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Non-sensory cells in the deafened organ of Corti: approaches for repair

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ABSTRACT After moderate cochlear trauma, hair cells degenerate and their places are taken by phalangeal scars formed by differentiated supporting cells. A short time after trauma, these supporting cells can respond to an induced expression of genes which signal hair cell differentiation during normal development and transdifferentiate into new hair cells. However, these non-sensory cells often lose their differentiated features after severe insults or prolonged hearing loss and become a simple, flat epithelium. The flat organ of Corti can serve as a substrate for gene- and stem cell-based therapies. Despite its prevalence, the flat epithelium is not well characterized. Recent data show that cells of the flat epithelium can divide and maintain the structural confluence of the membranous labyrinth. The mitotic potential of these cells should facilitate production of cells for therapies based on recapitulation of development or insertion of stem cells.

KEY WORDS: *hair cell, supporting cell, ototoxicity, regeneration, stem cell*

Types of cells and roles in normal ear

A critical component of the mammalian inner ear is a specialized epithelium called the organ of Corti, which is composed of two types of highly modified cells: sensory hair cells and non-sensory supporting cells. When the sensory hair cells degenerate, hearing is compromised. Research aimed at designing ways to replace these cells must focus on the surviving supporting cells. These supporting cells may remain in one of two states: (a) as tall columnar cells that continue to exhibit their specialized molecular and structural features, or (b) as short cuboidal cells that lack features of differentiated supporting cells. This chapter discusses potential therapies that may be applicable for each of these two types of supporting cells. Non-sensory cells that flank the organ of Corti should also be considered in designing hair cell replacement therapies. The chapter describes concepts related the potential therapeutic application of genes, molecules and signalling cascades described in other chapters of this Review Issue.

Roles of supporting cells in repair of non-mammal ears

Most epithelial tissues turn over and are able to repair after injury. Epithelia add new cells using a mechanism based on proliferation of stem cells. Examples include the epithelium of the intestinal brush border and skin. However, the auditory sensory epithelium found in birds and mammals is an exception in that stem cells have not been identified in these tissues. In birds, lost hair cells can be spontaneously replaced (Corwin and Cotanche, 1988, Ryals and Rubel, 1988). The structural recovery observed in the bird auditory epithelium is accompanied by impressive functional restoration (Adler *et al.*, 1993, Dooling *et al.*, 1997, Marean *et al.*, 1995, Niemiec *et al.*, 1994). The cellular basis for hair cell regeneration in birds is spontaneous transdifferentiation of supporting cells into new hair cells with or without mitosis (Adler and Raphael, 1996, Roberson *et al.*, 1996, Stone and Cotanche, 1994). The ability of mature differentiated cells to change their identity is rather uncommon. It does however point to supporting cells as a potential source for generating new hair cells in the mammalian ear. As such it is necessary to better understand the molecular mechanism initiating and regulating the phenotypic changes that occur when supporting cells spontaneously transdifferentiate to hair cells in birds.

Transdifferentiation in the basilar papilla may occur with or without mitosis (Adler and Raphael, 1996, Raphael, 1993, Roberson *et al.*, 1996; Stone and Cotanche, 1994). In cases of direct transdifferentiation (no mitosis) cells with a mixed phenotype can be seen in the sensory epithelium. Such cells exhibit features of hair cells and supporting cells (Adler and Raphael, 1996). The molecular signalling that regulates transdifferentiation in the basilar papilla involves Notch signalling and bears similarity to the developmental events (Stone and Rubel, 1999) and *Atoh1* is involved in the differentiation of the new hair cells (Cafaro *et al.,* 2007).

When the experimental lesion in the basilar papilla is extremely

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severe, hair cell regeneration is incomplete. In these cases cells from areas flanking the sensory epithelium may migrate into the sensory epithelium site (Cotanche *et al.,* 1995). This study demonstrated that the degree of damage to the non-sensory cells determines the ability of these cells to generate new hair cells and repair the epithelium and its function. It also showed that epithelial cells from areas adjacent to the sensory epithelium may migrate and contribute to the maintenance of the epithelial layer. Both of these facts are considered below for their relevance in the inducement of regeneration in the mammalian ear. On the whole, the ability of differentiated non-sensory cells to become new functional hair cells and restore hearing in birds provides a conceptual strategy for inducing a similar regenerative mechanism in the mammalian auditory epithelium. In pursuing this concept it is important to direct the focus of the experiments to the biology of the non-sensory cells that remain in the ear once hair cells are lost.

Lesions that leave differentiated non-sensory cells

Two methods are likely to be successful in generating new hair cells. The first is based on the use of developmental genes to reactivate developmental signalling cascades and induce transdifferentiation of non-sensory cells into new hair cells. The second involves the introduction of stem cells into the cochlea. Once the stem cells integrate in the host tissue they can differentiate and function as hair cells. Both methods rely on surviving non-sensory cells as the substrate for these therapies. Therefore, one of the most important goals for advancing this technology is the characterization of the recipient tissue, the target and substrate for the treatment. Several factors influence the fate of non-sensory cells that remain into the ear. Among the important factors are the type (etiology) and severity of deletion and the amount of time since the lesion occurred.

As hair cells degenerate after a moderate ototoxic insult, a highly regulated and complex mechanism of scar formation is initiated by the non-sensory cells that surround each dying cell (Forge, 1985, Leonova and Raphael, 1997, Raphael and Altschuler, 1991a, Raphael and Altschuler, 1991b). The immediate role of the scarring process is probably to prevent fluid mixing. It is especially important to prevent leakage of endolymph into the fluid bathing the basal domain of inner hair cells where terminals of the auditory nerve reside. Potassium-rich endolymph would depolarize the hair cells and the neurons, abolish hearing and most likely lead to additional trauma to the organ of Corti.

For a short time after an ototoxic lesion induced by kanamycin and ethacrynic acid, the supporting cells that form the phalangeal scars retain many of their differentiated features (Forge, 1985, Lenoir *et al.*, 1999, Raphael and Altschuler, 1991a). The cells remain tall, some of the cytoskeletal features remain intact and the apical surface of the epithelium remains well organized and resembles the normal organ of Corti except that hair cells are missing (Figs. 1A-C and 2A,B). A similar pattern of scarring may also appear after lesions that are less severe than the combination of kanamycin and ethacrynic acid. Overstimulation by noise or sound that results in hair cell loss may also leave the non-sensory cells differentiated (Abrashkin *et al.,* 2006). The transition from a normal organ of Corti to an epithelium containing differentiated supporting cells and no hair cells may occur within one or two days (Izumikawa *et al.*, 2005, Wang and Li, 2000).

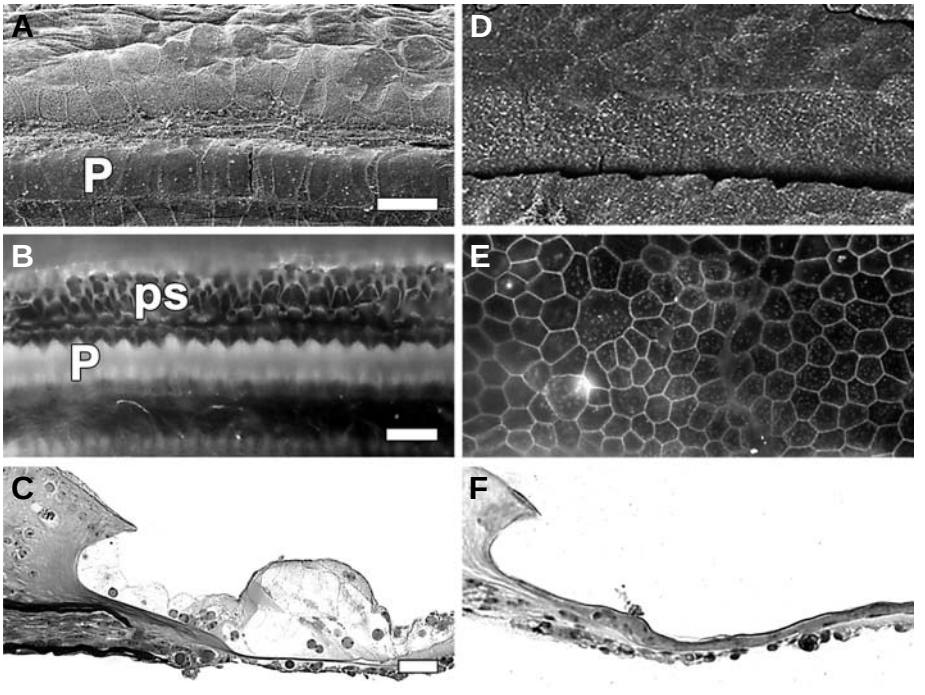

Fig. 1. The area of the organ of Corti after hair cells are eliminated using kanamycin and ethacrynic acid (A-C) or neomycin (D-F). (A) *SEM view of the surface showing lack of hair cells, while pillar cells (p) remain differentiated and well organized.* **(B)** *A whole-mount stained with phalloidin and photographed immediately below the reticular lamina showing differentiated pillar cells and well organized phalangeal scars (ps) in the former outer hair cell area.* **(C)** *A plastic section showing the auditory epithelium devoid of hair cells with surviving tall pillar cells and other supporting cells.* **(D)** *SEM image of the flat epithelium in which no organized cellular pattern can be discerned.* **(E)** *Phalloidin labeled whole-mount viewed at the reticular lamina showing a poorly organized epithelium with abundance of actin in intercellular junctions.* **(F)** *A plastic section showing a flat epithelium lining the scala media on the basilar membrane. Bars are 20 μm and apply to the paired image on the right.*

When non-sensory cells in the organ of Corti remain differentiated despite the loss of hair cells, cell proliferation or newly generated cells are not seen in this area (Roberson and Rubel, 1994, Wang and Li, 2000). However, these two studies showed that a small number of BrdU positive cells can be found in areas flanking the organ of Corti. Such cells are not found in ears that do not receive an experimental insult.

The condition of the sensory epithelium in ears that lose their hair cells due to hereditary disease in not well characterized in animal models, but at least in some cases, the epithelium becomes flat (Pawlowski *et al.,* 2006). In other cases pillar cells can be retained for several months despite the loss of hair cells (Hertzano *et al.*, 2004). In human ears, tem-

poral bone studies have mostly been done in ears with genetic disease that also received cochlear implants. In these ears the auditory epithelium was found to be flat in most cases"(Nadol, 1997, Nadol and Eddington, 2006, Nadol *et al.*, 2001). Temporal bone studies in aging ears of humans often show flat epithelium on the basilar membrane, but animal studies show that older animal ears may have differentiated supporting cells remaining.

Lesions that leave a flat epithelium

Severe lesions may lead to loss of hair cells as well as degeneration of supporting cells. Once differentiated supporting cells disappear, a layer of flat or cuboidal epithelial cells lines the scala media where the organ of Corti used to reside (Figs. 1 D-F and 2C). These short cuboidal cells have not been studied in detail, largely because there was no clear benefit to understanding their characteristics. However, recent progress in gene therapy and stem cell biology has created a need to understand these cuboidal cells and the flat epithelial layer that they form. Clearly, the flat epithelium will play a prominent role as the recipient of therapy. Therefore, the design of future cell-based and gene-based therapies for restoring hearing will need to consider the biology of the recipient tissue.

Flat epithelium replaces the differentiated supporting cells of the organ of Corti whenever the lesion is severe enough to cause degeneration of the supporting cells. In many cases, the epithelium flattens during the second stage in the pathology. First, hair cells die and the supporting cells form typical phalangeal scars. Then, once scars are formed and hair cells are gone, supporting cells are replaced by a simple epithelium. The transition from a normal organ of Corti to one that contains flat epithelial cells can take as short as two days (Kim and Raphael, 2007) or many months (Sugawara *et al.,* 2005). The latter study demonstrates that the long term outcome of an ototoxic insult may manifest as a differentiated non-sensory epithelium in the apical cochlea and a flat epithelium in the basal portion. It also demonstrates an important relationship between the condition of the non-sensory epithelium and the survival of the spiral ganglion.

It is presently unknown what cell type the flat epithelium is derived from. The two most likely possibilities are (a) the original supporting cells (Deiters and pillar cells and perhaps Hensen's cells and inner phalangeal cells) de-differentiate and become flat or (b) the original supporting cells die and cells from flanking areas migrate and replace them. Flanking cells could be from regions such as Hensen's cells, or the inner or outer sulcus and perhaps as far as Claudius cells or the interdental cells on the limbus.

Flat epithelial cells have been found in ears that lost hair cells due to severe ototoxicity, severe noise or sound overstimulation, infections and aging. Such cell have also been observed in ears with cochlear implant that were deafened by any of the factors listed above. The flat cells may appear cuboidal or completely flat. At present, little is known about the reasons for degradation of the auditory epithelium to the stage of a flat epithelium and about the biology of these cells. It is likely that many (if not most) patients who will seek hair cell replacement therapy for severe hearing loss will have cochleae with a flat epithelium.

Although quiescence is among the hallmarks of the sensory

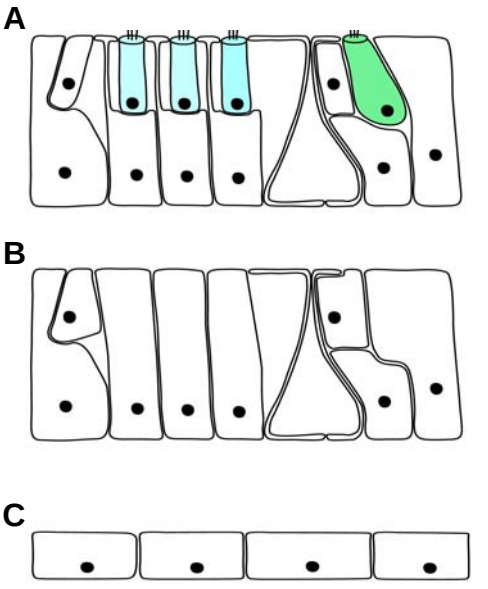

Fig. 2. Schematic depiction of the transition of the auditory epithelium *from the normal morphology* **(A)** *to the state where supporting cells remain differentiated despite the loss of hair cells* **(B)** *and to the state of the flat epithelium* **(C)**. **(A)** *The normal organ of Corti contains inner hair cells (green) and outer hair cells (blue), as well as supporting cells (white).* **(B)** *After hair cells are lost (at the short term and/or after a moderate insult), supporting cells remain as tall differentiated cells but their volume is increased as they fill the space vacated by the degenerated hair cells.* **(C)** *After severe lesions and/or long durations after the insult, the organ of Corti becomes a simple flat epithelium.*

epithelium of the normal cochlea, this may not hold for the flat epithelium. Rather, in mature guinea pig cochleae injected with neomycin, the remaining non-sensory cells become a flat epithelium which undergoes a robust proliferative response 4 days after the ototoxic insult (Kim and Raphael, 2007). As a result, the density of non-sensory cells that line the endolymph is increased and the epithelial layer maintains its confluence. This proliferative response presents opportunities for gene transfer and stem cells therapies. It is necessary to extend the findings on mitosis in the flat epithelium to other species and determine if additional superimposed insults can induce a renewed phase of proliferation.

Factors that influence the fate of supporting cells

The sequence of degeneration of different cell types may aid in understanding their interdependence. It has been shown that primary loss of auditory neurons does not lead to secondary degeneration of supporting cells and that presence of supporting cells in the inner hair cell area influences survival of the neurons (Sugawara *et al.,* 2005). Still, it is not clear why supporting cells survive in the differentiated state in some cases and become flat in others. At this point we can only speculate that supporting cells are directly sensitive to extreme levels of certain insults such as overstimulation or ototoxic drugs. The cause of supporting cell degeneration may be important for developing means to induce survival of these cells. However, once differentiated supporting cells are lost, the

reason for their loss may not be relevant for inducing regenerative process in the tissue.

Reparative strategy for differentiated supporting cells

When differentiated supporting cells remain in the deaf ear, forced expression of developmental genes such as *Atoh1* may yield generation of new hair cells by inducing transdifferentiation of the remaining supporting cells, with or without mitosis. Transdifferentiation is an uncommon phenomenon in which a differentiated cell changes its identity and becomes a new cell type (Call *et al.*, 2005, Li *et al.*, 2005). The finding that supporting cells in the avian basilar papilla divide and transdifferentiate into new hair cells after the original hair cells are eliminated (Raphael, 1992, Stone and Cotanche, 1994, Stone and Rubel, 2000) inspired attempts to induce transdifferentiation in the mammalian cochlea, where spontaneous transdifferentiation does not take place. Work *in vitro* and *in vivo* showed that in the mature organ of Corti, over-expression of a gene that induces hair cell differentiation during normal embryonic development can also induce transdifferentiation of differentiated supporting cells into hair cells (Izumikawa *et al.*, 2005, Shou *et al.*, 2003, Zheng and Gao, 2000). The forced expression of *Atoh1* in supporting cells can induce their transdifferentiation within a few days after hair cells are lost. The presence of ectopic hair cells after forced expression of *Atoh1* suggests that differentiated cells located outside the organ of Corti can also respond to the expression of this gene (Kawamoto *et al.,* 2003).

One problem associated with transdifferentiation therapy is the reduction in the number of supporting cells. Combining this therapy with enhancing mitosis in the epithelium may improve the structural and functional outcome this procedure. Another problem seen with the use of developmental genes is the limited time frame for their action. Transdifferentiation can be detected when forced *Atoh1* expression is induced 4 days after hair cells are lost (Izumikawa *et al.,* 2005), but at later time points this ability is diminished (our unpublished data).

Manipulation of cell cycle regulatory genes is another potential avenue for use in regenerating hair cells in the deaf auditory epithelium. As mentioned above, increasing the number of available supporting cells may enhance the repair afforded by *Atoh1*. It is also necessary to find ways that will lead directly from cell proliferation to regeneration of new hair cells. Mouse mutations in which supporting cells are released from quiescence display increased number of hair cells (Chen and Segil, 1999, Lowenheim *et al.*, 1999). While hearing in these mice is compromised, more research into the function of these ears may help improve the hearing and pave the way for using cell cycle genes for hair cell regeneration. Deficient expression in another cell cycle regulatory gene, Rb1, has also been shown to increase production of hair cells (Sage *et al.,* 2005). It appears that in normal ears, proliferation of non-sensory cells by itself does not induce generation of new hair cells (Minoda *et al.,* Raphael paper in revision). More work on the potential for cell proliferation to yield new hair cells in deaf ears is necessary.

Stem cell therapy is another option for placing new hair cells in the deaf auditory epithelium that retains differentiated supporting cells. Stem cells from several sources can be guided to differentiate into the hair cell phenotype (Li *et al.*, 2004, Li *et al.*, 2003). To integrate in the ear and become functional, these cells may either be differentiated *in vitro* and then implanted, or implanted and then guided to differentiate within the ear. At present both options appear challenging. Remaining supporting cells in the deaf ear have evolved to provide a tight seal of the endolymph and are not likely to facilitate integration of exogenous cells introduced into the ear. Designing the interface between the recipient tissue and the stem cells may help integration of the latter.

Reparative strategy for the flat epithelium

Two strategies can be conceptualized for restoring hearing in ears with flat epithelium. One possibility is to recapitulate development by applying a set of genes sequentially, in the order in which these genes are expressed during development. Many of the important genes have been identified (Fritzsch *et al.*, 2006). Such therapy should start with early developmental genes and culminate in the expression of *Atoh1*. It is possible that the number of genes necessary would not be too large if expression of some of them would spontaneously lead to expression of others. The ability of the flat epithelium to proliferate (Kim and Raphael, 2007) should facilitate use of large gene inserts with multiple promoters to accomplish such a complex gene expression manipulation.

The stem cell option is the other potential strategy for biological repair of hearing when the epithelium is flat. Better characterization of the flat epithelial cells will help design ways to integrate stem cells in this tissue. It is likely that both stem cells and the recipient cells will need to be engineered for such integration to occur. Use of viral vectors or other means for gene transfer may be necessary for preparing the flat epithelium to receiving the stem cells. Thus, the encouraging progress in obtaining and differentiating stem cells as hair cells will need to see a parallel progress in the successful integration of these cells in the tissue.

Nerves interaction with epithelial cells

New hair cells need to be innervated in order to function. As long as primary auditory neurons survive it is likely that the hair cells will attract them to their area so they may connect and form functional terminals. Neurites have been found to meander in the auditory epithelium devoid of hair cells (Bohne and Harding, 1992). Evidence for the ability of neurons to reach new targets in the auditory epithelium is provided by findings on innervation of ectopic hair cells (Kawamoto *et al.,* 2003) and by improvement of function after *Atoh1* therapy in deaf ears (Izumikawa *et al.,* 2005). If auditory neurons are absent, additional measures may be necessary, likely using stem cells (see Chapter by Edge). In human ears, auditory neurons survive in many cases of hair cell lesions and are expected to send new peripheral processes if new targets (hair cells) appear.

In the conceptual sequence of events described above, new hair cells will be expected to appear first and then attract neurons. This concept assigns no role for the neurons in the differentiation of the new hair cells, at least in the early stages. This is in line with findings that hair cells may develop in the absence of innervation (Fritzsch and Beisel, 2004, Fritzsch *et al.*, 2005).

Non-sensory cells that remain in the deaf ear can also be used for enhancing nerve survival and function so as to improve the outcome of the cochlear implant. These cells may be engineered

to secrete growth factors or other molecules that can positively influence neurons. It is therefore important to characterize the ability of the flat epithelium to be transduced by viral vectors or other means for expressing transgenes. Findings on the ability of the flat epithelium to proliferate (Kim and Raphael, 2007) suggest that viral vectors that integrate in the host DNA and yield long term gene expression can be used in this tissue.

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