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Concise Review: Inner Ear Stem Cells—An Oxymoron, But Why?

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Abstract

Hearing loss, caused by irreversible loss of cochlear sensory hair cells, affects millions of patients worldwide. In this concise review, we examine the conundrum of inner ear stem cells, which obviously are present in the inner ear sensory epithelia of nonmammalian vertebrates, giving these ears the ability to functionally recover even from repetitive ototoxic insults. Despite the inability of the mammalian inner ear to regenerate lost hair cells, there is evidence for cells with regenerative capacity because stem cells can be isolated from vestibular sensory epithelia and from the neonatal cochlea. Challenges and recent progress toward identification of the intrinsic and extrinsic signaling pathways that could be used to re-establish stemness in the mammalian organ of Corti are discussed.

Keywords

Adult stem cells; Aging; Nervous system; Notch; Tissue regeneration; Tissue-specific stem cells

Introduction

Mechanosensitive sensory hair cells represent an evolutionary successful concept used in many different mechanoreceptive organs ranging from the lateral line of aquatic animals to the complex inner ears of mammals with specialized vestibular and auditory organs. Despite the great morphological and functional variations of hair cell-bearing organs, the requirement of certain key genes for mechanosensory cell development is evolutionary conserved. The basic helix-loop-helix genes *atonal* and *atonal homolog 1* (*Atoh1*), for example, are essential for invertebrate chordotonal mechanoreceptor and vertebrate hair cell development, respectively [1]. Based not only on such genetic evidence but also on comparative anatomical studies, it is generally accepted that the inner ears, particularly those of amniotes, including reptiles and birds, as well as mammals, are homologous organs [2].

Despite their common ancestry, there is a crucial difference in the ability of adult vertebrate inner ears to regenerate lost hair cells. The most robust generation of hair cells happens in the vestibular organs of amphibians and fish that display permanent addition of new sensory

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cells leading to continuous growth of the sensory epithelial patches [3]. Mature avian vestibular sensory epithelia do not grow, but there is a robust turnover of hair cells as well as a robust regenerative response after induced hair cell loss [4, 5]. In contrast, mammalian vestibular sensory epithelia do not turn over hair cells and show only very limited mitogenic replacement of hair cells after drug-induced loss of hair cells [6].

The difference in regenerative capability becomes even more obvious in case of the auditory organs. The avian cochlea, also known as the basilar papilla, does not turn over hair cells, but it robustly responds to ototoxic insults with hair cell regeneration and functional recovery [7–9]. This regenerative capacity does not exhaust even after repeated deafening or at old age [10, 11]. The mammalian organ of Corti, conversely, does not replace lost hair cells. Continuous wear and tear, combined with the effects of aging as well as environmental threats such as loud noise and ototoxic drugs, result in an incessant diminishment of hearing at older ages. Approximately one-third of seniors over the age of 60 suffer from hearing loss (<http://www.nidcd.nih.gov>). Besides acquired hearing loss, approximately 2–3 out of 1,000 born babies are diagnosed with hereditary hearing loss and a similar high number of children lose their hearing before their teenage years (<http://www.nidcd.nih.gov>). This situation leads to an increasing health problem affecting hundreds of millions of patients worldwide. Undoubtedly, this number will continue to rise due to growing noise pollution, environmental factors, lifestyle choices such as listening to loud music, and worldwide increase of aminoglycoside use particularly in the third world, where these drugs are often the only affordable first-line treatments for life-threatening diseases such as tuberculosis [12].

In this review, we will compare the avian vestibular and auditory organs with their mammalian counterparts. We will start with describing anatomical and cellular differences as well as similarities. We will summarize the known regenerative mechanisms and the pathways involved in regeneration, and finally, we attempt to explain why supporting cells should be regarded as inner ear stem cells, how stemness is successively lost in the mammalian cochlea, and what options exist for re-establishing regenerative capacity in the adult mammalian cochlea.

Anatomical and Cellular Commonalities and Differences Between Avian and Mammalian Inner Ear Sensory Epithelia

In general, the sensory epithelia of the vestibular organs (utricle, saccule, and cristae) consist of a mosaic of sensory hair cells and surrounding supporting cells (Fig. 1A). Supporting cells reach from the apical surface to the basilar lamina. The hair cells, however, do not contact the basilar membrane and are basolaterally ensheathed by supporting cells. Anatomically, there are no major differences between avian and mammalian vestibular epithelia, but after hair cell loss, the differences become quite obvious. Avian vestibular hair cells readily regenerate, while proliferative hair cell regeneration in mammalian vestibular epithelia only happens at a very low rate [6]. This difference is also apparent in cultured chicken hatchling utricle sensory epithelia, which show high proliferative capacity [13], whereas cultured neonatal mammalian utricle sensory epithelia display only limited proliferative capacity [14].

Although avian and mammalian vestibular organs have similar anatomy, the anatomical differences between the basilar papilla and the mammalian cochlea are considerable. The avian hearing organ harbors a drawn-out patch of hair cells that is several millimeters long and has a width of more than 60 hair cells at its widest point (Fig. 1B). The hair cells are afferently innervated from the cochleo-vestibular ganglion underlying the auditory epithelium. Afferent nerve fibers connect to the sensory epithelium laterally, from the so-

called neural side, and innervate the cylindrical “tall” hair cells located toward the neural side. The shape of hair cells changes gradually across the avian basilar papilla and the abneural “short” hair cells are mostly innervated by efferent fibers [15]. It is presumed that the tall hair cells are equivalent to the inner hair cells of the mammalian organ of Corti that will be described in the next paragraph, whereas the short hair cells are presumably involved in feedback and gain control [15, 16]. Basilar papilla supporting cells are anatomically not substantially different from vestibular supporting cells and appear homogenous and without cytomorphological specializations.

In contrast, the organ of Corti, which is the sensory epithelium of the mammalian cochlea, has two highly specialized hair cell types and comprises a variety of supporting cell types with distinct cytomorphologies (Fig. 1C). A single row of afferently innervated inner hair cells extends from the base of the coiled cochlea to its apex. The inner hair cell row is accompanied by three rows of mainly efferently innervated outer hair cells, which fulfill amplification and frequency tuning functions. Highly specialized supporting cells that are organized in an orderly structured pattern are interdispersed between the hair cells [17]. Organ of Corti supporting cells appear to have evolved at least in part to provide mechanical support and filtering to the highly dynamic and actively moving tissue. Many supporting cells contain cytoskeletal specializations that are probably necessary for maintenance of cell shape.

Regenerative Responses in Avian Inner Ear Sensory Epithelia

Upon hair cell loss, the supporting cells of the basilar papilla regenerate hair cells [7, 8]. Within a day after induced hair cell loss, supporting cells start to re-express developmental genes that are normally found in prosensory progenitors [18, 19]. Many supporting cells do not re-enter the cell cycle but rather begin to differentiate directly into new hair cells (Fig. 2A). 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 re-enter the cell cycle and replenish the supporting cells lost due to transdifferentiation [20]. In parallel, supporting cells are able to respond to hair cell loss by asymmetric division, giving rise to pairs of replacement hair and supporting cells [21, 22] (Fig. 2B). This latter behavior resembles a bona fide somatic stem cell response. It is unclear which of the two regenerative processes is the dominating one in vivo, but it has been suggested that direct transdifferentiation might be a strong early phase regenerative response to massive hair cell loss [20, 23]. The degree of each regenerative mechanism in vitro appears to depend on the culture conditions and the nature of the ototoxic insult [13, 24]. In vivo, there is evidence for both processes happening in the basilar papilla after gentamicin-induced hair cell loss [20], whereas the major regenerative response in vestibular sensory epithelia appears to be happening via asymmetric supporting cell division [25]. Overall, it is clear that the regenerative processes that happen in the damaged basilar papilla are the direct result of activation of a resident population of stem cells. Plenty of questions, however, remain open. For example, it is not clear whether all supporting cells are stem cells or whether a subpopulation of stem cells exist. Likewise, the mechanisms that trigger direct transdifferentiation and asymmetric supporting cell division are unknown, although research on this topic is making progress as explained in the following paragraphs.

Coculture experiments of chicken utricle sensory epithelia with damaged chicken vestibular epithelia suggested that the regenerating epithelia secrete an activity that is able to trigger increased proliferation in the target tissue [26]. Likewise, the same study reported evidence for a soluble mitotic inhibitor that is secreted by the undamaged chicken utricle. A number of growth factors have been discussed as candidates for promoting proliferation in regenerating avian sensory epithelia [27, 28], and recent gene array analyses suggested the

possible involvement of Notch, transforming growth factor beta (TGF β), Wnt, activator protein 1 (AP-1), Pax, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and insulin-like growth factor 1 (IGF)/insulin pathways [18]. Using an RNA interference (RNAi)-based method for screening different transcription factors, Alvarado et al. [29] showed that inhibition of components of the AP-1 (Cebpg, Lrp5, JunD), Pax (Pax2 and Pax5), and Wnt (Wnt4) pathways, as well as c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) inhibitors were able to interfere with regenerative supporting cell proliferation. Downstream targets of TGF β signaling such as Cutl1 were also upregulated during sensory epithelia regeneration [18] and small interfering RNA (siRNA) to Cutl1 resulted in inhibition of supporting cell proliferation. Cutl1 is a suppressor of p27^{Kip1}, a cell cycle inhibitor that suppresses proliferation of supporting cells in the mammalian organ of Corti [30, 31]. Cutl1 has been put forward as a potential mediator of a regenerative response leading to downregulation of cell cycle inhibitors such as p27^{Kip1} at the onset of mitogenic hair cell regeneration in birds [18, 29].

Another pathway that has been implicated in triggering cell proliferation in the avian basilar papilla is mediated by protein kinase A, a direct target of cAMP. It has been demonstrated that increase of cAMP levels triggers a robust proliferative response in supporting cells of undamaged avian basilar papilla sensory epithelia [32]. Likewise, supporting cell proliferation in response to aminoglycoside treatment is strongly but not completely attenuated in cultured chicken basilar papillae when protein kinase A inhibitors were present. These observations suggest that one of the signals capable of triggering supporting cell proliferation in avian sensory epithelia could be acting either via receptor tyrosine kinases or via G-protein-coupled receptors leading to an increase in intracellular cAMP.

Besides secreted factors, there has been considerable interest in the role of cell-to-cell signaling mediated by the Notch pathway or more classic cell adhesion proteins. Notch signaling plays multiple roles in the avian inner ear such as during hair cell regeneration as well as in development, where the Notch pathway is important for early steps of prosensory specification, but also later on, in its classic role as mediator of lateral inhibition [33]. In the adult chicken basilar papilla, the Notch pathway is active during regeneration and manipulation of Notch signaling, for example, by inhibition of gamma secretase results in an overproduction of hair cells [34]. In contrast, in the mature mouse cochlea, Notch pathway genes become downregulated and remain silent, even after aminoglycoside-induced hair cell loss [35]. During development, Notch and its ligands are expressed in the emerging prosensory domains of the inner ear [36, 37], and activation of Notch in neighboring nonsensory regions appears to be sufficient for prosensory induction [38, 39]. Nevertheless, conditional disruption of the canonical Notch signaling mediator recombining binding protein suppressor of hairless J kappa in the developing mouse inner ear revealed that although Notch activation is sufficient, the RBPj κ -mediated canonical pathway does not appear to be essential for prosensory induction in the mouse cochlea [40]. In the avian inner ear, blockade of Notch activation leads to loss or reduction of prosensory domains, but induction of early prosensory markers such as Serrate1 does not appear to be dependent on Notch signaling [41].

Overall, it is obvious that the avian basilar papilla maintains a resident population of stem cells that are capable of fully regenerating the damaged auditory sensory epithelia. We are just beginning to understand the mechanisms how the regenerative potential of these normally quiescent cells is regulated, and how the cells become active after ototoxic damage leading to hair cell loss. Restored sensory epithelia are subsequently innervated and properly connected to the central nervous system, which functionally restores the auditory system [9, 10]. Nevertheless, an open question remains, which is whether the apparent stemness of supporting cells is a universal feature of avian vestibular and auditory supporting cells, or

whether the sensory epithelia maintain a specialized niche for a distinct somatic stem cell subpopulation [42]. The unequivocal identification of these stem cells and the unraveling of the ensuing mechanisms for regeneration are somewhat limited in the avian system, particularly because of the lack of routine genetic manipulations. A possible alternative model system for such studies is zebrafish. Hair cell regeneration in the zebrafish lateral line system, however, appears to follow yet another variation of regeneration program where a supporting cell divides symmetrically into two hair cells [43]; the lost supporting cell subsequently is very likely replaced by symmetric division of another supporting cell. Another open question, equivalent to the one raised in the regenerating chicken sensory epithelia, is whether lateral line supporting cells are randomly chosen to replace lost hair cells or whether there is a local niche maintained for a population of distinct stem cells.

Lack of Robust Regenerative Responses in Mammalian Inner Ear Sensory Epithelia

Evolutionary, it is inconceivable why the mammalian inner ear has lost its regenerative capacity. One argument that has been put forward is to achieve the structural specializations of the organ of Corti, which presumably extend the range of hearing into the higher frequencies, that the stemness and its ensuing regenerative potential of supporting cells was traded off for structural complexity [44]. Another argument is that, evolutionary, the preservation of regenerative capability was not under strong selective pressure because acquired hearing loss and ototoxic insults are mainly the product of the industrial revolution [45]. All these speculations, however, cannot explain why the mammalian vestibular system has such a restricted regenerative capacity when compared with birds, reptiles, and fish. Anatomically and functionally, the differences between the vestibular organs of mammalian and nonmammalian amniotes appear small. At the cellular level, however, either the signals that trigger regeneration or the factors that provide competence to the responding cells, or both, are no longer featured.

Although mitotic hair cell regeneration in adult mammalian vestibular sensory epithelia does only happen on rare occasions [6], there are some indications that adult vestibular supporting cells have regenerative capacity, which can be activated when the cells are dissociated and cultured in conditions that were originally developed to stimulate neurosphere formation from neural stem cells [46, 47]. Dissociated utricle sensory epithelium cells are able to grow clonally into spheres, albeit with low yields. Stringent tests showed that sphere-forming cells from the adult utricle sensory epithelium were self-renewing and able to give rise to cell types from all three germ layers [47], which indicates that the adult vestibular sensory epithelia harbors stem cells. Open questions remain. First, as with avian supporting cells, it is not clear whether stemness is a possible feature of all mammalian vestibular supporting cells or whether there is a subpopulation of sensory epithelial cells that maintain stemness and are the source of the limited regenerative capacity. Second, the number of stem cells with this ability is low: a few dozen per sensory epithelium, which, however, is more than the few mitotic cells that can be detected in vivo after an ototoxic insult [6]. This finding suggests that a group of supporting cells might be competent to respond to a regenerative trigger, but that the lack of appropriate signals or the presence of an inhibitor might contribute to the low regenerative capacity of adult mammalian vestibular epithelia. It is interesting in this regard that neonatal mouse balance sensory epithelia display a higher propensity for sphere formation than the adult tissue [46]. This suggests that young vestibular sensory epithelia harbor more cells that are able to re-enter the cell cycle provided an adequate trigger is supplied. Nevertheless, it is important to point out that presence of stem cells does not necessarily mean that the organ displays substantial regenerative capacity. In the mammalian central nervous system, for example, regenerative responses to injury or disease are limited, despite the existence of neurogenic

niches. Conversely, neural stem cells are not always and necessarily quiescent and have been shown to become active in certain situations [48]. It is consequently important to distinguish between stemness and regenerative potential, which not always go hand-in-hand.

Although the molecular nature of activators or inhibitors of mammalian hair cell regeneration are not known, some candidates are emerging. For example, brief exposure of explants of neonatal rat vestibular sensory epithelia to forskolin led to a significant increase of cell cycle re-entry of supporting cells, which indicates that tissue dissociation is not absolutely necessary to evoke S-phase re-entry [14]. Moreover, the S-phase re-entry in these cultures was only occurring in the presence of serum or mitogenic growth factors and was not observed when receptor trafficking to the plasma membrane was blocked. These observations suggest that transient elevation of cAMP levels in neonatal vestibular supporting cells very likely results in an increase of growth factor receptor density in the plasma membrane, which in turn leads to a higher number of supporting cells that are competent to respond to mitogenic stimulators in form of growth factors or serum components. Possible growth factor or cell contact-based signaling cascades involved in triggering S-phase reentry include the phosphatidylinositol 3-kinase cascade culminating in activation of mammalian target of rapamycin (mTOR) because inhibition of elements of this signaling cascade interferes with mitotic cell proliferation in neonatal rat vestibular sensory epithelia [49]. It appears that intact neonatal vestibular sensory epithelia *in vivo* do not contain sufficient amounts of mitogenic stimulators, hence the cell cycle quiescence of supporting cells that otherwise would be readily responsive to mitogens. Furthermore, maturing and aging supporting cells might lose growth factor receptors and consequently the competence to respond to mitogenic growth factors.

The adult mammalian organ of Corti completely lacks regenerative potential. In contrast, neonatal mouse organ of Corti-derived cells have a rather solid mitogenic capacity, which is reflected in their ability to give rise to clonal spheres or colonies [46, 50]. Mitogenic capacity, however, is not necessarily an indication whether neonatal organ of Corti-derived cells have the ability to generate progenitor cells that give rise to hair and supporting cells. Cell sorting experiments have shown that the cells with the highest capacity to give rise to hair cell- and supporting cell-marker positive cells are the pillar cells as well as the supporting cells that are most closely associated with hair cells [50, 51]. Nevertheless, other cell types that reside in the neonatal cochlea also have potential to proliferate and to differentiate into hair cells and supporting cells, albeit with less efficacy [51]. These observations are in support of the hypothesis that organ of Corti maturation and the distinct cytomorphological differentiation of cochlear supporting cells are accompanied by downregulation of signaling molecules and presumably also their receptors and intracellular signaling components. It is conceivable that the organ of Corti never establishes a proper stem cell niche and that the atavistic stemness found in neonatal cochlear supporting cells disappears when the cells become fully differentiated. Consequently, there is no regenerative capacity detectable in the mature organ of Corti. The question remains whether competence to respond to regenerative triggers can be restored in adult organ of Corti supporting cells. Research on other organ systems, such as the heart, is promising in this respect. The mammalian heart, like the inner ear, lacks robust regenerative capacity whereas the hearts of non-mammalian vertebrates such as fish can regenerate cardiomyocytes and can restore function [52]. Nevertheless, recent findings established a lineage relationship between stem cells that reside in the epicardial layer of the adult mouse heart and functional cardiomyocytes that differentiate *de novo* from the resident epicardial stem cells after myocardial infarction [53]. This mobilization and differentiation required pretreatment with thymosin β_4 , a peptide that has been previously shown to stimulate re-expression of developmental genes in presumptive stem cells in the epicardium [54]. Another example is the restoration of cell loss in a mouse model of stress-mediated muscle atrophy by treatment

with the food and drug administration (FDA)-approved drug losartan [55]. Cells with stem cell characteristics in these organs can evidently be tweaked to display a certain degree of regenerative potential, which is providing some reason for careful optimism. Nevertheless, particularly for the infarcted heart, many roadblocks need to be solved before functional restoration by activation of the regenerative potential of resident stem cells could become a feasible therapy option. Translated to the inner ear, it is plausible that the discovery of small molecule activators that evoke re-expression of developmental genes would be a promising route toward developing novel therapies for hearing loss [45, 56]. It would be interesting to investigate whether such a strategy would instigate localized developmental processes that lead to hair cell regeneration or even restoration of the anatomical intricacies of the organ of Corti. Although research in this regard is just beginning and translation into the clinic is probably decades away, it is obvious that a longer life paired with a lifetime of ototoxic insults causes a steady increase of the number of patients worldwide who await novel treatments for hearing loss. The apparent loss of stemness in the mammalian inner ear when compared to nonmammalian vertebrates remains puzzling, thereby making the term “inner ear stem cells” truly an oxymoron for patients who are dearly affected by the inability of the cochlea for self-repair.

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Figure 1.

Inner ear sensory epithelia. **(A):** A generic illustration of avian and mammalian vestibular sensory epithelia. **(B):** A drawing of the avian basilar papilla. **(C):** The mammalian organ of Corti.

Figure 2.

Hair cell regeneration. **(A):** A supporting cell differentiates into a hair cell. This process is also referred to as transdifferentiation. The lost supporting cell replaced via a mitotic division of another supporting cell. **(B):** An illustration of asymmetric supporting cell division giving rise to a new hair cell and a supporting cell.