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Recent insights into regeneration of auditory and vestibular hair cells

Stone, Jennifer S.; Oesterle, Elizabeth C.; Rubel, Edwin W.[a]

Author Information

Virginia Merrill Bloedel Hearing Research Center, Department of Otolaryngology and [a]Departments of Otolaryngology, Physiology, Neurosurgery, and Psychology, University of Washington School of Medicine, Seattle, Washington, USA
Correspondence to Edwin W. Rubel, PhD, Virginia Merrill Bloedel Hearing Research Center, CHDD Building, CD 176 Box 357923, University of Washington School of Medicine, Seattle, WA 98195-7923, USA Tel: +1 206 543 8360; fax: +1 206 616 1828

Abbreviations; EGF: epidermal growth factor; **FGF:** fibroblast growth factor; **IGF-1:** insulin-like growth factor 1; **TGF:** transforming growth factor;

Abstract

Advances in hair cell regeneration are progressing at a rapid rate. This review will highlight and critique recent attempts to understand some of the cellular and molecular mechanisms underlying hair cell regeneration in non-mammalian vertebrates and efforts to induce regeneration in the mammalian inner ear sensory epithelium. Curr Opin Neurol 11:17–24. © 1998 Rapid Science Ltd

Introduction

Mechanosensitive epithelia in the vertebrate inner ear are necessary for hearing, balance, and equilibrium. The most important cellular elements in these epithelial sheets are the hair cells, remarkably sensitive transducers of mechanical deformation into electrical potentials that are transmitted to the brain via the eighth cranial nerve.

In mammals, the hair cells of the inner ear, like most neurons, are generated during embryogenesis and must be sustained for the life of the organism to maintain normal auditory and vestibular sensitivity. There is a complex, yet relatively monotonic, relationship between the amount and location of hair cell damage and hearing deficits. The outcome in modern society is the loss of normal hearing in a massive and growing segment of the population because of congenital abnormalities, disease, noise exposure, environmental toxicants, therapeutic drugs, and the aging process itself. If we could find ways to permanently replace missing or damaged hair cells, a major segment of the population that suffers hearing and equilibrium problems could be effectively treated. Until 10 years ago, that possibility was largely discounted by biologists and medical science.

It has now become evident that combining the lessons from evolutionary biology with the power of modern molecular and cellular biology may yield solutions to this problems. Regeneration of hair cells, along with their supporting body structures, was studied in the early part of this century in urodeles [1], and post-embryonic hair cell production in vertebrates that increase in body size throughout life has been recognized for many years [2,3]. But 1 decade ago marked a turning point in this field with the publication of five papers on birds, advanced homeothermic vertebrates that share exquisite auditory sensitivity with mammals. These papers showed that: (a) when auditory hair cells are destroyed by exposure to intense noise or ototoxic chemotherapeutic drugs, they are replaced by increased mitotic activity and differentiation of the new cells [4–7]; and (b) new hair cells are continually produced in the vestibular epithelia of mature birds without apparent growth of the vestibular organs, suggesting continuous cell turnover [8].

A wave of activity ensued that addressed many of the initial questions posed by these original reports. What is the source of the new hair cells? Does the vestibular epithelium in birds upregulate mitotic activity after damage? Do the new hair cells have the same morphology as the original hair cells? Are the new hair cells innervated, and are they functional? These studies have been reviewed in a number of sources (e.g., [9–12]). In general, these questions were answered and, most importantly, it was found that regenerated hair cells do support recovery of auditory perception and vestibular function.

Although the questions above are important for understanding the biology of hair cell regeneration in birds, the question posed by the 1987–1988 discoveries that provoked the most interest in the biomedical community was whether this research could be parlayed into therapy for hearing and balance disorders in humans. Our laboratory and others have focused on developing strategies to address this issue. Fundamentally, two strategies have emerged: (a) to understand the cellular and molecular mechanisms underlying hair cell regeneraton in birds and other non-mammalian vertebrates; and (b) to induce regeneration in the mammalian inner ear sensory epithelium using growth factors and cytokines known to influence cellular proliferation and differentiation in other tissues. The rationale underlying the first strategy is that, by understanding the critical cellular and genetic pathways in birds and other non-mammalian vertebrates, we can discover what is missing in mammals and then surgically or medically replace it or induce it. The rationale underlying the second strategy is that maybe we can get lucky; there may be a single molecule that, when altered, will trigger the regenerative process or remove intrinsic inhibition of regeneration in mammals.

It was rapidly recognized that in-vitro organ and cell culture methods for mature inner ear sensory epithelium were essential for efficient progress [13–15], and markers for the various cell types needed to be found (e.g., [16–22,23••]). The advancement of these techniques has provided invaluable tools to study regulation of hair cell regeneration. Page limit allocations for this review do not permit an in-depth discussion of these methodological advances which underplays their importance. In addition, we are not able to cite many of the contributions that have led to the recent experiments discussed. Our purpose is to address some of the most burning issues and critique the current status of our field.

Hair cell regeneration in non-mammalian, vertebrates

Identity of hair cell progenitors

Regeneration of hair cells in mature non-mammalian vertebrates demonstrates that hair cell progenitors are preserved in inner ear epithelia after embryogenesis. The most likely hair cell progenitors in post-embryonic animals are support cells that reside in the sensory epithelium, near hair cells. In normal and damaged sensory epithelium, support cells divide and their progeny differentiate into new hair cells and support cells [24–32,33••,34••]. It is not known whether all support cells are capable of serving as hair cell progenitors. Proliferative support cells in the fish sacculus have no ultrastructural cytoplasmic features that distinguish them from nonproliferative support cells [34••]. Further, virtually all support cells in the chick basilar papilla (cochlea) seem to have the capacity to enter the cell cycle, because all support cells in the organ appear to leave growth arrest in response to a focal lesion [35]. However, when virtually all hair cells are destroyed in a portion of the bird basilar papilla, only a small portion of support cells (15%) actually progress to S phase [36••], and these proliferative cells are limited to the damaged region of the tissue. One wonders, therefore, if there are two or more classes of support cells or if there is an intrinsic signal restricting the proportion of cells that progress to mitosis following damage.

Recent studies suggest there may be an additional subset of support cells in non-mammalian species with the capacity to directly differentiate into hair cells without a mitotic event. This process has been termed 'direct transdifferentiation' [36••]. Evidence for direct trans-differentiation as a potential mechanism of hair cell regeneration was originally provided in amphibian lateral line organs [24,37]. In recent experiments [32], Jones and Corwin laser-ablated hair cells in the axolotl lateral line and monitored subsequent cellular events using video microscopy. They observed the development of hair cell properties within cells that did not appear to have recently undergone a nuclear division.

Additional evidence for direct transdifferentiation has been derived from recent studies in other species. Robertson *et al.* [36••] implanted an osmotic mini-pump containing 3H-thymidine into the perilymphatic space of chicks and then used ototoxic drugs to induce hair cell loss. Only a subset of new hair cells in the basilar papilla showed evidence of 3H-thymidine incorporation despite the continual availability of the nucleotide to all potential progenitor cells. Cells with morphological features of both hair cells and support cells in the non-mammalian inner ear have been detected by other researchers in the regenerating avian and amphibian sensory epithelium [21,38,39••,40••] and have been interpreted to be support cells in the process of converting into hair cells. Finally, new hair cells continue to be formed in the noise-damaged chick basilar papilla [38,40••] and the bullfrog sacculus [21,39••] when mitosis is partially attenuated by mitotic inhibitors.

Although these findings support the potential for direct transdifferentiation during hair cell regeneration in non-mammalian vertebrates, few studies have rigorously tested this possibility. To elucidate the contribution of mitotic versus non-mitotic hair cell production to regeneration, future studies will have to characterise the specific cellular events that lead to the formation of new hair cells under different experimental conditions and in different organs and species. There are emerging data that developing and regenerating hair cells resemble support cells during the course of differentiation; they have small microvilli, elongated cell bodies that appear to contact the basal lamina, and their nuclei lie in the support cell layer [23••,41,42,43••]. Experiments addressing the phenomenon of direct transdifferentiation would be strengthened by the combined use of nucleotide proliferation markers with antibodies or labels for each cell type to aid in preventing errors in cell identity. Many studies have led to the identification of more markers for non-mammalian support cells and native or regenerated hair cells (e.g., [16–22,23••,44–47]).

Triggers of progenitor mitosis in non-mammalian vertebrates

The cellular events that stimulate hair cell progenitors to divide and form new hair cells are not well understood. The onset of progenitor cell division is temporally and spatially correlated with the loss of hair cells from the sensory epithelium after drug-, laser-, or noise-induced damage in the chick basilar papilla [30,31,33••,35]. Spontaneous hair cell death occurs in inner ear organs that display continual mitotic activity and hair cell regeneration [8,48–51,52••,53,54], further linking hair cell loss with cell division. Several investigators have proposed that the contact between hair cells and support cells normally inhibits support cell division and that the change in cell contact induced by hair cell loss releases the mitotic inhibition that normally exists [4,24,55]. However, the nature of the intercellular signals between support cells and hair cells that are altered by hair cell loss remains to be determined before this hypothesis can be disproven.

In-vitro models have been developed to directly study the molecules that influence progenitor mitosis in non-mammalian vertebrates [13,23••,33••,39••,56,57••,58••] and have revealed that diffusible molecules are probably critical for hair cell regeneration [33••,59]. Insulin-like growth factor-1 (IGF-1) and additional growth factors and their receptors are expressed in the chick sensory epithelium before and after damage [60••], suggesting that they are candidate regulatory factors. Studies employing specific mitogens in culture have shown that IGF-1 upregulates progenitor mitotic activity in the mature avian vestibular sensory epithelium [58••]. Leukocytic cells that infiltrate the sensory epithelium in response to cellular damage may be an additional source of regulatory factors for the regeneration process [32,37,61]. A role for growth factor mediated activation of progenitors is suported by a recent study by Navaratnum *et al.* [57••], who found that stimulation of the cAMP pathway is sufficient to drive mitosis in hair cell progenitors in cultured chick basilar papilla.

We are far from understanding the complex cascade of intercellular and intracellular events that drive a support cell from quiescence to mitosis. The fact that this progression takes between 12 and 24 hours [25,28–31,33••] suggests that multiple cellular events are required, including new transcriptional activity and a variety of cytoplasmic changes. The increased application of molecular biological techniques on normal and experimentally damaged tissue will help identify additional molecules that regulate regeneration [60••,62••,63••].

Hair cell differentiation and tissue patterning during regeneration in non-mammalian species

Upregulation of mitotic activity is only the first step toward constructing or reconstructing a functional mechanosensory epithelium; there are a host of subsequent events that must involve complex cellular signaling and precisely choreographed cascades of gene regulation: the downregulation of cell division, progressive determination of cellular phenotype (e.g. hair cell versus support cell) and subcategory phenotype (e.g. tall versus short hair cell), and establishment of precise cellular arrays, quantitative characteristics (e.g. number and orientation of stereociliary bundles), and innervation patterns. The intercellular signals that regulate these events during development are beginning to be revealed (e.g. for review see [42,64–68]).

Hair cell regeneration in the in-situ amphibian and avian inner ear results in the formation of an epithelium that is remarkably normal in its cellular characteristics and intercellular patterns [23••,39••,69–72]. However, when there is massive hair cell loss regeneration does not seem to create a perfect replica of the normal sensory epithelium [4,73–77]. Relatively little work has been done toward evaluating the signaling cascades responsible for post-mitotic events during regeneration. It is usually assumed that the important steps will be the same as those that occur during development, but this assumption has not been rigorously tested, and some important differences exist. For example, hair cell differentiation, patterning, and organization can emerge quite normally in cultures of embryonic inner ear organs [78–83]. Although some post-mitotic cells express hair cell-specific markers in cultures from birds and amphibia [24,46], and a few hair cells appear to develop stereocilia bundles *in vitro* [13,15], the elegantly stereotypical array of hair cells and support cells that emerges during regeneration *in vivo* is often not present in regenerated tissue in culture (e.g. [23••]). Therefore, some of the genes that regulate mitotic and postmitotic events during hair cell regeneration are clearly not normally expressed, or their expression is not properly coordinated *in vitro*. These discrepancies remain to be understood.

Hair cell regeneration in the mammalian inner ear

It has generally been believed that mammalian hair cells are produced only during embryonic life and that they are not replaced when they die. The discovery of hair cell regeneration in birds suggested that this conclusion needed revisiting. However, examination of injured organ of Corti upheld this earlier dogma [84–86]. Results are not so clear with respect to the vestibular epithelium of mammals. Four years ago, two studies suggested that hair cell replacement might occur in the vestibular sensory epithelia of mature mammals [14,87]. Forge and colleagues demonstrated the appearance of immature-appearing stereociliary bundles *in situ* in the vestibular epithelia of adult guinea pigs and an increase in their numbers several weeks after aminoglycoside induced hair cell loss. Complementary studies performed *in vitro* with organotypic explants of the utricular macula from mature guinea pigs suggested that sensory epithelium cells can be induced to proliferate following aminoglycoside induced damage [14]. However, the small number of labeled sensory epithelium cells in cultures appeared insufficient to account for the number of immature stereociliary bundles seen *in vivo*. Further, experiments in adult guinea pigs that involved continual infusion of a cell proliferation marker into the inner ear after drug damage confirmed that a small amount of sensory epithelium cell proliferation is induced *in vivo* [43••,88]. However, the absence of labeled hair cells again led to concern over whether proliferating sensory epithelium cells have the potential to generate new hair cells [88].

In mature mammalian otolithic organs, the epithelium is distinctly laminated. Thus, the presence of cells in the hair cell layer that are labeled by proliferation markers following damage has been taken as one indication of regenerated hair cells. Such cells have been observed *in vitro* [14] and *in vivo* [43••,89,90]. Furthermore, a few cells that are double-labeled for a cell proliferation marker and a hair cell-specific marker have been detected *in vitro* [91••]. However, hair cells labeled with cell proliferation markers remain to be unequivocally identified *in vivo*, and the location of the nucleus is not a reliable phenotypic indicator for cell type in damaged epithelia, because of the disorganization of the tissue and potential mitotic activity that occurs at the lumen [29,30,92].

A recent study by Lopez *et al.* [93••] further supported the idea that there may be replacement of damaged vestibular hair cells in adult mammals following ototoxicity, but it did not provide insight about the origin of the new cells. Lopez and colleagues induced hair cell death with gentamicin administration and compared numbers of hair cells present in control versus drug-damaged chinchilla ampullae. Gentamicin treatment killed all type I hair cells and 93% of type II hair cells. Immature hair cells were seen 28 days after drug treatment, and the number of type II hair cells reached 55% of that seen in undamaged animals by 56 days, suggesting the production of new type II hair cells after damage.

Sources of new mammalian hair cells

There may be several mechanisms besides regenerative proliferation that acount for hair cell recovery in the adult mammalian vestibular sensory epithelium following damage [43••,88,91••]. Hair cells may arise directly from support cells without an intervening mitotic event – i.e. by direct transdifferentiation of support cells [21,27,32,36••,38,39,40••], as suggested by non-mammalian species. In thin sections of drug-damaged guinea pig utricles Li and Forge [43••] detected cells that appeared to possess features of both support cells and hair cells. They interpreted these cells as undergoing direct transdifferentiation from a support cell to a hair cell. The cells rest on the basement membrane and contact neural elements, and they have organized bundles of microvilli, similar to immature hair cell stereocilia, and thick bundles of microfilaments in their apical cytoplasm, similar to those seen in support cells. The authors noted that increasing maturation of the stereociliary bundles coincided with detachment from the basement membrane, consistent with progressive differentiation towards a hair cell phenotype. However, developing postmitotic hair cells have foot processes that appear to contact the basement membrane [23••,41,42], and support cells in the sensory epithelium can contact neural elements [94,95]. Thus, it is difficult to say with certainty that these cells are transdifferentiating support cells. This is an exciting area for future research, and additional studies combining the use of proliferation markers with hair cell- and support cell-specific markers will be helpful.

Repair of damaged hair cells is another mechanism that could account for hair cell recovery in the adult mammalian vestibular sensory epithelium. It has been postulated that non-lethally damaged hair cells may be capable of undergoing repair and replacing apical processes and hair bundles that have been lost through trauma. Evidence supporting this scenario has been reported for the developing organ of Corti *in vitro* [96,97,98••]. It remains to be determined if similar repair processes are at work *in vivo* in the mature mammalian auditory or vestibular sensory epithelium.

Triggers of mammalian hair cell production

Studies using organotypic cultures indicate that transforming growth factor (TGF)[alpha]; [99,100] and epidermal growth factor (EGF) supplemented with insulin [100] are mitogenic for support cells in the mature mammalian vestibular sensory epithelium. Messenger RNAs for the EGF receptor (which mediates the effects of TGF[alpha] and EGF), exist in adult rat utricular maculae [63••]. Unpublished results from our laboratory show that infusion of TGF[alpha] and insulin into the in-situ mature mammalian inner ear stimulates proliferation in the vestibular sensory epithelium [90]. Unfortunately, in-vivo infusion of factors stimulates proliferation in extrasensory tissues as well [101], highlighting the importance of future research for targeting the delivery of growth factors to specific cells or regions.

Messenger RNAs for several fibroblast growth factor (FGF) receptors are present in mammalian inner ear sensory epithelium (FGFR-3 [102]; FGFR-1 [63••]; FGFR-1 and 2 [103]), and vestibular hair cells contain basic fibroblast growth factor (FGF-2) [103], suggesting a possible role for the FGFs in regulating regeneration. FGF-2 stimulates mitosis in tissue cultures of partially dissociated neonatal rat utricular macula, and its mitogenic effect exceeds that seen with TGF[alpha] [103]. Interestingly, FGF-2 does not stimulate sensory epithelium cell proliferation *in vitro* in organotypic explants of utricles taken from normal adult mice [100].

Recently, IGF-1 was reported to stimulate cell proliferation in tissue culture preparations of neonatal (P4-5) rat utricular sensory epithelium [103]. IGF-1 and the IGF-1 receptor are expressed in rat utricular maculae [63••,103]. However, insulin and IGF-1 do not stimulate proliferation in normal utricles taken from adult mice [100], and insulin does not stimulate proliferation *in vivo* in undamaged adult rat utricles [90]. The discrepancies between these sets of results may be a result of the fact that mechanical dissociation of the sensory epithelium upregulates FGF and IGF-1 receptors, thereby enhancing the cells' response to IGF-1 and FGF. An alternative explanation is that neonatal tissue may respond differently to mitogens than adult tissue.

Sensory epithelial cell proliferation is inhibited by several members of the transforming growth factor-beta family of growth factors, namely, TGF1,2,3, and 5 [103]. It will be interesting to determine whether these factors act to stimulate differentiation, as well as to inhibit mitosis.

Cell proliferation has not been observed in normal or damaged organ of Corti from postnatal mammals, and factors capable of stimulating the proliferation of organ of Corti support cells have not been identified [84–86,97,98••]. However, microvillar tufts resembling immature stereociliary bundles were detected in young rats after drug-induced damage [104,105], suggesting that organ of Corti hair cells may attempt to regenerate but fail to complete their differentiation and eventually die. Further investigations are needed to determine the origin of these cells, whether they are, in fact, differentiating hair cells, and why they fail to fully differentiate.

Genetic manipulations of the mammalian inner ear

Methods for altering gene expression in the mammalian inner ear are beginning to provide valuable clues and methods toward understanding the regulation of mitotic activity and hair cell production in the inner ear. For example, FGFR-3 and FGF-3 knockout mice continue to produce auditory and vestibular hair cells, suggesting that FGFR-3 and FGF-3 are not critical for hair cell production and differentiation [106,107]. Gene transfer is a powerful technique with great potential for rescue hair cells [108] or spiral ganglion cells [109] from degeneration or damage, for treatment of hereditary inner ear disorders (e.g. [65,110,111]), or to stimulate hair cell regeneration. Successful gene transfer into the inner ear has been accomplished with adenovirus vectors [112–114] or adeno-associated viral vectors [115] into postmitotic cochlear cells *in vitro* [113] and *in vivo* [112,114]. Nearly all tissue types within the cochlea, including the organ of Corti and the spiral ganglion cells, can be successfully transfected. Additional detailed studies will help ascertain whether transfection can be achieved without interfering with the survival and normal functioning of the transfected cells and if the foreign genes can be stably expressed for long periods.

Conclusion

Past studies on non-mammalian vertebrates have shown that hair cells can be formed post-embryologically, enabling the return of normal structure and function in mature auditory and vestibular end organs after damage. Recent studies in non-mammals have provided insight into cellular mechanisms that may be used to stimulate hair cell regeneration in mammals, and it has been shown that the mammalian inner ear may possess cells that can be induced to divide. Evidence that nonmitotic regeneration may occur in several species has been generated, and molecules that upregulate regenerative proliferation in inner ear sensory epithelia have been identified in birds and mammals. Tools (e.g. cell culture methods, cellular markers, and genetic transfer methods) have been developed that will facilitate the identification of additional regulatory molecules and the assessment of their role in hair cell regeneration. Future sudies will also draw from the growing knowledge regarding mechanisms that guide the production and differentiation of hair cells during development.

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