Published in final edited form as:

Curr Opin Otolaryngol Head Neck Surg. 2009 October ; 17(5): 373–380. doi:10.1097/MOO.0b013e328330345b.

Regeneration of the mammalian inner ear sensory epithelium

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Abstract

Purpose of Review—This review will focus on “self-repair” of mammalian inner ear sensory epithelium including (1) recruiting the *in situ* proliferation and differentiation of endogenous cells at the damaged site and (2) the autologous transplantation

Recent findings—Self-repair refers to a favorable structural and functional outcome of damaged inner ear sensory epithelium. Our advanced ability of manipulating the fate of inner ear sensory cells makes *in situ* proliferation a possible candidate of hearing restoration. A practical alternative of the unavoidable immune rejection is to introduce autologous cells. Ependymal cells, induced pluripotent stem (iPS) cells, and olfactory sheath cells have been recognized as promising sources, which will spur ongoing efforts to evaluate the new cell sources for cell replacement therapy.

Summary—Further exploration of the innate advantages of *in situ* proliferation and using novel cell sources for autologous transplantation may serve as rehearsals for clinical trials in the near future.

Keywords

stem cells; inner ear; sensory epithelium; self-repair

Introduction

Sensorineural hearing loss is one of the major devastating ailments in industrialized countries. The suffering of hearing loss is devastating both emotionally and financially. Attempts at structural and functional recovery of the damaged inner ear have been tested extensively [1–12]. In this review, we will focus on “self-repair” of mammalian inner ear sensory epithelium via recruiting *in situ* proliferation and differentiation of endogenous cells at the damaged site, which may immediately rebuild the highly organized cytoarchitecture to reconstitute hearing. (2) Additionally, we will focus on autologous transplantation, which holds the great advantage of no disease transmission from donor to recipient and avoids the risk of immune rejection. Exploration of these approaches may serve as rehearsals for clinical trials in the near future.

Stratagems of regenerating sensory cells of adult mammalian inner ear

Regenerative medicine via cell-based therapy is growing into a widespread therapeutic modality. This modality, seeks to devise new therapies for patients with severe hearing loss,

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in which the physiological response of adult mammals does not suffice to restore functional sensory cells. The key issue here is to reconstitute the hair cells (HC) and spiral ganglion neurons (SGN), which do not regenerate after damage. Given the complicated pathogenesis of various types of hearing loss, it is unlikely that a single type of regenerative stratagem will fit all the degrees of tissue damage. The two essential stratagems are: (1) *In situ* self-repair therapy, where the remaining host cells, the endogenous stem or progenitor cells are mobilized to enhance repair. (2) Graft therapy, cells are derived from autogenic, allogeneic, or xenogeneic sources. Graft therapy has been investigated for years and the *in situ* therapy is an appreciated and promising approach. The ultimate goal of each stratagem is common: selection of correct cell types to reconstitute the dysfunctional inner ear. To this end, the origin of selected cells is probably of less consequence than if they exhibited the required functions.

1 *In situ* self-repair therapy

The following paragraphs will describe the detailed stratagems of *in situ* self-repair therapy.

1.1 *In situ* proliferation of inner ear stem/progenitor cells

Birds and amphibians regenerate their inner ear HCs throughout life [13,14], and it is also established that the adult mammalian vestibular system can regenerate HCs upon damage [15,16]. However, the inability of the mature mammalian cochlear system to regenerate damaged hearing in HCs leads to irreversible hearing impairment. Grafting to repopulate the lost HCs is confronted with a big challenge: the chance that grafted cells will integrate into key sites to fill the vacancy of lost hair cells is pretty low, if not at all. One alternative, is to recruit the *in situ* proliferation and differentiation of endogenous stem cells, which may immediately reconstitute the functional auditory HCs. Stem cells are defined by their ability to self-renew and by their potential to undergo multiple differentiations into functional cells if primed with the right conditions. Tissue-specific stem and/or progenitor cells have been isolated and characterized from different tissues. The hunting for the real stem or progenitor cells within the mature mammalian inner ear was cheered by a recent ground breaking study. This study demonstrated that pluripotent stem cells are identified from adult mammalian vestibular sensory epithelium and give birth to cell types of all three germ layers, including HCs and neurons [17]. The discovery of this true stem cell in the mature mice inner ear justified the *in situ* self-repair therapy. Proliferation and proper differentiation of quiescent endogenous stem cells may promise a prompt reconstruction of damaged structure and restoration of the failing function. Although hunting for the cochlear version of adult stem cells is still on its way, characterization of the niches needed to maintain and proliferate the vestibular stem cells, will help to establish a more specific and predictable program for native stem cell expansion. Identification of guiding molecules of each key differentiation stage will contribute to a better control of cell lineage commitment.

1.2 Conversion of supporting cells into HCs without mitosis

It is known that tissue injuries in many organs can cause phenotype changes, namely, metaplasia. This phenomenon is now considered as the disruption of a mechanism of developmental plasticity that is involved in non-oncogenic tissue repair [18]. Available evidence indicates that adult cells can be epigenetically reprogrammed into another closely related phenotype without cell division. Although the molecular mechanisms underlying this phenotypic plasticity need to be further unraveled, terminally differentiated supporting cells have achieved this phenotypic switch. This cell-type, not only provides structural support for hair cells, but can be induced to undergo a phenotypic switch into hair cells. The transcription factor, Math1, a key factor of hair cell differentiation, is essential for this phenotype conversion. Over-expression of Math1 is not only sufficient to induce ectopic supporting cells into hair cells during normal embryonic development, even terminally differentiated supporting cells

within mature organ of Corti can be trans-differentiated into hair cells. The striking feature of this apparent phenotypic plasticity has been confirmed by different research groups and a pilot *in vivo* study, has demonstrated partial functional restoration [19–22]. The common developmental lineage of supporting cells and hair cells may be the mechanism underlying this phenotypic switch. The major problem of this stratagem is that phenotypic conversion without mitosis leads to a reduced number of supporting cells. On the other hand, this forced phenotypic conversion stratagem is only effective within a limited time window following ototoxic insult [23].

1.3 Generating of new hair cells out of the terminal deafened sensory epithelium

Hair cells die upon ototoxic insult and eventually the remaining supporting cells also disappear, leaving only a layer of intractable flat epithelium. Until today, the putative endogenous stem cells were only identified in mature mice vestibular organs and although stem cells were found in neonatal auditory sensory epithelium [24,25], the similar endogenous stem cells in adult auditory epithelium have not been identified. As mature mammalian auditory sensory epithelium does not spontaneously turn on a regenerative response after damage, what can be done for end stage deaf patients?

Regeneration is not a single factor event. In mature mammalian tissue, homeostasis is maintained by multiple redundant mechanisms to keep functional stability and to prevent tumor genesis. Therefore, proliferative replacement of HCs may only be achieved by a prolonged supply of cell cycle regulatory factors to overbalance the innate and very determined homeostasis. These factors must be followed with supply of a set of regulatory factors, in the order of HCs cell development. However, the supply of cell cycle regulatory factors must be within a limited time window; otherwise, the regenerative attempt may end up with tumor formation. To this end, it is important to identify a relevant vector that does not integrate into the host genome and only produces the therapeutic factors for short period of time. Therefore, the adenoviral vectors which demonstrate strong tropisms to inner ear epithelium may appear as a powerful tool to deliver the regenerative and inductive signals to the damaged site [26, 27]. Additionally, the cochlea is isolated from the surrounding tissues by the bony capsule, which limits the spread of the vectors into neighboring tissues. This anatomical feature makes the cochlea a unique candidate for combined application of cell cycle re-entry, forced expression or silencing of particular genes and modification of DNA structures and chemical reagents. Jointly, these applications will enhance both the structural and functional outcome of the end stage deaf ear epithelium.

These significant steps forward in hearing rehabilitation depend on the advanced ability of manipulating the fate of inner ear sensory cells, which rely on the better understanding of development and regulation of the fate of inner ear sensory cells [28–30]. We thus highlight some recent understandings of this topic. The retinoblastoma (Rb) pathway regulates the transition from the proliferating stage to postmitotic stage of the mouse inner ear [31], and deletion of Rb leads to overproduction of hair cells [32]. Moreover, Notch signaling is involved in prosensory path formation, and the lateral-inhibition mediated differentiation of hair cells [33]. Loss of Notch signaling leads to an increase in the size of prosensory patches. The basic helix-loop-helix (bHLH) transcription factor Atoh1 (Math1), was revealed to play a key role in hair cell differentiation during development [34,35]. Interestingly, forced expression of Math1 alone in postnatal and adult stages would enhance ectopic hair cell formation near the organ of Corti [19,22]. A more recent study confirmed that putative hair cells induced by Atoh1 misexpression could generate functional hair cells, which highlight gene therapy to be a possible paradigm to ameliorate hearing loss [36]. The mammalian post-mitotic supporting cells have recently been recognized as a potential target for therapeutic manipulation. Since these cells can re-enter the cell cycle and trans-differentiate into new hair cells in culture, and

their proliferative capacity depends, in part, on the activity of cyclin-dependent kinase inhibitor p27^{kip1} [37]. Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells during inner ear development [38]. Inhibition of Notch signaling in the new born mouse organ of Corti with r-secretase result in the generation of new HCs [39]. Therefore, manipulation of the cell cycle regulatory genes and the key genes for HC commitment is another potential avenue for regenerating HCs from deaf auditory epithelium.

2 Autologous transplantation

When biopsies obtained from patient cells are able to expand and differentiate in culture and after transplantation into the same patient to heal a defect, autologous therapies are feasible. A direct biopsy is taken from tissue that is the same as the engineered tissue type. An indirect biopsy may be taken from a similar tissue when a direct biopsy would cause considerable injury. Autologous transplantation holds the great advantage of avoiding the risk of immune rejection due to differences in histocompatibility antigens. Therefore, immunosuppression is not required. However, there is a substantial practical appeal to “off the shelf” products that do not require the cost and time associated with customized preparation. Autologous transplantation has already achieved regulatory approval and reached the market for patients with life-threatening burns and for large articular cartilage lesions. These successful applications highlight the ongoing efforts to identify new autologous cell-based therapies. The updated opinion states that in addition to traditional bone marrow derived cells, ependymal cells, induced pluripotent stem (iPS) cells, and olfactory sheath cells have been recognized as promising sources. These sources will spur ongoing efforts to evaluate the new cell sources for cell replacement therapy.

A practical alternative to the unavoidable immune rejection is the introduction of autologous cells. Current viable cell sources for autologous inner ear sensory cell replacement are: ependymal cells, iPS cells, olfactory sheath cells, bone marrow cells and stem cells from amniotic fluid and placenta. These cells could be obtained from the recipient themselves via biopsy, as self-made cells share identical major histocompatibility complex with the recipient. This would enable autologous transplantation without immunosuppression.

The immune rejection may be overcome by isolation of donor cells for reintroduction into the same host. Autologous transplantation is a widely accepted and approved technique for regenerative medicine. It has an enviable profile of assets, including lack of immune rejection and is free of disease transmission from donor to recipient. Self-made cells also share identical major histocompatibility complex with the recipient, enabling autologous transplantation without immunosuppression. Exploration of these approaches may serve as preparation towards clinical trials in the near future.

One of the major disadvantages of autologous transplantation is the limitation in the amount of available tissue. Various researchers are striving toward hearing restoration with various substitute cell sources possessing featured properties, not necessarily a replica of the native inner ear sensory cells.

2.1 Ependymal cells

A recent study [40] reported extensive analyses using structural, molecular, and functional criteria to demonstrate that adult brain germinal zone cells, derived from the same neural ectodermal layer as the otic vesicle epithelial cells, demonstrate intriguing putative hair cells properties and may serve as a novel source of autologous cells for hair cell replacement. In the adult brain of both rodents and human, ependymal cells form a multi-ciliated single cell layer lining the lateral ventricles. These cells share many essential molecular, morphological, and physiological characteristics with inner ear HCs. Further they are polarized with actin-based

stereocilia and microtubule-based kinocilia, similar to vestibular hair cells of the inner ear. They can be identified by and commonly used HC markers such as: myosin VIIa (Figure 1), myosin VI, and CtBP2/RIBEYE. In a fashion similar to hair cells, endymal cells rapidly uptook the styryl dye FM1–43 that permeates hair cell transduction channels, suggesting that the endymal cells express similar large conductance, nonselective ion channels. Also notably important, these cells can incorporate into the cochlear sensory epithelia and have several defining electrophysiological characteristics of HCs. Moreover, they are electrically active, send synaptic input to target-deprived SGNs and are capable of releasing glutamate in response to membrane depolarization (Figure 2). A distinct advantage is that, unlike hair cells, endymal cells appear to retain the ability to proliferate and proliferative myosin VIIa positive endymal cells were identified both *in vitro* and *in vivo*. This raises their prospects for use in autologous replacement of nonrenewable HCs. Further examination of the differences between the proliferative capacity of endymal cells and the quiescence of hair cells may make endymal cells a valuable comparative model for understanding some basic biological questions.

Based on proof of principle experiments established with the functional test of endymal cells, we assert that endymal cells can undergo functional switches to assume functional characteristics of HCs. Thus, a broader view of the functional adaptation, or an inborn functional plasticity was just recognized. Some specialized cells may directly adapt their functions upon switching to a new environment, which may act as an alternative to the more complicated cell fate switch. Although the richness and complexity of this functional plasticity remains to be appreciated, an updated concept of essential multipotency of lineage phenotypes should be broadened to include the emerging recognition of multi-functional potential of stem cells.

Application for human therapy depends on further evaluation of their performance in animal models. The potential hurdle for human therapy is the age- and disease-related changes of endymal cells and safety concerns about collecting these cells. Nonetheless, as a tool for understanding the basic science of cell replacement in the inner ear, this work represents an important step forward [41]. Understanding of the mechanisms of functional plasticity may open a new window for cell replacement therapy.

2.2 Induced pluripotent Stem (iPS) cells

A successful allograft is accompanied by the insurmountable pitfalls of lifetime immunosuppression. An attractive solution to solve both the shortage of reproducible cells and immune rejection is the autologous source of stem cells, which are collected from the same patient. This will eliminate complications associated with immune rejection, and thus the deleterious side effects of immunosuppressive medications can be avoided. Limitations of harvesting the marketable autologous stem cells make this approach beyond the reach of clinical practice.

Recent ground breaking research has found that activation of a set of stem cell genes can reprogram terminally differentiated cells to acquire properties similar to those of embryonic stem (ES) cells [42–44]. The reprogrammed cells are namely induced pluripotent stem (iPS) cells. The iPS cells were similar to ES cells in morphology, proliferation, expression of some ES cell marker genes, as well as the multiple differentiations. This elegant proof of principle experiment shattered the established dogma, that terminally differentiated somatic cells could not be reprogrammed to the embryonic state.

Besides the general characteristics of ES cells, the unique features of iPS cells grant them additional advantages for cell replacement therapy. First, iPS cells could be generated from the customer patient, immune rejection is essentially overcome. Secondly, the application of

somatic cell derived iPS cells are free of ethical concerns regarding the use of human embryos. The potential of iPS cells is enormous and the iPS stratagem has been exponentially growing, and numerous iPS lines have been generated. Additionally, several iPS cell derived somatic cell types are being evaluated for regenerative medicine and pharmaceutical application.

Many obstacles remain before their medical and pharmaceutical applications can be fully realized. The hurdle of teratoma formation leads the transplantation stratagem to choose terminally differentiated cells and/or committed progenitor cells over primary iPS cells. However, the differentiation of HCs walks through a complex spatial and temporal pathway, where multiple interplays of various factors at a highly organized sequence may be essential to a fully committed HC. This complexity discourages the guarantee of a pure population of HCs, because the orchestrated differentiation usually requires cross-talk of signals from the co-differentiating neighbors, similar to the patterning *in vivo*. Thus, isolation of the desired differentiation derivative is required for obtaining a pure cell population. A better understanding of the signaling pathways guiding the development and differentiation of iPS cells will enable us to design a step-wise procedure employing a “correct” mixture of regulatory factors to achieve high-yield differentiation. The advancement of iPS techniques and the ability of manipulating HC differentiation may eventually enable us to design a biological “implants.” Such implants may precisely reverse the degeneration remaining HCs or supporting cells in the deaf ear.

Other obstacles must be addressed before application in clinical therapy. Among these are; 1) identification of the aberrant reprogramming, which is a co-product during iPS cell preparation. Since, it may result in impaired ability to differentiate and may increase the risk of immature teratoma formation after directed differentiation. 2) The promised silencing of transgene integration into the iPS cell genome is another concern. Reactivation or leaky expression of such transgenes (e.g. c-Myc) could lead to tumor genesis, creating a greater risk of immature teratoma formation [45].

2.3 Olfactory progenitors

The olfactory neuroepithelium in the mammalian nervous system is capable of generating new olfactory receptor neurons periodically throughout adult life, as well as the capacity to proliferate in response to acute injury. The unique proliferative feature results from the presence of multipotent stem cells in the olfactory neuroepithelium[46]. An *in vitro* differentiation of adult mouse olfactory precursor cells into HCs has been reported. These hair cell-like cells provide a potential autotransplantation therapy for hearing loss [47]. Precursor cells from mouse olfactory neuroepithelium were sphere-forming, and showed proliferative capacity. After co-culturing with cochlear cells and/or cochlear supernatant, the olfactory progenitor cells expressed several HC markers, including myosin VIIa, calretinin, and espin. These results demonstrated for the first time that adult olfactory precursor cells could differentiate into hair cell-like cells. Thus, olfactory neuroepithelium offers an abundant and easily accessible source of adult stem cells, which could be a potential autotransplantation therapy for hearing loss. However, the functional potential of this source remains to be addressed.

2.4 Bone marrow derived cells

Mesenchymal stem cells (MSC) from bone marrow have been reported to differentiate into multiple lineages, and have long been evaluated in clinical practice. An *in vitro* overexpression of the prosensory transcription factor, Math1, will induce MSC into inner ear sensory cells which expressed myosin VIIa, espin, Brn3c, p27Kip, and jagged2. Some of the daughter cells demonstrated F-actin-positive protrusions, a structural analog of HC stereociliary bundles. Hair cell markers were also induced in culture of mouse MSC-derived cells in contact with embryonic chick inner ear cells, and this induction was not due to a cell fusion event [48]. An

in vivo evaluation to test its restorative concept in inner ear repair, demonstrated the homing capability of bone marrow-derived cell (BMDC) to the deafened cochlea. However, the spontaneous transdifferentiation to any cochlear cell type after acoustic trauma was not observed [49]. These pilot studies suggest that pre-differentiation of bone marrow derived cells is required to achieve the possible hearing rehabilitation.

2.5 Stem Cells from Amniotic Fluid and Placenta

Since recent report has demonstrated the presence of stem cells with multiple potential in the amniotic fluid and placenta, they are now recognized as potential sources of stem cell therapy. Amniocentesis and chorionic villus sampling are widely accepted methods for prenatal diagnosis. Therefore, no ethical concerns will be aroused if embryonic and fetal stem cells would be taken from amniotic fluid and placenta before or at birth. Isolated human and rodent amniotic fluid-derived stem (AFS) cells express embryonic and adult stem cell markers. Undifferentiated AFS cells expand extensively without feeders. Interestingly, AFS cells are broadly multipotent. They can differentiate into cell types representing each germ layer, including neuronal, osteogenic, and hepatic phenotypes [50]. The demonstrated neuronal lineage indicates that AFS could be used for repopulation of inner ear sensory cells. Meanwhile, banking of these stem cells may provide a convenient source both for autologous therapy in later life and for matching of histocompatible donor cells with recipients.

3 Conclusion and perspectives in future research

Several issues still remain to be addressed before the novel stem cell stratagem grows into a bedside protocol. Although stable reconstitution of hearing sensation is still problematic and reconstruction of the entire cytoarchitecture of the cochlea sensory epithelium remains a big challenge, hearing rehabilitation could be achieved without the exact rebuilding of the delicate and highly organized cytoarchitecture. Various hearing rehabilitation approaches have blossomed from the combination of knowledge gained from past experiences, the joint work of molecular biologists, neuroscientists, bioengineers and neurosurgeons and will be the future model of hearing restoration. This model shares many common features with clinical application and may shed lights on clinical translation.

Acknowledgments

This research was supported by grants DC003826, DC007592 from the National Institute on Deafness and other Communicative Disorders (NIDCD) and grants RS1-00453, CIRMTG1-GSTDW from California Institute of Regenerative Medicine (CIRM)

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Figure 1. Ependymal cells demonstrate characteristics of HCs

a. Adult brain ependymal cells take the shape of polarized columnar epithelial cells and express myosin VIIA. (Scale bar, 20 μ m)

b. Arrow indicates an ependymal cell that was co-labeled with myosin VIIA and BrdU., suggesting the possibility of *in vivo* proliferation. (Scale bar, 20 μ m)

Scanning (c) and transmission electron microscopy (d) of the lateral ventricle region, demonstrated that ependymal cells are also equipped with structural profiles of stereocilia and kinocilia, similar to HCs. Panel (d) showing stereociliary appendages (arrow heads) and the characteristic 9 + 2 microtubule structure of kinocilia (arrows). Images are reproduced from Proc Natl Acad Sci U S A. 2008, 30;105(52):21000-5, with permission from PNAS.

Figure 2. Functional connections established between ependymal cells and SGNs

a1 Shown is a 3-D reconstruction image of an adult SGN (arrow) projecting neurites to an ependymal cell (arrowhead). (Scale bar, 20 μ m)

a2 Ultra structure of synapses was found between co-cultured SGNs and ependymal cells. The black arrowhead indicates the postsynaptic thickens. The ependymal cell can be identified by its signature structure; the clustered cilia (black arrows). The red arrow indicates a nerve ending of the SGN. (Scale bar, 7 μ m)

b Dual recording from the connected myosin VIIA-positive ependymal cell (in current-clamp mode; 0.4 nA current injection) and SGN (in voltage-clamp at -70 mV holding potential). The

synaptic current recorded in the SGN was sensitive to CNQX (dashed line), an AMPA receptor blocker.

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