Review

Recent progresses in stem cell research and hearing restoration

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Serious hearing and balance impairments can occur as a result of loss of hair cells related to aging, environmental stresses (such as noises exposure) or exposure to chemotherapeutic drugs (such as cisplatin and aminoglycoside antibiotics). Because a large portion of hearing impairment involves loss of hair cells, regeneration or replacement of these cells is a possible alternative to prosthetic devices[1]. Researchers have paid hot attention to the study of inner ear hair cell regeneration for hearing restoration for many years. Besides the important function of hair cells, there are many other contributors essential for normal hearing. Hair cell regeneration is one of the essential steps for total hearing recovery, but not the only one. In this article, we will review the progresses in research on hearing loss, stem cell application and hearing restoration.

New foci on factors contributing to hearing loss

The mammalian cochlea is a highly structured organ consisting of a wide variety of cell types. Although hearing loss is mainly attributed to the loss of hair cells, hearing impairment also results from dysfunction of many different cochlear cell types. For example, mutation of the connexin 26 gene, a cytoplasmic gap junction protein located in a variety of cochlear supporting cells[2], also result in hearing loss. Experimental data have showed that damage caused by noise exposure affects multiple cell types including those in the stria vascularis and supporting cells in the organ of Corti, in addition to hair cells [3].

Mammalian cochlear fibrocytes in non-sensory mesenchymal regions play important roles in cochlear physiology, including transportation of potassium ions to generate an endocochlear potential in the endolymph that is essential for the transduction of sound by hair cells[4]. It has been postulated that a potassium recycling pathway toward the stria vascularis via fibrocytes in the cochlear lateral wall is critical for proper hearing, although the exact mechanism has not been definitively determined [5, 6].

Fibrocytes within the cochlear lateral wall are divided into Types to V based on their structural features, immunostaining patterns, and general locations[7]. Types , and fibrocytes resorb potassium ions from the surrounding perilymph and from outer sulcus cells via the Na, K-ATPase. The potassium ions are then transported to type fibrocytes, strial basal cells, and intermediate cells through gap junctions and are secreted into the intrastrial space through potassium channels. The secreted potassium ions are incorporated into marginal cells by the Na, K-ATPase and the Na-K-Cl co-transporter, and are finally secreted into the endolymph through potassium channels [4, 7].

Degeneration and alteration of the cochlear fibrocytes have been reported to cause hearing loss without any other changes in the cochlea in the Pit-Oct-Unc (POU) -domain transcription factor Brain-4 (Brn-4) -deficient mouse[8] and the otospiralin-deficient mouse [6]. Brn-4 is the gene responsible for human DFN3, an X chromosome

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linked nonsyndromic hearing loss. Mice deficient in Brn–4 exhibit reduced endocochlear potential and hearing loss and show severe ultrastructural alterations. In humans, mutations in the connexin 26 (Cx26) and connexin 30 (Cx30) genes, which are expressed in cochlear fibrocytes and nonsensory epithelial cells, are well known to be responsible for hereditary sensorineural deafn–ess[9, 10]. These instances of deafness related to genetic, structural and functional alterations in the cochlear fibrocytes highlight the functional importance of these fibrocytes in maintaining normal hearing.

Degeneration of spiral ganglion neurons and cochlear nucleus neurons happens secondary to the loss of cochlear sensory epithelium. Moreover, the antero–ventral cochlear nucleus of mice shows a progressive loss of tissue volume (about 15 – 20%) and the number of neurons(about 25 – 35%) and a small decrease in the neuronal size [11, 12].

Therefore, studies of the cells of the organ of Corti besides hair cells, such as the supporting cells and fibrocytes, spiral ganglion cells and cochlear nucleus neurons are equivalently important for hearing restoration.

Stem cell properties of vertebrate inner ear cells

In fish, amphibians and birds, regeneration of sensory hair cells through asymmetric cell divisions of supporting cells can contribute to recovery of hearing and balance after hair cell loss caused by trauma or toxicity [13]. There is limited evidence for the presence of neurosensory stem cells in the mammalian ear that seem to be able to proliferate only under certain circumstances in vitro [14,15]. In mammals, the vestibular epithelia show restricted spontaneous hair cell regeneration [16]. Stem cells are found to reside in vestibular sensory epithelia of adult mice which can form new hair cells both in vivo and in vitro, revealing the route of vestibular hair cell regeneration in mammalian animals [15].

In a recent review [17], Duan et al pointed out that evidence has been presented for hair cell regeneration in cochlear explants from newborn mice and several groups have observed a moderate degree of spontaneous recovery after ototoxic damage to the inner ear in patients. Thus, there are grounds to believe that hearing disorders in mammals may, under certain circumstances, be successfully treated[17].

Isolation of cochlear stem cells can be challenging. Zhai et al[18] have presented an experimental technique that allows isolation and culture of hair cell progenitors from postnatal rat cochleae. These progenitor cells are isolated from the lesser epithelial ridge (LER, or outer spiral sulcus) area of pre–plated neonatal rat cochlear segments. They express the same markers as LER cells in vivo, including ZO1, Islet1, Hes1, and Hes5. When these cells are induced to express Hath1, they show the potential to differentiate into hair cell–like cells. These findings provide additional supporting evidence for the notion that LER cells are hair cell progenitors. This technique can be a useful tool in studying gene expression profiling and mechanisms of mammalian cochlear hair cell differentiation/regeneration[18]. Wang[19] also has showed that the newborn mouse cochleae contain sphere–form cells that have the capability to proliferate in culture and differentiate into cells that express hair cell markers. When treated with epidermal growth factors or basic fibroblast growth factors, the number of sphere shaped cells increase. The sphere cells express genes that are indicative of inner ear progenitor cells, including Otx2, BMP4, BMP7, and Islet1. During further differentiation, some cells grow hair bundle–like structures with hair cell markers myosin VIIA and espin. These observations indicate that newborn mouse cochlea contains highly proliferative progenitor cells that have features similar to normally developing inner ear progenitor cells [19]. This implies the possibility of using these sphere–form cells for reconstructing damaged cochlear hair cells [18, 19].

The difference between vestibular and auditory epithelia in mammals in terms of their stem cell properties is not well understood. Batts et al speculate that evolution of the auditory organ, which is younger than vestibular peripheral

sensory organs, involves extreme steps in differentiation of supporting cells that are incompatible with spontaneous regeneration [20].

Transdifferentiation and hair cell regeneration

Transdifferentiation usually refers to less dramatic transformations between closely related cell types which may occur with or without a step of cell division or proliferation. Direct transdifferentiation without cell division is often called "conversion"[20,22].

One outstanding example of successful trauma repair based on spontaneous transdifferentiation is found in the basilar papilla of birds. Mammalian cochlear hair cells, once lost, however, cannot be spontaneously replaced [20]. Studies in birds have shown that in response to the lesion, supporting cells divide and the daughter cells become new hair cells[22, 23]. In some cases, the transdifferentiation from the supporting cell to the hair cell phenotype appears to take place without mitosis [24]. Temporary de–differentiation is crucial for regeneration in the avian auditory sensory epithelium, yet this process is not well understood at the molecular regulatory level [20]. In many cases, transdifferentiation has been induced by inserting genes into target cells. Developmental genes, such as Atoh1, can be harnessed for inducing transdifferentiation in the mature mammalian sensory epithelium [20].

The number of hair cells and their proper organization is critical for normal hearing. As such, transdifferentiation without addition of new cells is unlikely to restore normal hearing. Izumikawa et al have speculated that secondary proliferation may occur once transdifferentiation is induced in supporting cells[25]. Evidence for the ability of supporting cells to divide in late developmental stages has recently been presented [26]. For more complete regeneration of the organ of Corti, it may be necessary to combine transdifferentiation therapy with treatment for inducing cell proliferation [20].

Experiments on transdifferentiation have been performed in vivo and in vitro in a variety of systems. In some cases, stem cells can also be induced to transdifferentiate and produce a different set of cell types than would normally develop. One example of such stem cell plasticity is seen with mesenchymal stem cells that can differentiate into cells that are not typical mesenchymal derivatives[27]. Interestingly, transdifferentiation is often partial or incomplete, leading to mixed phenotype (hybrid) cells [28].

Applications of Neural Stem Cells for cell replacement

Data suggest that the neurosensory precursors of the vertebrate inner ear may be composed of three populations:(1)neuronal precursors that form only neurons, (2) neurosensory precursors that form only hair cells or supporting cells, and (3) precursors that form both neurons and hair cells [29]. The existence of a population that can generate both hair cells and neurons from a single line of clonally related cells has therapeutic potential: it would allow for the transformation of neuronal stem cells that give rise to both neurons and hair cells out of the same stem cell [29].

Several murine and human stem cell lines have proven to be useful tools for cellular replacement experiments involving the brain and spinal cord. Moreover, these clonal neural stem cells (cNSCs) express several markers that are expressed in cochlear tissues, such as connexin 26 and the hair cell marker myosin VIIa [30, 31].

Parker[3] injected cNSCs into the scala tympani of sound–damaged mice and guinea pigs and allowed the animals to recover for up to six weeks. In both animal models, some of the cNSCs appeared to have migrated throughout the cochlea and demonstrated morphological, protein and genetic characteristics of neural cochlear tissue (e.g. spiral ganglion neurons, satellite cells and Schwann cells) and cells of the organ of Corti (pillar cells, supporting cells and hair cells). The different expression profiles exhibited by the cNSCs indicate that the cochlear microenviro– nment exerts an influence over cNSC develo– pment. It is hypothesized that once the cells of this neural stem cell line migrate into the cochlea, they receive signals from the microenvironment and the new balance of up and down regulation of the genetic cell fate programs expressed by local endogenous cells will

take place. These results support the developmental potential of stem cells. They also suggest that the mature mammalian cochlea retains the signals necessary to influence stem cell differentiation along a cochlear phen– otype, even though it cannot regenerate these cells from its own endogenous population [3].

Another source of neural–crest–derived stem cells was recently identified in sensory hair roots[32]. It is likely that these cells are related to the neural–crest–derived Merkel cells[33], a population of cells that express two genes essential for hair cell development, Pou4f3 and Atoh1 [34, 35]. These cells seem to retain their gene expression profile while proliferating. If so, these cells may readily differentiate into hair cells if implanted into ears; this appears to be possible with other stem–cell–derived precursors that are equally characterized by Atoh1 and Pou4f3 expression [36].

In another study [12], grafted neural precursor cells (NPCs) were implanted along the cochlear nucleus in the adult host followed by an augmented acoustic stimulation. NPCs were obtained from the olfactory bulbs at embryonic day 14 – 16 and were transplanted into the inside border of cochlear nucleus. The labeled cells survived at least 2 weeks, verified by Hoechst 33342 fluorescence, and by immunostaining for a neuronal marker. In some cases NPCs had migrated directionally to the root of the auditory nerve. This observation demonstrates the survival and migration of NPCs from the olfactory bulb (OB) along the adult auditory nerve in an augmented acoustic environment following implantation [12].

Therefore, NSC seems to be an appropriate tool for cellular replacement of hearing restoration.

Tracing and identification of transplanted stem cells

To trace transplanted cells, in a study by Parker, the injected male cNSCs (XY) were identified in female (XX) guinea pig hosts by FISH labeling of the Y chromosome [3]. Although, they were unable to completely rule out the possibility of cell fusion in these experiments, they never saw multiple sex chromosomes (i.e. XXXY rather than XX or XY) when labeling sections with both X and Y specific probes. The results indicate that FISH can be used for long–term detection of the Y chromosome in histological sections. One of the advantages of using FISH to detect xeno–transplanted cells is that it may also be used to detect cell fusion [37, 38].

Eliciting the in situ accumulation and persistence patterns of stem cells following transplantation would provide critical insight toward human translation of stem cell–based therapies. To this end, Politi [39] et al have developed a strategy to track neural stem/precursor cells in vivo using magnetic resonance (MR) imaging. They have found the optimal labeling with Resovist and carried out in vivo experiments to monitor the accumulation of Resovist–labeled NPCs following intravenous injection in mice with experimental autoimmune encephalomyelitis. With a human MR scanner, they were able to visualize transplanted cells as early as 24 hours posttransplantation in up to 80% of the brain demyelinating lesions. Interestingly, continued monitoring of transplanted mice indicated that labeled NPCs were still present 20 days post–injection. These studies provide evidence that clinical–grade human MR can be used for noninvasive monitoring and quantification of NPC accumulation in the central nervous system upon systemic cell injection.

Similar methods have been introduced to label and identify the transplanted stem cells [40, 41]. Cells are labeled with both SPIO and the fluorescent marker1, 1'–didodecyl–3, 3, 3', 3'–tetramethylindocarbocyanine perchlorate (DiI). DiI is a lipo–philic dye that labels cells by means of lateral diffusion through the plasma membrane, with an absorbance wavelength of 480 nm and emission at 565 nm. Labeled cells are identified with both fluorescence microscopy and Prussian blue staining. In vivo identification of transplanted cells is satisfied by the enhancement effects of SPIO particles under MR scanning [40, 42].

Factors that may regulate stem cell differentiate into hair cell

1. miRNAs

Some miRNAs have recently been shown to be se-

lectively expressed in hair cells [41] and may be important in consolidating cell cycle exit and maintaining differentiation of neurosensory aspects of the ear, but such functions require ear–specific conditional mutations of enzymes necessary for miRNA processing [43].

2. Retinoblastoma protein (pRb)

By profiling gene expression in developing mouse vestibular organs, Sage[13] et al have identified pRb as a candidate regulator of cell cycle exit in hair cells. Differentiated and functional mouse hair cells with a targeted deletion of Rb1 undergo mitosis, divide, and cycle, yet continue to become highly differentiated and functional. Moreover, acute loss of Rb1 in postnatal hair cells causes cell cycle reentry. Manipulation of the pRb pathway may ultimately lead to mammalian hair cell regeneration. These findings may have implications for regenerating other functional cells through manipulation of negative cell growth control genes [13].

Regeneration of spiral ganglion cells and cochlear nerve

The loss of spiral ganglion neurons (SGNs) is one of the major causes of profound sensorineural hearing loss[44]. It is considered that the number of functional spiral ganglion cells is of primary importance for the hearing capability in humans [45] and that a minimum number of SGN is required to achieve a high level of perception in cochlear implant patients [46].

Regeneration of the auditory nerve remains a challenge in restoring hearing [47]. Replacement of degenerated peripheral or central neurons by stem cell – derived neurons requires differentiation of the stem cell to the appropriate phenotype and directed growth of projections to reestablish functional neural circuits[48]. Regeneration of hair cells in patients with sensorineural hearing loss would also require an intact afferent innervation for function[25]. Indeed, in vitro experiments show that spiral ganglion neurons, transplanted into the organ of Corti after degeneration of afferent neurons, form connections with hair cells and express synaptic markers [49].

Allografts and xenografts have shown promise in the treatment of a variety of neurological diseases. Fetal dorsal root ganglion (DRG) neurons can extend functional connections in the rat spinal cord. Embryonic stem cells (ES cells) and adult neural stem cells (ANSC) have the potential to differentiate into neurons [50].

An interesting approach would be to use a cell replacement therapy with the potential to establish connections from the inner ear to the central auditory system. This hypothesis has been tested by xenografted (mouse to rat) implantation of embryonic dorsal root ganglion (DRG) neurons and ES cells along the auditory nerve in the adult host. In a study by Hu et al [47], DRG neurons were obtained at embryonic day 13 – 14 in transgenic animals expressing enhanced green fluorescence protein (EGFP). DRG neurons and ES cells survived for up to 9 weeks following transplantation along the auditory nerve of adult mammalians. The DRG neurons were found to migrate from the first cochlear turn to the internal meatus while ES cells were observed to migrate further centrally to the brain stem and close to the ventral cochlear nucleus. The results demonstrate not only the survival and migration of xenografted DRG neurons and stem cells along the adult auditory nerve but also the feasibility of a cell replacement therapy in the degenerated auditory system [47]. Hu [51] et al has also illustrated that ANSCs transduced with neurogenin 2 (ngn2) before transplantation can survive and differ enti– ate in the injured inner ear. In a similar exper– iment, Regala[50] et al have found that the vestibul –ocochlear nerve in adult rats and guinea pigs supports xenotransplants of embryonic DRG, ES cells and ANSC. The ability of xenografts to cross the transitional zone between the PNS and CNS is a necessary step for the formation of a functional neuronal conduit from the inner ear to the cochlear nucleus.

Matsuoka et al [44] tried to evaluate the survival of mouse MSCs transplanted into normal and oua-

bain–treated gerbil cochleae and to determine the migratory patterns of MSCs with two differing injection methods. The animals were deafened by ouabain, which damaged SGNs while leaving hair cell systems intact. They found that undifferentiated MSCs were able to survive in the modiolus both in the control and the ouabain–treated cochleae. The average number of profiles found in the modiolus was greater in the ouabain–treated cochleae than in the control cochleae. This difference was statistically significant. With the scala tympani injection, there were no profiles found in the modiolus either in the control or ouabain–treated cochleae, indicating that donor MSCs may need to be directly injected into the modiolus to replace injured SGNs. Finally, there was no evidence of hyperacute rejection in any of the gerbils despite the use of xenotransplantation.

Co–culture models provide a method to study differentiation under controlled conditions, with the advantage of being able to replicate some tissue–derived signaling[52]. Co–culture models have previously been used to successfully direct the differentiation of stem or precursor cells into neurons. Cochlear tissue for co–culture was isolated from post–natal day five Sprague–Dawley rat pups. Pre–differentiated mouse ESCs were added to auditory neuron cultures. Their results demonstrate the potential use of co–cultures, in particular hair cell explants, at directing the differentiation of ESCs into bipolar neuron–like cells. This may be the first report describing the differentiation of ESCs toward an auditory neuron lineage using in vitro models. Hair cell explant/embryoid body co–cultures hold promise for the identification of factors promoting auditory neuron differentiation in vitro. The maintenance of a healthy population of auditory neurons is important for cochlear implant function and future hair cell regeneration studies, both of which rely on critical numbers of surviving auditory neurons for their efficacy[52].

Prospect of cell replacement for hearing restoration

Currently, no therapy for restoring hearing is clinically applicable yet. Combination and sequential therapies that include genetic manipulation of cell cycle machinery, induction of new hair cells through gene therapy, and introduction of stem cells into damaged cochlea, cochlear nerve and cochlear nucleus neurons may be used to protect, repair and replace hearing structures. Since hair cells may potentially be regenerated from supporting cells, further understanding of mechanisms of renewal by transdifferentiation will greatly enhance the potential of such therapeutic interventions in treating hearing loss[20, 43].

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