

Fish n' Chicks: Model Recipes for Hair-Cell Regeneration?

Minireview

Jeffrey T. Corwin*[‡] and J. Carl Oberholtzer[†]

*Departments of Otolaryngology–Head and Neck Surgery and Neuroscience

University of Virginia School of Medicine
Charlottesville, Virginia 22908

[†]Department of Pathology and Laboratory Medicine
Division of Neuropathology
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

In nearly every waking moment, our brains respond to signals that originate from hair cells. These cells reside in six separate epithelia in our internal ears and are detectors of head rotation, gravity, and sound. Hair cells can be killed by loud sounds, certain antibiotics, and other drugs. Some are lost through infections and aging. Any loss is potentially significant, since hair cells are not added to the human ear after birth, according to the accepted view. "Nerve deafness," a permanent form of hearing loss, actually results from loss of hair cells in most cases, not from damage to nerves. Permanent balance dysfunctions also result from hair-cell loss in many cases. These conditions affect ~10% of the population and 25% of people over the age of 65, making hair-cell loss one of the most common neurological deficits. Unfortunately, despite considerable progress in understanding the physiology of hair cells and the neural basis of hearing and balance, most deficits that result from hair-cell loss have remained irreversible.

In contrast to the situation in humans, hair cells are produced throughout life in the ears of fish, amphibians, and birds. The discovery that hundreds of thousands of hair cells are added to the ears of postembryonic sharks led to the proposal that hair-cell loss might be repaired via regenerative replacement mechanisms that could form the basis for regenerative treatments. Hair cell regeneration does indeed occur in the ears of many vertebrates, even in organs such as the chick's cochlea, where cell production normally ends before birth. Treatments that cause permanent hearing and balance deficits in humans also kill hair cells in birds, fish, and amphibians, but in those species the loss of hair cells can evoke cell proliferation at the site of damage. The newly generated cells differentiate into supporting cells and replacement hair cells, which make synapses with surviving neurons. In nonmammalian ears, these repair processes can lead to rapid recovery from hearing and balance deficits that would be permanent in a human (references in Corwin and Warchol, 1991; Cotanche and Lee, 1994).

Supporting Cells Are Relatively Undifferentiated in the Most Plastic Hair-Cell Epithelia

Prototypical hair-cell epithelia contain only two resident cell types: hair cells and the supporting cells that cover

the basal lamina and extend to the apical surface between neighboring hair cells. Terminal neurites of afferent and efferent neurons and small numbers of roving leukocytes are also present. Proliferative, multipotential progenitor cells appear to be required for the initiation of regeneration in amphibian limbs and for most forms of regeneration in animals (references in Brockes, 1997). Consistent with that, supporting cells in fish, amphibians, and birds proliferate rapidly after hair cells have been lost, and those epithelia regenerate lost hair cells. In balance epithelia from rodent and human ears, small numbers of supporting cells will proliferate in culture after hair cells have been killed, but the response is much weaker than in nonmammals. All those epithelia contain relatively undifferentiated supporting cells that cannot be reliably subdivided on the basis of histological characteristics, so a relative lack of supporting cell differentiation and a capacity for regenerative proliferation may be linked (Table 1). Such a linkage is consistent with the apparent lack of plasticity in the organ of Corti, the auditory epithelium of placental mammals. Its supporting cells are structurally specialized as five differentiated subtypes, which are all effectively nonproliferative during postembryonic life. In rare cases of damage in cultures from neonates, the organ of Corti may be able to replace hair cells after birth, and that also can occur through a cell-fate change in embryonic organs (Kelley et al., 1996). One study of organs of Corti cultured from neonatal rats has reported dramatic and complete healing after hair-cell poisoning by an antibiotic, but other investigators have challenged that report. Partial healing responses in this organ have recently been reported.

Hair-Cell Regeneration in Chicks

Each chick cochlea contains roughly 10,000 hair cells that form a phenotypic gradient, wherein the cells differ progressively in size and shape along and across the epithelium, as do the numbers, dimensions, and geometric arrangements of the stereocilia in their hair bundles. There is little, if any, proliferation in the undamaged auditory epithelia, but a loss of hair cells evokes proliferation within ~200 μm of the site of loss, beginning after 16 hr (Warchol and Corwin, 1996). It appears that the division of a supporting cell can give rise to one cell that becomes a hair cell and to another that becomes a supporting cell (Jones and Corwin, 1996). Supporting cell divisions may also give rise to pairs of supporting cells and pairs of hair cells. One hypothesis proposes that new cells become hair cells by default unless they develop in contact with a cell already determined as a hair cell. Homologs of the Delta-Notch signaling system of *Drosophila* may control cell-fate choices in hair cell epithelia via lateral inhibition during development (Whitfield et al., 1997); lateral inhibition may also regulate the number of hair cells formed at sites of regeneration. Replacement hair cells acquire morphological phenotypes appropriate for their positions in the two-dimensional epithelium. Whatever signals are responsible for the establishment of hair-cell phenotypic gradients during development must have influences during hair-cell regeneration.

[‡]To whom correspondence should be addressed.

Table 1. Characteristics of Development, Plasticity, and Cellular Differentiation in Hair-Cell Organs of the Vertebrates

Type of hair cell epithelium	Embryonic Development	Postembryonic Development	Ongoing Plasticity	Duration of Plasticity	Degree of hair cell differentiation	Degree of supporting cell differentiation	Regenerative Ability
Lateral line organs of fish and amphibians	Cranial ectodermal placode origins. Epithelia form after migration.	HCs & SCs are added throughout life. Organs are added in postembryonic growth.	HC & SC populations turnover throughout life.	Regeneration throughout life. Cell turnover probably occurs throughout life.	One type recognized	Cytologically similar mantle, internal, and basal SCs can be distinguished by positions.	Organs are regenerated if amputated. HCs are regenerated from SC divisions.
Hearing and balance organs in fish and amphibian ears	First HCs produced soon after invagination of otocyst.	HCs & SCs are added throughout life in many species.	Possible slow turnover. Possible bundle repair.	Regeneration persists throughout life.	Multiple types recognizable as developmental stages.	Rather undifferentiated cells.	HCs are regenerated from SC divisions.
The balance epithelia in birds	Hair cells produced in the first third of incubation.	HC populations do not appear to increase post-embryonically	Active turnover of HC populations. Short HC life spans.	Regeneration is presumed to persist throughout life.	Two distinct types of HCs and innervation.	Rather undifferentiated cells.	HCs regenerated from SC divisions.
The hearing organ in birds	HCs & SCs all produced in the first half of incubation.	No increase in HCs and no measurable proliferation.	No measurable cell turnover.	Regeneration occurs in juveniles and in adults.	Two types of HCs, with phenotype gradients along the organ.	Rather undifferentiated cells.	HCs regenerated from SC divisions in the vicinity of damage.
The balance epithelia in mammals	HCs & SCs produced during the second half of gestation & for two days after birth.	No significant increase in HC populations.	Some rare HCs with small bundles in adult rodents. Possible bundle repair.	Trauma-evoked proliferation of SCs from adult rodents and humans. HCs numbers decline in elderly.	Two types of hair cells with distinct morphology and distinctly different patterns of innervation.	Rather undifferentiated cells.	Trauma-evoked proliferation in SCs at low rates. Bundles reappear after antibiotics.
The hearing organ in mammals.	HCs & SCs all produced in the second half of gestation.	No evidence of cell proliferation after birth.	No evidence for turnover.	No plasticity in adults. Considerable evidence for decline in HC populations with normal aging throughout life.	Two highly differentiated HC types, with phenotype gradients along and across the organ.	All the SCs are highly differentiated, with at least five types in a highly ordered arrangement.	OHCs killed in embryo are replaced via fate changes. Trauma-related divisions extremely rare if occurring at all.

Mechanoreceptive hair cells are present in three types of sensory organs in the vertebrates. The patterns of development of the sensory epithelia and the degree of differentiation of the supporting cells in those organs differ considerably between species. Differences in regenerative capacities appear to parallel some of those differences.

Unlike hair cells in the hearing organ, those in the chick's balance organs turn over throughout life, so losses are repairable via up-regulation of constitutive replacement processes. Under control conditions, hair cells in these organs live 20–29 days on average, according to estimates based on measures of proliferation and apoptosis. Factors that limit the life spans of these hair cells are currently unknown. Their identification might contribute to understanding the longevity of human hair cells, whose life spans have been presumed to extend to a century or more.

Hair-Cell Development Provides Some Leads for Understanding Regeneration

The understanding of inner-ear development is becoming more sophisticated as gene-expression patterns identify candidate signals for the control of morphogenetic patterns and cell fates. Some of the genes are expressed only transiently during development but may be expressed again during hair-cell regeneration. The expression of a few persists through late development into maturity. The products may play roles in growth or maintenance in mature hair-cell epithelia. Gene-expression patterns in developing ears have been reviewed

recently and expertly (Fekete, 1996; Whitfield et al., 1997) and are beyond the scope of this Minireview. However, we shall briefly review studies of two types of genes that are expressed early and in fully developed epithelia.

In chick embryos, the transforming growth factor β (TGF β) superfamily members BMP4 and BMP7 are expressed from the early otocyst stage throughout inner-ear morphogenesis. At hatching, BMP4 expression is restricted to hair cells in the cochlea and supporting cells in vestibular epithelia, while BMP7 expression is limited to supporting cells in the cochlea and has disappeared from the vestibular epithelia (Oh et al., 1996). The differing patterns of expression and the persistence in those cells suggest a signaling role.

In the rodent ear, the class IV POU-domain transcription factor Brn-3.1 (Brn-3c) is expressed in hair cells only, from the time of their differentiation to adulthood (Erkman et al., 1996). Null mutants for Brn-3.1 are deaf and have impaired balance, resulting from a complete absence of differentiated hair cells. Initially, both auditory and vestibular hair cells appear to develop, but they degenerate perinatally, so continued transcription of

Brn-3.1 appears to be required for maintenance of hair cells. Nearby supporting cells also dedifferentiate or die as a secondary consequence of the null mutation.

The Search for Triggers of Hair Cell-Regeneration

Nontumorigenic cells require the binding of mitogenic growth factors to specific receptors expressed on their surfaces before they will proliferate. Dozens of growth factors probably will be tested for effects on supporting cells. Recently, fibroblast growth factor 2 (FGF-2), a potent mitogen for many cells, has emerged as a candidate mitogen for supporting cells in chicks, according to preliminary results from culture supplementation (Corwin et al., 1996) and more extensive RT-PCR and immunocytochemical investigations (Lee and Cotanche, 1996). An antibody to FGF-2 labels the nuclei of all supporting cells in the chick cochlea, but an antibody to an FGF receptor produced heavy staining only at the expanded apical surfaces of supporting cells at sites of hair-cell loss. The results are consistent with a potential role for FGF-2 in the proliferative regeneration in chicks but are not yet conclusive.

Supporting cells in the chick's vestibular epithelia proliferate and give rise to new hair cells at approximately normal rates when cultured without serum or exogenous growth signals, indicating that the triggers for proliferation are present within the epithelium itself. Insulin-like growth factor 1 (IGF-1) and higher concentrations of insulin increase proliferation in cultures of avian vestibular epithelia; bombesin, epidermal growth factor (EGF), and TGF α do not.

The discovery that some supporting cells in the balance epithelia from mature mammals would proliferate after hair-cell loss led to explorations of growth factor enhancement. TGF α increases proliferation of supporting cells in cultured utricles from adult mice after antibiotics have killed hair cells, and it increases proliferation in undamaged epithelia, as does EGF when present together with insulin. Messenger RNAs for EGF receptor, FGF receptor-1, IGF-1 receptor, and insulin receptor are all present in hair-cell epithelia from damaged and undamaged utricles of rats, and platelet-derived growth factor α (PDGF α) receptor protein is most heavily expressed in hair cells (Saffer et al., 1996). Thirty different growth factors have been combined with serum in cultures of semi-dissociated sheets of utricle epithelia from neonatal rats to screen for mitogenic effects on supporting cells (Zheng et al., 1997). DNA synthesis increased in cultures that were supplemented with TGF α , EGF, IGF-1, and several FGF family members. FGF-2 produced the greatest increase, and the effects of TGF α and IGF-1 were additive when individually combined with FGF-2. Neutralizing antibodies to FGF-2 and IGF-1 reduced proliferation below control levels. An antibody to FGF-2 labeled hair cells but not supporting cells. Further *in vitro* and *in vivo* tests will be needed to evaluate the potential effects of FGF-2 on mammalian epithelia *in situ*. Many growth factors remain to be tested individually, in combination, and in specific sequences. The extracellular signals that control proliferation in hair-cell epithelia may be complex and redundant.

One intracellular signaling pathway has been revealed through studies of the chick's auditory epithelium. Agents that increase intracellular cAMP levels induce

proliferation of supporting cells and result in new hair cells (Navaratnam et al., 1996). Protein kinase A inhibitors decreased forskolin-induced stimulation of supporting cell proliferation and reduced the regenerative proliferation induced *in vitro* by hair-cell poisoning with an antibiotic. The results suggest that loss of hair cells may lead to elevation of cAMP levels in supporting cells and thereby stimulate proliferation. Elevation of cAMP levels might either result from the removal of a tonic signal emanating from hair cells that prevents the accumulation of cAMP in nearby supporting cells or from the generation of a positive signal that elevates cAMP levels during hair-cell loss. If the cAMP pathway plays a major role in regeneration *in vivo*, it might interact with the mitogen-activated protein (MAP) kinase cascade or other pathways via modification of intermediate signaling species. Alternatively, forskolin and elevated cAMP could influence proliferation by activating transcription of a growth factor receptor gene in supporting cells, making them responsive to a proliferation-signaling ligand already present under normal conditions.

The sources of the signals that induce supporting cells to proliferate might be identified more readily than the individual factors. Neighboring cells or tissues in the ear, roving macrophages that are present in the ear and attracted to sites of damage, dying hair cells, or the supporting cells themselves could all be potential sources of growth factors or other regulatory signals. Cell ablations via a laser microbeam have shown that supporting cells that are near to sites of hair cell loss, but not directly within the site, are some of the first to proliferate. That is inconsistent with a requirement for proliferation signaling via membrane-bound molecules (Warchol and Corwin, 1996). It is possible that growth-influencing signals such as second messengers are transmitted through the gap junctions that link supporting cells. The loss of hair cells causes immediate and dramatic changes in the shapes of nearby supporting cells. Under normal conditions, hair cells retain a characteristic shape. When hair-cell epithelia are dissociated, hair cells retain roughly the same shapes they had *in situ*, but supporting cells become spherical. It is presumed that higher internal pressure or stiffer cytoskeletons in hair cells cause the supporting cells to conform to the shapes of the small spaces left between neighboring hair cells. The loss of hair cells from these epithelia would allow supporting cells to spread into the region of loss and therefore become less constricted in shape. Spreading shape changes of this sort and mechanical tension changes have been shown to promote cell proliferation in endothelial cells (Chen et al., 1997).

What Is Responsible for the Lower Regenerative Responsiveness of Hair-Cell Epithelia in Mammals?

The correspondence between regenerative capacities of hair-cell epithelia and the degree of structural differentiation of their supporting cells is limited. Supporting cells in mammalian and nonmammalian balance organs are not recognizably different, but they differ markedly in the level of proliferation evoked by a loss of hair cells. Molecular differences that directly limit the capacity to dedifferentiate and become proliferative would not necessarily be evident in histology. The differences that

cause mammalian supporting cells to have more limited regenerative responsiveness than homologous cells in fish and birds should be investigated at the level of gene expression and posttranslational modifications. Identification of those differences just might hold the key for unlocking the regenerative potential of mammalian ears.

One hypothesis is that homologous supporting cells in mammals and nonmammals have such dramatically different proliferative responses to hair-cell loss because of differences in the expression or activities of tumor suppressor gene products. Highly differentiated, nonproliferative cells, such as pigmented epithelial cells from the iris and retina and chondrocytes, can dedifferentiate and become proliferative during regeneration. When regenerative blastemas are cultured, the multinucleate myotubes of newts also can be caused to dedifferentiate and proliferate, whereas those from birds and mammals are postmitotically arrested and do not dedifferentiate. The phosphorylation of Rb protein differs between those myotubes (Brookes, 1997). The hypophosphorylated form of pRb binds E2F transcription factors and prevents passage through the G₁-S restriction point of the cell cycle; thus, pRb prevents inappropriate proliferation of differentiated cells in animals other than salamanders. Such tonic negative signals may have to be nullified for significant regeneration to occur in mammalian ears. To our knowledge, tumors comprised of cells arising from hair-cell epithelia have not been reported, a situation quite unlike that for most epithelia. Suppression of cell proliferation also could be influenced by integrins and components of the ECM, by differences in the expression of growth factor receptors and ligands, and possibly by cytoskeletal components.

Perspectives

Permanent hearing and balance deficits often arise in humans via the loss of one type of cell, the hair cell. Histological simplicity and conservation across vertebrate species that shared common ancestry over 450 million years ago may help investigators who hope to learn how to control the regeneration of hair cells. Animal models for hearing and balance loss are representative of the human conditions, because the same treatments cause the hair cell losses, yet some of those models regenerate and recover function. It is advantageous that the epithelia of those models can develop and regenerate when denervated; such models should continue to be of profound value. In addition, mammalian ears must be studied directly. It appears likely that the low potential for dedifferentiation and proliferation of supporting cells is what limits regeneration in mammalian ears, but it is possible that stem cells play a role in some epithelia and might be missing from others. The small size and inaccessibility of hair-cell epithelia contribute to slow progress but are not insurmountable. The current absence of appropriate cell lines, the small selection of applicable cell markers, and the limited numbers of identified mutants have also been barriers, but these deficiencies of tools and reagents are being addressed. Efforts to develop regenerative therapies for balance dysfunctions certainly appear more tractable than approaches to hearing loss, although for the balance epithelia it seems reasonable to predict that the problems ahead are not all revealed and may be difficult and complex. There are, however, reasons for optimism. When

the molecular mechanisms that result in the regeneration of hair cells in model species are understood, that should contribute to new and fruitful approaches to their counterparts in mammals. Even before that, mammalian epithelia may reveal their own secrets in the proper forms of molecular sorcery. Limb of newt and ear of chick could be the start of the recipe.

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