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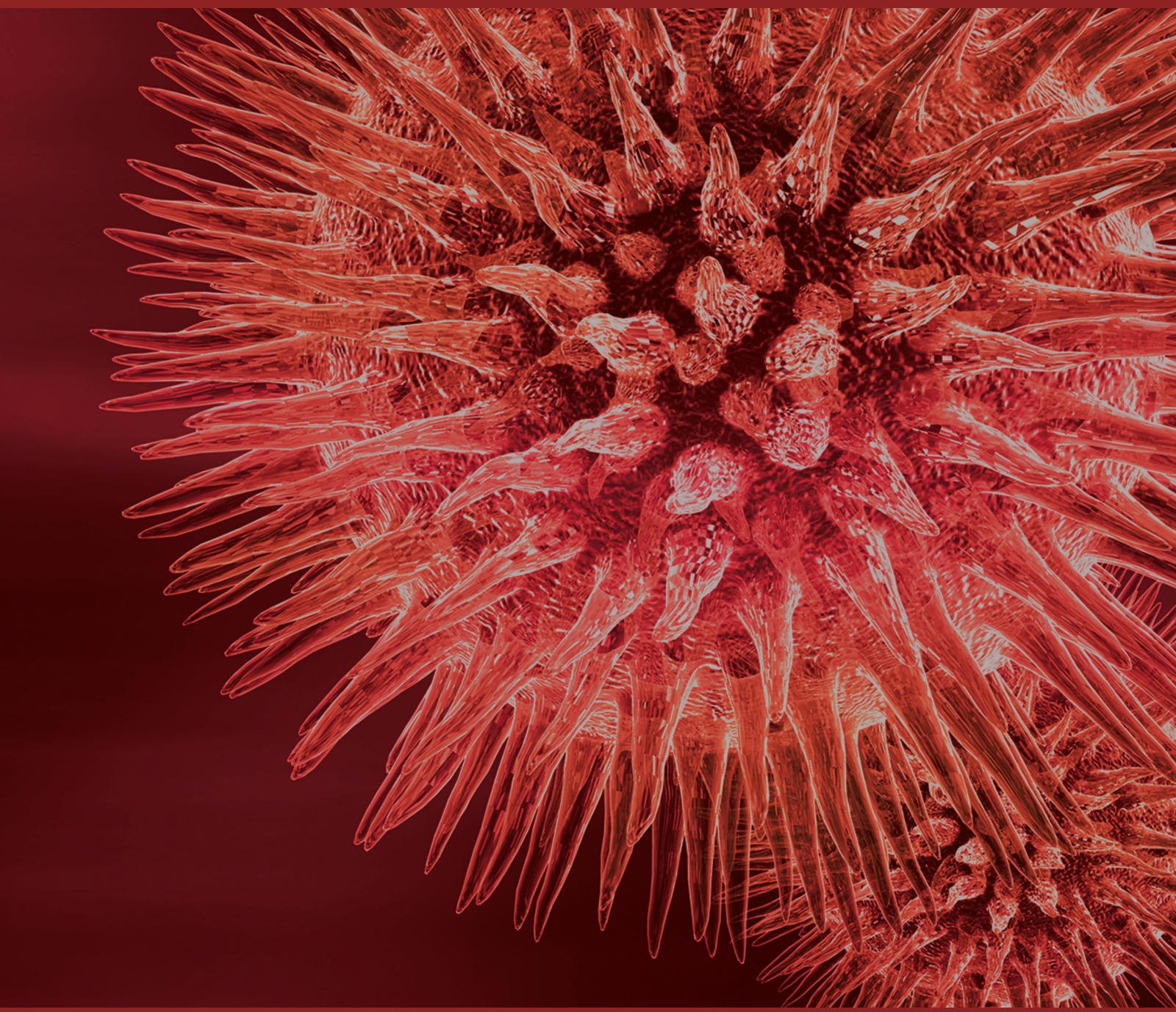
Advances in Molecular Genetics and the Molecular Biology of Deafness

Guest Editors: Shin-ya Nishio, Isabelle Schrauwen, Hideaki Moteki, and Hela Azaiez



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Editorial

Advances in Molecular Genetics and the Molecular Biology of Deafness

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Congenital sensorineural hearing loss is the most common sensory disorder, with approximately 1 in every 1000 newborns in developed countries suffering from severe-to-profound hearing loss. At least half of those cases are attributable to genetic causes with more than 90 causative genes identified to date reflecting the complex clinical and genetic landscapes of hereditary hearing loss [1, 2].

Recent advances in molecular genetics technologies, notably next-generation sequencing (NGS), have drastically accelerated the identification of novel genes involved in hearing mechanism and expanded the mutational spectrum of known deafness-causing genes [3–9]. In addition to NGS, recent progress in genome editing, embryonic stem cells, and induced pluripotent stem cells has opened a new gate to a fast and thorough characterization and understanding of the precise functions and mechanisms involved in the biology of hearing and deafness.

This special issue is to exhibit the advances and recent progress in the fields of molecular genetics and molecular biology of hearing and deafness.

The review paper by S. Kitajiri and T. Katsuno (Kyoto University) summarized the importance of the tricellular tight junction proteins (tricellulin, occludin, ILDR1, and the angulin family) in the inner ear by acting as a barrier separating the endolymphatic and perilymphatic spaces, which is essential for the generation and maintenance of the endocochlear potential.

M. Hosoya et al. described the cochlea distribution patterns of KIAA1199 proteins in a nonhuman primate, the common marmoset (*Callithrix jacchus*). *KIAA1199* has been reported as a cause of progressive hearing loss, but its spatial expression showed different and distinct patterns in mouse and rat cochlea. In this report, M. Hosoya at al. showed a more widespread KIAA1199 protein expression in the marmoset. These results are of importance for further investigation and elucidation of the functional role of KIAA1199 in primate cochlea.

Developing novel diagnostic tools that are tailored to specific ethnicities is crucial for a cost-effective genetic screening for deafness. In this issue, F. Zhang et al. described their multiplex genetic screening system "SNPscan assay" used to screen a total of 115 known mutations in *GJB2*, *SLC26A4,* and mtDNA 12SrRNA.

Cochlear implantation (CI) is the most important and effective treatment for patients with profound sensorineural hearing loss. However, outcomes vary among patients due to several reasons, one being the heterogeneous nature of the clinical as well as genetic etiology of hearing loss. H. Koyama et al. reported the CI outcomes in five patients with Waardenburg syndrome. They showed that CI is a good and suitable treatment option for Waardenburg syndrome cases.

Statins are inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase and widely used as cholesterol-lowering drugs. However, in the last decade, conflicting data about the effect of statins on neuronal cells and the auditory system has been published. K. Leitmeyer et al. studied the effect of simvastatin on spiral ganglion neurons explants in

vitro and showed its neurotoxic effect that seems to be at least partially mediated by the mevalonate pathway.

In this special issue, we collected both basic and clinical original research articles stimulating the continuing efforts to understand the mechanisms of deafness and hearing systems. It is our wish to increase interest in this field and further accelerate future treatment options based on solid basic research.

Acknowledgments

Finally, as guest editors, we thank all authors, the editors, and reviewers who have contributed to this special issue.

Shin-ya Nishio
Isabelle Schrauwen
Hideaki Moteki
Hela Azaiez

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Research Article

Distinct Expression Pattern of a Deafness Gene, *KIAA1199*, in a Primate Cochlea

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Deafness is one of the most common types of congenital impairments, and at least half of the cases are caused by hereditary mutations. Mutations of the gene *KIAA1199* are associated with progressive hearing loss. Its expression is abundant in human cochlea, but interestingly the spatial expression patterns are different between mouse and rat cochleae; the pattern in humans has not been fully investigated. We performed immunohistochemical analysis of a nonhuman primate, common marmoset (*Callithrix jacchus*), cochlea with a KIAA1199-specific antibody. In the common marmoset cochlea, KIAA1199 protein expression was more widespread than in rodents, with all epithelial cells, including hair cells, expressing KIAA1199. Our results suggest that the primate pattern of KIAA1199 expression is wider in comparison with rodents and may play an essential role in the maintenance of cochlear epithelial cells.

1. Introduction

Deafness is one of the most common types of congenital impairment, and at least half of all cases are caused by hereditary mutations. In some patients, hearing loss is progressive, with hearing loss developing gradually during childhood or youth after the acquisition of speech abilities. Mutation of the *KIAA1199* gene is one cause of hereditary hearing loss [1].

KIAA genes were identified by sequence analysis of human large (>4 kb) cDNAs in the Kazusa cDNA sequencing project [2]. *KIAA1199* was found in a sequence of cDNA clones of unknown human genes from human adult and fetal brain cDNA libraries in 1999 [3]. *KIAA1199* was later identified by cDNA microarray analysis as a cochlea-specific gene that was abundantly expressed in the human cochlea [4], and the protein it encodes has been found to play a central role in hyaluronan binding and depolymerization [5]. Recently, this gene became of interest in the cancer research field after clinical studies found it to have an involvement in cancer progression, metastasis, and poor prognosis of patients [6].

Signal cascade analyses revealed that *KIAA1199* is a likely target gene of the Wnt/β-catenin signaling pathway [7], which is also known to be a key pathway in inner ear development [8] and regeneration [9]. Mutations of *KIAA1199* cause progressive hearing loss with a downsloping pattern, and usually the hearing impairment starts after acquisition of languages [1]. In such postlingual hearing loss, in general, the auditory cortex has already developed and prevention of progressive hearing loss in the inner ear would be expected to be the most promising therapy for retaining long-term hearing ability; however, there is currently no such effective treatment for this condition. Thus, understanding the physiological functions of *KIAA1199* and its pathophysiology when mutated is an important issue.

Transgenic or knockout animal models are powerful tools for clarifying disease mechanisms. In many genetic disorders, including hereditary hearing loss, their mechanisms have been unveiled by using animal models, especially transgenic or knockout mouse models [10]. So far, no animal model harboring *KIAA1199* mutations or its knockout has been reported. Expression analysis of KIAA1199 protein in the cochlea has been performed in mice and rats [1, 11], where different distribution patterns for each species were described,

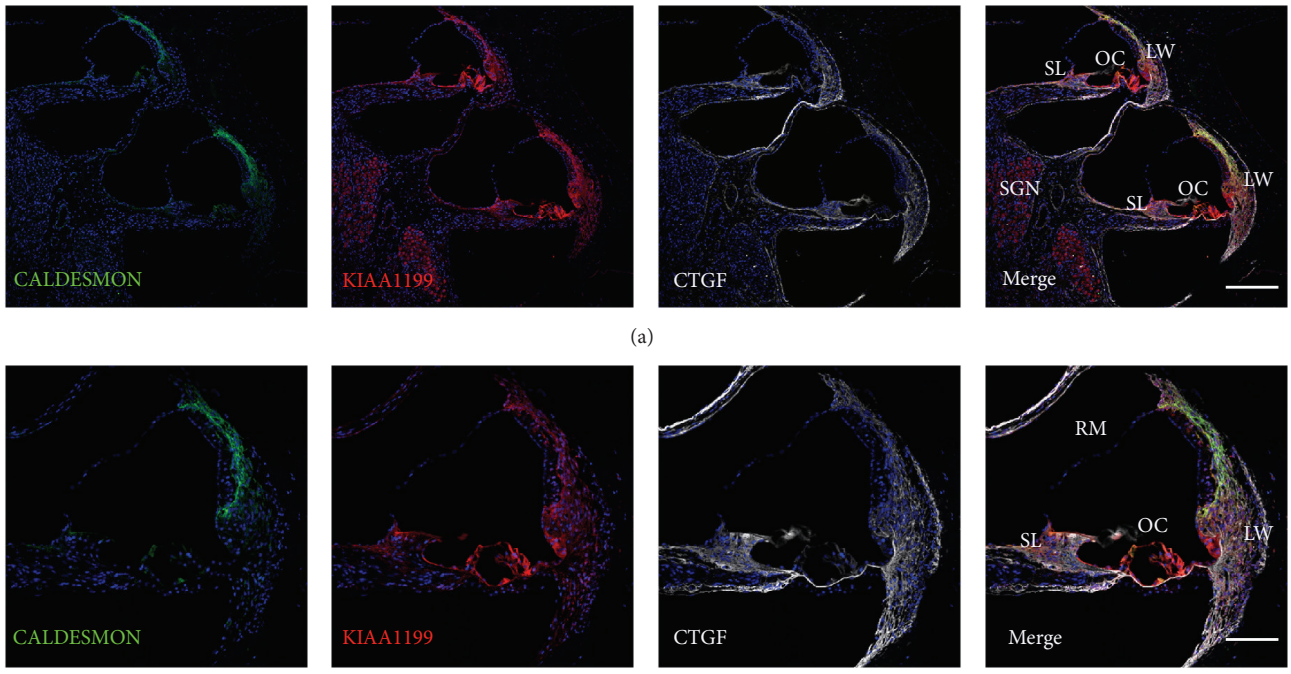

Figure 1: Expression of KIAA1199 in the cochlea of the common marmoset. (a and b) KIAA1199 expression is observed in the lateral wall of the cochlea, sensory epithelium, spiral limbus, and spiral ganglion neuron. No expression is observed in Reissner's membrane. LW: lateral wall of cochlea, OC: organ of Corti, SL: spiral limbus, SGN: spiral ganglion neuron, and RM: Reissner's membrane. The nuclei were counterstained with Hoechst (blue). Scale bar: 200 μm in (a) and 100 μm in (b).

suggesting the possibility of an even greater difference in primates. We therefore examined expression of KIAA1199 protein by immunohistochemistry in cochlea from a nonhuman primate, the common marmoset (*Callithrix jacchus*).

2. Materials and Methods

2.1. Specimens of the Common Marmoset. Fixed and decapitated cadaverous heads of newborn common marmoset (postnatal day 2) and 3–6-year-old common marmosets were kindly provided by Junichi Hata, Reona Kobayashi, Takahiro Kondo, Kimika Yoshino-Saito, and Seiji Shiozawa. Fixed skin samples were also kindly provided. The animal experiments were approved by the Ethics Committee of Keio University (number 11006) and were in accordance with the guidelines of the National Institutes of Health and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

2.2. Tissue Preparation. Temporal bones from newborn and young adult marmosets were dissected, fixed, decalcified with Decalcifying Solution B (Wako, Saitama, Japan) for 3-4 weeks, and embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) for cryosections. The 7 μm sections were used for immunohistochemistry. Skin samples from young adult marmosets were dissected and fixed.

Temporal bones from 3-week-old mice (C57BL/6) and 3-month-old rats were dissected, fixed, decalcified with Decalcifying Solution B for 3–10 days, and embedded in Tissue-Tek OCT compound for cryosection. The 7 μm sections were used for immunohistochemistry. These animal experiments using mice were approved by the Ethics Committee of Keio University (number 08020) and were in accordance with the guidelines of the National Institutes of Health and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.3. Immunohistochemistry. After a brief wash with phosphate-buffered saline (PBS) the sections were heated (80°C) in 10 mM citrate buffer (pH 6) for 1 h. After another brief wash, the sections were preblocked for 1 h at room temperature with 10% normal serum in PBS, incubated with primary antibodies at 4°C overnight, and then incubated with Alexa Fluor-conjugated secondary antibodies (Alexa488, Alexa555, and Alexa647) for 60 min at room temperature. The nuclei were counterstained with Hoechst 33342.

2.4. Whole-Mount Immunofluorescence. After 6 weeks of decalcifications of the cochlea of common marmoset, sensory epithelium containing organ of Corti was dissected. Sections were incubated with 10% normal serum in PBS for 1 h at room temperature and then with primary antibodies at 4°C overnight, followed by an incubation with Alexa Fluor-conjugated secondary antibodies (Alexa555 and Alexa647) and Phalloidin-conjugated Alexa488 (Thermo Fisher Scientific) for 60 min at room temperature. The nuclei were counterstained with Hoechst 33342.

2.5. Antibodies. The primary antibodies used in this study are as follows: anti-KIAA1199 (rabbit immunoglobulin G (IgG),

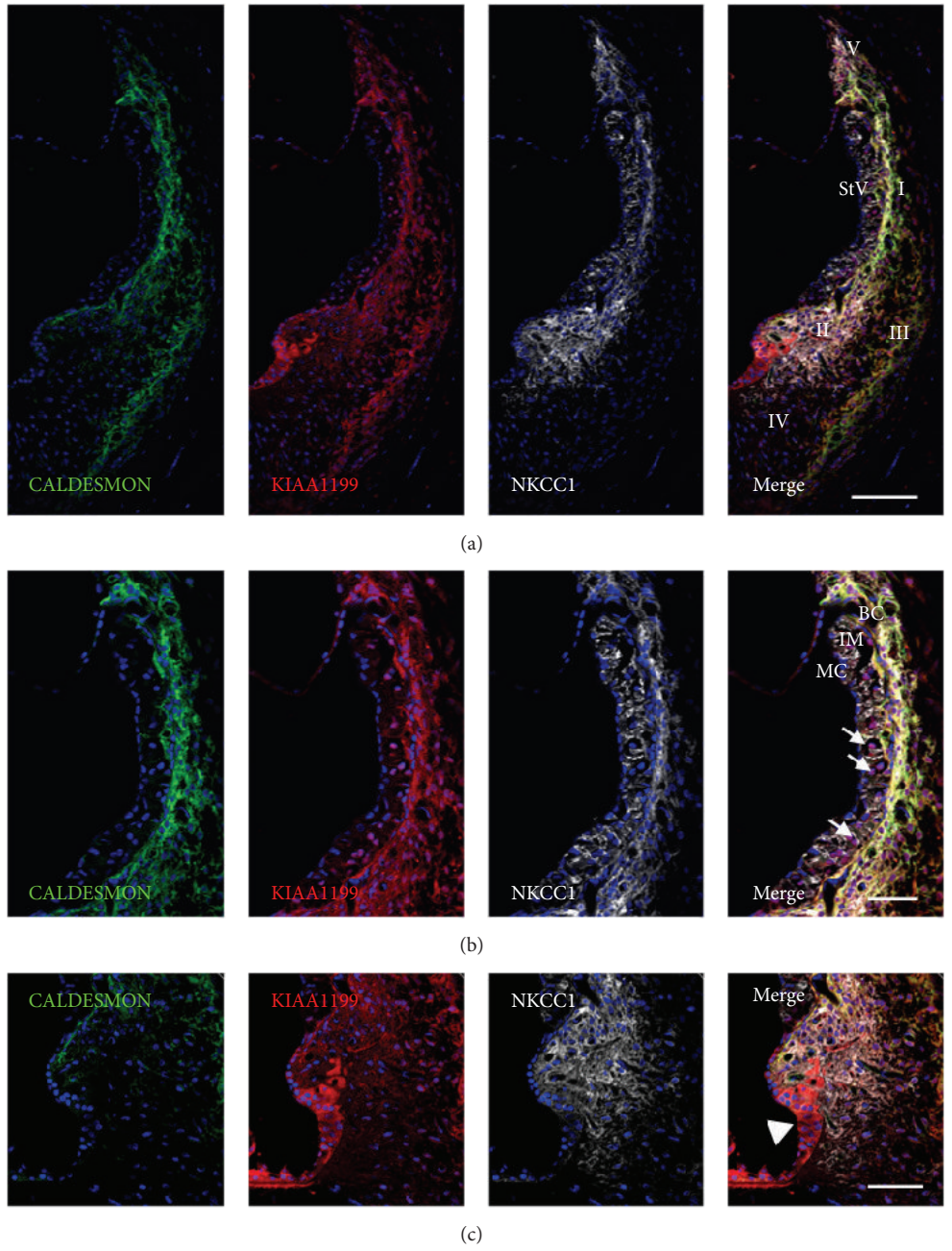

Figure 2: Expression of KIAA1199 in the lateral wall. (a) KIAA1199 expression is observed in type I, II, III, and V spiral fibrocytes. (b) KIAA1199 expression is observed in intermediate cells (arrow) and basal cells in the stria vascularis. No expression is observed in NKCC1 positive marginal cells. (c) KIAA1199 expression is markedly observed in outer sulcus cells (arrow head). No expression was observed in type IV spiral fibrocytes. StV: stria vascularis, I–V: type I–V spiral ligament fibrocytes, MC: marginal cells, IM: intermediate cells, and BC: basal cells. The nuclei were counterstained with Hoechst (blue). Scale bar: 100 μm in (a) and 50 μm in (b) and (c).

Proteintech, Manchester, UK, 21129-1-AP, 1 : 50, rabbit IgG, Cosmo Bio, Tokyo, Japan, CNP-IP-208, 1 : 50), anti-SOX2 (goat IgG, Santa Cruz Biotechnology, Dallas, TX, US, sc17320, 1 : 100), anti-CTGF (goat IgG, Santa Cruz Biotechnology, sc14939, 1 : 100), anti-CALDESMON (Sigma-Aldrich, C0297, 1 : 100), anti-NKCC1 (goat IgG, Santa Cruz Biotechnology, sc21545, 1 : 300), anti-MYOSIN7a (mouse IgG, DSHB, Iowa City, IA, US, 138-1-s, 1 : 30), and anti-β-III TUBULIN (mouse IgG, Sigma-Aldrich, T8660, 1 : 250).

3. Results and Discussion

Recently we reported a brief document about the basic morphology and feasible methods for immunohistochemical analysis of the cochlea of the common marmoset (*Callithrix jacchus*) [12], which is a useful animal in modeling human disease by generating transgenic monkeys [13]. The cochlea of marmosets has high similarity with that of humans in its basic morphology and protein distributions as defined

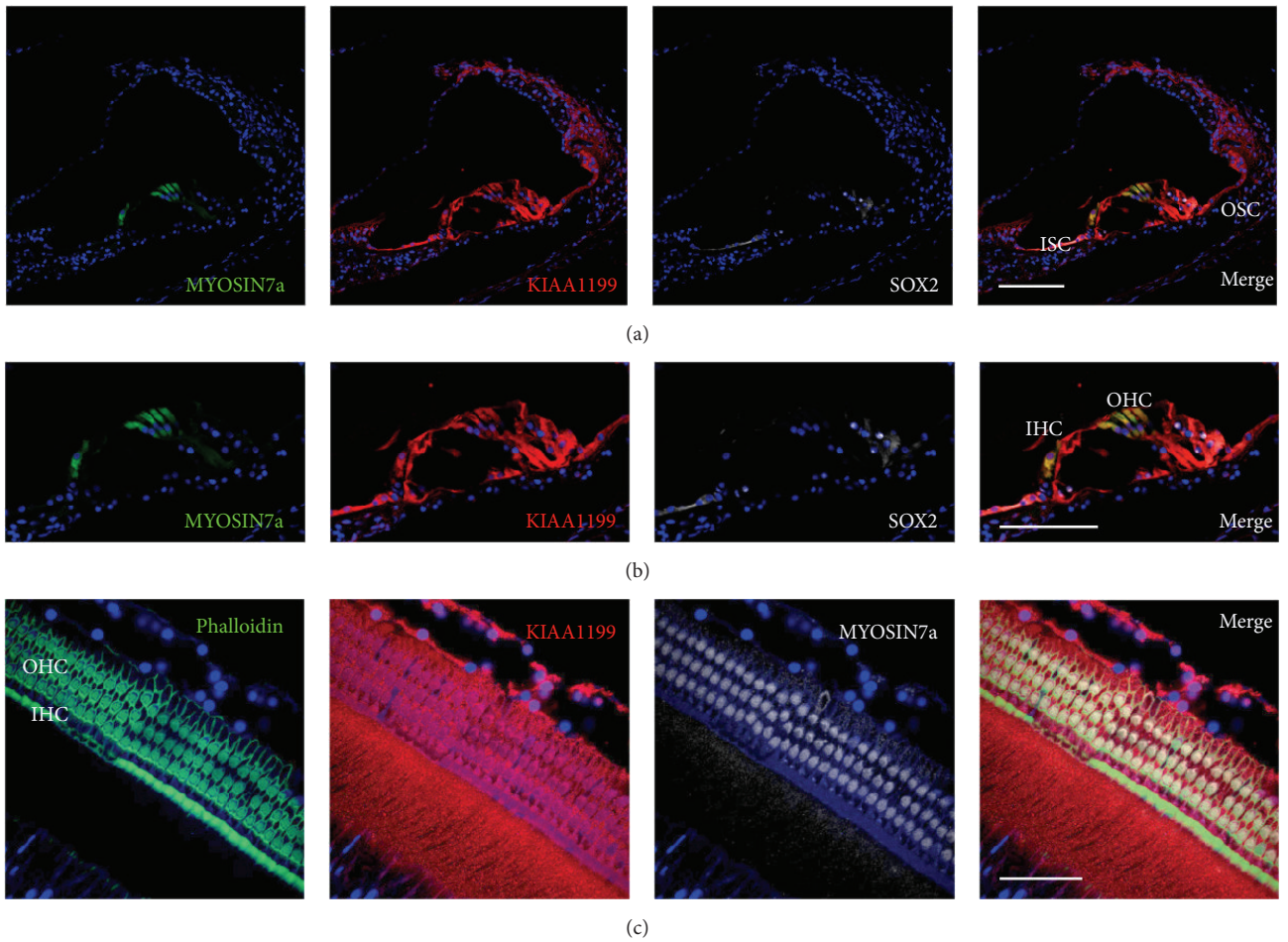

Figure 3: Expression of KIAA1199 in the organ of Corti. (a) KIAA1199 expression is observed in the organ of Corti. (b) KIAA1199 expression is observed in MYOSIN7a-positive hair cells and supporting cells between the outer and inner sulcus cells, including SOX2-positive supporting cells. (c) Whole-mount immunofluorescence also showed broad expressions of KIAA1199 in the organ of Corti. ISC: inner sulcus cells, OSC: outer sulcus cells, IHC: inner hair cells, and OHC: outer hair cells. The nuclei were counterstained with Hoechst (blue). Scale bar: (a) and (b): 100 μm, (c): 50 μm.

by immunohistochemistry. Our previous results identified discrepancies in protein expression patterns in the cochlea between primates and rodents, indicating the necessity of studying primates, especially in regard to the deafness genes, where the typical human symptoms cannot be reproduced in mouse models; for example, see *CX31* [14] and *CRYM* [15].

In the common marmoset, KIAA1199 protein expression is observed in the lateral wall spiral ligament, hair cells, supporting cells, spiral limbus, and spiral ganglion neurons (Figure 1). No immunoreactivity was observed in Reissner's membrane or beneath the basilar membrane.

In the spiral ligament, KIAA1199 expression was observed in type I, II, III, and V fibrocytes (Figure 2(a)). In the stria vascularis, KIAA1199 expression was observed in intermediate cells and basal cells, whereas no immunoreactivity was observed in NKCC1 positive marginal cells (Figure 2(b)). Notably, KIAA1199 immunoreactivity was relatively strong in the outer sulcus cell (Figure 2(c)).

In the organ of Corti, KIAA1199 expression was broadly observed in supporting cells between the inner and outer sulcus cells as well as the inner and outer hair cells (Figure 3). In the spiral ganglion neurons, KIAA1199 expression was observed in β-III tubulin positive neurons (Figure 4).

We also examined the expression of KIAA in the newborn of common marmoset. Immunoreactivities for KIAA1199 were observed in the lateral wall spiral ligament, hair cells, supporting cells, spiral limbus, and spiral ganglion neurons (Figure 5). The result indicates that this KIAA1199 expression pattern is maintained during their lifetime.

To validate the anti-KIAA1199 antibody (Proteintech) used in this study, we performed immunostaining in the skin of the common marmoset where both immunostaining and in situ hybridization were previously performed [16] (Figure 6(a)). Our results using the skin of common marmoset completely agreed with the previous reports of KIAA1199 expression in the skin of human. Next, we used another anti-KIAA1199 antibody (Cosmo Bio) to reconfirm our observations. The distribution patterns of the immunoreactivity for KIAA1199 using this other antibody was observed (Figure 6(b)). Furthermore, we stained the cochleae of mouse

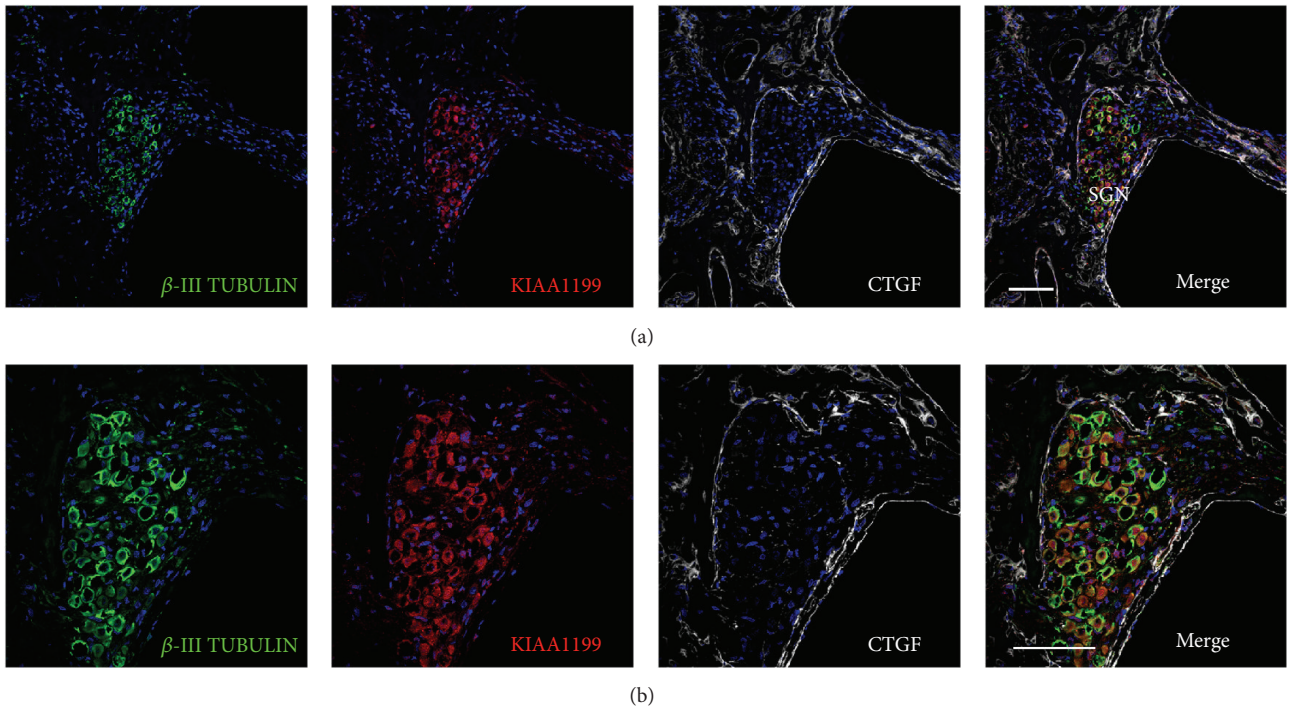

Figure 4: Expression of KIAA1199 in the spiral ganglion neurons. (a and b) KIAA1199 expression is observed in the spiral ganglion neurons. KIAA1199 expression is observed in β-III tubulin positive spiral ganglion neurons (SGN). The nuclei were counterstained with Hoechst (blue). Scale bar: 100 μm.

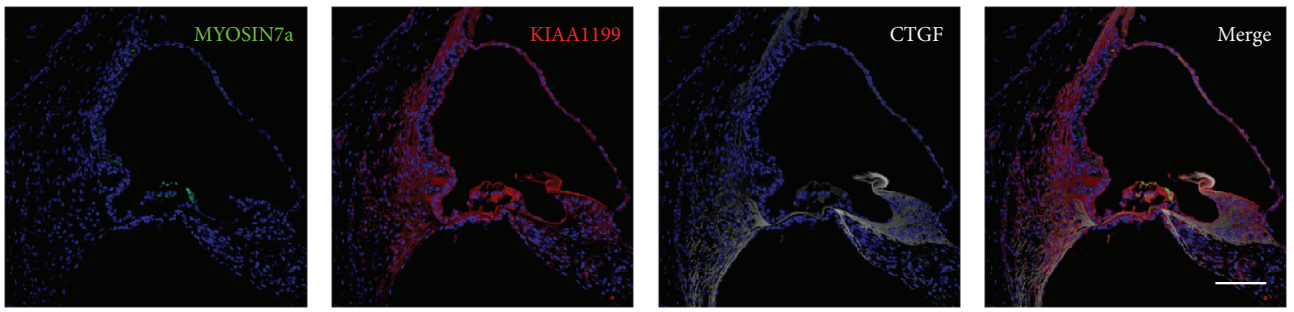

Figure 5: Expression of KIAA1199 in the newborn common marmoset. KIAA1199 expression is observed in the cochlea of newborn common marmoset (postnatal day 2). Immunoreactivities of KIAA1199 in the cochlear epithelial cells including hair cells, supporting cells, and outer sulcus cells were observed. Expressions were also detected in the lateral wall fibrocytes and stria vascularis. The nuclei were counterstained with Hoechst (blue). Scale bar: 100 μm.

and rat with the antibodies used in this study (Figures 6(c) and 6(d)). In the mouse cochlea, immunoreactivities were detected in the lateral wall fibrocytes and the spiral limbus as previously reported [1]. Of note, the presenting staining results in the rat cochlea with anti-KIAA1199 antibody completely matched those reported in a previous report [11].

The expression of KIAA1199 in the common marmoset was more widespread than that in the mouse or rat. Expression of KIAA1199 has been reported in the fibrocytes of the spiral ligament and the spiral limbus with a transient expression in Deiter's cells at P0 in the mouse [1] (Table 1). In the rat, KIAA1199 expression was observed in supporting cells; however, its expression was not observed in hair cells and outer sulcus cells where obvious expression was detected in the common marmoset cochlea. In short, there is a large species difference in the pattern of KIAA1199 expression in the cochlea across mice, rats, and primates.

To understand the pathophysiology of a progressive hereditary hearing loss in patients, it is necessary to understand the roles of the deafness gene in the maintenance of hearing. Recently, in cancer research, a relationship between the Wnt/β-catenin signaling pathway and KIAA1199 was reported [6]. KIAA1199 regulates the Wnt/β-catenin signaling pathway by interacting with ASCL2, LGR5, ITPR3, and Ca^{2+} signaling [6, 17, 18]. In the inner ear, Wnt/β-catenin signals are essential for the morphogenesis and regeneration

Table 1: Differential expression of KIAA1199 in marmosets and rodents.

	Expression patterns														
	Hair cells		Supporting cells												
	Inner hair cell	Outer hair cell	Inner sulcus cells	Inner pillar cells	Outer pillar cells	Deiter's cells	Hensen cells	Claudius cells	Outer sulcus cells	Spiral ligament	Spiral limbus	Stria vascularis	Reissner's membrane	SGN	Ref.
Mouse	−	−	−	−	−	+ (~P7)	−	−	−	+	+	−	−	−	Abe et al. 2003 [1]
Rat	−	−	−	+	+	+	−	−	−	NA	NA	NA	NA	NA	Usami et al. 2008 [11]
Marmoset	+	+	+	+	+	+	+	+	+	+	+	+	−	+	

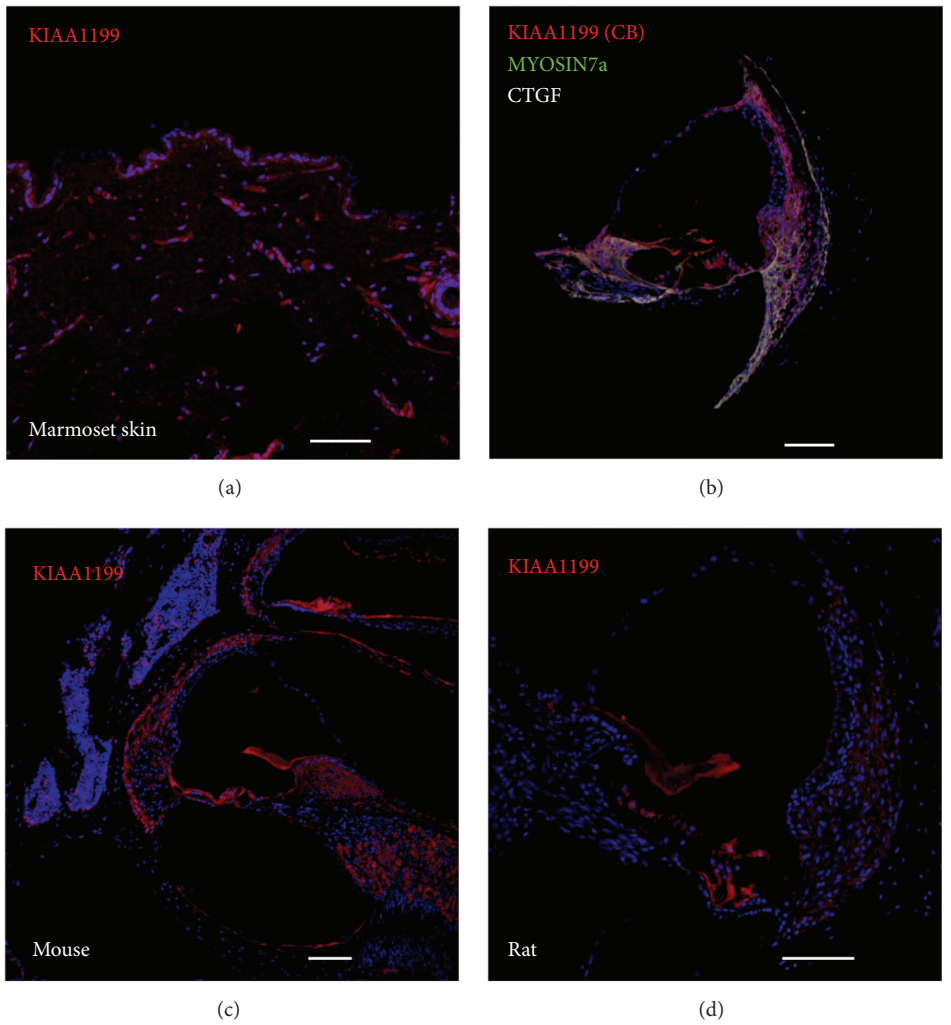

Figure 6: Validations of anti-KIAA1199 antibodies used in this study. (a) KIAA1199 expression is detected in the skin of the common marmoset. (b) Another anti-KIAA1199 antibody (Cosmo Bio: CB) revealed the same expression patterns of KIAA1199 in the common marmoset cochlea. (c) Immunohistochemistry with the cochleae of mouse. The immune reactivities in the lateral wall fibrocytes and spiral limbus were detected as reported previously. (d) Immunohistochemistry with the cochleae of rat. The immune reactivities were restricted in the supporting cells as reported previously. The nuclei were counterstained with Hoechst (blue). Scale bar: 100 μm.

of sensory and nonsensory epithelial cells [9, 19, 20]. The expression of KIAA1199 in the sensory epithelium in the marmoset, where Wnt activation is essential for its proliferation, may allow it to interact with the signal cascade and play an important role in cellular maintenance, as seen in cancer cells.

In a future study, functional analysis of KIAA1199 in the inner ear is necessary for understanding *KIAA1199*-related deafness. In such experiments, in general, generating transgenic or knockout mice is the first step. However, for KIAA1199, our observation showed large discrepancies of expression patterns in the cochlea between rodents and primates. Therefore, it is likely that a mouse model will fail to reproduce the hearing impairment observed in human patients. Thus, for an *in vivo* animal model, generating a transgenic primate model, such as a common marmoset, would be required.

4. Conclusion

KIAA1199 showed a primate-specific expression pattern in the cochlea. Future functional as well as mutation screening studies using primates will be crucial to understanding the mechanisms of *KIAA1199*-related hearing loss.

Competing Interests

Hideyuki Okano is a founding scientist and a paid member in Scientific Advisory Board of SanBio Co., Ltd.

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Research Article

The Hearing Outcomes of Cochlear Implantation in Waardenburg Syndrome

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Objectives. This study aimed to determine the feasibility of cochlear implantation for sensorineural hearing loss in patients with Waardenburg syndrome. *Method*. A retrospective chart review was performed on patients who underwent cochlear implantation at the University of Tokyo Hospital. Clinical classification, genetic mutation, clinical course, preoperative hearing threshold, high-resolution computed tomography of the temporal bone, and postoperative hearing outcome were assessed. *Result*. Five children with Waardenburg syndrome underwent cochlear implantation. The average age at implantation was 2 years 11 months (ranging from 1 year 9 months to 6 years 3 months). Four patients had congenital profound hearing loss and one patient had progressive hearing loss. Two patients had an inner ear malformation of cochlear incomplete partition type 2. No surgical complication or difficulty was seen in any patient. All patients showed good hearing outcome postoperatively. *Conclusion*. Cochlear implantation could be a good treatment option for Waardenburg syndrome.

1. Introduction

Waardenburg syndrome (WS) is a major cause of symptomatic sensorineural hearing loss (SNHL). It is an autosomal dominant disease characterized by dystopia canthorum, hyperplasia of the eyebrows, heterochromia iridis, white forelock, and congenital SNHL [1]. Clinically, WS is divided into 4 types based on the following clinical criteria [2]: the presence (type 1) or absence (type 2) of dystopia canthorum, additional upper limb anomalies and coarser facial characteristics (type 3), or Hirschsprung's disease (type 4). The genetic mutations differ among the types of WS (Table 1) [3].

The occurrence rates of SNHL in WS also differ among these types. About 60% of type 1 and type 3 children, and about 90% of type 2 and type 4 children, have SNHL [4]. While the hearing loss can be unilateral or bilateral and can vary in nature and severity, bilateral profound SNHL is the most common type of hearing loss.

Cochlear implantation (CI) is an option for patients with severe to profound bilateral hearing loss. However, few reports about CI in WS children have been published. The aim of this study is to describe the outcomes in five pediatric patients with WS who underwent CI at our institute.

2. Methods

A retrospective chart review was performed on patients who had undergone CI in the Otorhinolaryngology Department at the University of Tokyo Hospital from 1991 to 2014. Five patients were diagnosed as WS by their characteristic features or gene testing. Their clinical type, clinical course, preoperative hearing thresholds, high-resolution computed tomography (CT), and the course of hearing ability were evaluated. Meaningful auditory integration scales (MAIS), meaningful use of speech scale (MUSS), CI-2004 Japanese closed set three words' test, and 67-s monosyllable word tests were used to evaluate hearing performance after CI [5, 6].

3. Results

Table 2 shows the characteristics of the five children with WS who underwent CI. The average age at implantation was 2

Table 1: Classifications of Waardenburg syndrome.

	Clinical manifestations	The incidence rate of SNHL	Genetic mutation
Type 1	Dystopia canthorum, white forelock, white eyelashes, leukoderma, heterochromia iridis	60%	*PAX3*
Type 2	The absence of dystopia canthorum	90%	*MITF, WS2B, WS3B EDNRB, EDN3, SOX10 SNAI2*
Type 3	Type 1 + upper limb abnormalities	60%	*PAX3*
Type 4	Type 2 + Hirschsprung's disease	90%	*EDNRB, EDN3, SOX10*

Table 2: Characteristics of the patients.

Patient number	Operation age	Clinical classification	The type of hearing loss	Anomalies of the inner ear	Genetic mutation	Hereditary form
1	2 y 3 mon	Type 4	Congenital	None	None*	Sporadic
2	1 y 9 mon	Type 1	Congenital	IP2	N.A	Sporadic
3	2 y 2 mon	Type 1	Congenital	IP2	N.A	AD
4	2 y 2 mon	Type 1	Congenital	None	*PAX3*	AD
5	6 y 3 mon	Type 2	Progressive	None	N.A	AD

AD: autosomal dominant.
*Patient one was not tested for all Waardenburg genes.

years 11 months (ranging from 1 year 9 months to 6 years 3 months). Three patients were classified as type 1, one as type 2, and one as type 4. Four had congenital hearing loss and one had progressive hearing loss. The patient who had progressive hearing loss underwent CI at 6 years 3 months of age. Three patients (patients 3, 4, and 5) showed an autosomal-dominant pattern, and two patients (1 and 2) were sporadic. *PAX3* gene mutation was confirmed in one patient (4). Mutation was not detected in one patient (1). Three patients (2, 3, and 4) did not have gene testing. The average preoperative unaided threshold was 117.2 dB (105–135 dB) and the average aided threshold was 79.8 dB (60–84 dB). High-resolution CT of the temporal bone revealed that two patients (2 and 3) had incomplete partition type 2 while the other three patients had normal anatomy. No complication including a cerebrospinal fluid gusher during surgery was observed in any patient. All patients had full insertion of CI electrodes via scala tympani cochleostomy. Cefazolin or cefotiam was used as antibiotics for five days after surgery. No corticosteroids were used. CI24RE Contour Advance electrode was used for all patients. Facial nerve stimulation by CI was not seen in any of the patients.

Figure 1 shows the time course of MAIS and MUSS after the operation in four WS patients with congenital hearing loss. In all four patients, the MAIS scores increased immediately after CI and the MUSS scores rose slowly but surely.

Table 3 shows the results of postoperative thresholds and speech recognition tests. As it suggests, the average thresholds of cochlear implantation were below 40 dB for all patients. The average score of CI-2004 three words' tests is 78% and the score of 67-s monosyllable word tests in all three patients

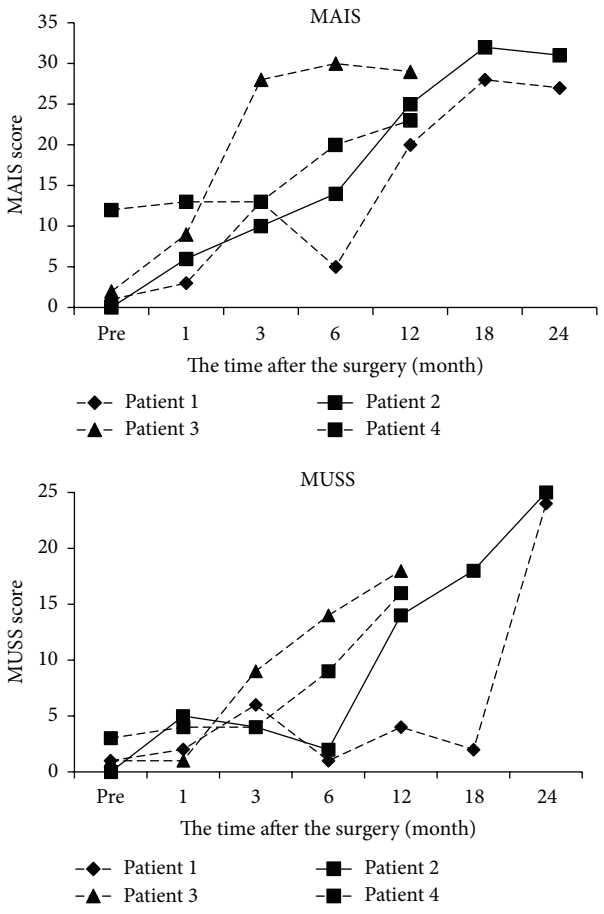

Figure 1: The results of MAIS and MUSS after CI.

Table 3: Postoperative thresholds and speech recognition score.

	The average postoperative thresholds of CI	CI-2004 three words' test	67-s monosyllable words test
Patient 1	32.5 dB	70%	95%
Patient 2	23.8 dB	92%	100%
Patient 3	26.2 dB	75%	N.A
Patient 4	35.0 dB	75%	N.A
Patient 5	30.0 dB	N.A	80%

(1, 2, and 5) who could perform the tests improved to more than 80%.

4. Discussion

In this study, we reviewed five WS patients who underwent CI. One showed a progressive pattern of hearing loss and four had congenital hearing loss. Two had a cochlear malformation and the others displayed no anatomical anomalies. There was no difficulty or complication during the CI surgery. The postoperative hearing performance was generally good in all patients.

WS was first described by Waardenburg and is now classified into four types [1]. WS 1, which is the original type, is characterized by dystopia canthorum, abnormalities of pigmentation (white forelock, white eyelashes, leukoderma, and heterochromia iridis), and SNHL. WS 2 differs from WS 1 by the lack of dystopia canthorum. WS 3, also called Klein-Waardenburg syndrome, has upper limb abnormalities accompanied by the same characteristics as WS 1. WS 4, also known as Shah-Waardenburg syndrome, has Hirschsprung's disease with the features of WS 2 [2, 7, 8]. The prevalence of WS is estimated at approximately 1 patient per 42,000 individuals, and WS accounts for 1%–3% of patients of congenital deafness [2]. WS is mainly inherited in an autosomal-dominant pattern but some patients show an autosomal-recessive pattern. Several genetic mutations have been reported according to the classification. WS 1 and 3 have been linked to a mutation in the *PAX3* gene on chromosome 2q35 [9]. One subtype of WS 2 has a mutation in the *MITF* gene on chromosome 3p12.3–p14.1 [10]. In addition, *WS2B*, *WS2C*, and *SNAI2* mutations have an association with WS 2, and *EDNRB*, *EDN3*, and *SOX10* are considered to be a cause of WS 2 and WS 4 [11–14]. In these mutations, *EDNBR* and *SNAI2* mutations can be a cause of autosomal recessive HL. We encountered *PAX3* gene mutation in one patient (patient 4) with clinical subtype of WS 1. This patient showed autosomal dominant HL and was consistent with other reports. The other patient (patient 1) was evaluated for *MITF* mutation at another hospital and was negative. Considering that this patient showed phenotype 4, other genes such as *EDNRB*, *EDN3*, and *SOX10* should have been investigated.

In WS, some patients show a progressive hearing loss pattern. Of all reported patients with WS who underwent CI, including our four patients, only four of 46 (8.7%) involved progressive hearing loss. Although some studies did not clearly describe the WS type in those with progressive hearing loss, such patients are reportedly limited to WS type 2. Our patient with progressive hearing loss was also type 2, which was consistent with a previous report. Other reports have also suggested that WS type 2 involves progressive HL [15, 16]. Pingault et al. stated that the genetic findings in WS 2 and 4 are more complex than those in WS 1 and 3, and that WS 2 and 4 are genetically heterogeneous [3]. These genetic varieties may lead to various clinical features in WS 2 and 4, including progressive hearing loss.

Previous studies reported abnormal radiological findings in the cochlea in WS. Semicircular canal dysplasia, an enlarged vestibular aqueduct, and dysplasia of the cochlea have been reported [17, 18]; the malformations found in our patients were incomplete partition type 2. Abnormal histopathological studies include degeneration of the organ of Corti, stria vascularis, and saccular macula, but abnormalities of the bony architecture of the cochlea and labyrinth have not been reported [19]. Oysu et al. stated that the rate of temporal bone malformations in WS type 1 is lower than that of children with congenital hearing loss in general [17]. These data suggest that, in WS patients, severe cochlear abnormalities that can result in poor results with CI [20–22] are rare, and that good performance can be expected from a cochlear structural aspect.

The postoperative performance in our patients was generally good, which was consistent with previous reports [23–25]. El Bakkouri et al. [26] compared 30 WS patients with 85 patients with the *GJB2* mutation and reported no difference in CI performance. Miyagawa et al. [27] reported satisfactory auditory performance after CI in those where genetic mutations including two cases of WS had been detected. All of these reports indicate that WS patients are also good candidates for CI. In spite of these results, some factors must be considered. Pau et al. [24] reported that some patients with WS have auditory neuropathy and these patients attain less benefit from CI. Some studies reported that WS is related to behavioral disorders, with developmental or cognitive impairment [25, 28]. No such disorder was seen in our patients, but closer consideration should be given to whether patients have other disorders or impairments when deciding on CI for WS.

5. Conclusion

Five patients of CI in WS in our institute were reviewed. One showed a progressive pattern of hearing loss. Two showed

cochlear malformations. There was no difficulty or complication during CI surgery. The postoperative performance was generally good in all patients. CI could be a good option for WS.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Mutation Analysis of the Common Deafness Genes in Patients with Nonsyndromic Hearing Loss in Linyi by SNPscan Assay

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Hearing loss is a common sensory disorder, and at least 50% of cases are due to a genetic etiology. Although hundreds of genes have been reported to be associated with nonsyndromic hearing loss, *GJB2*, *SLC26A4,* and *mtDNA12SrRNA* are the major contributors. However, the mutation spectrum of these common deafness genes varies among different ethnic groups. The present work summarized mutations in these three genes and their prevalence in 339 patients with nonsyndromic hearing loss at three different special education schools and one children's hospital in Linyi, China. A new multiplex genetic screening system "SNPscan assay" was employed to detect a total of 115 mutations of the above three genes. Finally, 48.67% of the patients were identified with hereditary hearing loss caused by mutations in *GJB2*, *SLC26A4,* and *mtDNA12SrRNA*. The carrying rate of mutations in the three genes was 37.76%, 19.75%, and 4.72%, respectively. This mutation profile in our study is distinct from other parts of China, with high mutation rate of *GJB2* suggesting a unique mutation spectrum in this area.

1. Introduction

Hearing loss or deafness is one of the most common neurosensory disorders in humans affecting about 1 to 3 in 1000 children worldwide [1]. It has been estimated that 30,000 newborns are detected with congenital hearing loss per 20 million live births every year in China [2], and approximately 50% of hearing loss is caused by a genetic etiology. At least 70% of all cases are classified as nonsyndromic hearing loss (NSHL) manifesting with isolated hearing loss without other associated clinical features. Of all NSHL, 75%–80% are autosomal recessive hereditary hearing loss, 15%–20% are autosomal dominant, 5% are X-linked, and 1% are inherited by mitochondrial genes [3].

Many previous genetic screening studies have shown that a large proportion of NSHL is caused by the mutations of a few genes, such as gap junction beta-2 protein (*GJB2*), solute carrier family 26 member 4 (*SLC26A4*), and *mitochondrial DNA* (*mtDNA*) *12SrRNA* [4, 5]. Therefore, mutation screening of these common deafness-causing genes is of vital importance during genetic testing and counseling of deafness.

China harboring the highest population in the world is consisting of 56 nationalities. The mutation spectrum of the common deafness genes may vary among different ethnic nationalities due to geographical and language separation. The city Linyi with the biggest population and the largest area in Shandong Province locates in the east part of China. Almost all the populations in this area are Han nationality. People in Linyi are always impressed by introversion and rusticity. Because of its desolate geographical condition, Linyi city is a relatively conservative area with a less migration of

population and an obstructive communication with other regions. Also, it has a less-developed economics for a long time. But to our knowledge, no systematic mutation analysis of deaf patients in Linyi has been reported previously. Therefore, there is a reason to believe that mutation spectrum of common deafness genes in patients with NSHL in this area must have a unique and special characteristic.

Up to now, many screening methods are used to detect the mutations of hearing loss, for example, direct sequencing, microarray, and PCR-restriction fragment length polymorphism (PCR-RFLP). These methods are either too expensive or time-consuming. Du et al. established a universal genetic screening system of hearing loss called the SNPscan assay which could detect 115 mutations of *GJB2*, *SLC26A4*, and *mtDNA12SrRNA* genes [6]. These selected mutations accounted for up to 90% of hearing loss caused by the most common deafness-causing genes in Chinese population.

In this study, we performed a comprehensive analysis of 3 common deafness-causing genes, *GJB2*, *SLC26A4*, and *mtDNA12SrRNA*, by SNPscan assay in 339 patients with NSHL from Linyi in eastern China. The aim was to investigate the molecular etiology in order to provide effective genetic counseling for hearing loss patients in this area.

2. Materials and Methods

2.1. Recruitment of the Subjects. A total of 339 subjects with NSHL from unrelated families were included in this study. They came from three different special education schools and one children's hospital in Linyi. Clinical questionnaire and informed consent were obtained from all subjects or their parents on their behalf. Clinical questionnaire showed basic information, including name, age, family history, health condition of the mother during pregnancy, and a clinical record of the patient, such as infections, possible head or brain injury, and the use of aminoglycoside antibiotics [7]. Physical and neurological examination was performed with special attention to renal, electrocardiac, and ophthalmologic differences to exclude those with syndromic hearing loss. An audiologic evaluation was performed including otoscope examination, tympanometry, and pure-tone audiometry (PTA). Auditory brainstem response (ABR) was employed in subjects when they were too young to accomplish the PTA test, and temporal bone computed tomography (CT) scan was also performed on participants for diagnosis of enlarged vestibular aqueduct (EVA) or inner ear malformation.

The onset of hearing impairment was categorized as prelingual/early (≤6 years) and late (>6 years). The degree of hearing impairment was calculated on the average of PTA in the speech frequencies 0.25, 0.5, 1, 2, and 4 kHz. Normal hearing was classified as PTA ≤ 25 dB nHL, mild hearing loss as PTA > 25 dB nHL and ≤40 dB nHL, moderate hearing loss as PTA > 40 dB nHL and ≤60 dB nHL, severe hearing loss as PTA > 60 dB nHL and ≤80 dB nHL, and profound hearing loss as PTA > 80 dB nHL. To enroll in this study, all subjects should have bilateral, permanent sensorineural hearing impairment. This study was approved by the Ethics Committee of Shandong Provincial Hospital.

2.2. DNA Samples. Genomic DNA for all 339 patients was prepared from 2 mL of peripheral blood with AxyPrep Genomic Blood DNA Extraction Kit (AXYGEN, USA). For the evaluation of the quantity and quality of extracted DNA, spectrophotometry (UNICO 2100, USA) and 1% agarose gel electrophoresis were carried out according to routine methods [8].

2.3. SNPscan for Mutation Detection. Two customized multiplex SNPscan assays from Genesky Biotechnologies Inc. (Shanghai, China) were designed to capture a total of 115 mutations of the three common deafness-causing genes as previously described (Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1302914): one is 53-plex and the other is 62-plex [6]. As a high-throughput and cost-saving SNP genotyping method, the SNPscan assay is based on double ligation and multiplex fluorescence PCR as described [9–11]. The SNPscan assays were done according to the detailed protocol described by Du et al. [6].

2.4. Statistical Analysis. The statistical analysis was performed using SAS 9.1.3 software (SAS, USA).

3. Result

3.1. Characterization of the Deaf Probands. A total of 339 probands with NSHL were recruited in this sequencing study, including 304 simplex and 35 multiplex probands. Simplex probands refer to the sporadic patients whose families have no one else suffering from hearing loss. Multiplex probands mean that there is at least one first- or second-degree deaf relative in the family. Clinical characterization of the deaf probands was summarized in Table 1. The patient cohort consisted of 196 males and 143 females, and they were all Han Chinese. The ages of the probands varied between a few months and 24 years (mean 8.9 years, 95% CI 8.3–9.5 years). Only one proband had late-onset, progressive, and moderate sensorineural hearing loss. All other probands had prelingual or early-onset sensorineural hearing loss. Hearing tests demonstrated that the level of hearing loss was severe to profound in 328 patients. The remaining 11 patients showed moderate hearing loss.

Among all these patients, 48.67% were identified with hereditary hearing loss by means of the SNPscan assay (Figure 1). There were 104 patients (30.68%) affected by *GJB2* mutations: 49 homozygotes and 55 compound heterozygotes. 45 (13.27%) patients carried two *SLC26A4* pathogenic mutations: fourteen homozygotes and 31 compound heterozygotes. 16 (4.72%) patients harbored *mtDNA12SrRNA* mutations consisting of 15 homoplasmic mutations and 1 heteroplasmic mutation.

Table 1: Deaf patients (n = 339) categorized by their clinical characteristics.

	Simplex (n = 304)	Multiplex (n = 35)
Sex		
Male	179	17
Female	125	18
Age at the test		
0–6 years	153	13
6–18 years	122	21
18–35 years	29	1
Age of onset		
Early onset (≤6 years)	303	35
Late onset (>6 years)	1	0
Severity of hearing impairment		
Mild	0	0
Moderate	11	0
Severe	56	5
Profound	237	30

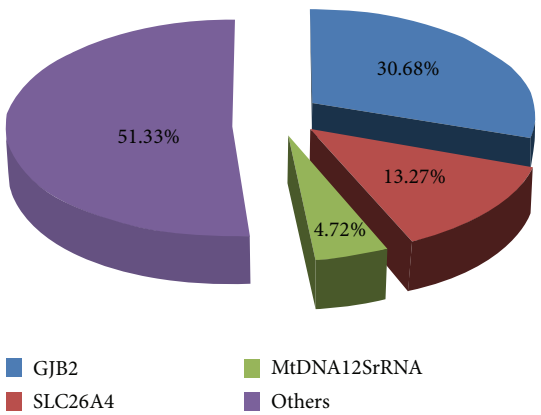

Figure 1: Distribution of the common deafness-causing genes in 339 NSHL patients.

3.2. Mutations in GJB2 Gene. Eleven variants were identified in this cohort. They were three frameshift deletions (c.176_191del16, c.235delC, and c.299_300delAT), two frameshift insertions (c.34_35insG and c.511_512insAACG), four missense mutations [c.109G>A (p.V37I), c.257C>G (p.T86R), c.427C>T (p.R143W), and c.571T>C (p.F191L)], one nonsense mutation c.9G>A (p.W3X), and one splicing site mutation IVS1+1G>A (shown in Table 2). Nine of them were pathological mutations which have been determined previously. The category of two nucleotide changes was unknown (c.9G>A, c.571T>C). The mutant alleles of *GJB2* accounted for 34.37% (233/678) of the total alleles in all patients (Table 3). The most common mutation allele of *GJB2* in this area was c.235delC with a mutant frequency of 20.65% (140/678). The second common was c.299_300delAT of 5.60% (38/678), followed by c.109G>A of 2.51% (17/678), c.34_35insG of 1.77% (12/678), c.176_191del16 of 1.33% (9/678), c.511_512insAACG of 1.18% (8/678), c.257C>G of 0.29% (2/678), c.427C>T of 0.29% (2/678), and IVS1+1G>A of 0.15% (1/678) (1/678) (Table 3).

A total of 104 patients (30.68%) were confirmed to be associated with hereditary hearing loss caused by *GJB2* mutation: 49 homozygotes (42 with the c.235delC allele, 4 with c.299_300delAT, and 3 with the c.109G>A allele) and 55 compound heterozygotes. Twenty patients (5.90%) had monoallelic variants in the heterozygous form: ten with c.235delC, eight with c.109G>A, one with c.299_300delAT, and one with c.511_512insAACG. Totally, 128 (37.76%, 128/339) patients had molecular defects in *GJB2* gene including three patients with c.571T>C mutation (Table 2).

The most common mutation allele of *GJB2* was c.235delC with a mutant frequency of 20.65% (140/678) affecting 98 patients. Among these patients carrying at least one c.235delC allele, there were 42 homozygotes, 46 compound heterozygotes, and 10 single heterozygotes.

3.3. Mutations in SLC26A4 Gene. Nineteen variants were identified in this cohort, including 12 missense mutations [c.147C>G (p.S49R), c.269C>T (p.S90L), c.563T>C (p.I188T), c.589G>A (p.G197R), c.1173C>A (p.S391R), c.1174A>T (p.N392Y), c.1225C>T (p.R409C), c.1226G>A (p.R409H), c.1975G>C (p.V659L), c.1985G>A (p.C662Y), c.2027T>A (p.L676Q), and c.2168A>G (p.H723R)], four nonsense mutations [c.235C>T (p.R79X), c.946G>T (p.G316X), c.1318A>T (p.K440X), and c.1540C>T (p.Q514X)], two splicing site mutations (c.919-2A>G, c.1707+5 G>A), and one frameshift insertion mutation (c.1547_1548insC) (Table 5). Combining the prediction of SIFT and Polyphen-2 with the results of previous studies, all variants analyzed in our cohort were considered pathogenic mutations except c.147C>G (p.S49R) for an unknown significant pathogenicity (Table 4).

There were 45 (13.27%) patients who were confirmed to have inherited hearing loss because they carried two *SLC26A4* pathogenic mutations: fourteen homozygotes (13 c. IVS 7-2A>G and 1 c.2168A>G) and 31 compound heterozygotes. And 22 (6.49%) patients who were identified carried one *SLC26A4* pathogenic mutation. Thus, the detection rate of *SLC26A4* mutations was 19.76% (67/339) in this patient cohort. All patients carrying two mutations were diagnosed with EVA syndrome by CT scan of the temporal bone. And 17 out of 22 patients carrying one pathogenic mutation were diagnosed with EVA syndrome.

The most common mutation allele of *SLC26A4* was c.919-2A>G with a mutant frequency of 9.59% (65/678) affecting 52 patients. Among these patients carrying at least one c.919-2A>G mutation allele, there were 13 homozygotes, 27 compound heterozygotes, and 12 single heterozygotes. The second common mutation allele of *SLC26A4* was c.2168A>G with a mutant frequency of 2.21% (15/678).

3.4. Mutations in mtDNA12SrRNA. Sixteen patients (4.72%) carried an *mtDNA12SrRNA* mutation, where all of which were the m.1555A>G mutation containing 15 homoplasmic

Table 2: *GJB2* genotypes of deaf patients from Linyi.

Allele 1			Allele 2			Number of patients
Nucleotide change	Consequence or amino acid change	Category	Nucleotide change	Consequence or amino acid change	Category	
IVS1+1G>A	Splicing site	Pathogenic	c.9G>A	p.W3X	Unknown	1
34_35insG	Frameshift	Pathogenic	109G>A	p.V37I	Pathogenic	1
34_35insG	Frameshift	Pathogenic	176_191del16	Frameshift	Pathogenic	1
34_35insG	Frameshift	Pathogenic	235delC	Frameshift	Pathogenic	8
34_35insG	Frameshift	Pathogenic	299_300delAT	Frameshift	Pathogenic	2
109G>A	p.V37I	Pathogenic	109G>A	p.V37I	Pathogenic	3
109G>A	p.V37I	Pathogenic	235delC	Frameshift	Pathogenic	1
109G>A	p.V37I	Pathogenic	299_300delAT	Frameshift	Pathogenic	1
109G>A	p.V37I	Pathogenic	—			8
176_191del16	Frameshift	Pathogenic	235delC	Frameshift	Pathogenic	8
235delC	Frameshift	Pathogenic	235delC	Frameshift	Pathogenic	42
235delC	Frameshift	Pathogenic	257C>G	p.T86R	Pathogenic	2
235delC	Frameshift	Pathogenic	299_300delAT	Frameshift	Pathogenic	22
235delC	Frameshift	Pathogenic	427C>T	p.R143W	Pathogenic	2
235delC	Frameshift	Pathogenic	511_512insAACG	Frameshift	Pathogenic	3
235delC	Frameshift	Pathogenic	—			10
299_300delAT	Frameshift	Pathogenic	299_300delAT	Frameshift	Pathogenic	4
299_300delAT	Frameshift	Pathogenic	511_512insAACG	Frameshift	Pathogenic	4
299_300delAT	Frameshift	Pathogenic	—			1
511_512insAACG	Frameshift	Pathogenic	—			1
571T>C	p.F191L	Unknown	—			3

Table 3: Allele frequencies of *GJB2* mutations in 339 deaf patients from Linyi.

Mutations	Consequence	Number of alleles	Allele frequency (%)
IVS1+1G>A	Splice site	1	0.15
c.34_35insG	Frameshift	12	1.77
c.109G>A	p.V37I	17	2.51
c.176_191del16	Frameshift	9	1.33
c.235delC	Frameshift	140	20.65
c.257C>G	p.T86R	2	0.29
c.299_300delAT	Frameshift	38	5.60
c.427C>T	p.R143W	2	0.29
c.511_512insAACG	Frameshift	8	1.18

mutations and 1 heteroplasmic mutations. Ten of these patients had a clear history of aminoglycoside antibiotic use.

4. Discussion

Currently, many studies have reported that *GJB2*, *SLC26A4*, and *mtDNA12SrRNA* genes are the most common causes in Chinese NSHL population [12–14]. In the present study we have screened for the common deafness gene mutations of 339 NSHL children from Linyi, east part of China. SNPscan assay was used to perform genotyping detection of these three common deafness-causing genes. Compared with other methods, this technique is accurate, rapid, and economically effective. As the novel mutations have been reported successively, more mutation alleles should be added in the SNPscan assay.

4.1. GJB2 Mutation Analysis. In this present study, *GJB2* mutations were detected in 37.76% (128/339) of all patients, including 30.68% (104/339) with two pathogenic mutations and 7.08% (24/339) with only one mutant allele. The c.235delC and c.299_300delAT were the most frequent mutations in the NSHL patients in Linyi, whereas the hotspot mutation in Xiamen, China, was c.109G>A [7].

The frameshift mutation c.235delC has been reported as the most common mutation causing premature protein termination in hearing impaired patients in East and Southeast Asia, while lower frequencies were reported in Europe and Oceania [15–24]. The c.235delC mutation allele frequency was 20.65% (140/678) in this work. In an earlier nationwide study, Dai et al. analyzed the *GJB2* mutation of 2063 unrelated NSHL students from 23 different regions of China, and the c.235delC mutation allele frequency was 12.34% (509/4,126)

Table 4: *SLC26A4* genotypes of deaf patients from Linyi.

Allele 1			Allele 2			Number of patients
Nucleotide change	Consequence or amino acid change	Category	Nucleotide change	Consequence or amino acid change	Category	
c.147C>G	p.S49R	Unknown	—			1
c.235C>T	p.R79X	Pathogenic	c.919-2A>G	Splice site	Pathogenic	1
c.235C>T	p.R79X	Pathogenic	—			1
c.269C>T	p.S90L	Pathogenic	—			1
c.563T>C	p.I188T	Pathogenic	c.919-2A>G	Splice site	Pathogenic	1
c.589G>A	p.G197R	Pathogenic	c.919-2A>G	Splice site	Pathogenic	1
c.919-2A>G	Splice site	Pathogenic	c.919-2A>G	Splice site	Pathogenic	13
c.919-2A>G	Splice site	Pathogenic	c.946G>T	p.G316X	Pathogenic	1
c.919-2A>G	Splice site	Pathogenic	c.1174A>T	p.N392Y	Pathogenic	2
c.919-2A>G	Splice site	Pathogenic	c.1226G>A	p.R409H	Pathogenic	3
c.919-2A>G	Splice site	Pathogenic	c.1318A>T	p.K440X	Pathogenic	1
c.919-2A>G	Splice site	Pathogenic	c.1540C>T	p.Q514X	Pathogenic	1
c.919-2A>G	Splice site	Pathogenic	c.1547_1548InsC	Frameshift	Pathogenic	1
c.919-2A>G	Splice site	Pathogenic	c.1707+5 G>A	Splice site	Pathogenic	3
c.919-2A>G	Splice site	Pathogenic	c.1975G>C	p.V659L	Pathogenic	3
c.919-2A>G	Splice site	Pathogenic	c.1985G>A	p.C662Y	Pathogenic	1
c.919-2A>G	Splice site	Pathogenic	c.2027T>A	p.L676Q	Pathogenic	2
c.919-2A>G	Splice site	Pathogenic	c.2168A>G	p.H723R	Pathogenic	6
c.919-2A>G	Splice site	Pathogenic	—			12
c.946G>T	p.G316X	Pathogenic	c.2168A>G	p.H723R	Pathogenic	1
c.1173C>A	p.S391R	Pathogenic	c.2168A>G	p.H723R	Pathogenic	1
c.1225C>T	p.R409C	Pathogenic	—			1
c.1226G>A	p.R409H	Pathogenic	c.1975G>C	p.V659L	Pathogenic	1
c.1226G>A	p.R409H	Pathogenic	c.2168A>G	p.H723R	Pathogenic	1
c.1226G>A	p.R409H	Pathogenic	—			1
c.1975G>C	p.V659L	Pathogenic	—			1
c.2168A>G	p.H723R	Pathogenic	c.2168A>G	p.H723R	Pathogenic	1
c.2168A>G	p.H723R	Pathogenic	—			4

[25]. Compared with 12.34% in his study, the difference in the c.235delC mutation allele frequency was significant ($P = 0.001$), reflecting a certain distinction of the c.235delC mutation frequency in Linyi and other areas. There was a higher carrying rate of c.235delC in the Linyi deaf population.

4.2. SLC26A4 Mutation Analysis. According to previous study, more than 200 mutations have been described in the *SLC26A4* gene with Pendred syndrome (PS) and enlarged vestibular aqueduct (EVA) syndrome (http://www.healthcare.uiowa.edu/labs/pendredandbor/) showing specific distinctions among racial backgrounds. Most of them are missense mutations, in addition to frameshift mutations, splice site mutations, insertions, or deletions [26].

The mutation hotspots of *SLC26A4* differed among different nations and areas. In the present study, the most common mutation in our patient cohort was c.IVS7-2A>G and the mutation allele frequency was 9.59% (65/678), whereas the mutation hot spots are p.T416P and c.IVS8+1G>A [27] in Northern Europe, p.H723R and c. IVS7-2A>G in South Korea [15], and p.H723R in Japan [28]. It was reported in a mutation study of a large cohort in Chinese deaf population that the most common *SLC26A4* mutation was also IVS7-2A>G (8.65%, 566/6,542) [12]. Although the mutation allele frequency was a little higher (9.59%) in our study, these frequencies of c.IVS7-2A>G are not significantly different ($P = 0.41$).

4.3. mtDNA12SrRNA Mutation Analysis. Patients with mutations in *mtDNA12SrRNA* can be affected with aminoglycoside antibiotic-induced deafness showing a hereditary model of maternal inheritance. And m.1494C>T and m.1555A>G are the most common mutations of this mitochondrial gene. Mutate rate of this gene varies among racial and geographic

Table 5: Allele frequencies of *SLC26A4* mutations in 339 deaf patients from Linyi.

Mutations	Consequence	Number of alleles	Allele frequency (%)
c.235C>T	p.R79X	2	0.29
c.269C>T	p.S90L	1	0.15
c.563T>C	p.I188T	1	0.15
c.589G>A	p.G197R	1	0.15
c.919-2A>G	Splice site	65	9.59
c.946G>T	p.G316X	2	0.29
c.1173C>A	p.S391R	1	0.15
c.1174A>T	p.N392Y	2	0.29
c.1225C>T	p.R409C	1	0.15
c.1226G>A	p.R409H	6	0.88
c.1318A>T	p.K440X	1	0.15
c.1540C>T	p.Q514X	1	0.15
c.1547_1548InsC	Frameshift	1	0.15
c.1707+5 G>A	Splicing site	3	0.44
c.1975G>C	p.V659L	5	0.74
c.1985G>A	p.C662Y	1	0.15
c.2027T>A	p.L676Q	2	0.29
c.2168A>G	p.H723R	15	2.21

origins in populations with NSHL, with a frequency of 0.6–2.5% in Caucasians [29–32], 1.8% in Turks [30], 0.7% in Germans, 1.8% in Hungarians, and 2.4% in Poles [29]. In our study, we found that 4.72% of the patients were identified to have NSHL caused by the m.1555A>G mutation of *mtDNA12SrRNA* gene which was close to that observed by Dai et al. (3.87%) ($P = 0.53$).

In summary, we performed the first genetic analysis of hearing loss patients from Linyi, eastern part of China. According to genetic detections of *GJB2*, *SLC26A4*, and *mtDNA12SrRNA* via the SNPscan assay, the fact that almost half of the patients with nonsyndromic hearing loss appeared to have a genetic etiology shows that the SNPscan assay is an available diagnosis tool for studies on genetic hearing loss. The results of the detection suggest that *GJB2* mutations appear to be a major cause of congenital hearing loss in Linyi. The prevalence of *SLC26A4* mutations and *mtDNA12SrRNA* mutations was very close to other previous studies in Chinese deaf population. However, about 50% of NSHL patients are still not identified with a molecular etiology. For this reason, copy number variations (CNVs) of the three common deafness-causing genes need to be detected. Also, next generation sequencing (NGS) will be employed in screening other deafness-causing genes in the future.

5. Conclusions

In this study, we used the SNPscan assay technique to detect the 115 mutations of the three common deafness-causing genes in 339 nonsyndromic hearing loss patients from Linyi, China. And we found that the mutation profile was distinct from other parts of China suggesting a unique mutation spectrum in this area. Also, the SNPscan assay was an available diagnosis tool for studies on genetic hearing loss.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Tricellular Tight Junctions in the Inner Ear

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Tight junctions (TJs) are structures that seal the space between the epithelial cell sheets. In the inner ear, the barrier function of TJs is indispensable for the separation of the endolymphatic and perilymphatic spaces, which is essential for the generation and maintenance of the endocochlear potential (EP). TJs are formed by the intercellular binding of membrane proteins, known as claudins, and mutations in these proteins cause deafness in humans and mice. Within the epithelial cell sheet, however, a bound structure is present at the site where the corners of three cells meet (tricellular tight junctions (tTJs)), and the maintenance of the barrier function at this location cannot be explained by the claudins alone. Tricellulin and the angulin family of proteins (angulin-1/LSR, angulin-2/ILDR1, and angulin-3/ILDR2) have been identified as tTJ-associated proteins. Tricellulin and ILDR1 are localized at the tTJ and alterations in these proteins have been reported to be involved in deafness. In this review, we will present the current state of knowledge for tTJs.

1. Introduction

Sound vibrations are converted into nerve action potentials in the inner ear [1]. For this process to occur, the strict compartmentalization of the cochlea is necessary. The structure of the inner ear can be broadly separated into two compartments: the endolymphatic space (endolymph) and perilymphatic space (perilymph). Within the cochlea, the endolymph and perilymph have an entirely different composition. The endolymph has a higher electrical potential (endocochlear potential (EP)) and a higher potassium concentration, when compared to the perilymph [2]. The barrier function of the epithelial cell sheet prevents paracellular permeability, and the separation of these two compartments is essential for the maintenance of their differences in composition [3]. Tight junctions (TJs) are intercellular junctions that play a major role in epithelial barrier function. TJs are formed by the TJ strand, which is a fibril-like structure consisting of tight junction-associated proteins from both of the adjacent plasma membranes [4–9]. The claudins are integral membrane proteins, identified as response molecule of barrier function, which form the TJ that seals the space between neighboring epithelial cells. To date, 24 claudins have been identified [10–12] and at least 10 of these are expressed in the inner ear [13]. Claudin-1, claudin-2, claudin-3, claudin-9, claudin-10, claudin-12, claudin-14, and claudin-18 are expressed in the organ of Corti. In addition, claudin-8 is expressed in Rissner's membrane, the spiral limbus, and the marginal cells of the stria vascularis and only claudin-11 is expressed in the basal cells of the stria vascularis [13]. The combination of these claudins is thought to be important for barrier function in the inner ear, and three of these claudins (claudin-9, claudin-11, and claudin-14) are reported to be critical for hearing [14–20]. Mutations in these proteins cause deafness in humans and mice. Claudin-11 (cldn-11) knockout mice demonstrate hearing loss as a result of reduced EP [14, 15]. Interestingly, mutations in human *CLDN14* cause profound, congenital deafness DFNB29 [16]. Both cldn-9 mutant mice and cldn-14 null mice are deaf, due to the rapid degeneration of cochlear hair cells shortly after birth, but do not display reduced EP [17, 18]. These phenotypes are considered to be associated with local disturbances in ionic balance within the inner ear. Therefore, the epithelial barrier that is formed by TJs is significantly involved in inner ear function.

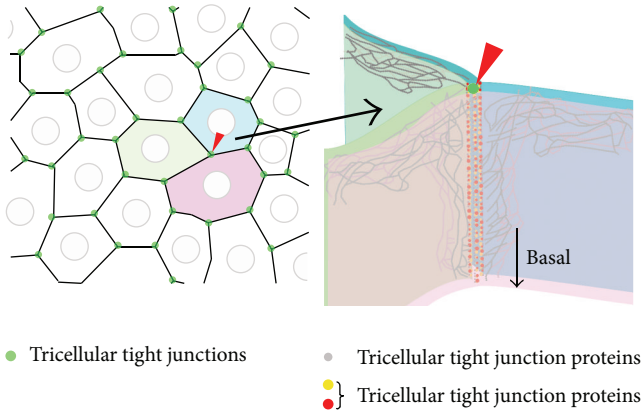

Figure 1: Schematic image of the tricellular tight junction (tTJ). The TJs between two cells (bicellular TJ, bTJ) function to seal off the intercellular space (black lines). However, the corner where three cells meet (green dots) cannot be occluded by bTJ proteins alone and a central "tube" may exist. tTJ proteins (red dots and yellow dots) are localized at this site and play a role in providing a barrier.

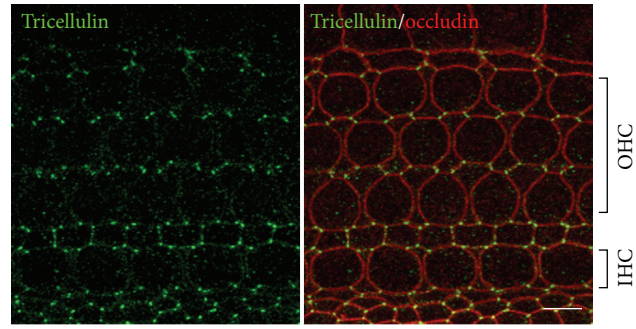

Figure 2: Localization of tricellulin and occludin in the organ of Corti. Double immunofluorescence microscopy of the organ of Corti of postnatal day 3 C57BL/6 mice using anti-tricellulin pAb (green) and anti-occludin mAb (red). Tricellulin is localized at tricellular contacts where three cells meet. Occludin, a tight junction-associated membrane protein, distributed to cell-cell contacts not only at tTJ but also at bicellular TJ. OHC: outer hair cell; IHC: inner hair cell. Bars, 5 μm.

2. The Tricellular Tight Junction (tTJ) in the Inner Ear

The epithelial cell sheet contains TJs between cells and functions as a barrier [19, 20]. However, at the apex of polygon-shaped cells, the corners of three epithelial cells abut against each other and this results in a structure that differs from normal TJs that are formed between two cells (Figure 1). At the apex of the three cells, the most apical strands of the horizontal TJs extend to the center of the corner, then turn, and grow in the basal direction. They are nearly attached to each other to form vertical strands. As a result, a narrow and long tube-like structure is formed in the extracellular space at the corner. This tubular structure is called a tTJ and is thought to reduce the free diffusion of solutes to ensure a sufficient paracellular barrier [21]. tTJs were first reported in the 1970s as structures visible in electron micrographs. However, the molecular entities comprising tTJs were unclear for a long time. The first molecule found to play a role in tTJs was tricellulin [22], and three more proteins, LSR/angulin-1, ILDR1/angulin-2, and ILDR2/angulin-3, have recently been identified [23, 24]. Tricellulin, LSR, and ILDR1 are each expressed in the inner ear and both tricellulin and ILDR1 are localized at the tTJ in the organ of Corti (Figure 2). These two genes are responsible for human deafness DFNB49 and DFNB42, respectively [25, 26].

3. Tricellulin and the tTJ

Tricellulin was first identified in a genetic screen for factors that are suppressed during the forced expression of the snail transcription factor, which is involved in the epithelial to mesenchymal transition in cultured mouse epithelial cells. Tricellulin is a four-pass transmembrane protein belonging to the MARVEL family, which also includes occludin (ocln), a TJ membrane protein that was identified before the role of the claudin proteins was determined [22]. Tricellulin is localized at the tTJ of epithelial cells in several locations, including those of the inner ear (Figure 2). Intercellular barrier function was found to be diminished following the RNAi knockdown of tricellulin expression in cultured epithelial cells, indicating that tricellulin is essential for sufficient barrier function [22]. Tricellulin also displays similar properties to the claudin proteins in providing a barrier against electrical conductance and permeability for ionic and uncharged solutes [22, 27, 28]. Mutations in the gene encoding tricellulin are also causative factors of human hereditary deafness DFNB49 [25]. Mice with a knockin of this human deafness mutation exhibit severe hearing loss caused by the degeneration of hair cells, similar to the phenotype observed with claudin-9 and claudin-14 deficiency [17, 19]. Furthermore, tricellulin was found to no longer be localized at the tTJ in the mutant mice. The morphology of the TJ can be visualized as a strand by the freeze-fracture replica method. In tTJs, the TJ strands extend deep into the basal side; however, in the organ of Corti of these mutant mice, the TJ strands remain separated from each other and the short TJ strands packed orthogonally are sparse. Therefore, tricellulin is thought to be necessary for the maintenance of the tTJ structure in the inner ear [29].

4. Occludin (Ocln)

Occludin is a member of the same protein family as tricellulin; however, it is localized to both tTJs and normal TJs [30, 31]. Occludin has been reported to affect the localization of tricellulin [32]. Occludin knockout mice exhibit a similar phenotype to tricellulin knockin mice, with apoptosis of the hair cells and degeneration of the organ of Corti. In these knockout mice, tricellulin is no longer localized to the tTJ, which is believed to be the cause of the observed deafness. This demonstrates that an interplay between the MARVEL family members is necessary for determining the localization of tricellulin and that the concentration of tricellulin at the tTJ is important for its function [33].

5. ILDR1 and the Angulin Family

Following the identification of tricellulin, a membrane protein known as lipolysis-stimulated lipoprotein receptor (LSR) was discovered. LSR was found through the expression cloning of an epithelium-derived cDNA library using localization to intercellular junctions as a marker and identified as a component of the tTJ [23]. RNAi knockdown of LSR expression in cultured epithelial cells was shown to decrease transepithelial electrical resistance (TER), suggesting that LSR is involved in barrier function. Interestingly, in LSR knockdown cells, tricellulin is dispersed across the cell membrane, rather than being concentrated at the tTJ, indicating that LSR regulates the localization of tricellulin to the tTJ [23]. LSR has two homologous genes known as immunoglobulin-like domain-containing receptor 1 and immunoglobulin-like domain-containing receptor 2 (ILDR1 and ILDR2). Together with LSR, these three proteins regulate the localization of tricellulin at the tTJ. These proteins form the angulin family, with LSR, ILDR1, and ILDR2 also known as angulin-1, angulin-2, and angulin-3, respectively [24]. Of these three angulin proteins, mutations in ILDR1 (angulin-2) have been identified as causes of human hereditary deafness [26]. By immunostaining, only ILDR1 was found to be expressed in the organ of Corti [24]. Because angulins can recruit tricellulin to the tTJ, the mutations in angulin-2/ILDR1 may cause hearing loss via changes in tricellulin localization. Surprisingly, however, tricellulin was found to localize at the tTJ in ILDR1 knockout mice, although its distribution along the depth of the tricellular contacts was subtly affected [34, 35]. Angulin-1/LSR is not normally expressed in the organ of Corti; however, its expression is seen when angulin-2/ILDR1 is deficient, and tricellulin is thought to be recruited to the tTJ as a result. This clearly demonstrates that compensatory functions exist between the angulin family members. However, the organ of Corti degenerated even when tricellulin was localized at the tTJ by angulin-1/LSR, indicating that there are also functional differences between the angulin family members [35].

6. EP Generation and Hair Cell Degeneration

Mutant mice have been shown to share deafness phenotypes with unchanged EP and hair cell degeneration all occurring within the same timeframe [17, 18, 29, 34, 36]. EP is likely to be related with hair cell degeneration in these mice. First, in explant culture condition, in which EP does not exist, the hair cell degeneration was not observed. Second, in double mutant mice with pou3f4-deficient mice, in which EP does not generate, hair cell degeneration was reduced [17, 18]. These results indicate that hair cell degeneration is triggered by changes to extracellular conditions. There may, therefore, be a common mechanism underlying deafness in these models, which is associated with local disturbances in ionic balance within the inner ear caused by leakage of K^+ and Na^+ ions or small molecules such as ATP [37]. In fact, it has reported that the concentration of K^+ ions in perilymph is slightly, but significantly, increased in cldn-9 mutant mice [18]. It might be involved in the viability of hair cells. In addition, the initiation of hair cell degeneration overlaps with the "rapid phase" of EP formation (Figure 3) [37–40]. There is difference in the timing of initiation of hair cell degeneration within "rapid phase" in mutant mice during EP formation. Furthermore, TJs not only play a role in providing a barrier function but also function as a selective barrier similar to a channel [6, 7, 22, 41–44]. This difference in timing for the initiation of hair cell degeneration might be caused by differences in paracellular permeability properties (the nature and amount of the molecules permitted through the barrier) associated with each of the TJ proteins.

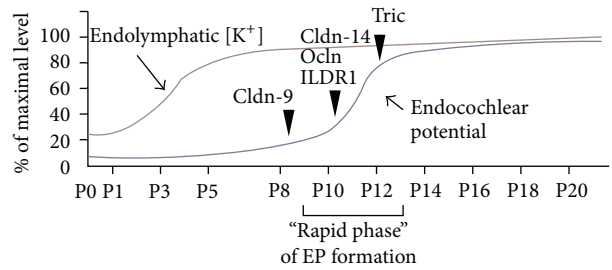

Figure 3: Time course of EP and endolymphatic $[K^+]$ elevation (modified from [38–41]) and hair cell degeneration in mice with mutations in TJ proteins. The arrowheads indicate the starting time for hair cell degeneration in each of the mutant mice models: cldn-9: claudin-9 mutant mice [18]; cldn-14: claudin-14 knockout mice [17]; ocln: occludin deficient mice [33]; ILDR1: ILDR1 null mice [34, 35]; Tric: tricellulin knockin mice [29] and knockout mice [36].

7. Conclusion

Hearing loss due to the failure of the normal bicellular TJ has been extensively investigated in both humans and mice. In addition, the new field of tTJ study has shown that this structure is also deeply involved in inner ear function. The phenotypes of mice with mutations in tTJ-associated proteins were found to be similar to those of mice with mutations in bTJ-associated proteins. At present, the organ of Corti is known to express two tTJ-associated proteins, namely, tricellulin and ILDR1. Both of these proteins have been associated with human deafness and mouse models with mutations in these proteins display hair cell degeneration. Further studies will be useful to evaluate the mechanism of this hair cell degeneration.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Simvastatin Results in a Dose-Dependent Toxic Effect on Spiral Ganglion Neurons in an *In Vitro* Organotypic Culture Assay

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Statins are inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an enzyme necessary for the production of mevalonate. They are widely used as cholesterol-lowering drugs. However, conflicting data about the effect of statins on neuronal cells has been published. To explore the effect of simvastatin on spiral ganglion neurons (SGNs), SG explants of 5-day-old rats were treated with increasing concentrations of simvastatin. In addition, SG explants were treated with mevalonate and with the combination of simvastatin and mevalonate. SGN number, length of the neurites, area of nonneuronal supporting cells, and neuronal survival were analyzed. Simvastatin treatment results in a significant dose-dependent decrease of SG neurite number, length of neurites, area of supporting cells, and SG neuronal survival compared to control. Interestingly, treatment with mevalonate in addition to simvastatin increased SG neuronal survival compared to simvastatin treatment only. However, treatment with mevalonate in addition to simvastatin did not influence SG neurite number, length of neurites, and area of supporting cells compared to simvastatin treatment only. Our results suggest a neurotoxic effect of simvastatin on SGNs *in vitro*. Neurotoxicity seems to be at least partially mediated by the mevalonate pathway. Therefore, caution is warranted to use simvastatin as a potential otoprotective drug.

1. Introduction

Sensorineural hearing loss is linked to degeneration and death of auditory hair cells (HCs) and their associated spiral ganglion neurons (SGNs), which is irreversible in mammals. Despite the progress made towards understanding the processes involved in HC and SGN death and survival, there is still no available cure for individuals with sensorineural hearing loss; only auditory prosthesis (e.g., hearing aids or cochlear implants) can offer some help to individuals with hearing loss. Therefore, developing therapeutic strategies for hearing loss prevention is one of the major goals of current auditory research. Among the potential otoprotective drugs are statins.

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and commonly used for the treatment of hyperlipidemia [1]. The HMG-CoA reductase is a rate-limiting enzyme in the cholesterol biosynthesis. Inhibition of HMG-CoA reductase results in a reduction of cholesterol in plasma and an increased expression of low-density lipoprotein (LDL) receptors [2]. Statins reduce the incidence of primary and secondary coronary heart disease in clinic trials and act by blocking the enzyme necessary for the production of L-mevalonate, an intermediary product in the synthesis of cholesterol [3, 4]. Moreover, statins also reduce the risk of atherosclerosis and have anti-inflammatory, immunomodulatory, and apoptotic effects [5].

During the past decade, statin treatment has been discussed to prevent or improve sensorineural hearing loss [6, 7]. Some clinical studies suggest that diet control and antilipid therapy improve sensorineural hearing loss associated with hyperlipidemia [8–10]. However, Olzowy et al. [11] did not find an effect of atorvastatin on progression of sensorineural hearing loss in the elderly in a prospective, randomized,

double-blinded clinical trial. Interestingly, Chung et al. [12] showed an association between previous statin use and sudden sensorineural hearing loss. It has been demonstrated that hyperlipidemia and atherosclerosis in apolipoprotein E knockout (ApoE-KO) mice resulted in structural and functional changes in the inner ear, which were associated with hearing loss in a time-dependent manner [13, 14]. Cai et al. [15] found that simvastatin treatment protects the hearing of ApoE-KO mice that were fed a high fat diet. They attribute these results to reduced atherosclerotic lesions and control of hyperlipidemia. Syka et al. [16] found a protective effect of atorvastatin on the inner ear. They explain their results by reduction of endothelial inflammatory effects which influence the blood supply to the inner ear. Our group recently demonstrated that simvastatin protects HCs from gentamicin-induced toxicity *in vitro* [17]. However, the effects of simvastatin have not yet been investigated on SGNs.

Given these conflicting data, we examined the effect of statins on SGNs *in vitro*. The aim of the present study was to evaluate the effect of simvastatin on SG neuronal survival, neuritogenesis, and neurite elongation *in vitro*.

2. Material and Methods

2.1. Preparation of Tissue Culture Plates. All experiments were performed as previously described by our group [18–23]. First, uniformly coated 24-well cell culture plates (Costar®, Corning Inc., Acton, MA, USA) were prepared and the wells were filled with 300 μL of 5 μg/mL poly-L-lysine (PLL) (Sigma-Aldrich, St. Louis, USA) in Dulbecco's modified Eagle's medium (DMEM) (Gibco by Invitrogen, Carlsbad, USA). Next, the culture plates were incubated at 37°C for 1 hour. The wells were then washed twice with phosphate buffered saline (PBS) and filled with 170 μL of primary attachment medium, containing DMEM (Gibco), 10% fetal bovine serum (Sigma-Aldrich), 25 mM HEPES buffer (Gibco), and 300 U/mL penicillin (Sigma-Aldrich).

2.2. SG Dissection. All animal procedures were carried out according to an approved animal research protocol (Kantonales Veterinaeramt, Basel, Switzerland). Neonatal 5-day-old Wistar rats (Harlan, Netherlands) were euthanized. The cochlea and the spiral ganglion were removed and further dissected similar to the method described by Van de Water and Ruben [24]. Briefly, the cochlear capsule was opened and the membranous labyrinth was removed from the modiolus. The spiral lamina containing the SG was carefully separated from the modiolus and transferred immediately into primary cell culture medium, where it was then cut into equal portions of 300 μm to 500 μm before being transferred to the prepared culture plates. Each explant was cultured in a separate culture well.

2.3. Cell Culture. First, explants were incubated for 24 h at 37°C in primary attachment medium, and the culture medium was subsequently changed to serum-free maintenance medium (DMEM (Gibco), 25 mM HEPES buffer (Gibco), 6 mg/mL glucose (Gibco), 300 U/mL penicillin (Sigma-Aldrich), and 30 μL/mL N2-supplement (Gibco)). Maintenance medium was supplemented with 10 ng/mL of recombinant BDNF for trophic support of SGN survival and optimization of neurite outgrowth (R&D Systems, Minneapolis, MN, USA). Cultures were kept in a humidified incubator at 5% CO_2 and 37°C for 72 h. Experimental cultures received various concentrations (1 μM, 10 μM, or 100 μM, resp.) of simvastatin (Sigma-Aldrich), mevalonate (10 μM, Sigma-Aldrich), or simvastatin and mevalonate (both 10 μM, Sigma-Aldrich). Culture media with DMSO only served as control. Simvastatin was dissolved in DMSO and the same DSMO concentration as in the samples treated with 100 μM simvastatin was used. Simvastatin was converted into the active acid following the protocol of Bogman et al. [25] prior to its use. Stock solutions of 10 μM simvastatin in DMSO were stored at −20°C. 20 SG explants were analyzed per experimental condition.

2.4. Immunohistochemistry. First, the explants were fixed with 4% paraformaldehyde for 20 min at room temperature (RT). Then, the explants were washed twice with PBS (Gibco) and permeabilized with 5% triton X-100 (Sigma-Aldrich) for 10 min. After permeabilization, the explants were again washed twice with PBS (Gibco) and then blocked for nonspecific antibody binding with 5% donkey serum (Sigma-Aldrich). Neurites were labeled for neurofilament using a mouse polyclonal 200 kDa anti-neurofilament primary antibody (1 : 400; Sigma-Aldrich). After primary antibody incubation overnight at 4°C, followed by two PBS washes, the neurites were visualized by 2.5 h of incubation with fluorescein isothiocyanate (FITC) conjugated secondary antibodies (1 : 100; Jackson Immunoresearch, West Grove, PA, USA) against mouse antibody.

2.5. Quantification of Neuronal Survival. To assess effects on neuronal survival, half turn SG explants were cultured as above for 72 hours, except that the explants were grown on glass cover slips. The explants were fixed as above, treated with 0.5% peroxide in methanol to block endogenous peroxidases, reacted with a mouse monoclonal antibody IgG against rat neurofilament 200 (Sigma-Aldrich), followed by a biotinylated secondary anti-mouse IgG, and developed by an avidin and DAB procedure (Vector Laboratories, Burlingame, CA). The tissue was cleared with citrosol (Fischer Scientific, Waltham, MA, USA) to allow visualization of the cell somas for evaluation of neuronal survival. 12 SG explants were studied per condition.

2.6. Data Analysis. Digital images for immunohistochemistry were obtained on a fluorescence microscope (Olympus IX71, Center Valley, PA, USA) and photographed with an AxioCam (Zeiss, San Diego, USA). Digital images for quantification of neuronal survival were obtained on an inverted microscope (Olympus BX63 Center Valley, PA, USA). For publication in this paper, images were optimized to achieve uniform brightness and contrast using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA). Neurite outgrowth from the SG was evaluated by measuring the number and lengths of the processes. Growth of supporting cells was evaluated by measuring the area of the skirt surrounding

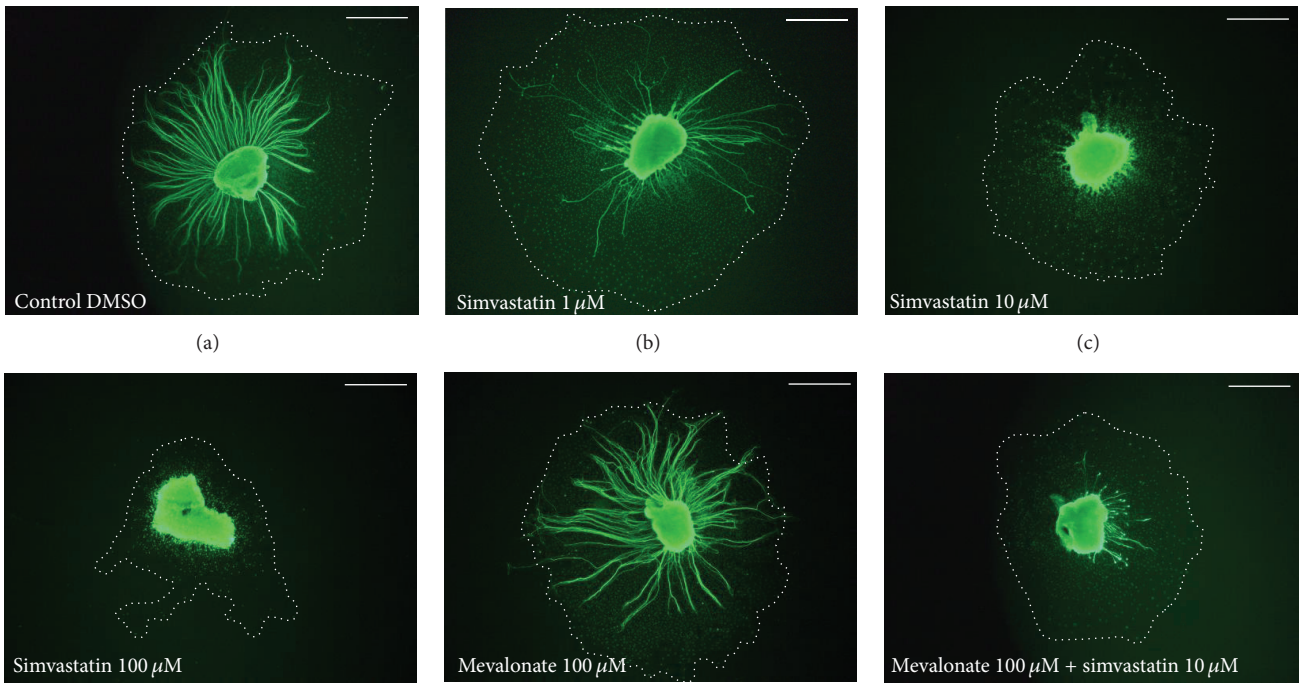

FIGURE 1: *Representative examples of rat SG explants stained with anti-neurofilament antibody*. (a) Representative example of a rat SG explant grown for 72 h in culture only. (b) Representative example for SG treated for 72 h with simvastatin 1 μM. (c) Representative example for SG treated for 72 h with simvastatin 10 μM. (d) Representative example for SG treated for 72 h with simvastatin 100 μM. (e) Representative example for SG treated for 72 h with mevalonate 100 μM. (f) Representative example for SG treated for 72 h with mevalonate 100 μM and simvastatin 1 μM. Dash lines indicate the area of supporting cells. Scale bar 250 μm.

the SGN. Images of the immunostained cultures were analyzed by using ImageJ software (NIH, Bethesda, MD, USA). Each neurite was traced and number of neurites, average lengths of neurites, and area of the supporting cells per explant were analyzed. Neuronal survival was analyzed by evaluating the number of neurons per 100 μm. A viable neuron fulfilled the following criteria: cell bodies with an intact cell membrane, no evidence of DNA fragmentation, and ultrastructurally homogeneous cytosol. Neurons with signs of apoptosis (DNA-fragmented nucleus, condensed chromatin, and membrane boiling or blebs or apoptotic bodies) were excluded [26, 27]. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey least-significant-difference post hoc test with Bonferroni correction. Data presented in the text and figures are means and standard deviations. Results were considered to be significant when the likelihood for a type 1 error was less than 5% ($p < 0.05$).

3. Results

3.1. Treatment with Simvastatin Results in a Dose-Dependent Decrease in SG Neurite Number. Simvastatin treatment results in a decrease in number of neurites per SG explant. The average number of neurites was decreased compared to control in all concentrations of simvastatin used in this study (1 μM, 10 μM, and 100 μM; ANOVA $p < 0.05$ for all conditions). This effect was dose-dependent (Figures 1 and 2).

3.2. Treatment with Simvastatin Results in a Dose-Dependent Decrease in Length of SG Neurites. Simvastatin treatment results in a decrease in length of neurites per SG explant. The average length of neurites was decreased compared to control in all concentrations of simvastatin used in this study (1 μM, 10 μM, and 100 μM; ANOVA $p < 0.05$ for all conditions). This effect was dose-dependent (Figures 1 and 2).

3.3. Treatment with Simvastatin Results in Decreased Area of Supporting Cells. Simvastatin also significantly decreased the area of nonneuronal cells, which have been previously identified as fibroblasts and Schwann cells [28] growing around the explant, as compared to the negative control. This effect was dose-dependent and significant for the two highest concentrations used in this study (10 μM and 100 μM; ANOVA $p < 0.05$ for both conditions) (Figures 1 and 2).

3.4. Simvastatin Decreases SG Neuronal Survival. The decreased number of neurites extending from SG explants could reflect the altered survival and/or neuritogenesis of SGNs. To assess this, we evaluated the survival of SGN cell bodies within explants. Simvastatin decreased SG neuronal survival when compared to controls in the two highest concentrations used in this study (10 μM and 100 μM; ANOVA, $p > 0.05$ for both conditions) (Figures 3 and 4).

3.5. Mevalonate Does Not Affect SG Neurite Number, SG Neurite Length, nor SG Neuronal Survival. Treatment with mevalonate did not influence SG neurite number, SG neurite

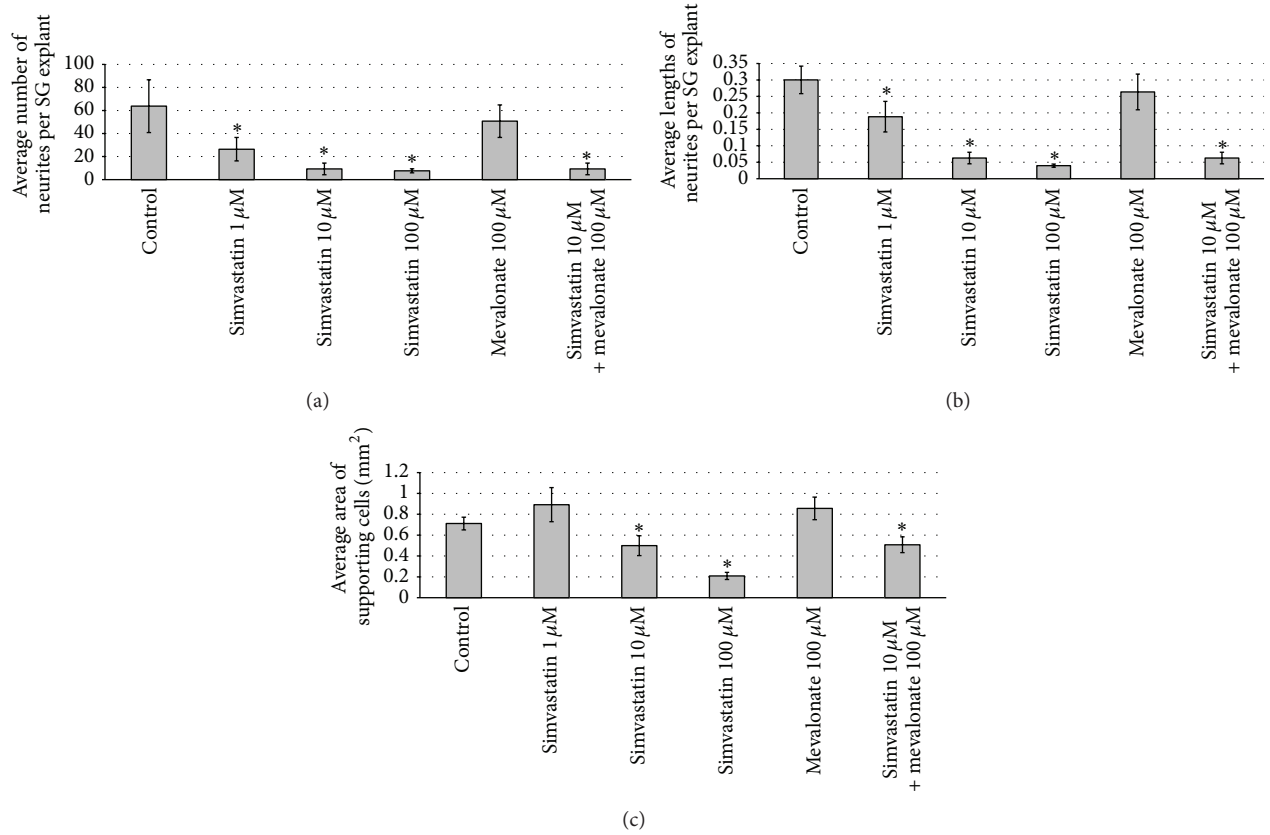

Figure 2: *Quantitative analysis of SG neurites number, SG neurites length, and area of supporting cells*. (a) Effect of simvastatin on the number of neurites from SG explants. There was a statistically significant decrease in the number of neurites in SG treated with simvastatin compared to control (ANOVA, $p < 0.05$). Mevalonate treatment had no effect on the number of neurites compared to control. The combination of simvastatin 10 μM and mevalonate 100 μM resulted in a decrease in the number of neurites per SG explant compared to control (ANOVA, $p < 0.05$). (b) Effect of simvastatin on the length of neurites from SG explants. There was a statistically significant decrease in the length of neurites in SG treated with simvastatin compared to control (ANOVA, $p < 0.05$). Mevalonate treatment had no effect on the length of neurites compared to control. The combination of simvastatin 10 μM and mevalonate 100 μM resulted in a decrease in the length of neurites per SG explant compared to control (ANOVA, $p < 0.05$). (c) Effect of simvastatin on the area of supporting cells. There was a statistically significant decrease in the area of supporting cells in SG treated with simvastatin compared to control (ANOVA, $p < 0.05$). Mevalonate treatment had no effect on the area of supporting cells compared to control. The combination of simvastatin 10 μM and mevalonate 100 μM resulted in a decrease in the area of supporting cells compared to control (ANOVA, $p < 0.05$). $*$ denotes statistical difference compared to control ($p < 0.05$).

length, or SG neuronal survival compared to control (Figures 1–4).

3.6. Treatment with Mevalonate in addition to Simvastatin Increases SG Neuronal Survival Compared to Simvastatin Treatment. Interestingly, treatment with mevalonate in addition to simvastatin increased SG neuronal survival compared to simvastatin treatment only (ANOVA $p > 0.05$; Figures 3 and 4). However, treatment with mevalonate in addition to simvastatin did not influence SG neurite number, SG neurite length, nor the area of nonneuronal cells around the SG explants compared to simvastatin treatment only (Figures 1 and 2).

4. Discussion

During the last decade, it has been hypothesized that statins might have a neuroprotective effect and therefore might be a potential drug for the treatment for sudden sensorineural hearing loss [6]. Recently, our group showed a protective effect of simvastatin on gentamicin-induced HC loss *in vitro*. We proposed that statins act by enhancing Akt activation and decrease the isoprenylation of small G proteins, such as Ras and Rho/Rac/Cdc42 [17]. However, we did not analyze the effect on SGN in our study. Cai et al. [15] discussed that statins prevent hearing loss due to reduction of atherosclerotic lesions and levels of glucose, cholesterol, low-density lipoproteins, and triglyceride. Moreover, Chang et al. [29] described a relationship between hyperlipidemia and hearing problems.

In contrast, Chung et al. [12] showed that sudden sensorineural hearing loss was significantly associated with previous statin use. Moreover, toxic effects of simvastatin are described in the inner ear [5]. The authors found neurodegenerative morphological changes and cell death after simvastatin treatment in cultured cochlear neuronal cells.

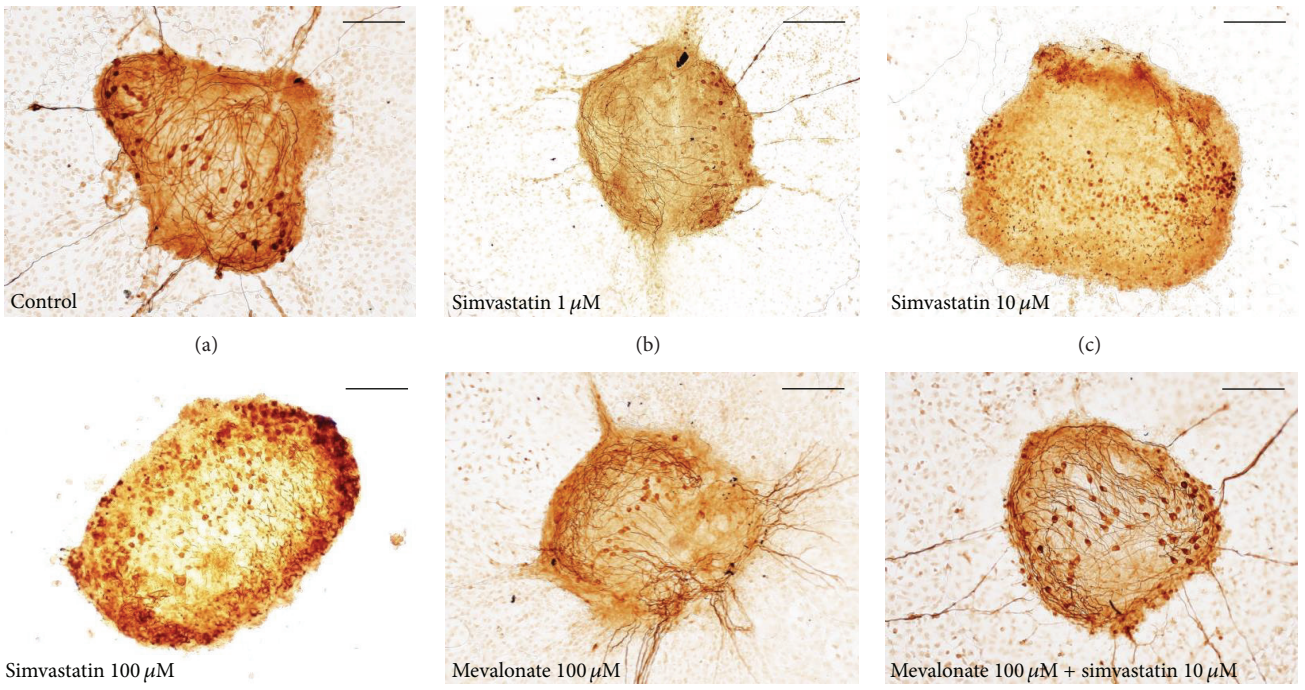

Figure 3: *Representative examples of rat SG explants stained to assess neuronal survival.* (a) Representative example of a rat SG explant grown for 72 h in culture only. (b) Representative example for SG treated for 72 h with simvastatin 1 μM. (c) Representative example for SG treated for 72 h with simvastatin 10 μM. (d) Representative example for SG treated for 72 h with simvastatin 100 μM. (e) Representative example for SG treated for 72 h with mevalonate 100 μM. (f) Representative example for SG treated for 72 h with mevalonate 100 μM and simvastatin 1 μM. Scale bar 150 μm.

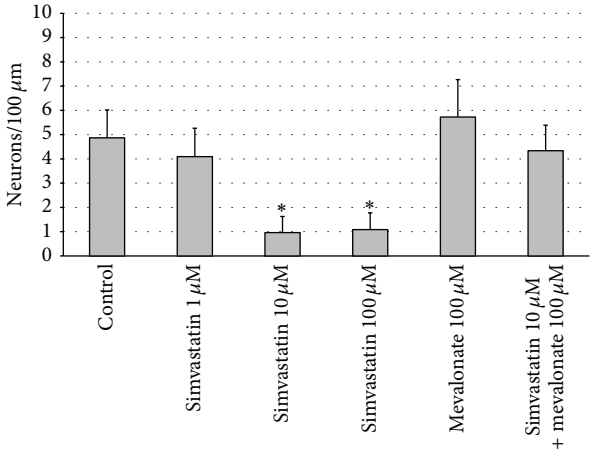

Figure 4: *Quantitative analysis of SG neuronal survival.* Treatment with simvastatin 10 μM and 100 μM resulted in decreased SG neuronal survival compared to control (ANOVA, $p < 0.05$). Treatment with simvastatin 1 μM, mevalonate 100 μM, and the combination of simvastatin 10 μM and mevalonate 100 μM did not influence SG neuronal survival compared to control. $*$ denotes statistical difference compared to control ($p < 0.05$).

The authors explain that this could be caused by a reduction of mevalonate pathway products, which are important antioxidants and membrane stabilizers. Simvastatin reduces the production of mevalonate by blocking the enzyme necessary for the production of mevalonate [3, 4].

Given these conflicting data, we evaluate the effects of simvastatin on SG neurites *in vitro*. Our data shows that simvastatin decreases the number of SG neurites, reduces the length of SG neurites, and also decreases the area of nonneuronal supporting cells around the SGNs (Figures 1 and 2). Moreover, we found that simvastatin reduced SG neuronal survival (Figures 3 and 4). Therefore, our results indicate that simvastatin is toxic for SGNs *in vitro*.

How can the toxic effect on SGNs of simvastatin be explained? Statins are inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an enzyme necessary for the production of mevalonate. Mevalonate is essential for the production of coenzyme Q10 and statins lead to a dose-dependent reduction in Q10 [30]. Q10 is a stabilizer of mitochondrial membranes and has an antiapoptotic effect [31]. Inhibition of the mevalonate pathway could be an explanation of our observations such that addition of mevalonate to simvastatin could rescue the SGN from simvastatin toxicity in our SGN neuronal survival experiments (Figures 3 and 4). We hypothesize that mammalian SGN may be more vulnerable to Q10 reduction by simvastatin than cochlear HCs. However, addition of mevalonate to simvastatin had no influence on SG neurite number, SG neurite length, and the area of nonneuronal cells around the SGNs compared to simvastatin treatment only. This indicates that the effect of simvastatin on SGNs and nonneuronal supporting cells is

only partially mediated by the mevalonate pathway and alternative mechanisms mediating the toxic effects of simvastatin have to be considered.

It should be noted that in our experiments we used organotypic explants from the cochlear SG that consisted of neurons and nonneuronal supporting cells, including fibroblasts and Schwann cells. Both cells might have influenced the observed reduction in neuritogenesis and length of neurites in our experiments. Moreover we could not distinguish between the dendrites and axons of SGN because there exist no differentiating markers. Two different subtypes with different functions and cellular interactions of SGNs are known, type I and type II SGNs [32]. The dendrites of type I cells are involved in afferent synapses exclusively with the IHCs, while the dendrites of type II cells exclusively interact with the OHCs [32]. It should be noted that in the present study we could not distinguish between type I and type II SGNs. 95% of SGNs are type I cells; therefore it seems likely that this subtype of neuron dominates our results. We used 5-day-old rat SGNs. In the rat cochlea onset of hearing approximately occurs on postnatal day 10 [33, 34]. We studied prehearing neurons because of the increased difficultly to culture older neurons. In addition, neurite development is still ongoing in 5-day-old rats [35, 36].

In summary, our data indicates a toxic effect of simvastatin on SG neuritogenesis, SG neuronal survival, and nonneuronal supporting cells *in vitro*. Therefore, caution is warranted to use simvastatin as a potential otoprotective drug.

Competing Interests

The authors declare that there are no competing interests.

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