Hair cell recovery in mitotically blocked cultures of the bullfrog saccule

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Hair cells in many nonmammalian vertebrates are regenerated by the mitotic division of supporting cell progenitors and the differentiation of the resulting progeny into new hair cells and supporting cells. Recent studies have shown that nonmitotic hair cell recovery after aminoglycoside-induced damage can also occur in the vestibular organs. Using hair cell and supporting cell immunocytochemical markers, we have used confocal and electron microscopy to examine the fate of damaged hair cells and the origin of immature hair cells after gentamicin treatment in mitotically blocked cultures of the bullfrog saccule. Extruding and fragmenting hair cells, which undergo apoptotic cell death, are replaced by scar formations. After losing their bundles, sublethally damaged hair cells remain in the sensory epithelium for prolonged periods, acquiring supporting cell-like morphology and immunoreactivity. These modes of damage appear to be mutually exclusive, implying that sublethally damaged hair cells repair their bundles. Transitional cells, coexpressing hair cell and supporting cell markers, are seen near scar formations created by the expansion of neighboring supporting cells. Most of these cells have morphology and immunoreactivity similar to that of sublethally damaged hair cells. Ultrastructural analysis also reveals that most immature hair cells had autophagic vacuoles, implying that they originated from damaged hair cells rather than supporting cells. Some transitional cells are supporting cells participating in scar formations. Supporting cells also decrease in number during hair cell recovery, supporting the conclusion that some supporting cells undergo phenotypic conversion into hair cells without an intervening mitotic event.

Hair cells (HCs), the sensory receptors of the vertebrate inner ear, are neuroepithelial cells characterized by apical bundles composed of a single true cilium—the kinocilium—and numerous actin-filled stereocilia (1, 2). These mechanosensory cells are lost with age and are highly susceptible to damage from noise, disease, and ototoxic drugs. This age and trauma-induced susceptibility is a profound health problem because a significant proportion of the human population suffers from deafness or balance disorders resulting directly from HC loss.

Until recently, it was thought that mammalian HCs were produced only during embryonic development and, once lost in mature animals, were gone forever (refs. 3 and 4; but see refs. 5 and 6). We now know that the auditory and vestibular organs of many nonmammalian vertebrates, including fish (7–9), amphibians (10–13), and birds (14–17), produce HCs at a low level throughout life. More importantly, these vertebrates retain the capacity to up-regulate their rate of HC production after acoustic (18–22) and aminoglycoside-induced (23–29) damage to existing HCs. This ability is not retained or is present only to a limited extent in mammals (30–34).

Mitotic HC Regeneration

It has been assumed that HCs and supporting cells (SCs) are derived from a common pool of mitotic precursors during mitotic regeneration (35). This assumption, while consistent with developmental studies demonstrating that HCs and SCs derive from a common progenitor (36), has not been tested by direct observation except in amphibian neuromasts (37–39). There is, however, good evidence that HC production in nonmammalian vertebrates is accomplished by the renewed proliferation of nonsensory SCs. SCs in the auditory (21, 40–43) and vestibular (44) organs leave growth arrest and reenter the G_1-phase of the cell cycle after acoustic or aminoglycoside-induced damage to existing HCs. It remains unclear whether all SCs are capable of serving as HC progenitors. SCs in damaged and undamaged regions reenter the G_1-phase of the cell cycle (45, 46), but only 15% of SCs progress from G_1-phase to S-phase after HC damage, and these cells are limited to the immediate vicinity of the damaged region (47). Proliferating SCs in the fish saccule have no immunocytochemical (48) or ultrastructural (49) features that distinguish them from nonproliferating SCs.

The cellular events that stimulate HC progenitors to divide are only partially understood. Progenitor proliferation is up-regulated by diffusible factors released by damaged inner ear organs (50, 51). Progenitors also continue to divide in serum-free medium, indicating that mitotic substances are intrinsic to the inner ear organs (52, 53). A number of fibroblast growth factors that bind and activate tyrosine kinase receptors are known to be present in inner ear organs (54, 55). The expression levels of many of these factors are changed after HC damage, although their mitogenic properties have not yet been determined. Leukocytes, which release growth factors and cytokines, are also likely to play a role in progenitor proliferation (38, 39, 56). The signal transduction pathways downstream of activated tyrosine kinase receptors are less well understood. Recent studies indicate, however, that activation of cyclic adenosine monophosphate (cAMP) leads to increased progenitor proliferation[†] (57) and that many signaling molecules in the cAMP pathway are expressed in the inner ear organs.[‡]

The daughter cells resulting from progenitor division have been presumed to differentiate as new HCs and SCs (19, 20). However, the sequences of events that follow progenitor division, including proliferation, down-regulation, and fate determination, are only beginning to be revealed (refs. 59 and 60; for recent reviews, see refs. 6 and 22). Although it is usually assumed that these postmitotic events mirror those that occur during normal development, this assumption has not been rigorously tested. Many disparities in patterning and organization exist between developing and regenerating inner ear organs, suggest-

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Abbreviations: C, control; GT, gentamicin-treated; MBC, mitotically blocked C; MBGT, mitotically blocked GT; HC, hair cell; SC, supporting cell; TC, transitional cell.

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[†]Bell, T., Bot, G. & Oberholtzer, J. C. (1999) *Assoc. Res. Otolaryngol. Abstr.* **22,** 130 (abstr.).

[‡]Bell, T., Matuai, H., Caruso, L. & Oberholtzer, J. C. (2000) *Assoc. Res. Otolaryngol. Abstr.* **23,** 240 (abstr.).

ing that the genes that regulate these events are not expressed or that their expression is not properly coordinated.

The fate of regenerated cells depends on the activity of transcription factors and other cell fate determinants. Although many transcription factors are expressed in the developing otocyst [see Brigande et al. (61) and Cantos et al. (62), this issue], there is limited evidence that any of them are critical for fate determination in the inner ear organs. Two notable exceptions are the helix–loop–helix transcription factor Math1 and the POU4 transcription factor Brn-3c, both of which are critical for HC development (63, 64). In knockout mice, Math1 deletion prevents HC production in the inner ear organs (63). HCs are transiently produced in Brn-3c knockout mice but do not develop bundles or migrate to their proper epithelial positions, suggesting that this gene plays an important role in HC differentiation, maintenance, or survival (64). The role of these and other genes in postembryonic HC production remains to be determined.

Contact-mediated signaling may play an important role in controlling cell fate during development and regeneration. Notch, an extracellular receptor activated by ligands on adjacent cells, is known to decrease proliferation and regulate pattern formation among sensory cells through lateral inhibition. This receptor, which has been extensively studied in the developing *Drosophila* nervous system, plays an important role in boundary formation and cell lineage decisions (reviewed in refs. 65 and 66). Studies of the developing (67–69) and regenerating (70) inner ear also implicate a role for Notch signaling in HC production. The role that Notch signaling may play in HC development and mitotic HC regeneration is discussed more fully in this issue in papers by Eddison et al. (71) and Stone and Rubel (72).

Nonmitotic HC Regeneration

Recent studies in regenerating nonmammalian (47, 73) and mammalian (32–34) inner ear organs have noted that progenitor proliferation, as determined by BrdUrd immunocytochemistry or tritiated thymidine autoradiography, is limited and that immature HCs in these organs are seldom labeled with immunocytochemical mitotic markers. These discrepancies remain true even when these markers are made continuously available to potential progenitor cells in inner ear organs (47). HC recovery is also sustained after acoustic (74) or aminoglycoside-induced (75) damage in inner ear organs in the presence of mitotic inhibitors such as arabinoside-C and in organotypic cultures under mitotically blocked conditions (73, 76). Taken together, these experiments indicate that HC regeneration can also occur by nonmitotic cellular mechanisms.

The cellular mechanism(s) underlying nonmitotic HC recovery are not as well understood as those underlying mitotic HC recovery. Sobkowicz *et al.* (77) were the first to suggest that damaged HCs could repair their bundles, demonstrating that cochlear HCs could withdraw from the lumenal surface and survive the loss of their apical structures. In later experiments, they demonstrated that laser-damaged HCs could regrow their bundles on regaining contact with the lumenal surface (78). It has recently been suggested that gentamicin-damaged HCs in the mammalian inner ear may be capable of a similar form of self-repair (34).

The possibility that some SCs might have the capacity to transdifferentiate, i.e., convert phenotypically into HCs without an intervening mitotic event, was first suggested by Jones and Corwin (39). In these experiments, Jones and Corwin observed the development of HC characteristics in SCs that had not undergone a nuclear division after laser-ablating HCs in the axolotl lateral line. Additional morphological evidence for SC conversion in nonmammalian (73–76) and mammalian (79, 80) inner ear organs has come from a number of other *in vivo* studies.

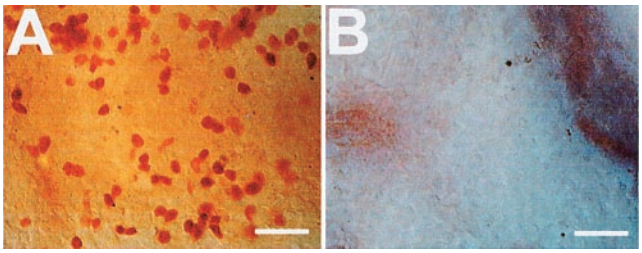

Fig. 1. Micrographs of GT (*A*) and MBGT (*B*) cultures incubated for 7 days in modified Wolfe–Quimby culture medium supplemented with 1 μM BrdUrd. Cultures were fixed for 2 h in 4% paraformaldehyde, permeabilized for 30 min in 1 M HCl containing 0.2% Triton X-100, and blocked for 30 min in PBS containing 10% normal horse serum, 2.5% BSA, and 0.2% Triton X-100. They were then incubated overnight at 4°C with anti-BrdUrd antisera (Caltag, South San Francisco, CA; IU-4) (1:2000), 1 h in biotinylated horse anti-mouse IgG (1:500), and 1 h in avidin-biotin peroxidase complex (1:500). BrdUrd-labeled nuclei were detected with standard diaminobenzidine (DAB) histochemistry. Note the presence of BrdUrd-labeled nuclei in GT (*A*) and absence of such nuclei in MBGT cultures (*B*). Bars = 25 μm.

In organotypic cultures of the bullfrog saccule, single SCs adjacent to damaged HCs up-regulate calcium-binding proteins after gentamicin ototoxicity, consistent with this hypothesis (76). There is also an *in vivo* loss of SCs in the bullfrog saccule (73) during HC recovery, suggesting that some SCs are converted to HCs during this process.

Normal and Mitotically Blocked Cultures of the Bullfrog Saccule

The bullfrog saccule, a sensor of gravity and substrate-borne vibration (10, 81), is a model system for studies of HC transduction (for recent reviews, see refs. 1 and 2). Saccular HCs also increase in number throughout adult life and regenerate after damage, making this organ an ideal system for studying HC development (10, 12, 82) and regeneration (28, 73, 76).

To isolate and study the mechanisms underlying nonmitotic HC recovery, we prepared normal and mitotically blocked saccular cultures by incubating control (C) and gentamicin-treated (GT) saccules in normal medium and medium supplemented with aphidicolin, an inhibitor of DNA polymerase alpha (83). One group of cultures was exposed continuously for 7 days to 1 μM BrdUrd, a thymidine analogue incorporated into proliferating cells. They were then processed for BrdUrd immunocytochemistry to identify proliferating cells and ascertain the effect of aphidicolin on cell proliferation.

Cell Proliferation in Saccular Cultures

BrdUrd-labeled nuclei in C (data not shown) and GT (Fig. 1*A*) cultures were found in the sensory macula and the macular margins. Within the sensory macula, the number of BrdUrd-labeled nuclei varied markedly, averaging 500 and 415 in C and GT cultures (Table 1). A small (<3%) number of solitary BrdUrd-labeled nuclei were observed. These nuclei were seen at

Table 1. BrdUrd-labeled nuclei in 7-day MBC and MBGT saccular cultures

Incubation	*n*	Total nuclei	Solitary nuclei	Paired nuclei
C	4	500.0 ± 383.3	4.8 ± 2.2	495.2 ± 381.4
MBC	4	5.0 ± 0.8	5.0 ± 0.8	0.0 ± 0.0
GT	4	415.0 ± 477.2	10.7 ± 5.5	404.3 ± 471.7
MBGT	4	10.8 ± 5.6	10.8 ± 5.6	0.0 ± 0.0

n, number of cultures; values, means ± SDs. C and MBC, control cultures incubated in normal (C) and aphidicolin-supplemented (MBC) medium; GT and MBGT, gentamicin-treated cultures incubated in normal (GT) and aphidicolin-supplemented (MBGT) medium.

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all epithelial positions and, when located in the apical third of the sensory epithelium, often had mitotic figures, indicating that they were undergoing mitotic division. Most (>97%) BrdUrd-labeled nuclei were found in cell pairs and did not have mitotic figures, implying that they were the progeny of progenitors that had undergone mitotic division.

Aphidicolin markedly decreased cell proliferation in mitotically blocked control (MBC) (data not shown) and gentamicin-treated (MBGT) (Fig. 1*B*) cultures, reducing the number of BrdUrd-labeled nuclei to <3% of their normal values. Paired BrdUrd-labeled nuclei were eliminated in MBC and MBGT cultures (Table 1), consistent with a blockage of mitotic division. Solitary BrdUrd-labeled nuclei in MBC and MBGT cultures were restricted to the apical third of the sensory epithelium and did not exhibit mitotic figures, implying that they were arrested in the G_2-phase of the cell cycle. Time-lapse studies of Hoechst-labeled MBGT cultures have confirmed that mitotic division did not result from G_2 to M-phase transitions, which would not be detected with BrdUrd immunocytochemistry (R.A.B., unpublished observations).

Morphology and Immunoreactivity in MBC Cultures

A separate group of 11 MBC and 17 MBGT cultures were incubated without BrdUrd up to 9 days to examine the mechanisms of nonmitotic HC recovery. After fixation, these cultures were labeled with phalloidin, a marker of filamentous actin, and immunolabeled with antisera against myosin VI, a known HC marker (84), and a single-chain antibody fragment against a novel cytokeratin expressed in bullfrog SCs (85). The morphology, ultrastructure, and immunolabeling of cells in these cultures were then examined with confocal and transmission electron microscopy.

MBC cultures, as revealed by myosin (red) and cytokeratin (green) immunolabeling, were composed of an organized mosaic of HCs and SCs (Fig. 2 *A* and *B*). HCs had round apical surfaces with myosin-labeled cell bodies and bundles emerging from a notched cuticular plate. Cytokeratin-labeled SCs, which lacked bundles and cuticular plates, had polygonal apical surfaces. The central region of MBC cultures was a pseudostratified columnar epithelium of cylindrical HCs restricted to the upper two-thirds of the sensory epithelium interspersed with elongated SCs spanning the sensory epithelium. The nuclei of these cells were organized into two layers, with the nuclei of SCs located at or near the basement membrane.

Mature HCs in the central saccule (*Bottom*, Fig. 2 *A* and *B*) had large apical surfaces and short kinocilia with prominent kinociliary bulbs. Immature HCs in the macular margin (*Top*, Fig. 2 *A* and *B*) had smaller apical surfaces, long, unbulbed kinocilia, and more elongated cell bodies than mature HCs. Unlike their more mature counterparts, immature HCs had both myosin and cytokeratin immunoreactivity throughout their cell bodies (right arrows) and in prominent perinuclear bodies (left arrows).

Morphology and Immunoreactivity in MBGT Cultures

Gentamicin treatment resulted in widespread damage to saccular HCs, the creation of scar formations, and the appearance of immature HCs in the central saccule. HCs were damaged lethally and sublethally by gentamicin treatment. Some lethally damaged HCs in MBGT cultures were extruded as a unit from the sensory epithelium. This process, although observed with confocal microscopy, was best appreciated by direct observation in MBGT cultures incubated without otolith membranes (R.A.B., unpublished observations). We also observed round epithelial holes in 3- and, to a lesser extent, 5-day MBGT cultures, presumably left by individual HCs extruding from the sensory epithelium.

HCs in MBGT cultures were also fragmented, i.e., broken into an apical remnant, consisting of the bundle and cuticular plate, and a basal remnant, containing the cell nucleus and remaining cytoplasm. HC fragmentation, although seen in the confocal microscope, was best appreciated with transmission electron microscopy (Fig. 3 *A* and *B*). Apical HC remnants, which always had intact bundles, were extruded from the sensory epithelium (top arrows). The basal remnants of fragmenting HCs (bottom

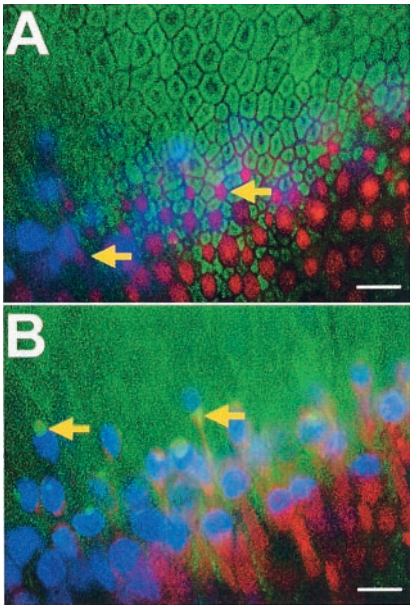

Fig. 2. Lumenal surface (*A*) and mid-epithelium (*B*) of myosin (red), cytokeratin (green), and Hoechst (blue) labeling a 9-day MBC culture, illustrating sequentially acquired with a confocal laser scanning microscope (Bio-Rad, Radiance 2000). In the central saccule (*Bottom, A* and *B*), HCs and SCs are immunolabeled, respectively, with myosin (red) and cytokeratin (green) immunoreactivity. Immature HCs on the macular margin are immunolabeled with both markers (right arrows, *A* and *B*), with cytokeratin immunoreactivity in the cytoplasm and perinuclear bodies (left arrows, *A* and *B*). Bars = 25 μm.

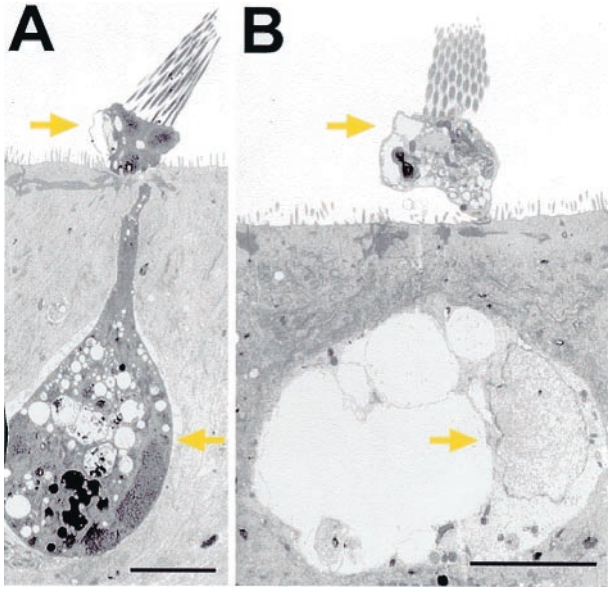

Fig. 3. Transmission electron micrographs (*A* and *B*) of apical (top arrows) and basal (filled arrows) remnants of fragmenting HCs in a 7-day MBGT culture. In *B*, the basal remnant is trapped inside a scar formation formed by the expansion of the apical processes of neighboring SCs. Bars = 10 μm.

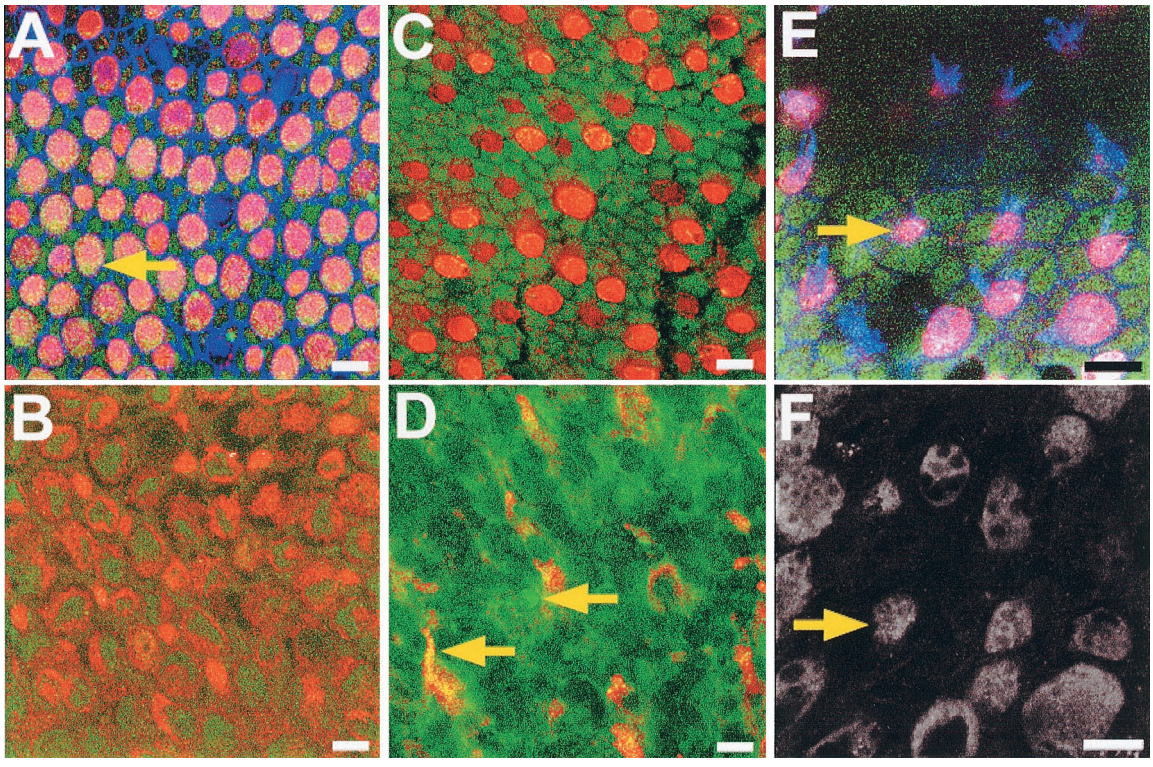

Fig. 4. Lumenal surface (*A*, *C*, and *E*) and mid-epithelium (*B*, *D*, and *F*) of 3-day (*A* and *B*) and 5-day (*C–F*) MBGT cultures, illustrating immunolabeling of sublethally damaged HCs. With the exception of their missing bundles, sublethally damaged HCs in 3-day cultures had normal morphology. They also retained their myosin immunoreactivity and, unlike undamaged HCs, had cytokeratin immunoreactivity around their basal bodies and cuticular plates (arrow, *A*). In 5-day cultures, these cells had degenerating cuticular plates (*C*), as well as elongated cell bodies, basal nuclei, and basal pseudopodia ending at or near the basement membrane (*D*). They also had cytokeratin immunoreactivity in their cytoplasm (left arrow, *D*) and perinuclear bodies (right arrow, *D*). Sublethally damaged HCs also had enlarged mitochondria and numerous autophagic vacuoles (arrows, *E* and *F*). Bars = 10 μm.

arrows) remained in the sensory epithelium, often under the scar formations formed by the expanded processes of neighboring SCs (Fig. 3*B*). Cytoplasmic remnants of these cells were highly vacuolated, and their nuclei were condensed or disrupted (Fig. 3*A*), suggesting that they were undergoing apoptotic cell death. Numerous apoptotic bodies were also seen in the sensory epithelium, consistent with this conclusion (A. Lysakowski and R.A.B., unpublished observations). Similar observations have recently been made in cultured explants of the mammalian vestibular organs (89).

The majority of HCs in 3- and 5-day MBGT cultures had partial or missing bundles. These sublethally damaged cells, except for the loss of their bundles, had normal morphology and myosin immunoreactivity and could be differentiated from SCs by their round apical surfaces (Fig. 4*A*), cuticular plates, and apical nuclei (Fig. 4*B*). Unlike undamaged HCs, sublethally damaged HCs also had cytokeratin immunoreactivity around their apical and lateral surfaces, particularly near their basal bodies (arrow, Fig. 4*A*). In 5-day MBGT cultures, sublethally damaged HCs had degenerating cuticular plates and smaller apical surfaces, implying that they were withdrawing from the lumenal surface (Fig. 4 *C* and *E*). They also had elongated cell bodies, nuclei between the HC and SC nuclear layers, perinuclear bodies (right arrow) and basal pseudopodia (left arrow) ending at or near the basement membrane (Fig. 4*D*). They also displayed, like immature HCs on the macular margins, both myosin and cytokeratin immunoreactivity (Fig. 4*D*). Sublethally damaged HCs, whether seen in confocal (Fig. 4*F*) or transmission electron microscopy (A. Lysakowski and R.A.B., unpublished observations), also had enlarged mitochondria and numerous autophagic vacuoles.

HC Loss and Recovery in MBGT Cultures

To document HC loss and recovery in MBGT cultures, we quantitatively analyzed HC and bundle density in the central saccule at varying time points after gentamicin treatment. Gentamicin treatment induced a rapid loss of bundles, with the density of undamaged bundles in MBGT cultures (filled circles) dropping by 3 days to $<5\%$ of the density in MBC cultures (open circles) before returning to $\approx 50\%$ of normal levels by 9 days after gentamicin treatment (Fig. 5 *Top*). HC density in MBGT cultures, determined from the density of myosin-labeled cells, dropped slightly in 3- and 5-day MBGT cultures before returning to its value in MBC cultures (data not shown).

Bundles in MBGT cultures were subdivided into mature (blue bars), damaged (red bars), and immature (yellow bars) bundles (Fig. 5 *Middle*). Undamaged mature bundle density was low in 3-day MBGT cultures but increased significantly by 9 days after gentamicin treatment, implying that large numbers of hair cells were being repaired. Damaged bundles were seen in large numbers in 3-day MBGT cultures but decreased rapidly at later survival times. The density of immature bundles was small at all survival times, presumably because they rapidly developed into mature bundles.

Transitional Cells and Immature Hair Cells in MBGT Cultures

Scar formations, consisting of four to eight SCs meeting at or near a common vertex, were first seen in 3-day MBGT cultures. These formations, created by the expansion of the apical projections of neighboring SCs into the epithelial spaces vacated by extruding or fragmenting HCs, also had prominent phalloidin-labeled rings, giving them a pie-shaped appearance (Fig. 6*A*). These rings, composed of actin segments in each of the SCs participating in the scar formation, consisted of a single layer,

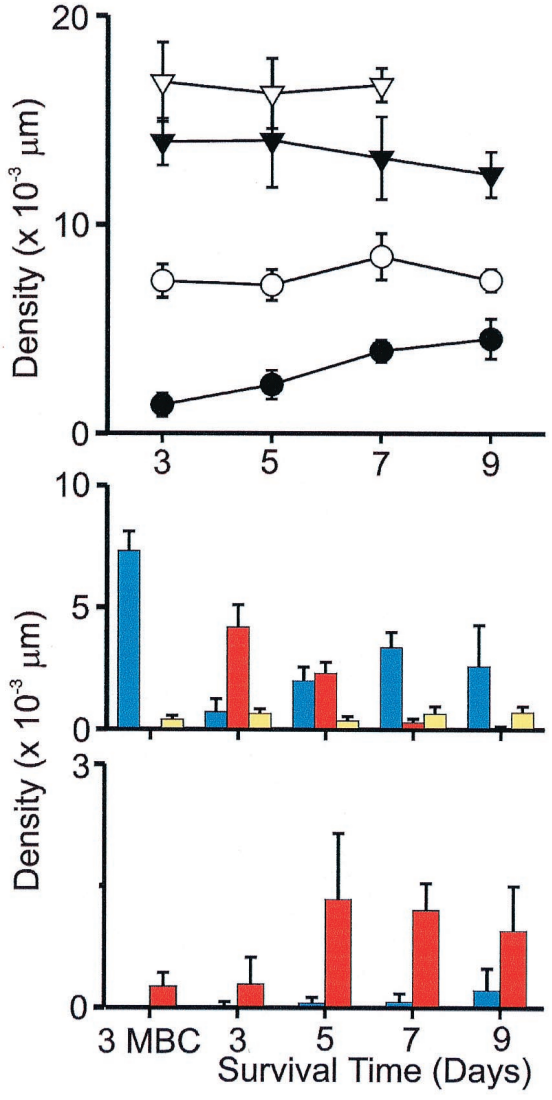

Fig. 5. (*Top*) Undamaged bundle density (●) and SC density (▼) in MBC (open symbols) and MBGT (filled symbols) cultures plotted vs. survival time. (*Middle*) Density of mature (blue bars), damaged (red bars), and immature (yellow bars) bundles in 3-day MBC cultures (*Left*) and MBGT cultures (*Right*) plotted vs. survival time. (*Bottom*) Density of repairing (blue bars) and nonrepairing (red bars) scars in 3-day MBC cultures (*Left*) and MBGT cultures (*Right*) plotted vs. survival time.

suggesting that they were surviving junctional structures from the interface between HCs and their neighboring SCs.

Most scar formations showed no signs of repair, i.e., SCs participating in these formations had cytokeratin, but not myosin immunoreactivity and no visible HC characteristics (left arrow, Fig. 6*A*). In other scar formations, transitional cells (TCs), coexpressing myosin and cytokeratin immunoreactivity, were seen as early as 5 days after gentamicin treatment (right arrow, Fig. 6*A*).)These cells, which we assume to be the precursors of immature HCs, had narrow, elongated cell bodies and nuclei below the HC nuclear layer.

Most (>90%) TCs had round apical surfaces, and they were found between the vertices and outer margins of scar formations (right arrow, Fig. 6*A*). They also had nuclei between the HC and SC nuclear layers (right middle arrow, Fig. 6*B*) and basal pseudopodia ending at or near the basement membrane (Fig. 6 *B* and *C*). Many of these cells also had unusual cell body morphology, traveling horizontally in the basal levels of the sensory epithelium before

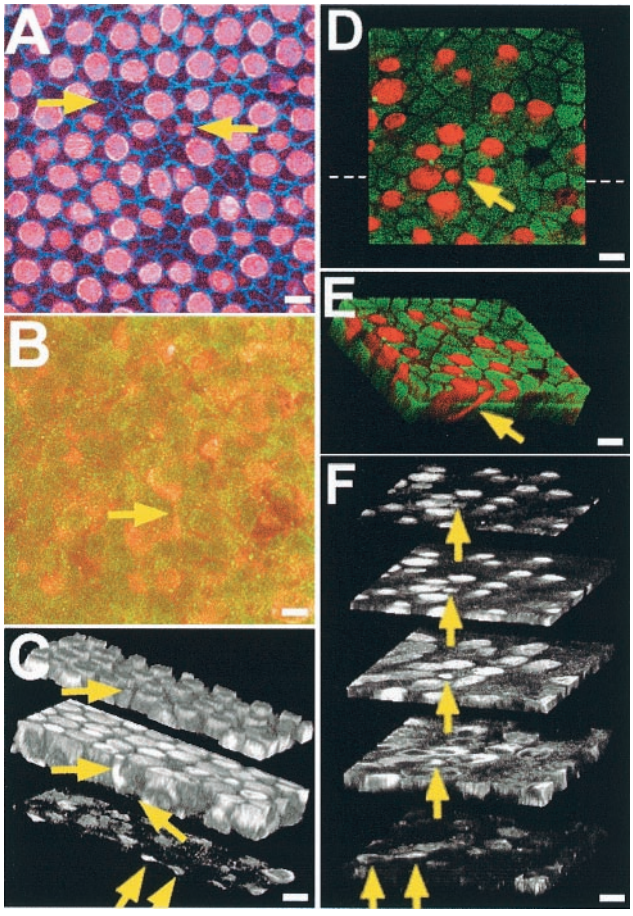

Fig. 6. Luminal surface (*A* and *D*), mid-epithelium (*B* and *E*), and cut-away views (*C* and *F*) of 5-day MBGT cultures, illustrating morphology and immunolabeling of TCs in repairing scar formations. Note round or triangular apical surface (arrows, *A* and *D*), elongated cell body (arrows, *B* and *E*), and basal nucleus (arrows, *C* and *F*). Bars = 10 µm.

turning apically to enter scar formations and reach the luminal surface (Fig. 6*C*). A small (<10%) number of TCs were actually single SCs participating in scar formations. These cells had polygonal or triangular apical surfaces (Fig. 6*D*), narrow, elongated cell bodies (Fig. 6 *E* and *F*), and nuclei on or near the basement membrane (lower left arrow, Fig. 6*F*).

Immature HCs with long, unbulbed kinocilia and short stereocilia were seen in the central regions of 9-day MBGT cultures (Fig. 7). Unlike TCs, these cells had myosin, but not cytokeratin, immunoreactivity and were not necessarily associated with scar formations. Most of these cells, like TCs, had small round apical surfaces (Fig. 7 *A* and *B*), nuclei between the HC and SC nuclear layers (middle arrows, Fig. 7*C*), and basal pseudopodia ending at or near the basement membrane (lower right arrow, Fig. 7*C*). A few SCs, with triangular apical surfaces and immature bundles, were also seen in scar formations. Immature HCs with more mature bundles, although commonly seen *in vivo*, were harder to document in MBGT cultures.

Scar Formation and SC Loss During HC Recovery

To examine the relationship between scar formation and scar repair, we quantitatively analyzed scar density in the central regions of MBGT cultures at varying time points after gentamicin treatment. Scar density increased 5-fold from its value in MBC cultures by 5 days after gentamicin treatment before leveling off at later survival times (Fig. 5 *Bottom*). The percentage of repairing scars continued to increase with survival time,

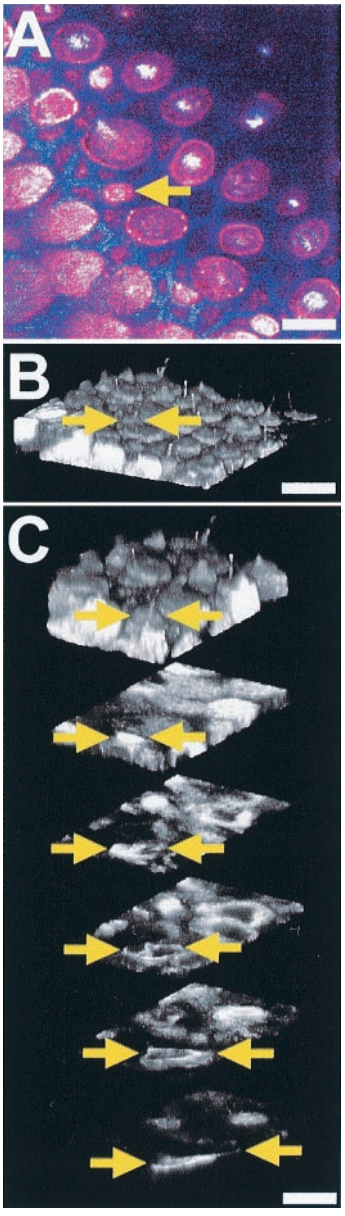

Fig. 7. Lumenal surface (*A*) and cut-away views (*B* and *C*) of 9-day MBGT culture, illustrating morphology and immunolabeling of immature HC. Note absence of cytokeratin immunolabeling, round apical surface (arrow, *A*), kinocilium and immature bundle (arrow, *B and C*), elongated cell body, basal nucleus (asterisks, *B and C*), and basal pseudopodia (pointer, *C*). Bars = 10 μm.

equaling ≈15–20% of all scars in 9-day MBGT cultures. The density of immature HCs was significantly larger than that of repairing scar formations at all survival times, implying that many immature HCs were not associated with repairing scars ($P < 0.01$).

Because both undamaged SCs and sublethally damaged HCs expressed cytokeratin immunoreactivity, we also measured SC density to determine whether HC recovery was accompanied by a loss in SC number (Fig. 5 *Top*). SC density did not change in MBC cultures (open triangles). In MBGT cultures (filled triangles), SC density decreased with increasing survival time, although its value was not significantly different in 3- and 9-day MBGT cultures ($P > 0.05$).

Conclusions

Using immunocytochemical markers at the light and electron microscopic levels, we have shown that it is possible to distinguish damaged HCs from mature HCs and SCs, a task difficult to accomplish solely on morphological grounds because of the cellular disorganization that occurs after gentamicin ototoxicity. Our results indicate that HCs are damaged lethally and sublethally by gentamicin ototoxicity. Lethally damaged HCs are extruded or fragmented; sublethally damaged HCs undergo early bundle loss and withdrawal from the lumenal surface, remaining in the sensory epithelium for prolonged periods. We were also able to identify precursor cells expressing HC and SC immunocytochemical markers, a task that has been difficult to accomplish in previous studies because of a lack of cell-specific markers.

Mechanisms of Nonmitotic HC Recovery. At least at the low gentamicin concentration used in this study, our results suggest that HC recovery in MBGT cultures is accomplished by two nonmitotic repair mechanisms, the self-repair of sublethally damaged HCs and the conversion of SCs. We have not examined the responses of MBGT cultures to higher gentamicin concentrations.

Hair Cell Repair. Our observation that large numbers of sublethally damaged HCs survive gentamicin treatment for prolonged periods provides a morphological basis for the self-repair of sublethally damaged HCs. Recent time-lapse studies by us (R.A.B., unpublished observations) confirm that sublethally damaged HCs withdraw from the lumenal surface. We have not yet observed by direct observation whether these cells survive to undergo self-repair. Although we have not measured the level of apoptotic cell death in our experiments, we feel that this possibility is unlikely. First, the number of damaged bundles is maximal in 3-day MBGT cultures and decreases markedly at later survival times. A similar result is seen in rat utricles, where gentamicin-induced apoptosis occurs mainly during the two days subsequent to gentamicin treatment (34). Second, extruded HCs were seldom seen by 5 days after gentamicin treatment, and fragmented HCs, unlike sublethally damaged HCs, had intact bundles. These remnants always had intact bundles, implying that HCs with missing bundles did not undergo fragmentation. Taken together, these observations imply that sublethally damaged HCs do not undergo extrusion or fragmentation at later survival times.

Although the origin of most immature HCs could not be determined with certainty in our experiments, three arguments suggest that they derive from sublethally damaged HCs. First, large numbers of sublethally damaged HCs are found in the sensory epithelium before the first appearance of TCs. Second, the morphological, ultrastructural, and immunocytochemical properties of sublethally damaged HCs are similar to those of TCs and immature HCs. Third, TCs and immature HCs have autophagic vacuoles indicative of cellular damage, indicating that they derived from damaged HCs rather than undamaged SCs. We therefore believe that most, if not all, sublethally damaged HCs undergo self-repair and that HC recovery in MBGT cultures is largely accomplished through this mechanism.

Supporting Cell Conversion. Our previous *in vivo* (73) and *in vitro* (73, 76) studies indicated that HC recovery in MBGT cultures was accompanied by morphological changes in single SCs, suggesting that nonmitotic HC regeneration was accomplished by the phenotypic conversion of SCs. These studies also indicated that single SCs participating in scar formations downregulated cytokeratin, a known SC marker (87), and upregulated calcium-binding proteins (76) known to be expressed in HCs. These immunocytochemical changes appeared to presage the development of morphological HC characteristics, including immature bundles and cuticular plates, in these SCs.

Our current results confirm that a small number of SCs participating in scar formations coexpress HC and SC immunocytochemical markers or display morphological HC characteristics. These morphological and immunocytochemical results indicate that SC conversion plays a role in HC recovery in MBGT cultures. The other possibility, that a cell other than a SC would join an existing scar formation or form a new scar formation, is not consistent with our *in vivo* and *in vitro* observations. More specifically, we have never seen a HC, damaged or undamaged, with a polygonal apical surface, an actin segment, or sharing a common vertex with neighboring SCs.

SC number also decreases in MBGT cultures after gentamicin treatment, consistent with the conclusion that SCs are lost as they undergo conversion during HC recovery. The loss of SCs, however, is significantly lower than the increase in immature HCs, indicating that this process plays only a minor role in HC recovery. The amount of this loss is also less than that seen *in vivo* (73), suggesting that our *in vivo* and *in vitro* gentamicin treatments are not equivalent or that SC conversion is not supported by our culture conditions.

Fate Commitment by Uncommitted Postmitotic Cells. It is possible that a population of uncommitted postmitotic cells could remain latent in the sensory epithelium after undergoing S-phase and change its developmental fate in response to the death of existing HCs. Kelley and his colleagues (88) have shown that embryonic cochlear cultures have a limited ability to repair damage to existing HCs and that cells retain a latent capacity to commit to the HC phenotype for up to 48 h after HCs begin to differentiate. It is not known whether cells in MBGT cultures have a similar latent capacity and, if so, how long this capacity is retained. These uncommitted cells, if they existed, would not be BrdUrd-labeled in our experiments. Nevertheless, we do not believe that shift in developmental fate plays a major role in HC recovery. Because proliferating cells, with the exception of this initial population, would be effectively blocked from entering G_2-phase, we would expect to see an increase in the number of new HCs by this mechanism only at early survival times. By contrast, the number of new HCs in MBGT cultures continued to increase with increasing survival time.

Morphological and Immunocytochemical Properties of HC Precursors. Our results indicate that sublethally damaged HCs in MBGT cultures acquire cytokeratin immunoreactivity on their lateral surfaces after the loss of their hair bundles. In previous studies, this pattern of cytokeratin immunoreactivity was ascribed to supporting cells (59). Whatever their origin, TCs down-regulate cytokeratin immunoreactivity as they differentiate into immature HCs. Cytokeratins are a family of polypeptides that constitute the largest and most complex class of intermediate filaments (58). In epithelial cells, they form a structural network that spans the cell cytoplasm, linking the plasma membrane, nucleus, and other cytoskeletal elements. This structural network is apparently achieved through a recently identified family of intermediate-filament-associated proteins that form cross-bridges between intermediate filaments and other cytoskeletal elements. Although the exact role of cytokeratin in the inner ear is unclear, detailed studies of its cellular distribution in mitotic and nonmitotic HC precursors should prove helpful in delineating the stages of HC differentiation.

Our results also indicate that the morphology and epithelial position of TCs and immature HCs are different from that of mature HCs. More specifically, the former cells have smaller apical surfaces, more elongated cell bodies, and more basal nuclei than the latter cells. Recent studies using calcium-binding proteins (59) and class III β-tubulin (60) as HC immunocytochemical markers also indicate that mitotic HC precursors have nuclei located well below the lumenal surface and appear to have physical connection with both the basement membrane and the lumenal surface. These morphological features suggest that mitotic HC precursors migrate from the lumenal surface to more basal positions before establishing their permanent positions. This migration may be necessary for mitotic and nonmitotic HC precursors to obtain positional cues for proliferation or differentiation from contact with other cells or the extracellular matrix of the basement membrane.

Maturation of mitotic and nonmitotic HC precursors coincides with a shortening of the basal pseudopodia and an apical migration of the cell nucleus, both of which remove these cells from the influence of the basement membrane. Interestingly, many TCs travel horizontally in the basal levels of the sensory epithelium before turning to reach the lumenal surface. They are also found on the margins rather than the vertices of scar formations, implying that they were not able to undergo further differentiation without obtaining patterning signals derived from contact with adjacent SCs. These patterning signals, which may be mediated by Notch and its associated ligands, might explain why multiple TCs were seldom seen in scar formations and ensure the faithful reproduction of the normal mosaic of HCs and SCs in repairing cultures.

Interactions Between Mitotic and Nonmitotic HC Recovery. Our results help to explain the early appearance of immature HCs and to reconcile differences between the number of proliferating and regenerating cells in previous studies (31, 32, 47). They also indicate that HC regeneration, at least in the bullfrog saccule, is accomplished by a combination of mitotic and nonmitotic mechanisms. It is not yet clear whether this is a general property of all auditory and vestibular organs. It is possible that using mitotic and nonmitotic mechanisms of HC regeneration allows inner ear organs to respond to ongoing and injury-induced HC loss with a higher degree of flexibility than could be attained by either mechanism alone. Our *in vivo* and *in vitro* results imply that nonmitotic HC regeneration creates new HCs more rapidly than mitotic HC regeneration (73). Nonmitotic mechanisms of HC regeneration might therefore be more suitable for producing larger numbers of HCs after catastrophic HC loss, where rapid HC production might be critical for preventing the subsequent degeneration of afferent neurons. Nonmitotic mechanisms of HC regeneration can also produce new HCs without the metabolic cost of maintaining a stem cell population or the cellular disruption associated with mitotic division. Unfortunately, nonmitotic mechanisms of HC regeneration, unlike mitotic division, are limited in their ability to restore HC and SC density to normal levels. Thus, a combination of mitotic and nonmitotic mechanisms of HC regeneration may normally be required to bring about full recovery.

Time-lapse experiments, using vital markers against the plasma membrane, cytoplasmic organelles, and ion channels and the immunocytochemical markers used in the present study, are underway in our laboratory to confirm the fate of sublethally damaged HCs and the origin of immature HCs in MBGT cultures (R.A.B., unpublished observations). Similar time-lapse experiments are being pursued by other laboratories.[§¶] These studies should provide much needed knowledge about the development of mitotic and nonmitotic HC precursors, including new stage-specific markers correlated with particular morphological and physiological characteristics and should lead to a better understanding of the basic mechanisms underlying proliferation and differentiation in these important sensory cells.

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