Cellular studies of auditory hair cell regeneration in birds

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A decade ago it was discovered that mature birds are able to regenerate hair cells, the receptors for auditory perception. This surprising finding generated hope in the field of auditory neuroscience that new hair cells someday may be coaxed to form in another class of warm-blooded vertebrates, mammals. We have made considerable progress toward understanding some cellular and molecular events that lead to hair cell regeneration in birds. This review discusses our current understanding of avian hair cell regeneration, with some comparisons to other vertebrate classes and other regenerative systems.

supporting cell | avian | basilar papilla

Hair Cells: Old and New

Hair cells are anatomically and functionally exquisite cells that serve as the mechanoreceptors for hearing, balance, and orientation in space. Their name is derived from the bundle of actin-filled stereocilia that protrudes from their apical surfaces into the fluid-filled cavities of the inner ear or lateral line organs. Each hair cell is surrounded by several supporting cells and forms synapses with the VIIIth cranial nerve, which encodes and transmits signals to and from the hindbrain nuclei. Auditory hair cells are highly susceptible to intense noise, ototoxic drugs, and aging, and many genetic defects lead to malformations of the peripheral auditory structures. The majority of hearing loss in humans is sensorineural in nature—i.e., derived from abnormalities in either hair cells or the VIIIth nerve. Unfortunately, hair cell loss in humans and most mammals is irreversible.

The first indication that warm-blooded vertebrates can regenerate hair cells in the inner ear came from relatively recent experiments aimed at addressing two completely different sets of problems in birds. Cruz *et al.* (1) were attempting to use the chicken cochlea (or basilar papilla) as a model system to study ototoxicity of aminoglycoside antibiotics. At the same time, Cotanche (2) was studying developmental changes in noise damage and tonotopic organization in the same tissue. Both groups reported preliminary indications that new hair cells were formed in the area of hair cell damage in the posthatch chick basilar papilla. Confirmation of this interpretation was provided when radioactive thymidine labeling was seen in both hair cells and supporting cells after noise damage in young chicks (3) and adult quail (4) (Fig. 1). At the same time, Jørgensen and Mathiesen (5) showed that there was mitotic activity and new hair cell production in the vestibular epithelia of untreated adult parakeets (Budgerigars). The vestibular epithelium's capacity for ongoing hair cell production is distinct from the avian auditory epithelium, in which there is no postembryonic mitotic activity (6) until hair cell damage is induced. Similar to basilar papillas, the rate of hair cell production is increased in avian vestibular organs after experimental damage (7–11).

Methods for Stimulating Hair Cell Regeneration in the Basilar Papilla

This paper focuses on hair cell regeneration in the avian auditory epithelium. Historically, two principal experimental methods have been used to induce the loss of auditory hair cells in birds: exposure to intense noise and treatment with ototoxic drugs.

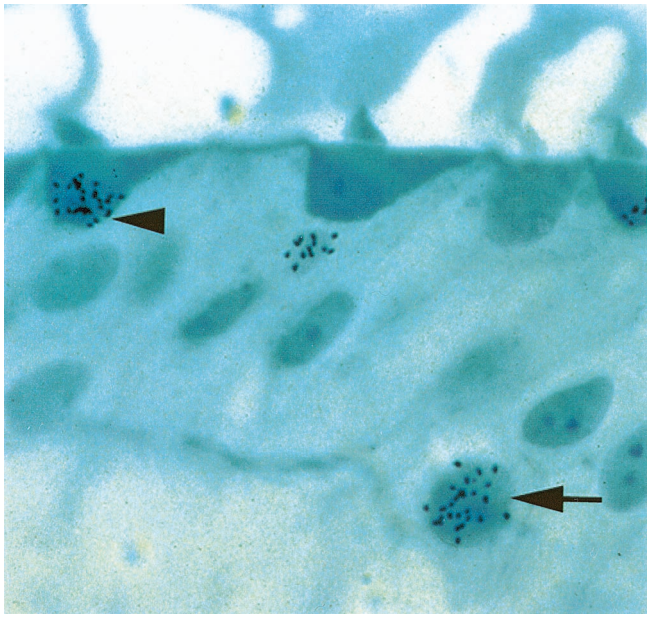

Fig. 1. Evidence of mitotic regeneration of hair cells and supporting cells in the avian basilar papilla. The nuclei of hair cells (arrowhead) and supporting cells (arrow) contain the radioactive nucleotide analog, tritiated thymidine, after noise damage in the mature quail, demonstrating they are the products of renewed cell division.

Exposure to intense pure-tone or broadband noise causes a lesion that varies with frequency along the tonotopic axis (length) of the epithelium, and that has a size and location along the neural axis (width) of the epithelium that change with intensity. The lesions that result from sound exposures at high intensities are composed of regions of complete hair cell loss as well as regions in which hair cells survive but appear damaged in a variety of ways (12–14). In most cases, noise exposure affects additional cell types besides hair cells (15–17).

The second method of inducing hair cell loss uses the ototoxic antibiotics, aminoglycosides (e.g., gentamicin, kanamycin, and streptomycin). Aminoglycosides target hair cells in the high-frequency, proximal portion of the basilar papilla. At low single doses, only hair cells at the proximal tip of the basilar papilla are

This paper was distributed at and is part of the papers presented at the National Academy of Sciences colloquium ''Auditory Neuroscience: Development, Transduction, and Integration,'' held May 19–21, 2000, at the Arnold and Mabel Beckman Center in Irvine, CA.

Abbreviations: FGF, fibroblast growth factor; IGF, insulin-like growth factor; FGFR, FGF receptor; RA, retinoic acid.

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killed (18). As dosage is increased, the region devoid of native hair cells expands toward the distal end. Recently, several laboratories have developed and standardized single-dosage, systemic, or local application paradigms for aminoglycosides (18–22). By carefully documenting the region of complete hair cell loss with each drug administration paradigm, it is possible to ensure that all hair cells in a particular frequency region of the basilar papilla are regenerated. Aminoglycosides generally cause complete hair cell loss throughout most of the lesioned area, with intermittent hair cell loss at the distal border of the lesion. At moderate doses they appear to induce little or no direct damage to nonhair cell elements of the avian sensory epithelium.

Avian Hair Cell Progenitors: Identity and Behavior

The observation that new hair cells and supporting cells are formed after noise exposure led to the hypothesis that supporting cells are the progenitors to new hair cells during avian regeneration (2–4). Direct evidence was derived from studying the earliest cell types to enter S phase after experimentally induced damage in chickens. After noise damage, both supporting cells and hyaline cells, which line the abneural edge of the auditory epithelium, incorporate exogenous nucleotides during early phases of regeneration (15, 23, 24). Subsequent analysis has ruled out hyaline cells as potential hair cell progenitors, because hyaline cell proliferation and migration into the region of hair cell extrusion after intense noise stimulation do not lead to the formation of new hair cells (25). Observations of the chick utricle, a vestibular organ (26), and the basilar papilla after laser ablation (27) or ototoxic drug treatment (20, 28) also revealed that supporting cells are the principal epithelial cells to enter S phase preceding hair cell regeneration.

Supporting cell entry into the cell cycle is detected by 18–24 h after the onset of the damaging stimulus to the basilar papilla (15, 23, 24, 27, 29, 30). This delay reflects the latency of the triggering stimulus for cell proliferation plus the time required for supporting cells to progress from the growth-arrested state to S phase. The level of supporting cell proliferation peaks within 2–3 days after the onset of the stimulus, and it returns to normal levels by 7–9 days (19, 23, 24, 30). Patterns of cell proliferation spatially and temporally mirror the progression of hair cell loss (31).

Although supporting cells appear to be the likely candidates for avian hair cell progenitors, it is not clear whether all supporting cells have this potential. After drug treatment, virtually all of the supporting cells in both the damaged and undamaged regions of the basilar papilla change their cell cycle status by leaving growth arrest and entering G_1 phase, as demonstrated by increased expression of proliferating cell nuclear antigen (19). This observation suggests that all supporting cells in some nonmammalian species have the potential to divide. Some support for this notion is provided by Presson *et al.* (32) in their study of the fish saccule. They showed that new supporting cells are recruited to divide during ongoing hair cell production after cytosine arabinoside (AraC) treatment, which kills progenitor cells in S phase (e.g., ref. 33). This finding demonstrated that the full component of progenitor cells was not actively dividing at the time AraC was administered and suggested that there is extended potential for proliferation among the quiescent supporting cell population.

Despite these observations, only progenitors in areas of the chick basilar papilla with obviously damaged hair cells progress as far as S phase and regenerate new hair cells (2–4, 19, 27, 34). Further, the cells that do reach S phase comprise only about 15% of the supporting cell population in those areas (35). These findings raise two questions. First, is the supporting cell population subdivided into cells with different proliferative potentials (i.e., terminally differentiated cells, stem cells, or committed precursor cells)? Second, what mechanisms trigger the transitions of supporting cells from growth arrest to S phase?

There have been few systematic studies addressing the proliferative potential of supporting cells in the avian auditory epithelium, and the results of these studies are equivocal. Results from one study (24) suggest that some progenitor cells act like stem cells, undergoing multiple rounds of cell division after noise damage. A single injection of the thymidine analog, BrdUrd, was administered to chicks early during the regenerative process, and animals were allowed to recover for varying periods after the injection. The total number of BrdUrd-labeled cells increased significantly, and clusters of labeled cells appeared to grow in size, suggesting growth of a colony of cells from one progenitor. However, a second study (30) suggests that progenitor cell recycling is in fact rare in the drug-damaged basilar papilla. We detected only very limited incorporation of two nucleotide analogs into the same progenitor cells when each nucleotide was administered separately at intervals estimated to mimic the average cycling time of mature eukaryotic cells. In contrast, recycling of progenitor cells occurs to a very high degree (70%) in the saccules of normal oscars (36) and to a more limited extent in the utricles of control chicks (30, 36).

Studies of the mammalian olfactory epithelium, which regenerates olfactory neurons on an ongoing basis and in response to damage, have shed considerable light on the profiles of neuronal progenitors in that tissue. Molecular analyses and studies of proliferative behavior of cultured olfactory epithelial cells have shown that at least three subtypes of progenitors exist among the supporting cells (reviewed in ref. 37). The neuronal colony-forming cells, which compose a small percentage of the progenitor pool ($<0.1\%$), resemble true stem cells in that they divide continually at a slow rate for a long period (38). Two additional cell populations with more limited proliferative potential exist: a Mash1-positive cell that is a committed amplifying cell (39) and its progeny cell, termed an intermediate neuronal precursor, which gives rise directly to olfactory neurons (40–42). Our field would benefit from analyses like these in the olfactory epithelium to elucidate progenitor subtypes. In the absence of this clarity, the term "progenitor cell" will be used in place of the term "supporting cell" throughout this paper to describe the cell that divides and gives rise to new cells in the damaged auditory epithelium.

Several laboratories have participated in the search for molecules that trigger progenitor cell proliferation in avian inner ear epithelia. This search has been facilitated by the development of culturing methods for auditory and vestibular end organs and for isolated sensory epithelia (8, 20, 43–46). Tsue *et al.* (47) found increased incorporation of radioactive thymidine into progenitor cells in single control utricles that were cocultured without contact with multiple utricles in which hair cell damage had been stimulated. Further, focal laser ablation of a few hair cells *in vitro* causes activation of progenitor cell proliferation up to 100 μm from the lesion site (27). These studies suggest that a diffusible stimulatory signal is released from inner ear epithelia at the site of hair cell injury.

The identity and source of the putative diffusible mitogen(s) responsible for these effects are not known. Supporting cells in cultured utricles continue to divide in the absence of serum (43, 44), suggesting that substances that are mitogenic for hair cell progenitors are intrinsic to the sensory organs. The VIIIth nerve synapses/fibers are damaged as a result of hair cell loss (48–50) and therefore seem to be good candidates for release of mitogenic substances. Such an effect appears to occur in other regions of the nervous system. For example, mitogens released from injured peripheral nerve processes are thought to induce Schwann cell proliferation (51). However, neural elements are not required for proliferation during hair cell regeneration, as mitotic activity proceeds in tissue cultures of auditory (20) and

vestibular (43, 52) epithelia isolated without the nerve. In addition, neural elements do not appear to play a critical role in the genesis of sensory epithelial cells. Transplantation of the otocyst without its connected ganglia does not appear to disrupt production of either hair cells or supporting cells (53, 54). In mice that are null for both brain-derived neurotrophic factor and neurotrophin-3, both hair cells and supporting cells form despite the complete absence of cochlear ganglion cells (55). It should be noted, however, that innervation may be required for normal differentiation of hair cells (e.g., see refs. 55–57) and for maintenance of hair cells once they are fully differentiated (e.g., see ref. 58).

What are potential mitogens for progenitor cells in the chick inner ear? Studies using reverse transcription–PCR, *in situ* hybridization, or immunolabeling have revealed that epithelial cells in the mature avian basilar papilla express the following potential diffusible mitogens: fibroblast growth factor (FGF)-1 (59), FGF-2 (59–61), and insulin-like growth factor (IGF)-1 (59). FGF-2 protein is present in supporting cell nuclei (61), and its levels appear to be highly up-regulated in the damaged area after noise exposure (60). These growth factors bind to and activate tyrosine kinase receptors, many of which also are expressed in the avian inner ear. PCR studies have shown that mRNAs for FGF receptor (FGFR)-1, epidermal growth factor receptor (erbB1), and IGF receptor-1 are present in the cochlear duct (61) and, more specifically, in the sensory epithelium (59). Further, *in situ* hybridization reveals abundant message for FGFR-3 in the supporting cells (unpublished observation). After hair cell damage, there are detectable changes in the levels of transcripts for FGF-1, IGF receptor-1, FGFR-1, and FGFR-3, but not for IGF-1, FGF-2, or ErbB1 (59, 61). At the protein level, FGFR-1 is present in hair cells in control tissue, and then it becomes elevated in supporting cells after hair cell damage (59, 61). However, the actual mitogenic properties of most of these growth factors in auditory hair cell regeneration have not yet been determined. A study from our laboratory has shown that addition of IGF-1 or insulin to cultured chick utricles causes progenitor cell proliferation to increase significantly (62), whereas it has no effect in the basilar papilla (E. Oesterle, personal communication).

The role of leukocytes as activators of progenitor cell proliferation also is being explored. In response to hair cell damage, there is substantial migration and proliferation of macrophages and microglia-like cells in the inner ear epithelia of chicks (11, 63) and other species (64–67). Leukocytes can produce growth factors and cytokines (68), which may act directly as mitogens or indirectly by stimulating growth factor production in target cells (e.g., ref. 69). Indirect evidence suggests that the secretory products of leukocytes also may stimulate mitotic activity during hair cell regeneration. Proliferation of leukocytes in the avian auditory and vestibular epithelia precedes the experimentally induced increase in progenitor cell proliferation (11). Further, tumor necrosis factor α, which is released by macrophages after tissue damage (68), induces an increase in progenitor cell proliferation when added to cultures of the chick utricular epithelium (70).

Binding of growth factors to extracellular receptors initiates a cascade of intracellular signals, leading to increased mitotic activity. One requirement for a full understanding of regulation of hair cell regeneration is to determine which signaling pathways downstream of the receptor are activated during progenitor cell proliferation. Exploration of these pathways is just beginning. Activation of cAMP leads to increased progenitor cell proliferation in the basilar papilla *in vitro* (45). Because cAMP activity has not been widely implicated in growth factor-mediated stimulation of cell proliferation in other systems, the manner in which increased cAMP signaling serves to stimulate mitosis in the chick sensory epithelium is not clear.

Recruitment of progenitor cells to the cell cycle also may occur in response to local changes in the cells' microenvironment, such as the alterations in direct cell/cell signaling and cell shape that occur during hair cell extrusion and the ensuing supporting cell expansion (71).

In addition to positive control of mitotic activity, antimitotic influences are likely to be at work in the avian auditory epithelium: (*i*) to prevent proliferation in the undamaged basilar papilla; (*ii*) to ensure that all supporting cells in the damaged area do not proliferate; and (*iii*) to down-regulate cell proliferation when the correct number of cells is regenerated. The fact that auditory hair cell regeneration requires the loss of hair cells has led to the hypothesis that healthy hair cells exert an inhibitory influence on the mitotic activity of progenitor cells that surround them (2). Accordingly, Belgian *Waterslager* canaries, whose basilar papillas sustain spontaneous low-level hair cell damage in all regions (72), display ongoing progenitor cell proliferation (73). As noted above, a low level of ongoing cell proliferation occurs in the normal chick vestibular organs (5, 7, 74). This activity is thought to occur in response to spontaneous continual loss of individual hair cells through programmed cell death (74).

Do putative mitotic inhibitors in the sensory epithelia of the avian inner ear act through diffusion or through direct cell–cell contact? Tsue *et al.* (47) found that progenitor cell proliferation is down-regulated in single drug-damaged chick utricles that are cocultured without contact with multiple control utricles, suggesting that an inhibitory molecule is secreted from control organs. The inhibitory factor thought to be active in that system has not been characterized. However, molecules with inhibitory potential have been identified. Addition of exogenous basic FGF-2 to cultures of control utricles and drug-damaged basilar papillas leads to significant down-regulation of cell proliferation in the sensory epithelium (75). As mentioned above, FGFR-1 (59, 61) and FGFR-3 (O. Bermingham-McDonogh, personal communication) are expressed by supporting cells in the basilar papilla, and both receptors can be activated by FGF-2. Interestingly, FGFR-3 expression is not seen in the avian vestibular epithelia, which displays continuous mitotic activity. Furthermore, FGFR-3 expression is down-regulated in the regenerating region of the basilar papilla after drug treatment (unpublished observations). These observations suggest that FGFR-3 may provide steady-state inhibitory influences over supporting cells in undamaged auditory tissues.

Contact-mediated signaling from hair cells to progenitor cells also may play a role in inhibiting proliferation, but there are currently no data to directly support this hypothesis. This phenomenon appears to occur in the regenerating rodent olfactory epithelium. Addition of differentiated olfactory neurons to cultures of olfactory neuronal progenitors induces decreased mitotic activity among the progenitor cells (38). The mechanism that guides this negative feedback of differentiated cells upon their progenitors has not been characterized. However, one molecule that may mediate this inhibition is the extracellular receptor, Notch.

Notch is an integral membrane receptor that binds and becomes activated by ligands on adjacent cells. Notch has been studied most extensively in the developing *Drosophila* nervous system, where it plays a role in lateral inhibition, as well as in cell lineage decisions and boundary formation (reviewed in ref. 76). There is emerging evidence that signaling through the Notch receptor is also critical for neurogenesis in vertebrates (reviewed in ref. 77). Recent studies have shown that Notch and some of its ligands are expressed in the basilar papilla during development (78) and regeneration (79), suggesting that cell–cell signaling via this receptor is important for hair cell genesis. Further, the developing sensory epithelia of mutant zebrafish (80, 81) and knockout mice (82) show abnormal cell fate specification and

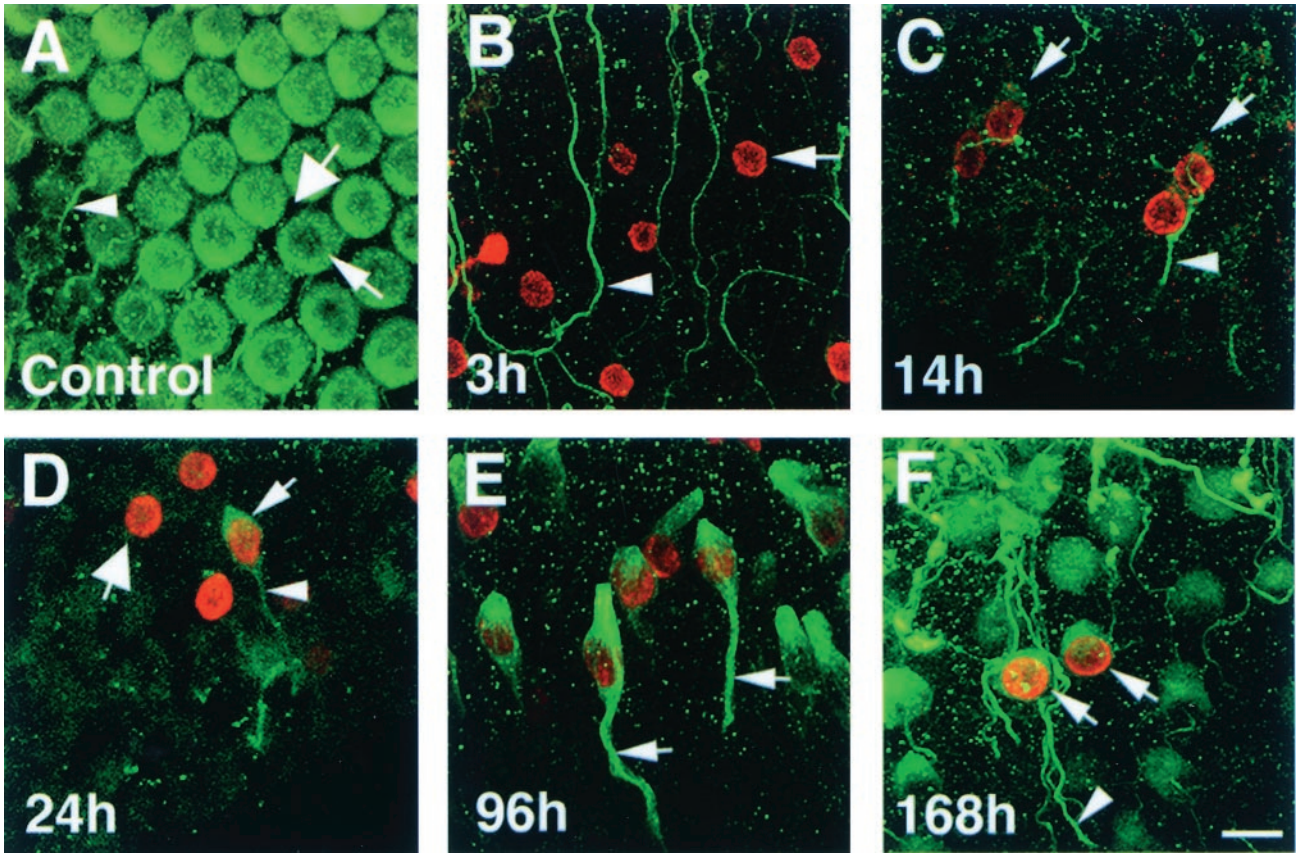

Fig. 2. Temporal progression of hair cell differentiation disclosed by antibodies to BrdUrd and the hair cell-selective protein, class III β-tubulin. Whole-mount preparations of the basilar papilla (BP) labeled with antibodies to BrdUrd (red) and/or β-tubulin (green). (*A*) In the control BP, β-tubulin is present in hair cells (thin arrow) and nerves (arrowhead), but not supporting cells (thick arrow). (*B–F*) Drug-damaged BPs taken from chicks at different times after a single BrdUrd injection at 3 days postgentamicin. (*B*) 3 h postBrdU. Progenitor cells in S or G_2 phase of the cell cycle (arrow) are labeled with BrdUrd, but not β-tubulin. β-tubulin is present in nerves (arrowhead) remaining after hair cell loss. (*C–F*) BrdUrd labeling in regenerated cells at progressively later stages of differentiation. (*C*) 14 h post-BrdUrd. Some rounded cells (arrows) near the lumen are double-labeled and represent new hair cells at an early stage of differentiation. These cells are associated with nerve processes (arrrowhead). (*D*) 24 h post-BrdUrd. Regenerated hair cells (thin arrow) are fusiform in shape and are associated with nerve processes (arrowhead). Some BrdUrd-positive cells are not labeled for β-tubulin (thick arrow); these cells are not differentiating as hair cells. (*E*) 96 h (4 days) post-BrdUrd. Regenerated hair cells have thick cytoplasmic processes that extend toward the lumen and basal lamina (arrows) of the epithelium. (*F*) 168 h (7 days) post-BrdUrd. Regenerated hair cells (arrows) resemble mature hair cells morphologically. Arrowhead points to nerve process. (Scale bar = 10 μm.)

tissue patterning, lending additional support for this hypothesis (see more in-depth discussion below).

A potential role of Notch in regulating mitotic activity in mature and developing tissues is beginning to emerge. Human gain-of-function mutations in *Notch* result in neoplasias in several tissues (reviewed in ref. 83), suggesting that Notch activation also may promote the proliferative state. Two recent studies report opposing effects of Notch on cell proliferation during formation of the *Drosophila* wing, depending on the stage of development. During early stages, activation of Notch stimulates cell proliferation (84), whereas during late development, activation of Notch leads to growth arrest (85). Clearly, functional studies of Notch in the avian inner ear are needed to test its potential role in regulating cell proliferation.

Repatterning the Mature Sensory Epithelium

The first newly formed hair cells appear in the regenerating avian basilar papilla by 3–4 days after the onset of a damaging stimulus (2, 15, 20, 28, 86) and continue to emerge in a manner that spatially and temporally mirrors hair cell loss (28, 86). Several weeks after damage the alternating array of supporting cells and hair cells is fairly well reestablished, with only minor deviations from the normal patterning, and hair cells appear fully differentiated (14, 15, 87, 88). At this time, the auditory nerve, which retracts after hair cell loss, has grown back to form synapses with the new hair cells (48, 50, 89–92). In the previous section, we discussed ways in which the appropriate number of new cells may be formed during hair cell regeneration in birds. This section addresses potential regulatory mechanisms for reestablishing the appropriate cell types and patterning in the avian auditory epithelium after damage.

A discussion of cellular differentiation during regeneration would not be complete without addressing the recent progress made toward identifying markers for mature and regenerating cells in the avian auditory epithelium. Cell-specific or cell-selective markers are critical for studies of tissue repair and regeneration for many reasons. They permit the analysis of early steps of hair cell differentiation, when mature morphological features of hair cells are not yet acquired. For example, a recent study from our lab using antibodies against the hair cell-selective protein, class III β-tubulin, has revealed that differentiation of regenerated hair cells resembles the embryonic process (93–95). New cells are mitotically generated at the luminal surface of the epithelium (96). Two to three days later they form extensions that reach toward the luminal and adlumenal surfaces of the epithelium (Fig. 2). By 7 days after mitosis they attain their mature globular shape. Information gleaned from such studies with cell-selective markers can be used to generate and test hypotheses about cellular and molecular interactions guiding cell commitment and differentiation during hair cell regeneration.

Markers also facilitate the characterization of the region of hair cell loss, allowing one to determine whether the damaging stimulus induces complete hair cell extrusion from the epithelium or only partial hair cell injury. This caveat has proven to be important in hair cell regeneration research, because some experimental paradigms in mammals induce stereociliary damage and repair rather than hair cell loss and scarring (97, 98). Markers are also useful for studying specific cell types in culture (20, 52, 98–101), because considerable cellular dedifferentiation often occurs *in vitro*, which makes it difficult to identify cell types solely by morphological criteria.

Several markers that are specific for mature and differentiating hair cells in chicks have been characterized in studies of the developing and mature auditory epithelium (20, 28, 78, 95, 102–107). Some of these markers have been used in culture paradigms to study hair cell regeneration (20, 52). A few antibodies and cDNA probes for supporting cell-specific proteins in the basilar papilla also have been identified (20, 108–110). However, these markers have not been used widely in studies of hair cell regeneration. Rather, supporting cells have been identified based on the exclusion of labeling for hair cell-specific antigens (e.g., refs. 28, 52, 79, and 111). Based on this ambiguity, we will use the term ''nonhair cell'' to describe cells whose identity has been inferred by using this approach.

The observation that the nuclei of both hair cells and supporting cells contain radioactive thymidine several days after it is injected after noise exposure led to the hypothesis that a common progenitor exists for both cell types in the mature inner ear of chickens (3). Recent studies have confirmed this notion in the developing basilar papilla as well as in the regenerating basilar papilla and utricle. Fekete *et al.* (112) used retroviral lineage analysis in the chick otocyst to show that hair cells and supporting cells in the basilar papilla often arise from a single progenitor cell division. Additional studies have shown that similar asymmetric differentiation predominates as the mode of cell production in the normal chick utricle (7, 30, 79) and the salamander lateral line organ (67). Asymmetric cell production is used during the ontogeny of many tissue types in a wide variety of species (reviewed in ref. 113). One advantage of forming cells with distinct phenotypes during each mitotic event is that the development of cellular diversity is ensured. In addition, if one of the daughter cells is a progenitor cell, then asymmetric cell production also ensures that cells with proliferative potential are maintained throughout the course of development and, in some cases, into the postembryonic period.

Interestingly, modes of cell production are quite variable in the regenerating basilar papilla after drug treatment. Double-labeling for BrdUrd and a hair cell marker has shown that individual mitotic events at 3 days postdrug treatment are equally likely to result in any of the following combinations of cell types: one hair cell and one nonhair cell, two hair cells, or two nonhair cells (28). This trend changes at a later stage of regeneration. Among cells born at 5 days postdrug treatment, the majority (71%) differentiate into two nonhair cells (J.S.S., unpublished data). These observations suggest that, across sensory epithelia and over the course of regeneration after damage in the basilar papilla, there are distinct differences in the differentiative potential of progenitor cells and/or the extrinsic factors that influence the fates of the daughter cells. Recent studies have begun to identify both intrinsic and extrinsic factors that may be important for proper tissue patterning during hair cell regeneration.

Cell fate outcomes are influenced in part by signaling from the progeny cell's environment. As discussed above, lateral inhibition via the Notch signaling pathway is perhaps the best-understood mechanism of extrinsic regulation of cell fate determination among sensory cells. Studies of the developing inner ear epithelia implicate a role for Notch and its ligands, Delta and Jagged, in hair cell specification (reviewed in refs. 77 and 114–116). In embryonic fish, birds, and rodents, transcripts for Notch 1, 2, and 3 and the ligands, Delta1 and Serrate1, are expressed in the otocyst (78, 80, 82, 117–123). Loss of function of the *Delta* homologue, *DeltaA*, in zebrafish leads to the overproduction of hair cells at the expense of supporting cells (81). In another zebrafish mutant, *mindbomb*, Notch signaling also is reduced, and a similar phenotype to the *DeltaA* mutant results (80). Further, in the developing mouse organ of Corti, the ligand Jagged-2 (a Serrate homolog) appears to be critical for proper cell patterning, as mice that are null for the gene generate supernumerary hair cells (82).

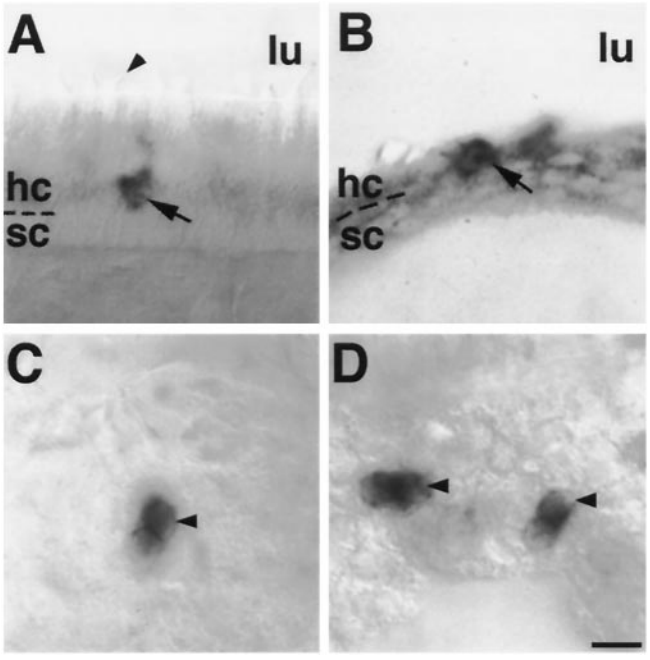

Fig. 3. Expression of *Delta1* mRNA in the chicken inner ear sensory epithelia. Cells expressing *Delta1* mRNA (black) are shown. Hair cells (arrows) in the untreated utricle (*A*) and drug-damaged basilar papilla at 3 days postgentamicin (*B*) contain high levels of *Delta1* mRNA. Arrowhead points to stereocilia at the luminal surface. Postmitotic sister pairs (arrowheads) in the untreated utricle (*C*) and drug-damaged basilar papilla at 3 days postgentamicin (*D*) have equal, high levels of *Delta1* mRNA. (Scale bar = 5 μm.) [Reproduced with permission from ref. 79 (Copyright 1999 The Company of Biologists Limited).]

Notch signaling in histogenesis in other adult tissues has been studied to only a limited degree (reviewed in refs. 83 and 124). In the regenerating inner ear epithelia of posthatch chickens, however, Notch signaling also may be critical for patterning the hair cells and supporting cells (79). Transcripts for Notch1 and its ligand, Serrate1 (chick's Jagged1 homolog), are abundant in nonregenerating inner ear tissues, whereas transcripts for *Delta1* are not. In mature chick utricles, and shortly after damage to the basilar papilla, *Delta1* mRNA is expressed in progenitor cells in S or G_2 phase, and it is symmetrically inherited by both daughter cells during mitosis (Fig. 3). During differentiation, *Delta1* mRNA levels appear to increase in cells acquiring the hair cell fate and disappear in cells acquiring the nonhair cell fate. During late stages of regeneration, *Delta1* mRNA expression decreases to its starting level. The effects of lateral inhibition may change over time in the regenerating basilar papilla and thereby influence the types of cells that are formed. For example, the increase in *Delta1*-expressing hair cells within the area of the lesion over time would lead to increased lateral inhibition and the generation of relatively more nonhair cells. Although the role of Delta1-Notch1 signaling in regenerating chick inner ear epithelia

remains to be tested experimentally, these observations suggest that the developmental role of Notch signaling appears to be preserved in the mature, regenerating chicken inner ear.

Signaling via diffusible factors is likely to be another important form of extrinsic regulation of cell specification during hair cell regeneration. One candidate factor, retinoic acid (RA), is a steroid hormone with long-range actions that is known to affect morphogenesis in many tissues (reviewed in ref. 125). RA, receptors for RA, and binding proteins for the precursor of RA, retinol, are present in the developing mammalian auditory epithelium (126–129). Addition of RA to cultures of the developing mouse organ of Corti causes an overproduction of hair cells (126), whereas inhibition of RA causes decreased hair cell specification (129). The role of RA in the organ of Corti of postnatal mammals remains controversial. LeFebvre *et al.* (130) reported that addition of RA to cultures of the drug-damaged cochlea of young rats stimulates regeneration or repair of hair cells, whereas Chardin and Romand (131, 132) detected no effect of RA in similar studies. In mature birds expression of RA receptors is present in the perinuclear region of hair cells (61), suggesting that RA signaling may be important for their differentiation. However, the role of RA in promoting cell specification in birds has not been tested.

The fate of newly regenerated cells depends on factors that are inherent to progenitor or progeny cells as well as extrinsic signaling molecules. These features include the activity of transcription factors and other cell fate determinants, which may be inherited during cell division (reviewed in refs. 113 and 133). Several transcription factors are expressed in the otocyst (reviewed in refs. 114, 134, and 135), but there is limited evidence that any of them is critical for cell fate determination or tissue patterning within the sensory epithelia. An exception is the helix–loop–helix transcription factor, Math1. Deletion of the *Math1* gene in mice leads to failure of hair cell production in both the auditory and vestibular epithelia, but supporting cells continue to be generated (136). Transfection of *Math1* in *in vitro* preparations of neonatal rat organ of Corti induces ectopic differentiation of hair cells in the region of the inner spiral sulcus (137). A role for Math1 in avian hair cell regeneration remains to be determined. A recent series of experiments from our lab provides preliminary evidence that *cProx1*, a chicken homolog for the DNA binding protein *Prospero* in *Drosophila* (138), may be involved in hair cell production during development and regeneration (J.S.S., unpublished observations). cProx1 protein is highly expressed in prosensory and proneural regions of auditory and vestibular portions of the avian otocyst, and expression is down-regulated by the time hair cell differentiation is complete. Interestingly, cProx1 protein becomes highly and transiently re-elevated in the mature auditory epithelium after experimental damage, first in progenitor cells and later in hair cells. Prospero and its homologs have emerged as an interesting set of transcription factors with diverse roles. In the *Drosophila* nervous system, Prospero is necessary for proper cell specification and/or differentiation in some lineages (e.g., refs. 139 and 140). Future investigations should test the hypothesis that *cProx1* is required for hair cell specification and/or differentiation during regeneration.

Numb is a cytoplasmic protein whose activity within some cells is sufficient to confer a neural fate. Studies in *Drosophila* first showed that loss-of-function *Numb* mutants underproduce neurons (141, 142). Recently, homologues for *Numb* have been identified in other vertebrates (143–146), and a critical role for *Numb* in neural specification has been demonstrated in chickens (146). Numb's effect on cell fate is determined at the time of cell division, as it is asymmetrically partitioned in the mother cell and distributed asymmetrically to daughter cells (reviewed in ref. 113). In recipient progeny, Numb acts by antagonizing Notch function (147–149). Based on the requirement for proper Notch signaling in hair cell specification, a potential role of Numb recently has begun to be explored in the developing and regenerating sensory epithelia of the inner ear. Numb protein is present in the mature chicken basilar papilla (J.S.S, unpublished observations). Further, Numb segregates asymmetrically within mitotic progenitor cells in this system. However, a critical role for Numb remains to be demonstrated.

Other cellular mechanisms, such as cellular reorganization (150) and programmed cell death (151), occur during development of the basilar papilla and may influence the numbers, types, or patterns of cells that are regenerated there in the mature bird after damage. Additionally, there is emerging evidence that hair cells may be produced by a nonmitotic conversion from supporting cells in mature frogs (152) and chickens (35, 153). This phenomenon, which is called direct or nonmitotic transdifferentiation, is addressed by Baird (161).

What's Wrong with Us?

Avian and mammalian auditory epithelia share many anatomical and functional features; they have similar types of specialized cell types, patterns of innervation, and mechanisms of sensory signal transduction. Despite these commonalities, these classes of vertebrates clearly possess critical differences with respect to their potential to form new cells after birth. The challenge lies in identifying where these differences exist. The most likely origin of divergence is the hair cell progenitor. In mammals, all cells in the auditory organ of Corti become terminally mitotic by embryonic day 14 (154). A similar phenomenon occurs in the avian basilar papilla by embryonic day 9 (155). Thus, the mature auditory organs in both classes of vertebrates exhibit mitotic quiescence. Despite this similarity, cells in the avian cochlear epithelium re-enter the cell cycle, divide, and differentiate into new hair cells after experimentally induced hair cell loss, but a similar stimulus causes no renewed mitotic activity in the mammalian organ of Corti (66). The failure of renewed proliferation in the mature mammalian auditory epithelium may be caused by persistent inhibition of mitotic activity among progenitor cells (156, 157), the absence of promitotic stimuli in response to hair cell loss (158–160), or the depletion of the hair cell progenitor during embryogenesis. The future's challenge is to identify which of these factors are the critical obstacles within the mammalian inner ear and to identify ways to overcome them. A growing understanding of the strategies used by animals with the capacity to regenerate inner ear hair cells, such as birds, will continue to provide insights into how this can be accomplished.

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