, and BM (Figure 3). Normalized to β -actin, there was a different expression of LaminB1 at protein level among the four groups in STV, SGC, and BM (one-way ANOVA, $F = 11.948, 7.935, \text{ and } 15.152, d.f. = 3, p < 0.001$). In BM, the LaminB1 protein at p0 and p7 was strongly expressed in comparison with p14 (SNK test, $p_0, q = 5.754, p < 0.01$ and $p_7, q = 3.053, p < 0.05$). Furthermore, protein level at p21 was significantly lower than that at p14 (SNK test, $q = 3.270, p < 0.05$). These data demonstrated that LaminB1 proteins peaked after birth in BM but gradually declined during development. The developmental patterns of LaminB1 protein in STV and SGC shared similarity with those in BM (Figure 3).

DISCUSSION

Recently, there has been growing evidence showing the presence and importance of LaminB1 in mammals. In the adult rat, LaminB1 was found in almost all the retinal neurons [16]. LaminB1 was down-regulated at mRNA and protein levels during mouse brain development [8]. In addition, LaminB1 expression was variable in human tissues, even absent in muscle and connective tissues [9]. When LaminB1 was depleted at midgestation, the nuclear integrity of retinal neurons was affected, and neuronal survival was impaired [17]. Further, LaminB1 mutant embryo survived but died shortly after birth with abnormal cranial structure and long bone ossification [4]. Consistently, the forebrain-specific LaminB1 knockout model showed a reduced forebrain with abnormal neural layering [18]. Moreover, a duplication of *Lmnb1* results in adult-onset autosomal dominant leukodystrophy (ADLD), the only human disease that has been associated with the mutation of LaminB1 [19]. In view of these, LaminB1 contributes greatly to proper tissues development and function.

The cochlea is a sophisticated sensory organ, responsible for perceiving sound and discriminating frequencies. Cochlear development during the first 3 postnatal weeks involves many typical changes, for example, the formation of hair bundles and the differentiation of sensory epithelia and STV regions [20]. Despite the fact that LaminB1

is required for normal development of many tissues, there was no published work providing information about the localization and function of LaminB1 in the developing cochlea.

In the present study, we have confirmed the presence of LaminB1 in cochlear tissues. Additionally, we have further analyzed the differential expression level of LaminB1 during postnatal development. Our experiment was conducted with several independent techniques. First, by immunofluorescence techniques (Figure 1), we determined the presence of LaminB1 in the OHCs, IHCs, SGC, and STV. The intensity of staining gradually decreased from p0 to p21. In the STV, a strong staining reaction for LaminB1 was detected in the basal cells, which constitute an extensive gap junctional communication with strial intermediate cells and with fibrocytes in the spiral ligament [21]. It has been established that postnatal development of gap junctional communication plays an important role in the generation and maturation of the endocochlear potential (EP) [22]. The gap junctional system is most likely the pathway for recirculation of cochlear K^+ ions from hair cells to marginal cells [23]. Interruption of this recycling, which may be due to abnormal basal cells, would deprive the STV of K^+ and result in hearing loss [24, 25]. Meanwhile, LaminB1 has been reported to have functions in the development and differentiation of certain cell types [3]. Hence, we suggested that the specific expression of LaminB1 in basal cells may promote the normal development of the gap junctional system and then contributes to the generation and maturation of the EP. Thereafter, we performed western blot (Figure 3) demonstrating that protein products were also present in cochlear tissues (STV, SGC, and BM) at different developmental stages. Finally, we used qRT-PCR and western blot (Figures 2, 3) to investigate the differential expression level of LaminB1 and revealed it altered at four developmental stages in STV, SGC, and BM. While both mRNA and protein levels of LaminB1 in STV and SGC were consistently down-regulated, the decreased mRNA expression in BM did not correlate with its decreased protein expression between p14 and p21. This discrepancy in the expression of LaminB1 at mRNA and protein levels could be due to post-transcriptional or post-translational regulation. The mRNA molecules generated from transcription

undergo a series of events leading to translation, including splicing, quality control, transport to the cytoplasm, and turnover [26-28]. Each step may be modulated to change the expression of protein from mRNAs. LaminB1 also undergoes some forms of post-translational modifications, such as farnesylation [29], phosphorylation, glycosylation, and sumoylation [30], which are linked to the significant conformational changes of their target proteins [31]. Therefore, the expression of LaminB1 exhibited differences between mRNA and protein levels from p14 to p21 (Figures 2, 3).

Our studies also have found that LaminB1 was decreased in individual cochlear tissues with postnatal time (Figures 1, 2, 3). We speculated that the following reasons are likely to explain the phenomenon. BM develops in a time-dependent manner, both structurally and functionally [32]. The reduction of LaminB1 in BM may be attributed to the thinning of the BM, development of the organ Corti, and reorganization of the epithelium [33]. We could also suggest that the loss of LaminB1 in BM in our study was coincident with the degeneration of Kolliker's organ. Our result in SGC was in agreement with previous finding that LaminB1 gradually decreased in the brain during development [8]. Further studies demonstrated LaminB1 as a negative regulator of myelin-specific proteins regulating oligodendrocyte development in ADLD brain tissues [8, 19]. Thus, down-regulation of LaminB1 might be associated with myelin formation in SGC. In addition, fluctuation of LaminB1 can alter nuclear ionic signaling and nuclear mechanics in somatic cells [34]. Therefore, we postulate that the loss of LaminB1 in STV may alter mechanotransduction signaling of the internal and external cochlea, affecting the generation and maturation of EP. Collectively, our study provided direct evidence that LaminB1 was expressed in the cochlea and was developmentally regulated, suggesting a possible role for LaminB1 in cochlear development.

CONCLUSION

In our current data and previous study demonstrating LaminB1 as a key regulator for development and differentiation in certain tissue and cell types, we may suggest that LaminB1 is important for cochlear development. Our findings provide a theoretical basis for further studies on the physiological function of LaminB1 in the cochlea.

Ethics Committee Approval: Ethics committee approval was received for this study from Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (IRB ID: TJ-A20171203).

Informed Consent: Informed consent is not necessary due to the animal experiments.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: The authors have no conflict of interest to declare.

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