Regeneration and replacement in the vertebrate inner ear

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Deafness affects more than 40 million people in the UK and the USA, and many more world-wide. The primary cause of hearing loss is damage to or death of the sensory receptor cells in the inner ear, the hair cells. Birds can readily regenerate their cochlear hair cells but the mammalian cochlea has shown no ability to regenerate after damage. Current research efforts are focusing on gene manipulation, gene therapy and stem cell transplantation for repairing or replacing damaged mammalian cochlear hair cells, which could lead to therapies for treating deafness in humans.

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Hair cells are the mechanoreceptors found within the inner ear that detect sound and head movements. Serious hearing and balance impairments can occur through the loss of hair cells by aging, environmental stresses, such as loud noises, or exposure to chemotherapeutic drugs, such as cisplatin or aminoglycoside antibiotics. At least 28 million Americans have a hearing impairment but only one out of five people who could benefit from a hearing aid actually wears one (www.nidcd.nih.gov). Because a large proportion of hearing loss involves the loss of hair cells, regeneration or replacement of these cells is a possible alternative to prosthetic devices.

Scientists once believed that warm-blooded animals had a full complement of hair cells at birth and, if lost, the damage was permanent. Over 15 years ago, several studies demonstrated that avians can regenerate their sensory hair cells [1–4]. Other studies demonstrated that the regenerated sensory hair cells were functional (reviewed in [5,6]). Nowadays, the regeneration phenomenon is better understood but the signaling mechanisms regulating hair cell regeneration remain unknown.

The sensory epithelium of the inner ear comprises two different general cell types: sensory hair cells and nonsensory supporting cells. There are several specialized types of hair cells in the mammalian inner ear: in the auditory system (inner and outer hair cells) and in the vestibular system (type 1 and 2 hair cells). These cells can be distinguished by their location in the organ, their morphologies and by the type of neurons that innervate the hair cell. There are also different types of supporting cells found within the mammalian auditory system (e.g. Deiters' cells and pillar cells) that each express unique structural and molecular signatures. However, in the vestibular system, the supporting cells appear to be relatively homogeneous, and scientists have yet to find morphological, molecular or physiological differences between the supporting cells.

Many events occur when the hair cells in the inner ear are damaged or killed. For example, the sensory epithelium is capable of repairing itself when hair cells in the sensory epithelium are damaged with a sub-lethal stimulus [7–9]. However, if the damage is more severe, it normally leads to the death of some or all of the hair cells in the sensory epithelium. Dying hair cells undergo programmed cell death (apoptosis) [10,11] and are either ejected from the sensory epithelia [12] or engulfed by

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neighboring cells. Following the death of the hair cells, the neurons from the VIIIth cranial nerve retract their synaptic terminals. In birds and lower vertebrates, a signal from the dying hair cell induces regeneration by triggering the neighboring supporting cells to either proliferate or transdifferentiate into an immature hair cell. Proliferating cells then respond to environmental, molecular or genetic cues to differentiate into hair cells or supporting cells. Finally, nerve fibers from the VIIIth cranial nerve reconnect the hair cell to the central nervous system so that the animal can process the sensory information.

Fish and chicks: 'lower vertebrate' model systems to study hair cell regeneration

In the past fifteen years, two different mechanisms have been proposed for sensory hair cell regeneration in avian and other non-mammalian species: mitotic proliferation and direct, nonmitotic, transdifferentiation. Many studies indicate that the supporting cells adjacent to a dying hair cell receive a signal to enter the cell cycle. The supporting cells that undergo mitotic proliferation migrate to the luminal surface of the sensory epithelium, duplicate their DNA and then divide into two daughter cells (reviewed in [6,13–15]). The daughter cells then proceed through symmetrical differentiation to produce two hair cells or two supporting cells [16–18], or through asymmetrical differentiation to produce one hair cell and one supporting cell [17–20]. Alternatively, hair cells can transdifferentiate from neighboring supporting cells by non-mitotic mechanisms [21–26]. Transdifferentiation is a switch in gene expression in the supporting cell so that it expresses markers that are characteristic of a developing hair cell. Although non-mitotic transdifferentiation of supporting cells is a simpler way of replacing lost hair cells, it results in the loss of the supporting cells; and often large numbers of hair cells and supporting cells are needed to repopulate the sensory epithelia. Mitotic cell division maintains the structural integrity of the organ by producing hair cells and supporting cells, whereas direct transdifferentiation results in a significant loss of supporting cells if they all transdifferentiate into hair cells.

Functional studies using avians

When it was discovered that birds could regenerate their sensory hair cells, the next logical question was: are the regenerated cells functional? Not only do the supporting cells need to differentiate into hair cells but the newly regenerated hair cells also need to be re-innervated by the VIIIth cranial nerve fibers. Moreover, the animal must use the new sensory information to produce behaviorally meaningful responses. Two recent reviews on functional recovery after sound and drug-induced damage have comprehensively covered these issues [5,6].

Although many studies have examined the physiology of recovered sensory hair cells, few have examined the complex properties of perceptual processing and behavioral plasticity. The recognition and production of vocal signals depend on hearing and are necessary for communication. Budgerigars, *Melopsittacus unduratus*, have been used to examine the renewal of vocal production and complex auditory perception after hair cell regeneration [27,28]. These birds mimic sounds and readily learn new vocalizations throughout life, which has been likened to language acquisition in humans. The birds were trained to match precisely their vocalizations to specific acoustic templates. Aminoglycoside treatment disrupted auditory perception and vocal production. Behavioral tests of auditory sensitivity showed that audiometric thresholds returned to near-normal levels (within 20 dB) within four weeks of deafening. More-complex perceptual tasks, such as vocal call discrimination and/or recognition, took up to five months to return to normal levels. Precision in vocal production initially declined but was restored to pre-treatment levels before the recovery of auditory function. Therefore, relatively little acoustic feedback from a few regenerated hair cells was necessary to guide full recovery of vocal precision.

Another series of studies examined complex communication behavior in male Bengalese finches, *Lonchura striata*, which learn a single sequence of 'syllables' early in life and reliably produce the same song throughout their lifespan [29–32]. After recording each bird's song and verifying its stability, they were treated with a combination of aminoglycosides and sound exposure to induce hearing loss and their songs rapidly deteriorated [33]. Once hearing was restored by hair cell regeneration [30], the song returned to its pre-exposure structure [31]. Restoration of hearing allowed each bird to access a stored 'template' of its own learned vocalization, and gradually match this new vocalizations to the stored memory.

Compensatory behaviors, such as gaze, oculomotor and postural responses, that occur during movement largely depend on a functioning vestibular system. The vestibular ocular reflex and the vestibular colic reflex disappear after the vestibular hair cells of birds are destroyed with aminoglycoside antibiotics [34–38]. However, these reflexes reappear as the hair cells regenerate [35–37,39]. Dickman and Lim [40] trained adult pigeons to run along a chamber and peck an illuminated key. Multiple behavioral measures assessing performance, posture, and head stability were quantified. Once normative values were obtained, the animals received aminoglycosides, which killed the vestibular hair cells and resulted in severe postural and head instability. As the regeneration process progressed, the tremor and head shakes diminished and spatial orientation and navigation ability improved to pretreatment levels.

Zebrafish: a new model system to study apoptosis and hair cell regeneration

Recently, there has been much interest in using zebrafish, *Danio rerio*, as an animal model for studying the inner ear. Zebrafish have sensory hair cells in the vestibular organs

that include two maculae (the utricle and saccule) and the three semicircular canals. Although the zebrafish does not have an auditory organ, such as a cochlea, *per se*, adult zebrafish can detect sound frequencies from ~200 to 4000 Hz [41,42]. Zebrafish also have a lateral line system, which comprises neuromasts that reside along the head and body in a stereotyped manner [43,44]. Each neuromast contains a central cluster of hair cells (surrounded by nonsensory supporting cells) that function to detect water currents relative to the animals' body [45,46].

Zebrafish embryos can be easily manipulated experimentally because they develop rapidly *ex utero* (the first hair cells can be detected 24 h after fertilization [47]). For example, 'small molecules' can be added to the aqueous environment to determine which molecules affect inner ear development, or whether these chemicals could have a protective effect against toxins, such as aminoglycoside antibiotics. This makes zebrafish a very amenable model for small-molecule chemical screens.

Zebrafish provide a genetically tractable vertebrate because progeny can be chemically mutated and the subsequent inner-ear mutants can be isolated with forward-genetic screens using behavioral assays and observation [48,49]. Many essential genomics resources, including genome maps, large-insert genomic libraries, radiation hybrid panels and expressed sequence tag databanks, have been developed for the zebrafish and continue to improve (www.zfin.org).

Several studies have examined the death and subsequent regeneration of hair cells in the lateral line [50–52]. In two studies, zebrafish larvae were treated with neomycin [51,52]. In one study, supporting cell proliferation increased 12 h after treatment and regenerated hair cells were observed 1–2 days later [51]. Future studies will use this preparation to identify genes that influence vertebrate hair cell death, survival and regeneration following ototoxic insults.

The mammalian cochlea: to grow where none has grown before

Although all non-mammalian vertebrates regenerate hair cells, two crucial questions remain about the mammalian cochlea: why have only mammalian vestibular hair cells shown any capacity for regeneration and why are hair cells (and supporting cells) in the auditory portion of the inner ear apparently unable to regenerate after damage? The supporting cells in the mammalian auditory system undergo terminal mitosis during embryogenesis, whereas the supporting cells in the mammalian vestibular system retain some limited capacity to regenerate in adulthood [53,54]. However, there is a window of time during embryonic development when additional hair cells can be induced to develop in the immature cochlea. Undifferentiated progenitor cells that normally give rise to the mouse organ of Corti form supernumerary hair cells if they are treated with retinoic acid between embryonic days 13 and 16 [55,56]. Furthermore, if existing hair cells are killed by laser ablation during this developmental period, adjacent uncommitted progenitor cells will change their fates and differentiate into hair cells to replace those that were lost. Surprisingly, labeling experiments with markers for DNA synthesis indicates that the additional and/or replacement hair cells do not arise from proliferation of the existing hair cells. Rather, it appears that existing progenitor cells within the developing organ of Corti are able to change their developmental fates in response to changes in their local environment. Thus, at least for a short time during cochlear development, the progenitor cells are multipotent and have the ability to develop into many different cell types.

It has been proposed that the mammalian cochlea shuts down its proliferative and regenerative capacity after embryogenesis to establish a more stable and complex auditory processor. Although this has functional benefits, it leaves the system vulnerable to genetic mutation and the more-recently evolved societal assaults of noise-induced hearing loss and ototoxic drug damage. Given that it appears that the mammalian cochlea is unable to regenerate on its own, scientists have developed three general strategies intended to artificially force the inner ear to regenerate or replace lost sensory cells, including: (1) the experimental manipulation (knockouts, transgenics) of genes that inhibit proliferation in the organ of Corti; (2) the intentional transfection (gene therapy) of cochlear cells with viruses carrying genes for inducing hair cell differentiation; and (3) the replacement of lost cochlear cells with either intrinsic or extrinsic (transplanted) stem cells.

Genetic manipulation in the mammalian inner ear

Several genes negatively regulate cellular proliferation in mature, highly differentiated tissues to stop uncontrolled growth (i.e. to stop them becoming cancerous). The *p27/Kip1* and *pRb* genes are activated in the mammalian cochlea at the time of terminal mitosis in the sensory epithelium [57–60]. Targeted deletions of these two genes lead to a prolonged period of mitotic activity in the sensory epithelium and an overproduction of hair cells and supporting cells [57–59]. Following an initial extended period of proliferation of progenitor cells in the *p27* knockouts, proliferation terminates by postnatal day six and is soon followed by extensive hair cell death [58] and the loss of auditory function [57,58]. The *pRb* knockouts exhibit a robust and continued proliferation of progenitor cells and an extensive overproduction of hair cells [59,60]. Moreover, the hair cells show clear mitotic activity [59,60], athough this often results in aberrant or multinucleated hair cells [60]. Unfortunately, these *pRb* mutants die at birth, so the effect of the overproduction of hair cells on cochlear function and the maturation of the organ of Corti are not known. Similarly, targeted deletion of *p19/ink4d*, another inhibitor of cell cycle progression, causes the initiation of DNA synthesis in hair cells but then leads to the precocious apoptosis of these cells and subsequent hearing loss [61].

Thus, these cell cycle inhibitors are used by the cochlear tissues to downregulate mitosis in the sensory epithelium and establish a mature, non-proliferating sensory epithelium. The elimination of these blockers initially leads to continued proliferation and overproduction of hair cells. However, these cell cycle inhibitors must also have another critical developmental function because eliminating them results in premature death of the hair cells or even the entire animal. Although these studies are a proof-of-principal that regeneration in the mammalian cochlea can be activated by elimination of specific mitotic blockers, there are additional consequences to the loss of these inhibitors that need to be understood before they can be used as a therapeutic approach to hearing loss.

Gene therapy in the mammalian cochlea

Recently, several studies have demonstrated that the mammalian homolog of the *Drosophila* transcription factor *atonal1* – *Math1* – is sufficient for hair cell genesis in inner-ear tissues. *Math1* is expressed in developing hair cells [62,63], and transgenic mice that are homozygous for the targeted deletion of the *Math1* gene lack vestibular and cochlear hair cells [63]. Moreover, transfection of *Math1* (or its human analog, *HATH1*) into mouse organ of Corti or utricular cultures [64,65] is sufficient to induce new hair cell development. Recent *in vivo* experiments have demonstrated that *Math1* transfected into the cochleas of guinea pigs deafened by kanamycin led to an extensive structural and functional recovery of the organ of Corti [66,67]. In these cases, the new hair cells appear to arise from direct transdifferentiation of existing supporting cells, rather than through the mitotic production of new cells. Although these studies are in their beginning stages, transfecting *Math1* into the deafened cochlea could prove to be a promising option for gene therapy. Target specificity is problematic in many gene therapy paradigms [68]. For example, when *Math1*-encoding viral particles were successfully introduced into guinea pig cochlea, there was an ectopic production of hair cells in places other than the organ of Corti [66]. Therefore, to re-establish the proper connections after damage, a model must be established where *Math1* can be more effectively delivered directly to the site of hair cell loss.

Stem cells in the inner ear

Stem cells have received considerable attention both in the scientific community and in the popular press because of their potential to repair or regenerate damaged cells and tissues in the human body. These cells have the capacity to give rise to any cell type in the body, and the fate of their progeny is determined by the microenvironment in which the stem cells reside. Thus, it has been proposed that if intrinsic stem cells in the inner ear could be activated or if extrinsic stem cells could be transplanted into the ear, they could produce new hair cells and/or neurons to replace those that have been lost.

Recently, a population of cells from the utricles of mice have been isolated that have been described as end-organ stem cells [69]. These isolated cells exhibit the capacity to self-renew and to produce cell types with the morphologies and biological markers of hair cells (myosin VIIa and Brn 3.1) and supporting cells (pancytokeratin and $p27^{Kip1}$). Additionally, these mouse cells were able to incorporate into muscle and liver when transplanted into the developing embryo of a chicken, which suggests a multipotency characteristic of stem cells. However, it is not completely clear that these stem cells were derived from cochlear epithelial cells, because there is a possibility that they could have arisen from mesenchymal or vascular stem cells in the tissue. It is also worth noting that, although these stem cells apparently reside in the vestibular sensory epithelia, there is no evidence that they contribute to recovery in the cochlea.

In other studies, researchers have manipulated stem cell populations to get them to express characteristics of neural or hair cell precursors. Mouse embryonic stem cells have been induced down a pathway that resembles neural progenitor cells [70]. When these cells were transplanted into an embryonic chick otocyst, they gave rise to new hair cells. Other investigators [71] were able to induce mouse embryonic stem cells to form neuron-like cells by overexpression of bHLH. Similarly, treating mouse bone-marrow stem cells with sonic hedgehog and retinoic acid enabled the stem cells to differentiate into neuron-like cells [72]. Strategies like these will allow readily available stem cells to be primed to have a more cochlear fate, and could be used for transplantation into damaged cochleas.

A few groups have attempted to transplant neural stem cells into the inner ears of mammals. Rat hippocampal stem cells have been injected into the cochleas of newborn rats, which exhibited some integration of the stem cells into the cochlear epithelium [73]. Adult neurospheres were transplanted into the cochleas of normal or neomycin-treated guinea pigs [74]. There was a higher survival rate of stem cells in the neomycin-treated cochleas, and it was reported that some of the surviving cells were beginning to express neuronal markers. Experiments in our laboratory [75,76] have transplanted an immortalized line of neural stem cells into the noise-damaged cochleas of mice and guinea pigs. These stem cells survived for up to six weeks, integrated into the spiral ganglion and organ of Corti in the damaged regions, and differentiated into neurons, glia, hair cells and supporting cells.

These various stem cell studies are all still preliminary but they do indicate that there might be a resident mammalian stem cell population, at least in the vestibular end organs. Moreover, the introduction of pluripotent stem cells into a damaged ear could result in these cells integrating into the remains of the sensory epithelium, thereby deriving the necessary signals for their differentiation into hair cells, supporting cells and neurons that

could repopulate and repair the damaged sensory epithelium. However, the same difficulties exist for transplantation as for gene therapy experiments. Integrating the transplanted cells to into damaged epithelium and generating the correct numbers of cells in the correct parts of the organ of Corti will be a challenge. Given that much of cochlear function depends on the mechanical properties of the organ of Corti, excess or inappropriately placed cells are likely to cause problems.

Summary

Regeneration in the vertebrate inner ear offers an exciting opportunity to explore the cellular, molecular and functional mechanisms that are involved in rebuilding the exquisitely sensitive sensory receptor complexes of the vestibular and cochlear end organs and their connections to the central nervous system. Regeneration occurs naturally or in response to damage or trauma in the bird cochlea and in the vestibular epithelia of mammals, birds and lower vertebrates. Thus, the regulatory pathways that control regeneration are readily available for defining and manipulation. Regeneration in non-mammalian systems offers a unique pharmacological opportunity to explore and modulate the pathways that lead to hair cell regeneration and could give important insights into the potential for inducing mammalian hair cell regeneration. The mammalian cochlea has not yet shown an innate capacity for regeneration but current techniques involving genetic manipulation, gene therapy and stem cell transplantation could enable us to unlock the cochlea's ability for structural repair and recovery of function. These research opportunities offer an exciting potential for developing cell- or drug-based therapies for treating hearing loss and balance disorders in humans.

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