Regenerative Medicine for the Inner Ear

Juichi Ito
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To my wife Noriko

Preface

Inner ear diseases such as sensorineural hearing loss (SNHL), tinnitus, and dizziness are very difficult to treat, especially in cases with highly severe hearing disturbance.

While the use of cochlear implants in profoundly deaf patients has been encouragingly useful, further improvement in the efficacy of this device is desirable. Recently, regenerative medicine has made great progress in application; however, clinical applications of regenerative medicine in the field of otolaryngology are limited as yet.

In this book, possible novel therapeutic strategies for the treatment of inner ear diseases, especially using regenerative medicine, are summarized. Most of the studies undertaken have been performed in the Department of Otolaryngology, Head and Neck Surgery of Kyoto University, Japan.

In early-phase inner ear damage, self-repair should be promoted to prevent inner ear cell death. Together with experimental results, recent findings of clinical trials using local drug application in the inner ear with neurotrophic factors have been established. In addition to induction of transdifferentiation as a possible next strategy, induction of cell proliferation is a useful alternative approach. In fact, cell transplantation therapy for the inner ear using embryonic stem cells and autologous cell sources, such as bone marrow stromal cells and induced pluripotent stem cells (as donor cells), have recently been initiated. Transplantation of these cells improves auditory function. Therefore, cell transplantation therapy is a useful method for the treatment of inner ear disorders. Apart from these approaches, a novel therapeutic method that involves implantation of an artificial auditory epithelium has been established for SNHL. This new artificial device is implantable and self-propelling by exploiting oscillations of the cochlear basilar membrane. A combination of these novel strategies may facilitate and improve the treatment of inner ear disorders and restoration of hearing ability in the near future.

Many of the issues proposed and discussed in this book are still controversial; however, the contributors are merely driven by their good intentions to contribute their recent findings in the hope of facilitating and realizing the important goal of treating inner ear diseases by medical caregivers.

It is my earnest hope that this book will serve as a useful beacon to shine light on the path of research for preclinical and clinical personnel involved in the pursuit of understanding inner ear function and treating relevant disorders. In addition, this work may also serve as a useful guide for doctors who deal with patients complaining of inner ear diseases.

Last but not least, I would like to thank all the authors for their valuable time and tireless efforts in contributing their findings to making the compilation of this book possible. The efforts and patience on the part of Springer Japan in publishing this book are much appreciated.

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Part I
Targets of Regenerative Medicine for the Inner Ear

Chapter 1
Anatomy of the Inner Ear

Tatsunori Sakamoto and Harukazu Hiraumi

Abstract The inner ear of mammals consists of the cochlea, the vestibule, and three semicircular canals. The macro anatomy of each component and the microstructure of each sensory region, especially all the components of the organ of Corti, are explained. The clinically available surgical approaches to the inner ear are also explained.

Keywords Cochlea • Crista • Hair cell • Macula • Saccule • Semicircular canals • The organ of Corti • Utricle

1.1 Morphology of the Inner Ear

The inner ear of mammals consists of the cochlea, the vestibule, and three semicircular canals. Because of its complex anatomy, the inner ear is referred to as a labyrinth. The inner ear is a double-walled structure. The outer wall is bony one, named otic capsule. The lumen of the otic capsule is called bony labyrinth. The inner membranous wall is known as a membranous labyrinth. The space between the bony and membranous labyrinth is called perilymphatic space and is filled with perilymph. Inside the membranous labyrinth is called endolymphatic space and is filled with endolymph. The perilymph is sodium rich and its component is similar to that of cerebrospinal fluid. The endolymph is potassium rich and very different from other fluids in the body.

The inner ear is accompanied by many vital structures. The inner ear is connected to the brain stem with the auditory nerve through the internal auditory canal. The facial nerve courses superiorly (labyrinthine and tympanic portion) and posteriorly (mastoid portion) to the cochlea and laterally (a junction between the

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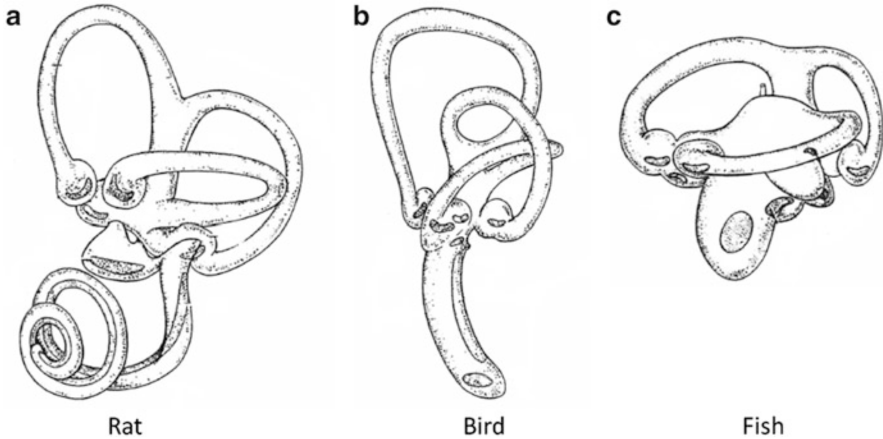

Fig. 1.1 Membranous labyrinth of vertebrates. Membranous labyrinths of a rat (**a**), a bird (**b**), and a fish (**c**) were shown. (Modified from "The Vertebrate body" by Romer et al. [1])

tympanic portion and the mastoid portion of the facial nerve) to the vestibule. Ventral to the cochlea, the internal carotid artery forms a knee. The jugular bulb of the jugular vein is positioned inferior to the cochlea.

1.1.1 The Cochlea

The cochlea is a hearing organ. The mammalian cochlea has a characteristic coiling morphology, where it has 2.75 turns in humans, 2 turns in mice, 4.25 turns in guinea pigs, and 2.75 turns in monkey [1, 2] (Fig. 1.1). The sensory epithelium of the cochlea is the organ of Corti, in which sensory hair cells stay in array (Figs. 1.3 and 1.4). In birds, the cochlea does not coil, and the sensory epithelium looks differently as basilar papilla [3] (Figs. 1.1 and 1.5). Fish and frogs do not have a hearing-specific organ; however, the sense of sound vibration is received by the saccule and the lagena (Fig. 1.1). The lagena is responsible also for linear acceleration and the magnetoreception [4].

The axis of the cochlea is known as the modiolus, in which cochlear nerve runs (Fig. 1.2). The cross section of the membranous labyrinth in the cochlea forms triangular shape bordered by the basilar membrane, the stria vascularis, and the Reissner's membrane (Fig. 1.3). The endolymphatic space inside the cochlea is called scala media or cochlear duct. The perilymphatic space facing the Reissner's membrane is called scala vestibuli, and the space facing the basilar membrane is called scala tympani. The scala tympani and scala vestibuli are connected through a small hole of the basilar membrane at the apex of the cochlea, called a helicotrema. Therefore, perilymphatic space is divided into two spaces by the cochlear duct. The basal portion of the scala tympani ends with a blind sac. The basal end of the scala

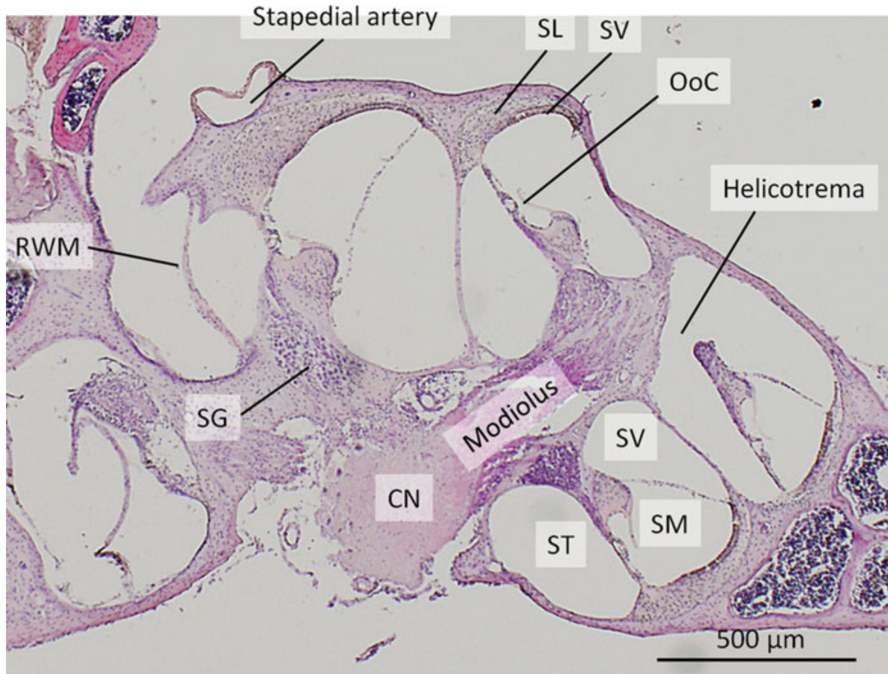

Fig. 1.2 Cochlea. Mid-modiolar section of an adult mouse cochlea, stained with hematoxylin and eosin. The cochlear nerve (CN) runs in the modiolus. Cell bodies of CN exist as spiral ganglions (SG) in Rosenthal's canal. The cochlear duct is seen as scala media (SM). Scala tympani (ST) and scala vestibuli (SV) are connected in the apical turn of the cochlea as helicotrema. Stapedial artery runs on the surface of the basal turn of the cochlea. Round window membrane (RWM), the organ of Corti (OoC), stria vascularis (SV), the spiral ligament (SL)

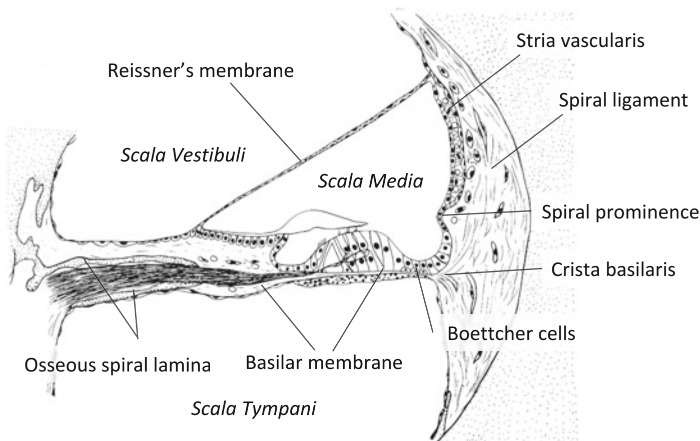

Fig. 1.3 The cochlear duct. (Modified from "Atlas of Otology" by Nomura et al. [5])

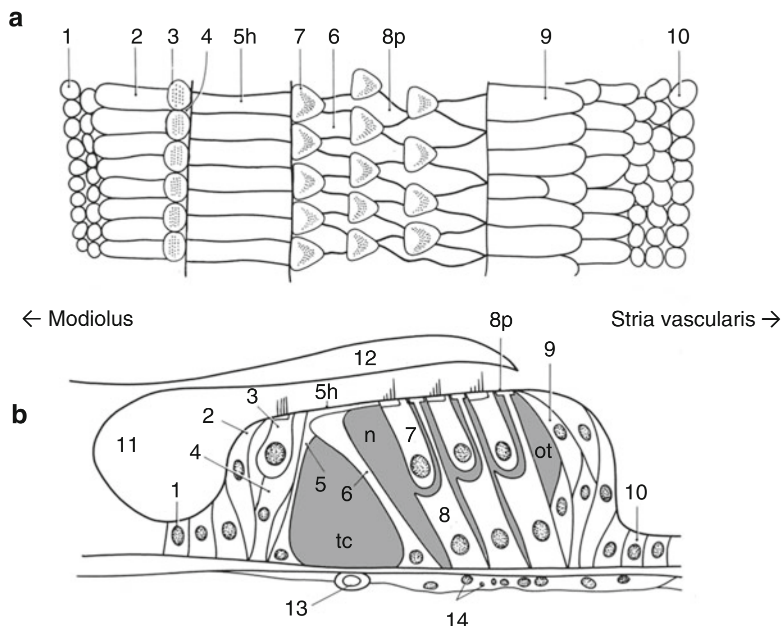

Fig. 1.4 The organ of Corti, surface view (**a**) and cross sectional view (**b**). The tunnel of Corti (**tc**), Nuel's space (**n**), and the outer tunnel (**ot**) are filled with cortilymph. *1* Inner sulcus cell, *2* border cell, *3* inner hair cell, *4* inner phalangeal cell, *5* inner pillar cell, *5h* the head of inner pillar cell, *6* outer pillar cell, *7* outer hair cell, *8* Deiters' cell, *8p* phalanx of Deiters' cell, *9* Hensen's cell, *10* Claudius' cell, *11* inner spiral sulcus, *12* tectorial membrane, *13* vessel of the basilar membrane, *14* tympanic border cell. (Modified from "Atlas of Otology" by Nomura et al. [5])

vestibuli is connected to the saccule via ductus reuniens. The basilar membrane is a resonant structure spreading between osseous spiral lamina and the crista basilaris. The basilar membrane in the basal turn is narrower and stiffer, leading to higher natural resonance frequency, than that in the apical turn. The organ of Corti is positioned on the scala media side of the basilar membrane.

The organ of Corti (Figs. 1.2, 1.3, and 1.4) is composed of hair cells and several types of supporting cells. When the sound vibration displaces the basilar membrane, the shearing force between the basilar and the tectorial membranes deflects the hair bundle at the apical surface of the inner hair cells and then is conveyed as the release of the neurotransmitter. Approximately 3,500 inner hair cells exist in one human cochlea [5]. The inner hair cells are innervated by type I afferent neurons with myelinated axons. The inner phalangeal cells are the direct supporting cells for inner hair cells. Border cells and inner sulcus cells are located next to the inner phalangeal cells. The outer hair cells appear as three rows, and 12,000 outer hair cells exist in one human cochlea [5]. These cells change their length through the function of the motor protein, prestin, in response to the sound stimuli [6, 7]. The outer hair cells are innervated by type II afferent neurons

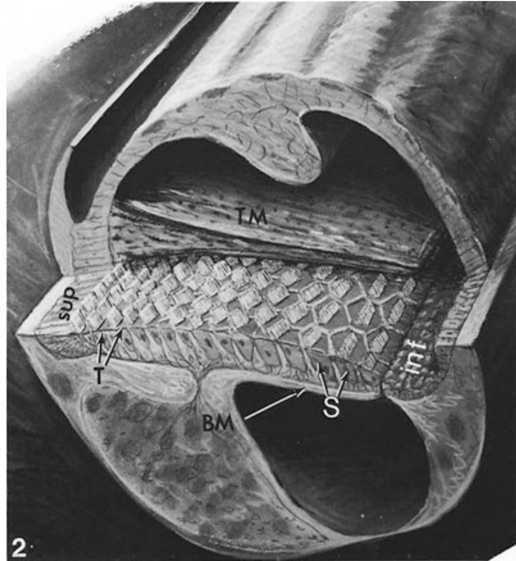

Fig. 1.5 The basilar papilla of a chicken. *T* tall hair cell, *S* short hair cell, *inf* inferior margin of the cochlea, *sup* superior margin of the cochlea, *BM* basilar membrane, *TM* tectorial membrane. (From Tilney and Saunders [3])

with unmyelinated axons. Deiters' cells support the outer hair cells. Deiters' cells have two parts: The cell body directly attaches to the basilar membrane and supports the basal side of the outer hair cell. The phalangeal process extends from the cell body to the apical surface of the sensory epithelium between outer hair cells and forms a part of reticular lamina. Inner and outer pillar cells exist between inner hair cells and the first row of outer hair cells and form a fluid-filled space called the tunnel of Corti. The space between outer pillar cells and the first row of outer hair cell/Deiters' cell is called Nuel's space. Hensen's cells are located next to the third row of the Deiters' cells and form several layers. Claudius' cells exist between Deiters' cells and the spiral prominence, forming outer sulcus. Boettcher cells are located beneath the Claudius' cells in the lower turns of the cochlea (Fig. 1.3). Tympanic border cells are cells lining the scala tympani side of the basilar membrane.

The loose connective tissue on the outer wall of the cochlear duct is the spiral ligament (Fig. 1.3). The stria vascularis is a vascular-rich structure on the spiral ligament spreading from the attachment of Reissner's membrane to the spiral prominence. The stria vascularis functions to maintain the high potassium concentration and positive potential of the endolymph (endocochlear potential (EP)) via Na/K-ATPase.

The basilar papilla is a sensory epithelium in the cochlear duct of birds, amphibians, and lizards, which functions like the organ of Corti (Fig. 1.5) [3]. Hair cells and supporting cells are compactly arranged on the basilar membrane. The tall hair cells exist near the superior margin of the basilar membrane, while the short hair cells exist near the inferior margin.

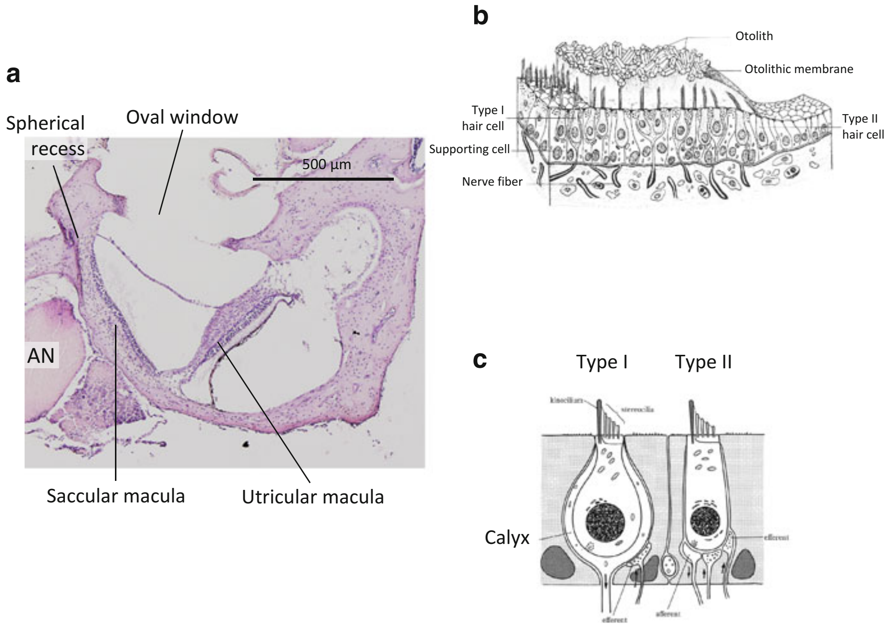

Fig. 1.6 The vestibule. (**a**) A section of adult mouse inner ear, stained with hematoxylin and eosin. The stapes was removed from the oval window. *AN* auditory nerve. (**b**) Macula. (Modified from "Modern Oto-rhino-laryngology" by Kirikae and Nomura [12]) (**c**) Type I and II vestibular hair cells. (Modified from "Atlas of Otology" by Nomura et al. [5])

1.1.2 The Vestibule

The vestibule (the utricle and saccule) is positioned superior to the cochlea and functions to sense linear acceleration (Fig. 1.6). In the vestibule, the membranous labyrinth forms two sacs which are connected each other. The sac near the semicircular canals is called the utricle, while the other located near the cochlea is called the saccule. The saccule is connected to the cochlea by a small channel called the ductus reuniens. The sensory epithelium of the vestibule is located in the macula of the utricle and saccule. The utricular macula is located horizontally with a free margin. It resembles a visor of a cap separating the utricle and perilymphatic space of the vestibule. The saccular macula is attached to the medial wall of the saccule. The bony labyrinth forms a shallow bowl called spherical recess at the attachment site of saccular macula. Each macula is overlaid by the otolithic membrane, which is a rigid layer composed of gelatinous extracellular matrix and otolith. The space between the otolithic membrane and the apical surface of the sensory epithelium is secured by the columnar filament layer [8]. Each macula is divided into two regions by the striola. In the utricle, hair cells are arranged in such a manner that the tall cilia face the striola. In the saccule, the tall cilia face the peripheral side instead of

the striola. Hair cells in the maculae are classified into type I and II hair cells [5, 9]. Type I hair cells have a bulbous shape and its basal part is surrounded by large socket-like afferent nerve endings called the calyx. Type II hair cells have a cylindrical shape and display simpler button-like afferent nerve contacts. Both type I and II hair cells receive efferent nerve contacts. Type I hair cells exist near the striola, and type II in the peripheral region. Type I and II hair cells are morphologically distinguishable, but the functional difference is not fully understood. Dark cells are responsible for the maintenance of endolymph through ion and water transport [10]. Dark cells exist around the edge of the utricular macula, but not the saccular macula [11].

1.1.3 Three Semicircular Canals

The three semicircular canals (lateral, superior, and posterior) are located postero-superior to the vestibule and cochlea. They form three perpendicular planes and work as detectors for angular acceleration. To be precise, the angle formed by two semicircular canals is not right angled and each canal is twisted; however the relative special arrangement of three semicircular canals contributes to the stimulus perception of three-dimensional head rotation. The superior and posterior semicircular canals share one canal at the insertion to the vestibule or the common crus. The semicircular canals are postero-superior to the facial nerve. The lateral semicircular canal is placed lateral to the vestibule adjacent to the facial nerve. The lateral semicircular canal forms an eminence in the middle ear, which is called the prominence of the lateral semicircular canal and is a very important landmark in ear surgery. The superior semicircular canal sometimes protrudes into the middle cranial fossa. The posterior semicircular canal is positioned between the posterior fossa dura and the mastoid portion of the facial nerve.

In each semicircular canal, the membranous labyrinth presents as a tubelike structure. At one end of each semicircular canal, the tube is dilated to form a spindle-like structure, which is known as the ampulla (Fig. 1.7). A crescent-shaped structure (crista) is located inside the ampulla. The crista is attached to the outer wall of the ampulla, and the hair cells of the semicircular canals are located in the sensory epithelium of the crista. In the crista, type I hair cells preferably exist at the central part, while type II hair cells are found in the peripheral part of the crista, which are equivalent to the vestibular maculae. The hair cells are surrounded and separated by supporting cells. The site next to the sensory epithelium is the transitional area and the dark cell area. The cupula is a gelatinous substance, which extends from the crista and stretches across the ampulla to the roof. Tips of the cilia of hair cells are embedded in the base of the cupula. When the rotating movement is applied to a semicircular canal, the relative movement of the liquid displaces the crista; thus the cilia will be bent.

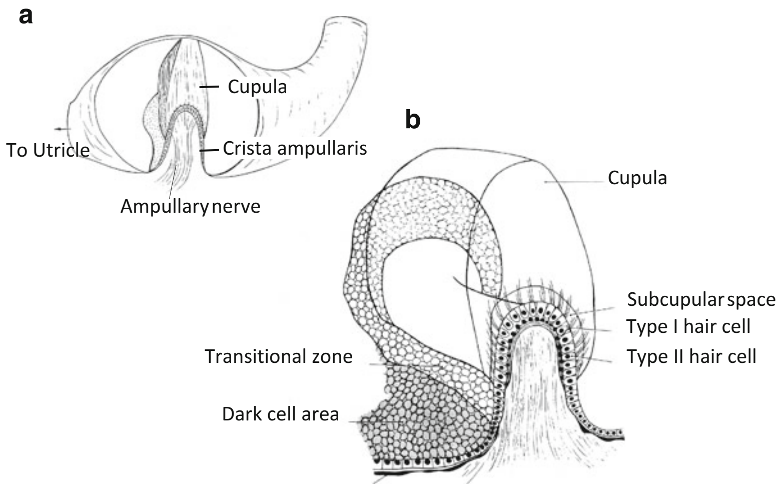

Fig. 1.7 Ampulla of the semicircular canal. (**a**) Membranous labyrinth of the semicircular canal. (**b**) Cupula and crista ampullaris. (Modified from "Atlas of Otology" by Nomura et al. [5])

1.1.4 Round and Oval Windows

The bony labyrinth has two windows facing the middle ear, i.e., the oval window and round window. The oval window is an opening in the vestibule, and the footplate of the stapes is attached to this window. The round window, which is an opening in the scala tympani of the cochlea, is closed with the round window membrane. The round window membrane is lodged deep in a pit called the round window niche, which is often closed at the opening by a membranous structure, called the pseudomembrane. The oval window faces laterally, and the round window faces inferiorly.

1.1.5 Endolymphatic Sac

The endolymphatic sac is a protrusion of the membranous labyrinth out of the bony capsule of the inner ear. The endolymphatic sac is positioned between the posterior semicircular canal and the posterior fossa dura and is connected to the utricle and saccule via the endolymphatic duct. In the other portion of the endolymphatic sac, the endolymphatic space is surrounded by the perilymphatic space. The endolymphatic sac is the only endolymphatic space that is accessible without violating the perilymphatic space.

1.2 Auditory and Vestibular Nerves

The medial side of the inner ear is connected to the auditory nerve, which comprises the cochlear, superior vestibular, and inferior vestibular nerves. The bone between the inner ear and the nerve forms a plate with small fenestrations, i.e., the cribriform plate. The superior vestibular nerve innervates the crista of the superior and lateral semicircular canals and utricle. The inferior vestibular nerve innervates the posterior semicircular canal and saccule. The nerve innervates the inferior semicircular canal branches to form he inferior vestibular nerve, which is called the singular nerve.

1.3 Blood Supply

The arterial blood supply to the membranous labyrinth is from the labyrinthine artery [13]. The labyrinthine artery is usually a branch of the anterior-inferior cerebellar artery. The labyrinthine artery branches to form the common cochlear artery and anterior vestibular artery. The common cochlear artery subdivides to form the main cochlear and vestibulocochlear arteries. The main cochlear artery supplies apical three-fourths of the cochlea. The main cochlear artery enters the modiolus and branches to form the external and internal radiating arterioles. The external radiating arterioles course over the scala vestibuli and ramify a capillary network of the stria vascularis and other structures in the outer wall of the cochlea. Internal radiating arterioles furnish blood supply to the spiral ganglion and structures above the basilar membrane. The vestibulocochlear artery supplies blood to the basal one-fourth of the cochlea and modiolus (cochlear ramus artery), the macula of the saccule, and crista of the posterior semicircular canal (posterior vestibular artery). The anterior vestibular artery provides blood circulation to the macula of the utricle and crista of the superior and lateral semicircular canal.

1.4 Surgical Approaches to the Inner Ear

The inner ear has a hard bony shell of the otic capsule, at sites of the two windows and endolymphatic sac. Therefore, a surgical approach to the inner ear needs drilling of the otic capsule or opening these two windows or the sac. There are three approaches to the inner ear: (i) transcanal, (ii) transmastoid, and (iii) extra-temporal bone.

Since the cochlea is positioned just behind the eardrum, the transcanal approach (i) is the most simple with minimal invasive method. By elevating the eardrum and removing some portion of the external auditory canal bone, the oval window and the round window niche can be well manipulated. This approach is used in the

surgery for otosclerosis and perilymphatic fistula. Recent advance in endoscopy enables access to the two windows via myringotomy. The endoscope provides excellent visualization of the round window membrane, since it is perpendicular to the eardrum and lodged deep in the round window niche. Drilling of the promontorium provides access to the cochlea. This approach is sometimes used in the cochlear implantation surgery.

The transmastoid approach (ii) is the most popular method in a clinical setting. This approach can be subdivided into two prongs: facial recess and retrofacial approach. The facial recess approach is widely utilized in the cochlear implantation surgery, where a bony portion between the facial nerve and chorda tympani is drilled to provide access to the oval window and the round window niche. By removing the lip of the round window niche, the round window membrane is accessible. Cochleostomy is also available for accessing the cochlea. The retrofacial approach is commonly used in the mastoid-endolymphatic shunt surgery. Using this approach, the endolymphatic sac is accessible with minimal damage to the inner ear. By incising the dura matter ventral to the sigmoid sinus, cisternal portion of the auditory nerve is visible (retrolabyrinthine approach). The otic capsules of the three semicircular canals are accessible with the transmastoid approach. By gently removing the otic capsule, the lumen of semicircular canal can be opened without causing sensorineural hearing loss.

The extra-temporal bone approach (iii) is extensively used in the surgery for vestibular schwannoma. In the middle fossa approach, the temporal lobe and dura matter are elevated from the temporal bone. By removing the bone above the internal auditory canal, it is possible to access the auditory nerve without violating the inner ear. This approach also provides good access to the superior semicircular canal and is used for surgery of the superior canal dehiscence syndrome. The retrosigmoid approach is another extra-temporal bone approach. By incising the dura matter behind the sigmoid sinus and depressing the cerebellum, a cisternal portion of the auditory nerve can be secured. Further removal of the bone dorsal to the internal auditory canal provides access to the whole part of the auditory nerve.

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Chapter 2
Therapeutic Targets and Possible Strategies for Regenerative Medicine for the Inner Ear

Takayuki Nakagawa

Abstract Regenerative medicine aiming for the functional recovery of the inner ear has several targets for therapeutics in tissue, cells, or cell organelle. Among various therapeutic targets, sensory hair cells have been paid considerable attention because of their importance in inner ear functions. At present practical methods for hair cell regeneration have not been developed. However, experimental studies have revealed possible strategies for regeneration according to development of new technologies. This chapter reviews therapeutic targets for regenerative medicine in the inner ear and possible strategies to realize regeneration of the inner ear.

Keywords Cell transplantation • Dedifferentiation • Self-repair • Technological regeneration • Transdifferentiation

2.1 Therapeutic Targets

The inner ear consists of two components from the point of view of function. The cochlea is an organ corresponding to hearing and the vestibules and semicircular canals are organs for vestibular function. In the cochlea, the sensory hair cell is included in main targets for the treatment of sensorineural hearing loss (SNHL). Hair cell regeneration has long been a central issue in research for inner ear regeneration, because hair cells are crucial for conversion of sound stimuli to neural signals, and studies of human temporal bones have demonstrated that the degeneration of hair cells is a main etiology for SNHL. The primary step for conversion of

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sound stimuli to neural signals is the tilt of stereociliary bundles located at the top of hair cells. Therefore, the stereocilia is a crucial cellular component of functional hair cells. In addition, for the tilt of stereocilia, the presence of the tectorial membrane is critical. The second step for conversion of sound stimuli to neural signals is the depolarization of hair cells, which requires a high concentration of potassium ions in the endolymph and several ion channels in hair cells. In the maintenance of high potassium in the endolymph, the function of the cochlear lateral wall including the stria vascularis and spiral ligament is inevitable. The third step is the release of neurotransmitters from hair cells to afferent dendrites of spiral ganglion neurons through synaptic contacts. At synaptic contacts between inner hair cells and afferent dendrites of spiral ganglion neurons, inner hair cells have specific organelle, ribbon synapses at their bottom. Considering regeneration of hair cells with normal function, regeneration of several cellular components of hair cells is necessary, and several cochlear components should be regenerated. All of these cellular components and cochlear components are included in therapeutic targets. An ultimate goal is complete regeneration of hair cells with necessary cellular components. However, even regeneration of one important component in hair cells would be efficacious for hearing recovery if other important components are maintained.

Beside hair cells, there are a number of cochlear components that are included in therapeutic targets. As mentioned above, the stria vascularis and spiral ligament in the cochlear lateral wall should be included in therapeutic targets. These components have a network of the gap junction, which is crucial for the maintenance of high potassium in the endolymph. Mutation of genes associated with the gap junction is the most frequent cause of congenital SNHL. Insufficient formation of the tectorial membrane also causes congenital SNHL. The spiral ganglion neuron is also an important therapeutic target. Spiral ganglion neurons play a crucial role in the transmission of auditory signals from hair cells to the central systems. Additionally, their loss diminishes clinical benefits of cochlear implantation, which is a medical device for restoration of hearing in patients with profound SNHL.

In the vestibular end organs, the hair cell is also a central player in their functionality similarly to the cochlea. Although adaptive frequencies of vestibular hair cells are quite different from cochlear hair cells, vestibular hair cells also covert mechanical stimuli to neural signals. The tilt of stereocilia in vestibular hair cells is induced by the movement of the cupula, a gelatinous component located above the stereocilia of vestibular hair cells. The otolith organs additionally have the otoconia, small particles composed of a combination of a gelatinous matrix and calcium carbonate on the cupula. The tilt of stereocilia in vestibular hair cells induces the depolarization of vestibular hair cells leading to the release of neurotransmitters into the synaptic contacts between hair cells and afferent dendrites of vestibular ganglion neurons. In the vestibular end organs, hair cells, cupula, and otoconia, vestibular ganglion neurons can be therapeutic targets. Synaptic contacts between hair cells and vestibular ganglion neurons are also included in therapeutic targets.

2.2 Possible Strategies

Recent studies have demonstrated that the mammalian inner ear has the capacity for regeneration, although it is limited. The presence of stem cell-like cells in the mammalian inner ear has been reported [1–4]. Some reports have demonstrated functional restoration of mammalian inner ear [5, 6]. However, the quality for the functionality of regenerated inner ears is not satisfactory. Further investigations are required before clinical application. Investigations for regeneration in the mammalian inner ear have been done referring to findings in other vertebrates including birds, in which hair cell regeneration occurs, or findings in developmental processes of the mammalian inner ear. Here we introduce possible strategies for induction of regeneration according to the stage or level of degeneration in the inner ear (Fig. 2.1). The sensory hair cells and spiral ganglion neurons have been primary targets for studies of inner ear regeneration. Therefore, regeneration of hair cells and spiral ganglion neurons is used as a model for discussion on possible strategies.

2.2.1 Self-Repair

Before hair cells or spiral ganglion neurons disappear, the induction of self-repair may be a pragmatic strategy. For this purpose, there are two possible strategies. One is the promotion of survival of hair cells and subsequent reconstruction of cellular components in hair cells by spontaneous activity. This can be expressed as the protection from cell death. Several agents for promotion of survival or protection from cell death have been reported. Some of such candidates have been examined for their efficacy and safety in clinical trials [7, 8]. On the other hand, there is no specific report describing the induction of reconstruction of cellular components in damaged hair cells. In spiral ganglion neurons, reconstruction of synaptic contacts with the inner hair cells or cochlear nucleus neurons is a key issue in regeneration of cellular components. For this purpose, further investigations should be required to reveal mechanisms for maturation of functional hair cells and spiral ganglion neurons.

2.2.2 Transdifferentiation

After hair cells have gone, three different possible strategies can be applied depending on the condition of the remaining supporting cells. If sufficient numbers of healthy supporting cells still remain in the sensory epithelium, the induction of transdifferentiation of supporting cells to hair cells can be a strategy for hair cell regeneration. Hair cells and supporting cells share a common progenitor in the development. In fate determination of progenitor cells in the sensory epithelium,

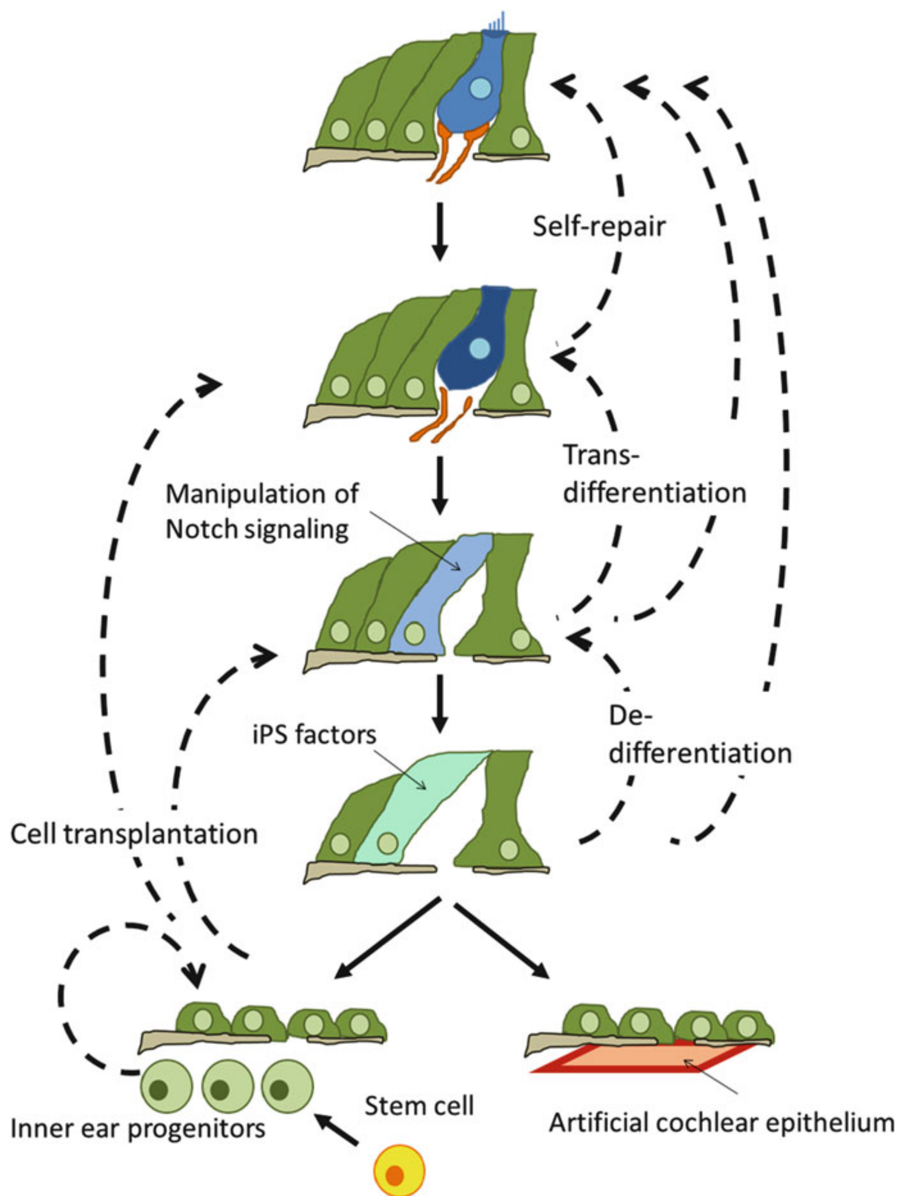

Fig. 2.1 Schematic drawing of the strategies for hair cell regeneration

the Notch signaling plays a key role [9, 10]. The manipulation of Notch signaling has been used for induction of transdifferentiation of supporting cells. Transdifferentiation of supporting cells to hair cells was firstly demonstrated by means of gene transfer. Introduction of Atoh1 gene into supporting cells using

adenovirus vectors induced transdifferentiation of supporting cells into hair cells [11, 12]. In general, the inhibition of Notch signaling increases the expression of Atoh1, and its activation suppresses Atoh1 expression. Next to gene transfer, pharmacological inhibition of Notch signaling was used for this purpose. Pharmacological inhibition of Notch signaling by gamma secretase inhibitors induced an increase of Atoh1 expression in neonatal cochleae, leading to excessive generation of hair cells [13]. The activity of Notch signaling weakens in the supporting cells according to maturation. In adult cochleae, virtually no expression of Notch ligands and receptors was identified in supporting cells. However, during certain duration after damage, temporal activation of Notch signaling was found even in adult cochleae [14, 15]. Topical application of gamma secretase inhibitors into cochleae resulted in hair cell induction [6]. However, at present hearing restoration by this approach is not satisfactory, and the therapeutic time window is limited.

2.2.3 Dedifferentiation

In case that insufficient numbers of supporting cells remain, transdifferentiation is not an effective strategy. Transdifferentiation of one supporting cells is equal to the loss of one supporting cells. In such a case, induction of proliferation in supporting cells is necessary. In the mammalian cochlea, cell proliferation rarely occurs after birth, while in the avian cochlea (basilar papilla) supporting cells proliferate in response to hair cell loss [16]. In the avian cochlea, both transdifferentiation of supporting cells to hair cells and proliferation of supporting cells followed by differentiation into hair cells occur [16]. To induce proliferation of supporting cells in the mammalian cochlea, the downregulation of cell cycle inhibitors is necessary. One of the major cell cycle inhibitors in mammalian supporting cells is p27, an inhibitor of cyclin-dependent kinase. Genetic deletion of p27 resulted in excessive generation of hair cells [17, 18]. Knockdown of p27 in supporting cells induced reentry of cell cycle in supporting cells, but the majority of supporting cells that had reentered cell cycle fell into apoptosis [19]. Thus, cell cycle reentry of supporting cells is not sufficient for regeneration of hair cells, suggesting that alterations in characteristics of supporting cells may be critical. Recently, challenges for induction of dedifferentiation in supporting cells have been reported [20, 21]. One possible strategy for dedifferentiation is introduction of transcription factors for generation of the iPS cell. Detailed analysis of mechanisms for alterations in characteristics of supporting cells in the avian cochlea after hair cell loss could provide useful information to induce dedifferentiation of mammalian supporting cells.

2.2.4 Cell Transplantation

Another option in case that supporting cells are severely damaged is the introduction of exogenous stem cells into the inner ear. In the early 2000s, the approach of cell transplantation for hair cell regeneration has gained considerable attention, because stem cells have been believed to accumulate in damaged sites and have the potential to repair damaged organs. However, migration of transplanted stem cells into damaged sensory epithelia of the inner ear rarely occurred [22]. In addition, the circumstance of the endolymphatic space in the inner ear is hard for the survival of transplants, because of its high concentrations of potassium. On the other hand, recent progress in research for induction of pluripotent stem cells for differentiation into inner ear cells has demonstrated that generation of sensory hair cells from pluripotent stem cells is possible. In the near future, specific guidance cues for hair cell induction from pluripotent stem cells would be discovered. As for spiral ganglion neurons, a cell transplantation approach is realistic comparing with hair cells. Recently, functional restoration of spiral ganglion neurons by transplantation of human ES cell-derived neural progenitors has been reported [5]. The iPS cells have a similar potential for regeneration of spiral ganglion neurons to ES cells [23]. However, the use of iPS cells involves the risk of tumor formation [24]. To realize cell-based therapy for regeneration of spiral ganglion cells, autologous transplants that are fully differentiated and completely eliminated undifferentiated cells must be used.

2.2.5 Technological Regeneration

As an alteration of biological approach for hair cell regeneration, an artificial cochlear epithelium has been investigated as technological regeneration. A cochlear sensory epithelium converts mechanical vibration to electrical signals. In response to mechanical stimuli, a piezoelectric material generates electricity and so could be used in place of a cochlear epithelium as a bionic cochlear epithelium. A thin membrane of a piezoelectric material framed with silicon generated electricity in response to sound stimuli after implantation into a guinea pig cochlea [25]. Problems to be resolved included insufficient power of the device to stimulate spiral ganglion neurons and limited sensitivity for sound frequencies. A combination of technological and biological approaches may be required to resolve these problems. Neurite induction from spiral ganglion neurons to the device by gene therapy might contribute to reduction of required electrical power for stimulation of spiral ganglion neurons.

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Chapter 3
Hair Cell

Norio Yamamoto

Abstract Hair cells are the main components of the inner ears that facilitate mechano-electrical transduction to perceive sound or change in body position. Hair cells are highly differentiated and have many specialized characteristics. Morphologically, hair cells have hair bundles on their apical side and receive innervation from the primary auditory nerve. Physiologically, deflection of hair bundles causes depolarization of membrane potential and as a result, hair cells transmit signals to the auditory nerve by releasing neurotransmitters. There are six different sensory epithelia and four different types of hair cells: inner and outer hair cells in the cochlea and type I and II hair cells in the vestibular organs. Each type of hair cell has different morphological and functional characteristics. Moreover, these hair cells are localized with other cell types (e.g., supporting cells) in specified patterns and polarity to function properly.

Owing to these highly differentiated characteristics, it is difficult to regenerate hair cells. Moreover, proliferation of mammalian cochlear hair cells is confined within embryonic period. However, recent advancement in the knowledge of developmental biology and the rapid progress in the field of stem cell biology are anticipated to resolve the problems that prevent the successful regeneration of mammalian hair cells.

Keywords Cochlea • Development • Mechano-electrical transduction • Stem cell • Vestibular organ

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3.1 Functions of Hair Cells

Hair cells are one of the main functional components of the inner ear that is located in the sensory epithelia and they facilitate the transduction of vibratory mechanical signals into electrical signals (mechano-electrical transduction (MET)). The mechanical vibration is induced by auditory signals that reach the cochlea from the outer and middle ear or by the movement of the fluid or otolith in the vestibular organs that contribute to the maintenance of the body equilibrium. There is one sensory epithelium in the cochlea and five sensory epithelia in the vestibular organ (Fig. 3.1a, b). The vestibular sensory epithelia are divided into two types, namely, the macula and the crista ampullaris. There are two maculae, namely, saccule and utricle, that detect the linear acceleration. Each of the three semicircular canals (superior, posterior, and lateral canals) contains crista ampullaris that detects rotatory acceleration.

Hair cells are named after their hair bundles (stereocilia and kinocilium) on their apical side. Once the stereocilia deflect toward a specific direction, cation channels called MET channels open to induce the influx of potassium ion, causing the depolarization of hair cells [1]. The structure that mechanically opens MET channels was identified in the stereocilia [2] and is called as tip-link. Hence, the presence of MET channel in the stereocilia is widely recognized. The depolarization of hair cells results in the opening of the voltage-gated calcium ion channels and the influx of calcium ion induces the release of neurotransmitter, glutamate [3, 4], from the basal end of hair cells to cochlear and vestibular nerves that innervate cochlear and vestibular hair cells, respectively. Molecular analyses of the important structures in

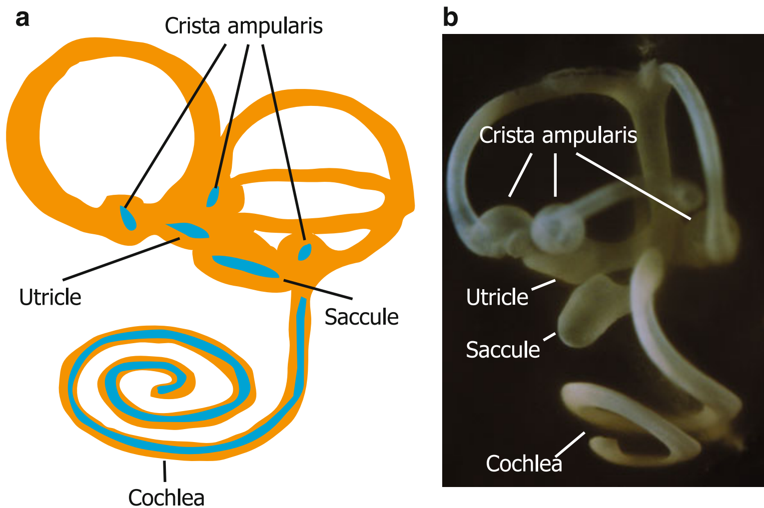

Fig. 3.1 Inner ear and sensory epithelia. (**a**) Schematic representation of the inner ear (*orange*) and its sensory epithelia (*blue*). There are six sensory epithelia in the inner ear. (**b**) Paint filling of E17.5 mouse inner ear

hair cells are in progress. Especially, the molecular components of MET channels have been investigated for a long time by several researchers. The candidate molecules that constitute MET channels were identified as transmembrane channel-like gene family 1 (Tmc1) and transmembrane channel-like gene family 2 (Tmc2) [5]. The molecular components that constitute the tip-link were determined as cadherin 23 and protocadherin 15 [6–8]. The genes for Tmc1, cadherin 23, and protocadherin 15 are causative genes of hereditary hearing loss, which indicate the importance of hair cells in hearing.

3.2 Characteristics of Each Hair Cell Type

There are two different types of hair cells in the cochlea (inner and outer hair cells) and in the vestibular organs (type I and II hair cells), respectively. Inner and outer hair cells are different in their position, ultrastructure, arrangement of stereocilia, innervation, and function. In the organ of Corti, inner hair cells (IHCs) are arranged in a single row adjacent to the modiolus. In contrast, outer hair cells (OHCs) are lined in three rows near the lateral wall (Fig. 3.2). IHCs are goblet-shaped (Fig. 3.3a) and their stereocilia are arranged in flattened U shape (Fig. 3.2). OHCs are long and cylindrical in their shape (Fig. 3.3a) and have stereocilia arranged in a W or V shape (Fig. 3.2). The innervation of IHCs is unique because IHCs are innervated mostly by afferent fibers that occupy 95 % of the cochlear afferent fibers [9]. Efferent fibers toward IHCs mostly make synapses with afferent fibers of cochlear nerve that innervate IHCs. OHCs are innervated by enormous efferent fibers and have only a few afferent fibers. The difference in the innervation pattern of IHCs and OHCs reflects the difference in the functions between both hair cells. IHCs mainly transduce auditory stimuli to the cochlear nerve as sensory end organs. In contrast, the depolarization and hyperpolarization of OHCs alter the length of the body of the OHC [10] rather than causing afferent nerve stimulation. This motility of OHCs contributes to the amplified vibration of the basilar

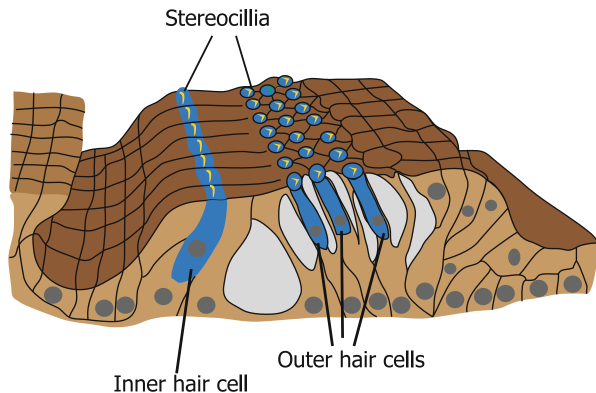

Fig. 3.2 Organ of Corti. Inner and outer hair cells are arranged in one and three rows, respectively. The stereocilia of inner and outer hair cells are arranged in U and V shapes, respectively

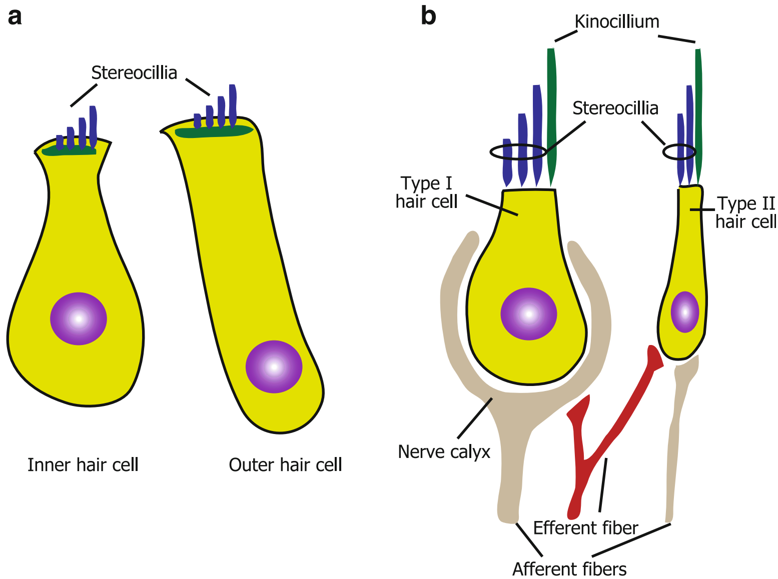

Fig. 3.3 Morphology of hair cells. (**a**) Cochlear hair cells. (**b**) Vestibular hair cells

membrane where IHCs and OHCs reside, resulting in the high sensitivity and frequency selectivity of hearing. Efferent stimuli from superior olivary complex to OHCs inhibit the electromotility of OHCs providing inhibitory modulation of the hearing [11]. The electromotility of OHCs is dependent on a novel motor protein, prestin [12]. Genetic disruption of prestin adversely affects the auditory brainstem response, distortion product of otoacoustic emissions, and cochlear microphonics in vivo as well as the electromotility of OHCs itself in vitro [13]. This, in turn, suggests the significance of OHC electromotility in the hearing of mammals.

Vestibular hair cells are morphologically similar to cochlear hair cells. However, vestibular hair cells have kinocilia (Fig. 3.3b) even at the postnatal stage, whereas cochlear hair cells lose kinocilia. Vestibular organs have two types of hair cells, namely, type I and II hair cells. Type I hair cells are flask-shaped and type II hair cells are cylindrical (Fig. 3.3b). Another morphological difference between these two hair cells is the difference in the innervation patterns of afferent fibers [14]. Type II hair cells have button endings of afferent fibers that is also seen in cochlear hair cells (Fig. 3.3b). In contrast, type I hair cells have calyx endings, which surrounds almost the entire basolateral surface of type I hair cells (Fig. 3.3b). The calyx-type afferent fibers have lower gains from head rotations compared to button-type afferent fibers [15], which reflect the functional difference between type I and II hair cells in the vestibular organs.

To distinguish cochlear and vestibular hair cells, which include the inner and outer hair cells, and type I and II hair cells, by using a molecular approach is still challenging. However, prestin is an OHC-specific motor protein and it is not

expressed in other types of hair cells [12]. Considering that partial inhibition of Hedgehog signaling causes ectopic vestibular hair cell-like cells in the cochlea [16], this signal pathway may have important roles in the differentiation of cochlear hair cells.

Both cochlear and vestibular hair cells are surrounded and mechanically supported by a number of supporting cells. In the cochlea, some supporting cells form rigid scaffolding surrounding the hair cells. Supporting cells separate the apical surface of IHCs and the entire cell bodies of OHCs from adjacent hair cells.

High-potassium environment must be established and maintained to induce depolarization of hair cells subsequent to the opening of MET channels. To maintain this environment, tight junctions are used in the cochlea. Tight junctions are present at the apical circumference of epithelial cells, and they effectively seal all cells within an epithelium together. In the cochlea, extensive tight junctions are formed between the apical surface of hair cells and supporting cells so that potassium ion can be kept from the basolateral sides of the hair cells. The molecular components of tight junctions in the cochlea included various types of claudins [17], some of which are causative molecule of hereditary hearing loss.

3.3 Arrangement and Planar Cell Polarity

As already described, cochlear hair cells are arranged as a single row of IHCs and three rows of OHCs. Once the arrangement is disrupted, hearing function of the cochlea is lost [16]. The organization of the hair cells in a precise arrangement is dependent on several molecular mechanisms. One of the mechanisms is Notch signaling [18, 19] that controls the cell fate determination in various organs. When Notch signaling is inhibited genetically [18, 19] or pharmacologically [20], the number of IHCs and OHCs increased at the expense of the number of supporting cells. This indicates that the strict cell fate determination between hair and supporting cells by Notch signaling determinates the arrangement of IHCs and OHCs. The arrangement of cochlear hair cells is also controlled by the elongation force of the cochlea. When extension of the cochlea is inhibited by pharmacological or genetic disruption of non-muscle myosin II, which controls the convergence and extensions of organs, the arrangement of cochlear hair cells is severely disrupted [21].

In addition to hair cell arrangement, the polarity of hair cells is tightly controlled. One example of this highly regulated process is the arrangement of stereocilia. The direction of the stereocilia is always stable and the vertex of the U or W is always directed toward the lateral walls (abneural direction) of the cochlea. The regulation of such polarity within a two-dimensional surface is called planar cell polarity (PCP). PCP was originally investigated using the orientation of the hair on the cuticle of Drosophila and several genes were identified to be involved in PCP. Disruption of PCP-related gene homolog in mammals induced disorientation of stereocilia [22].

3.4 Regeneration of Hair Cells

As described in this chapter, hair cells are highly differentiated and, as a result, they have many specialized characteristics in their function, morphology, and innervation. As a target of regenerative medicine, hair cells are challenging cells because of these highly differentiated characteristics. It is easy to characterize mature hair cells by their specific morphology, physiological characteristics, or several specific markers. However, to regenerate functional hair cells after their injury, supporting cells and innervation from afferent and efferent fibers as well as hair cells themselves must be regenerated and integrated as a functional organ. Moreover, in 1967, Ruben reported that mammalian cochlear hair cells stop their proliferation during developmental stages [23]. This suggests that mammalian cochlear hair cells never regenerate physiologically after birth because of the lack of stem cell population that usually proliferates slowly. It is important to describe how hair cells regenerate in the avian species that can regenerate hair cells continuously in the vestibular organs and after injury in the auditory organs. In the regeneration of avian hair cells, two mechanisms occur and supporting cells are the source of the regenerated hair cells in both mechanisms. One mechanism involves the proliferation of supporting cells [24, 25] and the other involves the transdifferentiation from supporting cells [26].

To overcome the limited regenerative ability of mammalian hair cells, induction of proliferation and/or transdifferentiation in the mammalian inner ears or transplantation of stem cells that are able to transform to any type of cells in the body is necessary. Induction of proliferation and transdifferentiation may result in the efficient regeneration of hair cells because this approach does not require the in vitro preparation of stem cells that sometimes take a long time. Both induction of proliferation and transdifferentiation have already been tried in neonatal mice. In these studies, growth factors or cell signaling pathways that play pivotal roles in the development of hair cells were used or manipulated. The induction of proliferation to repair mammalian hair cells was achieved with insulin-like growth factor 1 (IGF-1) [27]. Inhibition of Notch signaling with either genetic or pharmacological methods induced transdifferentiation of supporting cells into hair cells in the mammalian cochlea [28]. Although both experiments used neonatal cochlear organotypic cultures, these results suggest the possibility of hair cell regeneration even in the postnatal mammalian inner ears.

Pluripotent stem cells that can differentiate into any organs in the body are appropriate source of transplantation. Especially, induced pluripotent stem (iPS) cells are the preferable candidates because iPS cells can be established from the recipient [29, 30] and theoretically have no rejection reaction. Induction of hair cells from pluripotent stem cells has already been reported [31]. In this study, several hair cell marker-positive cells were successfully induced and these cells had stereociliary hair bundles, tip-links, and mechano-electrical transduction, indicating that highly differentiated hair cell-like cells were induced. However, their induction methods have two limitations. First, the authors used chicken utricle stromal cells to

promote the differentiation into hair cells, where the effective factors are uncertain. Second, the efficiency of hair cell induction was low. About 0.3 % of plated cells were induced to hair cells, indicating induced cells do not have enough numbers to form tight junctions or receive innervation from neurons. Another group also succeeded in the stepwise induction of vestibular-like epithelia from embryonic stem (ES) cells by using defined factors [32]. They induced neurons as well as sensory epithelia, and the epithelia had innervation from the induced neurons.

3.5 Conclusion

Regeneration of mammalian hair cells is still challenging because of the highly differentiated property of hair cells and specific interaction of hair cells with surrounding cells. Recent advancement of stem cell biology, developmental biology, and molecular biology is expected to enable the researchers in this field to overcome this issue.

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Chapter 4
Stereocilia

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Abstract The inner ear hair cells of mammals are remarkably sensitive mechanoreceptors, able to detect deflections of one atomic width. The root of this sensitivity lies in the stereocilia, apical specializations of the hair cells of protruded and bundled actin filaments. Stereocilia transduce sound stimuli into receptor potentials that are carried by the auditory nerve to the CNS. Almost half of all sensorineural deafness is caused by defects in the hair bundles. In this chapter we describe the molecular mechanisms underlying development and function of hair bundles. By exploiting this developmental knowledge it may be possible to generate hair cell-like cells and thus provide an alternative approach to regeneration-based cell therapies.

Keywords MET channel • Rootlets • Stereocilia • Tip links

4.1 Development of Hair Bundles and Stereocilia

Stereocilia is a specifically developed microvilli that bundles actin filament and forms protruded hair bundles on the apical membrane of each hair cell. Mammalian hair bundle morphogenesis commences during mid-gestation period of embryogenesis, in the mouse between E12.5 and E17.5 and in humans from the 7th/8th week, with vestibular hair cells beginning their differentiation before the hair cells of the cochlea [1]. Hair cell progenitors, like almost all other epithelial cells, have a

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microtubule-based cilium and actin-based microvilli on its apical surface. In this respect, the formation of hair bundles can be viewed as a modification and elaboration of preexisting subcellular structures [2, 3] and that one way to look at the developmental cues for hair cell specialization is that they put into place the machinery that allows the modification of the preexisting cilium and microvilli into the specialized kinocilium and stereocilia, respectively. The first evidence of specialization is the lengthening of the cilium, located at this point at the center of the cell [4, 5] to form the kinocilium. The kinocilium, at least in the mammalian cochlea, is a transient structure; however its function is critical for the morphogenesis of the hair bundle. The kinocilium moves to the cell periphery with the planar cell polarity (PCP) pathway coordinating the shift of kinocilia and ensuring that all hair cells are in register and face the same direction. This pathway makes use of core "PCP" signaling molecules such as Celsr1, dishevelled (Dvl), frizzled (Fzd), scribble (Scrb1), and Vangl2 [6–11]. The kinocilium also induces the adjacent microvilli to increase in height with the tallest immediately adjacent to the kinocilium, such that the stereocilia adopt a staircase configuration [5, 12]. The kinocilium is a microtubule-based cilium and as such has a slightly divergent microtubule cytoskeletal configuration (or axoneme). Close to the basal body nine doublets without a central pair of microtubules, typical of nonmotile cilia, are observed. More distally, a configuration consisting of eight doublets surrounding a central doublet is observed. Although the kinocilium regressed in the mammalian cochlea, vestibular hair cells retain them throughout life. Vestibular kinocilia act, in some cases, as attachment for the otolithic structures [13].

The transformation of microvilli in the stereocilia rods is characterized by an increase in both width and length, although the stereocilia are tapered, becoming thinner at the most proximal point, at the attachment to the hair cell proper. In addition apical membranous actin, that underlies the apical face of the hair cell, thickens to form the cuticular plate. The microvilli–stereocilia transformation requires specific proteins, such as espin, fimbrin, and fascin [14–20], to alter the properties of the actin filaments. In addition, the most centrally located actin filaments extend basally into the cuticular plate, forming rootlets. TRIOBPs were introduced as an actin-bundling protein associated with human hereditary deafness DFNB28 [21]. TRIOBPs are localizing to "rootlets" and provide the durability and rigidity for normal mechanosensitivity of stereocilia [22, 23].

The F-actin filaments in the stereocilia are polarized with their plus (barbed) end located at the tip of each stereocilium. The cytoskeletal core of the stereocilium had been thought be quite dynamic, with rapid turnover [24, 25], but recent evidence indicates a static and stable structure [26].

4.2 Functional Structure of Stereocilia

Depending on the species, the nature of the sensory organ, and the position with the sensory organ, the stereocilia of hair cells can vary in length, width, and number [27]. For example, even within the cochlear organ, stereocilia show a V or W shape

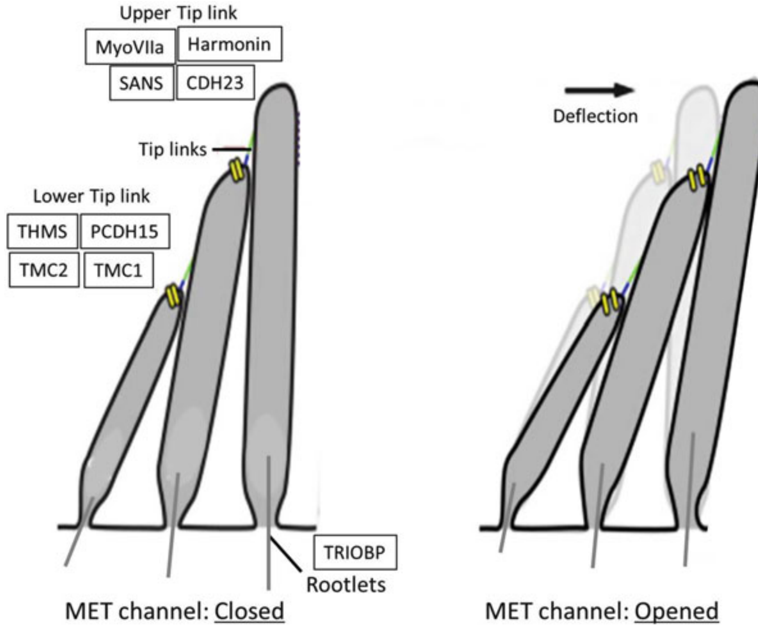

Fig. 4.1 Main molecules for the functional stereocilia. CDH23: cadherin 23, PCDH15: protocadherin 15, TMC: transmembrane channel-like protein (Modified from [36])

in outer hair cells and a flatter curved profile in inner hair cells. As for vestibular hair cells, brush-shaped stereocilia are seen, which possess taller and thicker bundles and a thicker cuticular plate in type I cells compared with type II cells [14, 28]. Despite the different hair bundle profiles, polarization of the stereocilia is generally seen in hair cells of the inner ear [12]. The kinocilia of hair cells in the otoliths are not oriented in a consistent direction, which point toward (in the utricle) or away from (in the saccule) a midline structure known as the striola [29].

Stereocilia possess a key role in mechanoelectrical transduction (MET), cochlear amplification, frequency selectivity, adaptation, and tuning. As shown in Fig. 4.1, MET channels are located near to the top of the second and third stereocilia row, rather than the larger first row [30]. The stereociliary tip links contain the gate cation channels, which open during deflection of the hair bundle and allow potassium and calcium to enter via these channels [31]. Recent findings suggest that MET channel is composed of transmembrane channel-like (TMC) proteins 1 and 2, but their precise function is still questionable and controversial [32–36]. There are a number of different links in stereocilia, tip links, lateral links, and ankle links [37, 38]. The distribution of links within the hair bundle and the types of link present can vary according to hair cell type and position within a given sensory organ, in the same way that the shape and form of the hair bundle can vary from one organ to another and within the same organ. During maturation of stereocilia, rootlets insert into the cuticular plate and adopt a tapered shape, enhancing the

rigidity of the structure to withstand the continuous deflection [15]. In addition, hair bundle polarity is an essential element of normal hair cell function [39].

MET channel is the essential component for the stereociliary function; however, "links," "PCP," and "rootlets" are also the indispensable components for the development, maturation, and function of the stereocilia.

4.3 Molecules for Functional Stereocilia

Many of the molecules that comprise the "tip link" are associated with Usher syndrome [37, 40]. Mouse models for Usher syndrome show perturbed stereocilia, mis-positioned kinocilium, and fragmented bundles with abnormal numbers, shapes, and orientation [41, 42]. The Usher type 1 proteins (cadherin 23 and protocadherin 15) are observed in the stereocilia tips with transient lateral links along stereocilia as early as E16.5 [41, 43–45]. The second type of link, ankle links, is formed [43] at birth and the Usher type 2 proteins (VLGR1, whirlin, and usherin) localize to these links [46–50]. As described before, the "tip link" itself is composed of homodimers of cadherin 23, bound at its carboxy-terminal ends to the upper tip link density (UTLD) and protocadherin 15, bound at its carboxy-terminal ends to the lower tip link density (LTLD). The two homodimers interact at their amino-terminal ends forming the structural link between adjacent stereocilia and between the tallest stereocilia and the kinocilium [51, 52] (Fig. 4.1). Myosin VIIA, SANS, and harmonin-b localize to the UTLD of mature hair cells and anchor the cadherin23 homodimer to the actin cytoskeleton necessary for the sensitivity of hair bundles to mechanical stimulation [53, 54]. In contrast to the well-known UTLD assembly, the molecular composition of the lower tip link density is less characterized. The cytoplasmic domain of protocadherin 15 is anchored to the LTLD, where it interacts with the tetraspanin TMHS. TMHS mutant mice are deaf, and fast adaptation is abolished. It has been proposed that TMHS may facilitate both the transport and assembly of the, as of yet unidentified, mechanotransduction channel which would be functionally coupled to the tip link apparatus via protocadherin 15 interaction [55, 56]. Although molecular assembly of the tip link and its connection to the actin cytoskeleton has been characterized step-by-step, its clear role in mechanotransduction is under question. Interestingly whirlin plays a role for the localization of both usherin and VLGR1 and for the stereocilia formation. Thus, some of the Usher proteins function to not only assemble the links but also transport link molecules and maintain the integrity of the links. Elucidating the exact roles of the Usher proteins may prove to be more difficult though. The protein themselves are large and exist as a number of isoforms [57–59]. Thus, for functional characterization, each isoform should be analyzed separately which necessitates the generation of isoform-specific reagents, such as mutants, antibodies, and over-expression models. Such elucidation is necessary if we want to recreate the machinery that generates stereocilia in cells.

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Chapter 5
Cochlear Lateral Wall

Takayuki Okano

Abstract The mammalian cochlea is the primary auditory sense organ which converts mechanical sound energy to electrical signals conducted by the nervous systems. The cochlear lateral wall, located laterally to the cochlear sensory epithelium in the cochlear duct, contributes auditory function and maintenance of homeostasis in the cochlear fluid through generation of endocochlear potential and K^+ recycling from perilymph to endolymph. Although our understanding of the basic mechanisms underlying auditory processing in the cochlea has increased significantly in the last two decades, the structure and function of the cochlear lateral wall seems to have been less appreciated during those periods. This chapter will focus on the cochlear lateral wall in terms of potential as a target for regeneration in particular. First, we will discuss the anatomy of the lateral wall reviewing the recent advance in this field. Next, we will discuss the function of the lateral wall, which is basically demonstrated through the advance of physiological studies, and then we will move on to the molecular basis of the cochlear lateral wall. Finally, we will discuss pathology of the lateral wall and possible strategies for hearing disorder caused by dysfunction of the lateral wall.

Keywords Endocochlear potential • Hereditary hearing loss • Ion recycling • Spiral ligament • Stria vascularis

5.1 Introduction

The mammalian cochlea is the primary auditory sense organ which converts mechanical sound energy to electrical signals conducted by the nervous systems. The cochlea comprises three sectioned tubular spaces filled with fluid: the scala

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vestibuli, scala media, and scala tympani. The scala vestibuli and scala tympani contain perilymph with high sodium and low potassium, whose composition is similar to that of ordinary extracellular fluid, and the scala media, on the other hand, is filled with endolymph with high potassium and low sodium. The cochlear endolymph holds an endocochlear potential (EP) of approximately +80 mV relative to blood plasma and perilymph. The existence of EP and specific ion composition is a distinct characteristic of cochlear endolymph, which is not observed in the extracellular fluids of any other organs in the mammal [1]. The epithelium and connective tissue set just laterally adjacent to the scala media is referred to as the cochlear lateral wall. The cochlear lateral wall is formed by a thick epithelium, the stria vascularis, covering the spiral ligament that comprises fibrocytes and epithelial cells, and a variety of types of cells organize both structures in a finely tuned way of cellular arrangement. The stria vascularis usually covers the whole lateral wall between the spiral prominence and the origin of Reissner's membrane and is a complex epithelium with numerous blood capillaries within it.

From a historical viewpoint, the plate from Reissner's 1854 publication illustrates the partitioning of the cochlea [2], suggesting that anatomists in that era would be aware of existence of three fluid-filled spaces in the cochlea. Early anatomists in the second half of the nineteenth century described in detail in their publication the cochlear sensory epithelium, the spiral ganglion, and the structure around sensory epithelium and neurons. Eponyms in the cochlea are still used in the field of basic and clinical science in modern era, as shown in the terminology, for example, Deiters, Hensen, Claudius, Rosenthal, Reissner, Nuel, and so on. However, little seems to have been known about the cochlear lateral wall at that time. In fact, the anatomy of the lateral wall has been revealed through electron microscopy in the 1950s, which is almost a hundred years later since the discovery of the organ of Corti. The early literature on the morphology of the stria vascularis by Nachlas and Lurie [3] and Johnson and Spoendlin [4] has shown morphological evidence which supports the theory that production of endolymph occurs in the stria vascularis. The stria vascularis was also believed to be a source of the positive endolymphatic potential.

Although our understanding of the basic mechanisms underlying auditory processing in the cochlea has increased significantly in the last two decades, the structure and function of the cochlear lateral wall seems to have been less appreciated during those periods. By comparison with the organ of Corti and spiral ganglion, relatively little is known about the roles and function of the cochlear lateral wall. This chapter will focus on the cochlear lateral wall in terms of potential as a target for regeneration in particular. First, we will discuss the anatomy of the lateral wall reviewing the recent advance in this field. Next, we will discuss the function of the lateral wall, which is basically demonstrated through the advance of physiological studies, and then we will move on to the molecular basis of the cochlear lateral wall. Finally, we will discuss pathology of the lateral wall and possible strategies for hearing disorder caused by dysfunction of the lateral wall. The authors would like to apologize in advance to any colleague whose work was not discussed or cited as a result of space limitation.

5.2 Anatomy of Lateral Wall

The lateral wall, consisting of the medially located stria vascularis and the laterally located spiral ligament, defines the lateral aspect of the scala media. Anatomy of the cochlear lateral wall has been intensively studied by immunohistochemistry and electron microscopy in the 1970s and thereafter. As mentioned above, the cochlea contains two segregated compartment filled with two different compositions of extracellular fluid. While the apex of hair cell faces the endolymphatic space, its lateral side of the cell body and basolateral surface are bathed in perilymph (Fig. 5.1). Acoustically evoked receptor potential is generated by the influx of potassium ions (K^+) from endolymph into hair cells. These K^+ are secreted basolaterally to the extracellular space and picked up by supporting cells. K^+ are transported from supporting cells to the stria vascularis through the cochlear sensory epithelium and the cochlear lateral wall. The marginal cells in the stria vascularis take up K^+ and release them back into the endolymph. Regarding ion transporting, cells in the cochlear duct are divided into two systems, epithelial cell gap junction system and connective tissue cell gap junction system, which are connected via intercellular junctions for electrical and ionic coupling among cells in each system. Gap junction channels connect the cytoplasm of adjacent cells, allowing the diffusion of ions and small molecules, which constitutes one of the most important pathways for intercellular communication. These gap junction systems play a key role in the maintenance of ionic and metabolic homeostasis in the cochlea. In the mature mammalian cochlea, five connexin isoforms have been

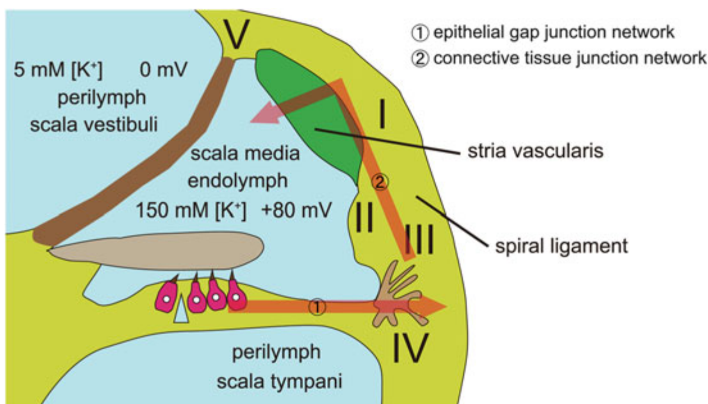

Fig. 5.1 Cochlear lateral wall and potassium ion recycling. The ionic composition with high potassium concentration and endocochlear potential are maintained by ion transport through the cochlear lateral wall. Potassium ion (K^+) influx from endolymph into hair cell is evoked by sound stimuli, followed by secretion of ion to extracellular space in the organ of Corti. K^+ are transferred through the epithelial gap junction network to the cochlear lateral wall, where the connective tissue gap junction network and the stria vascularis recycle and secrete K^+ into endolymph in the scala media. The area of each type of fibrocytes in the spiral ligament is indicated by I–V.

detected, connexin (Cx) 26, Cx29, Cx30, Cx31, and Cx43 [5, 6], which are associated with non-syndromic or syndromic deafness in human. Cx26 and Cx30 are present in all cells constituting the epithelial and connective tissue cell gap junction systems. Recent studies reported that Cx26 and Cx30 are differentially distributed within the non-sensory cells in the organ of Corti, suggesting unique roles of each Cx subtype [7, 8]. Cx31 is confined to the spiral limbus and fibrocytes in the spiral ligament and suprastrial zone and to the auditory nerve [9]. Cx29 is expressed in the Schwann cells surrounding the nerve fibers of spiral ganglion neurons [10, 11]. Cx43 is restricted to cells in the cochlear bony wall and the otic capsule [12, 13].

The fibrocytes in the spiral ligament form a network of ion-transporting connective tissue cell gap junction system that facilitates recycling of K^+ from perilymph back to endolymph. Spiral ligament fibrocytes are classified into five types according to characteristics in morphology and immunohistochemistry [14]. Type I fibrocytes are distributed between the stria vascularis and the bony wall of the cochlea and stained for carbonic anhydrase (CA) isozymes II and III and CK isozyme BB. Type I fibrocytes have few cellular processes and contained few cellular organelles in contrast to type II fibrocytes which possessed abundant mitochondria. Type II fibrocytes lie under the outer sulcus and spiral prominence and possess abundant cellular processes. Type II fibrocytes express ion transporters including Na^+,K^+-ATPase and Na^+,K^+,2Cl^- cotransporter, suggesting that type II fibrocytes are involved in regulation of the solute content in the cochlear lateral wall. Type III fibrocytes are located in the area adjacent to the cochlear bony wall in the inferior region of the spiral ligament. Type III fibrocytes contained not only CA II and III and CK isozymes BB and MM but also various types of cytoskeletal filament including actin, which indicates that type III fibrocytes play roles in metabolism as well as mechanical support for the basilar membrane. Type IV fibrocytes are placed facing to the scala tympani in the inferior part of the superficial spiral ligament. Type IV fibrocytes express Na^+,K^+-ATPase, CA II and III, and CK. Type V fibrocytes reside in the suprastrial area between the scala vestibuli and the cochlear bony wall. Type V fibrocytes appear heterogenous in terms of expression of ion transporter, the more superficial having predominantly ATPase and the deeper only expressing CA; however, morphological characteristics of type V fibrocytes resemble that of type II fibrocytes. Types II, IV, and V fibrocytes function to pump K^+ from the perilymph and produce a K^+ flow to type I fibrocytes which are electrically connected to the basal cells in the stria vascularis [15].

The stria vascularis is made up of three layers of cells: marginal cells in the luminal surface to the scala media, the intermediate cells, and basal cells adjacent to the spiral ligament (Fig. 5.2). The marginal cells are a layer of polarized epithelial cells that are derived from the epithelium of the cochlear duct and form the luminal surface of the scala media. Marginal cells are abundant in cytokeratin proteins and also include several molecules associated with ionic plumps and channels. The intermediate cells, which are probably derived from the neural crest, contain melanin and are referred to as melanocytes [16]. When melanocytes are missing in the stria vascularis, EP is not generated and hearing is severely impaired [17].

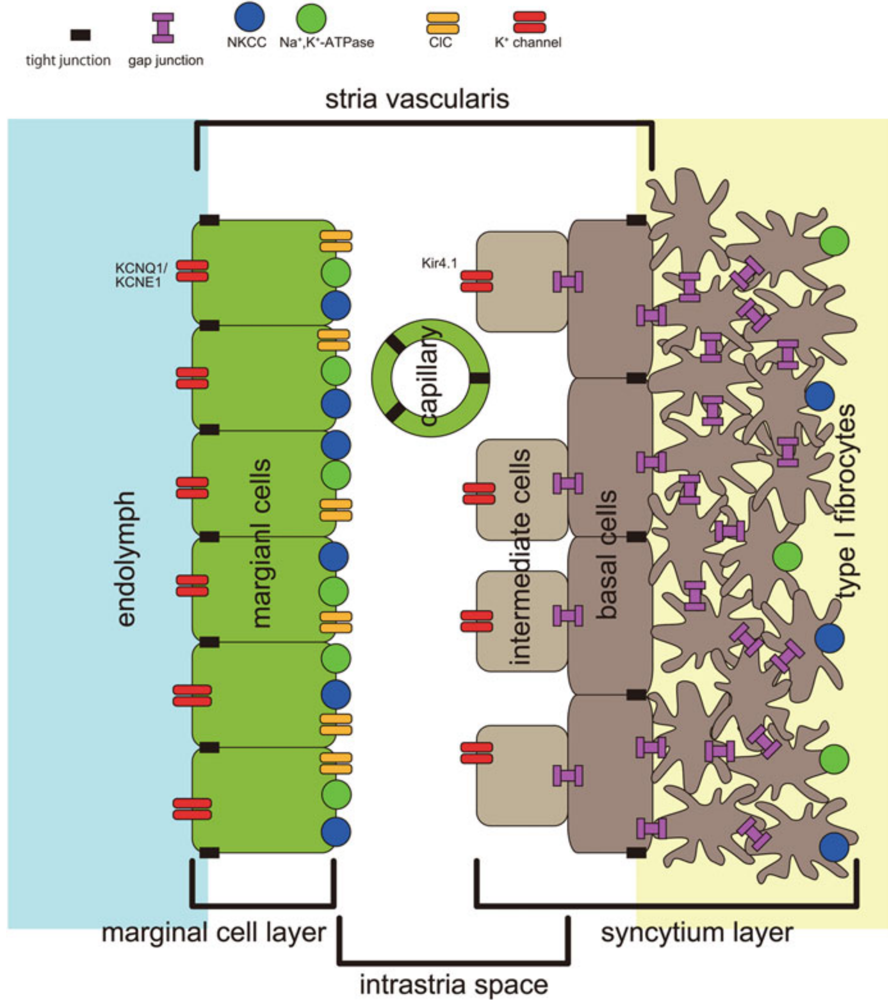

Fig. 5.2 Schematic of the two-pump model in the stria vascularis. Ion transporters and channels and three cell layers in the stria vascularis contribute generation of endocochlear potential and high potassium ion (K^+) concentration in endolymph. Intermediate cells, stria basal cells, and type I fibrocytes in the spiral ligament are electrically connected by gap junction. While stria intermediate cells express Kir4.1, K^+ channel, marginal cells express KCNQ1/KCNE1, K^+ channel, in the apical membrane. Tight junction blocks extracellular ion exchange between neighboring compartments both in the marginal cell layer and the basal cell layer. NKCC, Na^+,K^+, $2Cl^-$ cotransporter; ClC, ClC-K/Barttin-type Cl^- channel

Basal cells are located lateral to the intermediate cell layer. These flat cells form a continuous layer and a dense network of gap junction complexes with neighboring basal cells. It is not completely clear whether these cells are derived from mesodermal or neural crew origins.

At the lateral extremity of the epithelial cell gap junction system, root cells reside in partwithin the outer sulcus region. The root cells are named after characteristic fingerlike projection from their cell bodies and infiltrate between the mesenchymal fibrocytes of the spiral ligament. They display a graded variation of their gross morphological properties along the tonotopic axis of the cochlear spiral, particularly in terms of the number and size of the root processes. Also, the root cells possess the functional specialization including intercellular tight junction and gap junction [18, 19].

Melanocytes have been known to reside as intermediate cells of the stria vascularis. Intermediate cells express Na^+,K^+-ATPase and K^+ channels which are essential for production of EP. Intrastrial space is quite rich in blood vessels. The vessel wall constitutes the intrastrial fluid-blood carrier of specialized endothelial cells, which are surrounded by pericytes and melanocytes. Perivascular melanocytes in the intrastrial space maintain barrier integrity by controlling tight junction and adherence junction protein expression [20].

Bone marrow-derived cells also are widely distributed in the cochlear lateral wall [21, 22], most of which have phenotypes as tissue-resident macrophages. Specific roles of cochlear resident macrophages are largely unknown; however, macrophages play roles in protection of hair cells following application of ototoxic drugs or innate immune systems in the cochlea [23, 24]. In addition, Hirose et al. reported that bone marrow-derived cells are attracted to the cochlear sensory epithelium when the cochlea is insulted by acoustic overstimulation [25]. These observations suggest that bone marrow-derived cells play substantial roles in maintenance of cochlear function and homeostasis.

5.3 Physiology of the Cochlear Lateral Wall

The cochlea is the organ that continuously controls water and ion homeostasis for auditory perception. Like the retina, cochlea maintains continuous extracellular potential and ion gradients through transporting ions even in the absence of an acoustic stimulus, which was called “silent current” by Zidanic and Brownell [26]. In addition, these gradients are not generated by hair cells but in the stria vascularis, which benefits significantly for the detection of mechanical stimuli. As generation of EP and K^+ recycling from perilymph to endolymph are inseparably connected, we will discuss physiological roles and mechanisms of the cochlear lateral wall in terms of generation of EP.

As described above, the cochlear endolymph exhibits a positive potential of +80 mV, which is called the endocochlear potential. von Bekesy in 1952 first reported the DC potential in endolymph with a +80 mV positive potential to the perilymph [27]. Davis et al. measured the cochlear microphonic and found that the energy of the cochlear microphonic is derived from the polarization potential across the interface between the endolymph and the interior of the hair cells, which is called “battery theory.” In addition, they described that the movements of hair

bundles modulate the flow of electric current [28]. These batteries that drive auditory-evoked excitation in hair cell are comprised of electrochemical gradients across the apical surface of hair cell produced by EP. Since then, so many works have been done on the mechanism of generation of EP and high potassium concentration [29, 30]. Salt et al. found the intrastrial space with a positive potential and a low K^+ concentration through measurement of the potential and K^+ gradient in the stria vascularis using K^+-selective electrodes. The results also indicate that EP is not generated by the marginal cells alone but also by K^+ movement across the cell membrane of components in the stria vascularis [31]. The works by Salt et al. redefined the investigation for EP as research on cellular compartments and ion pumps that positively transport cation and anion across cellular membrane and intercellularly.

The two-cell model, also referred to as the five-compartment model [32], has been widely accepted as the mechanism for origin of EP [33]. In this hypothesis, the cochlear lateral wall comprises two epithelial compartments, the strial marginal cells and the syncytium that forms cellular complex of fibrocytes, strial basal cells, and strial intermediate cells. Type I fibrocytes, strial basal cells, and strial intermediate cells are interconnected by gap junction network, which means these cells are electrically equivalent. Type I fibrocytes in the cochlear lateral wall express both Na^+,K^+-ATPase and Na^+,K^+,$2Cl^-$ cotransporter. Vascular perfusion of either ouabain, an inhibitor for Na^+,K^+-ATPase, or furosemide, an inhibitor for Na^+,K^+,$2Cl^-$ cotransporter, dramatically reduces EP [34, 35], indicating that these two ion transporters are required for generation of EP. The strial intermediate cells express Kir 4.1 [36] which secretes K^+ into the intrastrial space. Vascular perfusion of Ba^{2+}, a potential blocker for the Kir family, remarkably suppresses EP [37], suggesting that the K^+ diffusion potential is primarily formed by Kir4.1 and critical for generation of EP. Tight junctions between n the basal cells make the syncytium a diffusional barrier and serve as the boundary of the apical surface composed of intermediate cell membranes and the basolateral surface comprising the fibrocyte membranes. Mice lacking Claudin11, a major constituent of tight junctions of basal cells, exhibit disruption of EP despite normal K^+ recycling, indicating the importance of basal cell tight junctions in the stria vascularis for generation of EP [38, 39]. The results also support two-cell model that EP is not generated by the marginal cells alone but the intrastrial space contributes generation of endocochlear potential. Expression of Na^+,K^+-ATPase and Na^+,K^+,$2Cl^-$ cotransporter in the basolateral membrane of the strial marginal cells sustains a low K^+ concentration in the extracellular fluid in the intrastrial space. Chloride ions exit from the marginal cells through ClC-Kb chloride channels, Barttin, which is supposed to be essential for K^+ recycling in the stria vascularis [40]. KCNq1/KCNE1 K^+ channels which are expressed in the apical surface of the marginal cells secrete K^+ into the endolymph contributing a K^+ diffusion potential and the dynamics of EP [41]. Whereas the basal cells in the stria vascularis express only Claudin11, the marginal cells express a variety of Claudin family including Claudin1, 2, 3, 8, 9, 10, 12, 14, and 18, suggesting importance of the barrier formation in the marginal cells [42]. Researches in the field of physiology and molecular biology have made

substantial advance to complete understanding for roles and mechanisms of the cochlear lateral wall. In the next section, we will discuss potential molecular targets for regenerative medicine in the cochlear lateral wall identified in either humans and mice or both.

5.4 Molecular Basis of the Function of Cochlear Lateral Wall

Several genes that are involved in ion transporting are reported to account for hereditary hearing loss. Mutations in GJB2 encoding connexin 26 are the most prevalent inherited source of congenital hearing loss in humans [43]. In most of these cases, the inheritance type is autosomal recessive; however, some cases of dominant inheritance are also reported. In *Gjb2* knockout mice, degeneration of the organ of Corti is observed as early as postnatal day (P)14 and their hearing are profoundly impaired [44]. Endolymphatic K^+ concentration and EP are much lower in *Gjb2* knockout mice than wild type, suggesting that Cx26 is essential for maintenance and function of the organ of Corti, but is not required for normal development of the cochlear sensory epithelium. A *Gjb6* knockout mouse model was also developed, and homozygous mice are hearing impaired and lack EP. Degeneration of the organ of Corti is observed from P18, which is similar to *Gjb2* mutant mice [45].

The SLC26 family is responsible for membranous transporting of anion, including chloride, iodide, and bicarbonate. SLC26A4 mutations are associated with syndromic hearing loss, Pendred syndrome, and non-syndromic hereditary hearing loss [46]. Most of the patients display radiologically detectable structural malformations of the inner ear, the most common feature of which is an enlarged vestibular aqueduct. Enlarged endolymphatic ducts are also observed in some patients with non-syndromic hereditary hearing loss due to *SLC26A4* mutations. In the mouse inner ear, Slc26a4 is expressed on apical surface of cells covered by the endolymphatic space [47]. *Slc26a4* knockout mice exhibit waltzer-like vestibular dysfunction and complete deafness with a severe dilatation of the endolymphatic duct and sac [48]. Functional analyses revealed that *Slc26a4* knockout mice gradually loss EP, beginning at P12, before normal onset of hearing despite normal concentration of K^+ in the endolymph.

In the cochlea, the essential segregation of endolymph from perilymph is achieved by tight junctions of epithelial cells bordering the fluid compartments. Tight junctions are composed of at least three types of transmembrane proteins: occludin, claudins, and the junction adhesion molecule family. Claudin proteins are widely expressed in the inner ear, with 10 types of claudin proteins of differential distribution or localization [42]. Recessive mutations of human *CLDN14* were identified as a source of non-syndromic hearing loss [49]. Claudin14 is detected in tight junctions of the reticular lamina in the organ of Corti in mice, and

Claudin14-null mice are deaf, whereas their EP stays normal [50]. Tight junctions in the basal cells of stria vascularis are primarily composed of Claudin11, and *Claudin11*-deficient mice show severe hearing loss without obvious degeneration of the organ of Corti [39]. In addition, EP in *Claudin11*-deficient mice is suppressed, while K^+ concentration in the endolymph is maintained at almost normal level. These findings indicate that establishment of the basal cell barrier in the stria vascularis is indispensable for hearing function through generation and maintenance of EP.

Kcne1, Kcnq1, and Kcnq4 encode for subunits of low-voltage-activated K^+ channel, which are the major determinants of cellular depolarization in excitable cells. Stria vascularis marginal cells secrete K^+ into the endolymph by K^+ channel composed of Kcnq1 and Kcne1 subunits. Mutations in *KCNE1* or *KCNQ1* in human induce syndromic hearing loss with cardiac symptoms, including prolonged QT interval in the electrocardiogram and arrhythmias (Jervell and Lange-Nielsen syndrome) [51–53]. *Kcne1* or *Kcnq1* knockout mice exhibit severe hearing loss and vestibular dysfunction [54, 55]. Although morphology of the cochlear duct is likely to be normal at birth, degeneration develops later after birth including a collapse of Reissner's membrane and a decrease in the volume of endolymphatic space. Kcnq4 is detected in the basolateral membrane of cochlear hair cells, suggesting that Kcnq4 channels are responsible for secretion of K^+ from hair cells to the perilymph [56].

Kir4.1, or KCNJ10, is expressed in the intermediate cells of stria vascularis [57]. K^+ concentration as well as EP and volume of the endolymphatic space is reduced in *Kcnj10* knockout mice, suggesting that the Kir4.1 channel provides the molecular mechanism for generation of EP in concert with other channels for K^+ secretion, as is indicated by the fact that *Slc26a4* knockout mice lack Kir4.1 expression in the stria vascularis [58].

In human, Bartter syndrome IV is an autosomal recessive disorder characterized by congenital deafness and severe renal salt and fluid loss, which is caused by mutations in Barttin, a beta-subunit of ClC-Ka and ClC-Kb chloride channels [59]. NKCC, Na^+,K^+,$2Cl^-$ cotransporter, and ClC, Barttin-type Cl^- channel are expressed in the basolateral membrane of the stria marginal cells. Barttin-knockout mice demonstrate severe hearing loss with a decrease of EP despite normal concentration of K^+ in the endolymph [60]. These observations indicate that Cl^- transport in the stria vascularis is also involved in the formation of EP.

Finally, DFN3, an X chromosome-linked non-syndromic mixed deafness, is caused by mutations in the POU3F4 gene, which encodes a POU transcription factor [61, 62]. *Pou3f4*-deficient mice were created and found to exhibit profound deafness with a dramatic reduction in EP. Histological analyses demonstrated a hypoplasia of fibrocytes in the cochlear lateral wall. The findings suggest that fibrocytes responsible for K^+ homeostasis in the lateral wall play a critical role in generating EP and auditory function as well. Taken together, these molecules described above could be potential targets for regenerative medicine to improve hearing in disorders of the cochlear lateral wall through regenerative therapy.

5.5 Targets of Regenerative Medicine

In human, some types of hearing impairment are clinically suggested to be caused by disorder or damage of the cochlear lateral wall. The most widely referenced scheme for describing age-related hearing loss is one attributed to Schuknecht, in which three major cochlear structures, afferent neuron, organ of Corti, and stria vascularis, can degenerate independently [63, 64]. In addition, an age-related loss of fibrocytes in the spiral ligament has been reported in the basal portion of the mouse cochlea [65]. Moreover, Ohlemiller et al. reported that degeneration of the strial intermediate cells plays essential roles in age-related hearing loss through providing melanin to both the marginal cells and the basal cells [66]. Taken together, the cochlear lateral wall is very likely to be affected in the pathology of presbycusis according to the data shown above.

Pathological changes in fibrocytes have been linked to noise-induced hearing loss as well as age-related hearing loss, where fibrocyte degeneration was shown to precede loss of hair cells and neurons [65]. Widespread degeneration of fibrocytes in the spiral ligament, especially among the type IV fibrocyte areas, was observed in aged mouse cochlea prior to hair cell loss or degeneration of spiral ganglion neurons, suggesting that pathological changes in the fibrocytes in the cochlear lateral wall might be responsible for hair cell degeneration in age-related hearing loss.

Sudden deafness, or sudden-onset unilateral hearing loss due to unknown etiology, is suggested to be caused, at least in part, by damage of the lateral wall. Whereas some studies focused on degeneration of the spiral ganglion or the organ of Corti in models of transient cochlear ischemia [67], recent studies have revealed that the cochlear lateral wall is also affected by cochlear ischemia or energy failure, which seems to be reasonable as vasculature and mitochondrial activity are abundantly distributed in the cochlear lateral wall. In a mouse model of acute mitochondrial dysfunction caused by application of 3-nitropropionic acid, cellular degeneration in the cochlear lateral wall primarily accounts for hearing impairment due to acute energy failure [68]. These clinical entities could be candidate diseases for regenerative medicine in the cochlear lateral wall, whereas much works should be done to elucidate the pathophysiology of each clinical condition in the future.

5.6 Conclusion

In this chapter, we presented a brief review on the cochlear lateral wall from the viewpoint of regenerative medicine. Recent progress in the field of research in the cochlear lateral wall in the last two decades has made our knowledge on the lateral wall biology remarkably improved. At the same time, we recognize that we are still at an early stage in our understanding of the molecular biology and physiology of the cochlear lateral wall. It would depend on further studies in the future to unveil

the precise roles and mechanisms of the lateral wall under normal and affected conditions and develop translational researches to connect basic biology to regenerative medicine.

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Chapter 6
Spiral Ganglion Cell and Auditory Neuron

Tetsuji Sekiya and Harukazu Hiraumi

Abstract Auditory nerve is a bundle of bipolar auditory neurons forming synapses peripherally with hair cells and centrally with cochlear nucleus cells. Spiral ganglion cells are the cell bodies of auditory neurons. The auditory neurons commonly degenerate both when hair cells are damaged initially and when axons of auditory nerve are injured primarily. Auditory neuropathic type auditory nerve degeneration, selective auditory neuronal degeneration with sparing of hair cells, exists much more than once thought. This pathological situation may become most suitable for cell transplantation intervention because survived hair cells can provide trophic factors to donor cells. The interface between the PNS (peripheral nervous system) and the CNS (central nervous system) portions is called the transitional zone (TZ). TZ may come to be a barrier against cell migration and centrally growing neurites from the distal side of TZ are sensitive to the repellent effects of astrocytes in the CNS portion of auditory nerve. It was revealed that glial scar is induced in the auditory nerve and/or cochlea nucleus regions not only in primary but also secondary auditory nerve degenerations. As glial scar strongly inhibits neuronal regeneration, our future approach to restore hearing should include a tactics to overcome inhibitory glial scar as in CNS neurodegenerative disorders.

Keywords Auditory nerve • Glial scar • Hearing • Spiral ganglion cells • Transitional zone

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6.1 Overview

Auditory nerve is a bundle of bipolar neurons (auditory neurons) spanned from the temporal bone to the brainstem through the cerebellopontine angle cistern [1] (Fig. 6.1). Spiral ganglion cells (SGCs), cell bodies of auditory neurons, are housed in Rosenthal canals and extend dendrites peripherally and axons centrally. Centrally, the auditory axons form synapses with second-order neurons in the cochlear nuclei and the peripheral processes (dendrites) with hair cells in the cochlea. The axons, their root entry zone, and a part of cochlear nucleus region are viewed in the cerebellopontine angle cistern through a retromastoid bone window. The other portions including dendrites and the cochlea are concealed within the temporal bone (Fig. 6.1). There are two types of auditory neurons in mammals. More than

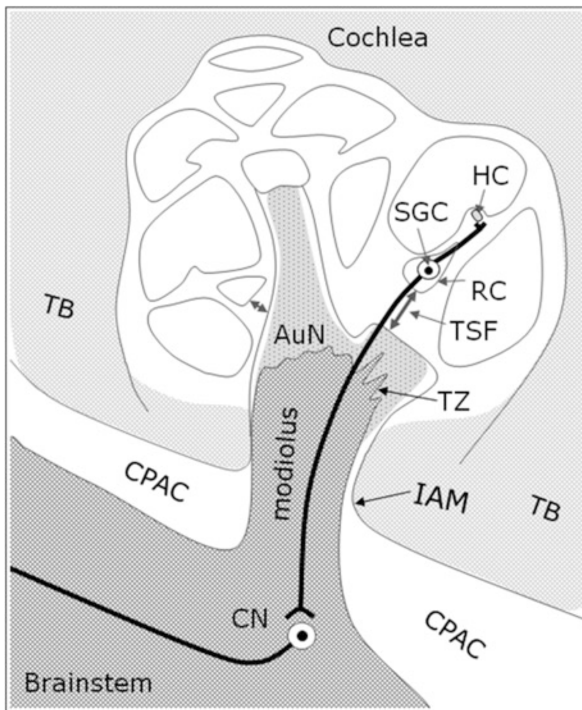

Fig. 6.1 Schematic illustration showing the anatomical relationships between the auditory nerve (AuN) and the surrounding structures. The auditory nerve is a bundle of bipolar neurons that form synaptic contacts with the hair cells peripherally and cochlear nucleus cells centrally. The cell bodies of the auditory neurons (spiral ganglion cells, SGC) are housed in the Rosenthal canal (RC). The tractus spiralis foraminosus (TSF) is an osseous canal through which the axons of the auditory nerve pass from the Rosenthal canal to the axis of the auditory nerve (modiolus). *CN* cochlear nucleus, *CPAC* cerebellopontine angle cistern, *HC* hair cell, *TB* temporal bone, *TZ* transitional zone. (From Sekiya et al. [9])

90 % of auditory neurons are classed as type I and less than 10 % are classed as type II [2]. Ten to 20 type I neurons converge on each inner hair cell. In contrast, each type II auditory neuron contacts about 30–60 outer hair cells [2]. A remarkably small number of hair cells, fewer than 16,000 in each human inner ear, generate receptor potentials that modulate spiking activity in the afferent auditory nerve fibers [3]. The auditory nerve contains efferent fibers from the olivary nucleus caudally and the dorsal nucleus of the trapezoid body rostrally to regulate the sensitivity of the inner and outer hair cells [4].

Auditory nerve that occupies one of the central places in hearing restorative medicine along with hair cells and other structures [1, 5, 6] (see Chap. 29) holds unique anatomical features in the scope of auditory nerve regeneration.

6.2 Transitional Zone (TZ) of the Auditory Nerve

The interface between the PNS (peripheral nervous system) and the CNS (central nervous system) portions is called the transitional zone (TZ) or the Obersteiner-Redlich zone [7, 8] (Fig. 6.1). Centrally from the TZ, myelin sheaths are formed by oligodendrocytes, and the supporting tissue is astrocytic. The peripherally convex shape of distal end of TZ is clearly visualized by an antibody to GFAP (glial fibrillary acidic protein) because of the presence of astrocytes only in the CNS portion of the nerve [9]. Peripherally, the sheaths are formed by Schwann cells that are enveloped in endoneurium [7]. The interface (TZ) is penetrated only by axons. The central portion of auditory nerve is exceptionally long among cranial nerves except olfactory and optic nerves [10]. This lengthy CNS portion of this nerve holds a crucial significance in investigations of hearing restoration [9, 11, 12]. Experimental results about cell migration trespassing TZ are diverse (see Fig. 29.2). A study demonstrated an occurrence of cell migration with peripheral to central direction trespassing TZ [13]. About central to peripheral cell migration trespassing TZ, the experimental results were conflicting [14, 15]. These discrepancies might have been due to the differences of donor cells, conditions of the host animals, and experimental settings. In the light of auditory nerve regeneration, however, it should be noted centrally growing neurites from distal side are sensitive to the repellent effects of astrocytes at TZ [16, 11], although peripherally growing neurites crossed the TZ [15].

6.3 Neurotrophins

BDNF (brain-derived neurotrophic factor) and NT-3 (neurotrophic factor-3) are synthesized in the hair cells and transported to the SGCs where their high-affinity receptors trkB and trkC are expressed and the survival and synaptogenesis of auditory neurons depend on these growth factors [17–19]. Auditory neurons receive

neurotrophins from several other sources, including the cochlear nucleus, and even SGCs themselves [18, 20]. Hair cells do not necessarily need the presence of auditory neurons for survival [19, 21–23]. Hair cells may become a crucial nutrient source for transplanted cells in the attempt to regenerate auditory neurons (see Chap. 29).

6.4 Target Pathologies for Auditory Nerve Replacement

When dendrites or axons of auditory neurons are damaged initially, auditory neurons degenerate but hair cells tend to be preserved to various degrees (primary auditory nerve degeneration) [5] (Fig. 6.2). In contrast, following the inappropriate use of ototoxic drugs or acoustic overstimulation, hair cells may degenerate and subsequently auditory neurons degenerate (secondary auditory nerve degeneration) (Fig. 6.2). Collectively, the auditory neurons commonly degenerate in either of

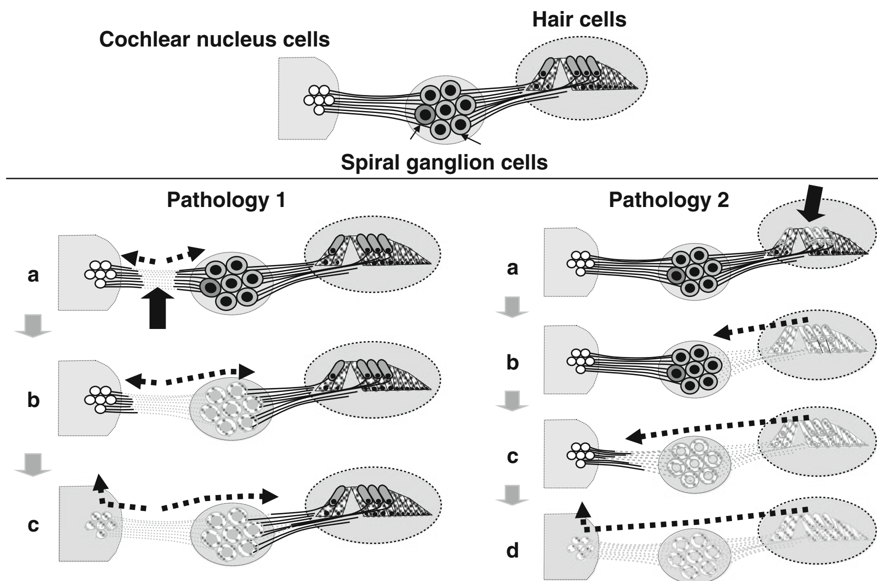

Fig. 6.2 Pathological processes occurred in deafness. In Pathology 1, the initial insult to the auditory nervous system occurs as a lesion in the dendrites or axons of the nerve (*A*, *solid arrow*). In time, the degeneration of the auditory neurons proceeds toward the cochlea and the brainstem (*B* and *C*, *dotted arrows*). In this pathology, the hair cells tend to be preserved to various degrees (*C*). In Pathology 2, the hair cells are damaged initially (*A*, *solid arrow*), and the dendrites and the auditory ganglion cells secondarily degenerate (*B*, *C*, *dotted arrows*). At the most advanced stage of this type of pathology, the degeneration of the cochlear nucleus cells might occur (*D*). The cell transplantation approach should cope with each of these pathologies properly. (From Sekiya et al. [5])

these pathological processes, indicating the auditory neurons occupy an indispensable part in reviving lost hearing.

Selective auditory neuronal degeneration with sparing of hair cells was recognized as auditory neuropathy where ABR (auditory brainstem response) was absent or profoundly distorted with otoacoustic emissions (OAE) and/or cochlear microphonics (CM) preserved [24]. Auditory neuropathic type auditory nerve degeneration is observed in various clinical disorders and elderly people [25–28]. Temporary threshold shift (TTS) has been assumed to indicate reversal damage to hair cell and auditory neurons without delayed auditory dysfunction, but a recent study demonstrated noise-induced primary auditory nerve degeneration occurred in a delayed fashion without hair cell damage in situation where TTS had been observed [29]. In small vestibular schwannomas, the hair cells are assumed to be well preserved [30–32]. Hearing preservation in vestibular schwannoma treatment still remains as an unresolved problem in neurosurgery [9]. Taken together, emerging evidence indicates auditory neuropathic type auditory nerve degenerations exist much more than once thought. This pathological situation may become most suitable for cell transplantation intervention because survived hair cells can provide trophic factors to donor cells [33, 34] (see Chap. 29).

6.5 Deafness, a Neurodegenerative Disorder

Whenever insults, such as mechanical trauma, ischemia, radiation, genetic disorders, or chemical insult, are imposed to the CNS, quiescent astrocytes resume proliferation, become hypertrophic, and upregulate GFAP and finally glial scar is formed along with the progression of neuronal degeneration [35, 36]. We demonstrated that compression of CNS portion of auditory nerve induced glial scar not only in the auditory nerve but also in the cochlear nucleus [9]. One recent report demonstrated that with hair cell damage glial scar was induced in the auditory nerve [12]. From these results, it has become apparent that glial scar is induced in the auditory nerve not only in primary but also secondary auditory nerve degenerations. Probably, glial scar formation may be more severe when the site of insult is closer to the brainstem, because we observed glial scar formation even in the cochlear nucleus region in direct compression to the CNS portion of auditory nerve [9, 37]. In every effort to restore lost auditory nerve function, glial scar in the auditory nerve and cochlear nucleus has to be overcome because glial scar is believed to strongly inhibit neural regeneration [38]. In our future investigations for hearing restoration, deafness should be regarded as a neurodegenerative disorder because it is fraught with the same glial scar problem as in other neurodegenerative diseases including spinal cord injury, Parkinson's disease, and amyotrophic lateral sclerosis (ALS).

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Chapter 7
Synaptic Contacts Between Hair Cells and Primary Neurons

Takayuki Nakagawa

Abstract Synaptic contacts between hair cells and primary neurons are included in important architectures for inner ear functions. Inner hair cells are innervated by afferent nerve fibers with characteristic morphology. Each type of inner hair cells has synaptic ribbons that are electron-dense structures at the presynaptic region. Synaptic ribbons play a crucial role in glutamate release to the afferent nerve endings. This chapter reviews basic anatomy of synaptic contacts between hair cells and primary neurons and discusses on the importance of ribbon synapses in inner hair cells of the cochlea as a therapeutic target.

Keywords Afferent nerve • Hair cell • Ribbon synapse

7.1 Overview

The inner ear consists of two sensory organs, the cochlea and vestibules. The cochlea is a sensory organ for auditory function. The vestibule has two otolith organs, utricle and saccule, and three semicircular canals. Otolith organs correspond to the magnitude and direction of linier motions, and semicircular canals detect angular movements of the head. Each sensory organ has a sensory epithelium, which contains sensory hair cells. Hair cells of the inner ear convert mechanical stimuli to neural signals. Hair cells are innervated by afferent nerve fibers of the primary neurons. Neural signals from hair cells are transmitted to afferent nerve fibers by release of neurotransmitters to synaptic clefts. Synapses between hair cells and primary neurons are crucial for the functionality of the inner ear, and their dysfunctions sometimes play a key role in inner ear diseases including sensorineural

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hearing loss and tinnitus. The afferent synapse of hair cells is characterized by an electron-dense presynaptic structure, known as the synaptic ribbon [1]. Ribbon synapses are found exclusively in sensory cells including hair cells. As function, ribbon synapses are possible to release neurotransmitters at high rates, which is crucial to sense and transmit stimuli over a broad range of stimulus intensities [2]. Regeneration of synapses between hair cells and primary neurons, in particular synaptic ribbons, is included in important therapeutic targets aiming highly functional restoration.

7.2 Cochlea

The cochlea has two types of hair cells, inner and outer hair cells. Both types of hair cells have a unique construction of afferent and efferent innervations (Fig. 7.1). The spiral ganglion neurons, primary auditory neurons, consist of two types of neurons, namely type I and II neurons. The type I neurons comprise approximately 90 % of the cochlear nerve. Most type I fibers contact only a single inner hair cell via a single terminal swelling [3], and single inner hair cells are innervated by 10 type I fibers [4]. The type II fibers contact 10–100 outer hair cells via 10–15 terminals [5]. Efferent nerve fibers originate from the superior olivary complex in the brain stem. Efferent nerve fibers project to afferent terminals or to hair cells.

Synaptic ribbons are electron-dense structures that are located at the base of hair cells and play a role as a pool of neurotransmitters [2]. From the point of view of auditory function, the synaptic contacts between the inner hair cell and type I afferent nerve fiber are particularly important (Fig. 7.1). Synaptic ribbons control release of neurotransmitters at various modes, rapid release of a large amount of neurotransmitters or slow and consistent release of a small amount of neurotransmitters depending on the intensity and duration of sound stimuli [2]. Synaptic ribbons at the base of the inner hair cells face to a single postsynaptic density at the postsynaptic membrane of type I afferent nerve terminal, of which contains large numbers of AMPA-type glutamate receptors [6]. A large number of glutamate receptors may contribute to secure from receptor saturation. Glutamate released from ribbon synapses to synaptic clefts is removed by amino acid transporters including the glutamate/aspartate transport GLAST in supporting cells [7].

7.3 Vestibule

The vestibular sensory epithelium also has two types of hair cells, type I and II hair cells. Type I and II hair cells differ in their afferent innervation patterns (Fig. 7.2). Type I hair cells are surrounded by the calyceal afferent nerve endings, and type II hair cells are innervated by bouton-type afferent nerve endings [8]. Type I hair cells have a single calyceal terminal, while type II hair cells have a number of bouton-type

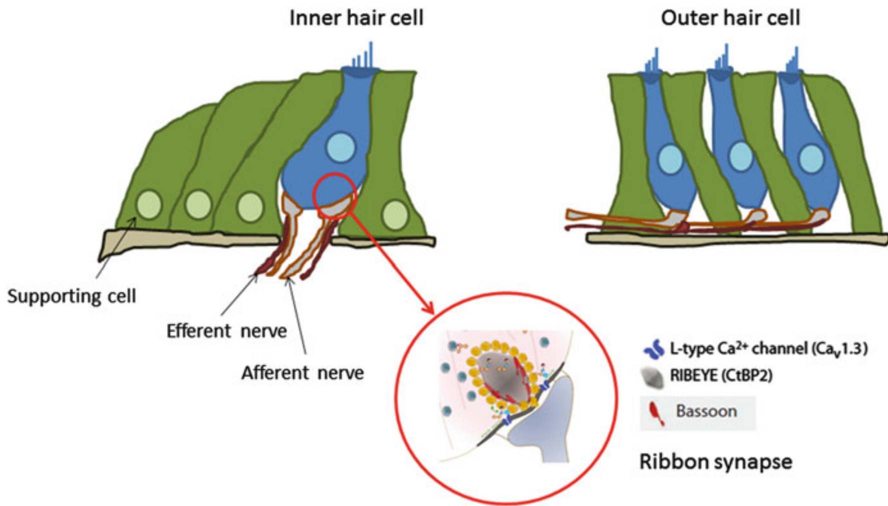

Fig. 7.1 Hair cell synapses in the cochlea [2]

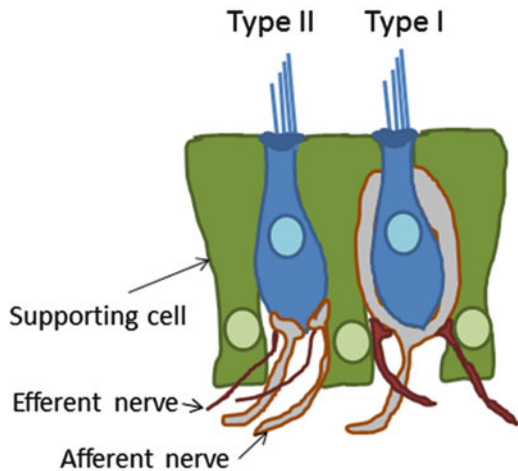

Fig. 7.2 Hair cell synapses in the vestibule

nerve endings. Also in the vestibular system, synaptic transmission between hair cells and afferent nerve fibers is mediated by ribbon synapses. The expression of AMPA-type glutamate receptors was also reported in the postsynaptic dense in afferent nerve endings in the vestibular epithelium [9]. Comparing with cochlear synapses, details of synaptic functions in vestibular hair cells have not been elucidated. Unlike mammalian cochleae, mammalian vestibular hair cells have the potential of spontaneous regeneration [10, 11]. In addition, the capacity of hair cell regeneration by gene transfer has extensively been investigated [12]. The majority of regenerated hair cells exhibited type II morphology.

7.4 Synaptic Ribbons and Disease

The dysfunction of synaptic ribbons in inner hair cells of cochleae causes hearing loss. Hearing impairment due to dysfunction of ribbon synapses showed threshold elevation of auditory brain stem response (ABR) thresholds and normal otoacoustic emissions [2]. Based on this, dysfunction of ribbon synapses is considered one of the causes for auditory neuropathy [13]. In animal experiments, the swelling of afferent dendrites of spiral ganglion neurons has well been known as one of the early events that occur in the cochlea after noise damage or exposure to excessive excitatory amino acids [14, 15]. In general, the swelling of afferent dendrites disappears within 7–14 days after the onset of noise- or drug-induced hearing loss. Therefore, this temporal degeneration at the afferent dendrites has been considered one of the causes for temporal thresholds shifts that are observed after noise exposure. In the process of hearing recovery from temporal threshold shifts, regeneration of ribbon synapses also occurred [16, 17]. Recent studies have demonstrated regeneration of ribbon synapses between afferent dendrites and inner hair cells [18, 19]. Actually, in our previous study to examine roles of prostaglandin E-type receptor 4 in mice, noise exposure after topical application of an antagonist prostaglandin E-type receptor 4 caused approximately 20 % loss of outer hair cells, but spiral ganglions and inner hair cells were well maintained [20]. Although of limited damage to outer hair cells, severe hearing loss was found in ABR measurements. In such a case, synaptic dysfunction between inner hair cells and spiral ganglion neurons might be involved. More recently, degeneration of ribbon synapses in the inner hair cells has been shown an initial change in age-related cochlear damage [21].

Proteins associated with the functionality of ribbon synapses have been reported. Myosin VI, bassoon, *vglut3*, Cav1.3, synaptotagmin 4, and otoferlin have close relation with ribbon synapse function, especially exocytosis [2]. The lack of myosin VI in mutant mice resulted in a dramatic decrease in synaptic exocytosis of inner hair cells [22]. Mouse mutants for the presynaptic scaffolding protein bassoon, which anchors synaptic ribbons at the active zone of inner hair cells, showed moderate hearing loss [23]. Inner hair cells in *vglut3*-null mice lack glutamate release, despite unaffected synaptic vesicular fusion [24]. In addition, *vglut3*-null mice showed degeneration of spiral ganglion neurons. Mutations in *vglut3* were detected in patients with auditory neuropathy [24]. Otoferlin has been proposed as a calcium ion sensor of synaptic exocytosis. In otoferlin-null mice, exocytosis of inner hair cells is defective [25]. Mutations in OTOF were also detected in patients with auditory neuropathy.

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Chapter 8
Otolith

Yosuke Tona and Akiko Taura

Abstract Otoliths or otoconia are located on the otolithic or otoconial membrane of both the utricle and the saccule. The utricle and the saccule detect linear acceleration in the horizontal and vertical planes, respectively. The otoconial membrane is divided into three layers: the subcupular meshwork, the gelatinous layer, and the otoconial layer. Otoconia are embedded in the loose filament network of the otoconial layer. Numerous proteins and genes are known to be related to otolith and otoconium formation. Otoconin 90 is the main core glycoprotein of otoconia for mammals and birds. Alpha-tectorin is needed for the normal formation of both the gelatinous layer and the otoconia as a whole. Otogelin and otoancorin provide instructions for the assembly and adhesion of the otoconial membrane proteins to the sensory epithelium. Factors affecting the structure of otoliths include drugs such as aminoglycosides, aging, and hypergravity. The interdependence of otoconia and semicircular canals is suggested, indicating the unrecognized importance of otoconia.

Keywords Otoconial membrane • Otoconins • Otolithic membrane • Saccule • Utricle

8.1 Anatomy

There are mineralized crystals on the sensory patches of the vestibular maculae, which are called otoliths in fishes and otoconia in higher vertebrates. Otoliths of fishes contain only three large mineralized crystals. In contrast, higher vertebrates have thousands of crystals, which are less than 10 μm. Otoliths or otoconia are

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located on the otolithic or otoconial membrane of both the utricle and the saccule. The utricle and the saccule are the sensors of linear acceleration in the horizontal and vertical planes, respectively. The utricle and the saccule are oriented perpendicular but never directly connected to each other. Both of them are filled with endolymph, which overflows to the endolymphatic sinus and the endolymphatic duct. The vestibule located outside the utricle and the saccule is filled with perilymph. The utricle communicates with the endolymphatic space of the three semicircular canals and is surrounded by connective tissue without bone. The saccule is anchored at the saccular macula by the spherical recess, situated at the concave of the bony labyrinth. The saccule narrows into the ductus reuniens, which opens into the cochlear duct.

The maculae of the utricle and the saccule are lined with sensory epithelium, containing among others, two types of hair cells [1]. Type I hair cells are corresponded to the inner hair cells of the organ of Corti and have a characteristic shape with a rounded bottom, thin necks, and wide heads. Type II hair cells are corresponded to the outer hair cells and have a cylindrical shape. The afferent fibers innervating macular hair cells are classified into three groups based on their peripheral terminals [2]. Calyx fibers terminate on type I hair cells, and bouton fibers, which have no calyx endings, innervate type II hair cells only. Dimorphic fibers innervate both type I and type II hair cells. The synapses formed between hair cells and afferent fibers are ribbon synapses. Studies in mutant mice revealed that resting discharge in primary afferent neurons occurs spontaneously in the absence of any external stimulation [3]. Efferent fibers rich in vesicles make synapses with both type II hair cells and afferent processes including calyces and boutons. The exposed surface of the hair cells has bundles of stereocilia and one kinocilium located at their periphery. Part of the motile cilia is covered with a gelatinous layer. The striola is a landmark that runs through the center of the macula, according to which the morphological polarity of individual hair cells relating to the kinocilia is altered [4]. At the utricular macula, the kinocilia of hair cells are facing the striola, whereas at the saccular macula, they are facing the reverse side of the striola.

The microstructure of the otoconial membrane is divided into three layers (Fig. 8.1) [5]. The subcupular meshwork (SM) is a dense reticular network of fibrillar proteins communicating with the process of hair cells and the surface of supporting cells. The layer lying directly over the SM is the gelatinous layer (GL), a firm tissue made up of a crosswise filamentous network. The GL houses the motile cilia in a small pore and has a structure similar to that of the tectorial membrane of the organ of Corti. The otoconial layer is located above the GL. The otoconial layer contains otoconia embedded in the loose filament network, which is vulnerable to physical stimuli. The loose connection of the otoconial layer results in isolation and discretion of the otoconia and leads to benign paroxysmal positional vertigo (BPPV) [6].

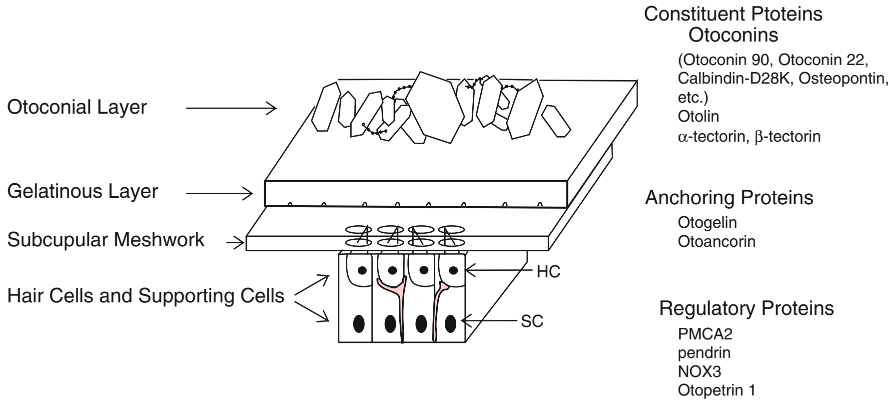

Fig. 8.1 Schematic representation of the sensory maculae and associated structures. Each hair cell (HC) is surrounded by supporting cells (SCs). Directly above the sensory epithelia, the otoconial membrane is composed of the subcupular meshwork, the gelatinous membrane, and the otoconia layer. Proteins that are revealed to influence the activity or structure of these layers are listed on the *right*

8.2 Otolith Formation

Proteins and genes relating to otolith and otoconium formation may be divided into four categories, as described below.

8.2.1 Formation of Normal Morphology

The formation of the otocyst and then the formation of sensory maculae are important steps in the development of otoliths and otoconia, as well as in the development of the normal morphology and function of the inner ear. Model animals in which *Otx1*, *fgf3*, *Eya1*, or *Cdh11* genes have been deleted develop abnormal otoliths or otoconia [7–10].

8.2.2 Constituent Proteins

Otoconins are part of the inner core matrix composed of glycoproteins and proteoglycans [11]. Some otoconins are unique to the core of otoliths or otoconia. Otoconin 90 is the main core glycoprotein of mammals and birds [12, 13], and otoconin 22 is the main core glycoprotein of amphibians and reptiles [14]. Both otoconin 90 and otoconin 22 are homologous to secretory phospholipase A2 [13, 14]. There are other minor otoconins, such as osteopontin and calbindin-D28K.

Osteopontin is a bone matrix protein that plays a role in the ossification of bone and teeth and in the formation of urinary stones and atherosclerotic plaques. In situ hybridization for the identification of osteopontin in the rat inner ear revealed osteopontin expression in sensory hair cells of the vestibular organs, suggesting its involvement in the maturation of otoconia [15]. Calbindin-D28K is a calcium-binding protein responsible for buffering the concentration of calcium ions [16]. In mammals, calbindin-D28K is expressed in the otolithic membrane of the sensory macula. Binding of calcium carbonate to calbindin-D28K is thought to be involved in the maturation process of otoliths and otoconia [16]. Otolin is a glycoprotein of less than 70 kDa expressed exclusively in the inner ear [17]. The C-terminus of otolin is structurally related to complement protein C1q and similarly enables the formation of oligomeric complexes. Zebra fish otolin morphants spontaneously develop decalcified and swollen otoliths. Otolin produced by supporting cells forms protein complexes with cerebellin-1 and otoconin 90. Alpha- and beta-tectorin have also been shown to be expressed in vestibular organs [18, 19]. Alpha-tectorin is strongly expressed in the peripheral area of the transitional zone and in the area where the accessory membrane originates in neonatal mice. Alpha-tectorin knockout mice display severe abnormalities in the extracellular matrix, leading to thinning of the gelatinous layer of the utricle and the saccule and agenesis or enlargement of otoconia in the saccule. In contrast, β-tectorin is expressed in the striola region. However, the contribution of β-tectorin to vestibular function is not well understood.

8.2.3 Regulatory Proteins

Plasma membrane calcium ATPase 2 (PMCA2) is the primary calcium ion pump of the endolymph, which serves to buffer the concentration of calcium ions. PMCA2 knockout mice have the agenesis of otoconia, despite normal sensory epithelium and gelatinous membrane [20]. The deletion of pendrin (Pds) leads to Pendred syndrome, an autosomal recessive hereditary disease characterized by deafness and thyroid goiter [21]. Pds knockout mice have large or no otoconia, in addition to other deformities including large vestibular aqueduct, dilated endolymphatic space, and degenerated stereocilia [22]. NADPH oxidase 3 (NOX3) forms compounds that will ultimately generate reactive oxygen species (ROS) in the inner ear. ROS are suggested to control the mitochondria and the endoplasmic reticulum, which are required to increase the concentration of calcium ions needed for mineralization of otoliths or otoconia [23]. Otopetrin-1 is a multi-transmembrane domain protein, the deletion of which results in severe balance disorder. Otopetrin-1 protein is localized in the granules secreted from supporting cells and in the otolithic membrane and the mutation of otopetrin-1 results in agenesis, deformation, misplacement of otoliths, and loss of a matrix [23].

8.2.4 Anchoring Proteins

Some proteins such as otogelin and otoancorin have been reported to provide instructions for the assembly and adhesion of matrix proteins at their right places in the inner ear. Otogelin is an *N*-glycosylated protein localized at the acellular membrane of the sensory patches such as the tectorial membrane in the cochlea, the gelatinous layer of the utricular and the saccular maculae, and the ampullary cupula, all of which affect mechanical transmission [24]. Otogelin knockout mice have both auditory and balance disorders. In the vestibule of otogelin knockout mice, the otoconial membrane is entirely detached from the sensory epithelium and found floating in the endolymphatic space. Otoancorin is an inner ear-specific protein, which is localized at the interface between the sensory epithelium and the gelatinous membrane [25]. In the vestibular systems, it is present on the apical surface of nonsensory cells beneath the otoconial membrane and cupulae. Otoancorin is thought to bind the gelatinous matrix to the underlying epithelial patches [25].

8.3 Factors Affecting the Structure of Otoliths

8.3.1 Drugs

The administration of streptomycin causes a decrease in the number of otoconia and changes the morphology of sensory cilia, which begins from the striola [26]. Moreover, streptomycin induces dark cells to absorb otoconia and to lower the calcium content of otoconia. The loss of otoconia was more prominently observed in the saccule, where no dark cells exist, suggesting a role for dark cells in maintaining the calcium concentration in otoconia. A previous study using guinea pigs showed that otoconia regeneration started at 6 weeks and was normalized at 10 weeks after administration of streptomycin [27].

8.3.2 Aging

Otoconia of the saccule start to degenerate in humans in the 6th decade of life [28]. It is known that otoconia of the saccule degenerate more remarkably than otoconia of the utricle. Low estrogen levels frequently detected in mid-aged and elderly women have been recognized to cause decalcification of otoconia, which in turn alters the electrolyte composition and pH value of both endolymph and perilymph. BPPV patients older than 50 years also present osteopenia or osteoporosis in 75 % of the cases [29]. Thus, altered hormone levels are suggested to induce vulnerability of otoliths and surrounding tissues.

8.3.3 Hypergravity

Anken et al. revealed that long-term exposure to 3 g hypergravity conditions causes a reduction in size and an increase in asymmetry of otoliths of cichlid fish compared to normal earth gravity controls [30]. Some of the animals subjected to hypergravity showed kinetotic behavioral abnormalities, such as spinning movements. Sondag et al. exposed golden hamsters to 2.5 g hypergravity conditions from uterine conception until 1–4 months after birth [31]. Hypergravity led to alteration of size distribution of otoliths, which persisted even after 8 months of exposure to normal gravity forces.

8.4 Otoliths and Semicircular Canals

Otoconia-deficient mutant mice are unable to swim and show impaired balance when challenged, but no disorientation [32]. "Head tilt" mutant mouse, which arose spontaneously at The Jackson Laboratory, have no phenotypic abnormality other than loss of otoconia [33]. Canal-only rotation stimuli produced attenuated vestibulo-ocular reflex responses in these "head tilt" mice. This suggests the interdependence of otoconia and semicircular canals and indicates the hitherto unrecognized importance of otoconia.

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Chapter 9
Tectorial Membrane

Yosuke Tona and Tatsunori Sakamoto

Abstract The mammalian tectorial membrane (TM) is an acellular gelatinous structure that connects to the organ of Corti at the tallest rows of the outer hair cell stereocilia. TM is divided into three zones: the inner limbal zone, the middle zone, and the outer marginal zone. TM contains different types of collagen, proteoglycans, and three non-collagenous glycoproteins, namely α-tectorin, β-tectorin, and otogelin. The cDNA sequence of both α-tectorin and β-tectorin contains a zona pellucida (ZP) domain, a module known to help in the formation of homopolymers and heteropolymers. Recent models suggest TM improves tonotopy and hearing sensitivity by coupling with outer hair cell bundles. Alpha- and beta-tectorin mutant mice display abnormal morphology of TM and hearing loss. Alpha-tectorin mutations are also known to cause hearing loss in humans, including stable moderate to severe hearing loss and progressive hearing loss. Abnormal TM morphology is also observed in patients with idiopathic sudden sensory hearing loss, suggesting the vulnerability of TM to inflammatory processes.

Keywords Otogelin • ZP domain • α-Tectorin (Tecta) • β-Tectorin (Tectb)

9.1 Anatomy

The tectorial membrane (TM) is one of the two acellular gelatinous structures in the inner ear, the other one being the basilar membrane (BM). Radially, the mammalian TM is extending from the spiral sulcus to the organ of Corti and extends longitudinally parallel to BM (Fig. 9.1). TM is divided into three zones: the inner limbal zone, the middle zone, and the outer marginal zone. The inner limbal zone is the thinnest one with the internal edge attached to the spiral limbus. The marginal zone

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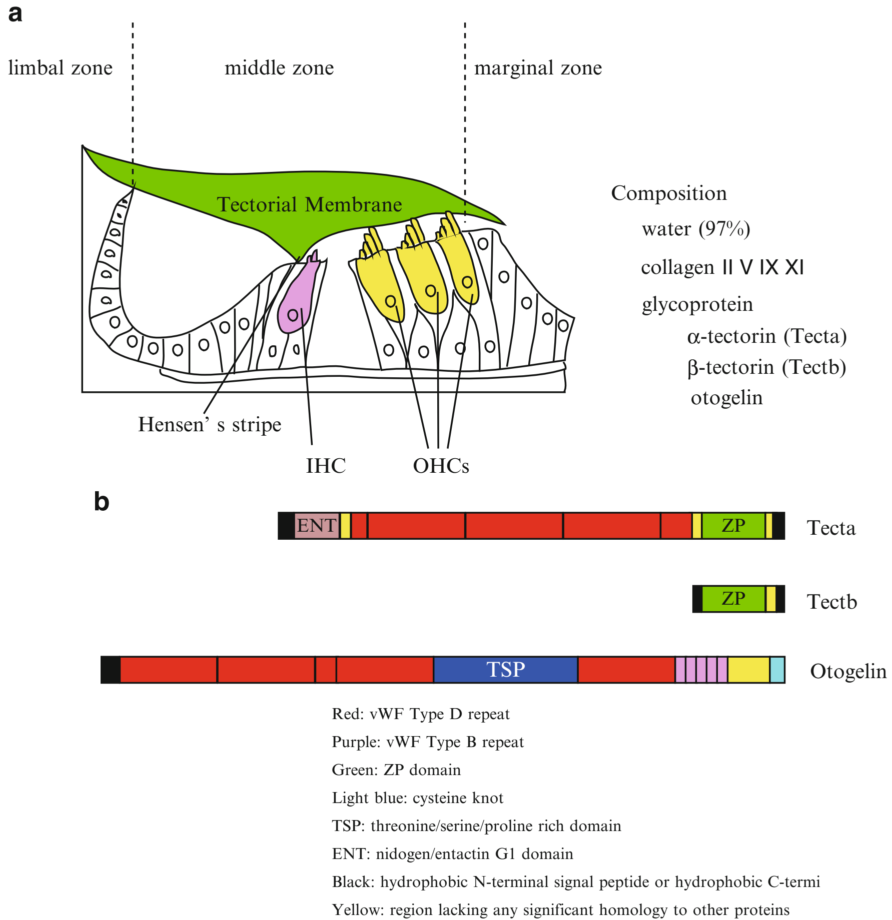

Fig. 9.1 (**a**) The schema showing the morphological relationship between the organ of Corti and TM. The *limbal zone* is the lesion where TM is attached to the spiral limbus, and the *middle zone* stretches over the internal sulcus and the organ of Corti, including inner hair cells (IHC) and outer hair cells (OHC). The *marginal zone* consists the outer edge of TM. The descriptions on the *right* show the components of TM. (**b**) Domain structures of the three non-collagenous glycoproteins of the TM. *Red* = vWF type D repeat, *purple* = vWF type B repeat, *green* = ZP domain, *C* = cysteine knot, *TSP* = threonine/serine/proline-rich region, *ENT* = nidogen/entactin G1 domain, *black* = hydrophobic N-terminal signal peptide (*right*) or hydrophobic C-termini (*left*), *yellow* = region lacking any significant homology to other proteins

is the thickest part of TM and the middle zone covers the internal sulcus and the organ of Corti. Hensen's stripe located in the subsurface of the middle zone of TM forms the attachment to the inner phalangeal cells and the border cells. Mostly, TM is revealed to be the consequence of secretion from the interdental cells [1]. The tallest rows of the outer hair cell stereocilia are embedded in the middle zone of TM [2, 3]. The marginal zone constitutes the outer edge of TM.

The morphology of the cochlear duct in birds is different from that in mammals [4]. The auditory sensory epithelium besides TM is called the basilar papilla, which is the long plain band containing multiple rows of hair cells. The tall narrow hair cells (THC) are located on the neural edge of the continuum, and the short wide hair cells (SHC) on its abneural edge.

9.2 Composition

TM is a gelatinous structure, 97 % of which is composed of water. About 50 % of the dry weight is formed by genetically determined collagens of type II, V, IX, and XI. The remaining components include proteoglycans (25 %) and non-collagenous glycoproteins (25 %), namely α-tectorin (Tecta), β-tectorin (Tectb), and otogelin [5]. Tecta, Tectb, and otogelin are specifically highly expressed in the inner ear. Tecta and Tectb form the striated sheet matrix, where they help organize the collagen fibers.

The cDNA sequence of Tecta contains an entactin G1-like domain at the N-terminal, five elements of von Willebrand factor (vWF) type D repeats, and a zona pellucida (ZP) domain at the C-terminal. Tecta protein is divided into three regions connected to each other via disulfide bonds. The cDNA sequence of Tectb is shorter than that of Tecta, containing a single ZP domain. The ZP domain is known to help in the formation of homopolymers and heteropolymers [6]. The sequence of otogelin contains several vWF type D repeats, a threonine/serine/proline-rich region, five elements of vWF type B repeats, and a C-terminal cysteine knot.

The central core of TM is composed of bundles of 20-nm collagen filaments that are embedded in an unusual striated sheet matrix [7]. This striated sheet matrix is composed of two types of thin filaments (7–9 nm), the light staining type and the dark staining type, coupled by staggered cross-bridges [7]. The upper surface of TM is covered by the covernet, a large network of anastomosing caliber fibrils.

9.3 Function

The mechanical role of TM in hearing is not fully recognized. In fact, it has been disregarded in previous cochlear models. More recent models, however, suggest the improvement of hearing sensitivity by strong TM radial coupling to outer hair cell bundles [8]. Frequency sensitivity was also shown to be improved by weak TM longitudinal coupling to outer hair cells [9]. These observations are supported by data obtained in transgenic mice [10–12], as described in the next paragraph.

9.4 Phenotype of Knockout Mice

Tecta$^{\Delta ENT/\Delta ENT}$ mice, which have the homozygous in-frame deletion of entactin G1 section of Tecta, lack almost all non-collagenous matrixes of TM. As a consequence, TM is almost completely detached from the organ of Corti and the surface of the spiral limbus [13]. *Tecta*$^{\Delta ENT/\Delta ENT}$ mice display moderate hearing loss of about 60–80 dB at 20 kHz and less severe hearing impairment at higher frequencies.

Mice with heterozygous mutation of Tecta Y1870C (the missense mutation detected in an Australian family with moderate to severe hearing impairment of about 60–80 dB) show an unusual TM of humpbacked shape with decreased attachment area, dilated space under TM, and loss of striated sheet matrix at the sulcus region [14]. *Tecta*$^{Y1870C/+}$ mice, however, demonstrated normal function of outer hair cells and BM motion. The compound action potential threshold of *Tecta*$^{Y1870C/+}$ mice was on average 55 dB higher than that of the *Tecta*$^{+/+}$ mouse, suggesting the essential role of TM in driving inner hair cells relating to motion of outer hair cell bundles.

Mice with homozygous mutation of the functional null deletion of *Tectb* gene have TM attached to the spiral limbus and the surface of the organ of Corti [15]. The knockout mice lack the organized striated sheet matrix which is characteristic in the wild-type mice. Instead, collagen fibrils are embedded in the matrix and dispersed randomly, and the abnormal filaments are formed by Tecta. The knockout mice of *Tectb* display severe hearing impairment at frequencies of 20 kHz or less, which is probably caused by the enlargement and bulging of TM at the apex of the cochlea.

Otogelin knockout mice have TM attached to the epithelia of the cochlea and almost normal microstructure with the atypical rodlike shape in the limbal zone [16]. The knockout mice have severe balance disorder and various degrees of hearing impairment. The mechanism underlying hearing dysfunction has not been elucidated yet.

Mice with homozygous mutations of the *Col11a2* and *Col9a1* genes, which form the collagen fibers inside TM, also have hearing impairment [17–19]. These mice lack the organization of the collagen fibrils, but it is difficult to evaluate the specific influence of the mutation in TM function, because *Col11a2* and *Col9a1* are broadly expressed in the multiple structures of the ear.

9.5 Known Mutation Causing Hearing Defects in Humans

A *Tecta* mutation associated with hearing impairment has been identified in a total of 15 families worldwide [20]. However, phenotypic differences of patients bearing *Tecta* mutations revealed the possible involvement of different genotypes [21]. All loss-of-function mutations of Tecta support its recessive heredity and cause stable, moderate to severe hearing loss from prelingual stage. All the missense mutations of Tecta involving cysteine residues are autosomal dominant and result in

progressive hearing loss [22]. The pathogenesis may be the disruption of disulfide bonds and the resultant instability of the matrix structures. Missense mutations involving amino acids other than cysteine cause stable hearing loss.

9.6 Clinical Implications

Linthicum et al. compared the temporal bone pathologies of patients with idiopathic sudden sensory hearing loss (ISSNHL) with those of known vascular impairment due to surgical interventions [23]. Histological analysis of tissues from ISSNHL patients more frequently revealed abnormal TM morphology, such as the separation of TM from the organ of Corti, than the postoperative vascular group. These findings draw attention to the unrecognized vulnerability of TM to inflammatory processes, which might result in sensory neural hearing loss.

9.7 Regeneration of TM

TM of chicks has been reported to regenerate after acoustic overstimulation [24]. Cotanche identified a series of new matrix substances that are secreted from the supporting cells at the basilar papilla of chicks after the acoustic trauma [24]. The regenerated area was only at the lower layer of the honeycomb-like matrix of TM, and the laterally oriented fibers of the upper layer were not regenerated. However, the regeneration of mammalian TM has not been reported yet. It is necessary to activate the interdental cells, other supporting cells, or stem cells to secrete the matrix proteins. It is also essential for functional recovery of the mechanical properties to reestablish the coupling between TM and stereocilia of hair cells.

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Part II
Development of the Inner Ear

Chapter 10
Development and Regeneration

Norio Yamamoto

Abstract Currently, regeneration of target organs is achieved by several strategies, including transplantation of stem cells and reprogramming of mature cells to the desired cell types (transdifferentiation). Transplantation of naïve stem cells can cause several problems such as teratoma formation, immunogenicity, and elimination of transplanted cells. These disadvantages can be avoided by induction of stem cells into the desired cell types. On the other hand, transdifferentiation of mature cells into the desired mature or differentiating cell types is another viable option for circumventing the numerous negative adverse effects of naïve stem cell transplantation. The selection of most of the factors required for the induction of stem cells or transdifferentiation is based on the progressively accumulating knowledge of developmental biology. These aspects include the determinants of dorsoventral or anteroposterior axis and germinal layers or the transcription factors specific for desired organs. By manipulating pluripotent stem cells using predetermined factors, these stem cells can be induced into all three germ layers (endoderm, mesoderm, and ectoderm) and into differentiated organs. To date, several organs have been successfully transdifferentiated from other types of mature cells.

Keywords Germ layers • Pluripotent stem cells • Reprogramming • Transdifferentiation

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10.1 Introduction

Until the concept of stem cells was proposed, transplantation of whole differentiated organs was the only method available to facilitate the functional recovery of the target organs. Transplantation medicine has achieved outstanding results with respect to various organs including the bone marrow, liver, heart, lung, kidney, pancreas, small intestine, and cornea. However, most transplantation therapies require immunosuppression to prevent the rejection of transplanted organs, causing a plethora of adverse effects. Regeneration from the patient's own tissues or stem cells has gained substantial attention and it is ideally a more preferable option for functional recovery of target organs. Several possible strategies exist for sustaining regenerative medicine: transplantation of pluripotent stem cells or somatic stem cells with or without induction of stem cells to desired cell types, induction of stem cells or progenitor cells in situ into desired cell types, and reprogramming of mature cells to desired cells or their stem/progenitor cells (transdifferentiation).

Among these strategies, using immature pluripotent stem cells in regenerative medicine seems most promising since these cells can contribute to multiple lineages. However, pluripotency is accompanied with a possible risk of teratoma formation since pluripotent stem cells are able to expand clonogenically into all kinds of germinal layers. In addition, even induced pluripotent stem cells (iPSCs) prepared from autologous tissues can cause immunogenicity [1], leading to adverse effects similar to those caused by tissue transplantation. However, iPSCs do not have immunogenicity after normal differentiation [2]. Although transplantation of untreated, naïve tissue stem/progenitor cells can lead to their migration and integration within host tissues without tumor formation [3, 4], this strategy is not useful for regeneration since these cells cannot survive in the recipient tissue in sufficient numbers. In addition, they cannot differentiate into desired cell types [4]. Moreover, primary sources of these cells are usually embryonic organs from other individuals, which also cause immunogenicity. Consequently, in order to employ pluripotent or somatic stem cells for regeneration, induction of stem cells (priming or preconditioning of stem cells) is necessary (Fig. 10.1). Another suitable strategy for achieving optimized regeneration is reprogramming of mature cells in situ (Fig. 10.1). These strategies require specific factors that play pivotal roles in the development of target organs (Fig. 10.1). These factors are involved in the regulation of differentiation, migration, proliferation, and apoptosis of various cell types that constitute the desired organs.

10.2 Pluripotent Stem Cells and Development

Induction of pluripotent stem cells has been used as an efficient strategy to achieve regeneration of several organs. However, induction protocols are based on results from previous studies of developmental biology. During development, cells choose

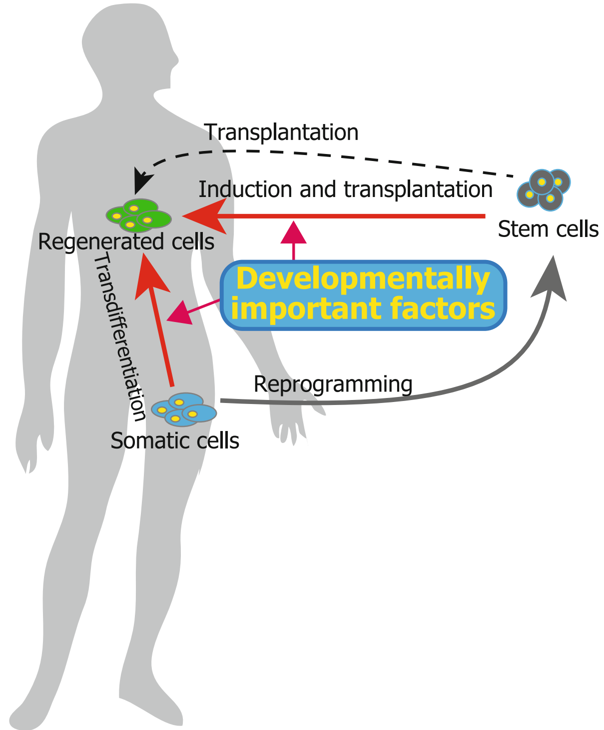

Fig. 10.1 Induction of stem cells or transdifferentiation requires developmentally important factors that regulate process of differentiation, migration, proliferation, and apoptosis

which germ layer fate to acquire (ectoderm, mesoderm, or endoderm) after fertilization, and they are then specifically induced into the desired cell type. The factors used to induce pluripotent stem cells into desired cells include those that determine early-stage dorsoventral or anteroposterior axis in development: the transforming growth factor-β (TGF-β) superfamily (Activin, Nodal, and bone morphologic protein), Wnt signaling proteins, and fibroblast growth factors (FGFs) [5] and their inhibitors. Currently, multiple types of organs and/or their tissue stem cells from all three germ layers can be induced from pluripotent stem cells.

10.2.1 Mesodermal Organs

Various mesodermal organs, such as those of the hematopoietic system and cardiomyocytes, can be successfully induced. Hematopoietic stem cells are induced from pluripotent stem cells by overexpression of a single transcription factor, HoxB4, and co-culture with OP9, a stromal cell line from mouse bone marrow [6, 7]. Transplantation of induced hematopoietic stem cells has also been shown to rescue a phenotype of sickle cell anemia in mouse models [6]. HoxB4 was selected for induction because it is specifically expressed in definitive hematopoietic stem

cells but not in the developing, early-stage organs or the primitive hematopoietic organ, yolk sac [7]. In case of cardiomyocytes, they were first generated from embryonic stem cells (ESCs) by spontaneous differentiation [8]. Since then, numerous protocols based on discoveries in developmental biology have been optimized. To begin with, markers of early cardiac induction were selected based on the study showing that platelet-derived growth factor receptor alpha (Pdgfr-α) and fetal liver kinase 1 (Flk-1) are co-expressed in the cardiac crescent of embryos at embryonic day (E) 7.0–7.75 and in the linear heart tube at E8.5 [9]. Next, the protocol to direct pluripotent stem cells toward the mesodermal lineage [10, 11] by adding Activin A [12–14], bone morphogenetic protein 4 (BMP-4) [15], and FGF-2 [16] that mimic the signaling environment of the primitive streak in a post-gastrulation embryo was determined. Cardiac differentiation was subsequently induced by inhibiting Wnt [17] and TGF-β signaling [18]. Cardiomyocytes thus derived from pluripotent stem cells have been integrated into infarcted guinea pig hearts to improve cardiac function [19].

10.2.2 Endodermal Organs

The induction of pancreatic cells as model of endodermal organs is well characterized. The protocol for pancreatic cell induction is divided in two stages: induction of definitive endoderm followed by the induction of a pancreatic progenitor. The study of vertebrate development demonstrates that Nodal induces embryonic differentiation into the endoderm [20]. Based on this result, an efficient protocol for generating definitive endoderm in vitro from pluripotent stem cells was developed [21]. In this protocol, Activin A, TGF-β family member, was used since it can be easily produced as a recombinant protein, and they can signal via similar downstream pathways with those of Nodal. The development of pancreatic endoderm from definitive endoderm is inhibited by sonic hedgehog (Shh) signaling [22], which in turn is inhibited by the signal from the notochord. This repression of the Shh signaling is essential for the development of pancreatic mesoderm [23]. In addition, retinoic acid signaling is necessary for the specification of the pancreatic and liver endoderm from the endodermal germ layer [24], and FGF10 is required for the expansion and branching of the pancreatic epithelium [25]. The combination of these three factors, namely, retinoic acid, the Shh inhibitor cyclopamine, and Fgf10, was found to successfully induce pancreatic epithelium in vitro from the definitive endoderm derived from pluripotent stem cells [26].

10.2.3 Ectodermal Organs

Several ectodermal organs such as the skin regenerate without treatment, while neurons or retinal tissues were considered to be incapable of regeneration. Currently, even these organs can be induced from pluripotent stem cells. To this end,

iPSCs are propagated in media supplemented with FGF-2 (basic fibroblast growth factor) to obtain neural precursors. The transplantation of these neural precursors has been shown to facilitate functional recovery in Parkinson's disease model and spinal cord injury [27, 28]. These studies are based on published research, which demonstrated that FGF-2-responsive progenitor cells can be isolated and cultured from the adult rat hippocampus [3]. Originally, FGF-2 was used to stimulate the proliferation of neuronal precursors from embryonic brain [29] or embryonic spinal cord [30]. Among the cell populations in the retina, retinal progenitor cells [31] and both photoreceptor cells as well as the retinal pigment epithelium (RPE) that support photoreceptor cells [32, 33] were induced from pluripotent stem cells by mimicking retinal developmental processes in a step-wise fashion. For the induction of retinal progenitors, ESCs were treated with Wnt and Nodal antagonists (Dkk1 and Lefty A) and Activin A [31]. These experiments utilized a plethora of transcription factors expressed at various developmental stages to define the cell populations obtained in the induction process.

10.3 Transdifferentiation and Development

In contrast to the induction of pluripotent stem cells, the transdifferentiation of mature cells is still less efficient. However, since pluripotent stem cells are induced from mature differentiated cells by overexpressing several factors that are specifically expressed in pluripotent stem cells [34], reprogramming has been tested in multiple types of organs. Even in cases of reprogramming of differentiated cells into other types of differentiated cells (direct conversion, or transdifferentiation), developmentally important factors have been utilized and some kinds of mature cells were successfully induced (see Chap. 21) [35–39]. The proteins that govern transdifferentiation are transcription factors that are specifically expressed in embryonic or matured target cells or their progenitor cells and that are important for the development, maintenance, or both of the desired cell type.

10.4 Future Direction

As described in this chapter, regeneration of organs recapitulates embryonic development in numerous aspects. Both processes involve the differentiation, migration, proliferation, and apoptosis of various cell types. Many of the same key signaling pathways that are activated during embryonic development are also activated during the regeneration process. Despite these similarities, there are a number of important differences between the molecular mechanisms that regulate regeneration and embryonic development, and these may be partly responsible for the inability of regeneration of organs into their original uninjured state.

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Chapter 11
Otic Induction

Hiroe Ohnishi and Tatsunori Sakamoto

Abstract Mechanisms of early inner ear development are overviewed and discussed in relation to current methods for the otic induction of pluripotent stem cells. In the embryo, after the three germ layers are formed, the ectoderm differentiates into neural and nonneural ectoderm, depending on the BMP concentration gradient. In the anterior region of the embryo, a horseshoe-shaped area of surface ectoderm adjacent to the neuroectoderm becomes competent as preplacodal ectoderm. Mutually opposing signals from BMP, Wnt, and their antagonists coordinately act to form the neural crest and preplacodal ectoderm. The preplacodal ectoderm is induced to become the otic–epibranchial placode via FGF signaling, and then the otic–epibranchial placode is divided into the otic and epibranchial placodes by Wnt. The otic placode then invaginates and pinches off to form the otocyst. The otocyst is then subdivided into several regions in response to three-dimensional morphogen gradients. Each region subsequently undergoes a different developmental fate to give rise to the endolymphatic sac, the membranous part of the cochlea, the vestibule, the semicircular canals, and the sensory areas and neurons of each substructure, respectively.

Keywords Ectoderm • Otic placode • Otocyst • Preplacodal ectoderm

11.1 Introduction

In developmental biology, "induction" is defined as the interaction between 2 or more cells or tissues through which one cell or tissue alters its shape, mitotic rate, and developmental fate in response to signals from the other. The inductive process is not binary but represents a gradual change that, at some point in time, becomes

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consolidated and irreversible. Development is a chain of these inductive processes. Molecular biology has provided information about intra- and extracellular molecules involved in induction and has enabled the investigation of each step of induction or differentiation. A signaling molecule that controls embryonic differentiation by forming a concentration gradient is called a morphogen.

In vitro differentiation systems using embryonic stem cells and induced pluripotent stem cells are considered useful sources of cells for regenerative medicine, in vitro models of diseases, and drug screening. Methods for the induction of inner ear hair cells from stem cells have also been studied for these purposes. Many differentiation methods using pluripotent stem cells, embryonic stem cells [1–6], and induced pluripotent stem cells [2] have been reported for otic induction. In addition, many of these studies have tried to mimic stages of inner ear development to facilitate inner ear induction from stem cells [1–4, 7, 8]. In this chapter, we will describe the stages of early inner ear development from fertilized egg to otocyst and detail the morphogens and marker genes responsible for each stage as they relate to techniques currently used for the otic induction of pluripotent stem cells (Fig. 11.1).

11.2 Formation of Epiblast and the Three Germ Layers

A fertilized egg divides several times and generates a trophoblast and an inner cell mass. The inner cell mass gives rise to the primitive endoderm (or hypoblast), which becomes the yolk sac, and the epiblast, which produces the embryonic tissues. In the epiblast, cells that delaminate from the primitive streak in the midline of epiblast give rise to the mesoderm and the endoderm. The remaining epiblast becomes the ectoderm.

For the in vitro differentiation of pluripotent stem cells, these processes are partly recapitulated by the formation of embryoid bodies (EBs). An EB is a pluripotent cell aggregate that is formed to induce stem cells to differentiate [9]. Generally, EBs are cultured in suspension or in nonadhesive culture dishes and are capable of forming resemblances of the three germ layers. EBs are also utilized for otic induction methods. Oshima et al. cultured EBs in medium supplemented with Dkk1 (a Wnt inhibitor) and SIS3 (a Smad3 inhibitor that inhibits TGF-β1-dependent Smad3 phosphorylation) to suppress mesoderm formation [2]. Koehler et al. also used EBs under conditions designed for the preferential development of ectoderm [4].

11.3 Differentiation into Neural and Nonneural Ectoderm

After three germ layers (ectoderm, mesoderm, and endoderm) are formed, the ectoderm differentiates into neural and nonneural ectoderm depending on the bone morphogenic protein (BMP) gradient [10, 11]. In the embryo, the ectoderm

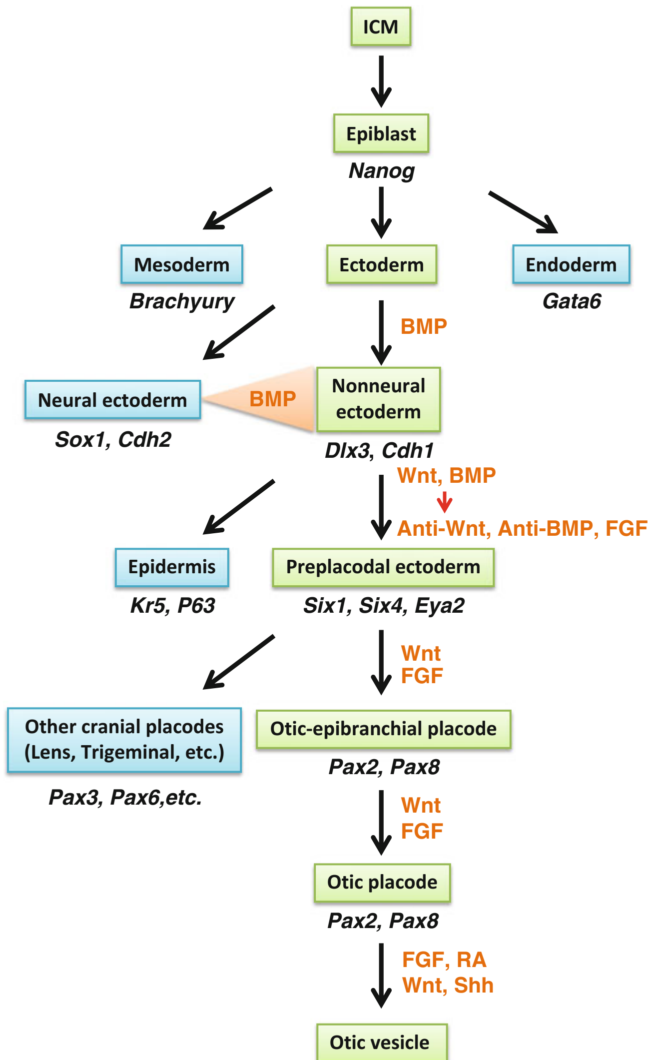

Fig. 11.1 Early inner ear developmental steps from the inner cell mass to the otic vesicle with morphogens and marker genes. *ICM* inner cell mass, *RA* retinoic acid

is a source of BMP, and the notochord, located in the midline of the mesoderm, is a source of BMP antagonists such as *noggin*, *chordin*, and *follistatin* [12]. The lateral part of the ectoderm that receives a high level of BMP signal gives rise to nonneural ectoderm, and the medial part of the ectoderm in which the BMP signal is suppressed by the antagonists gives rise to the neural ectoderm.

The membranous labyrinth of the inner ear is a derivative of nonneural ectoderm. BMP-4 supplementation has been used in various studies to induce nonneural ectoderm.

11.4 Induction of Preplacodal Ectoderm

In the head region, a morphologically indistinguishable horseshoe-shaped area of surface ectoderm next to the neuroectoderm called the preplacodal ectoderm (PPE) becomes competent to respond to placode-inducing signals at late gastrula or early neural plate stages [11]. The PPE and later on the placodes give rise to the lens, ear, olfactory epithelium of the nose, and trigeminal and epibranchial ganglia [11]. The PPE is characterized by the expression of *Six1*, *Six4*, and *Eya2* [13–16].

BMP signaling from the lateral ectoderm, Wnt signaling from the trunk ectoderm and mesoderm, FGF signaling from the mesoderm, and Wnt/BMP antagonists from the neural plate act in concert to form the neural crest and the PPE [13]. Although BMP signaling is required for the induction of nonneural ectoderm, subsequent BMP inhibition in conjunction with active FGF signaling is required for the formation of the PPE [13, 17–19]. Most of the studies concerning the induction of PPE have been conducted on Xenopus and fish rather than birds and mammals.

In addition to BMP, Wnt, and FGF signals, *Dlx3* and *Gata2* are also essential for the induction of the PPE, at least in Xenopus [17]. In addition, Six1 is sufficient to induce PPE at the expense of the neural crest and epidermis in Xenopus [20]. Furthermore, BMP levels need to be altered for PPE formation to take place [18].

The complicated process of PPE determination requires further investigation and is difficult to apply in the otic induction of pluripotent stem cells. The use of BMP-4 and SB431542 (a TGF-β inhibitor) followed by LDN-193189 (a BMP inhibitor) and bFGF in Koeheler's study may partly mimic this step [4].

11.5 Induction of the Otic Placode

The earliest visible primordium of the inner ear is the otic (or epibranchial) placode, which is visible as a thickening in the PPE. The otic placode becomes visible at 8.0–8.5 dpc in mice and at the 8/9 somite stage in chicks. *Pax2* is a widely used marker for the otic placode. In chicks, *Soho1* and *Nkx5.1* are also typically used [21, 22].

In the PPE, mesodermal FGF (more specifically Fgf3 and Fgf19 in chicks) determines the *Pax2*-positive otic competent region, called the otic–epibranchial progenitor domain, followed by Wnt (Wnt8c in chicks) and the attenuation of FGF, which further specifies the development of the otic placode over the epibranchial placode [21, 22]. In mice, neural Fgf3 and mesodermal Fgf10 are required for the induction of otic placodes [23, 24].

A recent study showed that Pax2 regulates proliferation rather than cell specification in the otic–epibranchial progenitor domain [25]. In mouse, *Pax8* is also an otic–epibranchial progenitor domain and otic placodal marker [26].

As in the embryo, FGFs, Wnt, and Pax2/8 are decisive factors for the formation of the otic placode from stem cells. For otic induction from pluripotent stem cells, bFgf as a universal Fgf or Fgf3/Fgf10 as more inner ear-specific FGFs are used. As Koehler et al. report, intrinsic Wnt in the cell aggregates in their method appears to function as an inducer for the otic fate [1]. The role of bFGF in the method developed by Oshima et al. may be attributed to the importance of FGF signaling in this step [2].

11.6 Induction of the Otic Vesicle and Axis Formation

The otic placode invaginates to form the otic pit or cup and pinches off from the surface ectoderm to form the otic vesicle. The otic vesicle further changes its morphology to form multiple inner ear substructures, including the semicircular canals, vestibule (the utricle and saccule), and cochlea. The process of the invagination and pinch-off to form the otic vesicle requires FGF, which was demonstrated by coculture of chick otic placode with FGF-soaked heparin beads [27].

During and after its formation, the otic vesicle is placed in the three-dimensional concentration gradient of various molecules, where it differentiates into several structures and cell lineages such as the endolymphatic sac, the membranous part of the cochlea, the vestibules, the semicircular canals, and the sensory areas and neurons of each substructure. The compartmentalization of the otic vesicle into these substructures is determined by three axes: the anterior–posterior axis, the dorsal–ventral axis, and the medial–lateral axis. Sensory organs and neurons are derived from the anterior region of the otic cup, and the posterior crista is derived from the posterior region. The dorsal region differentiates into the endolymphatic duct, semicircular canals, and utricle, and the ventral region differentiates into the saccule and cochlea. Neurons and most of the sensory epithelium are induced from the anterior region [28] (Fig. 11.2).

11.6.1 Anterior–Posterior Axis

Retinoic acid (RA) is a morphogen that acts in anterior–posterior axis formation. One study using RA-soaked beads showed that cells in the anterior otic cup that were exposed briefly to RA gave rise to neurons and most of the sensory organs of the inner ear, whereas prolonged and higher level exposure of RA in the posterior otic cup promoted the formation of non-sensory structures [29]. In mouse, *Tbx1* expression is localized in the posterior half of the otocyst [30]. In the *Tbx1*-null mutant mouse, expression of *NeuroD1*, *Lfng*, and *Fgf3* disappeared from their normal expression domains in the anterior region of the otocyst and instead

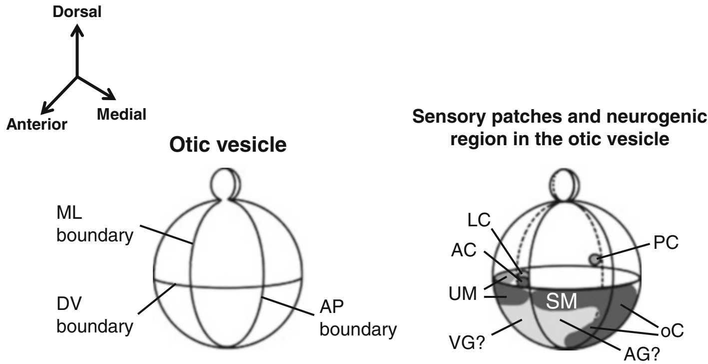

Fig. 11.2 The three axes in the otic vesicle and a possible distribution map of sensory organ primordia and neuroblasts. (Modified from Fekete and Wu [28].) *AC* anterior crista, *AG* auditory ganglia, *AP* anterior–posterior, *DV* dorsal–ventral, *LC* lateral crista, *ML* medial–lateral, *oC* organ of Corti, *PC* posterior crista, *SM* saccular macula, *UM* utricular macula, *VG* vestibular ganglia

expanded into the posterior region; in addition, the expression of *Otx1* in the posterior region of the otocyst disappeared [31]. These data suggest that *Tbx1* is also involved in anterior–posterior axis formation.

11.6.2 Dorsal–Ventral Axis

Wnts secreted from the dorsal hindbrain and Sonic hedgehog (Shh) secreted from the ventral floor plate and notochord are implicated in the patterning of the inner ear dorsal–ventral axis. Lithium chloride, a Wnt canonical pathway agonist, is able to restore, in part, otic vesicle gene expression patterns altered by ablation of the dorsal neural tube in otic explants [32]. *Wnt1*/*Wnt3a* double-knockout mice show defects in the dorsal region and in part of the ventral region of the inner ear [32]. The otic vesicles of *β-catenin* conditional knockout mice show severe shrinkage. This result indicates that Wnt signaling is required for otic vesicle formation [33]. The Wnt signal acts via the regulation of *Tbx1*, *Eya1*, and *Six1* [33].

One of the major downstream factors of *Shh* is the zinc-finger transcription factor *Gli3*. Based on the results of several studies on mice and chicks, *Shh* generates opposing gradients of *Gli3* repressor and *Gli2* and *Gli3* activators in the otic epithelium along the dorsal–ventral axis [34]. One study using the *Shh* conditional knockout mouse showed that the absence of *Shh* results in the disappearance of *Pax2*, *Otx2*, and *Gata3* and a morphological defect in the cochlea [35].

11.6.3 Medial–Lateral Axis

After the otic cup closes, the dorsal–medial region of the otic vesicle extends to form the endolymphatic duct. In contrast, the dorsal–lateral wall of the otic vesicle mainly gives rise to the vertical and horizontal pouches that form the three semicircular canals. The molecular mechanisms underlying the formation of the medial–lateral axis of the otic vesicle are not well understood.

11.7 Future Perspectives for Inner Ear Regeneration

The use of morphogens in a three-dimensional field for otic compartmentalization would be necessary for the more precise induction of cell types such as the cochlear hair cell in otic induction from stem cells. However, this information and the later steps in inner ear development, which are required for the induction of cochlea-specific cells or more mature hair cells, have not yet been successfully used in otic induction. As an alternative, it is anticipated that culture with otic mesenchymal tissues may promote hair cell maturation [2].

Current otic induction methods, although they have successfully induced inner ear sensory cells, are still not satisfactory in terms of induction efficiency and the quality of the product. Further investigation into inner ear development and progress in the development of technology for more precise application of developmental factors will improve methods of otic induction.

Direct lineage conversion is another approach for the induction of specific cell types [36]. Vierbuchen et al. reported the direct conversion of mouse fibroblasts into neuronal types by overexpression of the neuronal transcription factors *Ascl1*, *Brn2*, and *Myt1l* [37]. Ieda et al. reported that overexpression of three transcription factors (*Gata4*, *Mef2c*, and *Tbx5*) resulted in the direct conversion of mouse cardiac fibroblasts into cardiomyocyte-like cells [38]. To realize direct conversion to inner ear sensory cells, the master gene for the inner ear is required. *Atoh1* may be a candidate master gene for the inner ear sensory cells. However, *Atoh1* has been shown to be essential for the formation of cerebellar granule neurons, spinal cord interneurons, Merkel cells of the skin, and intestinal goblet cells, in addition to inner ear hair cells. Therefore, further studies are required to elucidate the cellular context underlying the competency of cells to respond to an instructive signal for inner ear sensory cell formation.

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Chapter 12
Cochlear Development

Tomoko Tateya

Abstract The sensory epithelium of the cochlear duct, called the organ of Corti, is the hearing organ of mammals including human. The organ of Corti is a masterpiece of cellular micro-architecture. The organ of Corti consists of two subtypes of hair cells, a single row of inner and three rows of outer hair cells, and several subtypes of supporting cells strategically positioned on the basilar membrane. The organization of these cells maximizes the extraction of sound energy by amplifying sound-induced basilar membrane motion and transmitting those movements, via the help of the tectorial membrane, to inner hair cells. In this section, an overview of cochlear development is presented and four important events during cochlear development are described: extension of cochlear duct, prosensory specification, cell-cycle exit, and cellular differentiation. Some of the most recent and enlightening results regarding the molecular mechanism underlying the formation of the organ of Corti are also discussed. Most data are from the experiments using mice, the most comprehensive model system for the developing mammalian cochlea.

Keywords Cochlear duct • Hair cells • Supporting cells • The organ of Corti

12.1 Extension of Cochlear Duct

The mammalian cochlear duct is a coiled structure resembling the shape of a snail, while the avian cochlear duct (basilar papilla) is relatively straight. In mice, the cochlear duct first arises as a ventral outpocketing from a posterolateral region of the developing otocyst beginning around E11 [1] and descends ventromedially

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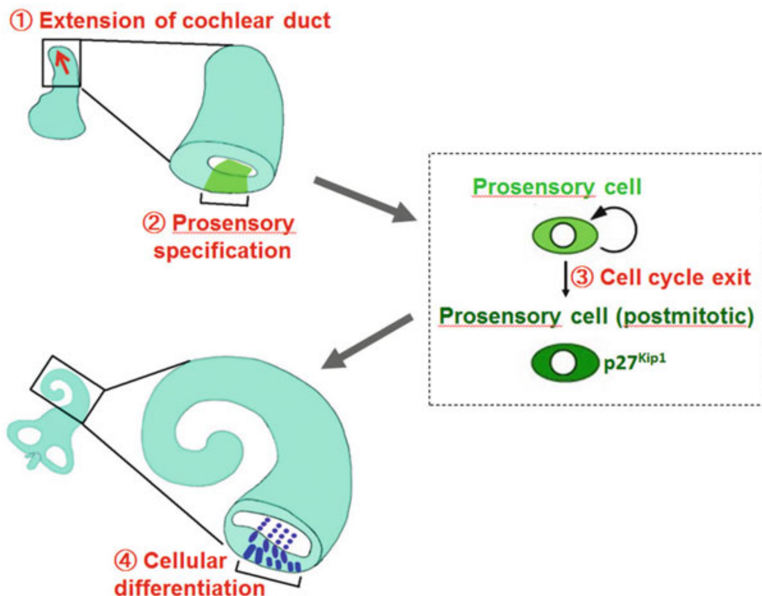

Fig. 12.1 Graphical abstract of Chap. 12. (Modified from [68])

forming an L-shaped organ (a half turn) by embryonic day 12. Then, the cochlear duct continues to extend and coil with the mature mouse structure consisting of one and three quarter turns (Fig. 12.1). Similar to other mammals, the mouse cochlear duct is tonotopically organized such that the base of the cochlear duct is most sensitive to high frequency sounds and the apical region to low frequency sounds [3].

12.1.1 Factors Regulating Cochlear Duct Extension

Multiple extrinsic factors regulate proper outgrowth and extension of the cochlear duct. One clear example is Shh secreted from the notochord and floor plate. Similar ventral phenotypes occur in $Shh^{-/-}$ ears and ears in chicken embryos in which the ventral midline has been ablated, including agenesis of the cochlear duct [4, 5].

Additionally, it is well established that a mesenchymal contribution to cochlear duct formation is also important [6]. Two transcription factors, Tbx1 and Pou3f4 (Brn4), of which the latter is expressed only in the otic mesenchyme, have been implicated so far. Lack of Tbx1 or Pou3f4 in the otic mesenchyme can lead to abnormal coiling or shortening of the cochlear duct, and these two pathways have been shown to interact genetically [7–9]. One possible mediator of these effects is RA, as both of these transcription factors are believed to induce expression of the RA degradation enzyme Cyp26 in the periotic mesenchyme [9].

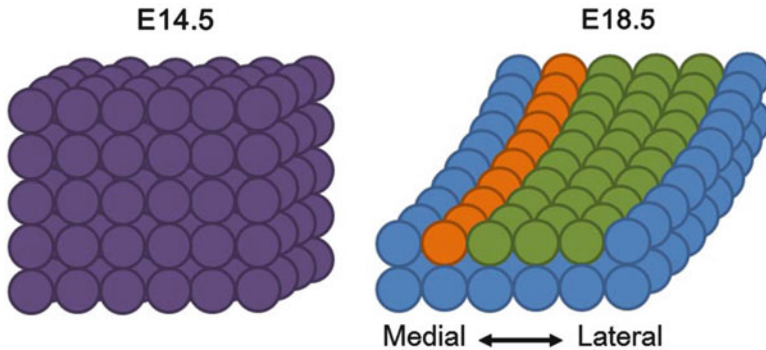

Fig. 12.2 Convergent extension

12.1.2 Convergent Extension

The cochlear duct is composed of pseudostratified epithelial cells derived from the otic placode. Even at the earliest time points following the initiation of cochlear outgrowth, the dorsal half of the duct, typically referred to as the floor, is already comprised of a notably thickened epithelium that contains five or six layers of cells [10, 11]. The organ of Corti arises from the floor of cochlear duct and the final pattern contains two layers of cells: a hair cell layer and a supporting cell layer.

Accumulating evidence suggests that the growth of the cochlear duct is regulated by not only proliferation of cells but also convergent extension movements [6, 12–14]. Genes within the planar cell polarity pathway such as Dishevelled1–3, Vangl2, and Celsr1 are thought to be involved in this process [15–17] (Fig. 12.2).

12.2 Prosensory Specification

The first step in the development of the organ of Corti is believed to be the specification of the prosensory domain [18] (Fig. 12.3).

Notch signaling seemed to the main effector of sensory specification, and other signaling pathways such as FGF [20–22] and Bmp signaling [23] also contribute to the sensory specification.

Before E12.5 all of the epithelial cells that compose the floor of the cochlear duct have a similar morphology; however, even at these early time points, a subset of cells express the prosensory markers Sox2, Jagged1 (Jag1, also referred to as Serrate1 in the chick), Lunatic Fringe (Lfng), Fgf10, and bone morphogenetic protein 4 (Bmp4), and they have been implicated in the specification of prosensory domain [1, 23] (Fig. 12.4).

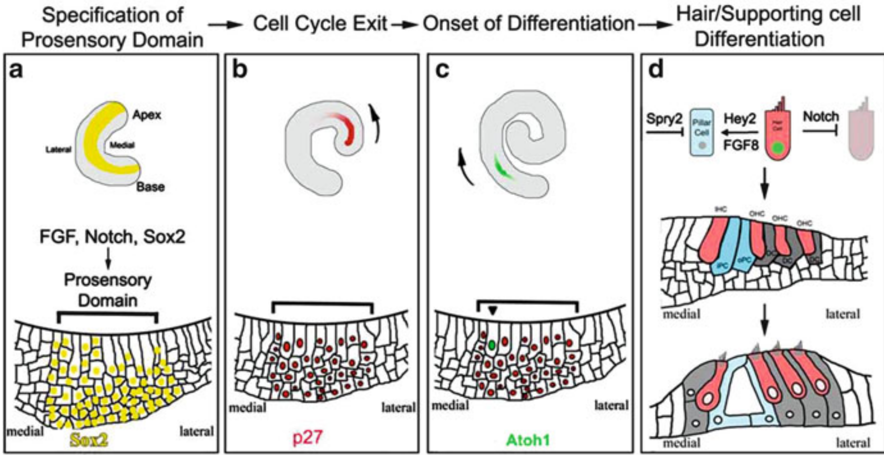

Fig. 12.3 Development of the organ or Corti. (Modified from [19])

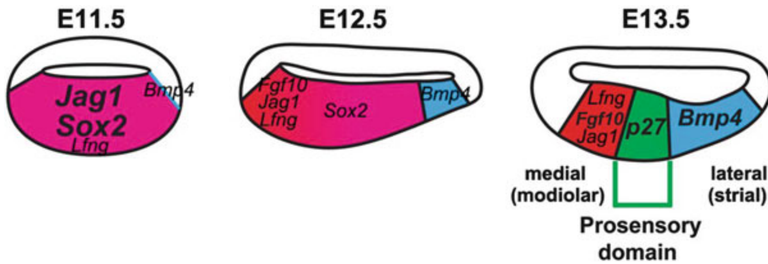

Fig. 12.4 Markers in prosensory formation. (Modified from [23])

12.2.1 Notch Signaling and Prosensory Formation

Jag1 and Lfng are expressed in patterns that are largely consistent with a role in specification of prosensory patches. Jag1 and Lfng are both components of the Notch signaling pathway, with Jag1 acting as a ligand for Notch, while Lfng modulates the activity of some Notch ligands [24, 25].

Lateral inhibition is the well-established role of Notch signaling in the regulation of cellular differentiation within prosensory domains and will be discussed in a subsequent section. But more recent experiments have illuminated an additional role for Notch signaling in the specification of prosensory domains, called Notch induction.

Although less well understood, the mechanism of inductive signaling differs from lateral inhibition and typically involves positive feedback rather than negative feedback [26].

Analyses of inner ears from Jag1-deficient mice reveal a decrease in the overall size of the sensory epithelia [27–31]. Similarly, deletion of Rbp-Jk, a transcriptional repressor that is required for all Notch function, leads to a complete absence of all vestibular epithelia and to a nearly complete loss of all cochlear hair cells as well [32].

To the contrary, overexpression of an activated form of Notch1, NICD (Notch-intracellular domain), in non-sensory regions of chick and mouse inner ear leads to the formation of ectopic sensory patches [31, 33–36]. Interestingly, ectopic activation of Notch can only induce ectopic sensory patches within a certain time window of development, suggesting other effectors may contribute to sensory specification [35, 36].

The HMG-box transcription factor Sox2 is thought to specify the auditory prosensory domain. Sensory precursors fail to develop in Sox2-deficient inner ears, leading to loss of hair and supporting cells [37] and forced expression of Sox2 in vivo induces ectopic sensory patches [36].

Sox2 has been suggested to be a downstream target of Jag1-Notch signaling. Sox2 expression is reduced in Jag1 conditional mutants [29] and activation of Notch leads to ectopic Jag1 and Sox2 expression [34, 36], suggesting that Jag1-Notch signaling acts upstream of Sox2.

12.2.2 FGF Signaling and Prosensory Formation

Fibroblast growth factors (FGFs) have been shown to be of critical importance for the development of the organ of Corti. In mice, FGF signaling has been implicated in the early inductive events of the otic vesicle (Chap. 11). In the next phase of cochlear development, at the sensory specification phase, FGF signaling is again thought to be required. Tissue-specific deletion of Fgfr1 results in severe defects in the development of both hair cells and support cells [20]. Subsequent studies have identified the likely ligand for this receptor as Fgf20 [21]. Fgf20 is specifically required for the differentiation of the lateral compartment of the cochlea (which includes OHCs and Deiters'cells) [22]. In vitro rescue experiments indicate that FGF signaling is downstream of Notch, as disruption of prosensory development by the Notch inhibitor g-secretase can be partially rescued by exogenous application of Fgf20 [38]. Interestingly, exogenous application of Fgf20 also restores Sox2 expression indicating that Fgf can independently control Sox2 expression [38]. However, the phenotype of Fgf20 knockout cochlea is relatively mild and the lateral compartment is partially formed [22]. Other ligands may be act for prosensory specification and Fgf10 is a good candidate because of the expression pattern in E11.5–E13.5 prosensory domain overlapping with Jag1 and Sox2 [23] (Fig. 12.4). Fgf10 knockout cochlea is shortened but otherwise normal indicating the possibility of compensation by other FGFs [39].

12.2.3 Bmp4 Signaling and Prosensory Formation

Bmp4 is one of the regulators for development of sensory domain in the cochlea. Bmp4 is expressed adjacent to the developing prosensory domain of the cochlea in cells destined to become Hensen's and Claudius' cells of the outer sulcus [1]. Ohyama et al. showed that BMP signaling is necessary for the development of the outer sulcus and the prosensory domain and that BMP4 suppresses medial markers but promotes lateral markers, suggesting that a gradient of BMP signaling is an important step in patterning the cochlea across its modiolar-to-strial axis.

12.3 Cell-Cycle Exit

Following specification of the prosensory domain, a subsequent important step is cell-cycle exit within this domain. Cells in the presumptive organ of Corti can first be distinguished by cell-cycle exit during mid-embryogenesis. This termination of mitosis occurs sequentially across the length of the cochlea, with cells at the apex exiting first from the cell cycle at E12 and cells at the base terminating mitosis last at E15 [40] (Fig. 12.3).

12.3.1 p27Kip1

The cyclin-dependent kinase inhibitor, p27Kip1, is involved in the maintenance of this nonproliferative state [41] (Fig. 12.3). p27Kip1 is one of the earliest known markers of the presumptive organ of Corti, and its expression correlates with the onset of mitotic exit [41, 42]. In p27Kip1−/− null mutants, hair and supporting cells continue proliferation after E15 resulting in extra hair and supporting cells [41].

p27Kip1 expression overlaps Sox2 expression and, as differentiation advances, Sox2 and p27Kip1 are gradually limited to supporting cells and become undetectable in HCs by birth [29, 37, 41, 43]. p27Kip1 has been shown to keep postnatal supporting cells quiescent [44, 45]. Induced ablation of Sox2 in postnatal inner pillar cells causes p27Kip1 downregulation and proliferation suggesting Sox2 is one of the upstream regulators of p27Kip1 to maintain the quiescent state of postmitotic inner pillar cells [46].

12.3.2 pRb

The retinoblastoma protein pRb is the protein product of Rb tumor suppressor gene and a key cell-cycle inhibitor. pRb is present in all cells in the E12.5 otocyst and its expression is prominent in hair cells during embryonic and adult ages [47]. Deletion of Rb in cochlear prosensory cells produces supernumerary hair cells and supporting cells and later apoptotic hair cell death [48]. Inducible inactivation of Rb in postnatal cochlear hair cells causes cell-cycle reentry and cell death [49]. Inducible inactivation of Rb in postnatal pillar cells and Deiters' cells results in cell-cycle reentry and maintain their supporting cell fate [50].

12.3.3 p21cip1 and p19ink4d

Two additional cyclin-dependent kinase inhibitor, p21cip1 and p19ink4d, are also expressed in the prosensory domain beginning between E14.5 and E16.5 [51, 52] and suggested to maintain the nonproliferative state. While deletion of p21cip1 has no apparent effect of maintenance of mitotic quiescence [52], loss of p19ink4d results in an increasing rate of spontaneous hair cell mitoses beginning in the postnatal period [51]. Deletion of both p19ink4d and p21cip1 results in initiation of mitosis in hair cells beginning on P3 [52].

12.3.4 Wnt Signaling and Lgr5

Wnt/b-catenin regulates proliferation within the mitotic prosensory domain of E12 mouse cochlea, and Wnt activity becomes reduced as development progresses [53]. Later in development, low-level Wnt reporter activity is still maintained at E17in pillar cells and in the third row of Deiters' cells [53].

Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), a Wnt target gene, has been shown to mark endogenous stem cells in rapidly proliferating organs [54, 55]. In the postnatal cochlea, Lgr5 expression is Wnt dependent and limited to supporting cell subtypes [56]. Lgr5-positive cells isolated by flow cytometry from neonatal Lgr5$^{\text{EGFP-CreERT2/+}}$ mice proliferated, formed clonal colonies, and differentiated into hair cells [57, 58]. Moreover, both in vitro and in vivo, Wnt signaling enhanced proliferation of Lgr5-positive cells [58]. These data indicate that Lgr5 marks Wnt-regulated sensory precursor cells in the postnatal cochlea and Wnt signaling can promote their proliferation.

12.4 Cellular Differentiation

Following specification of the prosensory domain and cell-cycle exit, individual prosensory cells become determined to develop as all of the unique cell types within the organ of Corti. The first indication of cellular differentiation within the prosensory domain is inner hair cells observed in the mid-basal region of the cochlea between E14 and E15. Inner hair cell differentiation then proceeds in a gradient that extends toward both the apex and the base of the cochlear spiral [59] (Fig. 12.4). The differentiation of outer hair cells and supporting cells is about 1 day behind inner hair cells and can be observed by E15 to E16.

12.4.1 Hair Cell Differentiation

The earliest known gene expressed in the prosensory domain associated with hair cells is the bHLH transcription factor Atoh1, which is first detected as a gradient that is strongest near the cochlear base and over time extends toward the apex [60, 61]. As development proceeds, Atoh1 expression is restricted to cells that will develop as hair cells.

Several factors that positively or negatively regulate Atoh1 have been identified. Sox2, which is expressed in all prosensory regions, is required for Atoh1 expression. However, Sox2 also seems to negatively regulate Atoh1, as prolonged expression of Sox2 inhibits the ability of Atoh1 to induce hair cell formation, whereas decreased expression of Sox2 leads to an increase in hair cell formation [43]. Other regulators of Atoh1 are the Ids (inhibitors of differentiation), a family of bHLH-related genes that act as antagonists of other bHLH genes [62]. Three of the four mammalian Id genes, Id1, Id2, and Id3, are broadly expressed in the developing cochlear duct but become downregulated in cells that will develop as hair cells [63]. Moreover, forced persistent expression of Id3 leads to an inhibition of hair cell formation, indicating that Ids act to negatively regulate Atoh1 [64].

Lateral inhibition of the Notch signaling pathway is involved in the regulation of hair cell development and Atoh1 expression. Within the inner ear, localization studies showed expression of Notch1 throughout the epithelium and expression of two Notch ligands, Jagged2 and Delta-like1, in developing hair cells [65, 66]. Moreover, several inhibitory bHLHs, including Hes1, Hes5, and Hey1, are expressed in developing supporting cells [2, 61, 67–69]. Deletion of different members of Notch signaling pathway results in varying increases in the number of hair cells, an effect that is very consistent with classic Notch-mediated lateral inhibition [65, 67, 68, 70–72].

12.4.2 Supporting Cell Differentiation

In the cochlea supporting cells take on several unique morphologies. Though the nature and developmental regulation of supporting cells is less well understood in comparison with hair cells, the FGF signaling pathway has been shown to regulate the formation of the pillar cells that give rise to the tunnel of Corti. Before pillar cell formation, Fgfr3 is expressed in the population of cochlear progenitor cells that will develop as pillar cells, outer hair cells, and Deiters' cells [73, 74]. At the same time, developing inner hair cells express FGF8, a ligand with a strong binding affinity for FGFR3. Deletion of either Fgf8 or Fgfr3 leads to a defect in pillar cell development, whereas deletion of Sprouty2, a molecule that has been shown to act as an FGF antagonist and is expressed in the cochlea, leads to an overproduction of pillar cells [75]. These results are consistent with the hypothesis that FGF8 secreted by inner hair cells binds to and activates FGFR3 in adjacent cells, leading to the formation of pillar cells (Fig. 12.4).

In addition to inducing prosensory cells to develop as pillar cells, FGF8/FGFR3 signaling also acts to prevent these same cells from developing as hair cells [76, 77]. This effect is mediated through activation of the inhibitory bHLH Hey2, a gene that is normally regulated through the Notch pathway. Interestingly, deletion of Hey2 alone does not lead to any changes in cell fate, but when Hey2 is deleted along with inhibition of Notch signaling, pillar cells will convert into outer hair cells [69].

12.4.3 Temporal Regulation of Cellular Differentiation

Cell-cycle exit in the cochlea occurs from the apex in an apical-to-basal gradient, while cellular differentiation begins from the base around E14.5, just after the cell-cycle exit has completed. It means that prosensory cells located in the apex of the cochlea that become postmitotic have to be undifferentiated until the cellular differentiation wave comes from the base. Recent works showed that this unique temporal pattern of sensory cell differentiation requires Hh signaling and the source of the signaling is Sonic hedgehog (Shh) from adjacent spiral ganglion [78–80]. In vivo activation and inactivation of the Hh effector smoothened (Smo) in the developing cochlear epithelium after prosensory domain formation revealed that Hh signaling inhibits prosensory cell differentiation into hair cells or supporting cells and maintains their properties as prosensory cells [81]. Smo conditional knockout (Smo CKO) mice showed that hair cell differentiation was preferentially accelerated in the apical region of the cochlea and exhibited hair cell disarrangement in the apical region, a decrease in hair cell number, and hearing impairment [81]. These results indicate that Hh signaling determines the timing of cellular differentiation and the basal-to-apical wave of hair cell development is required for the proper differentiation, arrangement, and survival of hair cells and for hearing ability [81].

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Chapter 13
Vestibular Development

Hiroko Torii and Akiko Taura

Abstract The vestibule consists of the semicircular canals and otolithic apparatus, which perceives the sense of equilibrium. The vestibule derives from the otocyst, which is mimicked by signaling information from its surrounding tissues to acquire its special disposition along three axes. The three semicircular canals and their cristae are derived from two evaginations of the otocyst. As the canal pouches increase in size, the opposing epithelia in the central portion of the structures merge toward each other to form a fusion plate. The fusion plates are eventually resorbed and the remaining edge of the pouch develops into a semicircular canal. The otolithic apparatus, which has characteristically uniform sensory epithelia, is also derived from the otocyst.

In this section, an overview of vestibular development and the molecular mechanism is described. Most of the data are from experiments using mouse, which has the most comprehensive model system for the developing mammalian cochlea.

Keywords Vestibule • Semicircular canals • Otolithic apparatus • Inner ear development

13.1 Introduction: Anatomy of the Vestibule

The inner ear consists of the cochlea and vestibule. The vestibule is adjacent to the cochlea and is the organ that perceives the sense of equilibrium. The vestibule consists of the semicircular canals and otolithic apparatus. The former has three orthogonally arranged canals and associated sensory structures or the ampullae,

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which house the sensory tissue, referred to as the cristae. The semicircular canals consist of an anterior, posterior, and horizontal semicircular canal, and working together they function to perceive angular head movements. The otolithic apparatus consists of the utricle and saccule, which together sense linear acceleration and gravity. Additionally, they have sensory epithelia called maculae. Both the cristae and maculae have hair cells (HCs) and they contribute to detecting acceleration with HCs of cristae lining one united polarity in contrast to the maculae of vestibule which contain a reversal zone, or line of polarity reversal (LPR), where orientation of the bundles can be rotated by 180°. The uniform alignment of a cellular structure within a plane of epithelial cells is referred to as planar cell polarity (PCP) [1, 2].

13.2 Morphological Development of the Vestibule

13.2.1 Early Stage Development of the Vestibule

This otic epithelium is influenced by signaling information from its surrounding tissues in acquiring its special orientation along the anterior–posterior (AP), dorsal–ventral (DV), and the medial–lateral (ML) axes. Wnts secreted from the dorsal hindbrain as well as sonic hedgehog (Shh) secreted from the ventral floor plate and notochord have been implicated in the alignment of the inner ear DV axis. Based on the Wnt1/Wnt3a double-mutant phenotypes, Wnts from the dorsal hindbrain are important extrinsic signals for the canal and crista formation [3]. Dlx5, in which one of the downstream genes responds to Wnt signaling, and Dlx5 deficiency affects the canal and crista formation [3–6]. Hmx3, which is also required for canal formation [7, 8], appears to be regulated by FGF rather than Wnt signaling [3, 9]. Shh from the ventral hindbrain and notochord is also required for canal formation. Due to deficiency in the lateral canal, the shape of the anterior and posterior canals becomes abnormal in the Shh−/− mutants. However, another study has suggested that this requirement of Shh for canal formation is a secondary effect, because the inner ears of mice with conditional knockout of smoothened within the otic epithelium have normal canals [10].

The mesenchyme surrounding the developing inner ear is important for shaping the canals. Replacing the otic mesenchyme (anterior to posterior) results in inner ears having a posterior canal and cristae with anterior characteristics [11]. Although the molecules involved are not known, studies in mice have implicated several mesenchymal genes, such as Pou3f4 (also known as Brn4) and Prx [12–14] in canal formation.

Fate mapping studies in chickens have shown the endolymphatic duct is derived from the dorsal region of the otic cup, whereas cells in the three semicircular canals are derived mostly from the posterolateral region of the otic cup [15].

Cells fated to develop as neurons delaminate from the anteroventral region of the otic epithelium, migrate short distances, and then coalesce as neurons of the cochleovestibular ganglion (CVG). Cells fated to give rise to sensory patches, referred to as prosensory cells, develop as sensory HCs and supporting cells.

13.2.2 Development of the Semicircular Canals

The three canals and their cristae are derived from two evaginations of the otocyst, viz., the vertical and horizontal canal pouches. The former gives rise to the anterior and posterior canals, while the latter forms the lateral canal. As the canal pouches increase in size, the opposing epithelia in the center portion of the structures merge with each other to form a fusion plate. Cells forming the fusion plate eventually are resorbed [16], and the remaining edge of the pouch develops into a tube-shaped canal. The common crus, a connecting structure between the anterior and posterior canals, is then formed as a result of this resorption process. Fate mapping the rim of the vertical canal pouch in chicken embryos has suggested that a majority of the cells contributing to canal formations originate from an area adjacent to the presumptive cristae, or the canal genesis zone [17]. Most cells in the canal pouch give rise to the common crus or disappear during resorption. Although the hypothesis that sensory tissues induce the formation of nonsensory structures is gaining support [18], our understanding of the molecular pathways remains incomplete. It is believed to involve Fgf and Bmp4 genes emanating from the presumptive cristae and Bmp2, which is expressed in the canal genesis zone [17, 19–21]. Another study has demonstrated that the vertical canal pouch responds to BMP signaling by changing the cells on its dorsolateral wall from a columnar to a squamous shape, thus expanding and increasing the size of the pouch [22]. Whether these cells respond to BMP signaling secreted from the hindbrain or within the otic epithelium, or both, is not clear. But specification of the canal tissue also requires the activities of various transcription factor genes, including dlx5, hmx2/3, lmo4, otx1, and sox10 [4, 6, 7, 23–30].

Furthermore, the molecular mechanism of the resorption process is also poorly understood, even though genes such as Netrin1 and Fgf9 are involved [31]. Considering the complexity of this developmental process and the many genes involved in the regulation of canal formation, it is often difficult to pinpoint the specific roles for those genes based on phenotype alone [32]. For example, the lack of a proper crista specification certainly affects canal formation [20, 33]. Failure to specify the canal pouch or the rim of the canal pouch also yields no canal formation. Furthermore, a reciprocal inhibition between the prospective canal and resorption regions has been shown [34]. Lrig3, an immunoglobulin superfamily transmembrane protein, has been shown to negatively regulate Netrin1 expression in the resorption domain. In Netrin1 mutants, Lrig3 expression remains expanded and is not properly restricted to the rim of the canal pouch, suggesting that Netrin1 also negatively regulates Lrig3. Based on this type of reciprocal inhibitory relationship between the

rim and central regions of the canal pouch, aberrant regulation of these domains could lead to a range of phenotypes, including no resorption, canal truncation (excessive resorption), or canals with larger or smaller calibers. Mice with truncations or thinning of canals commonly display behavioral deficits [35–37].

13.3 Epithelial Formation of the Vestibule

In the vestibular epithelial development, Notch signaling and its related genes have important roles. Many of genes, such as Lfng, Jag1, HES5, HES1, Math1, Delta1, and Jag2, are finally expressed in all sensory epithelia of the inner ear, although some of their expressions are restricted in vestibular epithelia at certain periods [38]. At E12, Jag1 is expressed in six regions, corresponding to the developing saccule, utricle, three cristae, and cochlea [39]. In the rudimentary cochlear duct, Jag1 transcripts are expressed in a broad band that extends the very short distance between its basal and apical turns. Transcription for Dll1 and HES5 is concurrently restricted to the developing cristae, while Math1 transcripts are only faintly detectable in the saccule [39–41]. By E13-14, Jag1, Lfng, and Math1 are expressed in all the sensory end organs of the ear, albeit HES5 and Dll1 expressions remain restricted to the vestibular epithelia, which are then beginning to assume their mature configuration. A third Notch ligand, Jag2, is detectable in the vestibular epithelia as well. As development progresses from mid to late gestation (E14 to 15), Lfng and Jag1 continue to be expressed in both the vestibular and auditory organs.

In the developing cristae of the mouse, hair bundles at the apex of the crest are usually more mature than those around its base, as might be expected from the pattern described for HC birth dates in these organs. Similarly, hair bundles in the maculae are more developmentally advanced in the central striolar portion than in the surrounding extrastriolar region, although immature bundles are seen alongside more mature ones in both regions.

13.4 Hair Cell Polarity Formation of in the Vestibule

The morphological characteristics of vestibular HCs and their organization within the vestibular maculae suggest that planar polarity is represented at three distinct anatomical scales (Fig. 13.1). Subcellular planar polarity is the polarized structure of the stereociliary bundle and the position of the kinocilium is unilateral of the apical cell surface in each HC (Fig. 13.1). Planar cell polarity (PCP) is manifest in the orientation of polarized cells within the two-dimensional surface of the epithelium. In the vestibular maculae, PCPs are evident in the coordinated orientation of stereociliary bundle polarity between neighboring cells (Fig. 13.2). Tissue polarity is the largest spatial scale within the organs or entire organism. In the maculae, tissue polarity can be observed in the division of vestibular HCs into two groups which are patterned around the LPR (Fig. 13.3). The developmental mechanisms regulating tissue polarity are the least understood [2].

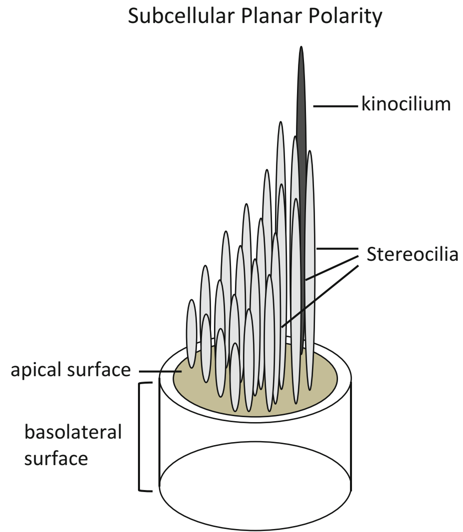

Fig. 13.1 Subcellular planar polarity. (Modified from Deans [2])

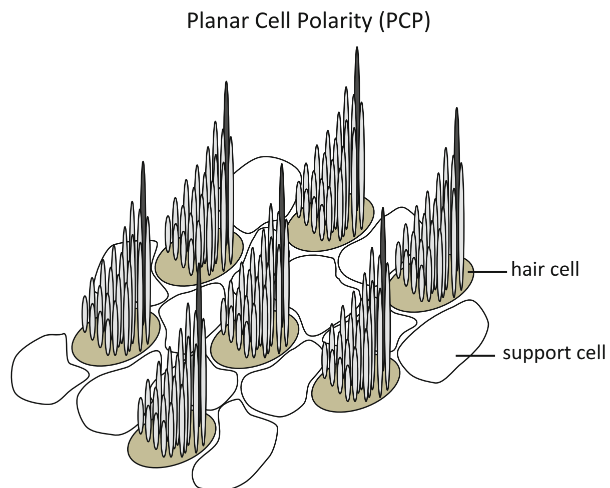

Fig. 13.2 Planar cell polarity (PCP). (Modified from Deans [2])

13.4.1 Subcellular Planar Polarity

At E12.5, the first HCs can be distinguished, and a single cilium emerges from the center of the cell surface. The cilium is surrounded by elongated microvilli [42, 43], and it lengthens to form the kinocilium to subsequently establish subcellular planar polarity as the cilium migrates to one side of the apical cell surface.

Polarization of the stereociliary bundle is cell intrinsic and occurs independently from mechanisms directing planar polarity at the level of PCP or tissue polarity. As described below, auditory and vestibular HCs in vangl2 knockout [44] and frizzled

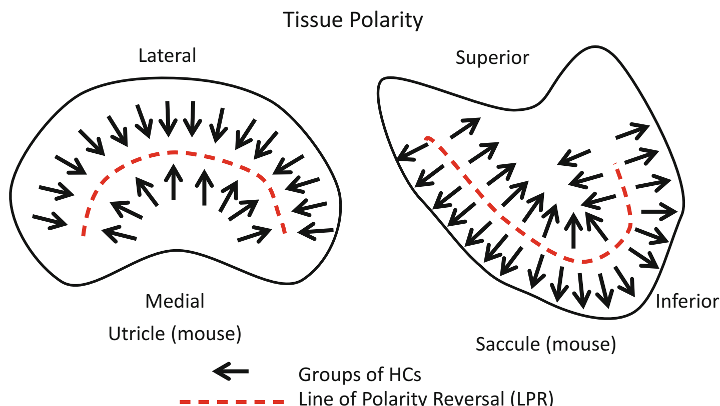

Fig. 13.3 Tissue polarity. (Modified from Deans [2])

3/6 double knockout mice [45] are incorrectly oriented relative to the neighboring cells, although their mutant HCs still form polarized stereociliary. Fewer mutations only affect subcellular planar polarity, while a conditional knockout mouse disrupting the function of IFT88/Polaris is an essential factor for intraflagellar transport and cilium formation [46]; protocadherin 15 mutants [47] have been reported to disrupt subcellular planar polarity construction.

Some of the molecular signals that direct formation of the polarized bundles are beginning to be uncovered. The function of P21-activated kinase PAK asymmetrically localized in the vicinity of the kinocilium [48] is further regulated by the small GTPase Rac1 which is itself a well-characterized regulator of actin dynamics [49]. GTPase Rac1 is, in fact, involved in certain functions of P21-activateed kinase PAK. The Rac1-PAK signaling pathway is also dependent upon the function of Kif3a. The Rac1-PAK signaling pathway is also dependent upon the function of Kif3a [50], a component of the Kinesin II motor complex that is necessary for plus-end microtubule trafficking and anterograde intraflagellar transport [51]. However, Kinesin II, whose identity is still unknown, is believed not to be the motor factor that translocates the kinocilium.

13.4.2 Planar Cell Polarity and the Coordinated Orientation of Adjacent HCs

Many essential molecules regulating this level of planar polarity have been identified through genetic screening in *Drosophila*, including the core PCP proteins Frizzled, Dishevelled, Van Gogh, Prickle, Diego, and Flamingo. The subcellular distributions of core PCP proteins are likely conserved between the mouse and *Drosophila* at the line of polarity reversal (LPR). Core PCP proteins are asymmetrically localized in the epidermal cells of developing *Drosophila* wings with

Frizzled and Dishevelled enriched at the distal cell boundary near the site of hair formation and Van Gogh and Prickle enriched at the proximal side. Flamingo is present at both cell boundaries. In the developing mouse utricle the core PCP proteins Frizzled6 (Fz6) and Prickle-like 2 (Pk2) are located on opposite sides of HCs and supporting cells: a pattern similar to the relative distribution of Frizzled and Prickle in *Drosophila*, suggesting that other PCP proteins are similarly distributed. The remaining core PCP proteins in mice are asymmetrically localized at the cell boundaries; however, proximal versus distal distributions have not been established. Pk2 is enriched at the same side of vestibular HCs, regardless of the stereociliary bundle orientation or cellular position relative to the LPR [52–56].

13.5 Innervation of Vestibular Hair Cell

Each vestibular afferent neuron has its cell body in the cochleovestibular (CV) ganglion and sends a peripheral axon toward the vestibular sense organs and a central axon to the vestibular nuclei in the brainstem. The CV ganglion consists of two cell clusters extending in a rostral–caudal direction in the internal auditory canal. It consists of a superior and inferior cell group related to the superior and inferior divisions of the vestibular nerve trunk, respectively. The superior division supplies the cristae of the superior and lateral canals, the macula of the utricle, and the anterosuperior part of the macula of the saccule. The inferior division supplies the crista of the posterior canal and the main macular portion of the saccule. The inferior division supplies the crista of the posterior canal and the main portion of the macula of the saccule. Medial to the vestibular ganglion, the nerve fibers of both divisions merge to form a single trunk, which enters the brain stem [57].

As one of the cranial peripheral nerves, the CV nerve is composed of both neurons and glia [58]. These two cell types arise developmentally from distinct sources, viz., the glial cells derived from neural crest cell (NCC) progenitors [59–61], while the neurons originate almost exclusively from the otic placode [59, 62, 63].

Apart from projecting central axons to the hindbrain, CV neurons also project peripheral neuritis to sensory targets developing within the otic epithelium [64]. The glial cells of the CV nerve are derived from NCCs that emigrate from the hindbrain at the level of rhombomere 4 (R4).

CV neurons extend peripheral neuritis to the developing vestibular sensory epithelium as early as E11.5 and extend central axons to the hindbrain as early as E12.5 [64, 65] and release some important factors related this neural development. In mice, disturbance of ERBB2 (a receptor that mediates neuron–glia interactions via the ligand Neuregulin1 [66]) compromises development of the CV nerve. Loss of ErbB2 results in altered migration of CV neuronal cell bodies, abnormal targeting of CV peripheral neurites, and reduced number of CV neurons [67]. Molecularly blocking Semaphorin/Neuropilin signaling in chicks disrupts

NCC migratory pathways and impairs the inward movement of epibranchial placodal neurons [68]. In certain studies, ablation of NCC migration by physical or molecular methods in chicks results in reduced numbers of neuroblasts migrating from the epibranchial ganglia and abnormal projection of central axons [69–71].

The maculae contain two types of HCs that are distinguished from each other by the synaptic structure formed with the afferent neurons. Type I HCs receive large calyx nerve endings that surround the cell body, while type II HCs make bouton-like contacts with nerve endings. The two HC classes are innervated by three types of afferent neurons. The calyx-only class of neurons contacts clusters of type I HCs in the striola region. Dimorphic neurons contact HCs throughout the sensory epithelia, forming calyxes with type I HCs and boutons with type II HCs. Bouton-only afferents only contact type II HCs located outside the striola. Each class of afferent neurons may contact multiple HCs, and the dimorphic and bouton-only neurons contact groups of HCs located on either side of the LPR. Despite the range of bundle orientations present in the utricle, afferent neurons only contact HCs with similar stereociliary bundle orientations. Although developmental mechanisms coordinating neuronal innervation and stereociliary bundle orientation are not well known, there is a single cell type that morphologically resembles the mature type II HCs prior to the obvious distinction of the two HC types. From the later appearance of morphologically distinct type I HCs during regeneration in the avian utricle, it has been suggested that type I HCs differentiate from the type II HCs in a serial progression of development. However, earlier studies of spatiotemporal patterns of HC birth have suggested that HCs in regions where type I HCs predominate in the mature sensory epithelium are born first, while hair cells in those regions where type II HCs predominate are born later, indicating that types I and II cells may be birth-specific.

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Part III
Cochlear Implants

Chapter 14
Cochlear Implant: Past, Present, and Future

Hiroshi Yamazaki

Abstract Cochlear implantation is accepted as an effective treatment to restore auditory perception in patients with bilateral severe to profound sensorineural hearing loss. During the early years of cochlear implantation, postlingually deaf adults with normal cochlear anatomy received a cochlear implant (CI); however, the frequency of cochlear implantation in congenitally deaf children, children with inner ear malformations, and children with multiple disabilities is gradually increasing as clinical reports demonstrating the safety and efficacy of CIs accumulate. Many studies show acceptable outcomes in these challenging populations. However, CI outcomes are often poor in patients with cochlear nerve deficiency (CND), which is defined by a small or absent cochlear branch of the vestibulocochlear nerve on magnetic resonance imaging, probably due to an insufficient number of spiral ganglion neurons (SGNs). Patients with CND who showed no improvement in auditory performance after cochlear implantation may be good candidates for auditory brainstem implant (ABI); however, patients with CI can maximally utilize the simple linear tonotopic organization in the cochlea and natural sound processing mechanisms in the cochlear nucleus which are theoretically more suitable for speech processing than direct stimulation at the brainstem by ABI. Thus, (re)generation SGNs combined with cochlear implantation, which may enhance CI-mediated stimulation, can be effective to improve auditory performance in patients with CND.

Keywords Bilateral cochlear implantation • Cochlear implant • Cochlear nerve deficiency • Inner ear malformation • Psychoneurological disorder

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14.1 Introduction

A cochlear implant (CI) is a surgically implanted electronic device designed to provide auditory sensation to patients with sensorineural hearing loss (SNHL). Cochlear implantation is accepted as an effective treatment to restore auditory perception in patients with bilateral severe to profound SNHL [1, 2]. SNHL is usually caused by dysfunction of or damage to cochlear hair cells. The CI bypasses the affected hair cells and electrically stimulates spiral ganglion neurons (SGNs), which are the secondary neurons, to provide afferent input to the auditory central nervous system. The CI is a highly successful artificial medical device that has brought the world of sound to and improved quality of life of hundreds of thousands of people since its inception. The 2013 Lasker-DeBakey Clinical Medical Research Award honored Graeme Clark, Ingeborg Hochmair, and Blake Wilson, three visionaries who contributed greatly to the development of the modern CI [3].

In this chapter, the general concepts of the CI are briefly explained, followed by a review of the clinical history and recent trends in cochlear implantation.

14.2 Overview of the Cochlear Implant System

A CI is composed of external and internal parts; the external part of the device includes a microphone, speech processor, and transmitter, while the internal part is implanted under the skin behind the auricle and consists of a receiver/stimulator and an electrode array. The speech processor analyzes the sounds collected by the microphone and sends electrical signals to the internal device through the transmitter. The receiver/stimulator, driven by the signals from the external device, activates electrodes that in turn stimulate the neural tissue in the cochlea to elicit auditory sensation. The design of a CI, the number of electrodes, and the shape of the electrode array differ depending on the manufacturer and the type of CI (including differences between devices made by the same company); however, all CIs share common fundamental concepts. The CI is designed to bypass affected hair cells and directly stimulate the SGNs. The normal cochlea exhibits exceptional capacity for sound analysis in terms of both frequency and intensity that is mainly achieved by the characteristic features of the basilar membrane, including the well-known tonotopic organization along the basal-apical axis [4] (Fig. 14.1). The CI is designed to reproduce this tonotopy by sequential frequency-electrode allocation along the electrode array in which the distal and proximal electrodes correspond to low- and high-frequency sounds, respectively. In the normal cochlea, characteristic features of the basilar membrane largely determine the best vibration position and the amplitude of the vibration [4], while in the CI, the speech processor determines these parameters on the basis of the coding strategies [1, 2].

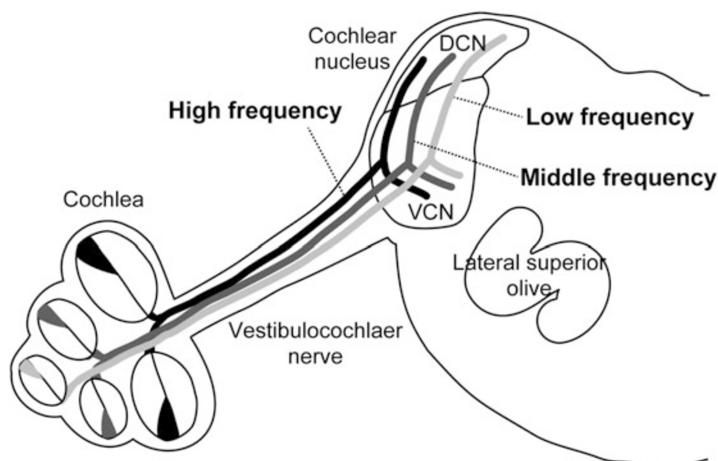

Fig. 14.1 Tonotopic organization in the cochlea and the cochlear nucleus. *Black*, *dark gray*, and *light gray lines* represent high, middle, and low frequencies, respectively. In the cochlea, a simple linear organization of the tonotopic map is observed, while the tonotopy in the cochlear nucleus has three-dimensional organization in which the characteristic frequency also changes in vertical direction to the surface of the brainstem

14.3 History of Cochlear Implants

According to written reports, attempts to treat deafness by electrical stimulation began in the eighteenth century. Benjamin Wilson and Alessandro Volta both used extra-auricular electrical stimulation to produce auditory sensation (in 1748 and 1800, respectively). The primitive electrical stimulation elicited disagreeable shocks in the head with some "auditory" sensation [5]. From 1940 to 1950, several clinical trials demonstrated that electrical stimulation at the promontory in the middle ear and direct stimulation of the auditory nerve provided some auditory sensation [5, 6]. André Djourno and Charles Eyriès, pioneers in this field, implanted an electronic neuronal stimulator in a deaf patient in 1957. Using the implanted device, the patient could discriminate between low and high frequencies and differentiate the intensity of the stimulation [1]. Djourno and Eyriès' device stimulated the residual stump of cranial nerve VIII following temporal bone resection in patients with large bilateral cholesteatomas [7]. This patient could not understand speech, but the results of this study inspired the clinicians and researchers who followed, and their work is usually referred to as the first cochlear implantation [1]. In 1961, William House and John Doyle performed single-channel cochlear implantation in two deaf patients by inserting a gold wire electrode into the scala tympani. Electrical stimulation of this single-channel electrode provided some auditory sensation to these patients [1]. Thereafter, a single-channel CI began to be implanted in deaf patients, mainly in the United States, and data supporting the safety and effectiveness of the single-channel CI began to accumulate, even though speech discrimination without lip

reading was difficult for patients with the device. Food and Drug Administration (FDA) approval for CIs was obtained in 1985. In 1984, Clark developed the popular multichannel implant with bipolar stimuli. The first pediatric cochlear implantation was performed by House in 1987 [1].

14.4 Historical Changes in Candidacy for Cochlear Implantation

During the early years of cochlear implantation, postlingually deaf adults with normal cochlear anatomy received CIs, but the frequency of cochlear implantation in congenitally deaf children, children with inner ear and/or internal auditory canal (IAC) malformations, and children with multiple disabilities gradually increased as clinical reports demonstrating the safety and efficacy of CIs accumulated. In this section, we focus on historical changes and recent trends in candidacy for cochlear implantation, as well as CI outcomes in challenging candidates, as we consider the effectiveness and limitations of CIs.

14.4.1 Age at Implantation

As mentioned above, the first pediatric CI surgery was performed in 1987, a quarter-century after the first adult CI surgery in 1961. The results of pediatric cochlear implantation demonstrated that CI-mediated auditory stimuli promoted speech and language development, even in prelingually deaf children who would have been obliged to use visual languages (such as sign language) without a CI. Several studies revealed that a younger age at implantation resulted in better language development [8]. Functional brain imaging studies demonstrated that visual stimuli (lip reading) increased regional blood flow and glucose metabolism in the auditory association area in deaf patients, an effect that was not observed in subjects with normal hearing [9, 10]. Interestingly, the congenitally deaf children who had used their CI for a long-term with appropriate auditory-verbal rehabilitation showed cortical activities similar to those of control subjects [9, 10]. These data suggest that CI-mediated auditory input prevents abnormal cross-modal reorganization in the temporal lobe in deaf children. Sharma et al. demonstrated that P1 latency, which is the indicator of maturation of the auditory system, was significantly shortened in children who underwent cochlear implantation at an early age when compared with children who underwent implantation after age 3.5 years [11]. Previous research identified the critical period (or sensitive period) in other primary sensory cortices such as a visual and somatosensory system [12], and Sharma's study, using an electrophysiological approach in deaf children with CIs, clearly demonstrated that a critical period also exists in the auditory system.

Based on results from these studies, age at cochlear implantation is becoming lower in the congenitally deaf population. In 1990, the FDA lowered the approved age for implantation to 2 years, then to 18 months in 1998, and, finally, to 12 months in 2000. From the viewpoint of mimicking the normal auditory experience during infancy, earlier implantation might be better to achieve sufficient auditory neural development. However, general anesthesia and operations in children less than 6 months old require special precautions [13], and precise evaluation of hearing level is usually difficult in infants. Therefore, cochlear implantation between ages 6 and 12 months might be most practical.

14.4.2 Bilateral Cochlear Implantation

Binaural hearing provides head shadow, squelch, and summation effects to improve speech discrimination in noise and is essential for sound localization to detect interaural time differences (ITD) and interaural level differences (ILD) [14]. Early in the history of cochlear implantation, improvement of speech discrimination in quiet was the main goal for implanted patients, but in recent years, the situation has changed dramatically. Significantly more congenitally deaf children with a CI attend mainstream kindergartens and schools, and these children have to listen to and understand speech sounds in noise. The single-sided CI user can use the head shadow effect, but this is usually not sufficient to facilitate learning at the same rate as students with normal hearing in noisy classroom conditions. Many studies have demonstrated that patients with bilateral CIs have better speech discrimination scores and sound localization ability in noise than patients with unilateral CIs, even though acquisition of these abilities seemed to depend on the age at second implantation and the time gap between the first and second operations [14]. The European Bilateral Pediatric Cochlear Implant Forum Consensus Statement, published in 2012, recommended that a deaf infant or child should receive bilateral CIs simultaneously as soon as possible after definitive diagnosis of deafness to permit optimal auditory development [15]. Even though some countries have not established an environment supporting bilateral cochlear implantation due to lack of financial support and health insurance coverage, simultaneous bilateral cochlear implantation at less than 1 year of age is the global trend in treatment for congenital deafness.

14.4.3 Inner Ear and Internal Auditory Canal Malformations

Inner ear malformations account for about 20–30 % of congenital severe and profound hearing loss. Although identification of a cochlear malformation was once considered a contraindication for cochlear implantation [2] due to the high

incidence of cerebrospinal fluid (CSF) gushers, facial nerve abnormalities, and poor CI outcomes, many children with an inner ear malformation currently undergo cochlear implantation [16, 17]. In 1987, Jackler et al. were the first to propose a classification system for inner ear malformations based on the hypothesis that termination of ordinary inner ear development leads to inner ear malformations. According to Jackler's classification, inner ear malformations are categorized into Michel deformity (labyrinth aplasia), cochlear aplasia, common cavity deformity (CC), cochlear hypoplasia, and incomplete partition, corresponding to each stage of inner ear development [18]. Later, Sennaroglu developed Jackler's classification system and further divided cochlear hypoplasia and incomplete partition into cochlear hypoplasia (CH) types I–III and incomplete partition (IP) types I–III, respectively [17, 19]. Sennaroglu's classification is used in many populations because of its effectiveness to predict surgical problems during implantation and postoperative CI outcomes [17, 20]. For example, patients with Michel deformity are not candidates for cochlear implantation because of no space for electrode insertion. IP-I, IP-III, CH-II, and a part of CC are susceptible to CSF gusher due to a communication between the IAC and the malformed inner ear, which is highly associated with the absence of the modiolus. On the other hand, cochlear implantation in patients with IP-II and large vestibular aqueduct syndrome is usually associated with good hearing outcomes. In addition to inner ear malformations, IAC malformations are also important because small diameters and stenosis of the IAC or bony cochlear nerve canal (BCNC) are highly associated with aplasia or hypoplasia of the cochlear branch of the vestibulocochlear nerve, also known as cochlear nerve deficiency (CND), which has a negative impact on CI outcomes [21]. When the diameter of the IAC or BCNC is smaller than 2 or 1.5 mm, respectively, it is diagnosed as narrow IAC (NIAC) [17] or hypoplasia of BCNC (HBCNC) [22]. In these groups, CI-mediated auditory response is usually poor because CND is associated with an insufficient number of SGNs, which are the target neurons for CI-mediated electrical stimulation. Therefore, patients with NIAC or HBCNC, in addition to those with Michel deformity and cochlear aplasia, may be candidates for an auditory brainstem implant (ABI), which is further discussed in Chap. 19.

Radiographic examinations using high resolution computed tomography (CT) and magnetic resonance imaging (MRI) are powerful tools to evaluate anatomical features of the inner ear and abnormalities of the cochlear nerve, but they cannot examine the physiological functions of the auditory system. In the population with inner ear and/or IAC malformations, especially CC or CND, CI outcomes vary widely even if the patients show similar radiographic findings [21, 23]. During this decade, several groups have proved the effectiveness of electrically evoked auditory brainstem response (EABR) testing, using the implanted device for prediction of CI outcomes as well as optimization of the electrode array placement and programing parameters in children with CC or CND [23–25]. The combination of radiographic and electrophysiological evaluations may be important to achieve the better CI outcomes in these populations.

14.4.4 Associated Psychoneurological Disorders

The incidence of psychoneurological disorders, such as intellectual disability, is higher in deaf children than in children with normal hearing [26, 27]. Congenital cytomegalovirus infection, which accounts for 25 % of congenital severe and profound hearing loss, also contributes to the high proportion of psychoneurological disorders in hearing-impaired children [28]. In patients with associated psychoneurological abnormalities, CIs can bypass the damaged hair cells in the inner ears, but cannot compensate for disorders of retrocochlear higher brain functions. Therefore, similarly to inner ear and IAC malformations, deaf children with developmental disorders (including psychoneurological disorders) were excluded as candidates during the early years of cochlear implantation. Recently, several studies demonstrated that cochlear implantation improves auditory performance and language development in patients with psychoneurological disorders, but the extent of the improvement is limited in comparison with implanted children without additional disorders [26–28]. However, it should be emphasized that the accumulated experiences of cochlear implantation in children with multiple disabilities, along with the development of rehabilitation programs for these patients, indicate that cochlear implantation definitely contributes to improving the quality of life for deaf children with multiple disabilities and their families [28].

14.5 Future Prospects

As CIs were proved to be safe and effective, candidacy for cochlear implantation was extended to patients with SNHL of various etiologies and most of these patients benefited from having a CI. In patients with CND, however, CI-aided auditory performance is often poor. This might be because the number of SGNs is not enough to elicit sufficient activation at the auditory brainstem in these patients. As described in Chap. 19, in spite of ongoing attempts to directly stimulate the central auditory system through auditory brainstem implantation, the ABI outcomes are also insufficient in these groups. The simple linear tonotopic organization in the cochlea and the natural sound processing mechanisms in the cochlear nucleus might contribute to the favorable outcomes for cochlear implantation. These speculations suggest that (re)generating approach for SGNs, in addition to standard cochlear implantation, may be effective to improve auditory performance in these challenging cases. For example, if pharmacological, cellular, or gene therapy approaches into the scala tympani during cochlear implantation can promote extension of the peripheral SGN fibers around the electrode array or induce (re)generation of SGNs, these anatomical changes may contribute to improvement of CI outcomes by decreasing impedance and widening the dynamic range in each electrode.

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Chapter 15
Recent Progress in Cochlear Implant

Harukazu Hiraumi

Abstract In modern cochlear implants, the electrode array has a small volume, is less stiff, and is positioned to prevent traumatic contact with the fine structure of the cochlea. With these improvements, the electrode array causes less trauma, and preservation of residual inner ear function is possible. Newly developed speech coding strategies are based on the psychology of hearing. The fine structure of speech is reproduced by mimicking the temporal excitatory patterns of inner hair cells. The role of the virtual channel is to excite the spiral ganglion neurons located between two electrode contacts by controlling the current spread from two neighboring electrodes. The psychoacoustic masking model is also utilized in channel selection. This model considers the physiological masking effect and reduces the number of stimulated channels without deteriorating the speech understanding. Due to the development of atraumatic electrode arrays, residual hearing at low frequencies can be preserved after cochlear implantation. The remaining hearing can be utilized to transmit low-frequency sounds, and the spiral ganglion neurons in the basal turn, which transmit high-frequency sounds, can be electrically stimulated (electric acoustic stimulation). Bilateral cochlear implantation is also a new trend. The use of two implants improves sound source localization and speech understanding in noisy environments.

Keywords Bilateral cochlear implantation • Electric acoustic stimulation • Electrode array • Speech coding strategy

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15.1 Introduction

The human cochlea contains approximately 3,500 inner hair cells, which are activated independently. A cochlear implant (CI) has a limited number of electrodes inside the cochlea, and they cannot be active at the same time. The auditory information available to CI recipients is much less than that of the normal-hearing population; therefore, until recently, the goal of cochlear implantation was to understand human speech in a silent environment. Now, this goal is accomplished in most cases with the use of multichannel electrodes and sophisticated speech coding strategies. In postlinguistically deaf CI recipients, the average speech intelligibility score was 41–66 % for consonants and 70–97 % for sentences in quiet conditions [1, 2]. The performance of prelinguistically deaf CI recipients is diverse because of various comorbidities. In children with normal development, the average speech intelligibility score in quiet conditions was 70 % for consonants and 84 % for sentences [3]. Currently, the goal of CI is to allow natural hearing.

15.2 Recent Progress in Electrodes

The emergence of multichannel electrodes has dramatically improved speech perception compared with single-channel electrodes. All currently available CIs are equipped with 12–22 intracochlear electrodes. A larger number of electrodes do not result in better speech understanding in the present CI systems. Scores on tests of consonant identification in quiet conditions saturate at 3 electrodes, and scores for the identification of consonants presented in competition with noise saturate at 5 electrodes. Scores for the recognition of sentences or words either in quiet or in competition with noise do not increase significantly with increases in electrode number beyond 6 [4]. This limitation is considered to be mainly due to current spread. The entire length of the electrode array is fixed because the length of the cochlea is limited. Therefore, an increase in the number of electrodes results in a decreased distance between the electrodes. The electrodes are not sufficiently electrically insulated, and two neighboring electrodes in an electrode array that is too condensed stimulate the same set of neurons because of the current spread. Therefore, current CI electrodes do not attempt to increase the number of electrodes. Instead, recent CI electrode arrays focus on decreasing the insertion damage to inner ear structures.

The scala tympani is surrounded by the modiolus, the spiral ligament, and the basilar membrane. Currently available electrode arrays attempt to avoid damage to these structures and to preserve residual inner ear function. Electrode arrays are either straight or pre-curved. A straight electrode array is advanced along the lateral wall of the cochlea during insertion. Therefore, a hard, straight electrode array can strip the spiral ligament. To avoid this, the standard electrode array produced by MED-EL contains wave-shaped wires, which makes the electrode array softer and

less traumatic. A pre-curved electrode array is held straight with a stiff stylet before it is inserted into the cochlea. After the removal of the stylet, the electrode array "hugs" the modiolus, which results in less electrical current needed to stimulate the auditory system [5].

Although inserting an electrode array with a stiff stylet has the potential to penetrate the basilar membrane, the overall incidence of insertion-related trauma is not greater than that with a straight electrode array [6]. The Contour Advance Electrode (Cochlear Ltd) is inserted with the Advanced Off-Stylet technique, which further reduces the damage to the cochlea [7]. The Contour Advance Electrode is a pre-curved electrode array with a soft tip. When the tip of the electrode array reaches the ascending portion of the basal turn, the stylet is held still and the electrode array is advanced deep into the cochlea. Using this technique, the electrode is advanced along the surface of the modiolus without hitting the lateral wall of the cochlea. Although this pre-curved electrode array preserves the lateral wall structures, the delicate structures of the modiolus may be harmed by the perimodiolar electrode array placement [8]. The HiFocus Mid-Scala Electrode (Advanced Bionics) is another type of pre-curved electrode. Its shape and insertion technique are similar to those of the Contour Advance Electrode. However, the HiFocus Mid-Scala Electrode is designed to be positioned in the center of the scala tympani, thus avoiding contact with any structures surrounding the scala tympani.

15.3 Recent Progress in Speech Coding Strategies

Speech coding strategies focus on how to extract the information that is essential for speech understanding. Progress in the field of microelectronics has facilitated the development of complex speech coding strategies. Speech coding strategies are based on a modification of continuous interleaved sampling (CIS) and n-of-m strategies, and an understanding of these two strategies is needed to understand current speech coding strategies. In CI, the acoustic input is divided into multiple frequency bands. In CIS, each electrode corresponding to each frequency band is stimulated sequentially. In the n-of-m strategy, bands exhibiting peak power (n) are selected from all the bands (m), and electrodes corresponding to the selected bands are stimulated. These strategies effectively extract formants essential for speech understanding.

Due to improvements in receiver-stimulators, current CIs are able to transmit more information than previously. The stimulus rates are continually increasing. The fine structure processing (FSP: MED-EL) strategy aims to code the fundamental frequency. Traditional speech coding strategies do not convey information regarding the fundamental frequency. Natural sound, including human speech, consists of sounds with a fundamental frequency and its integer multiples. In human vocalization, a glottal sound is created in the larynx. This glottal sound is modulated in the oral and nasal cavities, creating some peak frequencies. These peaks are called formants, and formants define the characteristics of voice.

The fundamental frequency determines the pitch of a voice. The average fundamental frequency of the human voice is 110–150 Hz in males and 220–270 Hz in females. In the present CIs, only one or two channels are assigned to this frequency range. Therefore, conventional CI users experience difficulty in detecting the pitch of a human voice. In addition, the fundamental frequency is important for auditory scene analysis. Under natural conditions, the world is full of complex sounds from multiple sources. The fundamental frequency is an important factor in the integration and discrimination of sounds from multiple sources.

As mentioned above, natural sound is complex, with multiple harmonic sounds. Sounds with the same fundamental frequency tend to fuse, and sounds with different fundamental frequencies are regarded as originating from different sources. This phenomenon facilitates the extraction of speech from background noise. Therefore, CI users experience difficulty in understanding speech in noisy environments. FSP was developed to code the fundamental frequency. In the healthy inner ear, the inner hair cells are excited when the basilar membrane is close to the tectorial membrane, and they are inhibited when the basilar membrane is apart from the tectorial membrane. Thus, the temporal excitatory pattern of the inner hair cell is synchronous to the input sound waveforms. This phenomenon, which is called "phase locking," is utilized to transmit sound frequency information to the brain, especially for low-frequency sounds. In FSP, the temporal stimulus pattern of the electrode is synchronous to the waveforms of the input sound at low frequencies. This stimulus pattern helps CI users detect the pitch of a sound and extract speech from the background noise [9].

Another approach for simulating natural hearing is to increase the number of electrodes. A number of physical electrodes greater than 6 do not improve speech perception, as is described above. Instead of increasing the number of physical electrodes, attempts have been made to create virtual electrodes by controlling the current spread. Recent CIs have multiple current sources and are able to stimulate two or more electrodes simultaneously. The activation of two physical electrodes can stimulate neurons between them (current steering) (Fig. 15.1). However, the simultaneous stimulation of multiple electrodes may stimulate unpredictable parts of neurons or an undesirably wide range of neurons. By using two or more intracochlear electrodes as active electrodes and ground electrodes, narrow regions of spiral ganglion neurons are stimulated (current focusing) [10]. The HiRes 90 K Implant, which employs the HiResolution strategy (Advanced Bionics), contains 16 physical electrodes and up to 7 virtual channels between each pair of physical electrodes; thus, the total number of electrodes is as great as 120. Unfortunately, the associated improvement in speech understanding was small [11]; further refinements of the current steering and focusing may improve the overall performance of this implant [12].

MP3000 (Cochlear Ltd) is a unique speech coding strategy that utilizes a psychoacoustic masking model. MP3000 employs n-of-m strategies, such as the advanced combination encoder (ACE) strategy. In the ACE, channels with the highest spectral energy are selected. Because natural sound exhibits energy peaks over a wide spectral range, the standard ACE strategy tends to select consecutive channels.

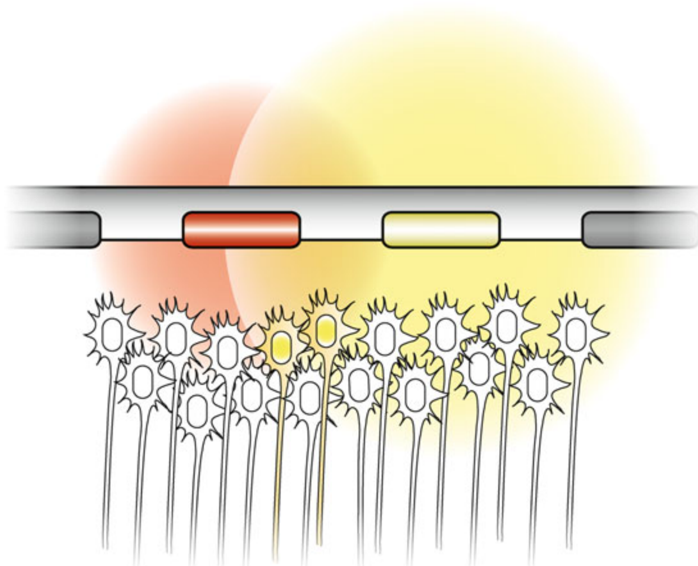

Fig. 15.1 A virtual channel is generated by the simultaneous stimulation of adjacent electrodes. The sum of the electric fields produces a peak in the overall field located between the electrodes. By controlling the current spread (current steering and current focusing), multiple virtual channels are created in a set of physical electrodes

In normal-hearing subjects, sounds near the spectral peak are masked and have little meaning. By using this psychoacoustic masking model, the MP3000 strategy reduces the number of activated electrodes without deteriorating hearing performance [13]. By reducing the activated electrodes, a high stimulation rate with low battery consumption is obtained. In addition, the activated electrodes are sparse in MP3000, which reduces the interactions caused by stimulating adjacent electrodes.

15.4 Electric Acoustic Stimulation (EAS)

Despite the recent progress in speech coding strategies, the sound information included in the fundamental frequency remains a serious limitation of CI. As described previously, modern electrode arrays are minimally traumatic. Because low-frequency sounds are arranged in the apical portion of the cochlea, residual hearing can be preserved and can be utilized to detect low-frequency sounds by using a short and low-volume electrode array (hybrid or electric acoustic stimulation (EAS)). In EAS, the sounds captured by the microphone are divided into low-frequency and high-frequency sounds. The low-frequency sounds are acoustically amplified and conducted to the external auditory canal using an earphone.

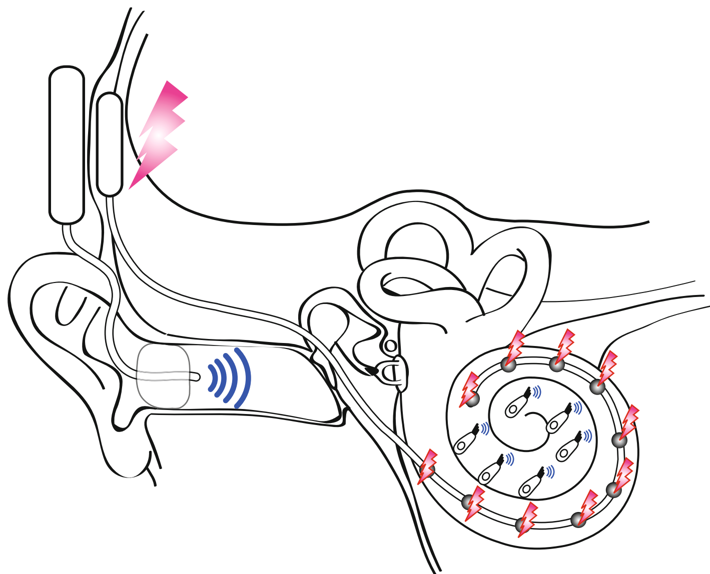

Fig. 15.2 In the electric acoustic stimulation system, high-frequency sounds are processed and delivered as an electric stimulation, and low-frequency sounds are conducted to the preserved inner hair cells

The high-frequency sounds are processed and electrically transmitted to the spiral ganglion neuron using the intracochlear electrodes (Fig. 15.2).

EAS utilizes specially developed electrode arrays. In FLEX series electrodes (MED-EL), the basal end contains seven paired electrodes, and the apical end contains five single electrodes, instead of 12 pairs of electrode channels (24 contacts). This design makes the tip of the electrode array soft and thin. The Slim-Straight Electrode and the Hybrid L24 Electrode (Cochlear Ltd) are also designed to preserve residual hearing. The Slim-Straight Electrode is thin and smooth. The Hybrid L24 Electrode is short to prevent damage to the superior turn of the cochlea. Using these electrode arrays, more than 90 % of patients sustained measurable hearing, and the average decrease in the pure-tone average at low frequencies was less than 15 dB [14, 15].

15.5 Bilateral CI

Recently, increasing numbers of patients receive CIs in both ears. Using two CIs improves sound source localization and may benefit language development. In condition with noise from the first CI side, the second CI may improve speech understanding [16]. In normal-hearing subjects, the sound information received by the two ears differs in intensity (the interaural level difference), arrival time

(the interaural time difference), and spectrum. These differences are detected by the superior olivary complex and the upper central nervous system, and they are used as major cues for sound source localization and speech extraction from background noise. Among these 3 cues, those with bilateral CI mainly utilize the interaural level difference, which is primarily derived from head shadowing effects. The interaural time difference and the spectral difference are barely perceptible because the electrode activation pattern does not have sufficient temporal and spectral resolution.

The Digisonic SP Binaural implant (Neurelec) may represent a solution for this problem. The Digisonic SP Binaural implant contains two intracochlear electrode arrays. One electrode array is inserted into the cochlea on the same side as the implant, and the other array is inserted into the other cochlea via a subcutaneous tunnel. The recipient wears two microphones in each ear, and the sound recorded in the two ears is transmitted to one speech processor. With this system, the temporal and spectral differences are digitally calculated and can be included in the speech coding strategy. The results of binaural CIs are still comparable to those of bilateral CIs [17]; however, future progress in microelectronics and speech coding strategies may improve the performance of this type of CI.

15.6 Problems with Modern CIs

Despite the recent progress described above, the pace of improvements in the performance of CIs has slowed, perhaps because the capacity for CIs to improve is approaching a limit. One problem with modern CI is the limited number of physical electrodes that are available. As described above, speech recognition does not improve significantly with increases in the electrode number beyond 6 [4]. The main reason for this limited electrode number is thought to be the current spread. One solution for this limitation is the induction of neurites from the spiral ganglion neurons to an electrode array made of biocompatible materials. This development may improve the insulation between the electrodes and reduce the current spread. Completely new devices may solve the current spread problems. Laser stimulation is also a candidate for stimulating a small area of auditory nerves [18]. Penetrating cochlear nerve implants have also been reported to provide high spectral resolution [19]. Piezoelectric materials attached to the basilar membrane can precisely stimulate the spiral ganglion neurons [20]. Inner ear implants composed of piezoelectric materials, a totally new concept in auditory devices, are discussed in the following chapters.

The other problem is that CI surgery inevitably causes inner ear damage. Improvements in the electrode array may reduce the insertion damage to the inner ear. Due to recent advances in the field of regenerative medicine, many doctors regard CI surgery as a chance to deliver therapeutic agents directly to the inner ear [21]. The application of regenerative medicine to CIs is discussed in the following chapters.

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Chapter 16
Regenerative Medicine in Cochlear Implantation

Norio Yamamoto

Abstract Cochlear implantation improves hearing and speech ability in patients with profound or severe sensorineural hearing loss. However, its effects are limited when there is a primary auditory neuron response deficiency. To overcome this limitation, several strategies have been developed for the preservation or regeneration of spiral ganglion cells, i.e., primary auditory neurons. Among those strategies, the administration of neurotrophic factors and the transplantation of neural stem or progenitor cells are two of the most promising. To preserve spiral ganglion cells, neurotrophic factors can be delivered into the cochlea by various methods including direct infusion, viral vectors, transplantation of neurotrophic factor-transfected cells, and transplantation of neural stem cells. To regenerate spiral ganglion cells, transplantation of stem or progenitor cells is required. The most efficient method is the transplantation of pluripotent stem cells that are induced toward neural or otic fate in vitro before transplantation.

In addition to overcoming the limitations of cochlear implantation, regenerative medicine plays a role in the extension of the cochlear implantation indication. Recently, even patients with residual hearing in the low frequency have received cochlear implantation. In this case, preservation of residual hair cells is mandatory. Several growth factors, including insulin-like growth factor (IGF-1), are useful for that purpose because they can protect hair cells from injury and even regenerate them.

Keywords IGF-1 • Neurotrophic factor • Regeneration • Residual hearing • Spiral ganglion cell

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16.1 Introduction

Since the introduction of multiple-channel devices in 1985, cochlear implantation has provided enhanced spectral perception and speech recognition to patients with profound or severe sensorineural hearing loss. When the indication of cochlear implantation was extended to earlier implantation in congenitally deaf children, cochlear implantation assisted children with hearing loss to develop speech production as well as hearing ability. However, some factors that limit the outcomes of cochlear implantation still exist. In contrast, the indication of cochlear implantation is further extended to those who have residual hearing in lower frequencies. Limitations in the outcome sometimes originate from the lack of the primary auditory neuron (Fig. 16.1) response to the cochlear implant. Further, the successful cochlear implantation in those who have residual hearing requires adequate numbers of hair cells that are often injured by the electrode insertion itself (Fig. 16.1). Regenerative medicine can contribute to resolving these two issues, achieving better outcomes and extending the cochlear implantation indication.

16.2 Spiral Ganglion Cells

16.2.1 Spiral Ganglion Cells as a Target of Regenerative Medicine

One of the most important factors that limit the outcome of cochlear implantation is the number of spiral ganglion cells, i.e., primary auditory neurons. Cochlear implant achieves its effects by stimulating spiral ganglion cells in the modiolus of the cochlea. Spiral ganglion cells are bipolar neurons that send two types of fibers in opposite directions. Longer central fibers, the primary auditory fibers, form the cochlear nerves and extend to the cochlear nucleus in the brainstem. Shorter peripheral fibers extend to inner and outer hair cells. The survival of spiral ganglion cells is dependent on the presence of hair cells and their release of neurotrophic factors. This explains why adult patients with short durations of deafness have better outcomes than those with long durations of deafness [1–3]. A longer duration of deafness causes more degeneration of spiral ganglion cells probably due to the lack of neurotrophic factors from hair cells [4]. Although a relationship between the number of survival spiral ganglion cells and the outcome of cochlear implantation was not found from postmortem histological studies using temporal bones of patients who underwent cochlear implantation [5, 6], 10 % of normal numbers of spiral ganglion cells were found to be necessary for successful cochlear implantation [5]. New-generation cochlear implant processing strategies that use current focusing and steering (e.g., HiRes 120 strategy) [7] may require more surviving spiral ganglion cells because current focusing and steering addresses many single

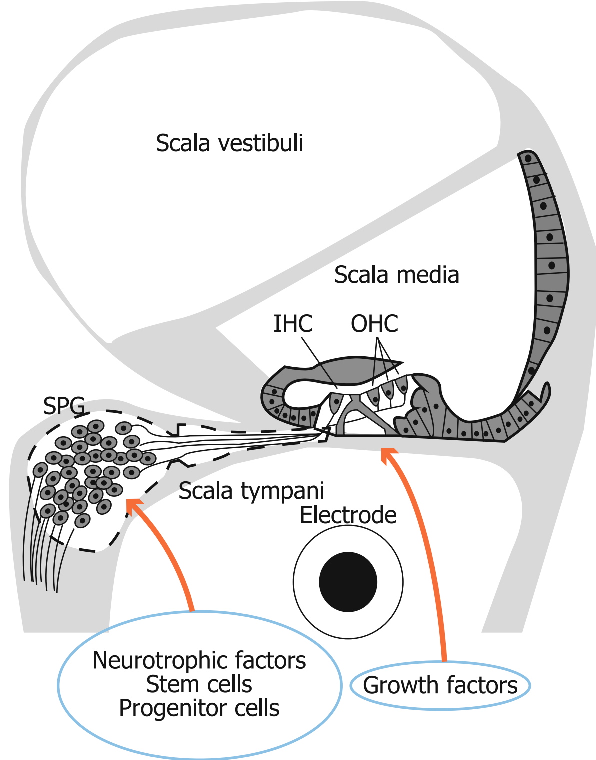

Fig. 16.1 Schematic representation of the targets and methods for the regenerative medicine in cochlear implantation. The electrode of cochlear implant was placed in the scala tympani. Neurotrophic factors, stem cells, or progenitor cells are administered to preserve or regenerate spiral ganglion cells. Growth factors are used for the preservation or regeneration of hair cells. Cells or agents are administered from the scala tympani in both treatments. *IHC* inner hair cell, *OHC* outer hair cells, *SPG* spiral ganglion cells

electrodes and virtual sites of stimulation along the length of the cochlea. Cochlear nerve deficiency (CND) is another etiology with few numbers of spiral ganglion cells. CND is defined as an absent or hypoplastic cochlear nerve detected by magnetic resonance imaging (MRI) and/or computed tomography (CT) imaging and its prevalence is 1–5 % of bilateral sensorineural hearing loss [8, 9]. CND is one cause of congenital hearing loss. The outcome of cochlear implantation for CND cases is poorer than that for non-CND cases [10], which is different from degeneration of spiral ganglion cells in adult patients.

16.2.2 Neurotrophic Factors

Several strategies have been developed to preserve or regenerate spiral ganglion cells. One strategy is to preserve spiral ganglion cells and regenerate peripheral neural fibers toward sensory epithelia of cochleae by using neurotrophic factors [11–17] (Fig. 16.1). Preventing spiral ganglion cells from degenerating will enhance the outcome of cochlear implantation as discussed in the previous section. In addition, extending peripheral neural fibers toward sensory epithelia enhances

the efficacy of cochlear implantation because cochlear implants are usually placed in the scala tympani where the electrodes cannot directly stimulate spiral ganglion cells. If peripheral fibers are regenerated from spiral ganglion neurons, direct stimulation of spiral ganglion neurons by cochlear implants will be accomplished [18].

Neurotrophic factors are produced by neurons, glial cells, sensory cells, and muscle fibers. Once neurotrophic factors bind their receptors, receptor cells show cell proliferation, cell differentiation, cell maturation, neuronal plasticity, axonal outgrowth or repulsion, and even apoptosis. Neurotrophic factors are possible factors to treat neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis through their various effects. Among several neurotrophic factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) are predominantly functional in the cochleae. They are secreted from hair cells and they control the fate of spiral ganglion cells both in the development and adult stages. Many studies have shown that exogenous application of neurotrophic factors protected degeneration of spiral ganglion cells and induced the regeneration of peripheral neural fibers that enhanced the effects of cochlear implants. Neurotrophic factors can be delivered into cochleae in various ways other than direct infusion using pumps [11–13, 15]. Viral vectors [14, 16] and transfected cells [19] are used to deliver neurotrophic factors into the cochlea, and they provide longer and more stable application of neurotrophic factors. Even the transplantation of neural stem cells into cochleae causes the release of neurotrophic factors (GDNF and BDNF) because of differentiation of neural stem cells into glial cells [20]. BDNF delivered by biodegradable gels significantly reduced the threshold elevation of electrically evoked auditory brainstem responses that reflect the function of spiral ganglion cells [21]. Cochlear implant electrodes themselves are also candidate carriers for delivering neurotrophic factors into the cochlea. Recent studies have shown the coating of the electrode with neurotrophic factors [22] or adding the delivering channels to the electrodes [23].

16.2.3 Stem Cells and Progenitor Cells

Neurotrophic factors can protect degeneration of spiral ganglion cells and induce the regeneration of peripheral neural fibers but they do not regenerate spiral ganglion cells. To achieve the regeneration of spiral ganglion cells, several studies have investigated the transplantation of neural stem cells or neural progenitor cells that are derived from pluripotent stem cells (Fig. 16.1). Transplantation of embryonic [24] or adult [25] neural stem cells into the cochlea showed survival of transplanted cells but showed no or very little differentiation of transplanted cells into neural fate. A more efficient method is the use of progenitor cells induced from pluripotent stem cells, which in most studied cases were embryonic stem (ES) cells. The transplantation of ES cells solely did not show efficient

differentiation into neurons. However, when they were transplanted with embryonic neuronal tissues, an efficient differentiation of transplanted cells into neurons was observed [26]. That study suggested the efficacy of neural induction of ES cells to achieve preferable regeneration of spiral ganglion cells. Many kinds of neural induction methods for ES cells have been described [27, 28] and those methods were applied to the transplantation of ES cells into cochleae. The results showed survival of neural cells derived from transplanted progenitors in the modiolus where spiral ganglion cells exist and functional improvement of auditory neurons was suggested [29]. Transplantation of ESC-derived otic progenitors into animals with viable hair cells and damaged spiral ganglion cells restores a population of spiral ganglion cells and auditory brainstem responses, thereby suggesting functional regeneration of spiral ganglion cells [30].

The most important problem when using neural stem cells or ES cells is the source of preparation. If the transplantation therapy is applied to the clinical situation, using neural stem cells is not realistic because those cells are prepared from embryonic or adult brain tissues. Induced pluripotent stem cells (iPSCs) that can be prepared from any tissues in the body [31] are a more suitable source of the regenerative medicine for spiral ganglion cells because iPSCs and ES cells are interchangeable.

16.3 Hair Cells

The regeneration and preservation of hair cells as well as spiral ganglion cells have become important in the clinical scene of cochlear implantation (Fig. 16.1). Since von Ilberg et al. reported the benefits of "electrical-acoustic stimulation" (EAS) for those who have residual hearing in low frequency [32], the functional preservation and even regeneration of hair cells have become important issues. The concept of EAS involves the combination of the acoustic stimulation of residual low-frequency hearing with hearing aids and the electrical stimulation of the high-frequency hearing with cochlear implants in the same ear, and this elicits more benefits, including hearing for speech in noise and music appreciation compared with the electrical stimulation only [33]. To obtain the benefits of EAS, functional hair cells are necessary. The development of soft and short cochlear implant electrodes [33–35] and minimally invasive surgical techniques [33, 36, 37] has contributed to the preservation of low-frequency hearing. However, the rate of low-frequency hearing preservation is variable [38] and sometimes it can be very low depending on the skill of surgeons. To obtain the benefits of EAS more consistently, treatment with pharmacological agents that can preserve or regenerate hair cells may need to be considered. Steroids are widely used in hearing preservation cochlear implant surgery [38] although the detailed mechanisms of hair cell protection with steroids are still unclear. Several promising reagents for the preservation or even regeneration of hair cells have also been reported. Most of them belong to the growth factor class (Fig. 16.1), including acidic fibroblast growth

factor (aFGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), transforming growth factor-beta1 (TGF-beta1), and GDNF [39]. IGF-1 has protective effects of hair cells in vivo in noise-induced hearing loss [40, 41] or ischemia-induced hearing loss [42]. In vitro study using neonatal mouse cochleae revealed that the maintenance of hair cell numbers in the injured cochlea was achieved through the inhibition of apoptosis [43]. Surprisingly, IGF-1 also induced proliferation of supporting cells in the cochlea, and this contributed to the maintenance of hair cell numbers. These results suggested that IGF-1 may induce the regeneration as well as the preservation of cochlear hair cells. A clinical trial for idiopathic sudden sensorineural hearing loss that is refractory to steroid therapy showed that IGF-1 has positive effects on the recovery of hearing [44]. In both basic and clinical research, IGF-1 is proving to be one of the most preferable agents for enhancing the hearing preservation rate in the cochlear implant surgery.

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Chapter 17
Artificial Cochlear Epithelium

Takayuki Nakagawa and Satoyuki Kawano

Abstract Conventional cochlear implants directly stimulate the spiral ganglion neurons via electrodes implanted in the cochlea. Conversion of sound stimuli to electric signals is performed by a speech processor and a transmitter as an external device. For electric stimulation of spiral ganglion neurons, en external battery is necessary. Previously, von Békésy proved his travelling wave theory using human cochleae from cadavers, indicating that even after complete loss of hair-cell function, the mechanical tonotopy of the cochlea for sound frequency remains. We hypothesized that if an implantable device that converts sound vibration to electric potential is fabricated using microelectromechanical systems, the sensitivity for specific sound frequency will largely be determined by the location where the device is implanted based on the travelling wave theory. Based on this hypothesis, we fabricated piezoelectric devices that were capable of converting sound stimuli to electric signals. In this chapter, the potential and limitations of piezoelectric devices for hearing restoration are discussed.

Keywords Basilar membrane • Cochlear implant • Piezoelectric material • Vibration

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17.1 Travelling Wave Theory

Sound stimuli are converted to neural signals in the cochlea. The cochlea has three compartments, the scala vestibuli, the scala tympani and the scala media, which are filled with a watery liquid. The former two compartments merge at the apex of the cochlea, and the scala tympani is a membranous duct containing the cochlear sensory epithelium. The vibration of otic ossicles, which transmit sound vibration from the tympanic membrane to the cochlea, makes the sound wave in the scala vestibuli that spreads to the scala tympani. The combination of vibrations in the scala vestibuli and the scala tympani causes vibration of the cochlear sensory epithelium on the basilar membrane, and the location of the largest vibration changes according to the sound frequency, which was named the "travelling wave" by von Békésy [1, 2]. High tones produce the largest vibration at the base of the cochlea and low tones produce the largest wave in the apex (Fig. 17.1). According to this mechanical tonotopy, high tones stimulate cochlear hair cells in the base portion of cochleae, and low tones stimulate hair cells in the apical portion of cochleae. This mechanical tonotopy of the cochlea is not enough for human sound recognition, especially for recognition of language or music. The mammalian cochlear hair cells, inner and outer hair cells, play crucial roles for highly sophisticated auditory functions of mammals [3]. Previously, von Békésy proved his travelling wave theory using human cochleae from cadavers, indicating that even after complete loss of hair-cell function, the mechanical tonotopy of the cochlea for sound frequency remains [1, 2]. This also persists in deafened cochleae.

The cochlear implant is a hearing device that restores hearing in deafened patients, of which development is worthy for Lascar prize. A stimulator of the cochlear implant, with an array of up to 24 electrodes, is surgically inserted into the cochlea, usually the scala tympani, and directly stimulates auditory primary neurons. Conversion of sound stimuli to electric signals is performed by a speech

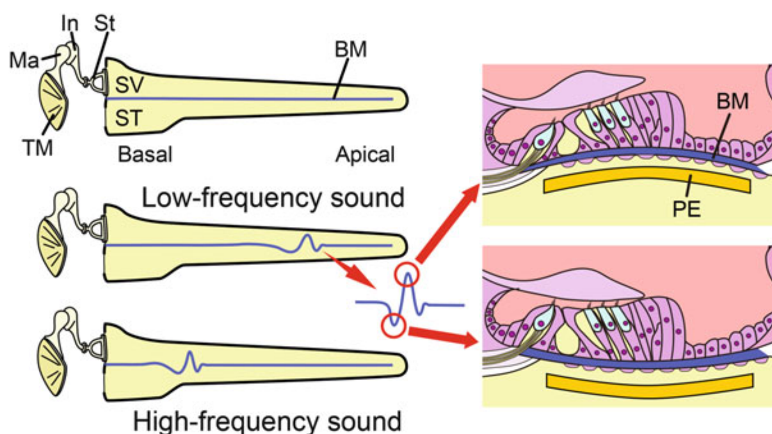

Fig. 17.1 Travelling wave theory and our hypothesis [4]

processor and a transmitter as an external device. According to the development of programs for a speech processor, clinical benefits of cochlear implants have remarkably improved. However, hearing obtained by the cochlear implant is still far from natural hearing. The cochlear implant utilizes the function of remaining spiral ganglion neurons in the cochlea, but does not utilize the mechanical tonotopy of the cochlea, which still remains in deafened cochleae. We have challenged to develop a new auditory device that utilizes the mechanical tonotopy of the cochlea without the use of an external battery (Fig. 17.1). We named this project "technological regeneration of the cochlear sensory epithelium" [4].

17.2 Piezoelectric Materials

Piezoelectric materials can generate the surface charge when it becomes mechanically stressed, and this phenomenon is the so-called piezoelectric effect. The inverse effect, which is the phenomenon that applying the voltage results in the expansion or contraction of the material, is also realized. Quarts, bones, lead zirconate titanate (PZT) and polyvinylidene difluoride (PVDF) are well-known materials which have the piezoelectric effect. Piezoelectric materials can be used for electric power generators and pressure sensors by the use of the direct piezoelectric effect. On the other hand, they can be used for actuators, which convert the input source to the mechanical motion, by the use of the inverse piezoelectric effect. Therefore, the direct piezoelectric effect can be used to generate electricity in response to sound vibration, and the inverse piezoelectric can be utilized as a sensor for sound vibration that might contribute to enhance the sensitivity for sound frequencies.

17.3 Non-Life-Sized Device

Our final goal is to develop a novel artificial cochlea which realizes both acoustic/electric conversion and frequency selectivity without an external energy supply. As the first step, the non-life-sized prototype device was developed for in vitro experiment [5] as shown in (Fig. 17.2) by the use of microelectromechanical systems (MEMS) technologies. The main part of the device comprises an artificial basilar

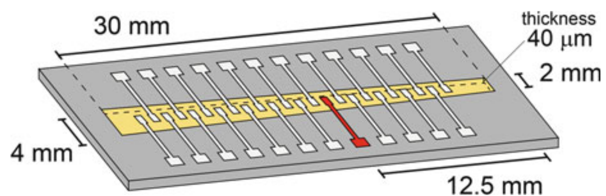

Fig. 17.2 Schematic drawing of a non-life-sized device [4]

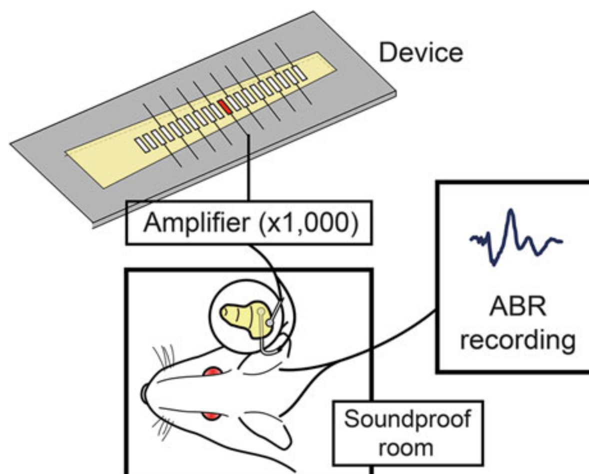

Fig. 17.3 ABR recording in guinea pigs using a non-life-sized device [4]

membrane made of piezoelectric material. It is a polyvinylidene difluoride (PVDF) membrane 40 μm thick fixed on a substrate with a trapezoidal slit.

The membrane over the slit is vibrated by acoustic waves and generates electric output due to the piezoelectric effect of PVDF, and the width is linearly varied from 2.0 to 4.0 mm along the longitudinal direction of 30 mm to change its local resonant frequency of $O(1)$–$O(10)$ kHz. A detecting electrode array with 24 elements of 0.50×1.0 mm rectangles is made of an aluminum thin film on the membrane, where they are located along the longitudinal direction with the gaps of 0.50 mm. The resonating place in the membrane vibrates with relatively large amplitude, the electric output there becomes high due to the large strain and the output at the other electrodes remains to be low. As a result, the electric signals from each electrode realize the function of frequency selectivity [5].

By the use of the prototype and non-life-sized piezoelectric device, the experiment of the generation of auditory brainstem responses (ABRs) in living guinea pigs [4] was made as shown in Fig. 17.3. The device was used as a transducer, and its electric outputs were amplified by 1,000-fold. Platinum–iridium ball electrodes were implanted into the scala tympani of the cochlear basal turn for stimulation of auditory primary neurons. As a result, typical ABRs in response to increased acoustic stimuli were recognized in our model animals as shown in Fig. 17.4 [4]. When acoustic stimuli of 104.4 dB sound pressure level (SPL) were applied to the piezoelectric device, the first positive wave of ABRs was clearly identified at a latency of 1.07 ± 0.05 ms, which was identical to the latency of the first positive wave in electrically evoked ABRs (0.98 ± 0.06 ms) in guinea pigs obtained in preliminary experiment as shown in Fig. 17.5 [4]. In normal ABRs, wave I is from the excitation of cells in the spiral ganglion of cochlea and wave II is from the excitation of cochlear nucleus. In general, the first positive wave of electrically evoked ABRs corresponds to wave II of normal ABRs. Compared with the latency of wave II of normal ABRs in normal guinea pigs ($n = 4$, 2.99 ± 0.11 ms), the

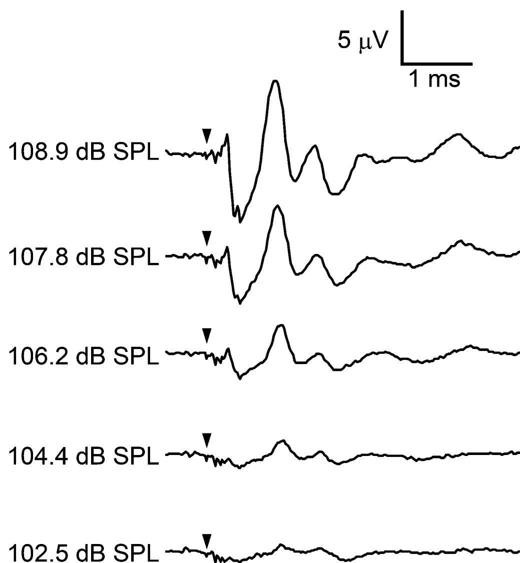

Fig. 17.4 ABRs obtained by sound exposure to a non-life-sized device [4]

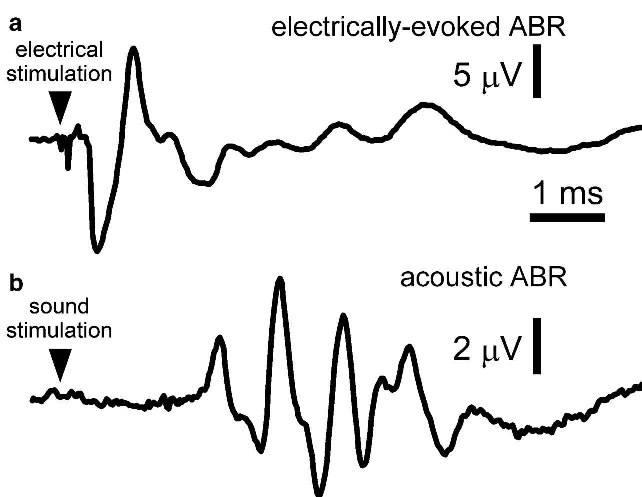

Fig. 17.5 Comparison of an electrically evoked ABR wave with an ABR wave obtained by sound exposure to a non-life-sized device [4]

latency of the first positive wave of piezoelectric device-induced ABRs was ~2 ms short as shown in Figs. 17.4 and 17.5 [4]. However, the latency of the first positive wave of piezoelectric device-induced ABRs was almost similar to the latency between waves I and II of normal ABRs (0.83 ± 0.04 ms) shown in Fig. 17.5. From these experimental analyses, it was found that the piezoelectric membrane generated biological ABRs by converting acoustic stimuli to electric signals.

17.4 Implantable Device

Based on the findings in non-life-sized devices, we fabricated a totally implantable device, which was designed for the basal turn of guinea pig cochleae (Fig. 17.6) [4]. A totally implantable device consisted of a silicon frame and a piezoelectric membrane. To investigate the potential of implantable devices, two types of devices were fabricated. One has no electrodes for aiming sound transmission from the external auditory canal to a device that was implanted in the cochlea. Another has electrodes that were extended to the outside of the cochlea for measurements of electric outputs from a device implanted in the cochlea.

Firstly, we measured electric outputs of the implantable device in vitro. In response to sound stimuli, an implantable device generated electric outputs, which was greater than non-life-sized devices. An increase of outputs was mainly caused by the difference in the thickness of piezoelectric membranes. In implantable devices, a piezoelectric membrane with 4 μm in thickness was used. The electric outputs from the device were able to generate ABRs in normal guinea pigs.

In the next step, we measured the vibration of a piezoelectric membrane in an implantable device after implantation into a guinea pig cochlea. A laser Doppler vibrometer was employed for detection of the vibration. As results, a piezoelectric membrane showed vibration similarly to the original basilar membrane of a guinea pig cochlea, although the amplitude of vibration in a piezoelectric membrane was smaller than that in the original basilar membrane (Fig. 17.7) [4]. These results indicated that sound stimuli from the external auditory canal were transmitted to a piezoelectric membrane that had been implanted into the cochlea.

The critical issue was whether an implanted device was able to generate electric outputs in response to sound stimuli. To measure electric outputs from an implanted device, an ex vivo model was used. A temporal bone of a guinea pig was excised and set on the stage. Sound stimuli were directly applied to the stapes in the middle ear. The electric outputs from an implanted device were recorded through electrodes extended outside of the cochlea. In response to sound stimuli to the stapes, an

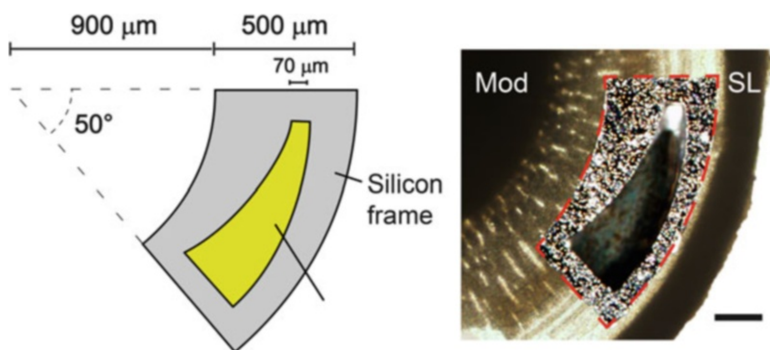

Fig. 17.6 Design of an implantable device for a guinea pig cochlea [4]. *Mod* cochlear modiolus, *SL* spiral ligament

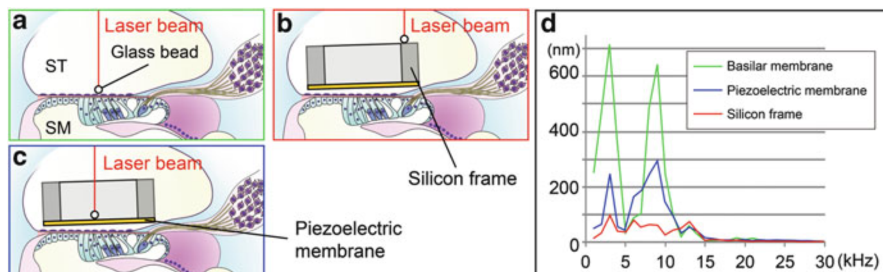

Fig. 17.7 Measurements of vibrations in a natural basilar membrane (**a**), silicon frame (**b**) and piezoelectric membrane (**c**) that are implanted in a cochlea by a laser Doppler vibrometer [4]. Amplitudes of generated vibrations according to sound frequencies are shown in (**d**)

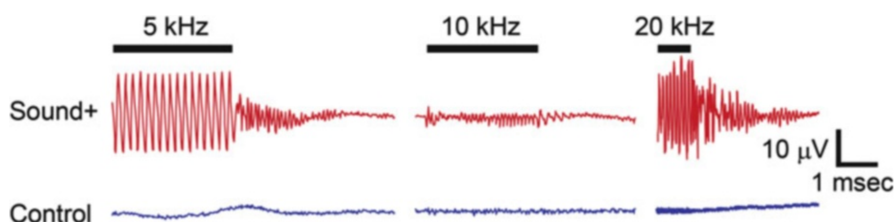

Fig. 17.8 Electric outputs from a device that is implanted in a guinea pig cochlea in response to sound stimuli (5, 10, 20 kHz at frequency). *Red waves* show outputs when sound stimuli are applied and *blue waves* show outputs without sound stimuli

implanted device generated electric outputs (Fig. 17.8) [4]. Importantly, electric outputs from the device gradually decreased after completion of sound application, which mimics a biological response. However, comparing with the intensity of electric stimuli from electrodes of cochlear implants, the magnitude of electric outputs from an implanted device was weak, which required 1000 times amplification to stimulate spiral ganglion neurons from the scala tympani.

17.5 Future Perspective

Our challenge to fabricate an artificial cochlear epithelium, a piezoelectric device that mimics the function of the inner hair cell and basilar membrane, achieved to generate electric outputs from a piezoelectric device that were implanted in the cochlea in response to sound stimuli. We believe that this is a great step for the development of novel auditory devices that are totally different from conventional cochlear implants. However, we should resolve following problems for practical use of piezoelectric devices. The first issue is an increase of electric outputs from a piezoelectric device. For this purpose, we are now investigating the potential of

various kinds of piezoelectric materials. The development of new electrodes that are located close to spiral ganglion neurons will contribute to reduce a required power of the device. The second problem is how to fix the device in the cochlea. For this purpose, we are planning use of electrodes of the device. The third issue is the selectivity for sound frequencies. To achieve natural hearing, the function of outer hair cells, an increase of tuning, is crucial. A piezoelectric membrane is used as a sensor for the vibration. Therefore, an addition of another piezoelectric membrane that works as a sensor might be a solution for this purpose. An alteration of the thickness and width of a piezoelectric membrane would also contribute to an increase of the sensitivity of a device.

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Chapter 18
Auditory Brainstem Implant

Hiroshi Yamazaki

Abstract An auditory brainstem implant (ABI) is an electrical device that stimulates the cochlear nucleus to provide auditory sensation. The ABI was originally developed for deaf patients with bilateral vestibular schwannomas associated with neurofibromatosis type 2 (NF2), but candidacy for an ABI has recently been extended to non-NF2 populations, including patients with congenital inner ear and/or internal auditory canal (IAC) malformations, severe cochlear ossification after meningitis or fracture of the cochlea, trauma-induced cochlear nerve disruption, and advanced otosclerosis. Different causes of hearing loss are associated with different hearing outcomes for ABIs, but the peripheral stimulation provided by cochlear implants (CIs) seems to result in better outcomes than central stimulation of the auditory neural system with ABIs. However, if cochlear implantation is contraindicated or fails to provide sufficient auditory sensation, auditory brainstem implantation may be the only solution. Since neural input and trophic support from spinal ganglion neurons (SGNs) to neurons in the cochlear nucleus are important for maturation or maintenance of neural circuits in the cochlear nucleus, auditory brainstem implantation in combination with preceding cochlear implantation and/or approaches for SGN regeneration or prevention of SGN degeneration might improve ABI outcomes.

Keywords Auditory brainstem implant • Cochlear implant • Cochlear nerve deficiency • Cochlear nucleus • Inner ear malformation • Internal auditory canal • Neurofibromatosis type 2 • Spiral ganglion neuron • Vestibular schwannoma

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18.1 Introduction

An auditory brainstem implant (ABI) is an electrical device that stimulates the cochlear nucleus to provide auditory sensation. The ABI was originally developed for deaf patients with bilateral vestibular schwannomas associated with neurofibromatosis type 2 (NF2). The first ABI with a single electrode was implanted by William House in 1979. In the 1990s, the single-channel implant was replaced by a multichannel implant that improved ABI-aided auditory performance. At first, candidacy for auditory brainstem implantation was limited to individuals diagnosed with NF2, but an increasing number of non-NF2 patients have received an ABI since 2002, when Colletti reported the first pediatric ABI cases with congenital malformations of the inner ear and internal auditory canal [1].

The fundamental mechanisms of an ABI are similar to those of a cochlear implant (CI). The ABI electrically stimulates the surface of the cochlear nucleus by a paddle-shaped electrode array, while the CI stimulates the spiral ganglion neurons (SGNs) through an electrode array inserted into the scala tympani. The cochlear nucleus is composed of the dorsal cochlear nucleus (DCN) and the ventral cochlear nucleus (VCN), with the VCN being further divided into anteroventral (AVCN) and posteroventral (PCVN) regions. The auditory nerve fibers, which are axons of the SGNs, branch to innervate these three subnuclei. Tonotopic organization is observed in each subnucleus [2]. In contrast to the linear tonotopic organization along the basal-apical axis in the cochlea, the tonotopy in the cochlear nucleus has a 3-dimensional organization in which the characteristic frequency changes in a vertical direction to the surface of the brainstem [3, 4]. Theoretically, the electrode array of the CI can reproduce the cochlear linear tonotopic organization, but the most commonly used surface electrode array of the ABI has difficulty reproducing the 3-dimensional tonotopic map of the cochlear nucleus, especially its vertical component [4]. To overcome this problem, an investigational penetrating ABI (PABI) having 2 arrays, a 12-electrode surface array plus a 10-electrode array with vertically penetrating needle microelectrodes, was developed to stimulate the cochlear nucleus in a more 3-dimensional manner [5]. The results of PABI were discouraging because less than 25 % of the penetrating electrodes resulted in auditory sensations, and the penetrating electrode array did not improve speech perception when compared with the use of a surface electrode array alone. The penetrating electrodes did contribute to a lower threshold, increased pitch range, and higher selectivity [5]. These results imply that, in addition to differences in tonotopic organization, we should focus on other functional differences between the cochlea and the cochlear nucleus. Inhibitory interneurons in the cochlear nucleus sharpen the neural representation of auditory stimuli by lateral inhibition [6]. Direct stimulation of neurons in the cochlear nucleus using ABI might disturb this lateral inhibition. Consistent with the theoretical disadvantages of ABI, several studies reported that the hearing outcomes of cochlear implantation are usually better than those of auditory brainstem implantation, as described below [7–9]. Auditory brainstem implantation is, however, the only effective treatment to restore hearing

in deaf patients if cochlear implantation is contraindicated or unsuccessful due to lesions in the inner ear and/or internal auditory canal (IAC).

18.2 Indications for Auditory Brainstem Implant

The ABI was originally designed to restore hearing in deaf patients with NF2. More recently, the ABI has been used in patients without NF2 who are not candidates for a CI or who have failed to receive benefits from a CI. Candidacy for an ABI is categorized as patients with and without NF2, and the latter category is further divided into congenital and acquired groups. Etiologies of deafness differ between the congenital and acquired non-NF2 groups: congenital inner ear and/or IAC malformations in the congenital non-NF2 group and meningitis-related ossification, trauma, and severe otosclerosis in the acquired non-NF2 group [10]. As mentioned in a 2011 consensus statement developed from outcomes in 61 non-NF2 children with ABIs [9], it is possible to use the ABI to stimulate the auditory system in a majority of patients with severe inner ear and/or IAC malformations, but widespread application of the ABI in prelingually deaf children would not be appropriate until longer-term effects and safety of pediatric auditory brainstem implantation have been established.

18.2.1 Patients with Neurofibromatosis Type 2

Neurofibromatosis type 2 is an autosomal dominant disorder characterized by the development of multiple nervous system tumors, especially schwannomas. Loss-of-function mutation of the *NF2* gene, which codes for the tumor suppressor protein called merlin, is reported to cause NF2 [10]. Diagnostic prevalence of NF2 is 1 in 100,000 people and its penetrance is almost 100 % by 60 years of age. Bilateral vestibular schwannomas are observed in 90–95 % of patients with NF2, who usually develop hearing loss that becomes noticeable between 20 and 30 years of age [10]. Unlike a unilateral non-NF2 vestibular schwannoma, vestibular schwannomas associated with NF2 tend to diffusely infiltrate adjacent nerves. The cochlear nerve is often sacrificed or severely damaged during the removal of the vestibular schwannoma, especially when the size of the tumor is large. In these patients, CI-mediated stimulation does not reach the brainstem and an ABI is the only solution to restore hearing. Radiotherapy for the vestibular schwannoma can precede the implantation of an ABI, but some reports have suggested that delayed deterioration of hearing outcomes after auditory brainstem implantation might be associated with radiotherapy-induced damage to the cochlear nucleus [9].

18.2.2 Congenitally Deaf Children

Congenitally deaf children have severe inner ear and/or IAC malformations that are associated with insufficient space for electrode insertion and hypoplasia of the cochlear branch of the vestibulocochlear nerve (VCN), also referred to as cochlear nerve deficiency (CND). Patients with the following inner ear and IAC malformations are potential candidates for an ABI: (1) Michel aplasia (complete labyrinthine aplasia), (2) cochlear aplasia, (3) common cavity deformity, (4) incomplete partition type 1, (5) narrow IAC (NIAC), and (6) hypoplasia of bony cochlear nerve canal (HBCNC), also called cochlear aperture aplasia or hypoplasia [9]. Among these malformations, only bilateral Michel aplasia is a definite indication for auditory brainstem implantation because there is no labyrinth available for cochlear implantation [8]. Patients with cochlear aplasia, NIAC, HBCNC, and isolated CND are usually good candidates for an ABI, but it should be noted that radiographic evaluations cannot exclude candidacy for cochlear implantation in these groups. Jeong and Kim reported two cases of patients with cochlear aplasia who showed favorable speech perception abilities with a CI. In these cases, computed tomography (CT) and magnetic resonance imaging (MRI) demonstrated the absence of a cochlea; nevertheless, clear auditory brainstem responses (ABRs) were electrically evoked using electrodes inserted in the malformed labyrinth [12]. Common cavity and incomplete partition type I malformations are sometimes associated with CND [13], but CI-mediated intraoperative electrically evoked auditory brainstem response (EABR) testing elicited an obvious evoked wave V in patients with these malformations [14, 15]. These results suggest that auditory nerve fibers are distributed in the malformed inner ear and transmit CI-mediated auditory signals to the central auditory system. In patients with NIAC, Song et al. demonstrated that the presence or absence of the VCN in preoperative imaging did not always correlate with the VCN status identified during auditory brainstem implantation, suggesting that the sensitivity of MRI is not high enough for a precise diagnosis of a hypoplastic VCN or cochlear nerve. Indications for auditory brainstem implantation in the population with inner ear or IAC malformations are under discussion, and more detailed information will be provided later in this chapter.

18.2.3 Acquired Deafness in Patients Without Neurofibromatosis Type 2

This category includes (1) severe cochlear ossification after meningitis or fracture of the cochlea, (2) trauma-induced cochlear nerve disruption, and (3) advanced otosclerosis [9, 10]. Among these, bilateral cochlear nerve disruption after trauma is a definite indication for auditory brainstem implantation because CI-mediated auditory signals cannot be transmitted to the auditory brainstem in individuals

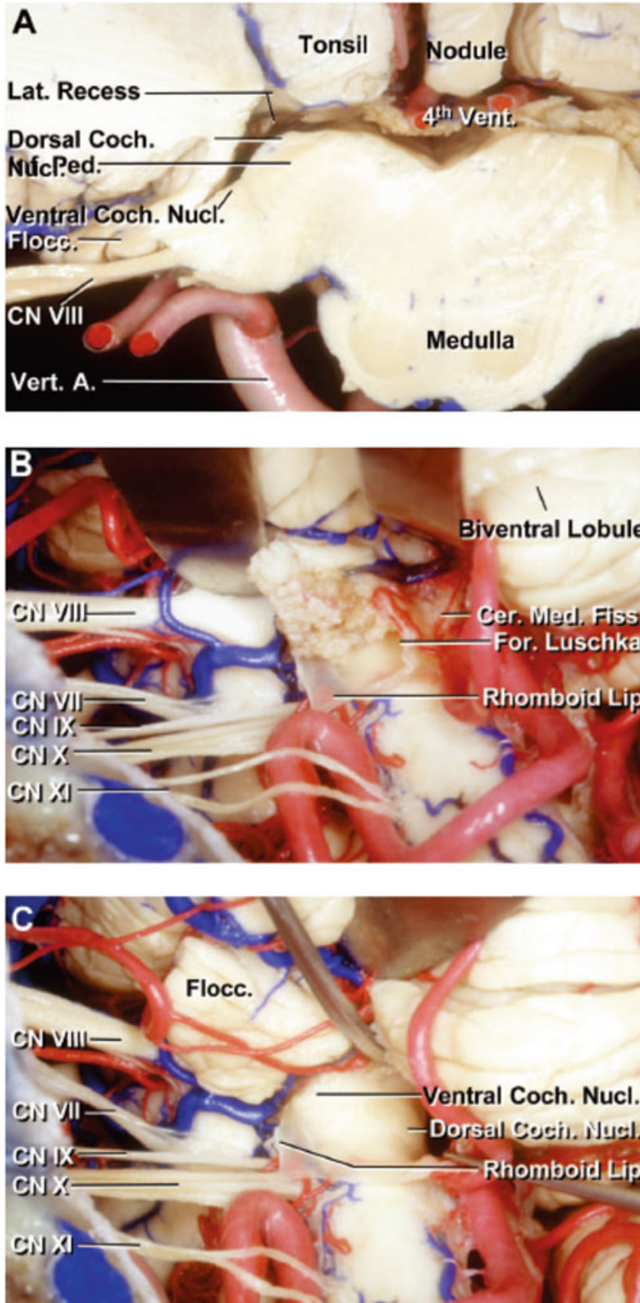
with this type of damage. Severe cochlear ossification and advanced otosclerosis may be indications for auditory brainstem implantation if a CI with the appropriate electrode arrays (e.g., the Contour and Double array electrode manufactured by Cochlear or the Compressed or Split electrode produced by MED-EL) failed to provide satisfactory outcomes or could not be inserted.

18.3 Surgical Procedures for Auditory Brainstem Implantation

In NF2 patients, the disc electrode is placed on the surface of the cochlear nucleus complex on the dorsolateral surface of the brainstem, immediately rostral to the pontomedullary junction, through a standard translabyrinthine approach following removal of the vestibular schwannoma. The cochlear nucleus is composed of the DCN and the VCN. As shown in Fig. 18.1, both the DCN and VCN are located adjacent to the lateral recess of the fourth ventricle near the foramen of Luschka. The cochlear nucleus has few visible surface landmarks; therefore, the surrounding structures, such as the root entry point of cranial nerve VIII, cranial nerve VII, the choroid plexus, cranial nerve IX, and the tela choroidea, are important guides for placing the electrode array accurately on the surface of the cochlear nucleus. Many surgeons prefer to place the electrode array of the ABI within the lateral recess to stimulate the DCN and the intraventricular part of the VCN (Fig. 18.1). In addition to the anatomical landmarks surrounding the cochlear nucleus, intraoperative monitoring of EABR using ABI-mediated stimuli is used to correctly place the electrode array on the cochlear nucleus. In non-NF2 patients, neurosurgeons prefer the retrosigmoid approach to the translabyrinthine approach because it is more familiar to them and the labyrinth and auditory nerve can be preserved.

18.4 Complications Specific to Auditory Brainstem Implantation

Compared to standard cochlear implantation, stimulation of nonauditory neurons, electrode migration, and cerebrospinal fluid (CSF) leakage are listed as ABI-specific complications. Up to 42 % of multichannel ABI users suffered from nonauditory sensations, including nausea, tingling in the throat, jittering of the visual field, and shoulder contraction, that were probably caused by activation of the vagal nerve, glossopharyngeal nerve, flocculus of the cerebellum, and accessory nerve, respectively [17, 18]. Incorrect positioning of the ABI electrode array is usually associated with nonauditory sensations. Accurate placement of the electrode array on the surface of the cochlear nucleus is essential in auditory brainstem implantation; however, a large tumor often compresses the pontomedullary



Abe and Rhoton 2006 [33], by courtesy of the author.

Fig. 18.1 (**a**) Cross section of pontomedullary junction at level of lateral recess of the fourth ventricle (4th Vent.). DCN (Dorsal Coch. Nucl.) and VCN (Ventral Coch. Nucl.) are located adjacent to the lateral recess (Lat. Recess) of the fourth ventricle. DCN underlies a prominence,

junction and creates difficulty in identification of the landmarks surrounding the cochlear nucleus. Changing the program of the speech processor, including deactivating the responsible electrodes and decreasing the amplitude of electrical stimuli, is usually effective to reduce these aversive ABI-mediated symptoms [17, 19]. Unlike the CI with an electrode array that is inserted into the cochlea, the electrode array of the ABI is placed on the surface of the brainstem with an unstable fixation. The fixation of the electrode array has been improved by the development of silicone backing, Dacron mesh, and nonelastic wire, but migration and dislocation of the electrode array still occur, especially in NF2 patients with large tumors compressing the brainstem. In these patients, the shape and position of the brainstem may change after tumor resection, which probably increases the risk for postoperative migration of the electrode array. Migration of the electrode array is usually associated with nonauditory sensations and deterioration of auditory perception; accordingly, these manifestations suggest the necessity of evaluating the position of the electrode array using high-resolution CT. The electrode lead of the ABI penetrates the meningeal dura and CSF may leak from the subarachnoid space to the mastoid air cells or the subcutaneous space along the electrode lead, regardless of the surgical procedure and choice of translabyrinthine or retrosigmoid approach [20, 21]. The percentage of CSF leakage in the ABI population is reported to range from 3.3 to 11 % [17, 19]. CSF leakage is usually controlled conservatively with or without lumbar drainage, but revision surgery is sometimes necessary.

18.5 Hearing Outcomes for Auditory Brainstem Implantation

During the last two decades, several groups have published the results of auditory brainstem implantation in NF2 and non-NF2 patients [17, 18, 21–24]. In general, hearing outcomes were lower in ABI recipients compared with outcomes for patients with CIs, but ABI-aided audiological performance varied widely according to the etiologies of deafness. As Colletti et al. established following long-term observation of 80 ABI adults with or without NF2 (Fig. 18.2) [23], trauma-induced cochlear nerve disruption and cochlear ossification were associated with favorable ABI outcomes and open-set speech discrimination scores greater than 50 %, while

Fig. 18.1 (continued) while VCN is more deeply imbedded in brainstem and does not produce as great a prominence on surface of brainstem. (**b**) Retrosigmoid view. Flocculus has been elevated to expose junction of vestibulocochlear nerve with side of brainstem at pontomedullary junction. Foramen of Luschka (For. Luschka), which is positioned dorsal to glossopharyngeal nerve (CNIX), is partially covered by choroid plexus. (**c**) Choroid plexus has been retracted rostrally to expose dorsal cochlear nucleus sitting in floor of lateral recess. Ventral cochlear nucleus is positioned in area between lateral edge of dorsal cochlear nucleus and junction of vestibulocochlear nerve (CN VIII) with brainstem [16]

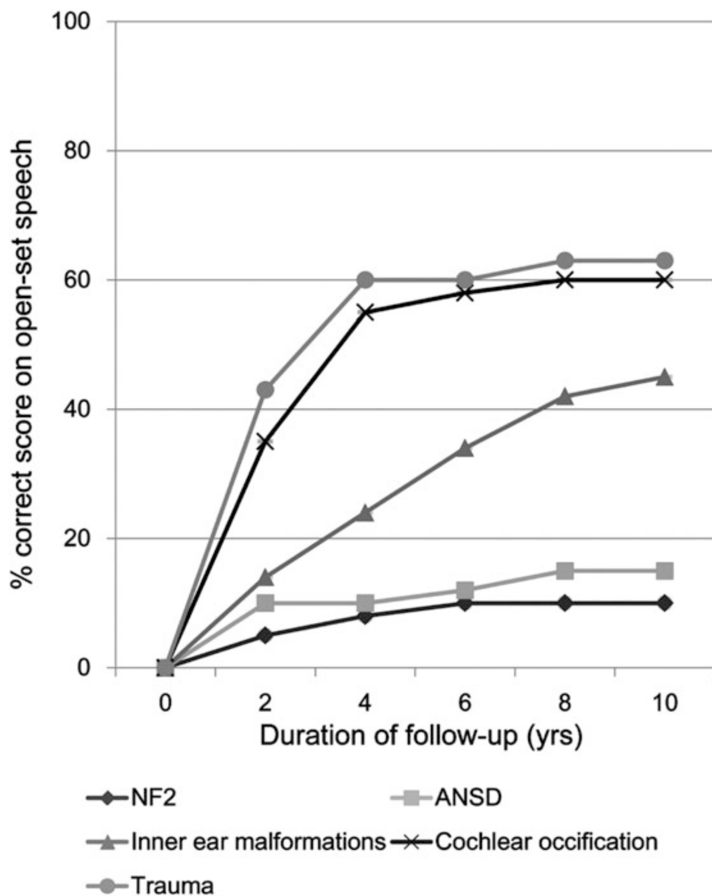


Fig. 18.2 Improvement of open-set speech discrimination scores (% correct) in patients with different causes of hearing loss after brainstem implantation [23]. NF2, neurofibromatosis type 2; ANSD, auditory neuropathy spectrum disorder; trauma, head trauma

subjects with NF2 and auditory neuropathy spectrum disorder (ANSD) showed only limited improvement of auditory performance, even though the majority of these patients had ABI-aided benefits in daily life, particularly in combination with lip reading. Patients with congenital inner ear malformations showed moderate ABI-aided speech discrimination scores that were better than those in the NF2 patients, but worse than those in patients with trauma-induced cochlear nerve disruption and cochlear ossification. Considering that both populations (NF2 patients and patients with cochlear nerve disruption and cochlear ossification) suffered from postlingual deafness, the limited benefit of ABI in patients with NF2, which was also reported by other studies [17, 22, 25], should be related to NF2-specific problems such as direct damage to the cochlear nucleus caused by chronic tumor compression on the brainstem, as well as surgical procedures for

tumor removal. ANSD is diagnosed by (1) absent or abnormal ABR and (2) present otoacoustic emissions and/or cochlear microphonics. Several reported etiologies causing sensorineural hearing loss meet these criteria, suggesting heterogeneity among patients with ANSD. Several studies have demonstrated that ANSD is highly associated with CND in the congenitally deaf population [26, 27]. In patients with an otoferlin-encoding (*OTOF*) gene mutation, synaptic release from inner hair cells is primarily affected, whereas demyelination and axonal loss may be responsible for hearing loss in systemic neurodegenerative diseases such as Charcot-Marie-Tooth disease. In Colletti's study, there was no description of the etiologies about ANSD; therefore, the exact reason for poor ABI-aided hearing outcomes in patients with ANSD was unclear. These results demonstrated that hearing loss of different etiologies was associated with varied hearing outcomes following auditory brainstem implantation.

18.6 Auditory Brainstem Implantation Compared with Cochlear Implantation

Previous studies showed that peripheral stimulation with CIs usually results in better outcomes than central stimulation of the auditory neural system using an ABI, suggesting that cochlear implantation should be tried before ABI whenever possible [10]. Utilization of the simple linear tonotopic organization in the cochlea and the natural sound processing mechanisms in the auditory nerves and neurons in the cochlear nucleus might contribute to favorable outcomes for cochlear implantation. The relatively high probability of ABI-related complications, including nonauditory stimulation, electrode migration, and CSF leakage, requires surgeons to carefully consider the indications for auditory brainstem implantation. As discussed previously, patients with Michel deformity or sacrifice of the cochlear nerve during tumor resection are definitely ABI candidates [8–10]. Trauma-induced bilateral cochlear nerve disruption is also an indication for auditory brainstem implantation, although this situation is unlikely to occur without the subject incurring fatal damage to the brain [8]. In patients with cochlear ossification and advanced otosclerosis, cochlear implantation may be followed by placement of an ABI [8]. For patients with cochlear aplasia or absence of the cochlear nerve or VCN, a more comprehensive analysis would be required to conclude the advantage of ABI. Due to the limitations of spatial resolution, high-resolution CT and MRI cannot exclude the possibility that a small number of cochlear nerve fibers innervate the inner ear, indicating the necessity for other functional evaluations. Since there are only a few reported cases of patients undergoing cochlear implantation followed by auditory brainstem implantation on the same side [28], the evidence for the advantages of an ABI compared with a CI for speech perception has not been fully established. In clinical application, a CI might be recommended for the initial operation in these patients. If the CI fails to provide sufficient auditory sensation,

then an ABI may be the only solution. It should be noted that the efficacy of auditory brainstem implantation may decrease after the critical period for development of auditory perception if effective auditory stimulation is not available during the first several years after birth. Therefore, prelingually deaf children should undergo implantation of an ABI as early as possible if an ABI is indicated [9]. To resolve the dilemma of the need for careful decision-making regarding the indications for ABI and the importance of brainstem implantation at an early age, electrophysiological evaluation of the auditory system might be effective. As described in Chap. 15, intraoperative CI-mediated EABR testing may be useful to predict outcomes of CI [29]; thus, electrophysiological examination may be an effective method to determine the necessity for an ABI during cochlear implantation.

18.7 Future Prospects

As described above, different causes of hearing loss were associated with different hearing outcomes following implantation of an ABI [23]. Since the ABI directly stimulates the cochlear nucleus, ABI outcomes should be influenced by the severity of pathological changes in the cochlear nucleus. Compared with SGNs, neurons in the cochlear nucleus are less susceptible to loss of hair cells, but transneuronal loss of neurons in the cochlear nucleus occurs when the cochlea is damaged during an early period of development [30]. Interestingly, prior to exposure to normal environmental stimuli during the developmental period, spontaneous neural firing is observed in the central auditory system. Similar to other sensory systems, these spontaneous neural activities are thought to facilitate maturation of auditory neurons and their synapses [31]. Therefore, the congenital hypoplasia of SGNs that is associated with CND can attenuate synaptic input from the cochlear nerve to the neurons in the cochlear nucleus, which may lead to disorganization or immature development of neuronal connectivity in the cochlear nucleus. In this respect, CI-mediated intracochlear stimulation preceding auditory brainstem implantation may be effective to maximize the use of the remaining SGNs and promote development of the central auditory system, even if a CI would provide only limited auditory sensation. As discussed in Chaps. 28 and 30, the combination of a CI with the SGN regeneration or extension of SGN afferent dendrites by pharmacological or genetic methods can increase the responsiveness of SGNs to CI-mediated electrical stimuli, which in turn increases synaptic input from the cochlear nerve to the cochlear nucleus.

Even after ABI surgery, it is possible that the remaining SGNs contribute to maintaining the functions of the cochlear nucleus. It is well known that a synaptic vesicle contains trophic factors in addition to neurotransmitters [32, 33]. Spontaneous synaptic release from the axon terminals of the remaining cochlear nerve fibers does not contain auditory information, but co-transmitted trophic factors can promote survival of neurons or maintain the dendritic protrusion in the

cochlear nucleus. CI-mediated electrical stimuli showed some trophic effects to SGNs [34], but a part of these effects may be induced indirectly by stimulation of glia and residual hair cells around the afferent dendrites of SGNs, rather than by direct activation of the SGNs. In the same way, ABI-mediated electrical stimuli can elicit synaptic release from the axon terminal of the residual SGNs, which may have positive effects to maintain the functions of the cochlear nucleus. Taking these results into consideration, both SGN regeneration and prevention of SGN degeneration may show some benefit to improve ABI outcomes, even though ABIs bypass the cochlear nerve to directly stimulate the cochlear nucleus. In patients with NF2, the cochlear nerve is often severely damaged. The cochlear nerve and the modiolus of the cochlear are accessible during removal of the tumor; therefore, pharmacological, cell biological, and gene therapy approaches to the remaining SGNs or cochlear nerve may be applicable during surgery.

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Part IV
Hair Cell Regeneration

Chapter 19
Hair Cell Regeneration in the Avian

Tomoko Kita

Abstract When hair cells are once damaged by disease, noise trauma, or aging, hearing loss occurs in a person. Although fish, amphibians, and birds exhibit the ability to grow new hair cells to replace damaged ones, mammals cannot regenerate hair cells by themselves. In this chapter, the regenerative mechanisms in avian inner ear are reviewed comparing with mammals.

Keywords Avian • Hair cells • Regeneration

19.1 Inner Ear Structure in Birds

Avian and mammalian vestibular organs have similar morphology; however, the anatomy of the basilar papilla (cochlear organ of lizards, amphibians, and birds) is considerably different from mammalian cochlea (Fig. 19.1). The avian sensory epithelia consist of the long-wide patch and are comprised of hair cells that are surrounded by relatively undifferentiated supporting cells. Although subclasses of avian supporting cells may be identified by differences in transcription factor expression [2], all supporting cells in the basilar papilla appear to have very similar morphologies like vestibular organs (Fig. 19.1b, c). In contrast, specialization of the mammalian organ of Corti for high-frequency hearing led to a reduction in hair cell numbers and a variety of distinct supporting cell phenotypes, which can be distinguished by both morphological [3] and molecular [4, 5] criteria (Fig. 19.1a).

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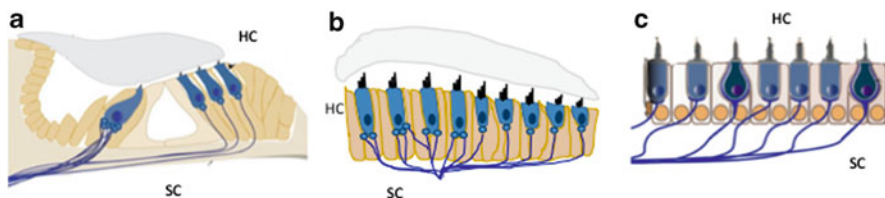

Fig. 19.1 Structure of inner ear sensory epitheria (modified from [1]). (**a**) Mammalian organ of Corti. (**b**) Avian basilar papilla. (**c**) Avian and mammalian vestibular epitheria

Another typical point of the structure for avian sensory epithelia is the weak cellular junction throughout the life, compared with the thick F-actin belts of the murine supporting cell from postnatal to adulthood [6, 7]. Generally adherens junction molecules associated with surrounding belts (E-cadherin, beta-catenin, or p120 catenin) translocate to the nucleus and alter transcription which regulate proliferation and differentiation in other epithelial cells [8–11]. It is therefore expectable in mammalian sensory epithelia that belt stiffness may restrict junctional molecules from transport to the nucleus, thereby limiting their ability to bind transcriptional elements that induce cell cycle entry and cell fate changes [12, 13].

19.2 Hair Cell Regeneration in the Avian Inner Ear

In contrast with mammals, mature birds can regenerate lost hair cell following sound exposure, aminoglycoside treatment, laser ablation, or genetic mutation [14–22]. Data from these groups strongly suggested that new hair cells were being produced in the damaged mature basilar papilla to replace those destroyed. In most cases, the hair cell loss induces non-sensory supporting cells to undergo either “proliferative” or “nonproliferative (direct transdifferentiation or self-repair)” mechanism for making new hair cells [21] (Fig. 19.2). On that time, the hair cell death and ejection from the sensory epithelium regulate the onset and progression of supporting cells producing hair cells [23–28], which was testified through the reduce of supporting cell mitosis after the rescues of damaged hair cells by apoptotic inhibitors [29].

In the first step of regenerative response, most of the supporting cells begin to reexpress developmental genes that are found in prosensory progenitors within a day after induced hair cell loss [26, 30, 31] (Fig. 19.2). It has been hypothesized that this process, which is also referred to as direct transdifferentiation, triggers a second response phase in which remaining supporting cells reenter the cell cycle and refill the supporting cells lost due to their transdifferentiation [32]. And in this second step, supporting cells are also able to respond to hair cell loss in parallel by asymmetric division, giving rise to pairs of replacement hair and supporting cells [33, 34]. The regenerative process in vitro is variable among culture conditions and degrees of ototoxic damage [35, 36]. Although it is still unclear which regenerative process is the dominating one, there is the strong evidence that both processes, “proliferative”

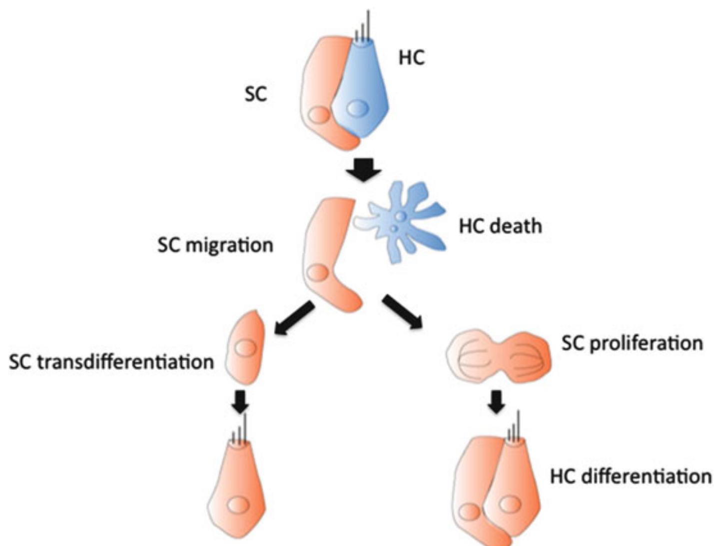

Fig. 19.2 Proposed mechanisms, proliferation and nonproliferation (transdifferentiation), of hair cell regeneration in the avian inner ear

and "nonproliferative," happen in in vivo damaged avian basilar papilla, where supporting cell mitotic proliferation is activated not only to make new hair cells but also to replace the supporting cells lost for direct transdifferentiation [28, 31, 32, 37–40]. Compared to the basilar papilla, asymmetric supporting cell division occurred in both damaged and undamaged avian vestibular epithelium [26]. The cause of spontaneous hair cell death remains a mystery; however, turnover of undamaged vestibular hair cells is quite widespread among vertebrates which strongly hallmarks an existence of "stem cell" population in these tissues. Further study for the clarification of the causes of hair cell death with high turnover rates and characterization of "stem/stem cell-like cell" population in vestibular epithelium may provide clues into the regeneration in mammalian inner ear [41].

19.3 Molecular Mechanisms of Avian Hair Cell Regeneration

Although the mechanisms of hair cell regeneration are not completely understood yet, some researchers' approaches led to the identification of several molecules with the capacity to stimulate or block hair cell regeneration in avian sensory epithelium as follows.

It was found in the coculture experiments with chick damaged vestibular epithelium or normal ones that a soluble factor released from the damaged vestibular epithelium stimulated proliferation and factor from normal tissue suppressed

mitotic activity [42]. From the subsequent experiments, this group suggests that robust regenerative proliferation of stem/stem cell-like cells in mature sensory epithelium requires a simultaneous release from negative regulation coupled with mitogenic signaling for epidermal growth factor (EGF) and/or insulin-like growth factor 1 (IGF-1)/insulin, but not with fibroblast growth factor 2 (FGF-2) [43–45]. Large-scale microarray analysis has performed for different damaging condition in chick sensory epithelium combined with the RNA interference (RNAi)-based method [30, 46]. Several distinct signaling pathways were identified at the regeneration stage by the use of the gene array, transforming growth factor beta (TGF-beta), Notch, Wnt, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB), IGF-1/insulin, and activator protein 1 (AP-1). Small interfering RNA (siRNA) of Cutl1 resulted in inhibition of supporting cell proliferation [46], which molecule is the downstream targets of TGF-beta and suppresses cyclin-dependent kinase inhibitor $p27^{Kip1}$. Cyclin-dependent kinase inhibitors control cell cycle entry, progression, and exit, so regulating cell cycle seems to be an important pathway to induce hair cell regeneration more directly [47, 48]. Other molecules including AP-1 (CCAAT enhancer binding protein (CEBPG), Jun-D, low-density lipoprotein receptor-related protein 5 (LRP5)), Pax (Pax2 and Pax5), and Wnt (Wnt4) were also suggested to regulate the supporting cell proliferation during chick hair cell regeneration. In addition to these secreted factors, cell–cell signaling mediated by Notch pathway or extracellular adherence proteins has been considerable. During hair cell regeneration Notch drives again lateral inhibition and the selection between hair cell and supporting cell fates [49–51]. As a consequence, the blockade of Notch signaling during regeneration results in the overproduction of hair cells at the expense of supporting cells [51, 52]. Notch1 and Jagged1 are expressed in the supporting cells of the adult basilar papilla [49, 51]. During regeneration Atoh1 is rapidly induced in the supporting cells (hours) [31, 52] and followed by Delta1 (days). Thereafter, hair cell differentiation markers are expressed and both Atoh1 and Delta1 downregulated [49]. Atoh1 is upregulated in the avian cochlear during hair cell regeneration [31, 51], suggesting that the regenerative mechanism in birds at least partially recapitulates aspects of embryonic hair cell development. Other pathways also have been implicated to trigger cell proliferative response in damaged and/or undamaged chick auditory epithelium and are mediated by cyclic AMP (and its regulated protein kinase A (PKA)), fibroblast growth factor receptor 3 (FGFR3), and Prox1 [53–55].

Because of the difficulty of gene manipulating for birds like transgenic chick, there is somewhat limitation to confirm the mechanism for regenerative response.

19.4 Functional Recovery of Hair Cell Regeneration

It is important to note that regeneration has been examined in several avian species (Gallus, Galliformes, Columbiformes, Passeriformes, and Psittaciformes) and appears to be essentially identical in all cases. Also for the function of regenerated

avian ear, both hearing and vestibular sensitivities return to near-normal levels after sufficient recovery times [56]. Such findings confirm that afferent neurons can reestablish functional synaptic contacts with regenerated hair cells. Studies of vocalizations in songbirds also suggest that higher level auditory functions are also restored after hair cell regeneration [57, 58]. In addition, regenerated hair cells develop with morphologies that are consistent with their position along the proximal-to-distal axis [59]. And tuning curves, while broad during the initial period of regeneration, ultimately sharpen to degrees that are close to their pre-damage levels [60]. These results suggest that positional information like tonotopic axis could be supplied by hair cells located adjacent to the region of damage or by the supporting cells that remain. Ultimately, the regenerating sensory epithelium must produce enough new hair cells and supporting cells to structurally and functionally repopulate the damaged region.

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Chapter 20
Self-Repair

Akiko Taura and Takayuki Nakagawa

Abstract Hair cells are known to be hard to regenerate in mammals once they are damaged. However, it is possible to repair even after damage, by blocking the degenerative pathway like an apoptosis. When hair cells are damaged, inflammatory responses are observed at first. At this stage, anti-inflammatory drugs such as glucocorticoid or cytokines are effective against hair cell damage. Also hair cells have proceeded to damage, mainly through a process known as apoptosis. During this process, anti-apoptotic drugs are effective for hair cell repair. Anti-reactive oxygen species (ROS), c-Jun-N-Terminal kinases (JNK) inhibitor, or caspase inhibitors are considered to be protective effect against hair cell damage. Neurotrophic factors and growth factors might be also effective for hair cells.

Keywords Apoptosis • Inflammatory responses

20.1 Introduction

Mammals show little capacity for generation of new hair cells in contrast to lower vertebrate such as avian. Cochlear sensory epithelium is known to be hard to regenerate in mammals; however, vestibular sensory epithelium has been reported to have some ability to regenerate [1–3]. Vestibular hair cells were able to regenerate morphologically and functionally after ototoxic drug damage and this self-repair was promoted by the application of glucocorticoids (GCs) [3]. GCs have been widely used as the clinical treatment of inner ear disease [4]. Several mechanisms may contribute to GCs effect. GCs have anti-oxidative effects,

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anti-inflammatory effects, anti-apoptotic effects, and so on. In this section, in terms of sensory epithelia, especially hair cells, the mechanism to be damaged by drugs is mentioned first, and next, some drugs can prevent irreversible damage of hair cells and help self-repair.

20.2 Mechanism of Hair Cell Damage

Once the inner ear has been damaged by several types of damage such as noise trauma or ototoxic drugs, lots of inflammatory cells infiltrate into the inner ear around 3–7 days after [5, 6]. Sato reported that excessive infiltration of hematogenous macrophages in the cochlea resulted in severe damage in the cochlea after ototoxic insult [7]. Interleukin 6 (IL-6) is well known as a pro-inflammatory cytokine that has lots of roles in immune responses and inflammations. In the central nervous systems, a strong involvement of IL-6 is pointed out on inflammatory responses. For example, in spinal cord injury, the inhibition of IL-6 reduced inflammatory cells and reactive astrogliosis and led to functional recovery [8]. In the inner ear, the upregulation of IL-6 was reported in ototoxic drug-treated models [9, 10]. Also TNF-α plays an important role as pro-inflammatory cytokines in ototoxic-induced cochlear damage [10, 11]. IL-6 and TNF-α activate NF-κB signal, leading to further production of pro-inflammatory cytokines. As another pathway, IL-4/IL-13 activates STAT6, leading to further production of pro-inflammatory cytokines [12]. These two pathways are considered to interact with each other and cause damage to sensory epithelium.

Also hair cells have proceeded to damage, mainly through a process known as apoptosis [13, 14]. Apoptosis is a very complex series of events when a cell faces a bad environment. Once apoptosis is initiated, the cells proceed to cell death. However, there is a period to go into apoptotic pathway or not. When the anti-apoptotic signals are dominant, the cell can escape from apoptotic pathway and avoid cell death. When the apoptotic signals are dominant, the cells go to cell death through the apoptotic cascade. In general, apoptosis is associated with phosphorylation of the transcription factor c-Jun (the activation of c-Jun-N-Terminal kinases (JNKs)) and the release of cytochrome *c* from the mitochondria and resulted in the activation of caspase.

Ototoxic drug such as aminoglycoside generates reactive oxygen species (ROS) which can lead to damage through reaction with many critical molecules [15]. The accumulation of ROS is an early step in hair cell death by ototoxic drug [16–18]. This early generation of ROS might cause the activation of JNKs in damaged hair cells. The JNKs, also known as "stress-activated protein kinases," are in the family of mitogen-activated protein kinases (MAP kinases). The activation of JNK occurs upstream of cytochrome *c* release from the mitochondria into the cytoplasm in hair cells exposed to ototoxic drug [19]. The release of cytochrome *c* from the mitochondria into the cytoplasm is thought to be an important step in apoptosis. The cytochrome *c* plays a role in apoptosis of vestibular hair cells

induced by aminoglycosides [20]. These events are followed by the activation of caspase-3. The activation of caspase-3 is regarded as the beginning of the terminal stage of cell death; however there is some period after the initial activation of caspase-3. There is a possibility for damaged hair cells to repair by the blockage of these signals in the apoptotic cascade.

20.3 Anti-inflammatory Drugs

20.3.1 Glucocorticoids

Glucocorticoid receptor expressions have been detected in all sensory epithelia of cochlear and vestibular tissues [21]. The cytokines released from cochlea as a result of noise exposure might be a target for GCs treatment for inner ear disorders [9]. In spinal cord injury, GCs inhibit production of IL-6 [22]. When dexamethasone (one of the GCs) was applied to the round window of the noise-treated guinea pig, outer hair cell loss reduced and the shift in ABR threshold decreased [23]. Dexamethasone was also effective in noise damaged rat cochlea [24] and damaged rat vestibule by ototoxic drug [3].

20.3.2 Inhibition of Inflammatory Cytokines

Anti-IL-6 receptor antibody was effective against noise damage in the cochlea [25]. The inhibition of TNF-α is also effective. For example, mannitol has otoprotective effects against TNF α-induced auditory hair cell (HC) loss [26].

20.3.3 Activation of Prostaglandin (PG)

The PGs during the promotion of inflammation is reported to be effective as nonsteroidal anti-inflammatory drugs (NSAIDS) [27]. In the inner ear, PGs are well known as the regulators of cochlear blood flow. PGE2 is the most abundant prostanoid in humans and involved in regulating many different biological functions [28]. PGE2 have four kinds of receptors, E prostanoid receptor (EP) 1–4. Among them, the activation of EP4 in cochlea significantly attenuates noise-induced damage [29] and the activation of EP2 and EP4 induces the formation of vascular endothelial growth factor in the cochlea [30]. Therefore the activation of EP signaling must lead to the development of novel therapeutic options for hearing loss in future [31].

20.4 Blockage of Hair Cell Apoptosis with Therapeutic Drugs

20.4.1 Anti-reactive Oxygen Species (ROS)

Up to now, lots of studies reported that an increase of ROS is an early event in programmed cell death [32]. ROS are also produced in hair cells in response to ototoxic drug exposure. So free radical scavengers can protect hair cells against ototoxic drug-induced death. Copper/zinc-superoxide and iron chelators can protect aminoglycoside-induced hearing loss in vivo [33]. And glutathione and hydrogen are also reported to be effective against hair cell damage by ototoxic drugs [34–36]. Bearing these in mind, anti-ROS therapies should be developed more in future for clinical use.

20.4.2 Anti-activation of JNK Signaling

JNK activation by phosphorylation has been shown to be important for neuronal cell death after trophic factor withdrawal in vitro and after injury in vivo [37, 38]. The activations of JNK in the inner ear by ototoxic drugs [39] and noise exposures [40] were also reported. The current study provides evidence that CEP-1347, an inhibitor of the JNK signaling pathway, may offer therapeutic potential to prevent death of both cochlear hair cells and neurons and to act against both acoustic and ototoxic trauma [39]. CEP-11004, indirect inhibitor of JNK activation, promoted the survival of hair cells after neomycin treatment [19]. The otoprotective efficacy of D-JNKI-1, a cell-permeable peptide that blocks the MAPK–JNK signal pathway, was also shown in vitro [41]. AM-111 is also reported as an otoprotective drug by blocking the JNK pathway [42–45]. In Germany, a prospective randomized clinical trial of the intratympanic AM-111 administration to acute acoustic trauma has been done and revealed the hearing recovery [46].

Heat shock proteins (HSPs) are part of an evolutionarily conserved stress response that is activated by changes in the cellular environment [47]. HSPs have strong cytoprotection and inhibit various apoptotic signaling proteins. Heat shock has a significant effect against both aminoglycoside- and cisplatin-induced hair cell death in the utricle preparation in vitro [48]. HSP is considered to inhibit proapoptotic JNK activation and cause hair cell death [49].

20.4.3 Anti-caspase Activation

Caspase constitutes a family of proteases that normally exist as inactive enzymes. Previous studies reported that general caspase inhibitors are able to promote hair cell survival after ototoxic drugs [50, 51]. There are 14 known caspases in

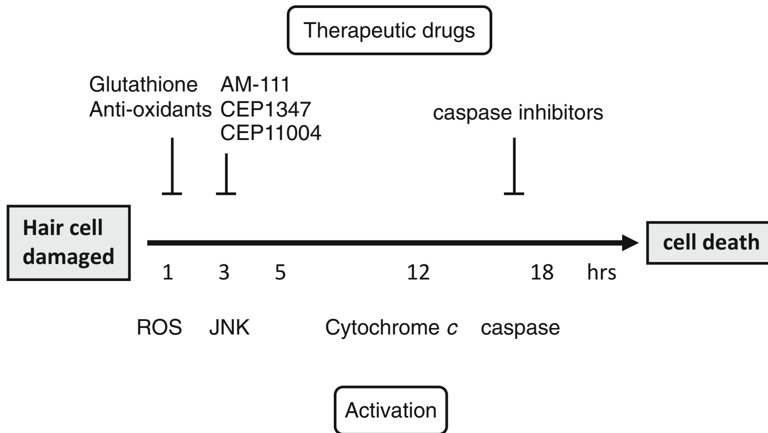

Fig. 20.1 Summary of critical events and therapeutic drugs after hair cell damage in the apoptotic process. Just after hair cell damage, ROS and JNK are activated. Several hours later, cytochrome *c* and caspase are activated. At each step, inhibitors such as anti-ROS, anti-JNK, or anti-caspase are effective against hair cell damages

mammals; some of them are thought to relate to apoptosis. Apoptosis-related caspases have been categorized into upstream initiator caspases and downstream effector caspases [52]. The upstream initiator caspases are caspase-8 and caspase-9. Caspase-9 activation requires the release of cytochrome *c* from the mitochondria. The inhibition of caspase-9 provided significant protection of hair cells exposed to neomycin [50]. Once activated these initiator caspases can activate downstream effector caspases, such as caspase-3. Effector caspases then carry out the apoptotic program by cleaving proteins necessary for cell survival. Caspase-3 activation appears at the same time that dying hair cells begin to bleb up and be ejected from sensory epithelium [53]. It is important to develop more specific inhibitors for clinical use in future (Fig. 20.1).

20.5 Other Therapeutic Drugs

20.5.1 Neurotrophic Factors

Neurotrophic factors are important for development, differentiation, or survival of neurons. In non-neuronal tissues such as inner ear, some neurotrophic factors are important for normal development and have a protective effect against hair cell damage. Brain-derived neurotrophic factor (BDNF) was effective for inner ear against drug-induced damage in vivo [54], and neurotrophin-3 (NT-3) is also important for developing auditory sensory epithelium. Both BDNF and NT-3

were found to be effective against ototoxic drug in rat auditory hair cells [55]. Although these neurotrophic protections of hair cells against drug and noise trauma appear to be statistically significant, the extent of protection is less than enough for clinical use in future.

20.5.2 Growth Factors

The insulin-like growth factor (IGF) signaling is very important in the inner ear development [56]. IGF-1 is effective against hair cell damage by noise trauma in vivo and by ototoxic drugs in vitro [57, 58]. IGF-1 maintained the hair cells in injured cochlea through the activation of both the PI3K/Akt and MEK/ERK pathways [59]. And the involvement of epidermal growth factor (EGF) and (TGF-α) in the inner ear is suggested. TGF-α and EGF (plus insulin) were reported to stimulate cell proliferation in mammalian vestibular sensory epithelium [60].

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Chapter 21
Transdifferentiation

Norio Yamamoto

Abstract Transdifferentiation is defined as the changing of one terminally differentiated cell type into another without producing a pluripotent intermediate. It was originally described in the conversion of fibroblasts to skeletal muscle cells and since then, it has been reported in various other tissues and even across different germinal layers. Transdifferentiation never occurs naturally and at a basic level, it requires overexpression of 2–5 selected transcription factors. These factors could be either expressed specifically in the target organ or those that have pivotal roles in the target cells.

In the regeneration of avian auditory sensory epithelia, supporting cells that are located around hair cells spontaneously transdifferentiate into hair cells after hair cell injury. Although postnatal mammalian hair cells never regenerate spontaneously, Atoh1 overexpression and the inhibition of Notch signaling have been reported to induce regeneration of hair cells through transdifferentiation of supporting cells in rodents. These results suggest transdifferentiation is a useful strategy for hair cell regeneration. However, the reported strategies manipulated only one factor and hair cell regeneration was inefficient. Therefore, investigating a more efficient combination of regeneration factors will be necessary to achieve clinically useful hair cell regeneration.

Keywords Atoh1 • Notch signaling • Transdifferentiation

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21.1 Transdifferentiation

21.1.1 Definition of Transdifferentiation

Transdifferentiation, or direct conversion, has been defined as the changing of one terminally differentiated cell type into another without producing a pluripotent intermediate [1] (Fig. 21.1). From the point of view of regenerative medicine, transdifferentiation is usually achieved by the exogenous expression of one, or a combination of several, transcription factor(s), and this is considered a form of reprogramming. Another typical example of reprogramming is de-differentiation to establish induced pluripotent stem cells (iPSCs). Exogenous expression of four critical transcription factors changes the differentiation status of well-matured cells into extremely immature pluripotent cells, iPSCs [2]. iPSCs can differentiate into any cell type, and thus, these cells have varied applications in regenerative medicine (Fig. 21.1b). The process of transdifferentiation is different from de-differentiation-mediated regeneration because transdifferentiating cells do not go through an immature pluripotent stem cell stage, but instead, they turn directly to a different type of cell (Fig. 21.1c). Transdifferentiation has been reported for various kinds of differentiated cells including skeletal muscle cells [3], cardiomyocytes [4], pancreatic β-cells [5], neurons [6], hepatocytes [7, 8], and Sertoli cells [9]. The transdifferentiation process can be of two types, depending on the differences between the original and the transformed cells. One is the conversion of cell types within the same germinal layer and the other occurs across different germinal layers. The former approach was demonstrated for the first time in 1987 with the conversion of fibroblasts into skeletal muscle cells using expression of the transcription factor myoblast determination (MyoD) [3]. Subsequent studies have also shown successful cell fate conversion within the same germ layer. In contrast, cell

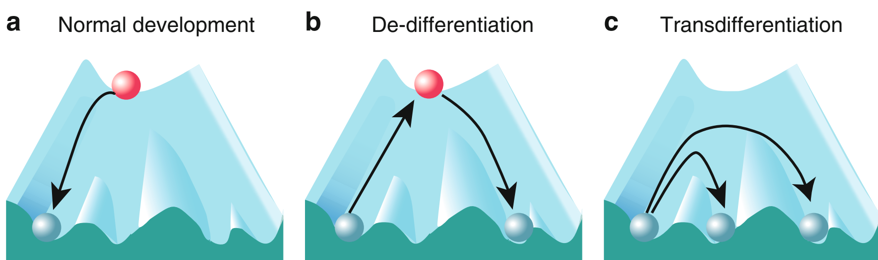

Fig. 21.1 Schematic illustration of normal development, de-differentiation, and transdifferentiation. The *top* of the hill represents an immature status of the cell, and the *bottom* represents a differentiated status. (**a**) In normal development, a pluripotent cell (a *red ball* on the *top* of the hill) rolls down to the bottom to differentiate into a mature cell (a *gray ball*). (**b**) A mature cell can be reprogrammed to become a pluripotent cell (induced pluripotent stem cell (iPSC)), which is represented by rolling up the hill. (**c**) A mature cell becomes another type of mature cell without producing a pluripotent intermediate, which is represented by jumping over the hill. This schematic illustration was modified from that presented in [1]

fate conversion across different germinal layers was not demonstrated until 2010 when the conversion of fibroblasts into neurons with a cocktail of three transcription factors was reported [6].

21.1.2 Transdifferentiation and Transcription Factors

Originally, transdifferentiation protocols used the overexpression of only 2–5 transcription factors, which are also called "reprogramming factors." To find a combination of the minimal set of transcription factors, most experiments used 5–20 transcription factors that satisfied the following criteria: (1) the transcription factors are specifically expressed in embryonic or mature target cells or their progenitor cells and are important for the development, maintenance, or both of the desired cell type and (2) target organs exhibit severe defects when the transcription factors are mutated [5–9]. After confirming that simultaneous transfection of all the transcription factors transformed fibroblasts or other types of cells into the desired cells, the effects of withdrawing individual transcription factors were observed to determine the minimal set of genes that can transform fibroblasts into the desired cells. For example, transdifferentiation of pancreatic β-cells requires three reprogramming factors: Ngn3, Pdx1, and Mafa [5]. All of them are important for the development of the pancreas and pancreatic β-cells, although there are many other factors that play pivotal roles in the development of pancreatic β-cells. However, the reasons why only these three factors are sufficient to induce pancreatic β-cells remain to be elucidated. Currently, a combination of microRNAs and transcription factors is also used to induce more efficient transdifferentiation [10].

21.1.3 Advantages and Disadvantages of Transdifferentiation

Although regenerative therapy using transdifferentiation is a promising method, there are some problems in its application. One problem is that no set of reprogramming factors has been shown to work robustly in both mouse and human cells, as observed in cardiomyocyte regeneration. This indicates that the results of animal in vivo experiments cannot be extrapolated to clinical applications. This situation is different from that of iPSCs, which can be induced by same set of reprogramming factors in both mice and humans. Enormous efforts will be necessary to understand the difference between animal and human studies in transdifferentiation.

Other problems include successful transdifferentiation only occurring with in vivo experiments [5] and problems with reproducibility [4, 11], indicating that more detailed analyses are still required. However, transdifferentiation will provide a more promising method for regenerative medicine than the stepwise

differentiation from pluripotent stem cells. To achieve regeneration by transplanting pluripotent stem cells (embryonic stem cells or iPSCs) that are induced to develop into desired cell types, sufficient numbers of cells must be prepared through efficient expansion. The induced cells must be efficiently delivered into the damaged organs and properly engrafted. Engrafted cells may require treatment to survive in the donor sites. Potential tumorigenicity of residual stem cells [12] and possible immune rejection also need to be considered. These pitfalls of cell-based therapies can be avoided by delivering reprogramming factors directly into damaged organs to induce regeneration through transdifferentiation.

21.2 Transdifferentiation and Hair Cells

21.2.1 Transdifferentiation and Avian Hair Cells

In the context of the regeneration of auditory or vestibular hair cells, transdifferentiation is an important phenomenon. In birds, vestibular hair cells regenerate throughout their life [13] and even auditory hair cells can regenerate after injury [14, 15]. Two underlying mechanisms of these regenerative processes have been identified. One is the proliferation of supporting cells that surround hair cells [14, 15]. Incorporation of ^{3}H-thymidine showed that supporting cells induced mitosis and daughter cells contributed to the regenerated hair cells. The other mechanism was the direct transdifferentiation of supporting cells into hair cells [16, 17]. Pharmacological blockade of mitosis with Ara-C or aphidicolin after hair cell injury in mature birds caused cell cycle arrest but did not prevent new hair cell production, indicating that newly regenerated hair cells were produced by mechanisms other than proliferation [16, 17]. Transdifferentiation was also morphologically confirmed by the identification of cells that had features of both hair cells and supporting cells [18]. Transdifferentiation may occur readily between hair and supporting cells in birds because both cells share common progenitors during their development [19].

21.2.2 Transdifferentiation and Mammalian Hair Cells

In mammals, hair cells in the postnatal stage never regenerate spontaneously because the proliferation of sensory epithelia ceases during the embryonic stage. This was proved by conducting experiment using ^{3}H-thymidine incorporation [20]. In addition, no spontaneous transdifferentiation from supporting cells to hair cells has been observed in mammals. However, with accumulating knowledge of inner ear development, overexpression of a transcription factor and the manipulation of signaling pathways have been attempted in order to induce the regeneration of hair cells.

21.2.2.1 Transdifferentiation and Atoh1

Among several transcription factors related to the development of hair cells, Atoh1 is the most promising factor for the regeneration of hair cells. Atoh1 is one of the proneural basic helix-loop-helix transcription factors that forms a dimeric complex with other basic helix-loop-helix transcription factors to activate transcription of target genes in order to maintain the cell cycle or to promote differentiation. Disruption of *Atoh1* with gene targeting caused complete loss of hair cells [21, 22], as well as supporting cells [22] in mice. On the basis of this result, Atoh1 was overexpressed in postnatal mammalian cochlea in vitro by using electroporation [23] and in vivo by using adenovirus vectors [24, 25], to induce transdifferentiation of hair cells. Atoh1 overexpression in rat cochlear explant culture showed induction of ectopic hair cell-like cells in the greater epithelial ridge [23], suggesting the transdifferentiation of epithelial cells in this region to hair cell-like cells. This study confirmed that the transfected cells expressed hair cell markers and that they had morphological characteristics of hair cells including cuticular plate and stereocilia although the functions of hair cells were not examined. Overexpression of Atoh1 in guinea pig cochleae in vivo using an adenovirus vector induced the development of ectopic hair cell that received neural axons from auditory nerves [25]. The source of transdifferentiated hair cells includes interdental cells, inner sulcus cells, and Hensen's cells. Moreover, when Atoh1 was overexpressed in the guinea pig cochleae after pharmacological hair cell injury, functional recovery as well as hair cell regeneration was observed [24]. In this study, the regenerated cells showed a mixed phenotype with features of both outer hair cells and supporting cells, as observed in transdifferentiation of hair cells in birds. However, Atoh1 overexpression in the cochlea using transgenic mice showed that the competency of hair cell transdifferentiation was limited to the neonate and juvenile stages [26, 27]. Moreover, Liu et al. showed that permanent expression of Atoh1 induced hair cell loss [27]. These results indicate that Atoh1 alone is insufficient as a reprogramming factor and Atoh1 should be combined with other transcription factors to induce a more complete transdifferentiation of hair cells.

21.2.2.2 Transdifferentiation and Notch Signaling

As a cell fate determinant, Notch signaling has important roles in various tissues including the central nervous system, hematopoietic system, and inner ears. Genetic disruption of *Jag2*, one of the ligands of Notch signaling, caused an increase in the numbers of hair cells and decreased the number of supporting cells [28], suggesting that cell fate determination between hair and supporting cells is regulated by Notch signaling. This was confirmed by more complete blockade using gene targeting of *Rbpj*, a common transcriptional regulator downstream of all Notch receptors [29, 30]. These studies using *Rbpj* mutant mice also showed that Notch signaling is necessary for the maintenance of sensory epithelium progenitors. On the basis of

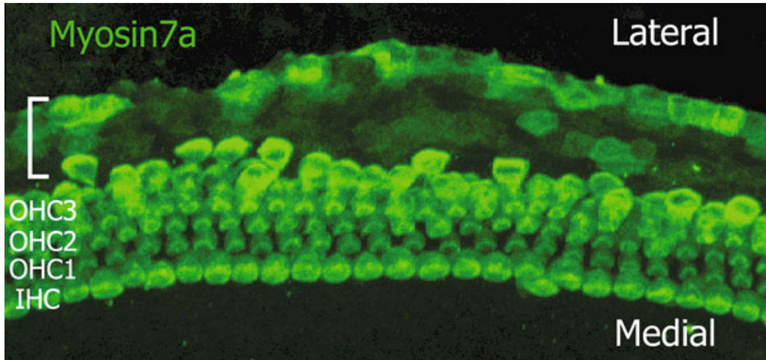

Fig. 21.2 γ-Secretase inhibitor induced ectopic hair cells. myosin7a (a hair cell marker) was visualized in *green*. Ectopic hair cells were observed (*bracket*) lateral to one row of inner hair cells (IHC) and three rows of outer hair cells (OHC1-3).

these findings, transdifferentiation from supporting cells to hair cells was examined by inhibiting Notch signaling. A conditional knockout of *Rbpj* in neonatal mouse cochlear explants showed that myosin7a-positive hair cell-like cells were induced from non-sensory epithelial cells in the cochlea by cell autonomous effects of *Rbpj* gene deletion [31], although the induced cells did not have stereocilia. Similar results were obtained through the pharmacological inhibition of Notch signaling by using a γ-secretase inhibitor [31] (Fig. 21.2). γ-Secretase inhibitor treatment on adult cochlea with injured hair cells caused the transdifferentiation of hair cells from supporting cells and functional recovery, although the hearing threshold change was very small [32].

21.2.3 Future Directions for Transdifferentiation-Mediated Hair Cell Regeneration

Currently, only a single factor has been manipulated to induce transdifferentiation of hair cells in the cochlea, and the induced hair cells are not identical to physiological hair cells. This indicates that a single factor is insufficient and that several factors should be combined to achieve transdifferentiation of hair cells. To achieve more complete hair cell regeneration, it will be important to find a minimum but promising set of reprogramming factors. Atoh1 and inhibition of Notch signaling will be candidates to achieve this. Proper estimation of induced hair cells is also necessary to determine the combination required for successful transdifferentiation. To evaluate the transdifferentiation of hair cells properly, it is important to delineate the key characteristics that define hair cells, which include gene expression

profiles, cellular organization of structural proteins, morphology, epigenetic marks, and functional attributes such as resting membrane potential and mechano-electrical transduction.

In previous studies, for the induction of transdifferentiation of hair cells, reprogramming factors were overexpressed in target cells such as supporting cells or epithelial cells adjacent to the organs of Corti. Among the various types of cells in the cochlea, supporting cells are the preferred targets for hair cell induction, as hair cells and supporting cells have common progenitors for their development [19], and supporting cells give rise to regenerated hair cells through cell division and transdifferentiation in birds [4–9]. Although supporting cells behave like progenitor cells in birds, other cell populations have also been reported to have stem cell- or progenitor cell-like characteristics. Those include tympanic border cells that reside in scala tympani and just beneath the organs of Corti. Tympanic border cells are slowly proliferating [33], which is one of the characteristics of stem cell. Moreover, they can contribute to the development of both hair and supporting cells in the cochlea [34]. In addition to these characteristics as progenitor or stem cells, tympanic border cells are easily reached from the round window, one of the two membranous windows in the bony cochlea. Therefore, tympanic border cells, as well as supporting cells, are the promising targets of hair cell transdifferentiation if their molecular profiles are characterized.

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Chapter 22
Dedifferentiation-Mediated Regeneration

Koji Nishimura and Takayuki Nakagawa

Abstract In several nonmammalian species natural regeneration occurs via dedifferentiation, where terminally differentiated cells revert back to a developmentally earlier stage. However, in mammals, once lost most terminally differentiated cells including cochlear hair cells do not regenerate. In this section, we introduce a novel strategy for hair cell regeneration in the mammalian inner ear, in which residual supporting cells are reprogrammed to otic progenitor cells that redifferentiate into hair cells. Induced pluripotent stem cell transcription factors (iPS cell TFs) and/or epigenetic modifications that intervene DNA methylation and histone acetylation are promising options to activate a cochlear supporting cell's endogenous regenerative potential.

Keywords Dedifferentiation • Epigenetics • Induced pluripotent stem cell transcription factors (iPS cell TFs) • Reprogramming

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22.1 Hair Cell Regeneration Strategies by Dedifferentiating Supporting Cells

Dedifferentiation is equivalent of cellular reprogramming in which terminally differentiated cells revert to a developmentally earlier state. Natural dedifferentiation is observed in several nonmammalian species: in amphibians, cells adjacent to the wound dedifferentiate after limb amputation, forming undifferentiated cells that redifferentiate to create all the components of the lost limb [1]; in zebrafish, cardiomyocytes dedifferentiate after amputation of ventricles and proliferate to regenerate the missing tissues [2]. Therefore, inducing dedifferentiation is a reasonable approach to regenerate mammalian tissues. Ultimate dedifferentiation of somatic cells was demonstrated by the generation of induced pluripotent stem (iPS) cells from fibroblasts by overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* [3]. By transient overexpression of iPS cell transcription factors (TFs), various lineage-specific precursor cells were generated: *OCT4*-transduced human fibroblasts gave rise to $CD45^{+ve}$ multilineage blood progenitor cells bypassing the pluripotent state [4]; iPS cell TFs along with a specific culture condition converted mouse fibroblasts into contracting cardiomyocytes via mitotically active cardiovascular progenitor cells [5] or neural progenitor cells [6], where a pluripotent intermediate was not involved. The hypothesis that various developmentally plastic intermediate cells are stochastically generated in the process of dedifferentiation with iPS cell TFs prompts us to test whether otic progenitor cells could be induced from cochlear epithelial cells as well. However, so far there has been no report of successful hair cell or cochlear progenitor cell induction with iPS cell TFs: Burns et al. reported that supporting cells of the adult mouse utricles remained postmitotic after ectopic expression of *Oct 3*/*4*, *Klf4*, or *Sox2* in culture [7]. Therefore, additional cues, including soluble signaling molecules, might be needed for otherwise quiescent supporting cells to be reprogrammed into a progenitor state.

The auditory hair cells in the bird show robust regeneration as supporting cells reenter the mitotic cell cycle after damage via acoustic trauma or ototoxicity [8, 9]. Although Cox et al. reported that spontaneous mitotic hair cell regeneration occurred in vivo within one week of age in mice [10], mammalian inner ear in general has a limited regenerative potential partly because the level of proliferation in supporting cells is extremely low [11]. One strategy to accomplish hair cell regeneration in the mature mammalian cochlea is to generate otic progenitor cells via dedifferentiation and to induce redifferentiation into hair cells (Fig. 22.1), as regeneration generally follows the normal pattern of development once the process has started [12]. Although initial dedifferentiation is independent of the cell cycle exit, in many cases, dedifferentiation is followed by proliferation [13]. In the avian inner ear, it is likely that partial dedifferentiation occurs in the process of supporting cell proliferation after injury [14]. Thus, even in the mammalian inner ear, induction of proliferation can increase the chance of dedifferentiation.

The cyclin-dependent kinase inhibitor, $p27^{kip1}$, is an early marker of presumptive sensory region that will generate hair cells and supporting cells [15]. Deletion of $p27^{kip1}$ led to an extension in the normal developmental limit in proliferation of cells

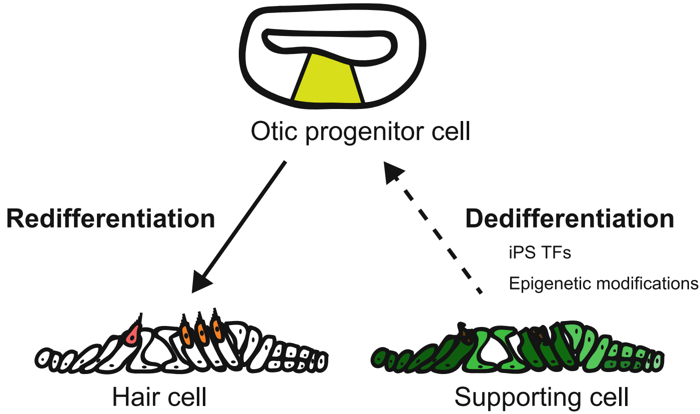

Fig. 22.1 Overview of dedifferentiation-mediated hair cell regeneration. Dedifferentiation-mediated hair cell regeneration involves a dedifferentiation step (*dashed arrow*) and a subsequent redifferentiation step (*arrow*). iPS transcription factors (TFs) and epigenetic modifiers would facilitate dedifferentiation of otherwise mature cochlear supporting cells

in the cochlea [16]. More recently, Ono et al. demonstrated that knockdown of $p27^{kip1}$ with short hairpin RNA-expressing vectors resulted in the cell-cycle reentry of post-mitotic supporting cells in the postnatal mouse cochleae ex vivo [17]. However, in parallel with the proliferation of supporting cells by knockdown of $p27^{kip1}$, there was an activation of the apoptotic pathway in proliferating supporting cells, suggesting that additional manipulations are required to achieve hair cell regeneration [17].

The *MYC* gene family members, which in mammals include *c-Myc*, *L-Myc*, and *N-Myc*, are basic helix-loop-helix leucine zipper transcription factors that play a prominent role in regulating cell proliferation and differentiation [18]. T58A variant of *c-Myc* initiated cell cycle reentry of postmitotic supporting cells of adult mouse utricles and a portion of the cells that reentered the cell cycle expressed the hair cell marker myosin VIIa [7]. If myosin VIIa-positive cells that reentered the cell cycle originated from the supporting cell population, ectopic *c-Myc* expression may be capable of dedifferentiating supporting cells into otic progenitor cells [7]. However, *c-Myc* gene therapy is not a realistic approach for stimulating regeneration in humans due to its potential oncogenic characteristics [19].

Multipotent otospheres, which are sphere-forming cells derived from dissociated cochlear supporting cells of neonatal mice, are similar to cochlear epithelial cells, in that both of them have the same methylation status of *Sox2* enhancers and the silenced *Nanog* and *Oct3/4* promoters [20]. Lou et al. demonstrated that *Oct3/4*, *Klf4*, *Sox2*, and *c-Myc* reprogrammed otospheres into iPS cells [21], suggesting the possibility of dedifferentiation of cochlear supporting cells at postnatal stages. What remains to be performed are detailed analyses of the process of reprogramming otospheres, which contributes to insights into dedifferentiation of

cochlear epithelial cells in tissue, characterizing cochlear progenitor cells, and identification of key molecules for otic induction. Needless to say, we need to take into account the difference between the artificially-produced otospheres and the original cochlear epithelial cells, as otospheres possess a proliferative capacity unlike postnatal cochlear epithelial cells in situ.

Several experiments have demonstrated that dedifferentiation of mature mammalian cells occurred in vivo: mature B cells were reverted to uncommitted hematopoietic progenitors by conditional Pax5 deletion in mice [22]; mature airway epithelial cells were dedifferentiated into stable and functional multipotent stem cells after the ablation of the airway stem cells [23]; and astrocytes were dedifferentiated into proliferative neuroblasts in the adult brain by overexpression of Sox2 [24]. More recently, Abad et al. demonstrated that transitory induction of iPS cell TFs in mice resulted in teratomas emerging from multiple organs, suggesting that even ultimate dedifferentiation can occur in vivo [25]. These precedents give us a proof of principle that cochlear supporting cells are dedifferentiated into otic progenitor cells in situ, which is an important step toward functional hair cell regeneration in the mammalian inner ear.

22.2 Epigenetics of Cochlear Sensory Epithelium

Epigenetics is a study of heritable changes in gene expression that occur without a change in DNA sequence [26]. Epigenetic regulation plays a pivotal role in cell differentiation and dedifferentiation. The two most well-known mechanisms of epigenetic alternations are DNA methylation and histone modifications. DNA methyltransferase-mediated methylation of cytosines in CpG islands recruits methyl CpG-binding proteins, histone deacetylases, and other associated proteins that change chromatin structure and prevent appropriate transcription factors from accessing the DNA, therefore silencing the gene expression as a result [27]. Mutai et al. demonstrated that an epigenetic regulatory mechanism, represented by DNA methylation and expression of *Dnmt3a* and *3b*, occurred in the mammalian auditory epithelium during postnatal development, suggesting a role of epigenetic regulation during postnatal inner ear development [28]. Two class I histone deacetylases were expressed in the sensory epithelium of the avian utricle and treatment with histone deacetylases inhibitors decreased in supporting cell proliferation in the avian utricle [29]. Thus, histone deacetylation is a positive regulator of regenerative proliferation in the avian uticle. Waldhaus et al. demonstrated that the degree of methylation of the otic *Sox2* enhancers NOP1 and NOP2 was correlated with the dedifferentiation potential of postmitotic supporting cells into otic stem cells, concluding that the stemness in the organ of Corti is partly dependent on the epigenetic status of NOP1 and NOP2 [20]. More recently, Wanda et al. reported that epigenetic factors involved in histone modification such as NuRD and PRC2 were present in the neonatal organ of Corti [30]. Cellular reprogramming by epigenetic modification in other system was demonstrated that 5-azacytidine, an

inhibitor of DNA methylation, caused fibroblasts to differentiate into muscle and fat cell [31]. Therefore, epigenetic factors expressed in cochlear sensory epithelium are targets for drug intervention to induce dedifferentiation of a supporting cell into a hair cell, by reactivating the cell's endogenous regenerative potential.

22.3 Concluding Remarks

Here we reviewed recent advances of research in cellular dedifferentiation and its potential application to hair cell regeneration. Although its impact on hair cell regeneration will be tremendous, further studies are mandatory to transfer basic findings gleaned from animal experiments to clinics as this field is still in its infancy.

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Chapter 23
Gene Therapy

Akiko Taura

Abstract Recently lots of genes are identified as the cause of hearing loss. So gene therapy is one of the promising future therapies for inner ear disorders. As vectors for gene transfer, viral vectors such as adenovirus are useful for gene delivery, but there still remain some problems about safety. In contrast, nonviral vectors have the advantage of reductions in toxicity compared to viral transduction, but the transduction efficiency is low. Up to now, gene transduction experiments revealed the hearing protection or regeneration of hair cells by gene transfer into hair cells, and gene replacement was effective to genetic disorders. In future, gene therapy for hair cells will improve our treatment for inner ear disorders.

Keywords Gene replacement • Protection • Regeneration • Vectors

23.1 Introduction

Due to the development of molecular biological techniques, lots of hereditary deafness have been identified and clinical diagnosis has become to be done. As the next step, the establishment of therapy based on genetic approaches is an urgent matter. The first gene therapy trials for genetic diseases were performed on two children with ADA-SCID 20 years ago [1]. So far, lots of clinical trials of gene therapy had been made mainly in life-threatening disease worldwide and some of them are reported the effectiveness of gene therapy [2]. However there still remain some problems about the safety and efficiency. Anatomically inner ear is well suited to local gene therapy, because inner ear is a closed system, thus allowing local application and remaining of vectors without spreading to whole body [3]. Lots of advantages have been reported using gene therapy for various types

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of hearing loss and vestibular disorder caused by ototoxic drugs and genetic and autoimmune disorders. For the hair cell regeneration, the transduction of specific gene related to hair cell induction such as *Atoh1* (*Math1* in the mouse) gene was reported to be effective [4]. For the genetic disorders causing congenital hearing loss, the optimal treatment is defective gene transduction. And for the dominant negative genetic disease, the inhibition of gene expression might be useful using RNA interference (RNAi) gene inactivation [5] or antisense oligonucleotides (ASO) [6]. These methods were also applied to the inner ear system [7–9]. Also there are several practical routes for delivering gene agents [10]. Considering above, gene therapy must be a promising tool for inner ear disorders in future when various problems such as safety or efficiency are resolved.

23.2 Vectors

23.2.1 Viral Vectors

Various kinds of viral vectors are useful for gene delivery. Adenovirus, adeno-associated virus, herpes simplex virus, vaccinia virus, retrovirus, helper-dependent adenovirus, and lentivirus have been tested for inner ear gene delivery.

23.2.1.1 Adenovirus (AdV)

Adenoviruses (AdVs) were discovered from human adenoids in 1953 [11]. AdV has an ability to infect both dividing and nondividing cells with higher efficiency compared to retroviral vectors. The short-term onset after infection and larger packaging capacity is also an advantage. However the expression is transient because the virus is not integrated into host genome, and there are side effects due to toxicity and immune response [12]. Experimental AdV for gene therapy is a serotype 5 [13] and has been deleted of sequences in the E1A, E1B, and E3 region, impairing the ability of virus to replicate. In inner ear, AdV can transfect into various cell types such as hair cells or spiral ganglion cells without severe side effects [14, 15]. However the efficiency of transgene expression in hair cells is not so high. Histone deacetylase inhibitor is useful for the increase of transgene expression in hair cells [16]. In order to transfect to specific cells, AdVs have been developed. For example, Cre-expressing recombinant AdV can control the expression of a gene in each regulating unit [17].

23.2.1.2 Adeno-Associated Virus (AAV)

Adeno-associated virus (AAV) readily integrates into the host genome, so trans-gene expression is lasting long term. The small packaging capacity of AAVs, the maximum gene insert size about 5 kb, limits their use to small genes. In inner ear,

various kinds of cell types such as hair cells, supporting cells, and spiral ganglion cells can be transfected with AAV without toxicity [18, 19]. Recently, novel viral vectors, such as recombinant adeno-associated virus (rAAV) vectors being non-pathogenic and the ability to produce prolonged gene expression or bovine adeno-associated virus (BAAV) having the potential for long-term gene expression with little or no side effects, have been developed [20, 21]. Using these rAAV vectors, the permeability of round window membrane can be increased by partial digestion with collagenase solution and the transfection efficiency was increased without severe side effects [20].

23.2.1.3 Lentivirus

Lentivirus provides long-term gene expression and large packaging capacity. In inner ear, lentivirus injected into rat scala tympani is limited to the cochlea without evidence of spread to the central nervous system. This is an important feature to minimize toxicity to tissues outside the cochlea [22].

23.2.2 Non-viral Vectors

Non-viral vectors such as plasmids or packaged within lipids have the advantage of reductions in toxicity compared to viral transduction. Cationic polymers such as polyethylenimine (PEI) were reported to be effective for gene delivery to the nucleus [23]. In inner ear, successful gene delivery was reported using PEI in vitro and in vivo [24]. However, the efficiency of gene transfer using these vectors is low and the transgene expression is also poor [25]. But a recent paper reported the effective gene transduction using hyperbranched polylysine particles into rat cochlea [26].

23.2.3 Non-viral Other Approaches

23.2.3.1 Electroporation

Electroporation uses electrical fields to create transient pores in the cell membrane that allow the entry of normally impermeable macromolecules such as DNA, RNA, and proteins into the cytoplasm [27]. Although this technique has been used to transfer DNA to bacteria or cells in culture, it has recently been applied to living animals. Up to date, electroporation has been adapted for use in various kinds of tissues such as skeletal muscle, liver, kidney, and so on. In inner ear, the gene transfer technique to the developing mouse inner ear in utero in the context of gain- and loss-of-function studies was developed [28].

23.2.3.2 Gene Gun

Gene guns have been used to deliver DNA to target cells of interest to achieve gene transfection and have an ability to infect both dividing and nondividing cells. This method is technically simple and requires less time for reagent generation than viral methods. But one of the problems of gene guns is that they need high operating pressures, which may result in direct or indirect tissue/cell damage. In inner ear, the transfection of hair cells was reported by using an improved biolistics (gene gun) method with more penetrating power and minimal tissue damage [29]. Recently various improved methods such as micro-needle-assisted gene gun are reported [30].

23.3 Gene Therapy Experiments

So far, lots of gene therapy experiments were reported. In this section, we introduce the gene therapy targeted on hair cells.

23.3.1 Protection

The overexpression or silencing of several molecules by gene transduction can be useful for protection of hair cells. Overexpression of GDNF and TGF beta by using AdV prevented hair cell degeneration [31] and overexpression of catalase protected hair cell damage against ototoxic drug [32]. Also the delivery of gene encoding inhibitor of apoptosis using AAV vector can protect against cisplatin-induced ototoxicity [33]. Silencing of NOX3, serving as the primary source of reactive oxygen species generation in the cochlea, protected the hair cells from cisplatin-induced damage [34].

23.3.2 Regeneration

Once hair cells are damaged by ototoxic drugs or noise trauma, regenerative treatments are needed. Some ectopic new hair cells were generated in mature guinea pig cochleae after *Atoh1* gene transfer with an AdV vector [35]. Furthermore, in vestibular system, *Atoh1* gene therapy was effective at generating new vestibular hair cells and restoring balance in mice lesioned with ototoxic drug [36, 37].

Table 23.1 Gene therapy experiments targeted to hair cell disorders

Gene	Vector	Recipient animal	References
Catalase	AdV	Guinea pig	[32]
Atoh1	AdV	Guinea pig	[4, 35]
Atoh1	AdV	Mouse	[36, 37]
GDNF/TGFbeta	AdV	Guinea pig	[31]
NOX3	(siRNA)	Rat	[34]
XIAP	AAV	Rat	[33]
Connexin 26	Liposome (siRNA)	Mouse	[9]
Connexin 26	BAAV	Mouse	[38]
Connexin 30	Electroporation (shRNA)	Mouse	[8]
Ush1c	ASO	Mouse	[7]
VGLUT3	AAV	Mouse	[39]

23.3.3 Gene Replacement for Genetic Disorders

For genetic disorders, optimal treatment is defective gene replacement. In a model of connexin 26-induced deafness (*GJB2* mutations), the expression of a dominant-negative connexin (Cx) 26 was successfully inhibited by siRNA silencing [9]. And transduction of connexin26 into Cx26Sox10Cre mice using a bovine adeno-associated virus vector (BAAV) restored connexin26 protein expression and rescued gap junction coupling in vitro [38]. The transuterine transfer of the Cx30 gene into the otocysts of Cx30-deficient mice rescued the lack of Cx30 expression in the cochlea and restored auditory functioning [8]. Also AAV-VGLUT3 gene transduction into the VGLUT3 mutant mice restored hearing level [39]. Treatment of neonatal Ush1c.216A mutation (216AA) mice with a systemic dose of ASO partially corrects USH1C.216G>A splicing, improves stereocilia organization in the cochlea, and rescues cochlear hair cells, vestibular function, and hearing in mice [7]. Also BAC transgene correction with wild-type Myo15a corrects structure and function of the inner ear in sh2/sh2J mice and the phenotypic rescue is stable for at least 6 months [40].

Up to date, lots of gene therapy experiments in inner ear have been reported. Gene therapy may become an effective treatment for inner ear disorders in future. However, in order to establish the definitely safe and effective clinical application, further improvements of vector and technology are needed (Table 23.1).

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Chapter 24
Cell Therapy

Takayuki Okano

Abstract Sensorineural hearing loss is one of the most common disabilities worldwide. In most cases, hearing loss in humans is believed to be caused by loss or dysfunction of hair cells in the cochlea, and when they are lost, hair cells cannot spontaneously regenerate, which enforce difficulty to cure most cases of severe or profound hearing loss. To date, cochlear implantation is the only way to rehabilitate hearing function in patients with profound hearing loss; however, normal hearing is not restored even with the best outcomes, in particular, in terms of music appreciation. Therefore, an alternative strategy would be much expected for the treatment on profound hearing loss. In this chapter, we will discuss on current understanding of hair cell biology as well as the initial studies aiming at transplanting cells into the inner ear for hair cell replacement. In subsequent part, we will highlight challenges for cell therapy for hair cell regeneration and discuss a couple of topics for cell transplantation into the inner ear.

Keywords Cell transplantation • Sensorineural hearing loss • Stem cell

24.1 Introduction

The cochlea is surrounded by bony wall except three parts: the internal auditory canal that the cochlear and vestibular nerves penetrate through, the oval window where the stapes fit, and the round window that separates the cochlea and the middle ear by thin membrane. These characteristics lead to the limitation of surgical access to apply drugs and/or cells inside the cochlea. The cochlea consists of three tubular spaces filled with fluid, which allows substance or migrating cells to freely move

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inside the cochlea. In the scala media filled with extracellular fluid of high potassium ion (K^+) concentration which is referred to as endolymph, mechanosensory hair cells, primary auditory receptor cells, and supporting cells, non-sensory cells surrounding hair cells, form highly organized cellular mosaic in the sensory epithelium (the organ of Corti) in the cochlea. The organ of Corti contains one row of inner hair cells and three to four rows of outer hair cells. Although there are wide variations among reports, the organ of Corti in a cochlea harnesses approximately 3,000 inner hair cells and 12,000 outer hair cells in human [1], which are much smaller number in comparison to the number of photoreceptor cells in the human retina [2, 3]. Hair cells themselves are not attached to the basement membrane, but lifted by supporting cells that adhere to the basement membrane of the cochlear sensory epithelium, which suggests that hair cells are literally maintained by supporting cells both mechanically and physiologically. Supporting cells are also highly differentiated components in the organ of Corti, and disruption in function of the supporting cells causes hearing impairment in some conditions. For example, while connexin26, a gap junction protein, is expressed in the supporting cells, deletion of connexin26 in both human and mice leads to hair cell degeneration [4], suggesting that supporting cells play key roles in the maintenance of functional hair cells.

On the other hand, hair cells in the cochlea are not homogenous but demonstrate a gradient from base to apex, which reflects longitudinal gradients within frequency tuning from high frequency in the base to low in the apex. Cell bodies of inner and outer hair cells are shorter in the base but longer in the apex. Inner and outer hair cells are located in specific position within the organ of Corti along the medial-to-lateral axis of the epithelium. Hair bundles on the luminal surface of hair cells show highly ordered organization along both the longitudinal axis and the medial-to-lateral axis. Bundles are short in the basal part of the cochlea and long in the apex, and the chevron shape of hair bundles on each hair cell orients laterally in a highly regulated manner, which is referred to as planar cell polarity. Although both inner and outer hair cells form synapses with dendrites of spiral ganglion neurons, the manner of synaptic formation is quite different between inner and outer hair cells. An inner hair cell receives innervation from more than ten of type I spiral ganglion neurons, whereas several outer hair cells receive innervation from a single type II spiral ganglion neuron. These characteristics are tightly connected to the precise tuning for frequency and temporal resolution in the mammalian auditory system. To date, none of the available methods for hair cell regeneration has achieved regeneration of heterogeneous hair cells compatible with tonotopy.

Hair cell damage has been clinically implicated in the pathology of hearing loss, including age-related hearing loss, drug-induced hearing loss, acoustic trauma, and a part of hereditary hearing loss [5]. However, since it is difficult to examine the cochlea in human through biopsy due to anatomical inaccessibility, most of pathological data in human have been obtained through autopsy. In addition, findings in experimental animals fill the gap in attempt to complete understandings for pathology of hair cell damage or degeneration. Substantial loss of hair cells has been reported in histological studies using experimental models on age-related hearing

loss, drug-induced hair cell damages, acoustic trauma, hereditary hearing loss, or cochlear ischemia. Hair cells, and most likely supporting cells as well, are not recovered after damage in the mature mammalian cochlea, although endogenous stem cells in the mammalian cochlea are also suggested in the previous works [6–8]. Activation of endogenous stem cells within the inner ear would probably be an ideal source for regeneration of the hair cells. However, cell transplantation into the cochlea would be an alternative strategy when there is not a stem cell population enough to regenerate damaged hair cells. This approach would offer several advantages, such as the ability to determine how many and/or what kinds of cell to implant as well as to activate and subject stem cells toward hair cell fate prior to transplantation.

Stem cells are prime candidates for cell-based therapies since they potentially can replace damaged cells when administered into a target organ. In a strict sense, cell-based medicine for hair cell regeneration would be applied only if pure form of hair cell damage takes place without any compromise in the function of cochlear lateral wall and spiral ganglion. However, in addition to the ability to differentiate into various types of functional cells, some stem cells secrete a broad spectrum of trophic growth factors, cytokines, and chemokines, release microvesicles or exosomes that facilitate the lateral transfer of organ-protective messages into targeted cells, and possess powerful immunomodulatory and anti-inflammatory functions to enhance the proliferation of endogenous stem cells and thus engage them in the repair of tissues. If there would be some cues for cell differentiation and synaptic formation in damaged area of the cochlea, transplanted cells could migrate to the site and are integrated into a functional sensory epithelium. In this chapter that follows, we will discuss on the current status of cell transplantation therapy for hair cell regeneration and future directions for clinical application of cell therapy for hair cell regeneration.

24.2 Studies Aiming at Cell Transplantation for Hair Cell Regeneration

During the last two decades, research for inner ear regeneration has evolved into a major current among scientists studying deafness and the auditory system. Several trials have investigated the effect of stem cell therapy in the inner ear using exogenous stem cells. In this section, we will review the history of experimental cell transplantation into the cochlea beginning with the pioneering report by Ito et al. [9]. In this study, neural stem cells derived from adult rat hippocampus were transplanted to the cochlea of neonatal rat at postnatal (P)0-2. As recipients grow as old as 4 weeks, histological analysis demonstrated that a part of transplanted cells survived in the cochlea. Moreover, some of the transplanted stem cells migrated into the organ of Corti and showed hair cell-like morphology. The observation suggests that the transplantation of exogenous cells to the cochlea could be an alternative strategy for the treatment of the damaged cochlea and sensorineural

hearing loss. Tateya et al. also studied the survival and differentiation of neural stem cells in the adult mouse cochlea damaged by the injection of neomycin [10]. Twenty-five days after transplantation, immunohistochemistry revealed that fewer parts of transplanted neural stem cells were found in the perilymphatic space and differentiated for hair cell fate expressing MyosinVIIa in the vestibular sensory epithelium, but not in the cochlea. Similar strategy was tested by Parker et al. [11] in the sound-damaged cochlea of mice and guinea pigs. Four to six after cell transplantation, transplanted neural stem cells were found in the organ of Corti and spiral ganglion. In addition, neural stem cells localized to outer hair cell region in the organ of Corti express MyosinVIIa, suggesting that exogenous stem cells could adopt cell fate commitment according to the surrounding environment. By contrast, two studies demonstrate another aspect of cell transplantation into the inner ear. Iguchi et al. transplanted neural stem cells in the mouse inner ear through horizontal semicircular canal and showed histological results indicating that transplanted neural stem cells express neurotrophic factors, such as BDNF and GDNF, 28 days after transplantation [12]. Hakuba et al. assessed protective effect of stem cell transplantation on auditory function in an ischemic cochlear model of gerbils [13]. Histological analysis demonstrated that some parts of transplanted cells were localized around the organ of Corti. Moreover, morphology of inner hair cells and auditory function in transplanted cochleae were preserved in comparison with controls. These data suggest that the transplantation of exogenous stem cells does not always lead to the differentiation of transplanted cells to hair cells, but induces protective effects on hair cells and/or surrounding supporting cells against insults in the cochlea.

Neural stem cells were preferably used in all studies shown above; however, a couple of studies indicates possible use of other cell sources for the transplantation of exogenous cells into the inner ear. Sakamoto et al. [14] transplanted mouse embryonic stem cells expressing eGFP into the aunt mouse inner ear damaged by neomycin prior to cell transplantation. Survived embryonic stem cells were found to express E-cadherin and NCAM, suggesting that transplanted cells differentiated into ectoderm cell fate; however, none of the transplanted cells were observed as hair cell phenotype. Kojima et al. [15] also tested the use of fetal rat otocyst cells on exogenous cell transplantation into the rat inner ear damaged by acoustic overstimulation. One month after transplantation, a few grafted fetal otic epithelial cells were integrated in the supporting cell layer of the damaged cochlea in host animals, whereas most of transplants were found in the perilymphatic space or attaching to the cochlear lateral wall. Revoltella et al. [16] examined the effects of hematopoietic stem cell transplantation on kanamycin- and/or noise-induced hearing loss. In this study, hematopoietic stem cells derived from human cord blood were intravenously transplanted into bone marrow-ablated Nod-Scid mice. Histological analysis revealed morphological recovery in the inner ear of transplanted animals. In addition, fluorescence in situ hybridization (FISH) analysis indicates small numbers of heterokaryons, presumably due to fusion of donor cells with endogenous cells in the cochlea up to two months after transplantation. In the study by Sullivan et al., adult stem cells were isolated from mouse tongue epithelium and

subsequently transplanted to the adult mouse cochlea damaged by noise exposure [17]. Auditory function was analyzed by determining auditory brainstem response (ABR) threshold shifts 4 weeks after cell transplantation, demonstrating protective effect of epithelial stem cell transplantation on noise-induced hearing loss. Histological examination showed that epithelial stem cells survive and integrate into the epithelial lining of the scala tympani expressing ion transporters, whereas no findings about the integration of transplants into the organ of Corti were described. Despite low rate of survival and integration into the organ of Corti, these four studies suggest that the damaged cochlea expresses some kind of cues which attract migrating cells and promote cell survival and differentiation.

Works reported to date include a variety of study design in terms of cell sources, animals, types for inner ear damage, and routes to access the cochlear spaces; however, cell transplantation was basically performed only once, and then a couple of weeks or months later, settlement and phenotypes in a part of transplanted cells were analyzed by immunohistochemistry in the studies previously reported, omitting vast majority which unsurvived or escaped out of the inner ear. Whereas some of the transplanted cells have been shown to develop as potentially hair cell-like cells, a far greater number of cells cannot be accounted for within several weeks of a transplant. In addition, in contrast to studies testing gene therapy for hair cell regeneration [6], functional studies have been poorly performed in studies designating cell transplantation for hair cell regeneration. Moreover, researchers found that much more transplanted cells were localized in the spiral ganglion than in the organ of Corti, forcing change of direction from hair cell regeneration to neuronal regeneration in the spiral ganglion as the main target. Based on the results from the studies shown above, we will discuss current status and future directions in the area of research for cell transplantation into the inner ear.

24.3 Challenges for Cell Therapy and Key Steps for Hair Cell Regeneration

As discussed above, the cochlea is one of the most morphologically and functionally specialized tissues in the body, which is quite different from tissues with simple cellular patterning such as muscle, lung, or liver. Based on the previous studies on cell transplantation into the inner ear, a successful transplantation would have to meet a number of requirements (Fig. 24.1). Transplanted cells should be delivered to all regions of the damaged cochlea, and the delivery of stem cells in the organ of Corti seems to be one of the most difficult tasks in the research of cell therapy for tissue repair. In the luminal surface of the organ of Corti, tight junction and reticular lamina against high potassium in endolymph are tightly formed, which makes it difficult for transplanted cells to migrate into the sensory epithelium. Therefore, the integration of transplanted stem cell into the tightly sealed and organized sensory epithelium is likely to be highly inefficient. An alternative approach would be using

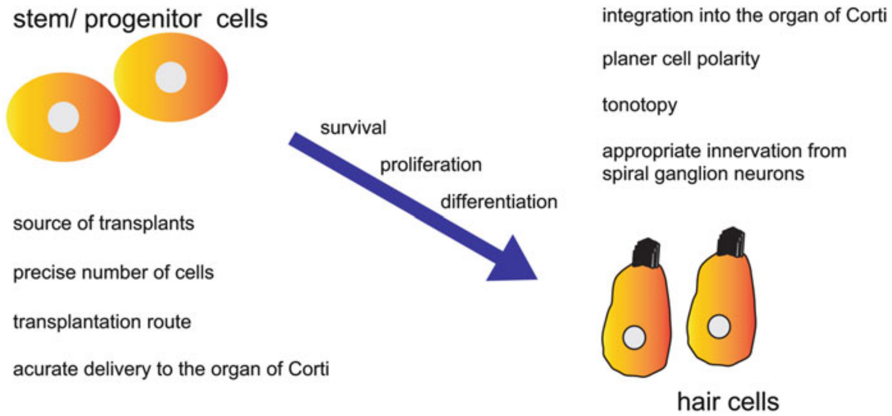

Fig. 24.1 Challenges to be overcome in cell therapy for hair cell regeneration. In *left hand*, requirements for preparation and transplantation of stem/progenitor cells are listed. In the *middle*, survival, proliferation, and differentiation should be tightly controlled after cell transplantation into the cochlea. In *right hand*, transplants-derived hair cells should be integrated into the organ of Corti and recapitulate native hair cells in terms of planar cell polarity, tonotopy, and innervation from the spiral ganglion neurons

molecular signals to attract stem cells so that transplanted cells would migrate and reside in an appropriate location. After transplanted cells are located in the site of interest, the control of the cell cycle in the cochlea has to be a major target to manipulate through cell therapy protocol. Providing precise number of hair cells or progenitor cells would be important to rehabilitate hearing since some of transgenic mice deficient of genes that regulates cell cycle show highly impaired hearing function despite overproduction of hair cells [18]. In addition to cell cycle regulation, transplanted cells should be controlled in terms of cell differentiation. It would be one of the biggest questions to date whether stem cells develop as functional hair cells after transplantation. Although Atoh1 has been proven to be a master gene for commitment for hair cell fate, we still don't know how to drive expression of Atoh1 in transplanted cells in vivo as well as what other genes are to be required for hair cell differentiation. Moreover, targets for regeneration are not only hair cells but also supporting cells in the organ of Corti. As shown above, the dysfunction of supporting cells also leads to hearing impairment and hair cell degeneration, so it would be important to consider the regeneration of supporting cells as well as hair cells. Finally, innervation from spiral ganglion neuron to hair cells and synaptic formation should be required for functional regeneration of hair cells. As mentioned above, tonotopy along longitudinal axis of the cochlear duct determines frequency resolution; thus, hair cells generated from transplanted stem cells should receive innervation from appropriate neurons responsible for specific frequency. These issues would be solved through basic research on stem cell biology both in vitro and in vivo in the future. In this section, we will discuss some other points that appear to be useful for cell-based regenerative medicine for hair cells.

24.3.1 How to Apply Exogenous Cells and Possibility for Tissue Implantation into the Cochlea

On cell-based medicine generally, including in cochlea and other organs or tissues, cells are multiplied in culture or used with minimal manipulation after harvesting and then delivered by one of the two major strategies. In one hand, cells are suspended in medium and directly injected into the defective tissues. The transplanted cells are expected to home to the damaged area or the site of interest. In any cases, little guidance is provided to the cells by the transplant system before and after transplantation. Alternatively, cells may be allowed to adhere to a material carrier in vitro, be allowed to proliferate and differentiate within a bioreactor, and subsequently be implanted on the material including functional cells to a specific anatomical site. The material serves as a template to guide tissue formation and typically is designed to degrade in concert with the deposition of new extracellular matrix and cell proliferation [19]. However, our knowledge is still limited at this moment about when and how much amount of stem cells should be transplanted into the inner ear. It should be also clarified how frequently stem cells should be administered into the inner ear. Regarding cell transplantation into the cochlea, the strategy to perform single injection of exogenous cells has been solely adopted in the studies reported to date. In the previous works, direct transplantation to the cochlea is most commonly used, however, the site of cell injection varies among studies. Cochleostomy in the lateral wall [14, 20], the round window [21], the lateral semicircular canal injection [12], the internal auditory meatus [22], and utriculostomy [23] have been tested.

Transvascular delivery of stem cells would be another option to have transplanted cells reach the cochlea, although efficiency would be very small without any manipulation after transplantation since the inner ear is quite a small organ in the body in terms of weight, cell number, and blood flow, so that most part of transplanted cells could escape to other organs or tissues in the body. The concept for organ-specific “ZIP code” would solve these issues on transvascular stem cell delivery. The general idea is that each organ and pathology has a unique vascular “ZIP code” that can be targeted with affinity ligands [24]. Organ- or pathology-specific delivery of drugs or nanoparticles containing small nucleotides has been explored for treatment of cancer, congenital enzyme defects, or regenerative medicine [25–27]. These strategies employ carrier molecules that bind to receptors exposed on the cell surface in target tissues or pathology. If “ZIP codes” in the cochlea would be identified, cell delivery based on tissue-specific “ZIP code” would become available, and transvascular delivery of stem cells to the cochlea could be powerful tools for regenerative therapy for hearing loss because no surgical approaches are required to have stem cells reach to the cochlea.

Finally, although there would be many challenges to transplant new tissues into the cochlea, it seems to be worth to try tissue implantation into the cochlea. Cell sheet formation with cellular mosaic of hair cells and supporting cells has not been achieved even in in vitro stem cell experiments. Hair cell-like cells have already

been induced from ES cells or iPS cells [28]; however, the formation of a cell sheet of inner ear sensory epithelium has never been accomplished in vitro. If cell sheet formation that mimics organ of Corti would be accomplished, then next target would be the three-dimensional culture of stem cell-induced sensory cells. Three-dimensional culture from stem cells to cochlear spiral seems to be quite attractive. Indeed, Koehler et al. demonstrated a stepwise method for the differentiation of mouse embryonic stem cells to inner ear sensory epithelia [29]. In this study, clusters of embryonic stem cells recapitulate in vivo development with precise temporal control of signaling pathway and form sensory epithelia that look like the vestibular end organs in three-dimensional culture system.

24.3.2 Stem Cell-Based Regenerative Medicine and Artificial Organ

Artificial organs versus cell-based regenerative medicine would be another major issue in debate when cell-based medicine would be clinically applied. Various artificial organs have been subjects of intensive research and development and now widely used in patients. Cochlear implants are one of the most successful artificial organs, and approximately 324,200 people worldwide have received cochlear implants according to the Food and Drug Administration in the United States [30].

Artificial organs are roughly categorized into three groups as follows: (1) mechanically engineered artificial organ, (2) in situ tissue engineering, and (3) organ designed by stem cell-based regenerative medicine. In addition, organ transplantation from the brain dead would be the fourth in a broad sense. As for mechanically engineered artificial organs, there are two subgroups of artificial organs, ones are for life support while awaiting an organ transplantation or regeneration process and the others are for improvement of the patient's ability. Artificial lung and heart are sometimes used for patients while awaiting organ transplantation. In addition, artificial dialysis is also widely used in clinic to replace the function of kidney in chronic renal failure patients. However, devices that release patients from continuously tethering to stationary resources or power supply have not become available in these artificial organs. On the contrary, some of artificial organs have prevailed these days for the improvement of the patient's ability. Artificial lens after cataract removal, or artificial joint prosthesis, are clinically used in common while these artificial organs work without any power supply. In contrast, cardiac pacemaker and cochlear implant are the most successful mechanically engineered artificial organs to date since these are working for more than ten years without replacing power supply. Clinical indication and characteristics of cochlear implants are discussed in the Chap. 27 by Yamazaki in this book.

In situ tissue engineering is applied for tissues or organs that possess abilities to regenerate themselves, such as skin, bone, trachea, or blood vessels. A typical

approach uses biodegradable scaffolds that attract endogenous stem cells and growth factors to regenerate tissue of interest. It has been also accepted that inflammation is not only a detrimental response to biomaterials, but, when harnessed properly, it can also be exploited to induce a natural regenerative response. To the best of our knowledge, in situ tissue engineering for cochlear regeneration has not been achieved yet to date. Alternatively, transplantation of organs designed by stem cell-based regenerative medicine appears to be the third option for regenerative medicine using artificial organs. Indeed, in vitro studies by Oshima et al. showed that both mouse embryonic stem cells and iPS cells can be induced to commit ectodermal cell fate and differentiate into hair cell-like cells in mammalian inner ear [28]. Moreover, Koeler et al. demonstrated a three-dimensional culture system that subjects embryonic stem cells to form otocyst-like cell clusters, which finally form sensory epithelia that resemble the vestibular end organs [29]. These findings indicate that the formation of artificial cochlea designed by stem cell-based regenerative medicine would be a promising strategy for regeneration of cochlear sensory epithelium. Accurate innervation could be a next mark to accomplish once transplantation of artificial cochlea would be realized in vivo.

Organ transplantation from donors of brain dead is widely performed in the cases with chronic organ failure, including kidney, lung, heart, and liver. However, it has not been the case in the cochlea or inner ear to date, since excision and transplantation of the inner ear are technically challenging.

These categories listed above are not independent, but overlap in part one another and mutually interact to develop and improve function in the organ of interest. It does not matter whether these tissues are cultivated inside or outside the patient's body in order to regenerate functional organs or tissues. Therefore, it would be very important to clarify what situation would be suitable for cell-based regenerative medicine and what would be the contraindication in the future.

24.3.3 In Vivo Tracking of Stem Cells After Transplantation

In the last, but not least, in vivo tracking of behavior of transplanted stem cells should be of interest even though it is a challenge under currently available technology. Tracking the dynamic behaviors of transplanted stem cells has been a long-standing research goal of biologists, medical scientists, and engineers. As mentioned above, the cochlear duct is surrounded by the bony wall that prevents target tissues or cells from being directly observed by optical microscope. The development of the way to trace transplanted cells in intact cochlea would be a major advance in the area of stem cell research in the inner ear. The ideal tracer molecule should fulfill a number of criteria: biocompatibility, absence of genetic modification to stem cells, single-cell detection at any atomic location, quantification of cell number, minimal dilution with cell division, minimal transfer of contrast agent to non-stem cells, noninvasive imaging in the living subject over months to

years, and absence of requirement for ejectable contrast agent [31]. Radionuclide labels possess a number of characteristics that account for their preferential use in clinical practice. First they are relatively safe, and second, radionuclide methods are highly sensitive and quantifiable. However, the tracking of labeled cells is limited by temporal loss of radio tracer. Moreover, physiological accumulation and excretion of free radio tracer may interfere with the activity in labeled stem cells. In the study by Bos et al., rat mesenchymal stem cells were labeled with superparamagnetic iron oxide (SPIO), administered intravenously, and tracked with magnetic resonance (MR) imaging [32]. MR imaging of SPIO-labeled stem cells offers a noninvasive evaluation of stem cell engraftment in host organs; however, excessive iron load from SPIO labeling impairs differentiation of stem cells. Some of the recent studies, including one by Nejadnik et al., have explored the way to reduce iron exposure and improve efficacy for the differentiation of stem cells [33]. Real-time imaging and longitudinal tracking of stem cells in in vivo three-dimensional tissue environments have been explored with an integrated optical microscope. The integrated microscope combines multiple imaging functions derived from optical coherence tomography (OCT) and multiphoton microscopy (MPM), including optical coherence microscopy (OCM), microvasculature imaging, two-photon excited fluorescence (TPEF), and second harmonic generation (SHG) microscopy [34]. Stem cells labeled with fluorescence protein would be traced using these techniques with smallest surgical invasion to make a pinhole for observation inside the cochlear duct.

24.4 Concluding Remarks

In this chapter, we briefly presented the anatomy of the cochlea and hair cells and discussed the current status and future directions of cell-based therapy for hair cell regeneration. Despite recent advances in the area of regenerative medicine in the inner ear, the exploration of replacement of damaged hair cells by cell transplantation has just started. Clearly, there are still many concerns and challenges in biological, clinical, and ethical aspects to overcome before clinical application of cell-based regenerative medicine in the inner ear. Indeed, recent progress of stem cell biology provides a variety of source for transplanted cells [35, 36]. Delivering cells directly to the organ of Corti would be ideal, but its small size and relative inaccessibility should be major obstacles. Techniques to detect survival and differentiation of transplanted cells in vivo should be developed. Although there will be much work to be done, we believe the future of cell-based regenerative medicine for cochlear disorder would be bright.

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Part V
Spiral Ganglion Neuron Regeneration

Chapter 25
Clinical Background

Harukazu Hiraumi

Abstract Spiral ganglion neurons (SGNs) conduct auditory information from the inner hair cells to the cochlear nucleus. Most patients with a loss of SGNs exhibit severe degeneration of the hair cells. In the clinical setting, therefore, the drawback of an SGN deficit is a poor outcome after cochlear implantation. Now, cochlear implantation is indicated for forms of hearing loss other than inner hair cell loss, including auditory neuropathy spectrum disorder, vestibular schwannoma, and congenital hearing loss. The number of SGNs required for cochlear implantation is estimated to be approximately 5,000, or approximately 15 %, of the number in the normal population. Patients with a sufficient number of SGNs benefit from cochlear implants, but fewer SGNs result in an unsatisfactory outcome. Even in patients with an adequate number of SGNs, the age-related degeneration of SGNs may lead to deterioration in speech discrimination. This deterioration can be problematic, especially in children with congenital hearing loss, whose average number of SGNs is approximately 40–60 % of the number in normal children. In patients with an insufficient number of SGNs, auditory brainstem implantation is another option; however, the performance of auditory brainstem implants is inferior to the average performance of cochlear implants.

Keywords Auditory neuropathy spectrum disorder • Congenital hearing loss • Inner ear malformation • Vestibular schwannoma

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25.1 Introduction

Spiral ganglion neurons (SGNs), along with hair cells, are indispensable for successful auditory perception. The number of SGNs is approximately 35,000 in the first decade, and it decreases by approximately 1,000 SGNs per decade in subjects with a histologically normal organ of Corti [1]. A total loss of SGNs results in profound hearing loss, but the effect of partial loss of SGNs has not been well elucidated. In an experimental animal model, the hearing threshold did not increase even after the ablation of 50–90 % of the cochlear nerves [2]. Clinically, however, an SGN deficit is usually accompanied by various degrees of hair cell loss, resulting in mixed cochlear and retrocochlear types of hearing loss [3].

Recently, cochlear implants (CIs) have been reported to be useful in patients with retrocochlear hearing loss. Before the CI became popular, retrocochlear hearing loss has been considered a result of problems in the SGNs and the auditory nerve. A CI substitutes for the inner hair cells, and retrocochlear hearing loss was traditionally regarded as a contraindication for CI. Therefore, the success of CIs in such patients has been surprising. This success is partially due to some patients with retrocochlear hearing loss preserving an adequate number of SGNs. Now, the pathologies of retrocochlear hearing loss are thought to be diverse (Fig. 25.1), including pathology of the synapses (synaptopathy). The other reason for the success of CIs is that the auditory information transmitted by CIs is processed and simplified so that a limited number of SGNs can transfer the necessary information to the brain. The minimum number of SGNs required for satisfactory CI results is estimated to be approximately 5,000 [4], or approximately 15 %, of the normal SGN population. In patients with fewer SGNs than this minimum required number, full speech understanding may not be obtainable with CIs. In this section, we illustrate the clinical appearance of major diseases associated with the degeneration or congenital hypoplasia of SGNs.

25.2 Auditory Neuropathy Spectrum Disorder

Historically, a diagnosis of SGN loss was based on negative recruitment phenomena, positive temporal threshold shift, and poorer speech understanding than that predicted from the pure-tone audiogram threshold. Although these tests retain some importance in the evaluation of retrocochlear hearing loss, they are not applicable to small children and patients with severe cochlear damage. Recently, a group of patients with impaired speech discrimination was found to exhibit a discrepancy between the otoacoustic emission and the auditory brainstem response. These patients are categorized as having an auditory neuropathy spectrum disorder (ANSD). ANSD is defined by (1) absent or atypical auditory brainstem responses and (2) recordable otoacoustic emissions and/or cochlear microphonics, suggesting that the outer hair cells function normally, but the inner hair cells and/or spiral

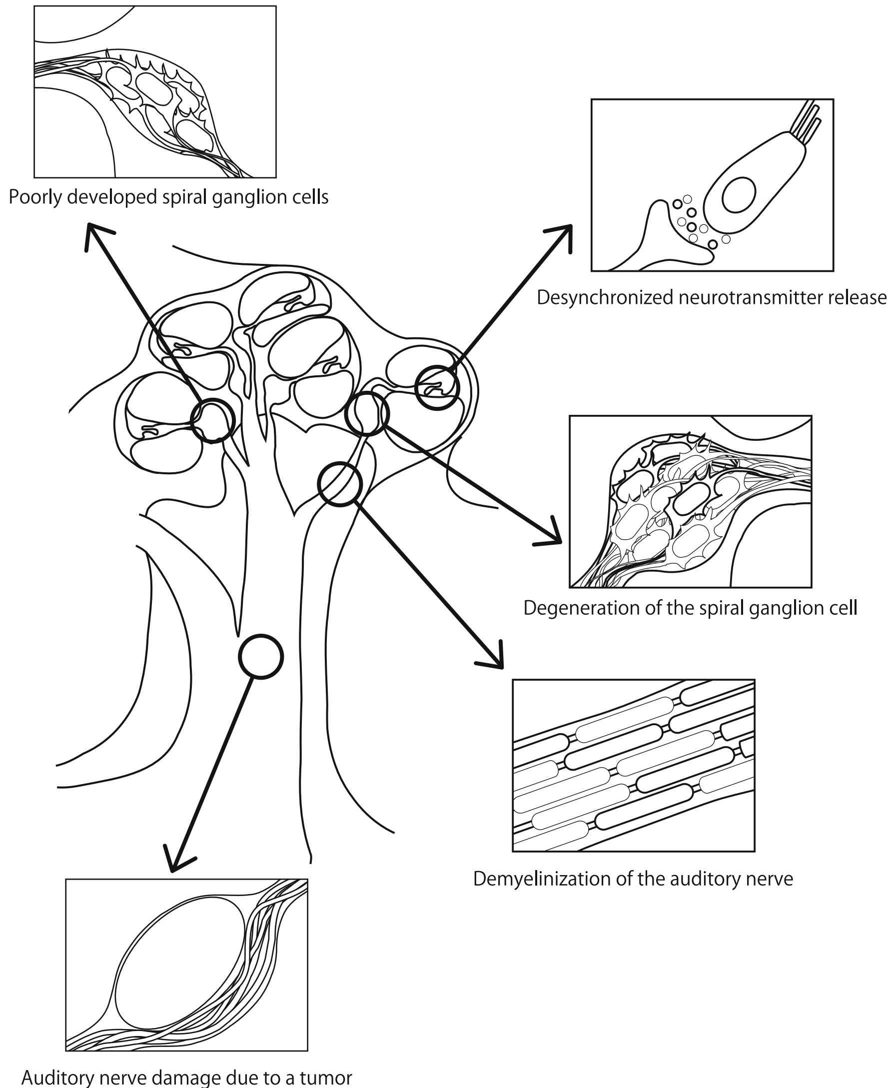

Fig. 25.1 Diverse causes of the clinically diagnosed "retrocochlear" type of hearing loss. The precise pathology of each case is often difficult to determine clinically. Note that "synaptopathy" refers to a dysfunction of the synapses between hair cells and afferent SGNs, which is audio logically diagnosed as the "retrocochlear" type of hearing loss

ganglion neurons fail to activate the auditory neural system in the brainstem [5, 6]. The incidence of ANSD is estimated to be 10–15 % in children diagnosed with severe to profound sensorineural hearing loss [7]. Audiometric findings widely vary among patients with ANSD, ranging from congenital profound hearing loss to post-lingual mild hearing loss. In addition to an increase in the pure-tone threshold,

ANSD is characterized by speech understanding that is worse than that predicted from the pure-tone audiogram, impaired sound localization, and difficulty in speech perception in a noisy environment [3, 5, 6]. The ANSD is thought to be heterogeneous because the diagnostic criteria for ANSD are not based on the etiology of the disease.

Recent studies have demonstrated a substantial overlap between patients with ANSD and those with cochlear nerve deficiency (CND), especially in the pediatric population [8–11]. As described below, hypoplasia of the bony cochlear nerve canal is often observed in ANSD patients with CND, suggesting that certain types of congenital malformations in the temporal bone are responsible for ANSD in at least part of the pediatric population [11]. Other than congenital malformations of the temporal bone, a considerable number of these cases are caused by dysfunctions in the release of neurotransmitters from the hair cells. Other pathologies include the degeneration of the spiral ganglion cells (ganglionopathy) and demyelinating and/or axonal diseases of the auditory nerve (auditory neuropathy sensu stricto) [3] (Fig. 25.1). These pathological lesions can result in desynchronized auditory nerve activity. In ANSD patients with optic atrophy, a delayed auditory cortical response is detectable despite a negative auditory brainstem response [12]. ANSD patients are now regarded as good candidates for CI [13, 14], but some of these patients experience unsatisfactory results. The severe loss or congenital severe hypoplasia of SGNs is hypothesized in such patients. There have been various attempts to characterize such patients preoperatively.

Imaging studies may supply information about the remaining number of SGNs because the number of SGNs is highly correlated with the diameter of the cochlear nerve [15]. MRI with thin-slice T2-weighted imaging can illustrate the cochlear nerve clearly. ANSD patients with normal cochlear nerves exhibit better speech performance than those with abnormal cochlear nerves [16]. In congenital cases of ANSD, high-resolution CT scans are also useful in the evaluation of anomalies of the cochlear nerve, which is discussed in the following section. Another tool to determine the remaining number of SGNs is the electrophysiological study. ANSD patients with good postoperative speech perception attain significantly higher scores on the electrically evoked auditory brainstem response than those with poor speech perception [16], although the results of electrophysiological studies have not correlated well with those of histological investigations.

25.3 Vestibular Schwannoma

Hearing loss is the most common symptom in vestibular schwannoma patients [17]. Even in patients with preserved hearing, the residual hearing may be lost after treatment, including microsurgery and radiosurgery, or even during careful observation. The pathology of the hearing loss may occur in the cochlea or the auditory nerve, depending on the degrees of compression of the labyrinthine artery and direct damage to the cochlear nerve. Hearing preservation is an unresolved problem

in the treatment of vestibular schwannoma patients. Treating neurofibromatosis type II is particularly challenging because these patients may lose bilateral hearing. In such patients, the restoration of hearing is very important. Auditory brainstem implants (ABI) are generally indicated for such patients. Recently, CI was proposed as another option for vestibular schwannoma patients with preserved cochlear nerves. CI surgery is an established and relatively safe procedure, and it can be performed at the time of surgery to remove the vestibular schwannoma via the translabyrinthine approach or after the surgery through the middle cranial fossa or retrosigmoid approach. Unfortunately, however, the performance of CI is widely variable, even among patients with grossly preserved cochlear nerves [18]. This finding indicates that the anatomical preservation of the cochlear nerve does not necessarily indicate its functionality. Damage to the cochlear nerve is unpredictable. The number of SGNs shows only a weak correlation with tumor size, and the pure-tone threshold and speech discrimination scores do not predict the remaining number of SGNs [19].

25.4 Congenital Hearing Loss

Congenital profound sensorineural hearing loss is a main target for CI, and most children with this condition benefit from CIs [20]. In patients with various causes of congenital hearing loss, the average number of SGNs is 14,000–20,000 [21]. This number fulfills the minimum requirement for CI. However, some children have a very small number of SGNs. Histopathological studies have demonstrated a severe loss of SGNs in patients with Cockayne syndrome [22] and xeroderma pigmentosum [23]. These diseases are DNA repair disorders, which can cause severe loss of SGNs [23]. In children with trisomy 13 syndrome [24] and DiGeorge syndrome [21], a total or near-total loss of SGNs have also been reported. These patients are predicted to show a poor outcome after CI, although this has not yet been reported. Children with CHARGE association have traditionally not been regarded as good candidates for CI because the outcome was poor in a considerable proportion of these cases. CHARGE children frequently exhibit inner ear malformations. The typical anomalies include hypoplasia of the cochlea and the absence of the semicircular canals [25, 26]. The histology of CHARGE association is variable. The inner ear may be normal [27], or the modiolus and Rosenthal's canal may be hypoplastic [28]. This variety may be the reason for the variable outcomes observed after CI in children with CHARGE association [29].

Congenitally deaf children with CND experience poor outcomes after CI. CND is strongly associated with hypoplasia of the bony cochlear nerve canal and narrowing of the internal auditory canal [30]. The fundus of the internal auditory canal forms a pit at the junction with the cochlea. In patients with congenital cochlear nerve deficiency, this structure sometimes becomes narrow and resembles a canal on CT scans. This radiological finding is called a "bony cochlear nerve canal" (Fig. 25.2). A recent study revealed that patients showed poor results after CI

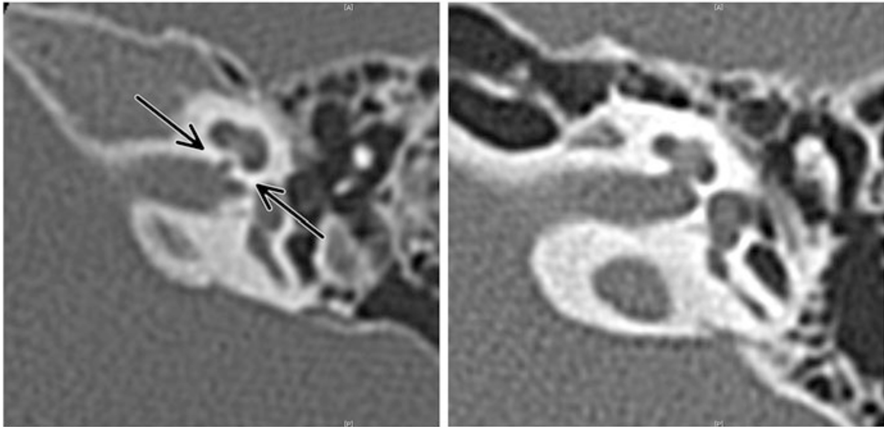

Fig. 25.2 CT images showing narrow (*left*) and normal (*right*) bony cochlear nerve canals. Normally, the bony cochlear nerve canal does not resemble a canal. In patients with poorly developed cochlear nerves, however, this structure becomes constricted and resembles a narrow canal (*arrow*)

when the diameter of the bony cochlear nerve canal was smaller than 1.4 mm [11]. In CHARGE association patients, 77 % of the ears demonstrated cochlear aperture atresia [31]. This finding may be related to radiological findings of a narrow bony cochlear nerve canal, although this has not been demonstrated histologically.

The rate of age-related SGN degeneration is approximately 1,000 per decade in individuals with a normal organ of Corti [1], but the degeneration is accelerated in patients with hair cell loss due to a lack of tropic support. A survey of hearing loss patients with various etiologies revealed a rate of SGN degeneration of approximately 2,100 cells per decade [32]. CIs are very effective in congenital SNHL children during early stages of life, but the long-term results remain unknown. It is possible that the effectiveness of CIs decreases later in life due to the loss of SGNs. Intracochlear electrical stimulation suppresses the apoptosis of SGNs, although postmortem histopathological studies have not supported this in human patients. The remaining number of SGNs is smaller in the implanted ear than in the non-implanted ear [33].

25.5 Limitations of Auditory Brainstem Implants in Patients with SGN Degeneration

In patients without an ample number of viable SGNs, auditory brainstem implantation is the only option for the restoration of hearing; however, the results of this procedure have not been satisfactory [34]. Patients with neurofibromatosis type II exhibit particularly poor speech understanding and most of these recipients

require visual cues to understand speech. The outcome of auditory brainstem implantation in patients without tumors is superior to that in neurofibromatosis type II patients, but it is inferior to the outcome of CI [35]. In addition, auditory brainstem implants are associated with a risk of electrode migration and malstimulation of the vagus nerve, which can cause lethal complications. Therefore, CI is the preferred auditory implant for patients who may have a sufficient number of SGNs. In patients with a poor performance after CI, an auditory brainstem implant can be regarded as an option. However, the outcome is not promising, as described above. The following chapters discuss attempts to regenerate SGNs, which may improve the performance of CI.

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Chapter 26
SGN Development

Koji Nishimura and Norio Yamamoto

Abstract Auditory information is received by hair cells in the cochlea and then transmitted to cochlear nucleus neurons in the brainstem via spiral ganglion neurons (SGNs), which are the primary auditory neurons. Cochlear implants, which are mostly the only solution for those with profound sensorineural hearing loss, rely on the function of residual SGNs. Thus, regeneration of SGNs is important for advancing the outcome of cochlear implants as well as biological solutions to deafness. Having used mainly genetically modified mice, researchers unveiled several transcription factors that orchestrate SGN neurogenesis and several neurotrophic factors that regulate SGN maturation and survival. With cellular and molecular knowledge of SGN development in hand, we can develop novel methodologies to regenerate lost SGNs in the mammalian cochlea.

Keywords Cochleovestibular ganglion • Delamination • Neurogenesis • Spiral ganglion neuron

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26.1 Overview of Spiral Ganglion Neuron Development

Spiral ganglion neurons (SGNs), which are primary auditory neurons, mediate the perception of sound by transmitting the sound information from cochlear hair cells to the cochlear nucleus. Neural precursors of SGNs are specified for a proneural fate as neuroblasts delaminate from the anteroventral otic vesicle between embryonic day (E) 9.5 and E12.5 in mouse [1, 2] (Fig. 26.1a, b). Neuroblasts coalesce to form the cochleovestibular ganglion (CVG), from which SGNs and vestibular ganglion neurons (VGNs) are differentiated [4]. Most CVG neurons originate from the ectodermal cell-derived otic placodal cells [5]. Once SGNs have differentiated from the CVG, they coil along the lengthening cochlear duct and extend afferent dendrites toward the inner and outer hair cells as well as sending processes to the cochlear nucleus in the brainstem (Fig. 26.1c).

26.2 Neurogenesis

Neurogenin1 (Neurog1) and Neurod1 are members of the basic helix-loop-helix (bHLH) family of transcription factors. Neurog1 is necessary for SGN formation; deletion of Neurog1 in mouse causes complete loss of SGNs [2]. Neurod1 is necessary for SGN differentiation and survival; in mice that lack Neurod1, neurons are lost just after delamination and have completely disappeared by E13.5 [6]. Although either Neurog1 and Neurod1 is sufficient to induce neuronal identity in cochlear non-sensory epithelial cells at embryonic stages in vitro, ectopic expression of Neurog1 fails to induce Neurod1 expression. Therefore, Neurog1 and Neurod1 might function in different signaling cascades or Neurog1 requires specific cofactors to induce Neurod1 expression [7].

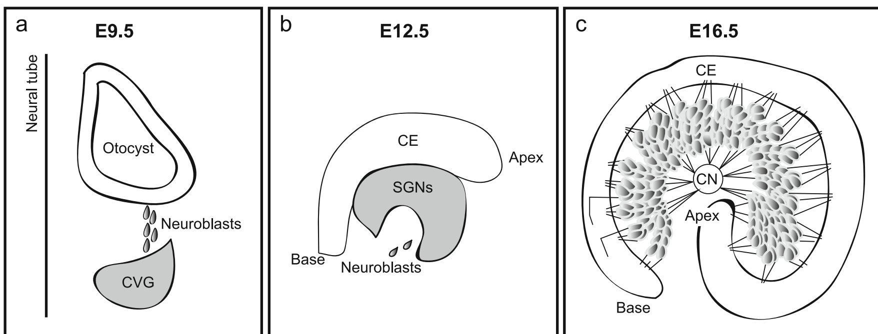

Fig. 26.1 Schematic representation of the developing SGNs in mice, modified Coate and Kelley [3]. (**a**) Neuroblasts delaminate from the otocyst and form the CVG. (**b**) The last wave of neuroblasts populates the SGNs. (**c**) SGNs coil along the lengthening cochlear duct. Type II SGNs extend into the OHC region at the base. *CVG* cochleovestibular ganglion, *SGNs* spiral ganglion neurons, *CN* cochlear nucleus, *CE* cochlear epithelium

bHLH transcription factors such as Neurog1 and Neurod1 interact with LIM-HD proteins to synergistically regulate gene expression [8, 9]. Specifically, Islet1 is an LIM-HD gene that plays a role in development and differentiation of the proneurosensory domain at E12 in mouse. As the sensory epithelium starts to differentiate, Islet1 is downregulated in the entire cochlear epithelium but is maintained in cells following a neuronal lineage. Therefore, Islet1 is an early marker for the proneurosensory domain and a later marker for SGNs and plays a potential role in the inner ear-specific sensory and neuronal cell development [10]. The expression of these proneural factors such as Neurog1, Neurod1, and Islet1 is maintained by the ATP-dependent chromatin remodeling enzyme CHD7, which is mutated in human CHARGE syndrome [11].

Sox2 is not only a universal stem cell marker but also one of the earliest definitive markers of the proneurosensory domain within the developing inner ear [12]. Sox2 plays an important role in maintaining a pool of multipotent progenitors and controlling differentiations of neurons and sensory epithelial cells [7, 12, 13]. Sox2 is downregulated in nascent hair cells by E16 in mouse, but remains expressed in supporting cells and spiral ganglion cells [7, 13]. Mutations in SOX2 lead to sensorineural hearing loss in humans [14]. Light coat and circling (Lcc) mutant mice that do not express Sox2 in the inner ear exhibit a loss of neurons [7] and a loss of hair cells and supporting cells [12], suggesting Sox2 is necessary for SGN and sensory cell formation. Sox2 is also sufficient to induce neurons in non-sensory regions of the cochlea at embryonic stages [7].

The murine eyes absent (Eya) and homeobox Six gene family are essential for inner ear development, which is arrested at the otocyst stage in either Eya1- or Six1-null mutants [15–17]. Haploinsufficiency for human EYA1 and SIX1 causes branchio-oto-renal syndrome [18, 19]. Specifically, in Eya1- or Six1-null mutants, neurogenesis is initiated, but neuroblasts do not form the CVG, suggesting both genes are required for differentiation and maintenance of sensory neurons [20]. More recently, it was shown that Eya1 and Six1 together with the SWI/SNF ATP-dependent chromatin remodeling protein complex activate Neurog1 and Neurod1, and Sox2 cooperates with these factors to mediate neuronal differentiation [21].

26.3 A Potential Role of Insulin-Like Growth Factor-1 (IGF-1) in SGN Survival and Maturation

IGF-1 plays an important role in murine inner ear morphogenesis [22] and postnatal cochlear maturation [23]. Igf1r is expressed in SGNs at E13.5 and E16.5 demonstrated by in situ hybridization [22]. Igf1r is also predominantly expressed in SGNs compared to VGNs both at embryonic and postnatal stages according to Goodrich's lab microarray database [24]. The number of SGNs in Igf1−/− mutants decreases as a result of apoptotic cell death beginning after P20 [23], implying that IGF-1 plays a role in SGN survival and maturation. However, SGN loss in

Igf1−/− mutants might be secondary due to insufficient production of neurotrophins by cochlear hair cells. Additionally, the number of SGNs in Igf1r−/− mutants at embryonic stages does not appear to be different from that of heterozygotes (Takayuki Okano, personal communication). Conditional deletion of Igf1r using an inner ear-specific driver is necessary to elucidate a potential role of IGF-1 in SGN survival and maturation, because Igf1r−/− mutants die at birth, preventing a subsequent analysis of inner ear [22].

26.4 Spiral vs. Vestibular Ganglion Neuron Specification

SGNs and VGNs are generated during two overlapping but distinct waves of neurogenesis; SGNs derived from a Neurog1 lineage are born later than VGNs [1], in line with the previous birth-dating studies that VGNs are born before SGNs [25]. Additionally, neuroblasts giving rise to SGNs and VGNs delaminate from distinct regions of the otocyst [26]. The zinc finger transcription factor Gata3 is initially expressed at the otocyst, but its expression is progressively restricted to SGNs and the organ of Corti compared to VGNs [26, 27]. Haploinsufficiency of GATA3 causes human hypoparathyroidism, sensorineural deafness, and renal dysphasia (HDR) [28, 29], indicating GATA3 has a profound role in cochlear neurosensory development. Conditional deletion of Gata3 in SGNs leads to precocious extension of their peripheral axons and aberrant path finding to cochlear epithelium [27]. Expression profiling of Gata3-null SGNs revealed a broad shift in gene expression toward a more differentiated state, implying Gata3 serves as an intermediate regulator that guides SGNs through differentiation and preserves the auditory fate [27]. At present, upstream factors that restrict Gata3 to the auditory division in the CVG remain to be seen.

26.5 Type I and Type II Spiral Ganglion Fate Specification

The type I neurons, which comprise 90–95 % of the SGNs, terminate on inner hair cells. Each afferent dendrite contacts only a single inner hair cell, but each hair cell directs its output to multiple nerve fibers [30]. Type II neurons comprise 5–10 % of the SGNs and a single type II neuron contacts 10–100 different outer hair cells [31, 32]. Type I and type II SGNs are different even at embryonic stages demonstrated by the following observations: a small number of type II SGNs in the base of the cochlea at E16.5 extend past the row of inner hair cells and innervate directly to outer hair cells [1] (Fig. 26.1). A subset of SGNs at E18.5 is labeled peripherin [33], which marks type II SGNs in the adult [34]. On the other hand, SGNs develop through a process of superfluous outgrowth of afferent dendrites followed by pruning at postnatal stages [35]. Therefore, although type I and type II SGNs are specified at embryonic stages, refinement of projections occurs during later stages of circuit assembly.

26.6 Tonotopy in the SGNs

In SGNs, there is tonotopic distribution of firing features, voltage-gated ion channels, and synaptic proteins: SGNs isolated from apical, low-frequency regions showed greater levels of slow accommodation compared to those isolated from basal, high-frequency regions [36]; large conductance calcium-activated potassium channels, Kv1.1 and Kv3.2, are enriched in basal neurons, contributing to abbreviated firing features [36]; the presynaptic protein synaptophysin is enriched in the apical neurons while the postsynaptic AMPA receptor subunits GluR2 and GluR3 are abundant in the basal neurons [37]. Neurotrophin-3 (Ntf3) is expressed preferentially in the apical SGNs, whereas brain-derived neurotrophic factor (Bdnf) is expressed predominantly in the basal SGNs [38]. Regionalized concentrations of NTF3 and BDNF partly determine tonotopic features that characterize SGNs in the fully developed ear [39]. Characteristics of basal SGNs is enhanced by exposure to BDNF and reduced by NTF3, whereas that of apical SGNs is enhanced by NTF3 and reduced by BDNF [39]. "Yin-yang" regulatory effects of Ntf3 and Bdnf on voltage-gated ion channels and synaptic protein composition in SGNs indicate that Bdnf and Ntf3 activate distinctly different elements of trk-based signaling pathways [39].

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Chapter 27
Gene Therapy for Regeneration and Preservation of Spiral Ganglion Neurons

Hiroshi Yamazaki and Takayuki Nakagawa

Abstract Spiral ganglion neurons (SGNs) are the targets for electrical stimulation from cochlear implants (CI), and SGN lesions can affect CI-mediated auditory performance. Primary diseases in SGNs are relatively rare; however, loss of hair cells, which is the most common cause of sensorineural hearing loss, usually leads to the secondary degeneration of SGNs. Therefore, most of the attempts at gene therapy for SGNs have been intended to prevent degeneration of SGNs following loss of hair cells in order to improve CI outcomes. Previous studies showed that direct administration of exogenous peptide neurotrophic factors into the cochlea enhanced the survival of SGNs after noise- or aminoglycoside-induced loss of hair cells; however, this effect was transient. Other studies demonstrated that gene therapy using viral-derived vectors, especially adenoviral and adeno-associated viral vectors, successfully achieved the long-term expression of a gene encoding neurotrophic factors and promoted SGN survival and neurite extension of SGNs in ototoxically deafened guinea pigs and congenitally deaf mice with a genetic mutation. Inoculation of viruses expressing glial cell-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) into the scala tympani decreased thresholds for electrically evoked auditory brainstem responses, suggesting the clinical importance of gene therapy using neurotrophic factor-expressing viruses to improve CI outcomes.

Keywords Auditory nerve • BDNF • Cochlear implant • Gene therapy • Neurotrophic factor • Neurotrophin • NT-3 • Regeneration • Spiral ganglion neurons • Viral vector

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27.1 Introduction

Approximately 95 % of spiral ganglion neurons (SGNs) receive synaptic input from inner hair cells and transmit these signals to neurons in the cochlear nucleus. Abnormalities in SGNs lead to retrocochlear sensorineural hearing loss. As described in Chap. 26, primary lesions in SGNs are mainly categorized as acoustic tumors, auditory neuropathy spectrum disorder, and congenital malformations of the cochlear nerve. The proportion of retrocochlear lesions among the deaf population is relatively low, and sensorineural hearing loss is usually caused by damage to the hair cells in the cochlea due to genetic mutation, congenital viral infection, presbycusis, chronic otitis media, Meniere disease, and other disorders [1, 2]. Even if the pathological lesion is originally limited to the hair cells, the loss of these most peripheral neurons is followed by degeneration of the secondary neurons, SGNs [3–5]. Previous studies demonstrated that neurotrophins, specifically neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), are essential for maintaining the peripheral fibers and cell bodies of SGNs in the mature cochlea [6–13]. During the early postnatal period, mRNA for NT-3 and BDNF is expressed in the inner hair cells (NT-3) or both inner and outer hair cells (BDNF), but only expression of NT-3 mRNA is sustained in the adult cochlea [7, 14]. The mRNA of glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β superfamily, is also expressed in developing inner and outer hair cells and in mature inner hair cells [15]. SGNs express the neurotrophin receptors, TrkB and TrkC, as well as the GDNF family receptor α-1, indicating that SGNs are responsive to NT-3, BDNF, and GDNF [11, 16]. Results of these studies imply that loss of hair cells leads to decreased expression of neurotrophic factors in the cochlea, which in turn may cause degeneration of SGNs.

Cochlear implantation is accepted as an effective treatment to restore auditory perception in patients with severe or profound sensorineural hearing loss due to cochlear lesions. Since a cochlear implant (CI) directly stimulates SGNs to provide auditory signals to the central auditory system, degeneration of SGNs must affect CI-mediated auditory performance. In fact, several studies using animal models demonstrated that the loss of SGNs and their peripheral fibers increased the threshold of electrically evoked auditory nerve responses [17, 18]. Considering that retrocochlear hearing loss caused by primary lesions in SGNs is rare, most attempts at gene therapy for SGNs have been intended to prevent degeneration of SGNs following the loss of hair cells in order to enhance outcomes of cochlear implantation [19, 20]. In this section, we will review recent studies reporting gene therapy to prevent degeneration and promote regeneration of SGNs following damage to the hair cells.

27.2 Direct Administration of Peptide Neurotrophic Factors

Direct infusion of exogenous peptide neurotrophic factors (including GDNF, BDNF, and NT-3) into the cochlea, individually or in combination of two or three, promoted survival of SGNs after noise- or aminoglycoside-induced loss of hair cells [15, 21–24]. In addition to preventing the secondary loss of SGNs, neurotrophic factors also promoted SGN fiber extension [25, 26]. In these studies, however, the effects were transient and persisted no longer than 4 weeks, even though an osmotic pump was used to infuse the peptides into the cochlea [23, 24, 27]. It should be noted that cessation of the administration of exogenous BDNF resulted in an accelerated following loss of SGNs, suggesting that neurotrophin must be supplied continuously during the patient's life span to preserve SGNs in clinical situations [24].

27.3 Viral-Mediated Gene Delivery for Long-Term Expression of Neurotrophic Factors

Gene therapy using viral-derived vector is suitable to achieve the long-term expression of a gene encoding neurotrophic factors. As described in Chap. 24, a variety of viruses are available for gene therapy. Each virus has characteristic target cell specificity, and the combination of the type of virus with its delivery routes appears to be essential for successful delivery of exogenous genes into the target cells. Moreover, duration of transgene expression and cytotoxicity are important factors in selecting a vector appropriate for the clinical application. For the purpose of SGN gene therapy, herpes simplex 1 virus (HSV-1), adenovirus serotype 5 (Ad5), and adeno-associated virus serotype 2 (AAV2) were infused into the scala tympani or scala media (Table 27.1).

27.3.1 SGN Survival by Viral-Mediated Overexpression of Neurotrophic Factors

The first model for viral-mediated gene therapy for SGN degeneration was reported by Staecker et al. [28]. In this study, BDNF-expressing HSV-1 vector was infused into the scala tympani in mice after complete destruction of the auditory hair cells by exposure to an aminoglycoside. Inoculation with BDNF-expressing HSV-1 vector resulted in a significant improvement in neuronal survival of SGNs at 4 weeks after infusion. Stable expression of the exogenous BDNF was observed 4 weeks after injection, suggesting that HSV-1 vector-mediated expression of BDNF contributed to SGN survival. The following studies, published from 2000 to 2004, demonstrated that different

Table 27.1 Previous studies using neurotrophic factor-expressing virals to enhance SGN survival in model animals

References	Vector	Promoter	NTFs	Delivery route	Effects	Animal
Staecker et al. [28]	HSV-1	HSV-1 IE 4/5 promoter	BDNF	Scala tympani	SGN survival	Ototoxically deafened mice
Yagi et al. [29]	Ad5-CMV	CMV	GDNF	Scala tympani	SGN survival	Ototoxically deafened guinea pigs
Kanzaki et al. [30]	Ad5-CMV	CMV	GDNF	Scala tympani combined with chronic ES	SGN survival Decrease in EABR thresholds	Ototoxically deafened guinea pigs
Lalwani et al. [31]	AAV2	CMV	BDNF	Scala tympani	SGN survival	Ototoxically deafened guinea pigs
Nakaizumi et al. [32]	Ad5-CMV	CMV	BDNF	Scala tympani	SGN survival	Ototoxically deafened guinea pigs
Rejali et al. [33]	Ad5-CMV	CMV	BDNF	Insertion of the electrode coated by transfected cells	SGN survival	Ototoxically deafened guinea pigs
Chikar et al. [34]	Ad5-CMV	CMV	BDNF	Scala tympani combined with chronic ES	SGN survival Decrease in EABR thresholds	Ototoxically deafened guinea pigs
Shibata et al. [35]	AAV2-CMV	CMV	BDNF	Scala media Scala tympani	Peripheral fiber regrowth	Ototoxically deafened guinea pigs
Wise et al. [36]	Ad5-CMV-GFP IRES	CMV	BDNF, NT3	Scala media Scala tympani	SGN survival peripheral fiber regrowth	Ototoxically deafened guinea pigs
Atkinson [37]	Ad5-CMV	CMV	NT-3+ BDNF	Scala media	SGN survival Peripheral fiber regrowth	Ototoxically deafened guinea pigs
Fukui et al. [38]	Ad5-CMV	CMV	BDNF	Scala media	SGN survival Peripheral fiber regrowth	Pou4f3 KO mouse
Takada et al. [39]	Ad5-CMV	CMV	BDNF	Scala media Scala tympani	SGN survival	Gjb2 conditional KO mice

combinations of a neurotrophic factor and a viral vector (GDNF-expressing Ad5, BDNF-expressing Ad5, and BDNF-expressing AAV2) also enhanced SGN survival in ototoxically deafened guinea pigs at 4 weeks after inoculation into the scala tympani, indicating that viral-mediated long-term expression of neurotrophic factors is effective in preventing cell death of SGNs following the loss of hair cells [29, 31, 32]. Transgene expression was observed mainly in SGN cell bodies, suggesting that an autocrine mechanism may account for increased SGN survival. Regarding the delivery route of the virus, Wise et al. demonstrated that in comparison with scala tympani inoculation, injection of virals into the scala media resulted in greater SGN survival in the basal turn of the cochlea, which was associated with consistent transgene expression within the partially degenerated organ of Corti. However, since fewer SGNs survived in the middle and apical turns following injection into the scala media when compared with scala tympani inoculation, further experiments are necessary to reach a definite conclusion regarding the best injection site. Paralleling these gene therapy studies, researchers were attempting to combine cochlear implantation with viral-mediated overexpression of a neurotrophic factor to maintain the number of SGNs and improve CI outcomes. Rejali et al. coated the CI electrode with allogeneic fibroblasts transfected by BDNF-expressing Ad5, instead of using a neurotrophin-eluting biopolymer. Insertion of the BDNF-expressing electrodes preserved significantly more SGNs in the basal turns of the cochlea in guinea pigs for at least 48 days following implantation when compared to control electrodes [33]. Since direct infusion of virus into the inner ear may induce aversive immune responses, this ex vivo transfection approach has the potential to accomplish long-term growth factor secretion with minimal side effects.

27.3.2 Neurite Extension of SGNs by Viral-Mediated Overexpression of Neurotrophins

Previous studies reported that peptide neurotrophic factors infused into the scala tympani using a mini-osmotic pump promoted SGN neurite extension and enhanced SGN survival [21, 24, 25]. In 2010, focusing on the effects of gene therapy on neurite extension of SGNs, two groups demonstrated that viral-mediated overexpression of BDNF and/or NT-3 resulted in resprouting of peripheral SGN fibers [35, 36]. It should be noted that regrowth of SGN fibers induced by an osmotic pump-mediated administration of neurotrophin peptides was disorganized, and looping back within the osseous spiral lamina and lateral projection along the basilar membrane were often observed [25]. The peptide infusion into the perilymph led a high concentration of administrated neurotrophins throughout the cochlea, which may have disturbed the original local gradient of neurotrophins released from the organ of Corti and caused the disorganized regrowth of SGN fibers. Consistent with this conclusion, inoculation of the neurotrophin-expressing virus into the scala media resulted in more localized transgene expression within

the organ of Corti compared with scala tympani infusion and was associated with sprouting of SGN fibers to neurotrophin-expressing cells [35, 36]. The organized regrowth of SGN fibers induced by gene therapy clearly contrasts with the disorganized growth induced by osmotic pump-mediated administration of neurotrophin peptides, suggesting the advantage of viral-mediated gene therapy through scala media inoculation to promote regrowth of SGN fibers.

27.3.3 Prevention of SGN Degeneration in Deaf Mice with a Genetic Mutation

In the aforementioned studies, aminoglycoside-treated guinea pigs or rats were used to study gene therapy to prevent SGN degeneration secondary to ototoxic drug-induced hair cell loss. Recently, BDNF-expressing gene therapy was applied to *Pou3F4* or conditional *Gjb2* knockout mice, which are animal models for congenital genetic hearing loss [38, 39]. In particular, pups of *Pou3F4* knockout mice have no cochlear hair cells [38]; therefore, these mice serve as a valuable model to evaluate whether viral-mediated BDNF expression can induce nerve fiber regeneration and SGN preservation in ears with hereditary deafness. Inoculation of BDNF-expressing AV into the scala tympani or scala media enhanced SGN survival in the basal turn of the cochlea in both strains of mutant mice. Regenerative sprouting of peripheral SGN fibers into the auditory epithelium was observed in the treated *Pou3F4* knockout mice. These results suggest that congenitally deaf children with a hereditary genetic mutation can be candidates for gene therapy to improve outcomes of cochlear implantation.

27.4 Effects of Gene Therapy on Electrical Stimulation in Model Animals

In clinical application, the primary goal of gene therapy using neurotrophic factor-expressing viruses is to improve CI outcomes by preserving SGNs, which are the targets of CI-mediated electrical stimulation [20]. As described above, inoculation of BDNT, NT-3, and/or GDNF-expressing viruses into the scala media or scala tympani has been proven to prevent SGN degeneration and to enhance resprouting of SGN fibers in ototoxically and genetically deaf animals. However, it is important to determine whether these histological changes contribute to functional benefits in patients receiving CI-mediated electrical stimulation.

Chronic electrical stimulation in the cochlea alone significantly reduced deafness-related loss of SGNs when compared with unstimulated ears [40]. Electrical stimuli induced elevation of intracellular Ca^{2+} concentrations and release of synaptic vesicles containing neurotrophins and neurotransmitters from presynaptic

terminals on SGNs via voltage-gated calcium channels, which may have prevented SGN degeneration [41, 42]. When the administration of BDNF peptide into the scala tympani using a mini-osmotic pump was combined with chronic electrical stimulation in ototoxically deafened guinea pigs, electrical stimulus thresholds were significantly lower than those measured in the ears receiving electrical stimulation alone, suggesting functional advantages of the infusion of BDNF peptide in cochlear implantation [43]. Consistent with this data, inoculation of GDNF- or BDNF-expressing virus into the scala tympani enhanced SGN survival and decreased EABR thresholds [30, 34]. The difference in the electrically evoked auditory brainstem response (EABR) thresholds was significant in the gene therapy experiment using BDNF-expressing Ad5 [34]. While continuous administration of neurotrophic peptides using a mini-osmotic pump carries the risks of infection, degradation of peptides in the pump, and cannula clogging, only a single injection is necessary to achieve long-term transgene expression in viral-mediated gene therapy, suggesting the advantage of gene therapy in clinical applications to improve CI outcomes. However, inoculation with viruses involves risks of an adverse immune response and toxicity, especially when using the virus the second time [44]. Accordingly, more extensive investigations are necessary before this technology is safe for clinical application in humans.

27.5 Comparison of Results for Patients with CIs with Animal Models

Theoretically, an increased number of surviving SGNs would increase sensitivity to electrical stimuli and contribute to improved CI outcomes. Surprisingly, histopathological studies using temporal bones of patients with CIs did not show a definite relationship between the number of surviving SGNs and CI-aided auditory performance, and patients with a surviving SGN population of only 10–15 % showed sufficient speech discrimination [45, 46]. Since EABR testing was not performed in these studies, it is difficult to compare these data with BDNF- or GDNF-induced improvement of EABR thresholds in animal studies; nevertheless, there are some discrepancies between the human and animal studies [30, 34]. Since the ability to discriminate speech depends on higher brain functions rather than auditory brainstem responses, individual differences in duration of CI usage, duration of deafness before implantation, and cognitive functions might influence speech discrimination scores more than the number of surviving SGNs in patients with CIs.

Regarding the regenerative sprouting of SGN fibers, no peripheral SNG fibers were observed in the temporal bones of patients with CIs, suggesting that SGN cell bodies rather than peripheral fibers are the targets for CI-mediated electrical stimulation [45, 46]. In theory, closer contact between electrodes and the peripheral neuronal fibers of SGNs can decrease impedance of electrical stimulation and prevent current spread to achieve a more restricted current field, which may

contribute to better pitch perception. In this respect, regrowth of SGN fibers as well as prevention of SGN degeneration induced by neurotrophic factor-expressing viruses has the potential to improve CI outcomes.

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Chapter 28
Cell Therapy for Regeneration of Spinal Ganglion Neurons

Tetsuji Sekiya and Masaaki Ishikawa

Abstract Cell transplantation is regarded as one of the potential therapies to replace diseased auditory neurons and experimental studies have been compiled to revive auditory neurons during the last decade. Revitalizing auditory neurons are crucial to accomplish rewiring of peripheral auditory system in toto as they are essential for the survival and maintenance of cochlear nucleus cells. We reviewed the relevant literature with the object of functional replacement of auditory neurons. After many types of cell lines have been investigated as donor cells so far, now our studies should be directed to conclusively clarify which cell line(s) are most suitable as donor cells and when the optimal differentiation stage is to harvest donor cells. Cell transplantation site is critical for successful cell transplantation and should be selected so that growth factors are efficiently available for cell survival and differentiation. Auditory nerve is a favorable transplantation site as neurotrophins synthesized in hair cells directly flow down into auditory nerve trunk. Total replacement of auditory neurons might be ideal but currently a distant goal. However, it is likely adding even a small number of auditory neurons to existing residual neurons would be clinically significant to patients' hearing.

Keywords Auditory neuron • Cell transplantation • Hearing restoration

28.1 Background

Although there are several potential therapeutic options to rebuild lost auditory nerve function such as encouraging neurite extension from damaged auditory neurons and recruiting neural cells from other locations or endogenous inner ear stem cells [1–3], they are still in the earlier developmental stage. Hence, in this

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chapter we focus our discussion solely on exogenous cell transplantation, especially in vivo studies, to clear what we should do in our future studies to fulfill our final goal, functional rewiring of peripheral auditory system.

28.2 Differentiation Stage of Donor Cell

Over the last decade since the first experimental study of inner ear regeneration [4], in vivo experimental studies attempting auditory nerve replacement have been compiled [5] (see a comprehensive list in Needham et al. [5]).

For successful reconstruction of auditory neurons, it would be logical to guide transplanted cells to recapitulate the embryological developmental process [6, 7] (Fig. 28.1). Currently, however, it remains controversial what cell line(s) are the most suitable as donor cells. Likewise, the optimal timing of harvesting donor cells is still in dispute. Otic progenitor cells derived from human ES cells transplanted into the auditory nerve trunk of auditory neuropathic gerbils extended neurites, resulting in forming functional synapses at both sides of donor cells [8]. In a study of retinal regeneration, post-mitotic precursor cells were integrated into the host more sufficiently than proliferating progenitors and functional visual function was regained [9]. Transplanted auditory precursor cells adopted a bipolar shape within 4 days after transplantation to the auditory nerve (Fig. 28.1), but mouse ES cells did not show the same morphological response in the same experimental setting [6, 10]. These studies imply ontogenetic stage-/region-restricted differentiated cells have some built-in internal molecular program to dominate the initial phase in forming the cell morphology and in deciding the final cell fate and are more sensitive to the local environmental cues [6, 10]. The more undifferentiated cells we choose as donor cells including ES cells and iPS cells [11, 12], the more advanced techniques to control cell differentiation and cell fate should be sought.

28.3 Neurite Extension, a Prerequisite for Auditory Nerve Regeneration

One crucial prerequisite in the study of auditory nerve regeneration is that donor cells extend definite neurites like default bipolar shape or at least two from multipolar processes of donor cells, toward hair cell and cochlear nucleus regions. Without such elongated neurites, synaptic formations could not be expected to be formed at both sides of transplanted cells.

To promote neurite extensions from donor cells, growth factors are indispensable. Donor cells need two different nutrient sources for survival and differentiation; one is peri-grafting and the other is post-grafting sources. In peri-grafting period, usually there is no other alternative but to provide immediate nutrients

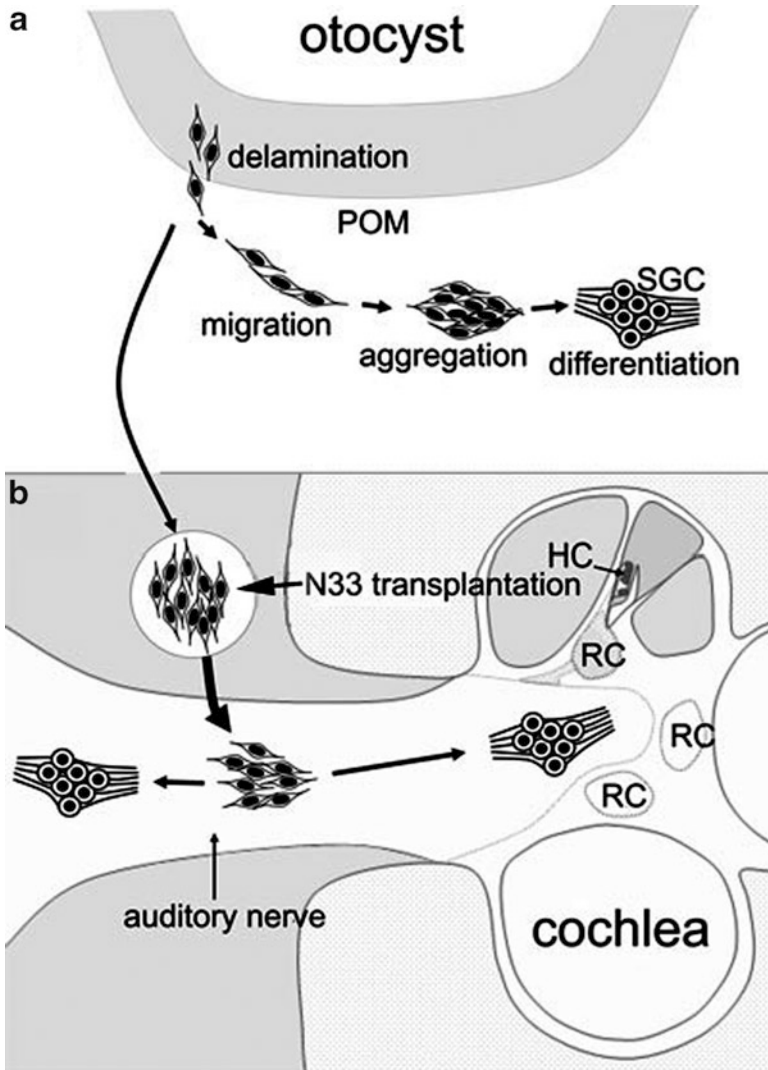

Fig. 28.1 (**a**) During normal development, sensory neuroblasts delaminate from the otocyst epithelium and migrate through the periotic mesenchyme (POM). During this phase there is a period of proliferation called transit amplification. The cells then aggregate and start to differentiate. The process occurs over a period of approximately 4 days and migration occurs over a distance of up to 500 μm. (**b**) Transplanted precursor cells of auditory neurons (N33) recapitulate the process of migration, aggregation, and morphological differentiation in the adult rat auditory nerve tract. *RC* Rosenthal's canal, *SGC* spiral ganglion cells, *HC* hair cells (cited from Sekiya et al. [6])

externally, such as donor cells transplanted with nutrient-rich medium. In post-grafting period, donor cells should settle down in a location where nutrients such as neurotrophic factors are constantly and permanently provided (see Chap. 7).

It cannot be expected that sustained supply of neurotrophins is provided to donor cells in the scala tympani [2, 13, 14], because the composition of perilymph is similar with the cerebrospinal fluid that hardly contains an ample amount of growth factors [15]. To compensate such a drawback of perilymphatic fluid, co-grafting of growth factor-producing cells or infusion of growth factors to transplanted site was performed with success [2, 13]. Neuronal differentiation and survival rate of mouse ES cells transplanted into the scala tympani were greatly enhanced only when GDNF was continuously infused into the scala tympani [2]. When ES cells were transplanted into the scala tympani with NGF-producing dorsal root ganglion (DRG) cells, the transplanted cells extended neurite-like processes toward the host SGCs and their peripheral nerve processes [13].

28.4 Auditory Nerve as an Avenue for Donor Cell Migration/Differentiation and Route for Trophic Factors Supply

Emerging evidence indicates auditory nerve trunk is more favorable as a site for cell transplantation than any other places in attempting auditory nerve regeneration [6, 10, 16–19]. It is likely neurotrophins synthesized in hair cells directly flow down into auditory nerve and "showering" of these factors on transplanted cells within auditory nerve trunk enhances their migration and neurite elongation. ES cells after neural induction had been applied were transplanted into the basal portion of auditory nerve. Four weeks after transplantation, the donor cells extended neuritic processes markedly both peripherally and centrally [16]. ES cells converted to neural progenitor cells were infused into auditory nerve trunk in deafened gerbils. By 2–3 months, the donor cells had extended neurites abundantly into the organ of Corti through empty Rosenthal's canal (RC) [17]. A study demonstrated auditory precursor cells transplanted into auditory nerve trunk extensively migrated both distally and proximally [6]. ES cells placed at the internal auditory meatus portion of the auditory nerve that had been previously injured by compression migrated along the auditory nerve, passed through empty RC and the habenula perforata, and finally reached the scala media, a suitable place to form the synapse with hair cells, indicating the transplanted cells can reach there without breaching the membranes sealing the peri- and/or endolymphatic spaces [10]. These results indicate auditory nerve can be used as a useful avenue for donor cell migration/differentiation and a route for trophic factor supply, especially in auditory neuropathic-type auditory nerve degeneration where hair cells are intact (see Chap. 7).

There were reported various cell transplantation techniques into the cochlea [5]. To directly reach the CNS portion of auditory nerve, it is necessary to enter into

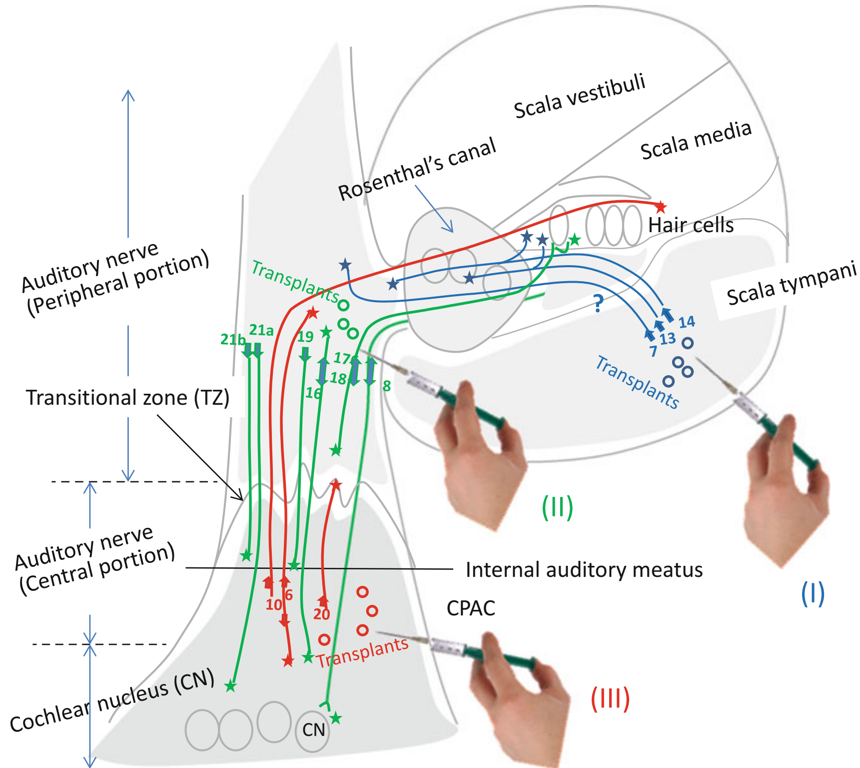

Fig. 28.2 Reported results of cell transplantation experiments to rebuild lost auditory neurons. The reports in which neurite extensions were confirmed are mainly listed. (*I*), (*II*), and (*III*) indicate routes of cell transplantation. (*I*) scala tympani approach (*blue*); (*II*) trans-round window/perilymphatic space approach (*green*); (*III*) retromastoid auditory nerve approach (*red*). *Numbers* denote reference numbers. Trajectories of cell migrations (*blue*, *green*, and *red curved lines*) are not necessarily confirmed in all cases as is representatively shown by a *question mark*. *One sided* and *bidirectional arrows* indicate initial transplanted sites and stars farthest point that donor cells reached. See the detailed results in the text. *CPAC* cerebellopontine angle cistern

intracranial space (Fig. 28.2) after incising single membrane, the dura mater [6, 20]. In trans-round window/perilymphatic space approach, one more membrane sealing the scala tympani has to be incised before the dura mater is incised that lines the inner surface of internal auditory meatus within which auditory nerve trunk resides (Fig. 28.2). Breaching of the membrane sealing perilymphatic spaces induces inevitable disturbance of the homeostasis of the inner ear fluid environment, thus placing hearing at risk [21–23].

28.5 Commitment of Auditory Neurons for Regeneration in Cochlear Nucleus Cells

In a study with embryonic DRG neurons cocultured with brainstem slice, numerous neurites from DRG neurons extended toward cochlear nucleus (CN) region, indicating an endogenous attractive factors were released from the cochlear nucleus [24]. Survival of cochlear nucleus cells intimately depends on the auditory neurons; innervation by auditory neuron and subsequent synaptic activity are essential for normal development of the cochlear nucleus cells [25]. With reinstalling of auditory nerve spike activities using a cochlear implant in deafened cats, the endbulb of Held was rescued [26]. These results indicate regeneration of auditory neurons is prerequisite for regeneration in CN and related structures.

A study showed statoacoustic ganglion cells transplanted in the auditory nerve migrated into the CN in the animals deafened with beta-bungarotoxin [20]. DRG neurons and ES cells transplanted in transected auditory nerve migrated close to ventral cochlear nucleus [21]. Similarly, auditory precursor cells transplanted into the auditory nerve migrated not only peripherally but also centrally [6]. However, one potential dilemma in this regard is that the more proximal the donor cells migrate toward the CN region, the more they become remote from the hair cell region and vice versa. It has not been clarified yet whether single donor cell can bridge between hair cell and cochlear nucleus cell as in default condition or multiple neurons with interneurons are needed as was demonstrated in a study where severed pyramidal tract fibers were reconnected using multiple neurons [27].

28.6 Is “Massive” Regeneration of Spiral Ganglion Cells Needed for Hearing Restoration?

Studies indicate that there is no significant correlation between clinical performance of cochlear implant (CI) and the residual absolute number of auditory neurons and hence it is believed that some other unidentified clinical variables are related to CI performance [28–31]. Theoretically, however, some minimal number of auditory neurons should be left functional. In one postmortem study of patients with CI, the least number of the SGCs of the patient was 1,443 that is approximately less than 5 % of total number of SGC, 30,000 [31]. Coincidentally, it is reported that preservations of axons and myelination are not proportional to the degree of neurological recovery in spinal cord injury [32] and effective locomotion was found to recover when only of 5–10 % of the original axonal population was maintained [33]. Based on these observations, there seems to be a possibility even if regenerated auditory neurons were small in number, its effect would be significant to patients’ hearing. To clarify this important point, morphological studies corroborated by functional evaluation are needed in our future experiments [8].

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Chapter 29
Afferent Dendrite and Axon

Takayuki Nakagawa

Abstract In the last decade, a number of reports on the cell-based therapy for regeneration of the spiral ganglion neurons have been published. Various types of cells can be a source of regenerated spiral ganglion neurons. Considering the functionality of regenerated neurons, there are several requirements. One is the projection to the cochlear nucleus, which is necessary for transmission of auditory signals to the central auditory systems. Another is the projection to the peripheral targets including inner hair cells and electrodes of the cochlear implants. Future investigations are required for elucidation of molecular mechanisms for projections from spiral ganglion neurons to both the central and peripheral auditory systems.

Keywords Cochlear nucleus • Guidance • Projection • Spiral ganglion neuron

29.1 Regeneration of Spiral Ganglion Neurons

In the last decade, there have been a number of publications on the cell-based therapy for regeneration of the spiral ganglion neurons. Regeneration of spiral ganglion neurons is most close to clinical application among cell transplantation approaches for the inner ear. Comparing with hair cell regeneration, the induction of glutamatergic neurons from stem cells is realistic. In addition, anatomical characteristics of spiral ganglion neurons are included in advances of regeneration of spiral ganglion neurons in cell-based therapy. The soma of the spiral ganglion neuron is located in the bony canal in the cochlea, Rosenthal canal. Therefore, a

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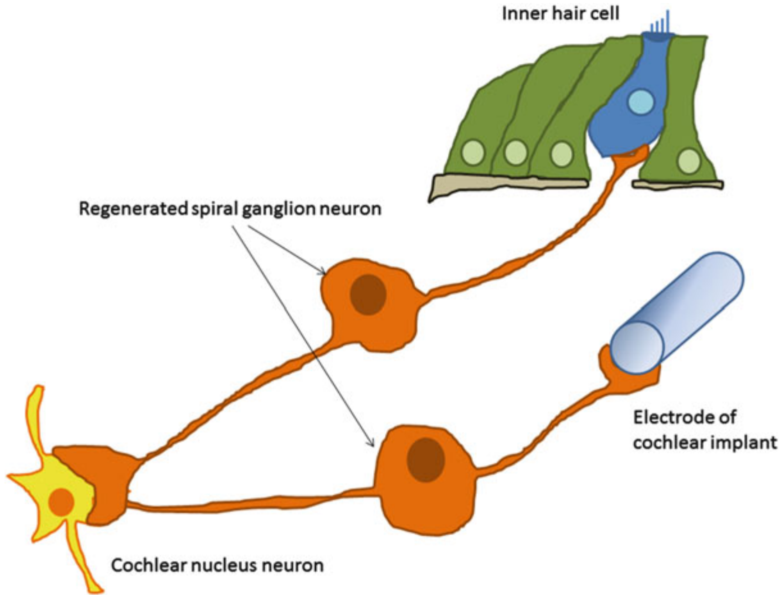

Fig. 29.1 Projection of regenerated neurons to the peripheral targets including inner hair cells and electrodes of cochlear implants and to the central target, the cochlear nucleus neuron

target site for injection of transplants is evident and easy to reach. Cochleostomy, which is a common surgical approach for cochlear implantation, can be utilized for cell transplantation to Rosenthal canal. In fact, the majority of publications on cell therapy for spiral ganglion neurons used this surgical approach. Considering clinical settings, this surgical approach is practical.

Various types of cells including ES cells, iPS cells, and mesenchymal stem cells can be a source of regenerated spiral ganglion neurons. Considering the functionality of stem cell-derived neurons in the cochlea, there are several requirements. One is the projection to the cochlear nucleus, which is necessary for the transmission of auditory signals to the central auditory systems (Fig. 29.1). Regeneration of spiral ganglion neurons might contribute to the promotion of clinical benefits of cochlear implantation. In case of the combination of cell transplantation and cochlear implants, the capacity of regenerated neurons for projection to the central auditory systems is critical. Another is the projection to the peripheral targets including inner hair cells and cochlear implant electrodes in the scala tympani (Fig. 29.1). In case of selective loss of spiral ganglion neurons, which is an extremely rare situation, the projection of regenerated neurons to inner hair cells followed by synapse formation is crucial. The projection of regenerated neurons to electrodes of the cochlear implant might contribute to the reduction of required electrical stimuli from electrodes (Fig. 29.1).

29.2 Projection to the Central

The projection of regenerated neurons to the cochlear nucleus is an inevitable term of conditions (Fig. 29.1). However, there have been limited publications showing the central projection of transplant-derived neurons located in the cochlea [1, 2]. The potential of stem cell-derived neurons for projection to the cochlear nucleus has been demonstrated using brain stem slice cultures [3]. One of the major obstacles is limited knowledge of molecular mechanisms for central projections of spiral ganglion neurons. In the development of spiral ganglion neurons, after delamination of immature neurons from the otocyst, immature neurons form the central projection to the cochlear nucleus [4]. The potential of netrin-1 for axon guidance in spiral ganglion neurons has been reported [5, 6]. The netrin-1 receptor, deleted in colorectal carcinoma (DCC), is expressed in spiral ganglion neurons [5, 6]. Investigations using ES cell-derived neurons showed the projection of ES cell-derived neurons that had settled in the cochlear modiolus to the cochlear nucleus [1, 2]. Netrin-1 also mediated axon guidance in ES cell-derived neurons [7]. Therefore, netrin-DCC signaling might contribute to the central projection of transplanted ES cell-derived neurons in the cochlea. On the other hand, loss of inputs from spiral ganglion neurons induced an increase of insulin-like growth factor-1 (IGF-1) in the cochlear nucleus leading to the reorganization of synaptic contacts [8, 9]. In addition, upregulation of netrin-1 by exogenous IGF-1 application has been reported damaged cochleae [10]. Hence, these molecules could contribute to form synaptic contacts between the cochlear nucleus neurons and regenerated spiral ganglion neurons. Further investigations, especially for axon guidance in developing spiral ganglion neurons, are crucial to realize highly functional regeneration of spiral ganglion neurons.

29.3 Projection to the Peripheral

Comparing with the central projection of spiral ganglion neurons, mechanisms for peripheral projection are well understood [11]. Neurotrophins play key roles for the guidance of afferent dendrites of spiral ganglion neurons. Among neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are crucial for the guidance of afferent dendrites [12–16]. These neurotrophins are secreted by inner ear hair cells. Previous reports have demonstrated neurite projections from ES cell-derived neurons to cochlear or vestibular hair cells [17–19]. In addition, the formation of ribbon synapses between inner hair cells and ES-derived neurons has been demonstrated [17]. Gene transfer of BDNF into the cells located in the scala tympani resulted in neurite elongation of spiral ganglion neurons into the scala tympani [20]. This technique may be utilized for the induction of neurites from spiral ganglion neurons or transplanted neurons to the electrodes of the cochlear

implant. Recently, the efficacy of several small molecules to stimulate BDNF receptors has been reported in models for Huntington's disease [21, 22]. These small molecules might be utilized for the induction of neurite outgrowth from transplanted neurons.

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Part VI
Stem Cells

Chapter 30
Inner Ear Stem Cells

Mirei Taniguchi and Norio Yamamoto

Abstract Mammalian inner ear has limited regenerative ability, and functional recovery does not occur after damage. However, recent studies indicated that the cells within the inner ear have the characteristics of stem cells, namely, capacity for self-renewal and pluripotency. Since the specific markers for inner ear stem cells have not been found, several methods have been used to detect inner ear stem cells, including sphere-forming assay, fluorescence-activated cell sorting (FACS), side population study, and analysis of slow-cycling cells or Wnt signaling in the inner ear. The potential candidates of cochlear stem cells are the supporting cells, the cells at lesser epithelial ridge (LER), the cells at greater epithelial ridge (GER), and the tympanic border cells. The number of stem cells in the inner ear is estimated to be very low and is reported to decrease dramatically with maturation. It is necessary to elucidate the regulatory mechanisms of inner ear stem cells, clarify the reasons behind the quiescence of inner ear stem cells, and identify the causative factors that influence the decrease in the number of inner ear stem cells with maturation, in order to facilitate future regeneration therapy.

Keywords Inner ear • Regeneration • Stem/progenitor cells

30.1 Introduction

Stem cells are defined by their capacity for self-renewal, the ability to give rise to new stem cells, and pluripotency, which is the ability to differentiate into more than one type of cells. Although mammalian inner ear has limited ability to regenerate, several groups have reported the existence of stem cells within the mammalian inner ear.

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Since specific markers for inner ear stem cells have not been found, several methods have been used to detect inner ear stem/progenitor cells so far. The major approach involves the in vitro generation of cell-derived floating spheres. Using this technique, mammalian inner ears were reported to contain cells that had the ability to proliferate clonally and to differentiate into several types of cells, including hair cells in vitro [1–4].

Fluorescence-activated cell sorting (FACS) was also used to isolate specific type of cells and analyze their stemness potential [5–10].

The location of inner ear stem cells is very important in order to analyze their environmental regulatory mechanism. However, dissociation distorts the micro-anatomy of the inner ear, making it difficult to determine the precise location of stem cells. To overcome this disadvantage of dissociation-based study, an in vivo study was performed using the slow-cycling nature of stem cells [11].

Several groups analyzed the cells expressing Wnt target genes and identified stem/progenitor cells in the cochleae by using both in vivo and in vitro models [8–10].

In this section, we would like to review these studies reporting stem/progenitor cells within the mammalian inner ear.

30.2 Inner Ear Stem Cells

It has been reported that damaged mammalian postnatal vestibular organs generate new hair cells (see Part IV "Hair Cell Regeneration"). Li et al. [1] first demonstrated that cells from an adult mouse utricle sensory epithelium contained cells that displayed characteristic features of stem cells. These cells grew clonally and formed floating spheres in vitro. In addition, they had the capacity for self-renewal, and they gave rise to immature spheres that expressed various genetic markers of immature developing inner ears, including nestin, Pax2, bone morphogenetic protein (BMP)-4, and BMP-7. Sphere-derived cells differentiated into cells of all three layers, ectodermal, endodermal, and mesodermal linage, which is the main characteristics of stem cells. Using the spheres, cells were generated with hair-cell phenotype, positive for myosin7a, with espin- and F-actin-positive stereociliary bundle-like structure.

Although regeneration was reported to occur in vestibules, the mammalian organ of Corti in the inner ear does not regenerate in vivo after damage. Therefore, it is noteworthy that White et al. [5] showed that mammalian cochlear supporting cells contained cells that had the ability to transdifferentiate into hair cells. They purified neonatal cochlear supporting cells by fluorescence-activated cell sorting (FACS) from transgenic mice expressing green fluorescent protein (GFP) under the control of the p27kip1 promoter. p27kip1 protein is a cyclin-dependent kinase inhibitor that functions as an inhibitor of cell cycle progression. It is first expressed in the primordial organ of Corti and is downregulated during subsequent hair-cell differentiation, but it persists at high levels in differentiated supporting cells of the mature organ of Corti [12].

The GFP expression was observed in different supporting cells, including interphalangeal cells, pillar cells, Deiters' cells, and Hensen's cells. They cultured purified p27kip1-GFP-positive supporting cells with periotic mesenchymal cells and found that they retained the ability to divide and transdifferentiate into myosin 6-positive hair cells in vitro. This suggested that mammalian cochlear supporting cells possessed at least progenitor capabilities. They reported that, among the different types of supporting cells, pillar and Hensen's cells were found to have greater potential to form hair cells by using p75NGFR+ as their marker in FACS purification.

Zhang et al. [3] isolated greater epithelial ridge (GER) cells from neonatal rat cochleae enzymatically and mechanically. GER cells formed proliferative spheres, and they had the ability to generate myosin7a-positive hair cells and p27kip1-positive supporting cells. Therefore, they considered GER cells as targets for regenerative therapy of the inner ear.

Oshima et al. [4] isolated sphere-forming stem cells from early postnatal mouse inner ear and analyzed their gene expression and efficiency for sphere formation. They showed that spheres from the organ of Corti and vestibular sensory epithelial cells expressed multiple hair-cell markers including myosin7a and espin, and expressed functional ion channels, reminiscent of nascent hair cells. They reported that the capacity for sphere formation of cells in the mouse cochleae decreased sharply during the second and third postnatal weeks, which was much faster than that of the vestibular organs.

Savary et al. [6] performed side population (SP) analysis, using Abcg2/Bcrp1 as one of the markers for stem/progenitor cells. The SP phenotype has been used to isolate putative stem cell populations. It is based on the unique ability to efflux Hoechst dye in an ATP-binding cassette (ABC) transporter-dependent manner [13, 14]. Abcg2/Bcrp1 is a member of the ATP-biding cassette family of cell-surface transporter proteins, and it is considered to be one of the stem cell markers [15]. Savary et al. reported that Abcg2 transporter was expressed in supporting cells with other stem/progenitor cell markers, nestin and musashi1, in the postnatal mouse cochleae. They purified SP supporting cells by FACS and found that the SP cells differentiated into the colony expressing myosin7a or p27kip1 in vitro. Therefore, they concluded that supporting cells should be regarded as hair-cell progenitors.

Sinkkonen et al. [7] used antibodies to cell-surface proteins to label dissociated cells of the neonatal organ of Corti. They purified different cell types by FACS analysis and found that CD326+/CD146low/CD271low cells in lesser epithelial ridge (LER) and supporting cells gave rise to more myosin7a-positive cells in vitro than in the other non-sensory epithelial cells. Cells at LER in rat cochlea were also reported to have the ability to form spheres and differentiate into myosin7a-positive cells by Zhai et al. [2].

Taniguchi et al. [11] identified slow-cycling cells, one of the characteristics of stem cells, in the mouse cochleae in vivo. Stem cells normally proliferate at a slow rate in mature organs [16]. The so-called label-retaining cells, or slow-cycling cells, of the skin and prostate have been recognized as stem cells [17, 18]. They used the

exogenous proliferation marker 5-bromo-2′-deoxyuridine (BrdU) in combination with the endogenous proliferation marker Ki-67 and identified tympanic border cells, located beneath the basilar membrane, as slow-cycling cells of the mouse cochlea in vivo. Immunohistochemical analysis indicated that these cells stained positive for immature cell marker nestin. The number of slow-cycling cells in the tympanic border cells decreased dramatically in about two weeks after birth as the cochlea matured. This decrease coincides with other reports on inner ear stem cells [4].

Wnt signaling plays a critical role in regulating tissue homeostasis, including the maintenance of somatic stem cells [19]. Several groups have been studying Wnt target genes in the inner ear to detect inner ear stem/progenitor cells.

Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) is one of the Wnt target genes. Chai et al. [8] and Shi et al. [9] reported that they purified Lgr5+ supporting cells from postnatal mouse cochlea by FACS and that they formed spheres and differentiated into myosin7a-positive hair cells in vitro.

Jan et al. [10] reported that they found transient but robust Wnt signaling and proliferation in tympanic border cells during the first 3 postnatal weeks. They used the Axin2lacZ reporter mouse, as Axin2 is a downstream target and feedback inhibitor of Wnt pathway, whose active signaling marked endogenous stem cells in many tissues [20]. In vivo lineage tracing showed that a subset of hair cells and supporting cells was derived postnatally from Axin2-expressing tympanic border cells. In vitro, Axin2lacZ cells formed clonal colonies and differentiated into hair-cell-like (myosin7a positive) and supporting cell-like (e.g., sox2 positive) cells. They concluded that Axin2-positive tympanic border cells had the potential to act as precursors of sensory epithelial cells.

30.3 Conclusion

In summary, although the inner ear has limited ability to regenerate, there is evidence indicating the existence of inner ear stem cells. Potential candidates of stem cells are supporting cells [2, 4, 5, 7–9], cells at LER [2, 4, 7], cells at GER [3], and tympanic border cells [10, 11] (Fig. 30.1). The total number of them in the inner ear is estimated to be very small and reported to decrease as the inner ear matures [4].

The microenvironment surrounding the stem cells is called as niche [21]. It has a significant role in the maintenance of stem cells and enables them to keep proliferate and differentiate into mature cells. However, the niche for inner ear stem cells is unknown. It is necessary to elucidate the regulatory mechanisms of inner ear stem cells, clarify what keeps the inner ear stem cells in quiescent condition, and identify the reason for the decrease in the number of inner ear stem cells with maturation. If these questions are addressed to activate inner ear stem cells in vivo, it will be a great step in regeneration therapy.

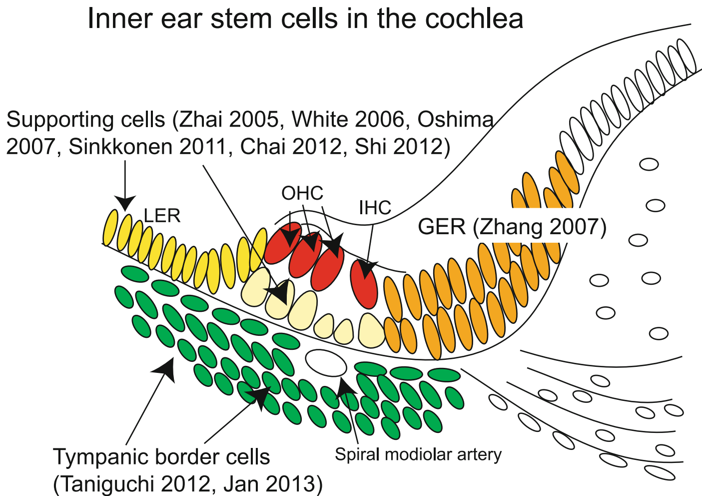

Fig. 30.1 Inner ear stem cells. Potential candidates of stem cells are supporting cells [2, 4, 5, 7–9], cells at LER [2, 4, 7], cells at GER [3], and tympanic border cells [10, 11]. *IHC* inner hair cells, *OHC* outer hair cells, *LER* lesser epithelial ridge, *GER* greater epithelial ridge

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Chapter 31
Pluripotent Stem Cells

Tatsunori Sakamoto, Koji Nishimura, Hiroe Ohnishi, and Takehiro Iki

Abstract Embryonic stem cells (ESCs) are pluripotent stem cells that are derived from early embryos. Induced pluripotent stem cells (iPSCs) are somatic cell-derived reprogrammed cells that have equivalent characteristics to ESCs. Disease-specific iPSCs have started to be used for the investigation of and development of the treatment for several diseases. These cells have been intensively used for the regeneration of inner ear. In this chapter, we summarize the background of ESCs and iPSCs, introduce recent advances in disease-specific iPSCs, and discuss the difference between ESCs and iPSCs in terms of clinical application. Then, the major achievement for the induction of inner ear sensory cells and the transplantation of ESC- and iPSC-derived neural precursor cells are overviewed.

Keywords Disease-specific iPSCs • Embryonic stem cells • Induced pluripotent stem cells • Neural induction • Otic induction • Pluripotent stem cells • Transplantation

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31.1 Introduction

One possible strategy for the inner ear regeneration is the use of stem cells. Pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are attractive tools for it. ESCs and iPSCs are cultured cells that have two unique characteristics: self-renewal and pluripotency. In other words, it is possible for us to maintain and induce differentiation of these cells in vitro into all types of cells of the body. This technology is pushing forward the research for the developmental biology, understanding pathophysiology of diseases, and development of the treatment and/or drugs and is applied to regenerative medicine.

In the research field of inner ear regeneration, pluripotent stem cells have been studied to replace two targets, hair cells and spiral ganglion neurons (SGNs), both of which do not regenerate in the mammalian cochlea. Degeneration of either hair cells or SGNs causes sensorineural hearing loss, one of the most prevalent chronic disabilities all over the world.

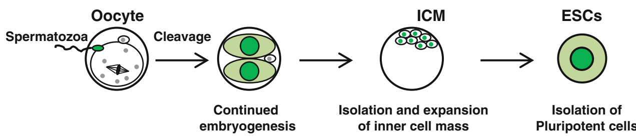
In this chapter, we would like to explain the background and the use of pluripotent stem cells in the inner ear regeneration.

31.2 Pluripotent Stem Cells

31.2.1 Embryonic Stem Cells (ESCs)

A fertilized egg of the vertebrates cleaves several times to produce 16 or 32 cells. A morula is an embryo at this embryonic stage. The cells in the morula, then, start to aggregate and become arranged into a cystic form (blastocyst). A clump of cells in the blastocyst, or inner cell mass, will form the whole body. ESCs are cultured cells derived from this inner cell mass (Fig. 31.1). The first establishment of ESCs was shown in mice [2, 3], followed by many other species, rat [4], hamster [5], rabbit [6], pig [7], calf [8], medaka [9], and so on. The first primate ESCs were established from rhesus monkey [10], and the first human ESCs were established in 1998 [11].

These unique cells are used in many research aspects. One big achievement in the ESC study is the generation of the genetically modified animals. The genome of ESC can be modified by random mutagenesis, homologous recombination, or gene insertion (transgene). When ESCs are introduced into another blastocyst, the resulting animal, which is called chimera, consists of two kinds of cells, one derived from the original inner cell mass and another from the exogenous ESCs. If the ESC contributes to the germ cells, this animal is called germ-line chimera, and this phenomenon is called germ-line transmission [12]. All the cells in the animal that is derived from ESC germ cells have the same genome as the ESC. By this strategy, ESCs are widely used for the generation of knock-out/knock-in animals, chimeras, and clones. Nowadays, these genetically modified animals are indispensable tools for biological research and animal industry.



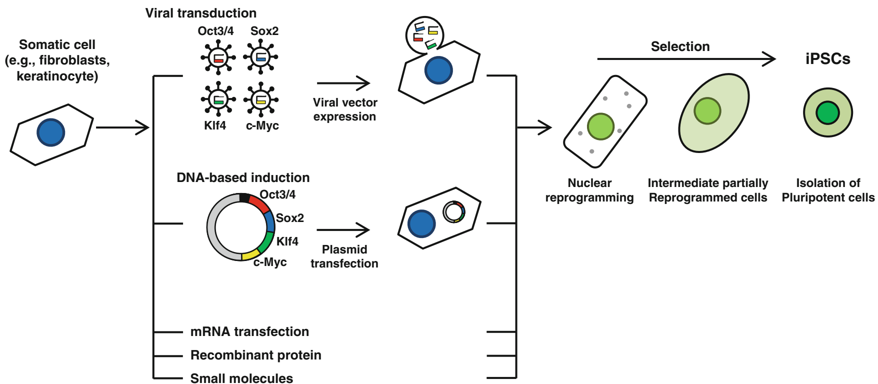


Fig. 31.1 Establishment of pluripotent stem cells. (**a**) ES cells are derived from inner cell mass. (**b**) iPS cells are derived from somatic cells, using viral transfection, plasmid transfection, mRNA transfection, recombinant protein introduction, and small-molecule introduction, followed by selection. (Modified from Narsinh et al. [1])

Another achievement of ESC-related research is the investigation on the "stemness": essential characteristics of a stem cell that distinguishes it from differentiated cells. Several factors that are related to the stemness have been identified through the study of ESCs [13]. Among them, *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*, which are predominantly expressed in ESCs, were identified as the sufficient set of factors to reprogram somatic cells into ESC-like character, named iPSCs [14].

The most challenging part of ESC study is the understanding mechanisms of differentiation and controlling it. ESCs derived from the inner cell mass are a good in vitro model for the study of cellular differentiation in early embryogenesis. Extending this study, ESCs were induced to differentiate into a wide variety of cell types including hepatocytes, pancreatic islet cells, blood cells, chondrocytes, cardiomyocytes, dopaminergic neurons, Schwann cells, retinal pigmented epithelium, and so on, and expected to be applied in clinics [15].

31.2.2 Induced Pluripotent Stem Cells (iPSCs)

iPSCs are somatic cell-derived stem cells similar to ESCs in terms of self-renewal and pluripotency. iPSCs were first established by Takahashi and Yamanaka in 2006 [14] (Fig. 31.1). They introduced *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* using retrovirus

vector into mouse embryonic fibroblasts and selected using the *Fbx15* reporter system, which is specifically expressed in mouse ESCs and early embryos, but is dispensable for the maintenance of pluripotency and mouse development. Resulting cells were similar to mouse ESCs in many points such as an expression of stem cell-specific genes and surface glycoproteins, chromatin methylation patterns, and differentiation potency into three germ layers in vivo and in vitro. However, these cells were not able to contribute to adult chimeras. To improve the quality as pluripotent cells, fully reprogrammed cells were selected by the expression of Nanog, which is required for maintaining pluripotency, and germ-line chimeras were obtained as a result [16]. In the next step, human iPSCs were established by using the same set [17] or another set of transcription factors (*OCT3/4*, *SOX2*, *NANOG*, *LIN28*) [18]. These human iPSCs also fulfilled the same criteria as mouse iPSCs and ESCs, except for confirmation of chimera formation and germ-line transmission, which is obviously ethically unacceptable.

After the generation of iPSCs from fibroblasts [17], iPSCs have been generated from various types of cells to demonstrate a proof of principle that all somatic cells can be reprogrammed into pluripotency: murine liver and stomach cells [19], pancreatic β-cells [20], terminally differentiated lymphocytes [21], cord blood cells [22], third molar [23], keratinocytes of plucked hair [24], and neural stem cells (NS cells) [25, 26]. iPSCs were generated also from cochlear epithelial cell-derived otospheres of neonatal mice [27]. Although otospheres resemble multipotent NS cells in their overall gene expression pattern and epigenetic status [28], the reprogramming efficacy of NS cells by four factors was approximately 10 times higher than that of otospheres [25, 27]. Differences in demethylated patterns of NS cell-specific Sox2 enhancers may partly cause the difference in reprogramming efficacy.

More recently, it was reported that less factors are required to reprogram somatic cells into pluripotency. iPSCs were generated from mouse and human fibroblasts without *Myc* [29] and from keratinocyte and mesenchymal stem cells by three factors: *OCT3/4*, *SOX2*, and *KLF4* except *c-MYC* [30, 31]. Additionally, iPSCs were generated from human NS cells by introduction of *OCT3/4* and *KLF4*, or only *OCT3/4*, suggesting that endogenous expression of the reprogramming factors can complement exogenous factors [32].

31.2.3 Disease-Specific iPSCs

The genetic mutation in a genome of a patient is taken over to iPSCs, when somatic cells of the patient are reprogrammed to iPSCs. These cells, so-called disease-specific iPSCs, provide opportunities to develop novel strategies to elucidate the pathological mechanisms of disease, together with physiological mechanisms by comparing with wild-type cells. Differentiated cells from disease-specific iPSCs into the target or precursor cells serve as in vitro disease models, that is, recapitulation of the disease phenotype at cellular level, and are used to reveal molecular

mechanisms of the disease. These differentiated cells are also useful to develop new treatments. Soon after the establishment of human iPSCs [17], mutant iPSCs were successfully generated from several diseases including amyotrophic lateral sclerosis (ALS) [33], muscular dystrophy, Parkinson disease, and Huntington disease [34], followed by the research for cell survival and function of spinal muscular atrophy patient iPSC-derived motor neurons [35]. Since then, disease-specific iPSCs have already been generated to establish disease models, from neurological disorders [36–46], cardiovascular diseases [47–51], metabolic abnormalities [36, 52, 53], and hematological insufficiency [54–56].

The major advantage of the use of disease-specific iPSCs owes much to the ability to generate a specific tissue/cell type from the cells obtained from another tissue. For example, the pathogenetic mechanism in Alzheimer's disease (AD) comes from chronic accumulation of abnormal amyloid-β peptide (Aβ) oligomers in the brain tissue. However, brain biopsy for the diagnosis or research is not easy, and, before the time of diagnosis of AD, neural tissue has been extensively modified due to the chronic accumulation of Aβ. By using several lines of iPSCs derived from AD patient's skin fibroblasts (AD-iPSC), Kondo et al. [42] induced AD-iPSC-derived neurons. They successfully detected stress responses as an early cellular phenotype and showed that docosahexaenoic acid (DHA) treatment alleviated the stress responses in the AD-iPSC-derived neurons from a subset of patients. This not only clarified the early mechanism of the disease, but also may help select patients who are responsive to DHA treatment. iPSCs derived from skin fibroblasts of dilated cardiomyopathy (DCM) patients [50] also provided a good opportunity to perform cellular study, because cardiac tissues from DCM patients are difficult to obtain and do not survive in long-term culture.

Disease- or patient-specific iPSCs are used for drug screenings in vitro to identify candidate chemical compounds for the therapeutic purpose [39, 40, 42, 50, 57] or to test toxicity of drugs [48, 51]. For example, a histone acetyltransferase inhibitor called anacardic acid was identified that rescued the phenotype of abnormal motor neuron differentiated from ALS-iPSCs [39].

Mutations in mitochondrial DNA (mtDNA) are implicated in numerous human diseases. Artificial modification of mtDNA would be one possible strategy to treat diseases due to mutations in mtDNA. However, the modification of mtDNA is difficult because mtDNA is encapsulated in the mitochondrial outer and inner membrane, and multiple copies of mtDNA exist in each cytoplasm. Mitochondria in each somatic cell are maintained during the process of reprogramming for the generation of iPSCs. Thus, resulting iPSCs that carry the mutant mtDNA serve as an in vitro model for these diseases. Disease-specific iPSCs have been established from those patients including mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [58, 59] and Pearson marrow-pancreas syndrome [60].

As genomic DNA of iPSCs (and ESCs) has an accessible chromatin state, genomic modification is relatively easier compared to other somatic cells. Thus, recent precision gene modification techniques such as the helper-dependent adenoviral vector [61], zinc-finger nucleases (ZFN) [62], transcription activator-like

effector nucleases (TALEN) [63], and CRISPR-Cas9 system [64, 65] are suitable to be applied to modify a genome of pluripotent stem cells. For example, ZFN was used to introduce XIST (the intrinsic X chromosome-inactivating gene) into a trisomic chromosome 21 in Down's syndrome patient-derived iPSCs, followed by successful trisomy silencing [66]. These techniques will provide a new therapeutic concept of those intractable diseases in the future.

As described above, disease-specific iPSCs are remarkable powerful tools and resources for both research and therapy. The inner ear tissues of a living human are difficult to obtain because the inner ear is encapsulated in the hard bone, and the puncture to that capsule for biopsy results in the serious deterioration of the inner ear function. Furthermore, the amount of the inner ear tissue obtained from the biopsy would be limited and it would be difficult to maintain the obtained tissues in a long-term culture. Disease-specific iPSC technologies will be powerful tools to establish inner ear disease models for the investigation of the pathogenic mechanisms, drug discovery, and invention of new treatment strategies. A wide variety of diseases related to inner ear dysfunction of monogenic, chromosomal, or mitochondrial etiology will be the target of entities that are modeled by patient-specific iPSCs.

31.2.4 "Can iPSCs Replace ESCs?"

There are several advantages of iPSCs over ESCs for research and future clinical applications [1]. The first point is the ethics. The generation of ESCs is ethically problematic because it usually accompanies with the destruction of embryos, which are continuous to the living individual. Some researchers justify the generation of human ESCs because they are generated from the fertilized eggs that are destined to be discarded after in vitro fertilization. For those who do not accept this justification, the generation of ESCs from blastomeres [67] or from preimplanted morula [67, 68] might be acceptable because it does not involve the activity of destroying the whole embryos. However, there is no consensus on propriety of intervening embryos to generate ESCs. On the other hand, iPSCs do not raise this ethical problem because they are derived from somatic cells. Secondly, disease-specific iPSCs can be easily obtained from patients as mentioned above. Theoretically, ESCs generated by nuclear transfer (ntESCs) [69, 70] enable the generation of pluripotent stem cells that code genomic information of transferred nucleus. However, it is costly to prepare for every ntESC, where the nucleus originates from each patient. Thirdly, the premise that iPSCs and their progeny would not cause any immune response in the host due to self-tolerance was supported by two reports that syngeneic iPS-derived cells did not elicit immune responses [71, 72]. But there is an opposing report that teratomas from syngeneic murine iPSCs were immunorejected by a host, whereas those from mouse ESCs were immunotolerant [73]. Kaneko and Yamanaka rebutted this report maintaining that immunorejection of syngeneic iPSCs may have been caused by gene integration [74].

However, human ESC research is still important because there is a serious concern about the stem cell-related malignancy. The generation of iPSCs originally accompanied with gene integration. Such genomic modification is a risk factor for insertional mutagenesis, which may cause malignant cell transformation [75]. To reduce this risk, non-integrating gene delivery methods for the expression of defined pluripotent factors have been reported (Fig. 31.1): iPSCs generated with adenoviruses [76], sendai viruses [77], plasmid vectors [78], removable transposon systems [79], direct delivery of recombinant reprogramming proteins [80], and synthetic modified messenger RNA [81]. The use of a proto-oncogene c-Myc is also a concern, which now can be replaced with L-Myc [82] or eliminated [29]. Genetic defects in iPSCs result in not only tumorigenesis but also differentiation defectiveness. Koyanagi-Aoi et al. [83] identified low-quality iPSC lines out of a relatively large number of human iPSC and ESC lines. iPSC lines of low quality were marked by higher expression levels of several genes expressed from long terminal repeats of specific human endogenous retroviruses and molecular signatures such as RNA expression and DNA methylation patterns. Those iPSCs were prone to form teratoma when neurally induced and transplanted into mouse brains. Thus, human iPSCs have considerable variation in terms of complete differentiation and tumorigenesis.

In terms of quality control, ESCs are still superior, because clinical grade human ESC lines have already been established [84]. Therefore, the use of iPSCs in the clinic will require much more research and understanding, and human ESCs will provide an accurate reference for iPSCs, which will allow them to be standardized for their use in clinical applications.

31.3 The Use of Pluripotent Stem Cells for the Regeneration of the Inner Ear

Since Ito et al. first demonstrated the regeneration of the auditory pathway by transplants of fetal brain tissue [85], several stem cell-based approaches to cure deafness using mesenchymal stem cells, neural stem cells, ESCs, and iPSCs have been adopted. Current major targets are cochlear hair cells and SGNs, which are primarily compromised and unregenerable cells in sensorineural hearing loss.

31.3.1 Specific Induction to the Inner Ear Sensory Fate

Inner ear hair cells of the mammals have very little capacity of regeneration [86, 87]. This is one major reason for the difficulty in recovering from permanent profound hearing loss. One suggested modality for the recovery from the hearing impairment is the regeneration of hair cells. In vitro differentiation of inner ear hair

cells from pluripotent stem cells is regarded as an important milestone to realize stem cell-based rescue of inner ear hair cells.

Developmental biology of inner ear has given clues for stepwise differentiation of pluripotent stem cells toward the inner ear sensory fate. Currently, there is no universal method to induce auditory and vestibular sensory cells from pluripotent stem cells at high conversion rates. As many researchers struggled for the in vitro induction of inner ear hair cells from stem cells [88], Li et al. first reported the induction of hair cell-like cells from mouse ESCs [89] (Table 31.1). Mouse ESC-derived embryoid bodies were cultured with EGF and IGF-1 on adhesive culture dishes and then supplemented with bFGF to obtain otic progenitor cells. These cells were further differentiated and became positive for hair cell markers and possessed hair bundle-like F-actin staining at the apical surface.

Oshima et al. improved the stepwise method and tested electrophysiological property of the induced cells [90]. Embryoid bodies from mouse ESCs or iPSCs were cultured with Dkk1 (Wnt inhibitor), SIS3 (selective inhibitor of Smad3 that interferes TGF-β signaling), and IGF-1 to induce rostral ectodermal linage, followed by the treatment with bFGF on adhesive culture dishes to enrich otic population using Pax2 as an otic marker. These cells were cocultured with chicken utricle stromal cells to further differentiate into hair cell-like cells, with the expression of multiple hair cell markers including Atoh1 and Myosin7a, and stereocilia- and kinocilia-like structures. These hair cell-like cells responded to mechanical stimulation with currents that were reminiscent of immature hair cells.

Chen et al. showed that human ESCs also have the capacity to differentiate into hair cell fate [91]. Human ESCs were plated as monolayer and supplemented with FGF3 and FGF10, which are synergistically required to induce otic placode fate in mice. Epithelial cell-like colonies were manually picked up and further differentiated in the media supplemented with all-*trans*-retinoic acid and EGF. Resulting culture included hair cell marker-positive cells with immature stereocilia-like structures.

Koehler et al. further broke down the early developmental steps and applied to mouse ESCs utilizing serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) system [92]. Mouse ESC aggregates were treated with BMP4 and TGF-β inhibitor (SB-431542), and then their outer epithelium showed definitive ectoderm markers (AP2 and Ecad). Next, BMP-/SB-treated aggregates were exposed to BMP inhibitor (LDN-193189) and FGF2, and then thickened patches were formed in the outer epithelium, mimicking the otic placode in terms of morphology and marker expression (Pax8 and Ecad). After further differentiation culture, otic vesicle-like structures are formed in the BMP-/SB-FGF-/LND-treated aggregates. Wnt inhibitor from days 8 to 10 significantly reduced the number of vesicles, indicating the role of endogenous Wnt for the formation of otic vesicles. By day 16, the vesicles were lined with hair cell-like Myo7a(+) Sox2(+) cells with F-actin-rich stereocilia-like structures. Hair cell-like cell layer was tightly arranged with Sox2(+) supporting cell-like cells. The rapid uptake of FM1-43 dye and the diversity of voltage-dependent currents

Table 31.1 Methods for differentiation of ESCs/iPSCs into inner ear hair cells

Authors	Cell source	Method	Specific factors and/or conditions	Methods of testing hair cell identity/quality
Li et al. [89]	Mouse ES cell	EB formation, followed by adherent culture	Serum-free conditions EGF, IGF-1, bFGF	Hair cell marker expression
Oshima et al. [90]	Mouse ES cell Mouse iPS cell	EB formation, followed by adherent culture and coculture with stromal cells	Dkk1, SIS3, IGF-1 bFGF Chicken utricle stromal cells	Hair cell marker expression Morphology by SEM Electrophysiological analysis
Chen et al. [91]	Human ES cell	Adherent culture	FGF3, FGF10 Retinoic acid, EGF	Hair cell marker expression Morphology by SEM Electrophysiological analysis
Koehler et al. [92]	Mouse ES cells	Cell aggregate formation by SFEB method, followed by sequential exposure to differentiation factors	BMP4, TGF-β inhibitor BMP inhibitor, bFGF	Hair cell marker expression Morphology by TEM Electrophysiological analysis FM1-43FX uptake
Ouji et al. [93]	Mouse ES cells	EB formation, followed by culture in conditioned medium of a stromal cell line cells	Conditioned medium by a stromal cell line cells	Hair cell marker expression Morphology by SEM FM1-43FX uptake
Ouji et al. [94]	Mouse ES cells	EB formation, followed by forced expression of Math1 gene	Forced expression of Math1 gene	Hair cell marker expression Morphology by SEM FM1-43FX uptake

EB embryoid body, *SEM* scanning electron microscopy, *TEM* transmission electron microscope

demonstrated that induced hair cells were functional. From the expression of calbindin 2, these hair cells seemed to be equivalent to vestibular type II hair cells.

Approaches above are stepwise differentiation mimicking the embryonic inductive signals. Another approach is the direct induction. Ouji et al. [93] used a conditioned medium by a stromal cell line (ST2) to culture mouse ESC-derived embryoid body, and up to 20 % of outgrowth from the embryoid body expressed several hair cell markers, and its induction rate has been improved by Atoh1 overexpression [94].

As introduced here, hair cell induction from pluripotent stem cells has gradually become a reliable method. This method will directly be applied to patient-specific iPSCs to elucidate the mechanism of inner ear diseases or for the drug screening. However, hurdles still exist before the realization of hair cell regeneration. It is still necessary to improve the induction rate and to develop the method to specifically induce cochlear hair cells. More fundamental difficulty is the in vivo application of induced hair cells. It is not realistic to transplant mature hair cells and to arrange them beautifully in the organ of Corti [95]. Other approaches would be necessary. Introduction of inner ear inducers shown above in a time- and concentration-controlled manner to initiate differentiation of inner ear stem cells, introduction of stem cells which are programmed to differentiate to inner ear sensory fates, and introduction of stem cell-based factor-releasing cells may also be other possible strategies.

31.3.2 Neural Induction and Transplantation

SGNs are also an important target of the inner ear regeneration. For patients who do not have residual hearing, a cochlear implant (CI) has been the only solution to restore their hearing. As CIs function through direct stimulation of SGNs, restoration of hearing by CIs depends on the remaining SGNs. However, only a small fraction of remaining SGNs is required for CIs to perform [96]. The minimum required number of SGNs for CIs to effectively function is estimated as few as 3,500 [96, 97], whereas the number of SGNs in normal adult human is about 35,000 [98]. Therefore, the regeneration of even a small number of SGNs in a damaged ear has a significant clinical impact in relation to cochlear implants. Compared to the hair cells, the regeneration of SGNs seems realistic, because the transplantation of neural precursors derived from mouse [99] and human [91] ESCs has shown to improve the auditory brain stem responses.

In earlier trials, undifferentiated ESCs were transplanted with or without co-transplanted neural tissue [100–103], showing survival in the cochlea, partial differentiation into ectodermal/neural fate, and migration in the inner ear.

To be more specific to neural tissue regeneration, neural induction methods were adopted including simple monolayer culture [104, 105], stromal cell-derived inducing activity (SDIA) method (coculture with PA6 cells, a stromal cell line derived from skull bone marrow) [99, 106–109], or embryoid body formation followed by

specific growth factors [91]. Okano et al. transplanted SDIA-induced neural progenitor cells derived from mouse ESCs into the modiolus of normal and deafened guinea pigs and showed the functional recovery by the improvement of ABR threshold [99]. Nishimura et al. first reported the transplantation of iPSC-derived neural progenitors into mouse cochlea, using SDIA as a neural-inducing method [108, 110]. Of note, a subset of transplants expressed vesicular glutamate transporter 1 (vGluT1), which marks glutamatergic neurons in the SGNs. Chen et al. showed functional recovery by the transplantation of human ESC-derived neural progenitor [91]. Human ESC-derived embryoid bodies were exposed to FGF3 and FGF10 and then replated and cultured on gelatin-coated dishes in the presence of bFGF and Sonic Hedgehog, followed by the supplementation of neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) to induce otic neural progenitors (ONPs). These ONPs were transplanted into the modiolus of auditory neuropathy model gerbils and gained partial improvement of ABRs.

The formation of synaptic connection is important, although it is unclear if transplanted neural progenitor contributed to the reconstruction of the neural connection between inner ear and brain stem. Synaptic connection between ESC-derived neural progenitors and inner ear hair cells was shown in vitro [111, 112] and in vivo [91]. However, the central extension of neurites and the synaptic connection with the cochlear nucleus in the brain stem have not been adequately shown.

To realize better auditory functional recovery by the cell-based strategy, the induction of neurons that more closely resemble SGNs may help. Reyes et al. transiently overexpressed neurogenin 1 in mouse ESCs followed by supplementation with BDNF and GDNF and effectively induced glutamatergic neurons in vitro and in vivo [113]. Purcell et al. also used neurogenin 1 overexpression and BDNF and showed that mouse ESCs are specifically induced to KCNQ4-positive SGN-like cells [114]. Lee et al. reported that the combination of several small-molecule inhibitors (a BMP-mediated SMAD inhibitor, an activin-mediated SMAD inhibitor, a GSK3 inhibitor, and a gamma-secretase inhibitor) with retinoic acid induced human iPSCs into neural progenitors [115]. These cells were further treated with NT-3, BDNF, NGF, GDNF, ascorbic acid, and dibutyryl cAMP, and a significant number of cells showed sensory neuron characteristics such as the expression of peripherin and Brn3a and susceptibility to varicella-zoster virus infection. This method may be modified to generate auditory sensory neurons.

31.4 The Next Step for the Stem Cell-Based Regeneration of Inner Ear

Inner ear hair cells are now generated in vitro from pluripotent stem cells, and the transplantation of neural progenitors derived from pluripotent stem cells improved hearing threshold in deafened animals, although both require further development

for the clinical application. In order to use pluripotent stem cells in the clinics, other hurdles still exist. It is not realistic to establish pluripotent cell line from each patient. iPSC bank project has just started to handle this difficulty. To minimize the variation and enable assurance/validation as clinical grade product, the process of the production needs to comply with the good manufacturing practice (GMP) standard. These activities have just started.

In addition, stria vascularis and spiral ligament, which are required for the maintenance of inner ear homeostasis, may also be a target of regenerative medicine of the inner ear, but not much has been studied for their regeneration as well as their development compared with hair cell and SGN counterparts.

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Chapter 32
Somatic Stem Cells

Takayuki Nakagawa

Abstract A number of somatic stem cells have been used in inner ear research. In this chapter, two stem cells, mesenchymal and hematopoietic stem cells, are the focus, because these stem cells have already utilized in clinical settings. Of mesenchymal stem cells, the potential of bone marrow- and adipose tissue-derived stem cells for the treatment of the inner ear is reviewed. As for hematopoietic stem cells, their contribution to the maintenance of inner ear cell circumstances is introduced.

Keywords Adipose tissue-derived stem cells • Bone marrow-derived stem cell • Hematopoietic stem cell • Immune suppression • Macrophage • Spiral ganglion neuron

32.1 Introduction

The discovery of somatic stem cells led to a revolution of therapeutic strategies and a proposal of novel therapies. Among a variety of somatic stem cells, transplantation of mesenchymal and hematopoietic stem cells is widely utilized in a variety of fields. Hematopoietic stem cell transplantation is included in the standard protocol for the treatment of bone marrow malignancies. The autologous transplantation of mesenchymal stem cells is increasingly employed for the treatment of various diseases. These two widely used stem cells are the focus of this chapter, and their contribution to inner ear biology will be introduced.

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32.2 Mesenchymal Stem Cells

32.2.1 Bone Marrow-Derived Stem Cells

Bone marrow stromal cells or bone marrow-derived stem cells (BMSCs) have been investigated in a variety of fields of regenerative medicine. Firstly, the potential of naïve BMSCs has been investigated in the field of the inner ear. Naito and his colleagues examined the fate of autologous BMSCs after transplantation into chinchilla cochleae [1]. Autologous BMSCs showed robust survival in the cochlea, and a few of them differentiated into neurons. In addition, transplanted BMSCs migrated into various portions of the cochlea (Fig. 32.1). Transplantation of mouse BMSCs into the cochlea also showed similar findings [2]. Interestingly, transplanted BMSCs massively migrated into the spiral ligament, of which characteristic was utilized to regenerate specific fibrocytes in the spiral ligament [3]. These findings suggest the potential use of BMSCs themselves for the treatment of sensorineural hearing loss due to damage on the spiral ligament-specific fibrocytes. The problem is how to diagnose sensorineural hearing loss that is

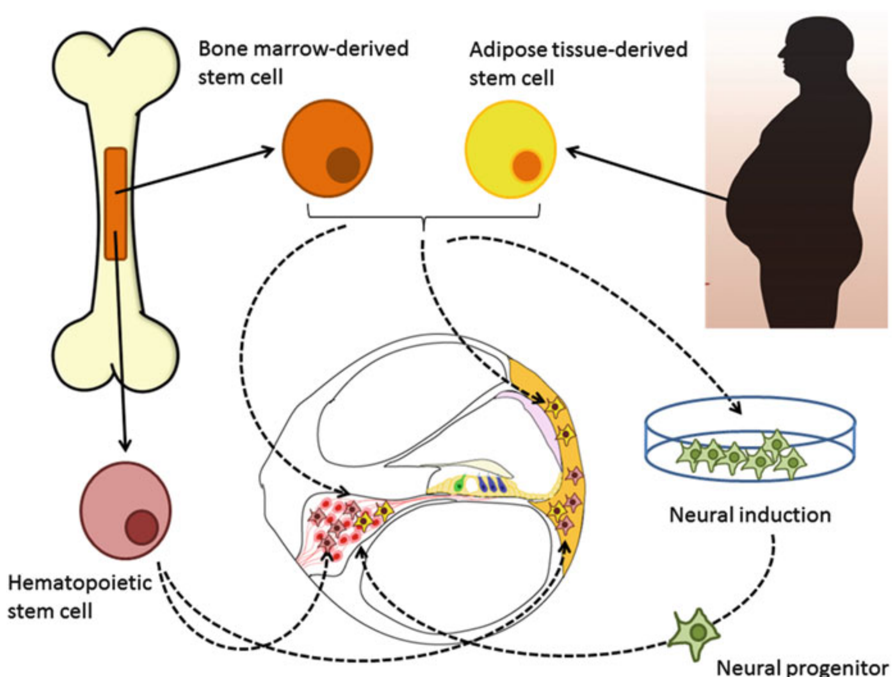

Fig. 32.1 Bone marrow- and adipose tissue-derived stem cells and hematopoietic stem cells in the cochlea. From bone marrow and adipose tissue, stromal or stem cells are available. They have a potential for forming in the stromal tissues in the cochlea. They can be a source of neural progenitors and express a marker for macrophages

susceptible for the treatment. At present we have no diagnostic tool to determine degeneration of the spiral ligament in patients. Recently, the use of the optical coherence tomography for imaging cochlear tissues in living animals has been reported [4]. However, the quality of imaging is not satisfactory to determine histological damage in the spiral ligament.

BMSCs have gained particular attentions as a source of neuronal cells for transplantation (Fig. 32.1). Several methods for neural induction of BMSCs have been established [5–8]. Ogita and his colleague transplanted neurospheres derived from BMSCs into damaged cochleae and demonstrated the survival of BMSC-derived neurons in cochleae [9]. Kondo and her colleague established an induction method of BMSCs into sensory neuron phenotypes using Wnt1 [8]. They transplanted BMSCs into the cochlea followed by local application of neural induction medium and Wnt1, resulting in the survival of BMSC-derived neurons in the cochlea [8]. However, the hearing restoration by transplantation of BMSCs or BMSC-derived neural precursors has not been demonstrated unlike ES cell-derived neural cells [8, 9]. The capacity for neurite outgrowth and synaptogenesis could be key elements for functional restoration. The ability of BMSCs for differentiation into inner ear hair cells has also been reported [10, 11]. However, the efficient induction required Atho1 gene transfer. In 2010, the presence of multilineage-differentiating stress enduring (Muse) cell was discovered [12, 13]. Muse cells can be purified by FACS sorting with SSEA-3 and CD105 from mesenchymal stem cells including BMSCs. However, there has been no report on the potential of Muse cells for differentiation into inner ear lineages and for a source of transplants for the treatment of inner ears.

BMSCs have unique effects on modulation of immune responses [14–16], which is the focus of intensive investigations. BMSCs altered cell surface marker expression in T cells and decreased the expression of cytokines including interferon gamma and TNF-alpha. Several clinical trials, including large-scale placebo-controlled phase III clinical trials, are currently underway evaluating the therapeutic potential of BMSCs for the treatment of catastrophic inflammatory diseases, including steroid-refractory graft-versus-host disease, multiple sclerosis, and Crohn's disease. Such a characteristic of BMSCs can be utilized for the promotion of transplanted cell survival. For successful cell therapy, the survival of sufficient numbers of transplants is included in key elements. Therefore, BMSCs may be used for suppression of immune response after cell transplantation into the inner ear.

32.2.2 Adipose Tissue-Derived Stem Cells

The adipose tissue is also a source of mesenchymal stem cells. In general, adipose tissue-derived stem or stromal cells (ADSCs) have the similar potential including neural differentiation and immune suppression to bone marrow-derived stem cells [17–19]. However, there are few publications on ADSCs for the treatment of the inner ear.

ADSCs have the capacity for secreting chemokine and growth factors similarly to BMSCs [18, 19]. Therefore, paracrine effects of ADSCs for the protection of hair cells were examined. ADSCs secreted several growth factors including hepatocyte growth factor (HGF) and insulin-like growth factor 1 [20], which are known to have protective effects on hair cells. Coculture of mouse cochlear explants with mouse ADSCs showed significant protection of cochlear hair cells against an aminoglycoside [20]. Interestingly, coculture with ADSCs showed superior protective effects to supplementation of HGF alone [20]. Therefore, combinations of several growth factors may have additive effects. At present, limited numbers of growth factors are available in clinical setting. From this point of view, ADSC transplantation can be an alternative for growth factor treatment.

As for immune modulation by ADSCs, therapeutic effects of ADSCs on a model with autoimmune hearing loss were investigated [21]. Immune suppression by ADSCs attenuated hearing impairment in this model [21].

32.3 Hematopoietic Stem Cells

Hematopoietic stem cell (HSC) transplantation was the first example of a successful stem cell therapy and is widely utilized for treating various diseases [22]. HSCs also consist of a heterogenous population of multipotent stem cells that collectively possess the potential to differentiate all blood cell types. HSCs can be purified from bone marrow cells by cell surface markers including Sca1 and c-Kit. HSC transplantation is commonly used in therapy for blood- or bone marrow-related malignancies.

In the field of inner ear biology, the presence of HSC-derived cells in the inner ear and their possible roles have been investigated (Fig. 32.1). Lang and colleague have demonstrated the presence of HSC-derived cells in the adult cochlea by analysis of murine HSC chimeras [23]. HSC-derived cells were most abundant in the spiral ligament. HSC-derived cells were also found in other locations normally occupied with fibrocytes and mesenchymal cells in the inner ear. Sato and colleague have demonstrated that the majority of HSC-derived cells in the cochlea are macrophages and that noise exposure induces accumulation of HSC-derived macrophages in the cochlea [24]. Okano and colleague have revealed that HSC-derived macrophages gradually turn over for several months during steady-state replacement by HSC-derived cells [25]. Not only in the cochlea but also in the vestibular peripherals HSC-derived cells are present, especially in the endolymphatic sac [26]. No spontaneous differentiation of HSCs into the hair cells, supporting cells, and neurons was identified in these studies. Therefore, HSCs are not candidates of a source for cell therapy, but play an important role in the immune systems of the inner ear.

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Part VII
Future Perspective

Chapter 33
Regenerative Medicine for the Inner Ear: Summary

Juichi Ito

Abstract There are more than 400,000 deaf or highly hearing-impaired people in Japan. In fact, one out of 1,000 newborns is deaf. Therefore, recovering the hearing ability of the deaf has always been one of the most important priorities in the field of otolaryngology.

Sensorineural hearing loss (SNHL) is very difficult to restore, especially in cases with severe hearing disturbance. The possible therapeutic strategies for treatment of SNHL are summarized below. In early-phase cochlear damage, it is crucial to rescue auditory cells from cell death and promote self-repair and cell activity. Induction of transdifferentiation (e.g., induction of transdifferentiation from supporting cells to cochlear hair cells) is the next useful strategy, or an alternative approach is to induce proliferation. Several challenging studies manipulating cell cycle regulators to regenerate inner ear hair cells have previously been attempted. However, if no cell sources remain in the inner ear, cell transplantation then becomes the only choice to restore cell growth through regeneration. Using bionic materials is another remaining possible alternative approach. Worthy of mention is a device (microimplant)—without battery and extracorporeal equipment—that has recently been developed.

Keywords Artificial auditory epithelium • Drug delivery system • Sensorineural hearing loss • Stem cell • Transdifferentiation • Transplantation

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33.1 Strategy 1: Rescue from Cell Death and Induction of Self-Repair

For the treatment of inner ear diseases, a novel drug delivery system (DDS) has been developed. The major problem in developing therapeutic strategies for inner ear disease treatment is the difficulty of effectively delivering drug into the target site (i.e., inner ear). Neurotrophic factors or some other chemicals have been proved to protect inner ear hair cells and spiral ganglion neurons (SGNs) from ototoxic drugs and aging [1, 2]. However, only a few viable ways to apply drugs into the inner ear DDS have been successfully developed. There have been some reports employing the use of osmotic mini-pumps; however, this invasive approach requires middle and inner ear surgeries. Although the use of viral vectors for drug delivery is also an effective approach, the risk of virus toxicity remains a major issue in clinical use.

The purpose of this study is to establish a novel inner ear drug delivery system (DDS). In this study, a biodegradable hydrogel derived from pig skin type I collagen is used as a carrier of therapeutic drugs. Gelatin polymers are electrostatically complexed with drugs to form gelatin polymers that release drugs. Clinical significance includes the following factors: (1) It is safe and is currently being used clinically; (2) the release-rate of drug is adjustable; and (3) no extracorporeal devices are needed. A currently useful drug employed is insulin-like growth factor-1 (IGF-1).

Basic experiments have to be first performed before proceeding to clinical challenge. The experimental aim was to examine possible protective effects on inner ear hair cells (HCs) and SGNs against ototoxic treatments using biodegradable hydrogel with IGF-1. Noise stimuli-induced deafness rats and guinea pigs were tested. After exposure to loud noise, IGF-1-soaked hydrogel was applied on the round window membrane (RWM) of the cochleae. In the control group, physiologic saline-soaked vehicle gel (in place of IGF-1) was placed on the RWM. Histological and functional evaluations as well as functional assessments of the electrically evoked auditory brainstem response (eABR) were performed. The eABR thresholds of IGF-1-treated group were significantly lower than those of controls. One month after IGF-1 treatment, histological evaluation was performed. In the control group, many outer hair cells (OHCs) of the cochlea were damaged after noise exposure. On the contrary, OHCs in the IGF-1 gel group remained intact. Based on the other preclinical results, clinical trials were subsequently performed. This is the world's first cell growth factor application on the inner ear. Table 33.1 shows the protocol for phase I–II clinical study [3, 4].

After tympanostomy, the exact RWM location was confirmed using a fine 0.7-mm-diameter endoscopic fiberscope before applying IGF-1-immersed hydrogel on the RWM. Of the 25 patients recruited, the hearing ability of 15 patients (60 %) improved significantly. A clinical trial demonstrating the successful development of a novel DDS strategy for local drug delivery to the cochlea using biodegradable hydrogel is summarized (Table 33.2).

Table 33.1 Protocol for phase I–II clinical study

Subjects: unilateral acute profound hearing loss; systemic steroid treatment application is ineffective within 1 month after deafness onset
Single application (5 mg) of IGF-1 containing hydrogel on the RWM
Observation period: 2 months
Evaluation: pure tone audiometry and OAE

IGF-1 insulin-like growth factor-1, *RWM* round window membrane, *QAE* otoacoustic emission

Table 33.2 Summary of a novel DDS strategy to the cochlea

Phase I–II clinical trials of local IGF-1 treatment using gelatin hydrogels for acute SNHL resistant to systemic glucocorticoid application
Recovery of hearing ability was established in 50 % of patients
No significant adverse drug effects were observed

SNHL sensorineural hearing loss

Table 33.3 Results of a novel delivery drug system (DDS) using lidocaine-loaded polylactic/glycolic acid (PLGA) microparticles

Lidocaine-loaded PLGA microparticles offered sustained delivery of lidocaine to the cochlea
Mild sensitivity reduction of the auditory system
No vestibular dysfunctions observed
No severe inflammations in the middle ear mucosa

Another subject of interest was the attenuation of tinnitus. Systemic or intratympanic application of lidocaine has been known to be effective against tinnitus, albeit the effect was short lasting and there was the need of close risk monitoring of adverse drug effects. The aim of this study was to develop a novel DDS for sustained local delivery of lidocaine into the cochlea. Therefore, the use of lidocaine-loaded polylactic/glycolic acid (PLGA) microparticles for the sustained delivery of lidocaine in vitro and in vivo was investigated. The results of a novel delivery drug system using lidocaine-loaded PLGA microparticles are summarized in Table 33.3. Investigations of the efficacy of lidocaine in animal tinnitus models are now under way.

33.2 Strategy 2: Regeneration via Transdifferentiation and Regeneration via Cell Proliferation

Induction of transdifferentiation, which is a possible inner ear HC regeneration strategy, has been reported by transdifferentiation of supporting cells (SCs) to HCs using Atho1-gene transfer with adenoviral vectors [5]. In our study, a

chemical-genetic approach—using small molecules for regulation of targeted proteins—was adopted. Notch inhibition by gamma-secretase inhibitor was investigated in an ex vivo system. The gamma-secretase inhibitor generated numerous HCs in E17.5 mice and ectopic HCs in P3 mice in an ex vivo system. These findings suggest that possible transdifferentiation from supporting cells to hair cells by chemical agents may be established.

Induction of cell proliferation is an alternative approach to regenerate HCs. Manipulation of cell cycle regulators has been used for HC regeneration. A cyclin-dependent kinase inhibitor, such as p27, has been reported to negatively regulate the cell cycle in SCs to regenerate into HCs. Therefore, this gene therapy may serve as a possible choice for hearing loss treatment.

33.3 Strategy 3: Regeneration via Cell Transplantation

If only a few cell sources remain in the inner ear, cell transplantation becomes a possible choice to restore cell growth through regeneration. This then serves as another strategy for the development of cell therapy for the treatment of inner ear disorders. We initiated our research using rodents and embryonic stem cells (ES cells) and then progressed to primates using autologous cell sources such as bone marrow stromal cells (BMSCs) and induced pluripotent stem cells (iPS cells). There are three major target sites for cell therapy of the inner ear: (i) the auditory epithelium (especially HCs and SCs); (ii) the spiral ganglion neurons (SGNs); and (iii) the cochlear lateral wall. Note that these are crucial and sensitive sites for auditory function and their deficits result in hearing impairment (Fig. 33.1).

The target site (i) is the auditory epithelium, especially HCs. Recent reports have demonstrated that regeneration of the components of the sensory organs (including the retina and inner ear) can be derived from pluripotent stem cells such as ES or iPS cells. Progenitors of retinal cells are induced by inhibition of the Wnt signal and Nodal. Inner ear progenitors are also induced by the inhibition of Wnt and TGF-beta signals. These progenitors can differentiate into mature sensory cells. From the progenitors to mature sensory cells, different induction strategies are used. Although both the retinal and inner ear mature sensory cells can be derived from ES or iPS cells, their efficiencies are quite different. Among the several types of mature retinal cells, the induction efficiency of the rod photoreceptor cells is 8–25 %; however, that of inner ear HC-like cells is only 0.03 %. The efficiency difference between the retinal and inner ear cells is almost in the range of two orders. Figure 33.2 shows the schemes of cell fate specification and specific markers of the cell fates in the development of the retina and inner ear. Retinal development involves all relevant specific markers in various steps and cell types. In contrast, only a few markers are involved in the inner ear development, especially at early-stage development.

Inner ear development and structures are complex. On embryonic day 13 (E13), all cells in the cochlea are morphologically identical. As the development

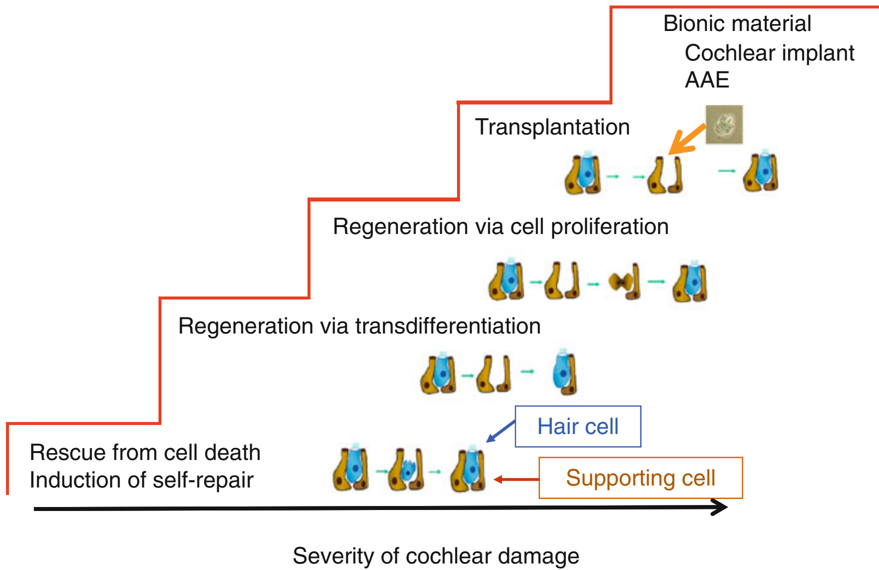

Fig. 33.1 Therapeutic strategies for sensorineural hearing loss

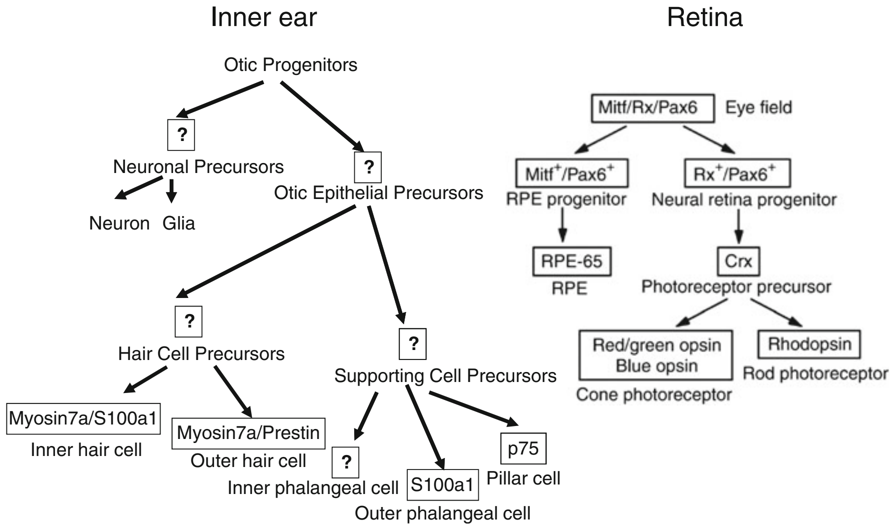

Fig. 33.2 The cell fate specification and specific markers of the cell fates in the development of the retina and the inner ear

progresses, many types of specific cells with respective characteristic morphologies, such as IHCs and OHCs, evolve. Even before E13, morphologically identical cells have different gene expression profiles. Due to these complex structures and various components, gene expression profiling of single cells is required to identify

the relevant important markers in each step of the inner ear development. A single cell is dissociated from the embryonic inner ear, and RNA from the cell is then extracted and amplified. Comprehensive analysis of the gene expression based on a microarray approach is performed. Collecting the data from many cells, it is possible to perform cell classification based on gene expression profiling to eventually identify the specific markers for each group of the inner ear.

The second target site (ii) is the SGNs, which transmits auditory stimuli from IHCs to the central nervous system. In addition, SGNs are essential to realize the clinical benefits via cochlear implantation, where the mechanism is to stimulate SGNs by the cochlear implant electrodes. SGNs are surrounded by bony walls, thus facilitating the placement of cell transplants on intended sites for treatment purposes. Moreover, neural progenitor cells for mimicking SGNs are comparatively easy to obtain.

In our study, the first ES cell-derived neural progenitors were used as donor cells for SGN regeneration. ES cells were generated into neural progenitor cells by the stromal cell-inducing activity (SDIA) method. Briefly, mouse ES cell-driven EGFP genes of the CAG promoter were cocultured with PA6 cells for 5–6 days before isolating the premature neural cells. ES cell-derived neural progenitors display high neural differentiation with prominent neuritis outgrowth. Using guinea pigs as the recipient, SGNs were destroyed by applying some ototoxic agent (ouabain) topically onto the cochlea. One week later, mouse ES cell-derived neural progenitors were transplanted into the cochlear modiolus. Auditory function was monitored by electrically stimulated auditory brain stem response (eABR) measurements. ES cell-derived neural progenitors were then directly injected into the cochlear modiolus. Control animals received injection of the culture medium alone. When histological analysis was performed 4 weeks after transplantation, transplanted ES cell-derived neurons were found abundantly in the cochlear modiolus. Most of the grafted cells demonstrated neurite extensions and were of the neuronal phenotype of beta III tubulin immune-positive cells. Functional restoration after cell transplantation was also evaluated. The difference in eABR thresholds was statistically significant between the transplanted and sham-operated animals at 2 and 4 weeks after transplantation. These data indicate that transplantation of ES cell-derived neurons contributes to functional restoration in the auditory nervous system.

Based on the favorable results in rodents, preclinical experiments using primates were performed. The cochleae of cynomolgus monkeys were locally treated with a topical single-bolus cisplatinadministration, which efficiently destroyed the spiral ganglion neurons (SGNs). One month after cisplatin application, monkey simian ES cell-derived neural progenitors were transplanted together with cochlear implantation. Control animals received the culture medium alone with cochlear implantation. The auditory function was evaluated by measuring the eABR at 1- or 2-month intervals using the cochlear implant electrode. Measurements of eABR demonstrated significant improvements of the auditory function via cell transplantation. Functional restoration of hearing ability was obtained by cell transplantation (Fig. 33.3).

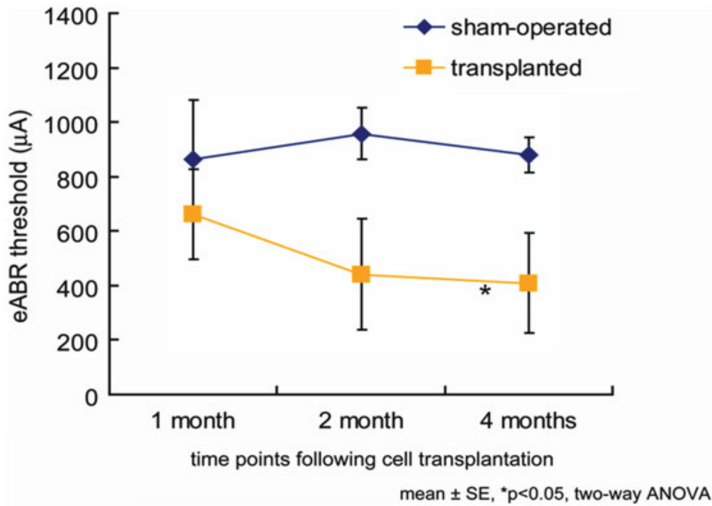

Fig. 33.3 ABR measurements following cell transplantation. Transplanted animals demonstrated gradual improvements in thresholds of eABR following cell transplantation

Once hearing ability had been restored, transplantation experiments using induced pluripotent stem cells (iPS cells) started. Although preferred—ES cells have pluripotency and self-renewal characteristics and differentiate into various cell types including neuron and hair cells—the use of ES cells poses unavoidable ethical problems for therapeutic application, i.e., destruction of fertilized ova.

Instead, the recent use of iPS cells has been focused. The iPS cells can be generated from any adult or embryonic cells using the skin, liver, stomach, and other tissues/organs. By the retroviral expression of 4 factors—Oct3/4, Sox2, Klf4, and cMyc—iPS cells mimic ES cell-like pluripotency and self-renewal [6]. Obviously, the problem of ethics is now negligible compared with ES cells. Furthermore, unique approaches such as the use of disease- or patient-specific iPS cells have already been adopted. It is of utmost importance and benefit that iPS cells can be differentiated and cultured into neural fates of interest. Then, iPS cell-derived neural progenitors were transplanted into the SGN region of deafened animals, which have previously yielded viable outcome similar to that using ES cell-derived neural progenitors. These results indicate a current trend that iPS cells can and will replace ES and other stem cells in the field of inner- ear regeneration.

The target site (iii) of cell transplantation in restoring hearing loss is the use of spiral ligament (SL) fibrocytes, which play an important role in K+ ion recycling and in maintaining the endocochlear potential. Several reports have demonstrated that genetic abnormality in SL fibrocytes causes profound hearing impairment. We have actually used bone marrow stromal cells (BMSCs) as the transplants for SL regeneration. BMSCs, available from the bone marrow, are easy to culture and exhibit multipotency. To investigate the potential of BMSCs for SL regeneration, a mouse SL degeneration model was established, which effectively degenerated type

Table 33.4 Results of cell transplantation in preclinical studies

ES cell-derived neural progenitors implanted in the cochlear modiolus and differentiated into neurons after transplantation
Transplantation of ES cell-derived neural progenitors to the cochlear modiolus improved auditory function
iPS can and will replace ES and other stem cells in the field off or inner ear regeneration
Transplanted BMSCs first migrated and then proliferated flourished in the spiral ligament after transplantation
BMSC transplantation improved endocochlear potential
ES cell transplantation contributes to the functional recovery of auditory structures

ES embryonic stem, *BMSCs* bone marrow stem cells

II and IV SL fibrocytes. As a result, the endocochlear potential is significantly reduced. After SL degeneration, BMSCs were transplanted into the inner ear via the lateral semicircular canal. Four weeks after transplantation, many transplanted cells were found proliferating in the cochlea (including the lateral wall region). Therefore, significant recovery of the endocochlear potential was next investigated. BMSC transplantation improved EP.

The results of cell transplantation studies are summarized in the Table 33.4.

33.4 Strategy 4: Using a Newly Invented Bionic Material

A new hearing device that takes the place of cochlear implantation has recently been developed. The aim of this project is to develop a novel therapeutic method for sensorineural hearing loss (SNHL) using a newly invented artificial sensory organ. This venture is named as the *HIBIKI* Project (Hibiki means sound or echo in Japanese). The development of a new and totally implantable device is accomplished by an incorporation of nanotechnology, cochlear micromechanics, and tissue engineering, together with regenerative medicine for the inner ear. The new device is named as artificial sensory epithelium (AAE) or Hibiki device, since this device imitates the functions of auditory sensory epithelia. The AAE implantation involves: (1) surgically exposing the bony wall of the scala tympani to insert into the cochlea and placing it on the surface of the basilar membrane; (2) when a sound reaches the cochlea, the basilar membrane vibrates; (3) thus vibrating the AAE placed on the basilar membrane; (4) AAE distortions caused by vibrations then generate electricity via piezoelectric effect (an effect which converts mechanical movements into electric signals) to stimulate the auditory nerve; and (5) acoustic input is next transmitted to the central nervous system (Fig. 33.4).

AAE, as its name implies, imitates the functions of the sensory epithelia. This means that the elements required for AAE transform vibratory movements into electric signals with frequency characteristics. To confirm that these required elements can be accomplished by AAE, a prototype of AAE was fabricated to

① Open the wall of scala tympani and place AAE on the basilar membrane

② The basilar membrane vibrates by the sound stimulation

③ AAE vibrates with the movement of the basilar membrane

④ AAE generates electricity from piezoelectric effect

⑤ The auditory nerve is stimulated by the electricity

⑥ Information of the sound is transmitted to the central nervous system (CNS)

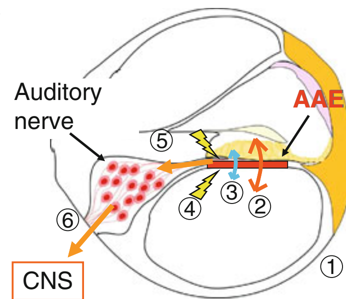

Fig. 33.4 The idea of how AAE works

verify the validity of the basic mechanisms. This prototype of AAE has a gradient in its width, which is also a characteristic of the basilar membrane. The output voltages of the piezoelectric device are considered to be enough to stimulating neurons. More details are written in Chap. 17 of this issue [7].

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