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Morphological Correlates of Regeneration and Repair in the Inner Ear

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1. Introduction

The loss of hair cells is a major cause of disabling hearing impairments that affect approximately 250 million persons worldwide and a contributor to inner ear balance disorders that can lead to falls late in life. Normal healthy human inner ears contain approximately 16,000 hair cells in the sound sensing cochlea, around 8000 hair cells in each of the three rotation sensitive semicircular canal cristae, and 18,000–33,000 hair cells each in the gravity sensing utricle and sacculus (Fig. 2.1; Rosenhall 1972; Wright et al. 1987). The majority of people will lose some of those hair cells as they mature and age and many will develop age-related deficits of hearing called presbycusis (Fig. 2.2; Bredberg 1968; Rosenhall 1973). Presbycusis and age-related deterioration of vestibular reflexes correlate with declines in the numbers of cochlear and vestibular hair cells, respectively (Fig. 2.2; Bredberg 1968; Rosenhall 1973; Paige 1992).

Hair cells can be damaged and killed by loud sounds, infections, head trauma, and autoimmune disorders. The clinical use of aminoglycoside antibiotics and certain chemotherapeutic agents such as cisplatin can also cause hair cell loss. As life expectancies have lengthened, the occurrence of hearing and balance disabilities has grown, because hair cell losses in humans and other mammals are permanent and cumulative.

In contrast to mammals, many nonmammalian vertebrates produce hair cells throughout life and regenerate replacements for hair cells that have been lost. The replacement cells become innervated, which leads to the restoration of hearing and balance sensitivity, usually within a matter of weeks. Hair cell regeneration also occurs in the lateral line neuromasts of fish and amphibians, which share many aspects of tissue structure and organization with the hearing and balance end organs of the ear (Fig. 2.3).

This chapter reviews and discusses current understanding and highlights unresolved questions pertaining to morphological aspects of the mechanisms that

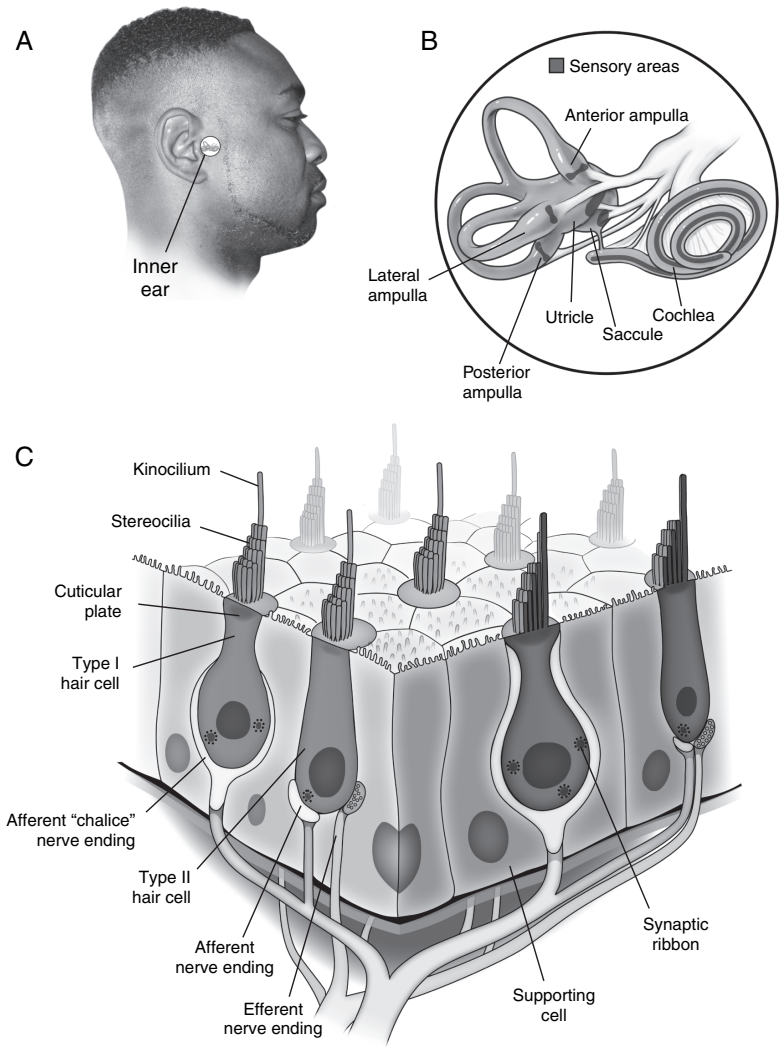


FIGURE 2.1. Schematic diagrams of the human inner ear. (A) Approximate size and location of the sensory organs of the inner ear. (B) Schematic drawing of the auditory and vestibular labyrinth. The sensory patches within each organ are indicated by the dark coloration. (C) Schematic representation of a section of the sensory epithelium from a vestibular organ to demonstrate the general structure and organization of the sensory epithelium.

underlie hair cell regeneration and repair in vertebrates. It also reviews investigations that have explored the potential for hair cell regeneration in the ears of mammals.

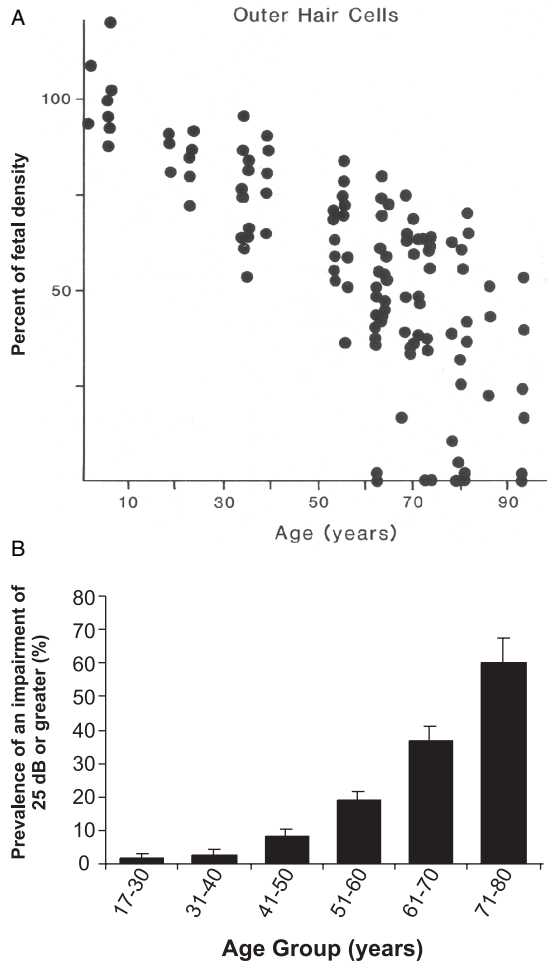


FIGURE 2.2. Loss of hair cell number and function in the human cochlea with increasing age. **(A)** Density of outer hair cells in the cochlea decreases with age, expressed as a percentage of the fetal density (100%). **(B)** Prevalence of hearing impairment of 25 dB or greater in the better hearing ear among individuals of different ages. The increasing prevalence of hearing loss at increased age correlates strongly with the loss of cochlear hair cells. **(A)** Modified from Bredberg (1968). **(B)** Modified from Davis (1989).

2. Ongoing Production of Hair Cells in Nonmammalian Vertebrates

In many nonmammalian vertebrates, the production of hair cells is not limited to embryonic development (Corwin 1981, 1983, 1985; Popper and Hoxter 1984; Lanford et al. 1996). For example, the ears of sharks and rays add hundreds of thousands of hair cells in the ear and increase in sensitivity as juveniles grow into

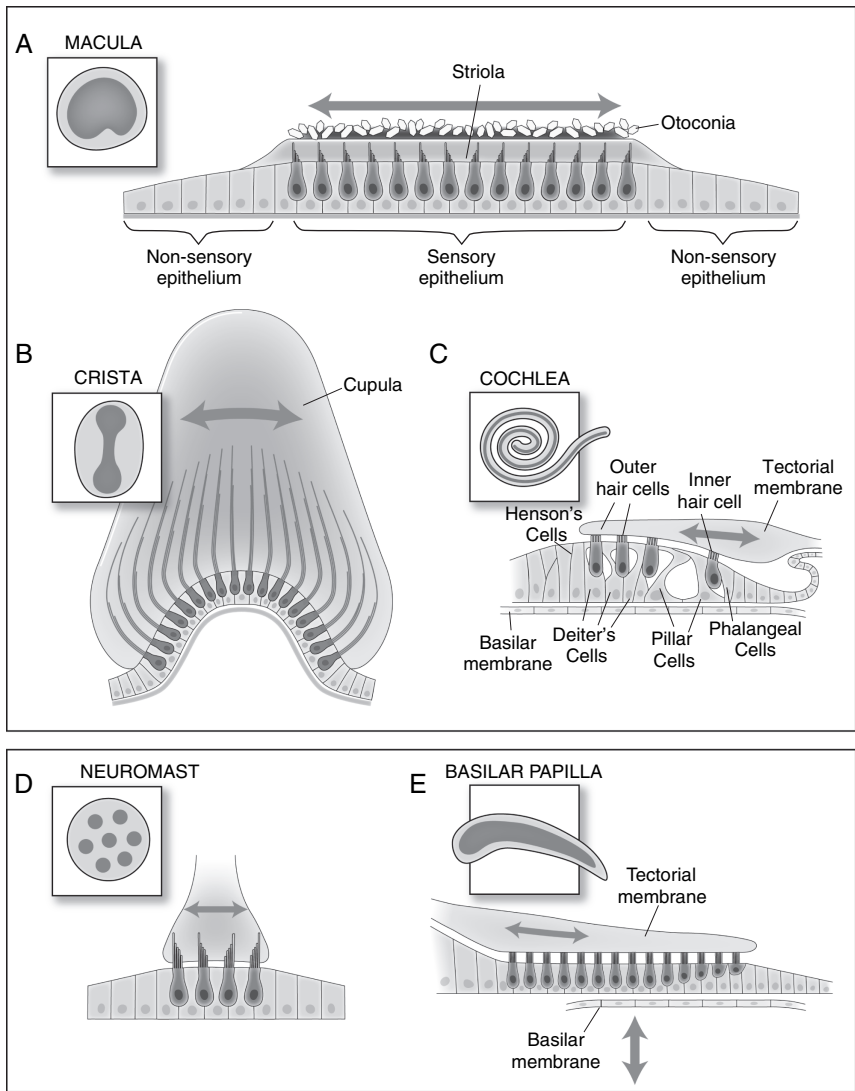


FIGURE 2.3. Schematic diagrams showing general structural organization of sensory epithelia. (A) Organization of otolith macular organs. The sensory patch, indicated by the darkened color in the inset, is comprised of hair cells and supporting cells in a mosaic pattern. The hair cells insert their hair bundles into a gelatinous matrix covered by otoconial crystals that provide inertial weight to acceleration of the head (arrow). The sensory epithelium is surrounded by a nonsensory epithelium, made up of supporting-like cells. (B) Organization of the semicircular canal cristae. The barbell-shaped sensory patch is organized very similar to that of the otolith macular organs. The hair bundles, however, are inserted into a large cupula, which transduces rotational movement of fluid through the canal to the hair bundles (arrow). (C) Organization of the mammalian cochlea. In contrast to the generally uniform population of supporting cells in vestibular organs,

adults (Fig. 2.4a; Corwin 1981, 1983). The ears of bony fish and amphibians also add thousands of hair cells during postembryonic growth (Lewis and Li 1973; Li and Lewis 1979; Popper and Hoxter 1984; Corwin 1985; Lombarte and Popper 1994; Lanford et al. 1996).

2.1 Postembryonic Hair Cell Production

These new hair cells are the product of postnatal proliferation, as [^3H]thymidine labels both newly produced hair cells and supporting cells (Corwin 1981). The distribution of proliferating cells and newly produced hair cells in elasmobranchs and amphibians is strongly biased toward appositional growth, with cells added at the outer edge of the sensory epithelium (Fig. 2.4b,c), but some interstitial proliferation and addition of new hair cells occurs in the central regions of the maculae as well (Lewis and Li 1973; Li and Lewis 1979; Corwin 1983, 1985).

Appositional expansion is not the only pattern for ongoing growth in the ear. In teleost fish, interstitial addition outweighs appositional growth at the outer margin (Popper and Hoxter 1990; Lombarte and Popper 1994; Lanford et al. 1996). There is also ongoing proliferation and production of hair cells within the vestibular maculae of chickens occurring throughout the epithelium (Jørgensen and Mathiesen 1988; Roberson et al. 1992; Kil et al. 1997).

Thus, the ears of many nonmammalian vertebrate classes show signs of normal continued production of hair cells throughout life. The permanent loss of hair cells that occurs in mammalian ears appears to be unique, as hair cell epithelia retain progenitors in nonmammalian vertebrates that can produce hair cells.

3. Generation and Regeneration of Lateral Line Hair Cells

Hair cells are also continually generated in the sensory organs of the lateral line. Lateral line neuromasts are small collections of hair cells and supporting cells on the heads and bodies of fish and aquatic amphibians. The hair cells in the lateral line are morphologically similar to hair cells from the inner ear. They show expression of the same molecular markers as inner ear hair cells,



FIGURE 2.3. the cochlear supporting cells (e.g., Deiters', pillar, and phalangeal cells) show discrete morphological specializations to form the unique structure of the organ of Corti. (D) The lateral line neuromasts found along the body of fish and amphibians is organized similar to vestibular organs, though there are many fewer hair cells per organ. (E) The basilar papilla of birds shows some similarity to the vertebrate cochlea, though the supporting cells do not form morphologically discrete subtypes or show high levels of specialization.

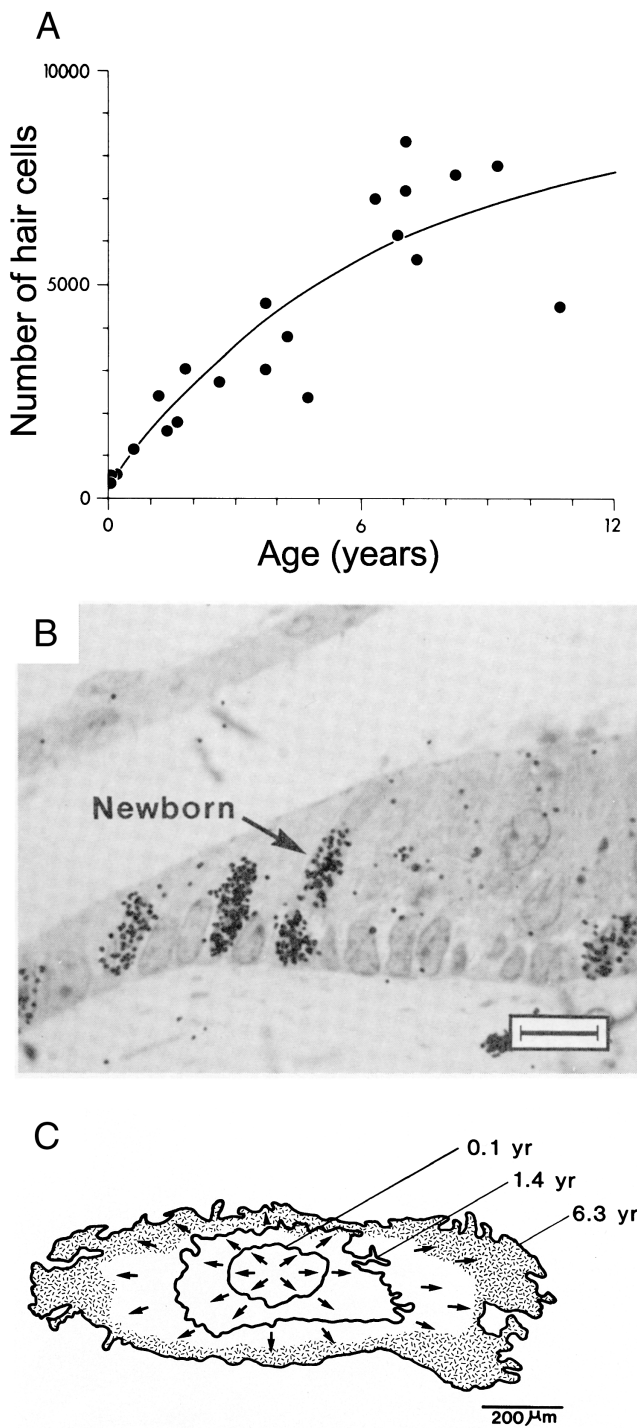


FIGURE 2.4.

including homologs of atonal (Itoh and Chitnis 2001) and myosin VIIa (Ernest et al. 2000), and are thought to be homologous. During the life of the animal, the lateral line organs bud off accessory neuromasts that lie dorsal or ventral to the primary organ and gradually develop into collections of up to 30 neuromasts (Stone 1937; Ledent 2002). The accessory organs begin as a few supporting cells around a single hair cell, but grow to include several hair cells, indicating that the cells of the neuromasts continue to produce new sensory structures and hair cells (Ledent 2002).

3.1 Regeneration of Hair Cell Epithelia After Amputation

In amphibians, neuromasts also can be replaced during regeneration after amputation of the tail. Cells in the last neuromast on the tail stump divide to give rise to a regenerative placode that is morphologically similar to the embryonic placode that first produces the lateral line organs in embryos and larvae. The regenerative placode migrates along the regenerating tail producing replacement neuromasts similar in number to those lost through amputation (Stone 1933, 1937; Speidel 1947; Wright 1947). These replacement organs develop hair cells with normal morphology and become reinnervated (Stone 1933, 1937; Speidel 1947; Wright 1947; Jørgensen and Flock 1976). Normally, the regenerating placode forms from the posterior edge of the last neuromast, but grafting experiments that reversed the orientation of the neuromasts demonstrated that production of a regenerating placode and neuromasts could develop from the anterior edge as well (Stone 1937). This suggests that the marginal cells closest to the wound are able to respond to some signal from the wound to initiate the regenerative process.



FIGURE 2.4. Ongoing production of hair cells continues throughout life in the inner ear of many vertebrate classes. (A) A plot of the number of hair cells within the macula neglecta of a ray versus age of the animal. The slope indicates that approximately three hair cells are added per day in the first years of life and approximately one hair cell is added per day beyond 6 years of age. (B) A micrograph of the edge of the sensory epithelium of a toad's saccule. A [³H]thymidine labeled newborn hair cell is indicated at the edge of the epithelium, and three labeled supporting cells lie just beyond the last hair cell. The transition from the sensory epithelium to the nonsensory epithelium is the primary location for addition of new hair cells in cartilaginous fish and amphibians. (C) Superimposed outlines of the sensory epithelium from the macula neglecta of rays at different ages showing the increase in size of the sensory patch. The arrows indicate the orientation of the hair bundles pointing away from a central point of symmetry. The shaded portion indicates the region of hair cells with immature hair bundles at the outer edge of the epithelium. (A, C) From Corwin (1983); (B) from Corwin (1985).

3.2 Identifying the Progenitors of Replacement Hair Cell Epithelia

It was suggested decades ago that for both neuromast budding and regenerating placodes, the likely source of the cells was the supporting cells of the neuromast (Stone 1933, 1937). Time-lapse microscopy confirmed the identity of progenitor cells of the regenerating placode as mantle-type supporting cells that reside at the edge of the neuromast, adjacent to the internal supporting cells and hair cells (Jones and Corwin 1993). The location of the regenerating cells at the border between the sensory epithelium and surrounding epidermis is similar to the location of the proliferative cells that produce new hair cells throughout the lives of elasmobranchs and amphibians (see Section 2.2). Neuromasts formed from a regenerative placode have the ability to form a new regenerating placode in response to reamputation of the tail (Speidel 1947). The progenitor cells, therefore, can make all of the differentiated cell types within the neuromast, and can reproduce latent precursors satisfying two defining characteristics of stem cells: self-renewal and multipotency.

3.3 Regeneration of Lateral Line Hair Cells In Situ

The lateral line can also generate replacement hair cells within individual neuromasts after selective destruction of individual hair cells (Balak et al. 1990; Song et al. 1995). In response to loss of hair cells within a neuromast, the surrounding supporting cells become proliferative and can generate both replacement hair cells and supporting cells (Jones and Corwin 1996). Thus, within the lateral line sensory organs, regeneration can replace lost hair cells or develop multiple new neuromasts, with all of their sensory and nonsensory cells, during tail regeneration.

4. Hair Cell Regeneration in the Inner Ear

Similar to the findings of ongoing addition of hair cells in anamniotes and the regenerative capacity of the lateral line, nonmammalian vertebrates such as fish, amphibians, reptiles, and birds are capable of significant regeneration of hair cells after damage to the inner ear.

4.1 Morphological Recovery in the Avian Hearing Organ

The full complement of hair cells in the chicken basilar papilla sensory epithelium is produced embryonically and the cells all become quiescent after embryogenesis (Tilney et al. 1986; Katayama and Corwin 1989). In response to tonal acoustic overstimulation, hair cells within the region of the basilar papilla tuned to the stimulating tone are lost or suffer significant damage, while hair cells in regions tuned to other frequencies are spared (Fig. 2.5a; Cotanche 1987).

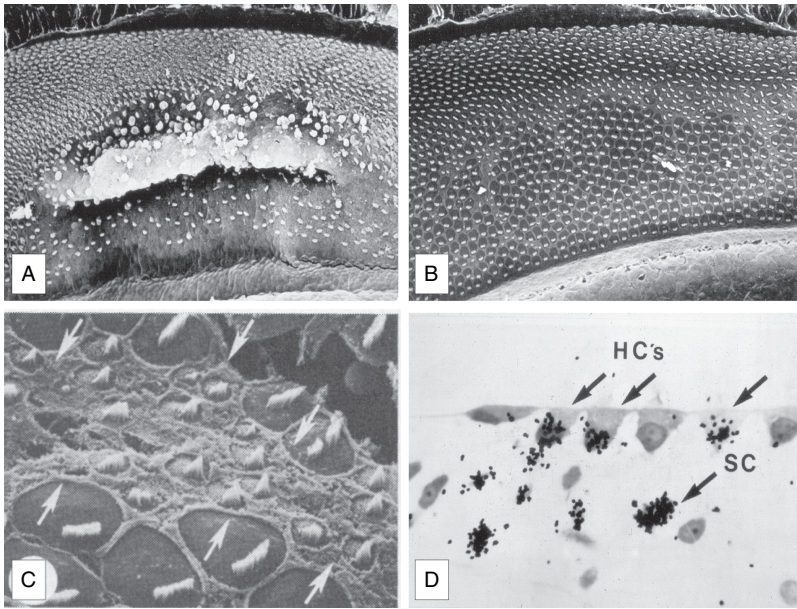


FIGURE 2.5. Hair cell regeneration in the chicken basilar papilla. (A) After 48 h of acoustic overstimulation at 1.5 kHz, hair cells within the region of the cochlea tuned to 1.5 kHz are lost and can be seen extruding from the epithelium. (B) After a 10-day recovery after acoustic overstimulation, the epithelium has been repaired. Hair cells can be seen throughout the formerly damaged regions. (C) Close-up micrograph of hair cells in the damaged region of a chick cochlea 6 days after damage shows a number of new hair cells with small bundles and small apical surfaces. (D) Injection of the birds with tritiated thymidine during the 10-day recovery period reveals labeling of both hair cells (HC) and supporting cells (SC), indicating that the new hair cells are progeny of dividing supporting cells. (A–D) From Corwin and Cotanche (1988).

This is followed by proliferation at the lesion site, and over 6–10 days there is a dramatic recovery in the number of hair cells (Fig. 2.5b; Cotanche 1987; Corwin and Cotanche 1988). Many of the hair cells within the damaged region exhibit small hair bundles reminiscent of immature hair bundles on developing hair cells (Fig. 2.5c). The ability to stimulate proliferation and regeneration of new hair cells was similar in young chickens and adult quail (Corwin and Cotanche 1988; Ryals and Rubel 1988; Ryals and Westbrook 1990). Thus, cells within the avian cochlea retain the capacity to reenter the cell cycle and generate new hair cells throughout life, even though they normally become mitotically quiescent after embryogenesis. The newly differentiating cochlear hair cells take on the distinct positional morphologies of the cells they replace (Cotanche 1987), suggesting that positional identity such as tonotopy can be passed on to the regenerating cells. Regeneration of hair cells also occurs in birds in response to aminoglycoside toxicity in both the auditory and vestibular systems (Lippe

et al. 1991; Weisleder and Rubel 1993). After regeneration of hair cells in birds, the replacement cells become innervated and there is significant recovery of hearing and vestibular function (see discussion by Dooling, Dent, Lauer, and Ryals, Chapter 4).

4.2 Ongoing Proliferation in the Avian Vestibular System

Although the cells in the avian basilar papilla are normally mitotically quiescent until damage induces cell cycle reentry, in the avian vestibular system there is ongoing proliferation throughout life. However, there is also a low level of ongoing cell death, approximately 90% of which are hair cells within the vestibular system (Kil et al. 1997). This suggests that the continual production of new cells in the vestibular system may be in response to the dying cells and serves to replace lost hair cells rather than promote continued growth of the epithelium. Consistent with that hypothesis, inhibition of cell death in the utricle limits ongoing proliferation (Matsui et al. 2002). The mechanism and reason behind the continued loss and regeneration of hair cells in the avian vestibular system remain unclear.

4.3 Regeneration in Fish and Amphibians

The sensory epithelia from fish and amphibians also show substantial damage-induced proliferation and regeneration of hair cells. New hair cells are produced after aminoglycoside treatment in fish (Lombarte et al. 1993) and in bullfrogs (Baird et al. 1993), and after destruction of hair cells in the lateral line (Balak et al. 1990; Song et al. 1995). This generation of hair cells is accompanied by increased proliferation (Balak et al. 1990; Presson and Popper 1990; Baird et al. 1993; Presson et al. 1996; Avallone et al. 2003), similar to that seen in the avian organs after damage. In summary, fish, amphibians, and birds are all capable of significant regeneration of hair cells after damage to the inner ear. The replacement hair cells in these species occurs via cell divisions that occur at the sites of damage.

4.4 Regenerative Responses in Mammalian Ears

The hair cells and supporting cells in the vestibular system of mammals are similar to their counterparts in birds, fish, and amphibians (Fig. 2.3). In contrast, hair cells and supporting cells in mammalian cochleae are highly specialized, without direct counterparts in the auditory organs of other vertebrates. This high degree of differentiation and specialization suggests that regeneration may be more limited in the mammalian cochlea. However, the similarity between mammalian and nonmammalian vestibular cells paired with the robust regeneration in nonmammalian ears suggests that hair cell regeneration may be more likely in mammalian vestibular epithelia.

4.4.1 Morphological Recovery in the Mammalian Vestibular Organs

After aminoglycoside-induced hair cell loss, guinea pigs allowed to recover for 4 weeks developed small immature hair bundles in the damaged regions of the utricular epithelium (Fig. 2.6; Forge et al. 1993, 1998). These cells were reminiscent of the appearance of regenerating hair cells in chickens (Cotanche 1987). Utricles from adult guinea pigs maintained *in vitro* after aminoglycoside treatment had small numbers of [^3H]thymidine-labeled cells throughout the sensory epithelium, showing that hair cell damage can trigger proliferation in vestibular organs of mammals (Warchol et al. 1993). Profiles of putative [^3H]thymidine-labeled hair cells were also found in those epithelia. When utricles obtained during otologic surgery on adult human patients were treated similarly, that resulted in at least 100 [^3H]thymidine-labeled cells in each utricle (Warchol et al. 1993). Thus even supporting cells from the sensory epithelium of adult humans can proliferate after damage.

However, the tens to hundreds of dividing cells in mammalian utricles after damage are considerably less than the thousands that would be seen in avian

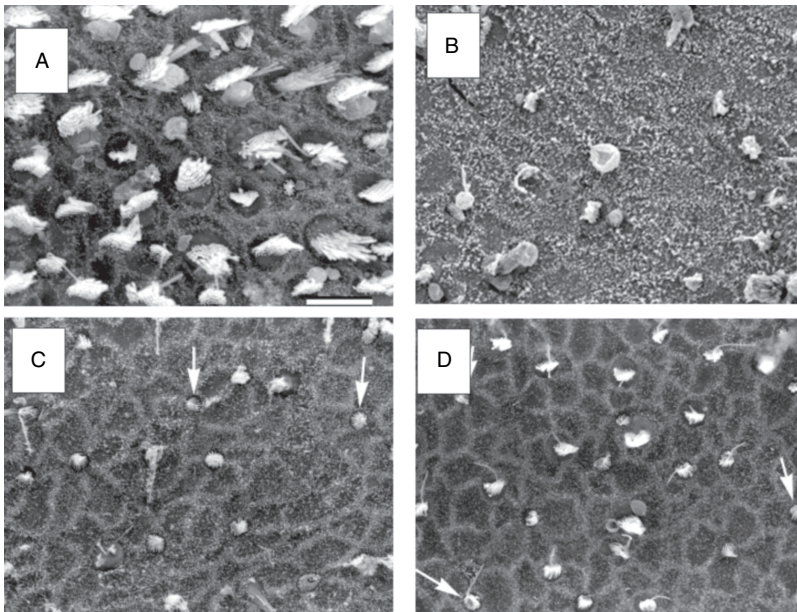


FIGURE 2.6. Regeneration in the mammalian vestibular epithelium. (A) Scanning electron micrograph of the striola region of a control utricle from a mature guinea pig. (B–D) Damage and recovery of hair bundles in guinea pig utricles after aminoglycoside toxicity. (B) The striola of a utricle 1 week after the end of aminoglycoside treatment, showing loss of most of the hair bundles. (C) The striola of a utricle 2 weeks after the end of aminoglycoside treatment showing initial recovery of hair bundles (arrows). (D) The striola of a utricle 4 weeks after the end of aminoglycoside treatment showing continued replacement of hair bundles (arrows). (A–D) From Forge et al. (1998).

utricles after similar damage (Warchol et al. 1993; Matsui et al. 2000). There was also a notable quantitative mismatch between the large numbers of recovering hair bundles reported by Forge and colleagues (1993, 1998) and the limited number of proliferating cells found by Warchol et al. (1993). There may be a substantial difference in the level of damage to the utricles *in vivo* versus *in vitro*, which may make it difficult to compare the response between these studies. The studies showing substantial recovery of hair bundle numbers were done *in vivo*, wherein aminoglycoside nephrotoxicity limits the concentrations that can be used without severe systemic repercussions, while the investigations of proliferation were performed *in vitro*, wherein high levels of aminoglycoside ensure the death of most of the hair cells. Thus, whether the difference in the magnitude of proliferation versus hair bundle recovery is due to the level of damage, or whether it reflects a nonproliferative mechanism of regenerating hair cells remains an open question.

4.4.2 Minimal Hair Cell Regeneration in the Mammalian Cochlea

In contrast to the mammalian vestibular system, there is, as yet, little evidence for proliferative regeneration within the mammalian cochlea, where the supporting cells show significant morphological specialization. While there have been reports that the cells of the organ of Corti can be stimulated to proliferate and regenerate hair cells after aminoglycoside treatments (Lefebvre et al. 1993), confirmation of those results has proven difficult (Chardin and Romand 1995). The specialization and differentiation of supporting cells into discrete cell types with distinct morphologies within the organ of Corti may limit their ability to reenter the cell cycle.

In summary, while regeneration in the mammalian inner ear does not appear to occur at the robust level as in nonmammalian vertebrates, there is evidence for a limited regenerative response, at least in the vestibular system. By further studying both the strong regenerative processes in nonmammalian systems and the limited regeneration in mammals, it may be possible to override the limitations in the mammalian regenerative response and promote clinically significant recovery from hair cell loss.

5. The Source of Regenerating Hair Cells

The continual production of new hair cells throughout life and the regeneration that follows damage both appear to depend on proliferative precursor cells. The morphological identity of the cells that produce regenerating hair cells within damaged epithelia has been a key question in establishing a mechanism for the observed recovery of functional hair cells. Proposed sources have included the hyaline cells that lie at the margin of the chick basilar papilla (the same position as the cells that result in appositional growth in elasmobranchs and neuromast regeneration in amphibians), the differentiated supporting cells within the sensory organs, or a specialized reserve pool of distinct stem cells.

During damage-induced regeneration, proliferating cells can be labeled with tritiated thymidine or 5-bromo-2-deoxyuridine (BrdU). In the avian ear, supporting cells within the sensory epithelium are the first cells to be labeled by mitotic markers, and this is followed by the production of new, labeled, hair cells (Fig. 2.5d; Corwin and Cotanche 1988; Ryals and Rubel 1988; Raphael 1992; Roberson et al. 1992; Hashino and Salvi 1993; Stone and Cotanche 1994). Hyaline cells, which reside at the abneural edge of the sensory epithelium, will also migrate into the wounded region and proliferate after extensive damage to the basilar papilla (Girod et al. 1989; Cotanche et al. 1995). However, proliferation of hyaline cells has not been associated with hair cell regeneration (Corwin et al. 1991; Raphael 1992; Cotanche et al. 1995; Warchol and Corwin 1996). In addition to the indirect evidence of BrdU- or [^3H]thymidine-labeled supporting cells pointing to them as the source of new hair cells, direct observation via time-lapse microscopy has shown that supporting cells within salamander lateral line neuromasts divide and the progeny differentiate into replacement hair cells and replacement supporting cells (Jones and Corwin 1996). Thus, in response to damage, cells within the epithelium that show the morphological characteristics of supporting cells appear to be the primary cells that reenter the cell cycle to produce replacement hair cells and supporting cells (Fig. 2.7).

5.1 Reserve Stem Cells versus Dedifferentiation of Supporting Cells?

Although cells classified as supporting cells have been identified as the primary source for regenerating hair cells, there are two hypotheses about the nature of those cells. They may be normal functional supporting cells that dedifferentiate, return to the cell cycle, and produce replacement hair cells and supporting cells, or they may be a discrete population of reserve stem cells that are morphologically similar to supporting cells.

The possibility that there may be a discrete population of proliferative cells within the sensory epithelium separate from the supporting cells has been considered (Presson and Popper 1990), though more recent studies have

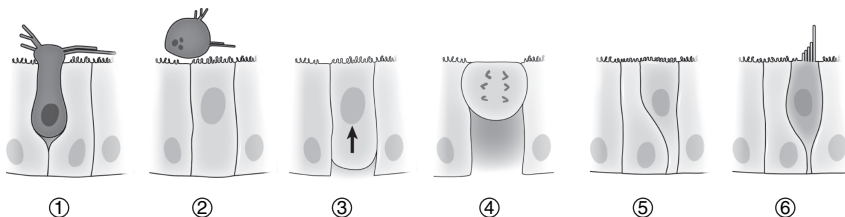


FIGURE 2.7. Schematic diagram of regenerative proliferation by supporting cells. In response to loss of a hair cell (1) the supporting cell nucleus migrates toward the epithelial surface (2–3) where it undergoes mitosis (4). The progeny of that division then go on to form replacement hair cells or supporting cells (5–6).

concluded that dividing precursor cells are morphologically indistinguishable from other supporting cells in the fish inner ear (Presson et al. 1996). Further, destruction of the proliferating precursor cells with cytosine arabinoside (Ara-C) is followed by a new wave of proliferating cells that replace the lost cells and generate new hair cells (Presson et al. 1995). After damage in the chick inner ear, the majority of supporting cells downregulate expression of G_0 markers and begin expressing markers consistent with passage to late G_1 phase (Bhave et al. 1995). These data suggest that regeneration does not depend on a discrete population of reserve cells, but rather originates from a larger collection of supporting cells that have the capacity to dedifferentiate, reenter the cell cycle, and produce replacement hair cells.

The heavy bias of proliferation and addition of hair cells at the lateral edge of the epithelium in elasmobranchs and amphibians strongly suggests that a class of stem cells resides at the moving junction between the sensory epithelium and the nonsensory epithelium in these animals. A similar population of stem cells is found in the ciliary marginal zone in the retinas of fish, amphibians, and birds, contributing to growth and regeneration of the retina (Straznicky and Gaze 1971; Johns 1977; Wetts and Fraser 1988; Fischer and Reh 2000). Growing knowledge about these stem cells may be applicable to questions about the proliferative cells at the edges of inner ear epithelia. Although they do not have similar ongoing growth of the organs, the auditory organs of chickens and the vestibular organs of mammals initially develop in a pattern similar to the radial addition of cells in many anamniote ears. Proliferation and cell differentiation occur first near the center of the sensory epithelium and progress radially outward from there until the whole sensory epithelium has been produced (Sans and Chat 1982; Mbiene and Sans 1986; Katayama and Corwin 1989). Perhaps the mechanisms that control ongoing hair cell production at the margin of sensory epithelia in anamniotes may also be active in amniotes during development, but normally suppressed after maturity.

6. What Triggers the Regeneration Response?

Understanding the stimulus that triggers the regenerative response in supporting cells of nonmammalian vertebrates may provide some insight into how to stimulate similar proliferation in the mammalian ear. The potential molecular signals that may underlie this response are discussed in much greater detail by Oesterle and Stone in Chapter 5, but several elements relate more specifically to anatomical and morphological concerns.

6.1 The Triggering Effects of Hair Cell Loss

The loss of hair cells is an important stimulus for regeneration of new hair cells. In undamaged avian cochleae, there is little or no supporting cell proliferation (Corwin and Cotanche 1988; Ryals and Rubel 1988; Katayama and

Corwin 1989), but individual laser ablation of a patch of hair cells is sufficient to stimulate regenerative proliferation in the area around the wound (Warchol and Corwin 1996). The necessity for hair cell loss and the restriction of proliferation to the damaged area suggests that hair cells may suppress proliferation in supporting cells (Corwin et al. 1991). Similarly, along the outer margin of an anamniote's sensory epithelium, the outermost row of cells may not contact hair cells and thus may be relieved from this suppression of proliferation. One hypothesis is that hair cells express proteins along their basolateral membrane that prevent adjacent supporting cells from reentering the cell cycle (Corwin et al. 1991). Interaction of cadherins, transmembrane proteins that mediate cell–cell adhesion at adherens junctions, can suppress proliferation (Caveda et al. 1996), and is thought to mediate contact-inhibition of proliferation through sequestration of β -catenin (Fagotto and Gumbiner 1996). Changes in cadherin-mediated cell–cell contact, such as loss of hair cell-supporting cell junctions, could be a trigger for reinitiation of proliferation. Another cell–cell signaling pathway that may play a role in maintaining inhibition of supporting cell proliferation is the Notch pathway.

Notably, however, while the proliferative response in damaged chick basilar papillae is restricted to the area around the lesion, dividing supporting cells can be seen up to 180 μm away from the edge of the wound, in apparently undamaged regions (Warchol and Corwin 1996). Thus, while loss of hair cells is necessary to stimulate regenerative proliferation, it is not yet established whether loss of direct cell–cell signaling, such as through cadherins or notch–delta, is a necessary trigger for proliferation of supporting cells, or whether broader, more diffusible signals play the critical role.

6.2 The Potential Influence of Immune Responses to Damage in the Inner Ear

Several studies suggested the possibility that immune responses may influence regeneration within hair cell sensory epithelia. After tail amputation in axolotls, macrophages are recruited to the posterior side of the posteriormost neuromast, which will give rise to the regenerative placode (Jones and Corwin 1993). This suggests that they may play a role in the regenerative placode formation, for example, breaking down the glycocalyx that surrounds the neuromast, allowing mantle supporting cells to migrate out to form the migratory placode. Similarly, macrophages and microglia are recruited to sites of damage in the avian utricle and basilar papilla (Warchol 1997, 1999; Bhawe et al. 1998) and in rat organ of Corti (Wang and Li 2000). The exact role of macrophages and microglia in repair and regeneration of damaged sensory epithelia is unknown, but they are known to have significant effects during epidermal wound healing (Gailit and Clark 1994; Martin 1997). Among their reported roles are removal of dead or dying cells and cellular detritus, production of mitogenic cytokines, and modulation of extracellular matrix composition. In hair cell epithelia, macrophages will scavenge and remove debris, dead or damaged hair cells, and

even progeny from recent cell divisions. Macrophages likely also play a role in production of growth factors. Treatment of aminoglycoside-damaged chick utricles with dexamethasone to block macrophage cytokine production significantly reduces the number of proliferating supporting cells without significantly altering the number of macrophages (Warchol 1999). This indicates that chemical signaling from macrophages may be more important than their direct reparative effects on the epithelium. An important open question is whether the immune response to damage in the mammalian inner ear is similar to that in the avian inner ear or axolotl lateral line, as the extent of macrophage invasion into the damaged mammalian organ of Corti may be limited (Fredelius 1988; Fredelius and Rask-Andersen 1990; Hirose et al. 2005).

6.3 The Potential Importance of Cellular Shape Change

Cell shape has a direct impact on the ability of cells to proliferate (Folkman and Moscona 1978). In cultured endothelial cells, cells allowed to spread out on micropatterned substrates were able to proliferate, while cells forced to maintain compact cell shapes did not proliferate (Chen et al. 1997; Huang and Ingber 1999). Several experiments have suggested that there may be an important role for cell spreading in control of proliferation in hair cell sensory epithelia. In isolated cultures of chick utricular sensory epithelium, cells at the edge of the epithelium that spread out proliferate well while central cells that remain columnar and tight packed do not proliferate (Warchol 1995, 2002; Witte et al. 2001). Within the inner ear, one of the initial responses to loss of hair cells is expansion of the supporting cell surface to cover the space of the missing hair cells (Forge 1985; Cotanche 1987; Cotanche and Dopyera 1990; Marsh et al. 1990; Li et al. 1995). The significant expansion of the supporting cells in the avian basilar papilla coincides with the time during which they return to the cell cycle, and their return to normal surface dimensions occurs as replacement hair cells and supporting cells differentiate (Corwin and Cotanche 1988; Marsh et al. 1990). In mammalian utricular epithelia, cellular shape following damage is strongly correlated with cell-cycle entry, with tall, columnar cells rarely entering the cell cycle while most flattened, spread cells returned to the cell cycle (Meyers and Corwin 2007). These data are all consistent with the hypothesis that morphological shape change in supporting cells may be an important trigger in stimulation of regeneration, though further experimentation is necessary to clarify the specific role of such shape change on initiating proliferation.

7. Nonproliferative Restoration of Hair Cells

Although much of the morphological recovery from damage in nonmammalian vertebrates appears to be from proliferation of supporting cells, there is growing evidence that not all of the recovery is due to proliferation. The first hair cells to appear following damage to the chick are not labeled with BrdU, though

subsequent generation of hair cells is predominantly from proliferative means (Roberson et al. 2004). Similarly, morphological recovery in the newt (*Notophthalmus viridescens*) appears to occur in the absence of proliferation, as new hair cells are not BrdU labeled and still arise in the presence of mitotic inhibitors (Taylor and Forge 2005). In the mammalian vestibular system, there is a quantitative mismatch between the large number of hair bundles that reappear at the epithelial surface and the small number of dividing cells that can be observed (Warchol et al. 1993; Forge et al. 1998; Berggren et al. 2003). Further, differentiated hair bundles in bullfrog (*Rana catesbeiana*) and chick organs reappear after damage even in the presence of mitotic inhibitors (Adler and Raphael 1996; Adler et al. 1997; Steyger et al. 1997; Baird et al. 2000; Gale et al. 2002). Two potential mechanisms that have been proposed for this nonproliferative recovery are repair of damaged hair cells and phenotypic conversion of a supporting cell directly into a hair cell without intervening mitosis.

7.1 Sublethal Hair Cell Damage and Repair

Acoustic overstimulation, aminoglycoside antibiotics, platinum-based chemotherapeutics, loop-diuretics, head trauma, or infection can all lead to damage or death of hair cells. In many cases, the specific manner in which these insults lead to damage or death of hair cells is unknown, but many recent advances have been made in understanding the mechanisms of damage and to develop potential strategies to protect hair cells from such damage (see Forge and Van De Water, Chapter 6).

7.1.1 Hair Cell Responses to Damage

Generally, the initial response of hair cells to low levels of furosemide, aminoglycosides, cisplatin, or mechanical overstimulation involves disruption of the tip-links connecting adjacent stereocilia, followed by splaying of the bundle and/or fusion of the stereocilia (Fig. 2.8a,b; Engstrom et al. 1983; Pickles et al. 1987a, b; Osborne and Comis 1990b; Clark and Pickles 1996). More extensive insult can lead to loss of stereocilia at their point of insertion into the cuticular plate and loss of the hair bundle (Fig. 2.8c; Pickles et al. 1987a; Osborne and Comis 1990b; Gale et al. 2002). At higher levels of insult, hair cells are killed and lost from the epithelium, either via physical extrusion of the entire cell, or apoptosis within the epithelium (Fig. 2.8d,e; Li et al. 1995; Nakagawa et al. 1997). Any of these levels of damage will produce a loss-of-function, either from disruption of mechanosensation in surviving cells or from loss of the sensory cells themselves.

Because hair cells can exhibit a range of morphological effects depending on the level of insult, careful analysis of the level of damage is necessary to ensure that later regenerative steps are appropriately identified. For example, many studies have used hair bundle counts as the primary assay for loss of hair cells, but hair cells may be able to lose their stereociliary bundles and survive. In fact,

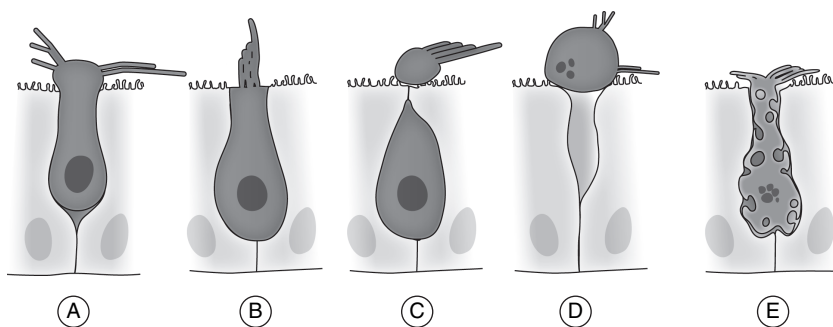


FIGURE 2.8. Hair cells can have a number of morphologic responses to insult. (A) In response to light to moderate acoustic overstimulation or ototoxic insult, the hair bundle may fragment and the stereocilia may become splayed. (B) At higher levels of damage, stereocilia may fuse together and portions of the hair bundle may be lost. This is often accompanied by swelling or blebbing of the apical surface of the hair cell. (C) Hair cells may also completely pinch off their damaged hair bundle along with a bit of apical cytoplasm, resulting in a bundleless hair cell. In response to higher levels of damage, hair cells may be fully extruded from the epithelium (D) or become vacuolated and degenerate within the epithelium (E).

alterations to the hair bundle and stereociliary loss are often the initial responses to traumatic insult, and may in fact serve to protect the cell from further insult. Thus a critical step in analysis of regeneration is solid determination of the level of damage to the epithelium.

7.1.2 Repair and Replacement of Damaged Hair Bundles

The mechanosensory function of hair cells is dependent on the presence of a functioning hair bundle, but often the initial response to insult is damage to the hair bundle. Tip-links are extracellular filaments that connect stereocilia and are proposed to act as the gating spring that opens sensory transduction channels at the tips of the stereocilia in response to movement of the hair bundle (Pickles et al. 1984; Ricci et al. 2006). Tip-links can be damaged by moderate acoustic overstimulation (Clark and Pickles 1996; Husbands et al. 1999), treatment with calcium chelators (Assad et al. 1991), or elastase (Osborne and Comis 1990a). Breakage of tip-links, such as by calcium chelation, leads to a loss of mechanotransduction consistent with the hypothesis that the tip-link gates the mechanotransduction channels in response to hair bundle movement (Assad et al. 1991). There is rapid morphological recovery of tip-links after calcium-chelation-induced breakage, accompanied by a physiological recovery of mechanotransduction (Zhao et al. 1996). There is similar recovery in the number of tip-links after acoustic overstimulation in tall hair cells of the chick basilar papilla within 1–4 days after the damage (Husbands et al. 1999). Thus, the fine structure of the hair bundle can be readily repaired after light damage, and this repair returns functionality to the hair cells.

In cases where damage to the hair bundle has been more extensive, resulting in fusion or loss of stereocilia or even loss of the entire hair bundle, regeneration of the damaged bundle would be necessary to permit functional recovery (Fig. 2.9a). In bullfrog (*Rana catesbeiana*) saccules treated with low doses of aminoglycoside antibiotics, a number of cells lose a large portion of the bundle, but retain some of their stereocilia, often fused together. Within a week after the damage, some of these hair cells rebuild a hair bundle with the traditional staircase pattern adjacent to the remnants of their damaged bundle (Gale et al. 2002). This is suggestive of reconstruction of a hair bundle in mature hair cells. Hair cells can also completely lose their hair bundles and survive as bundle-less hair cells within the epithelium for days in both bullfrogs and rats (Zheng et al. 1999; Gale et al. 2002). There also can be recovery of hair bundle number, though the number of hair cell bodies remains constant, in the presence of mitotic inhibitors to prevent new cell birth (Zheng et al. 1999; Gale et al. 2002). Thus, even after relatively significant damage, such as complete loss of their bundle, hair cells are able to recover and repair, which may lead to recovery of function within the damaged organ even in the absence of proliferation.

Such plasticity of the hair bundle may be a normal part of its physiology. Studies examining the actin composition of hair bundles have found that actin is continually added to the tips of stereocilia and removed at the base, resulting

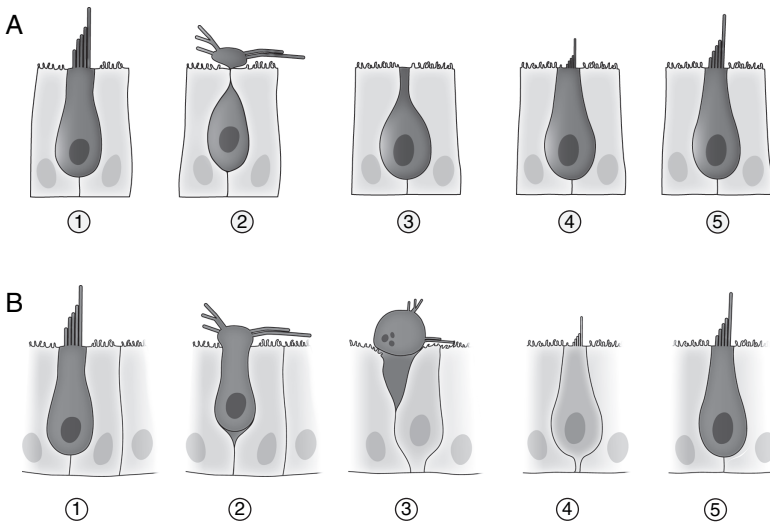


FIGURE 2.9. Schematic diagrams of two proposed mechanisms of nonproliferative regeneration. **(A)** Repair of damaged hair cells. In response to damage, the hair cell loses its hair bundle and apical cytoplasm (1–2), but over time is able to regenerate a new bundle that matures into a normal hair bundle (3–5). **(B)** Direct conversion of a supporting cell into a hair cell without an intervening mitosis. When a hair cell is lost (1–2), one of the neighboring supporting cells, no longer suppressed by an adjacent hair cell (3), is able to redifferentiate into a hair cell and develop a morphologically mature hair bundle (4–5).

in a complete turnover of the actin in each stereocilia every 48 hours (Schneider et al. 2002). Thus hair bundles are not static, but rather are dynamic with the cytoskeletal elements of the stereocilia continually recycled. It is not unreasonable, then, to suggest that after damage to the bundle, alterations in the actin dynamics may lead to repair or reconstruction of stereocilia. Mouse hair cells that have their bundles mechanically disrupted are able to rebuild a kinocilium, leading to reorganization of the cuticular plate and reinitiation of stereocilia formation (Sobkowicz et al. 1995). Together, these data suggest that if hair cells survive the insult, they may have a significant capacity for repair that could lead to morphological, and possibly functional, recovery. Such repair may account for some of the nonproliferative regeneration observed in many systems.

7.2 Phenotypic Conversion into Hair Cells

A second hypothesis that has been put forth to account for the nonproliferative generation of new hair cells, is that supporting cells are able to convert directly into hair cells without undergoing an intervening mitosis (Fig. 2.9b). This process has been termed either transdifferentiation or phenotypic conversion. The term phenotypic conversion will be used herein to eliminate any confusion with transdifferentiation, as that term has also been used to describe the conversion of one tissue type to another.

7.2.1 Evidence for Phenotypic Conversion

The evidence in support of this hypothesis includes the disparity in the number of dividing cells after injury and the number of new hair bundles or hair cells seen in the epithelium (Forge et al. 1998), a lack of incorporation of S-phase markers (e.g., BrdU) in hair cells after recovery (Roberson et al. 1996, 2004; Li and Forge 1997; Berggren et al. 2003; Matsui et al. 2003), and recovery of hair cells even in the presence of a mitotic inhibitor (Adler and Raphael 1996; Adler et al. 1997; Steyger et al. 1997; Baird et al. 2000; Taylor and Forge 2005). Cells that have an intermediate morphology between hair cells and supporting cells (i.e., nuclei in the basal layer, contact with the basal lamina, apical specializations and microvilli similar to hair bundles) can be found during recovery from damage (Adler et al. 1997; Li and Forge 1997; Matsui et al. 2003), consistent with supporting cells in the process of redifferentiating as hair cells. Time-lapse recordings of regeneration in axolotl lateral line have shown occasional hair cells differentiating from cells identified as supporting cells without an intervening cell division (Jones and Corwin 1996).

7.2.2 Developmental Window for Conversion in the Mammalian Cochlea

In the embryonic mammalian cochlea, shortly after terminal mitosis, newly differentiating supporting cells can convert into hair cells after ablation of adjacent hair cells, but the supporting cells rapidly lose this capacity as they

mature (Kelley et al. 1993). Thus, there may be a limited window during differentiation when mammalian cochlear supporting cells are plastic enough to convert into hair cells, though mature cochlear supporting cells with their morphological specializations are unlikely to do so.

In the mature mammalian organ of Corti, with its highly differentiated supporting cells, there have been several reports of morphological changes in supporting cells after hair cell loss. Deiters' cells, which surround the three rows of outer hair cells, develop dense bundles of microvilli that resemble immature hair bundles, and are occasionally contacted by efferent and afferent fibers after loss of the outer hair cells (Romand et al. 1996; Lenoir and Vago 1997; Daudet et al. 1998, 2002). While they are able to develop actin-rich microvilli bundles, the bundles never mature into stereocilia, the cells do not develop a tall kinocilium, they do not acquire morphological or molecular features of hair cells, and they retain features of supporting cells including gap junctions between adjacent cells (Daudet et al. 1998, 2002). Over time, most of the atypical cells and neighboring supporting cells are lost from the sensory epithelium and replaced by cells from the outer sulcus (Daudet et al. 1998). Thus, mature Deiters' cells attempt to undergo morphological change, but are unable to complete the process and are removed from the epithelium.

7.2.3 Conversion into Hair Cells by Gene Manipulation

There is evidence that the cells that lie just outside the organ of Corti, including the Hensen's cells and tectal cells adjacent to the outer hair cells, and cells of the greater epithelial ridge adjacent to the inner hair cells, though not in the sensory epithelium, are nonetheless capable of being converted into hair cells. Application of retinoic acid to the mouse cochlea causes the production of supernumerary hair cells next to both the inner and outer hair cells without any additional cell divisions, but once again only if applied during a short developmental window between E14 and E18 (Kelley et al. 1993). Similar results have been reported for treatment with epidermal growth factor (EGF) and transforming growth factor- β (TGF- β) in neonatal rat cochleae (Chardin and Romand 1997; Lefebvre et al. 2000). The nonsensory epithelial cells adjacent to the organ of Corti thus retain the ability to convert into sensory hair cells when pushed by application of exogenous growth factors, though this capacity seems to be lost as the cells mature.

In cochlear explants from neonatal rats, exogenous expression of *Atoh1* (the atonal homolog also known as *Math1*) in cells of the greater epithelial ridge (GER) is sufficient to convert nonsensory epithelial cells there into a hair cell phenotype: expressing myosin VIIa, a molecular marker of hair cells, taking on the morphology of hair cells, and developing hair bundles (Zheng and Gao 2000). Similarly, the use of adenovirus to transfect *Math1* into mammalian inner ear organs leads to formation of ectopic hair cells in the nonsensory epithelium around the organ of Corti in vivo in mature guinea pigs and may convert supporting cells from damaged adult organs of Corti and adult utricles into hair cells (Kawamoto et al. 2003; Shou et al. 2003). More recently, there is suggestion

that transfection with *Atoh1* may be able to promote a reconstitution of the organ of Corti after aminoglycoside damage by converting surviving supporting cells into hair cells, allowing a limited recovery of cochlear function (Izumikawa et al. 2005). If the surprisingly accurate reconstitution of the epithelium after damage by *Atoh1* can be replicated, this would suggest that the supporting cells of the cochlea are not only capable of being converted into hair cells by *Atoh1*, but also that the positional cues that specify the identity of the newly differentiating cells are maintained. Such genetic manipulations should continue to provide information about control of cell fate in the inner ear, even if it remains unlikely that genetic therapy will be a viable approach for inducing clinical regeneration.

7.2.4 Evaluating the Current Evidence for Supporting Cell Conversion

The role that phenotypic conversion plays in normal regeneration is unclear at present, as much of the indirect evidence supporting conversion could also be consistent with repair of damaged hair cells, such as recovery in the presence of mitotic inhibitors and recovery of hair bundle numbers. Supporting cell conversion in the absence of proliferation notably requires a decrease of supporting cell number as they convert into hair cells. However, decreases in the number of supporting cells are smaller than the number of cells that develop new hair bundles (Forge et al. 1998; Zheng et al. 1999). To establish whether significant supporting cell conversion occurs, it will be necessary to do specific and conclusive experiments that demonstrate conversion and exclude other mechanisms such as repair. Nonetheless, supporting cells or a subpopulation of less differentiated (e.g., recently produced) cells can convert into hair cells and contribute to morphological recovery, particularly in epithelia where ongoing cell addition means that cells at different states of differentiation may be present.

7.3 Mitotic Contribution of G_2 Cells

An additional mechanism that may contribute to apparent nonproliferative regeneration is mitosis and differentiation of cells that have already passed S-phase. Such cells will not be blocked by many mitotic inhibitors, which often block passage into S-phase, nor will they label with mitotic labels, which are incorporated in S-phase. In bullfrog (*Rana catesbeiana*) saccular cultures blocked with aphidicolin, occasional pairs of immature bundles were found throughout the epithelium, consistent with mitotic production in the absence of the cells passing through S-phase (Gale et al. 2002). In particular, in epithelia where there is constant addition of cells, there may be a fraction of cells residing in G_2 -phase that can contribute to regenerative recovery. G_2 -arrest of precursors is another potential source for mitosis in the absence of S-phase passage. During salamander limb regeneration, myotubes arrest in G_2 (Tanaka et al. 1997), and during differentiation of *Drosophila* eye and wing development, cells arrest in G_2 in a Wnt/Notch-dependent manner (Kimura et al. 1997; Johnston and

Edgar 1998). The potential contribution of G₂-phase cells to nonproliferative recover seems worthy of further investigation, such as screening for cells that have twice the DNA content of neighboring cells.

7.4 Nonproliferative versus Proliferative Recovery

There is a strong possibility that conversion, repair, and proliferative regeneration all contribute to morphological recovery of hair cells after damage. Importantly, only in mammals is regenerative proliferation strictly limited, and only in mammals are sensory deficits attributable to loss of hair cells permanent. Nonproliferative mechanisms of regeneration, while they may contribute significantly to morphological recovery from low levels of damage, may not be sufficient to bring about functional recovery in response to significant hair cell loss in the mammalian ear. Also, without proliferation, the number of supporting cells that can be converted while still maintaining sufficient supporting cell number for epithelial function will be limited. Generation of clinically significant numbers of replacement hair cells may therefore require returning mature mammalian supporting cells to a proliferative state, as occurs in other vertebrates.

8. Can Regeneration Restore Hair Cells in Mammals?

As discussed in the preceding text, the number of hair cells in the mammalian inner ear decreases with age, few cells are regenerated in the vestibular and auditory system after damage, and clinically, loss of hair cells in humans leads to permanent sensory deficit. The ongoing proliferation and robust regenerative proliferation that occur in nonmammalian vertebrates therefore do not occur in mammals at sufficient levels to be physiologically relevant. However, there may be low levels of repair and regenerative processes that can be stimulated to lead to functional regeneration in mammals.

8.1 Developmental Production of Hair Cells in the Mammalian Inner Ear

In mammals, the hair cells in the auditory and vestibular organs become fully established during embryonic development. In rodents, nearly all of the progenitor cells in the organ of Corti and vestibular system undergo terminal mitosis during mid to late embryonic development, followed by hair cell differentiation producing functional hair cells by birth (Ruben 1967; Kaltenbach and Falzarano 1994; Kaltenbach et al. 1994; Geleoc and Holt 2003). While a full cohort of cells is produced embryonically, and the cells are thought to become postmitotic, low ongoing rates of proliferation could occur and remain well below the sensitivity limits of the tests used to look for production in postembryonic mammalian ears. In fact, examination of hair bundles within the utricle maculae

from mature guinea pigs has shown on average one cell per thousand that has a small, immature bundle characteristic of newly differentiating hair cells, and six to seven cells per thousand that have intermediate size hair bundles (Lambert et al. 1997). Occasional immature-looking hair cells have also been reported in the sensory epithelia of the bat (Kirkegaard and Jørgensen 2001). If there is even a small level of ongoing proliferative production of cells, as occurs in most nonmammalian vertebrates examined, such a mechanism may provide an entry point into initiating a large proliferative response in mammals.

8.2 Reserve Stem Cells versus Supporting Cell Proliferation in Mammals

In contrast to the robust supporting cell proliferation in nonmammalian vertebrates previously discussed, studies of proliferation in response to damage in mammals suggest that few cells contribute to the regenerative response. Does this represent a distinct mechanism of replacing lost hair cells between mammals and other vertebrates, such as the presence of a few stem cells rather than a large population of potential progenitors?

Consistent with this hypothesis, a small fraction of cells from the adult mouse utricular epithelium act as colony-forming stem cells when dissociated, and the progeny of these cells can differentiate into many cell types including cells that express proteins in common with hair cells (Li et al. 2003). These colonies each have one to three cells that can be subcloned to form their own spheres in cultures. The demonstrated multipotency and self-renewal of these progenitors are the hallmarks of a stem cell population, suggesting that there may be a handful of isolated stem cells within mature mammalian vestibular epithelia. At this point, the identity or other characteristics of these potential stem cells are unknown.

What, then, of the capacity for the many supporting cells within the epithelium to reenter the cell cycle as occurs in nonmammalian vertebrates? The majority of supporting cells from perinatal rodents can be stimulated to proliferate in culture with growth factor and pharmacologic treatment, in a manner similar to, and using the same intracellular cascades as, avian supporting cells (Gu et al. 1996; Montcouquiol and Corwin 2001a, b; Witte et al. 2001). Thus the proliferative capacity of perinatal utricular epithelia is consistent with the hypothesis that most supporting cells are capable of proliferating. This is in contrast to the limited proliferation seen in mature organs in response to damage. However, when mature supporting cells are stimulated to spread and flatten following a large lesion, nearly all spread cells reenter the cell cycle, suggesting that most supporting cells retain the capacity to reenter the cell cycle, but are restricted from doing so under normal conditions (Meyers and Corwin 2007). Further experimentation will be necessary to determine whether a few reserve stem cells exist in the mature mammalian inner ear, or whether all mature supporting cells can serve as a potential, though only rarely stimulated, progenitor. It seems unlikely that mammals would retain only a few stem cells, in particular too few to bring about functional repair, while all other vertebrates can utilize any

supporting cell as a regenerative precursor. Rather, it may be that the cellular control of proliferation has been significantly tightened in mammals and that only rarely can a mature supporting cell overcome the strong inhibition.

8.3 Loss of Mammalian Proliferative Capacity with Age

Notably, the capacity to stimulate supporting cells to reenter the cell cycle is critically dependent on the age of the animal. While large numbers of supporting cells from perinatal animals can be stimulated to proliferate, few to no cells enter S-phase in cultures from mature animals (Gu et al. 1997; Hume et al. 2003; Gu et al. 2007). Thus, the proliferative mechanisms that operate in cultures from the neonatal inner ear are turned off or suppressed in more mature epithelia, consistent with the limited proliferation found in mature guinea pig or human utricles after damage. Coincident with this change in proliferation, there are changes in the components of the basal lamina and cytoskeleton that affect the ability of supporting cells to change shape and reenter the cell cycle (Davies et al. 2007). Pharmacological treatments that alter the cytoskeleton show promise in promoting supporting cell proliferation in mature utricles (Davies et al. 2007; Meyers and Corwin 2007). The fundamental nature of the cytoskeletal and other changes that underlie this change in proliferative capacity are unknown, but future studies should help elucidate the mechanisms that repress proliferation in mature epithelia (see also Oesterle and Stone, Chapter 5).

8.4 Challenges and Promises of Mammalian Regeneration

The restricted proliferative response to damage in the mammalian inner ear suggests that significant numbers of new cells are not produced as they are in nonmammalian vertebrates, consistent with the clinical permanence of auditory and vestibular deficits associated with hair cells loss and the continual decrease in hair cell number throughout the life of mammals. There may be some non-proliferative mechanisms that allow morphological recovery, and more work needs to be done to assess whether this could contribute to functional recovery. While mammalian supporting cells appear to lose their capacity to reenter the cell cycle as they mature, the presence of some supporting cells that reenter the cell cycle and immature hair bundles in the adult mammalian vestibular system indicate that the cellular processes necessary for regeneration can occur in mammalian sensory epithelia. Work has begun to point to genetic changes and cellular pathways that may underlie the loss of proliferative capacity, but the fundamental changes that restrict supporting cell proliferation in mammalian epithelia remain unclear. Further investigation into the control of proliferation in the mammalian inner ear and the mechanisms that underlie the robust proliferation in nonmammalian vertebrates may lead to strategies that can upregulate proliferative regeneration, bringing about morphological and functional recovery from damage.

9. Levels of Cell Differentiation

One recurring theme in this chapter has been that cells that become too differentiated become refractory to regenerative processes, whether that is cell cycle reentry or conversion of cell fate. Framed more broadly, the plasticity of the inner ear to respond to damage may be tied into the level of specialization and differentiation of the cells within the sensory organ (Fig. 2.10). In particular, the most specialized cell type within the ear, the hair cells, are terminally differentiated and under normal conditions do not reenter the cell cycle. Genetic manipulation of *retinoblastoma* control of the G_1 – S transition can force differentiating hair cells to stay in the cell cycle (Sage et al. 2005), but normal hair cells are not thought to contribute to regeneration, except via self-repair. In the mammalian cochlea, where supporting cells show the highest degree of morphological specialization and differentiation, the normal proliferative response is minimal, and capacity for cell fate conversion is minimal outside a narrow developmental window. Supporting cells in the mammalian vestibular organs show fewer specializations than those in the cochlea, and are occasionally able to reenter the cell cycle and may be capable of redifferentiating into a hair cell by phenotypic conversion. Supporting cells in nonmammalian sensory organs also show few morphological specializations and appear generally capable of reentry into the cell cycle as

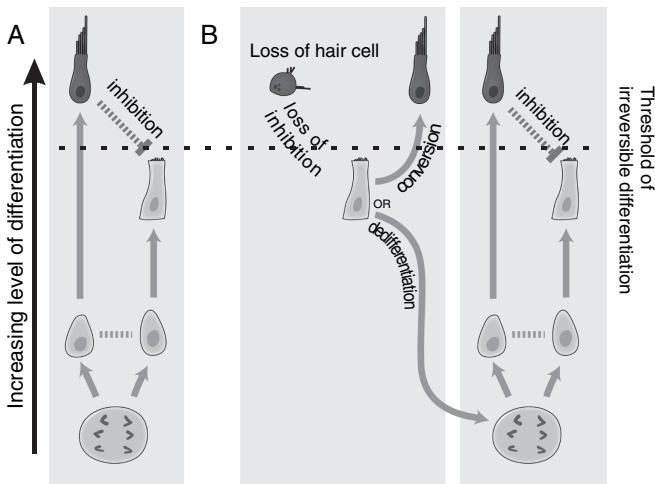


FIGURE 2.10. Schematic of how levels of differentiation may affect hair cell regeneration. (A) Multipotent progenitors (bottom) have no differentiated characteristics and can divide to produce both hair cells and supporting cells. As the hair cell differentiates, it crosses a threshold beyond which it is incapable of returning to the cell cycle or respecifying its fate. An inhibitory cue from the hair cell holds the supporting cell in a less morphologically differentiated state. (B) When the hair cell is lost, the inhibition of the supporting cell is relieved, allowing it to either directly differentiate into a hair cell (conversion) or dedifferentiate into a multipotent progenitor that returns to the cell cycle.

well as potentially able to convert directly into hair cells. Notably, in sensory epithelia that have ongoing proliferation, such as the lateral line or the avian, fish, or amphibian vestibular system, a sizable population of recently produced, partially differentiated supporting cells may reside and be capable of phenotypic plasticity or cell cycle reentry.

As the supporting cells in the mammalian cochlea and vestibular organs differentiate, they become less capable of reentry into the cell cycle as well as less capable of converting into a hair cell in response to hair cell ablation or pharmacological treatment. One could therefore imagine a hierarchy of differentiation, with the original progenitor cells for the hair cells and supporting cells serving as an undifferentiated, multipotent cell (Fig. 2.10). The progeny of that cell begin to differentiate, with the hair cell quickly crossing a threshold of differentiation beyond which the cell cannot reenter the cell cycle or change its fate (potentially controlled by the *retinoblastoma* pathway among other signaling mechanisms). In nonmammalian vertebrates, the supporting cell differentiates, but retains enough plasticity either to alter its fate directly or to return to a multipotential progenitor state. In elasmobranchs and amphibians, multipotent progenitors may also be retained at the margin between the sensory and nonsensory epithelium. In the mammalian vestibular system, the supporting cells retain plasticity for a short time after they are produced, but gradually become refractory to reentering the cell cycle or converting their fate to become hair cells. In the mammalian cochlea, the supporting cells again initially show some plasticity, but quickly specialize to a point where few can return to a multipotent progenitor or change their fate without direct genetic manipulation. As more is discovered about the genes involved in supporting cell differentiation and maintenance of a multipotential progenitor fate, several aspects of this hypothesis can be more completely tested.

10. Reinnervation of Hair Cells

In addition to the challenges faced in recovering an appropriate number of hair cells after damage, these hair cells must become integrated with the nervous system for there to be restoration of function. The recovery of hearing and vestibular function in birds in the days to weeks after loss of hair cells indicates that once hair cells regenerate, sufficient reinnervation occurs to enable communication between the sensory cells and the central nervous system (see Saunders and Salvi, Chapter 3). Ten days after acoustic overstimulation in adult quail, both efferent and afferent terminals with defined synaptic specializations were seen on regenerated hair cells (Ryals and Westbrook 1994), lagging about 3 days behind the regeneration of hair cells (Wang and Raphael 1996). In contrast, in studies of gentamicin-treated chicks, few synapses were present on regenerating hair cells after 10 days, though terminals gradually formed over several months, matured, and developed specializations, though not matching the complexity of normal synaptic terminals (Hennig and Cotanche 1998; Zakir and Dickman 2006). The difference in speed of neural reconnection may be due to the more complete

lesion of hair cells and the broader systemic effects from aminoglycoside antibiotics, such as kidney damage, compared to acoustic overstimulation, which would be restricted to the basilar papilla. Nonetheless, reinnervation in the avian inner ear can recapitulate the original innervation and lead to functional recovery, suggesting that substantial plasticity is retained in the regenerating afferents and efferents. Importantly, such reinnervation requires healthy neurons. Studies in the chicken found that low doses of kainic acid would damage the glutamatergic cochlear afferent synapse but allow normal reinnervation and return of cochlear function, while high doses killed the cochlear neurons and led to irreversible loss of the synapses and cochlear function (Sun et al. 2000, 2001).

10.1 Reinnervation in Mammals

Although significant spontaneous hair cell regeneration does not occur in the mammalian cochlea, the neural fibers do show signs of plasticity that suggest that reinnervation is possible. After acoustic overstimulation, the fibers innervating the organ of Corti degenerate, though it is unclear whether the loss of the fibers is due to direct excitotoxicity from the overstimulation (Puel et al. 1994) or is secondary to the loss of hair cells (Lawner et al. 1997). While some neurons in the spiral ganglion die after overstimulation, after 1 year, nerve fibers can be seen throughout the damaged regions of the cochlea, doubling back toward the spiral ganglion, traveling laterally along the basilar membrane, or spiraling around the basilar membrane (Bohne and Harding 1992). Some of the regenerating fibers terminate on cuboidal or squamous epithelial cells while others migrate into remnants of the organ of Corti and terminate on supporting cells or surviving hair cells (Bohne and Harding 1992).

The regenerating fibers within the cochlea appear to be exclusively afferent neurites (Strominger et al. 1995), though neurons within the cochlear nucleus will sprout small axonal processes between 2 and 8 months after overstimulation-induced degeneration (Bilak et al. 1997). Thus, while hair cells are not replaced after loss in the mammalian organ of Corti, the innervating neurons can regenerate. Paired with the finding that functional recovery follows reinnervation of regenerated hair cells in the avian inner ear, this suggests that if new hair cells could be produced in mammals, they are likely to be reinnervated, at least by afferent neurons, which may lead to functional recovery.

11. Summary

Morphological evidence of regeneration and repair in the lateral line and inner ear sensory organs of vertebrates has accumulated since the 1930s. After trauma to their lateral lines, aquatic amphibians regenerate entire sensory epithelia and the individual hair cells within them. This regeneration begins when supporting cells reenter the cell cycle, replicate their chromosomes, divide, and produce new cellular progeny that differentiate as replacement hair cells and supporting cells.

Supporting cells in the ears of fish, amphibians, and birds also divide and produce new hair cells and supporting cells throughout life, contributing to significant growth of the hair cell populations in the ears of some species, continual turnover of hair cells in others, and most significantly to the regenerative replacement of hair cells that have been killed by trauma or toxicity. Reptiles have not been widely investigated, but at present it appears that mammals alone lack the capacity for effective replacement of hair cells.

Research aimed at the discovery of treatments that may stimulate regeneration of hair cells in mammals has yielded considerable progress. In mammals, there is strong evidence for recovery in the number of hair bundles after damage to the vestibular system, indicating that some process of repair or regeneration can occur *in vivo*. While the proliferative responses are limited in mature animals, some supporting cells divide in response to damage, even in epithelia from 50-year-old humans.

Mammalian supporting cells share many morphological characteristics with the supporting cells that are the key to the replacement of hair cells in nonmammals, but the mammalian cells become quiescent early in the postnatal maturation. Before this, the proliferation of supporting cells in neonatal mammalian vestibular epithelia can be greatly stimulated by appropriate growth factor treatments and by direct activation of intracellular signal cascades. In addition, it has been shown that the forced expression of the transcription factor *Atoh1* will result in the differentiation of new hair cells in mammalian ears. Thus, the machinery for the regeneration of mammalian hair cells is present and appears to be capable of functioning under experimental conditions.

11.1 Open Questions and Future Directions

While much progress has been made in identifying the underlying mechanisms for regeneration in the inner ear, many open questions remain. We would like to briefly summarize a few of what we feel are important morphological questions.

1. Is there a population of stem cells at the margins of the elasmobranch and amphibian sensory epithelia? Do these cells recapitulate the original developmental processes, or are the cells distinct from the original progenitors that lay out the sensory epithelium? Do the cells at the margin of the mammalian epithelia retain any of the properties of these ongoing progenitors?
2. What is the trigger for stimulating regeneration? Is this trigger missing or decreased in mammals or is it the response to the trigger that is decreased?
3. What processes underlie the dedifferentiation of supporting cells allowing them to return to the cell cycle?
4. What is the contribution of nonproliferative mechanisms to recovery following damage? Are there limits to how much hair cells can repair damage? Can protective treatments preserve hair cells and allow sufficient post-damage repair to bring about return of function? Does supporting cell conversion contribute significantly to recovery? In addition, because these mechanisms are believed to occur in mammals, but mammals do not have significant

functional recovery from significant damage, what physiological relevance do these mechanisms have in mammals versus nonmammalian vertebrates? As the mechanisms underlying nonproliferative repair are elucidated, can these mechanisms be augmented to stimulate functional recovery in mammals?

5. What is the primary limiting factor for stimulating regenerative proliferation in mammals? Is it that mammalian supporting cells are too highly differentiated to return to the cell cycle, and only a handful of stem cells remain? Or do all supporting cells retain the capacity to reenter the cell cycle if appropriately stimulated?

11.2 Conclusions

Continued research into the mechanisms of hair cell regeneration at all levels from species that produce hair cells throughout life to those where proliferation is activated only in response to damage, to mature mammalian cochleae where little regeneration occurs, is likely to provide further insight into the factors that limit regeneration. Four steps must be achieved to turn hopes of regeneration in mammalian into reality. Quiescence of supporting cells must be reversed or suspended, supporting cell proliferation must produce new cells, the new cells must be induced to differentiate as replacement hair cells, and those cells must become reinnervated and functionally integrated into the nervous system. Progress has been made toward the achievement of each of those steps, and continued research ultimately holds the promise of developing treatments to help the millions of people affected by hearing loss and vestibular dysfunctions.

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