Temporal, Spatial, and Morphologic Features of Hair Cell Regeneration in the Avian Basilar Papilla

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ABSTRACT
Hair cell–selective antibodies were used in combination with the nucleotide bromodeoxyuridine (BrdU) to examine the temporal, spatial, and morphologic progression of auditory hair cell regeneration in chicks after a single gentamicin injection. New hair cells are first identifiable with an antibody to class III beta (β) tubulin (TuJ1) by 14 hours after BrdU incorporation, but progenitor cells in S phase and M phase are TuJ1-negative. TuJ1 labeling reveals that new hair cells are first detected at 3 days after gentamicin, in the base, and the emergence and maturation of regenerating hair cells spreads apically over time. Differentiation of regenerating hair cells consists of a progressive series of morphologic changes. During early differentiation (14 hours to 1 day after BrdU), regenerating hair cells are round or fusiform and remain near the lumen, where they are generated. During intermediate differentiation (2–4 days after BrdU), regenerating hair cells resemble support cells; their somata are elongated, their nuclei are in the support cell layer, and they appear to contact both the lumenal surface and the basal lamina. The 275-kDa hair cell antigen is first expressed in regenerating hair cells during this period. During late differentiation (7 days after BrdU and later), TuJ1-positive cells acquire the globose shape of mature hair cells. Labeling with antibodies to hair cell antigen, calmodulin, and ribosomal RNA confirms this morphologic progression. Examination of sister cells born at 3 days post-gentamicin reveals that there is equal likelihood that they will assume the hair cell or support cell fate (i.e., both asymmetric and symmetric differentiation occur).[1] J. Comp. Neurol. 417:1–16, 2000. © 2000 Wiley-Liss, Inc.

Indexing terms: β-tubulin; calmodulin; chick; hair cell; differentiation

Hair cells are highly specialized cells that are located in the inner ear organs and the lateral line neuromasts. They serve as the sensory receptors for hearing, equilibrium, and motion detection. In birds, vestibular hair cells are produced continually throughout life (Jörgensen and Mathiesen, 1988; Roberson et al., 1992). In the mature avian auditory epithelium, or basilar papilla, hair cells are produced only after hair cell death is induced by noise exposure (Cotanche, 1987a; Corwin and Cotanche, 1988; Ryals and Rubel, 1988), ototoxic drug treatment (Cruz et al., 1987; Lippe et al., 1991), or laser ablation (Warchol and Corwin, 1996). Avian hair cell regeneration is accomplished by mitosis of support cells, which surround hair cells in the sensory epithelia (Girod et al., 1989; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Tsue et al., 1994; Warchol and Corwin, 1996a; Gleich et al., 1997). The resulting cell progeny differentiate into new hair cells and support cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988), and the normal epithelial array is reconstituted with a high degree of fidelity (for review, see Cotanche et al., 1994). There is emerging experimental evidence that regeneration of hair cells in mammals and lower vertebrates may also occur by means of a process termed "phenotypic conversion" or "direct transdifferentiation," whereby support cells convert into hair cells without an intervening mitosis (Baird et al.,

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1993, 1996; Adler and Raphael, 1996; Roberson et al., 1996; Steyger et al., 1997; Forge et al., 1998).

Although many molecules that regulate proliferation of postembryonic hair cell progenitors have been identified (reviewed in Stone et al., 1998), the signals that control cell fate decisions and differentiation during hair cell regeneration remain unknown. One of the factors that has hampered the identification of cellular and molecular regulators of cell fate decisions during hair cell regeneration is the paucity of markers for cell types in the early stages of regeneration.

TuJ1, an antibody to class III beta-tubulin (βIII tubulin), is a useful marker for identifying developing neurons in vivo as well as in vitro (e.g., Moody et al., 1987; Lee et al., 1990; Easter et al., 1993; Memberg and Hall, 1995). Mature and regenerating hair cells in the posthatch chick basilar papilla also express high levels of βIII tubulin, as disclosed by the TuJ1 antibody (Stone et al., 1996a). In addition, early developing hair cells in both the auditory and vestibular epithelia of chicks are heavily labeled with TuJ1 by embryonic days 6–7 (Molea et al., 1999). This corresponds to the first time they are distinguishable by either SEM (Cotanche and Sulik, 1984) or immunofluorescence for the 275-kDa hair cell antigen (Richardson et al., 1990; Bartolami et al., 1990). In our experience, TuJ1 does not label hair cells in the mature mammalian organ of Corti (Stone et al., 1996b).

In this study, we used TuJ1 and other antibodies to examine the temporal, spatial, and morphologic features of hair cell regeneration in whole-mounts of the drug-damaged chick basilar papilla. Our study reveals four important aspects of cellular differentiation during regeneration of avian auditory hair cells: (1) by using the TuJ1 antibody, regenerating hair cells can be distinguished from postmitotic support cells as early as 14 hours after S phase; (2) regenerated hair cells pass through a stage of differentiation, during which they are highly elongated and appear to contact both the lumenal surface and the basal lamina, thereby resembling support cells; (3) there is equal propensity for postmitotic pairs of cells to differentiate either symmetrically, as either two hair cells or two support cells, or asymmetrically, as a hair cell and a support cell; and (4) hair cell differentiation is initiated in the basal tip and spreads apically over time.

MATERIALS AND METHODS

Animal care

Fertilized eggs or 1-day-old hatchlings of White Leghorn chickens were received from H & N International (Redmond, WA). Eggs were placed in a humidified incubator at 37°C until hatching. Hatchlings were stored in heated brooders with ample food and water in the Animal Care Facility at the University of Washington. All procedures that are described were approved by the University of Washington's Animal Care Committee and conform to NIH guidelines for vertebrate animals.

Gentamicin treatment

Chicks between 6 and 9 days after hatching (40–70 g) were given a single subcutaneous injection of the ototoxic antibiotic gentamicin (400 mg/kg; Lyphomed, Deerfield, IL). Afterward, chicks recovered from the transient systemic toxicity induced by gentamicin in a heated chamber for 3–5 hours and were returned to the Animal Care Facility until killing.

Bromodeoxyuridine injections

At 3 days after gentamicin injection, chicks received a single intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) dissolved in sterile 10 mM phosphate buffered saline, pH 7.4 (PBS) at a dose of 100 mg/kg. The 3-day time point was chosen because it is the time at which the highest number of cells in the basilar papilla enter S phase after this gentamicin treatment (Bhave et al., 1995).

Tissue preparation

The following tissues were used in this study: (a) basilar papillas from control chicks; (b) basilar papillas from chicks killed at 3, 4, 5, 6, or 7 days after gentamicin injection; and (c) basilar papillas from chicks that received a single BrdU injection at 3 days after gentamicin treatment and were killed at 3, 6, or 14 hours, or 1, 2, 3, 4, or 7 days after the BrdU injection. At least four basilar papillas were examined for each experimental variable.

Chicks were killed by overdose of pentobarbital (100 mg/kg; intraperitoneal injection) and decapitated. Basilar papillas were dissected from the temporal bone and placed in cold Hanks' buffered saline solution (HBSS; Gibco/BRL, Grand Island, NY). The tegmentum vasculosum was removed with fine microforceps, and the remaining tissue was placed in 0.01% Type I collagenase (Sigma Chemical Co.) in HBSS for 3 minutes. The tectorial membrane was removed by grasping it with microforceps at the apical end of the organ and pulling it off the entire length of the sensory epithelium. Basilar papillas were fixed for 30 minutes to 2 hours in 4% paraformaldehyde, rinsed in PBS, and stored at 4°C in PBS until labeling.

Semi-thin sections

Plastic semi-thin sections of control basilar papillas were prepared as described previously (Stone et al., 1996a). Sections were mounted on chrome-alum–subbed microscope slides and stained with toluidine blue.

Immunofluorescence

The following monoclonal antibodies were used: TuJ1 (diluted 1/1,000, gift from Dr. Anthony Frankfurter, University of Virginia, Charlottesville, VA), Y10B (diluted 1/500, obtained originally from Dr. Joan Steitz, Yale, New Haven, CT), anti-calmodulin (diluted 1/500, clone 6D4, Sigma Chemical Co.), anti–hair cell antigen (anti-HCA; diluted 1/1000, gift from Dr. Guy Richardson, University of Sussex, UK), and anti-BrdU (diluted 1/100, Becton Dickenson, San Jose, CA). All primary antibody incubations were performed overnight at 4°C, except for the anti-BrdU, which was sometimes performed for 2 hours at room temperature. All antibody and blocking reactions were performed in antibody dilutant, which consisted of 0.05% TritonX-100 in PBS. Each step was followed by thorough rinses in PBS. The primary antibody was omitted from reactions as a negative control.

For single antigen labeling experiments, organs were blocked with 10% normal horse serum in antibody dilutant for 20 minutes and incubated in primary antibody as described above. Organs were placed in Bodipy FL–conjugated goat anti-mouse IgG (1/300; Molecular Probes, Eugene, OR) for 2 hours at room temperature. After im-

munofluorescence to detect TuJ1, some samples were counterstained with propidium iodide (diluted at 10 μg/ml in PBS; Sigma Chemical Co.) for 15 seconds or with rhodamine phalloidin (diluted 1/50 in antibody dilutant; Molecular Probes) for 1 hour.

The method we used to double label basilar papillas for BrdU and either βIII tubulin, calmodulin, or ribosomal RNA closely followed the protocol described by Memberg and Hall (1995). Basilar papillas were reacted with either TuJ1, anti-calmodulin IgG, or Y10B overnight at 4°C, then with Bodipy FL–conjugated goat anti-mouse IgG (1/300; Molecular Probes) for 2 hours at room temperature. Tissue was then post-fixed for 20 minutes with 4% paraformaldehyde, rinsed with PBS, and reacted with 2N HCL in antibody dilutant for 1 hour at room temperature. After numerous rinses with PBS, tissue was reacted with anti-BrdU IgG for 2 hours followed by lissamine rhodamine–conjugated donkey anti-mouse IgG (1/300; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hour.

Triple labeling to detect βIII tubulin, HCA, and BrdU was performed as follows: TuJ1 was applied first and detected by using Bodipy FL–conjugated goat anti-mouse IgG; anti-HCA was applied next and detected using Cy5-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories). Tissues were then post-fixed in 4% paraformaldehyde for 20 minutes, and BrdU was subsequently detected as described above for the double labeling.

Analysis of tissues

Tissues were mounted onto microscope slides, coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and examined with an MRC-1024 confocal laser scanning microscope (BioRad, Hercules, CA) at 60× by using LaserSharp Version 2.1 (BioRad). Confocal slices through the sensory epithelium in a plane parallel to the lumenal surface (Z series) were collected at 0.5- or 1-μm steps. Data were processed digitally with NIH Image and Photoshop (Adobe, Mountain View, CA), and images were printed with a Phaser IISDX dye-sublimation printer (Tektronix, Beaverton, OR).

For the analysis of modes of cell differentiation (asymmetric vs. symmetric), a total of 91 BrdU-positive sister cell pairs were examined across six basilar papillas. Sister cell pairs were defined as *distinct* if they were the only two labeled nuclei within approximately six nuclear diameters. Pairs were considered to be *closely associated* if they were located within approximately two nuclear diameters of each other. When locating and identifying pairs, the entire depth of the epithelium in the lesioned area was scanned.

RESULTS

Biology of the normal and regenerating basilar papilla

The anatomy of the mature basilar papilla, which is the avian auditory epithelium, has been described in many elegant studies (e.g., Tanaka and Smith, 1978; Manley, 1990). It is flat and elongated, and high-to-low frequencies are encoded along its length from basal-to-apical regions, respectively. The sensory epithelium of the basilar papilla is composed primarily of hair cells (auditory receptors) and nonsensory support cells (Fig. 1A). Hair cells have long bundles of actin (Tilney and Saunders, 1983) called stereocilia that project from their lumenal surfaces. In the undamaged organ, the cell bodies of hair cells are confined to the lumenal portion of the epithelium; they do not contact the basal lamina. Hair cell nuclei are round and arranged in a monolayer close to the lumen. Several support cells surround each hair cell such that hair cells do not normally form contacts with each other. Support cells span the entire depth of the epithelium, contacting both its lumenal and ablumenal surfaces. Support cell nuclei are oval and smaller than hair cell nuclei, and they are typically located within the ablumenal two-thirds of the epithelium. Support cells in the normal posthatch basilar papilla are mitotically quiescent (Ryals and Rubel, 1988; Corwin and Cotanche, 1988; Oesterle and Rubel, 1993; Bhave et al., 1995). However, after experimentally induced hair cell damage, they divide (Girod et al., 1989; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Tsue et al., 1994; Warchol and Corwin, 1996), and daughter cells differentiate into new hair cells and support cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988).

Time-course of class III β-tubulin labeling in regenerating hair cells

TuJ1, an antibody to class III β-tubulin (βIII-tubulin), labels hair cells in the basilar papilla of developing (Molea et al., 1999) and posthatch chicks (Stone et al., 1996a; Fig. 1B). It is an excellent marker to demonstrate patterns of hair cell loss and regeneration in mature avian tissues as well (Stone et al., 1996a). After a single injection of gentamicin at 400 mg/kg, hair cells are lost from the basilar papilla in a progressive manner, starting at the basal tip and moving apically. Complete hair cell loss occurs in the basal one-fourth to half of the basilar papilla by 2–3 days (Stone et al., 1996a; Fig. 1C). This pattern of hair cell loss is also evident with scanning electron microscopy (Bhave et al., 1995; Janas et al., 1995). Many newly formed hair cells are detectable with TuJ1 by 4 days after gentamicin, and the stereocilia of regenerating hair cells are first seen around 5 days after gentamicin (Bhave et al., 1995; Janas et al., 1995). Hair cell regeneration appears to be nearly complete by 10–15 days after gentamicin injection (Stone et al., 1996a).

We used TuJ1 to determine when βIII-tubulin protein is expressed in regenerating hair cells as they differentiate, relative to the DNA synthesis (S) phase of the cell cycle. Regeneration was stimulated by a single injection of gentamicin. At 3 days after gentamicin, which represents when cell proliferation in the basilar papilla peaks after gentamicin injection (Bhave et al., 1995), chicks were given a single injection of the immunologically detectable nucleotide BrdU (Gratzner, 1982). This nucleotide becomes incorporated into proliferative cells in S phase and is passed to daughter cells during mitosis. Progenitor cells that enter S phase after BrdU is cleared from the cochlea (by at least 6 hours after injection; Stone and Cotanche, 1994) are not labeled with the nucleotide. Basilar papillas were harvested at different times after the BrdU injection, and the organs were double-labeled to detect βIII-tubulin (with TuJ1) and BrdU. This method allowed us to detect proliferative progenitor cells as well as postmitotic cells at various stages of differentiation. For this analysis, we focused on BrdU-positive cells that are in the middle of the lesioned area. There was some variation in the morphol-

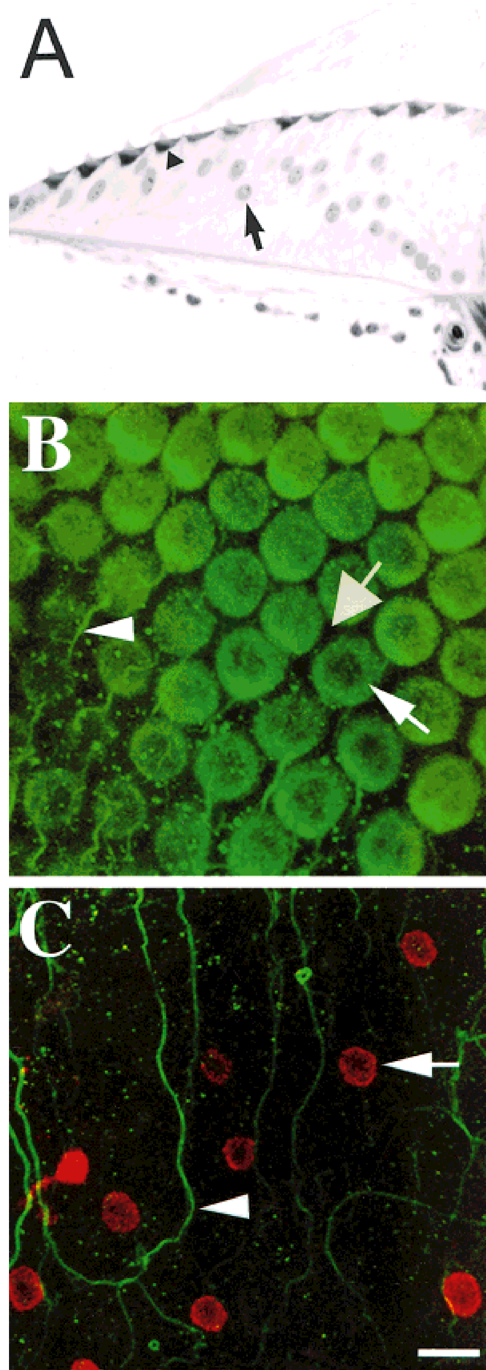

Fig. 1. Anatomy of the damaged and undamaged basilar papilla. **A:** A toluidine blue-labeled cross-section demonstrates the normal architecture of the mid-basal region of the basilar papilla. Hair cells (arrowhead) are darkly stained and reside near the lumen. Support cells appear unstained and span the entire depth of the epithelium; their nuclei (arrow) are located ablumenal to the hair cells. **B,C:** Stacked confocal slices through 66–100% of the depth of the lesioned epithelium in whole-mount basilar papillas, with slices parallel to the lumenal surface. The relative depths of different structures in the epithelium are not evident. B: TuJ1 labeling in hair cells in the mid-basal region of a control organ. The hair cell cytoplasm (thin arrow) and nerve processes (arrowhead) are labeled; support cells (thick arrow) are not labeled. C: At 3 hours after bromodeoxyuridine (BrdU) in a drug-damaged organ at 3 days after gentamicin, unpaired BrdU-labeled nuclei (red label, arrow) are scattered throughout the lesion. The TuJ1-positive hair cells are missing. BrdU-labeled cells are TuJ1-negative, but neural processes (green label, arrowhead) are TuJ1-positive. Scale bar = 22 μm in A, 10 μm in B,C.

ogy of BrdU-labeled cells within the damaged area at each time point, which will be discussed later. Here, we report the morphologies that are most prevalent.

At 3 hours after BrdU (equivalent to 3 days after gentamicin) (Fig. 1C), BrdU-labeled nuclei are detected throughout the lesioned basal end of the papilla. An average of 221 (±45 [SD], n = 3 organs) BrdU-positive nuclei are present within the lesion. All BrdU-positive nuclei are unpaired, and most appear to be located in the ablumenal two-thirds of the epithelium (data not shown). These observations were expected, because labeled nuclei are probably in S phase or early Gap 2 (G2) phase of the cell cycle at 3 hours after BrdU (Stone and Cotanche, 1994; Tsue et al., 1994). No double-labeled cells are detectable at this time. However, there are many TuJ1-positive neural processes that course through the support cell layer of the damaged epithelium (Fig. 1C).

At 6 hours after BrdU, BrdU-labeled mitotic figures (approximately 3–10 per epithelium) are scattered throughout the lesioned area (data not shown). BrdU labeling is also seen in unpaired cells as well as pairs of postcytokinetic cells (data not shown). βIII-Tubulin is not immunodetected in any BrdU-positive cells at this time (data not shown). Based on these findings, hair cell progenitors in the posthatch basilar papilla are not TuJ1-positive.

At 14 hours after BrdU (Fig. 2A), low levels of βIII-tubulin labeling are evident in a small subset of BrdU-labeled cells whose nuclei reside in the lumenal one-third of the epithelium. In such cells, TuJ1 immunoreactivity is confined to the lumenal portion of the cytoplasm, forming a cap-like label over the nucleus. At 1 day post-BrdU (Fig. 2B), the pattern of βIII-tubulin immunoreactivity in BrdU-positive cells is similar to that seen at 14 hours post-BrdU, but the intensity of the labeling in the lumenal portion of the cell is considerably higher. F2

Beta III tubulin labeling in BrdU-positive cells changes dramatically between 1 and 4 days post-BrdU. The general morphology of double-labeled cells is similar at 2 days (Fig. 2C), 3 days (Fig. 2D), and 4 days (Fig. 2E) post-BrdU. The nucleus is located deeper in the epithelium than at earlier times, either at the interface of the hair cell and support cell nuclear layers, or in the support cell nuclear layer. The cytoplasm lumenal to the nucleus is elongated (Fig. 2C,D,E), forming a neck-like region that is highly TuJ1 immunoreactive. In some cells (Fig. 2E), a TuJ1-positive cytoplasmic process extends toward, and may contact, the basal lamina (also see Fig. 3). Many of the morphologic features of double-labeled cells between 2 and 4 days post-BrdU resemble those of mature support cells. At 7 days after BrdU (Fig. 2F), double-labeled cells morphologically resemble mature hair cells. The nucleus has relocated to the hair cell nuclear layer, and the cell bodies are round and larger than at earlier times.

As early as 14 hours post-BrdU, TuJ1-positive processes appear to approach and/or contact double-labeled cells (Fig. 2A,B,F). These processes are similar in diameter to the neural fibers noted in the hair cell-free epithelium at 3 days after gentamicin (Fig. 1C), and they are considerably thinner than the TuJ1-positive descending process on regenerating hair cells at 3–4 days post-BrdU (Fig. 2E). On the basis of these observations, we believe that such processes are neural in nature. Alternatively, they may be the structural precursors to the hair cell descending processes seen after 3 days post-BrdU. F3

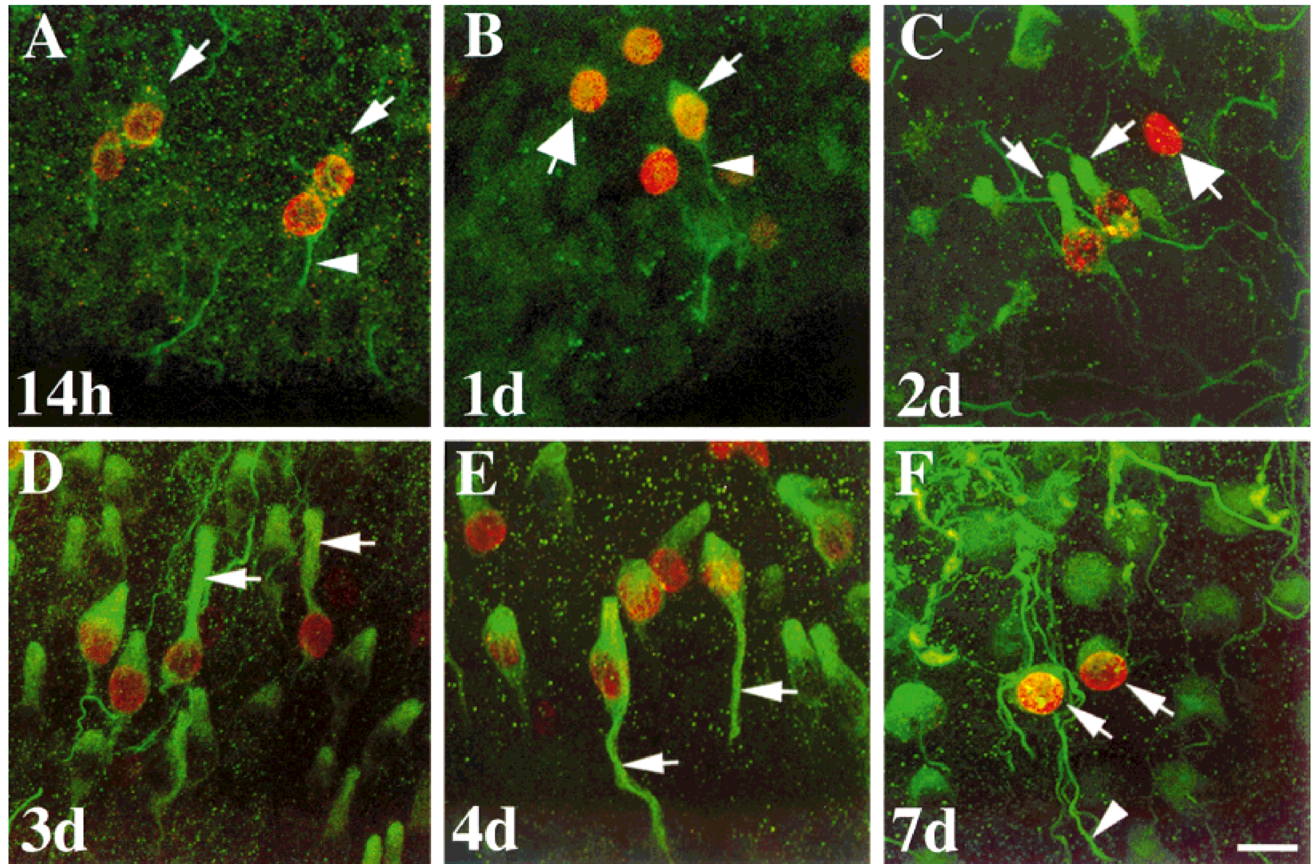

Fig. 2. Time-course of class III β-tubulin expression in regenerating hair cells. Stacked confocal slices through 66–100% of the depth of the lesioned epithelium in whole-mount basilar papillas, with slices parallel to the lumenal surface. The relative depths of different structures in the epithelium are not evident. TuJ1 labeling is green and BrdU labeling is red. The time after bromodeoxyuridine (BrdU) injection, hours (h) or days (d) is indicated in the lower left corner of each panel. **A:** Two pairs of BrdU-labeled nuclei at 14 hours after BrdU are shown. Weak TuJ1 immunolabeling is evident in at least one cell (arrow) in each pair. **B:** At 1 day after BrdU, TuJ1 labeling in BrdU-positive cells (small arrow) is more intense than at 14 hours. **C:** At 2 days after BrdU, TuJ1 immunoreactivity is very strong in a pair of BrdU-positive cells, especially in the lumenal, neck region (arrows). **D:** At 3 days after BrdU, double-labeled cells have highly elongated necks (arrows). **E:** At 4 days after BrdU, many TuJ1-positive cells have descending processes (arrows) as well as elongated necks. **F:** At 7 days after BrdU, double-labeled cells (arrows) appear large and round. Neural processes (arrowheads in A, B, and F) are closely associated with double-labeled cells. The large arrows in B and C point to BrdU-positive/TuJ1-negative cells. Scale bar = 10 μm in F (applies to A–F).

Although we focused on analyzing BrdU-positive/TuJ1-positive cells in the process of differentiating into hair cells, we also detected many BrdU-positive/TuJ1-negative cells at 14 hours post-BrdU and at the later time points (Fig. 2B,C). Presumably, such cells are in the process of attaining the mature support cell phenotype or re-entering the cell cycle. We also noted many TuJ1-positive/BrdU-negative cells at many time points. This result was expected, because only a subpopulation of progenitor cells is in S phase during the short period when BrdU is available for incorporation after a single BrdU injection. Thus, cells that are born before or after this period would not contain BrdU.

Some regenerating hair cells are elongated and may contact the basal lamina

TuJ1-positive/BrdU-positive regenerated hair cells between 2 and 4 days post-BrdU injection appear elongated, contacting the lumen, and extending a process toward the basal lamina. To view the extent of the ablumenal process, we examined TuJ1-labeled basilar papillas between 5 and 7 days post-gentamicin (equivalent to 2 and 4 days post-BrdU), when the elongated hair cell phenotype was common. Basilar papillas were counterstained with propidium iodide, a fluorescent dye that labels nucleic acids, to help visualize the hair cell and support cell nuclear layers.

Figure 3A is a stacked Z series of the regenerating region of the basilar papilla at 5 days post-gentamicin, with all of the layers of the epithelium collapsed into one plane. Individual slices from different depths within the same field shown in Figure 3A were arranged into a montage (Fig. 3C–H). The approximate location of each slice is indicated in Figure 3B. One TuJ1-positive cell in particular bears the elongated phenotype. The lumen-contacting portion of the cell is evident in Figure 3C. The nucleus of the cell (Fig. 3D) is located in a deeper slice. The cell's ablumenal process, which is distinguishable from neural elements, because it is considerably thicker in diameter,

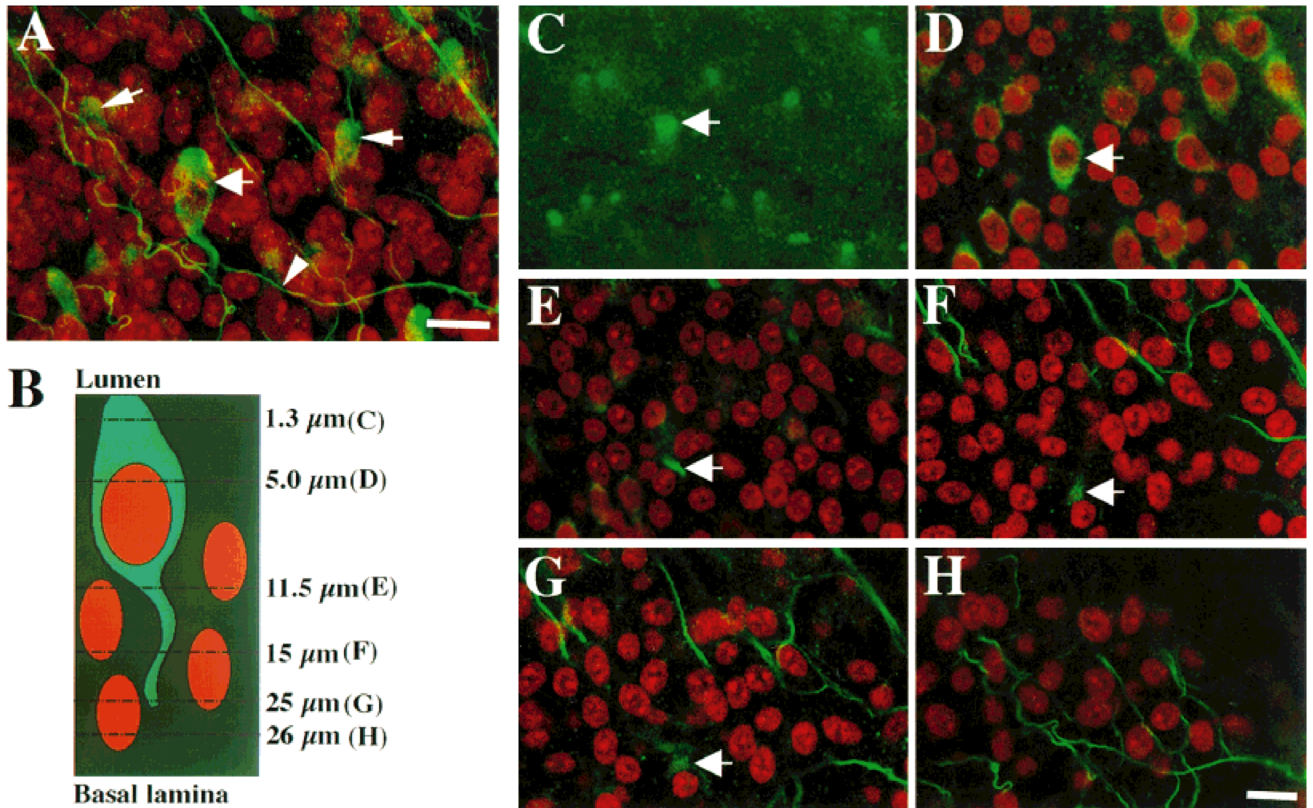

Figure 3

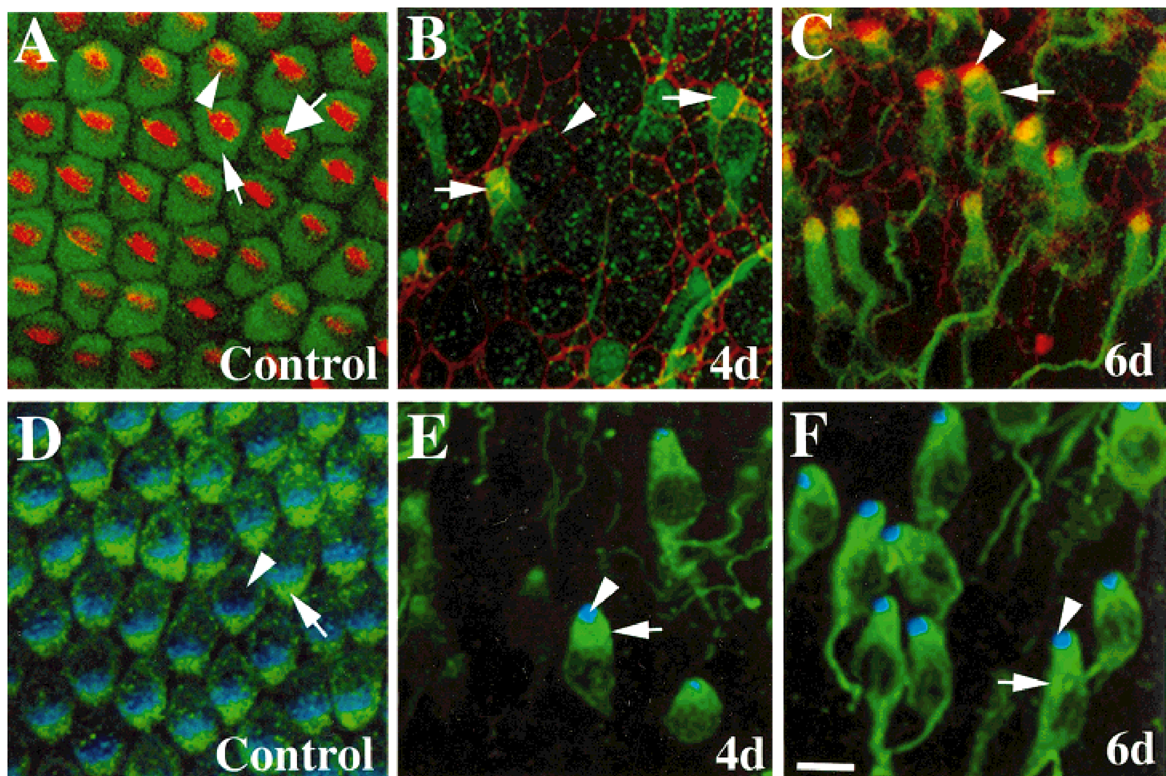

Figure 4

descends into the support cell nuclear layer as far as 25 μm from the lumen (Fig. 3E–G), approximately 3 μm from the basal lamina. In the 26-μm slice (H), we were unable to distinguish the ablumenal process. Thus, it was not possible for us to ascertain whether the ablumenal process actually contacts the basal lamina.

Differentiation of apical hair cell specializations relative to class IIIβ-tubulin

Between 2 and 4 days post-BrdU incorporation (which correspond to 5 and 7 days post-gentamicin, respectively), TuJ1-positive/BrdU-positive cells morphologically resemble support cells (Figs. 2 and 3). To determine whether the TuJ1-positive, elongated cell types are differentiating hair cells or support cells, we examined the presence of developing stereocilia on such cells by using HCA and phalloidin labeling. Basilar papillas from control and gentamicin-treated chicks were double labeled to detect βIII-tubulin and phalloidin or βIII-tubulin and the hair cell antigen (HCA). Phalloidin labels filamentous actin in the stereocilia, cuticular plates, and intercellular junctions of mature avian hair cells (e.g., Raphael, 1993), and it robustly labels the stereocilia of developing avian cochlear hair cells by embryonic day 12 (E12) (Bartolami et al., 1990). In the basilar papilla, HCA is a hair cell–specific antigen that is present on the surface of stereocilia of mature hair cells (Richardson et al., 1990) and on immature stereocilia of developing auditory hair cells as early as E6.5 (Bartolami et al., 1990).

Double labeling with phalloidin and TuJ1 is shown in Figure 4A. In the undamaged mature basilar papilla, βIII-tubulin is present throughout the hair cell cytoplasm, whereas phalloidin binds actin in the stereocilia and cuticular plate. At 4 days post-gentamicin, there is no evidence of phalloidin labeling of the stereocilia or cuticular plates within TuJ1-positive cells (Fig. 4B). In contrast, at 6 days post-gentamicin, phalloidin labels an apical structure resembling immature stereocilia on many TuJ1-positive cells (Fig. 4C). Most TuJ1-positive/phalloidin-labeled cells at 6 days post-gentamicin are highly elongated and have their nuclei in the support cell nuclear layer (data not shown).

Double labeling for βIII-tubulin and HCA is shown in Figure 4D. In the undamaged mature basilar papilla, βIII-tubulin is present throughout the hair cell cytoplasm, whereas HCA is present on the stereocilia. At 4 days post-gentamicin, HCA labeling is seen within the short, immature stereocilia of TuJ1-positive cells (Fig. 4E). At this time, the necks of TuJ1-positive cells are slightly elongated, and, in some cells, the nucleus resides within the support cell nuclear layer (data not shown). HCA immunoreactivity within the stereocilia of TuJ1-positive cells is more robust at 6 days after gentamicin (Fig. 4F) than at 4 days post-gentamicin.

This analysis confirms that the TuJ1-positive cells seen to span the depth of the sensory epithelium between 5 and 7 days post-gentamicin (Fig. 3) or between 2 and 4 days post-BrdU (Fig. 2D,E) are hair cells. It also shows that phalloidin binds actin in regenerating hair cells by 6 days post-gentamicin, which is approximately 2 days after βIII-tubulin is first detectable. In contrast, HCA appears to be initially expressed at 4 days post-gentamicin, which is around the time that βIII-tubulin is also first detected.

We sought to determine when, relative to DNA synthesis, HCA is expressed in differentiating hair cells. Similar to our analysis of TuJ1 labeling, we opted to examine this in cells born at 3 days post-gentamicin, the time of maximal cell division. Chicks were given a single injection of BrdU at 3 days post-gentamicin, and they recovered for 1, 2, or 3 days after the BrdU injection. Basilar papillas were triple labeled to detect TuJ1, HCA, and BrdU. At 1 day post-BrdU (or 4 days post-gentamicin), cells that are double labeled for TuJ1 and BrdU are present throughout the damaged region (Fig. 5A). At this time, we did not detect cells that are double labeled for HCA and BrdU or cells that are triple labeled for TuJ1, BrdU, and HCA. In contrast, at 2 days post-BrdU (or 5 days post-gentamicin), faint HCA labeling is detected on the stereocilia BrdU-positive/TuJ1-positive cells (Fig. 5B). And, by 3 days post-BrdU (or 6 days post-gentamicin), the intensity of HCA labeling in triple-labeled cells had increased dramatically (Fig. 5C). These findings demonstrate that, relative to S phase, βIII-tubulin is expressed in regenerating hair cells approximately 1 day before HCA. (We were unable to perform a similar study to determine when, relative to S phase, phalloidin binds the hair cell stereocilia, because the BrdU and phalloidin labeling protocols are incompatible.)

In our triple-labeling study (Fig. 5), the earliest time that we detect TuJ1-labeled cells that are also HCA-

Fig. 3. Some TuJ1-positive differentiating cells may contact the basal lamina. **A:** Stacked confocal slices through the entire depth of the lesioned epithelium of a whole-mount immunolabeled basilar papilla at 5 days after gentamicin injection, with slices parallel to the lumenal surface. Nuclei are labeled with propidium iodide (red), and cells (arrows) and nerves (arrowhead) are labeled with TuJ1 (green). The relative depths of different structures in the epithelium are not evident. **B:** Schematic illustration that shows the location of each slice shown in C–H. **C–H:** Individual slices at increasingly deeper levels in the same region of the epithelium shown in A. Arrows point to portions of the cell indicated in A by the thick arrow, at different depths within the epithelium. This cell's descending process extends to at least 25 μm from the lumenal surface, nearly contacting the basal lamina, which is at 28 μm. Scale bars = 10 μm in A, 10 μm in H (applies to C–H).

Fig. 4. Differentiation of apical hair cell specializations relative to class III β-tubulin immunoreactivity. Stacked confocal slices of the lesioned epithelium of whole-mount immunolabeled basilar papillas with slices parallel to the lumenal surface, starting from 3 to 5 μm above the epithelium (in the lumen) and extending through two-thirds of the depth of the epithelium. The relative depths of different structures in the epithelium are not evident. The days (d) post-gentamicin treatment are indicated. A–C show basilar papillas double-labeled with phalloidin (red) and TuJ1 (green). **A:** In hair cells in control basilar papillas, phalloidin labels the stereocilia (thick arrow) and cuticular plate (arrowhead), while TuJ1 labels the cytoplasm (thin arrow). **B:** At 4 days post-gentamicin, no phalloidin labeling of stereocilia or cuticular plates is evident on TuJ1-positive cells (arrows). Phalloidin labels the points of cell/cell contact (arrowhead). **C:** At 6 days post-gentamicin, phalloidin binds immature stereocilia (arrowhead) on TuJ1-positive cells (arrow). D–F show basilar papillas double labeled with anti–hair cell-specific antigen (HCA) antibodies (blue) and TuJ1 (green). **D:** In control basilar papillas, HCA is present in stereocilia (arrowhead) on TuJ1-positive hair cells (arrow). **E:** At 4 days post-gentamicin, HCA is detectable at low levels in immature stereocilia (arrowhead) on TuJ1-positive cells (arrow). **F:** At 6 days after gentamicin, HCA levels (arrowhead) are elevated in TuJ1-positive cells (arrow) relative to 4 days after gentamicin. Scale bar = 10 μm in F (applies to A–F).

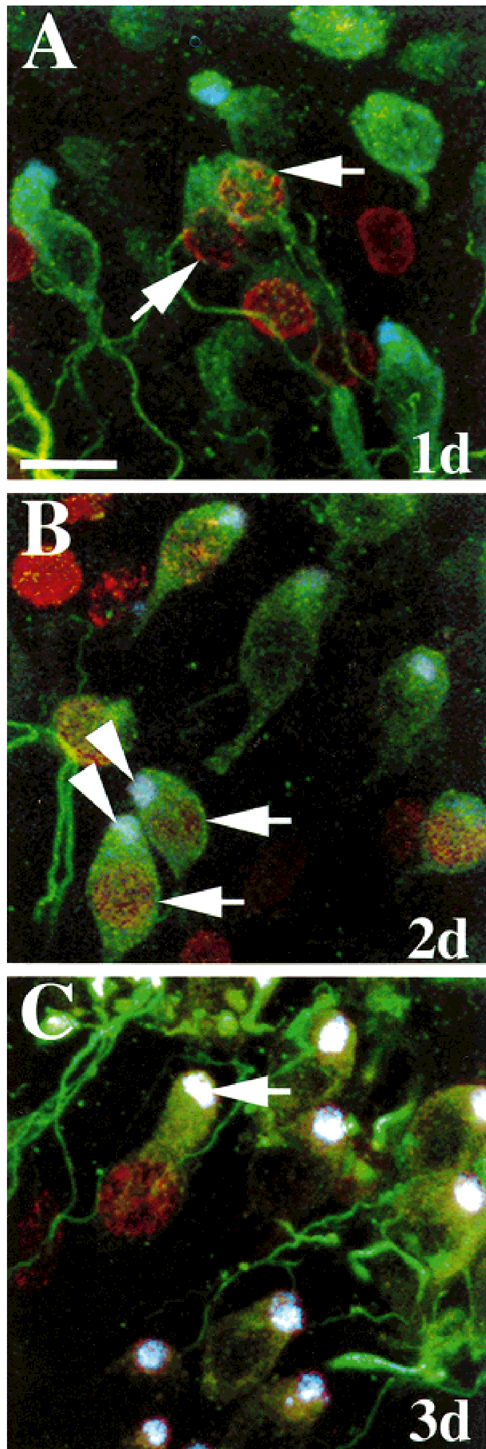

Fig. 5. Timing of hair cell–specific antigen (HCA) immunoreactivity in regenerating hair cells. Stacked confocal slices of the lesioned epithelium of whole-mount immunolabeled basilar papillas, with slices parallel to the lumenal surface, starting from 3–5 μm above the epithelium (in the lumen) and extending through two-thirds of the depth of the epithelium. The relative depths of different structures in the epithelium are not evident. The days (d) after bromodeoxyuridine (BrdU) are indicated. A–C show basilar papillas triple labeled with antibodies to BrdU (red), HCA (pale blue/white), and βIII-tubulin (green). **A:** At 1 day post-BrdU, cells that are double labeled for BrdU and TuJ1 (arrows) are HCA-negative. **B:** At 2 days post-BrdU, some BrdU-positive/TuJ1-positive cells (arrows) are faintly HCA-positive at their apical tips (arrowheads). **C:** At 3 days after BrdU, HCA labeling of the apex of TuJ1-positive cells (arrow) is robust. Scale bar = 10 μm in A (applies to A–C).

positive is at 5 days post-gentamicin, which is 2 days after their birth dates, at 3 days post-gentamicin. This result might suggest that regenerated hair cells first become immunoreactive for HCA at 5 days post-gentamicin. However, we noted HCA labeling in some TuJ1-positive cells at 4 days after gentamicin (Fig. 4D–F). The most likely explanation for this apparent disparity is that the HCA-positive/TuJ1-positive cells that we detected at 4 days post-gentamicin are born between 2 and 3 days after gentamicin, i.e., before the BrdU injection. However, because we did not examine cells born at this time, we were unable to resolve this disparity.

Immunoreactivity to calmodulin and ribosomal RNA over the course of hair cell differentiation

To confirm our findings regarding the time course of TuJ1 labeling in regenerating cells, we examined the expression of two other antigens, calmodulin and ribosomal RNA, in conjunction with BrdU labeling. The experimental paradigm resembled that described for TuJ1/BrdU double labeling (Figs. 1, 2). Antibodies to calmodulin label differentiating hair cells as early as 1 day post-BrdU incorporation (Stone et al., 1996a). The antibody to ribosomal RNA, Y10B (Lerner et al., 1981), labels all cells in the brain, but it gives reliably larger signal in neurons than in other cell types (Garden et al., 1994, 1995). Y10B has not been reported previously to label hair cells selectively, but the electron density of mature and regenerating hair cell cytoplasm (Duckert and Rubel, 1990) suggested that it might be useful.

In the undamaged basilar papilla, anti-calmodulin labeled the cell bodies as well as the stereocilia of hair cells, but it does not appear to react with support cells (Stone et al., 1996a; Fig. 6A). At 3 hours post-BrdU injection (3 days post-gentamicin injection), the majority of BrdU-positive cells are not colabeled with antibodies against calmodulin. However, a small subpopulation (approximately 10%) of BrdU-labeled cells contain weak calmodulin immunoreactivity in their cytoplasm (Fig. 6B). Double-labeled cells have nuclei in the support cell layer of the epithelium and are highly elongated in shape, with cytoplasmic extensions both lumenal and ablumenal to the nucleus. No pairs of double-labeled cells are seen at this time, suggesting that double-labeled cells are premitotic, in either S or G2 phase.

At 6 hours post-BrdU (Fig. 6C), numerous BrdU-positive mitotic figures are present throughout the damaged area, and all of them are calmodulin-positive. Calmodulin appears to be present within the mitotic spindle. Calmodulin labeling is also detectable in mitotic figures throughout the developing chick basilar papilla at E6 (J. Stone and E. Rubel, unpublished observations), which is a highly proliferative phase (Katayama and Corwin, 1989). To determine whether all mitotic cells, or only a subpopulation, are calmodulin-positive, basilar papillas at 3 days post-gentamicin ($n = 4$) were immunofluorescently labeled with antibodies to calmodulin and counterstained with propidium iodide, enabling us to identify mitotic chromosomes. All mitotic figures within the damaged area of the sensory epithelium are calmodulin-positive (data not shown), suggesting that calmodulin is present in all progenitor cells during mitosis.

At 14 hours post-BrdU (Fig. 6D), many BrdU-positive cells are immunoreactive for the anticalmodulin antibody. Double-labeled cells are rounded and located in the hair cell layer. From 2–3 days post-BrdU (Fig. 6E), calmodulin-

positive/BrdU-positive cells are elongated, similar to TuJ1-positive cells at this time of differentiation.

The Y10B antibody labels robustly the cell bodies of mature hair cells in the undamaged basilar papilla, and it is not strongly reactive with support cells (Fig. 6F). At 3 hours (Fig. 6G) and 6 hours (data not shown) post-BrdU, Y10B immunoreactivity is not detected in BrdU-positive cells. Therefore, Y10B does not strongly label progenitor cells. At 14 hours post-BrdU (Fig. 6H), many BrdU-positive cells are strongly immunoreactive for Y10B, but there is also some diffuse immunoreaction in the surrounding tissue. Strongly labeled cells appear round or fusiform and are located in the hair cell layer. From 2–3 days post-BrdU (Fig. 6I), highly Y10B-positive/BrdU-positive cells appear morphologically similar to those at 14 hours post-BrdU. This observation differs from our observations of BrdU-positive cells that were labeled with either TuJ1 or anticalmodulin antibodies, which appear highly elongated rather than round or fusiform at this time of differentiation. This is probably because Y10B immunoreactivity is concentrated in the perinuclear cytoplasm in regenerating hair cells.

At 14 hours post-BrdU and at the later time points, we detected BrdU-labeled postmitotic cells that did not label with either antibodies to calmodulin or to ribosomal RNA (e.g., Fig. 6E). These cells were presumably in the process of differentiating into support cells.

F6&7

Cells are regenerated by means of asymmetric and symmetric modes of differentiation

Between 14 hours and 7 days post-BrdU, closely associated pairs of BrdU-positive cells with similar levels and patterns of BrdU labeling are common (Figs. 2A,C,F, 6D,H,I). We considered such cells postmitotic sister cells from a single cell division that had not migrated far from each other. In some cases, sister cells have similar morphologies and similar, positive levels of the hair cell marker (Figs. 2C,F, 6D,H,I), suggesting that they are differentiating synchronously as hair cells (i.e., differentiating symmetrically). We also noted sister cell pairs that are composed of a βIII-tubulin–negative cell and a βIII-tubulin–positive cell, as well as those that are composed of two TuJ1-negative cells (data not shown). In these cases, it appeared as if the sister cell pairs are differentiating as a support cell and a hair cell (i.e., differentiating *asymmetrically*) or as two support cells (i.e., differentiating *symmetrically,* respectively.

These findings suggest that both symmetric differentiation (generating two like cells) and asymmetric differentiation (generating two unlike cells) occur during hair cell regeneration and led us to examine quantitatively the identity of sister cells in other samples. Chicks received a single injection of BrdU at 3 days post-gentamicin and survived for 3 additional days after the injection to allow time regenerating hair cells to express βIII-tubulin (see Fig. 2). Basilar papillas were dissected and double labeled for BrdU and βIII-tubulin. All distinct and closely associated pairs of BrdU-positive cells in the damaged area were analyzed for βIII-tubulin immunoreactivity (see Materials and Methods section for details).

Figure 7 shows the three potential combinations of pairs in one region of the basilar papilla. Of the 91 pairs of cells that we examined, 33 ± 9% (SD) are composed of one βIII-tubulin–positive cell and one βIII-tubulin–negative cell; such pairs were considered to have differentiated asymmetrically. Thirty-six ± 14% are composed of two βIII-tubulin–positive cells, and 31 ± 13% are composed of two βIII-tubulin–negative cells. In both of these latter cases, pairs appeared to have differentiated symmetrically.

Positional variation in the phenotypes of regenerating hair cells

The progression of hair cell differentiation that we have described here (Figs. 2, 4, 5, 6) does not occur in a synchronous manner throughout the lesioned area. Rather, there is marked variation in the morphologic features of new hair cells across different positions within the lesion. To study this difference, we examined TuJ1 labeling among cells in opposite ends of the lesion at 3, 5, and 7 days after gentamicin injection.

TuJ1 labeling discloses the length of the lesion, which we define as the area of cellular disruption, regeneration, or both. By 3 days post-gentamicin, the lesion extends from the basal tip of the epithelium to 870 μm (±311 μm [SD]; n = 2 organs) from the basal tip (Fig. 8A), or approximately one-third the length of the papilla. TuJ1-positive cells are present in the basal tip (Fig. 8B), but no TuJ1-positive cell bodies are detected in the apical half of the lesion (Fig. 8C). Some TuJ1-positive cells are round and smaller than mature hair cells, whereas others are elongated with tapered necks. Throughout the damaged area, nerves are evident, and some appear to contact TuJ1-positive cells.

F8

By 5 days post-gentamicin, the lesion has spread apically to approximately 1,250 μm (±127 μm; n = 4 organs) from the basal tip (Fig. 8D). Immature-looking TuJ1-positive cells are present throughout the lesion. In the basal half of the lesion (Fig. 8E), most labeled cells are small and round, and they appear more densely packed than at 3 days post-gentamicin. In the apical half of the lesion (Fig. 8F), many labeled cells are elongated, resembling those in the basal tip of the lesion at 3 days after gentamicin. In addition, a few TuJ1-positive cells in the apical half of the lesion are large and round. Such cells are presumed to be damaged, native hair cells that had not yet been killed by the gentamicin treatment at the time of tissue fixation. In both regions, new TuJ1-positive cells appear more numerous and tightly packed than at 3 days post-gentamicin.

By 7 days after gentamicin, the lesion extends to approximately 1,338 μm (±160 μm; n = 4 organs) from the basal tip (Fig. 8G). In the basal half of the lesion (Fig. 8H), the vast majority of TuJ1-positive cells are round and appear more numerous, larger, and more evenly distributed than cells at 5 days post-gentamicin. In the apical half of the lesion (Fig. 8I), many cells have an elongated, immature phenotype.

These data demonstrate a basal-to-apical progression in the appearance and differentiation of new hair cells after a single gentamicin injection. However, it should be noted that this does not necessarily demonstrate that new hair cells formed in the basal part of the lesion undergo an accelerated rate of differentiation relative to cells formed in the apical part of the lesion (see Discussion section).

DISCUSSION

In this study, we used hair cell–selective antibodies in combination with the S phase marker BrdU to examine temporal, spatial, and morphologic features of hair cell regeneration in the drug-damaged chick basilar papilla. We confirmed that the TuJ1 antibody, which binds βIII-

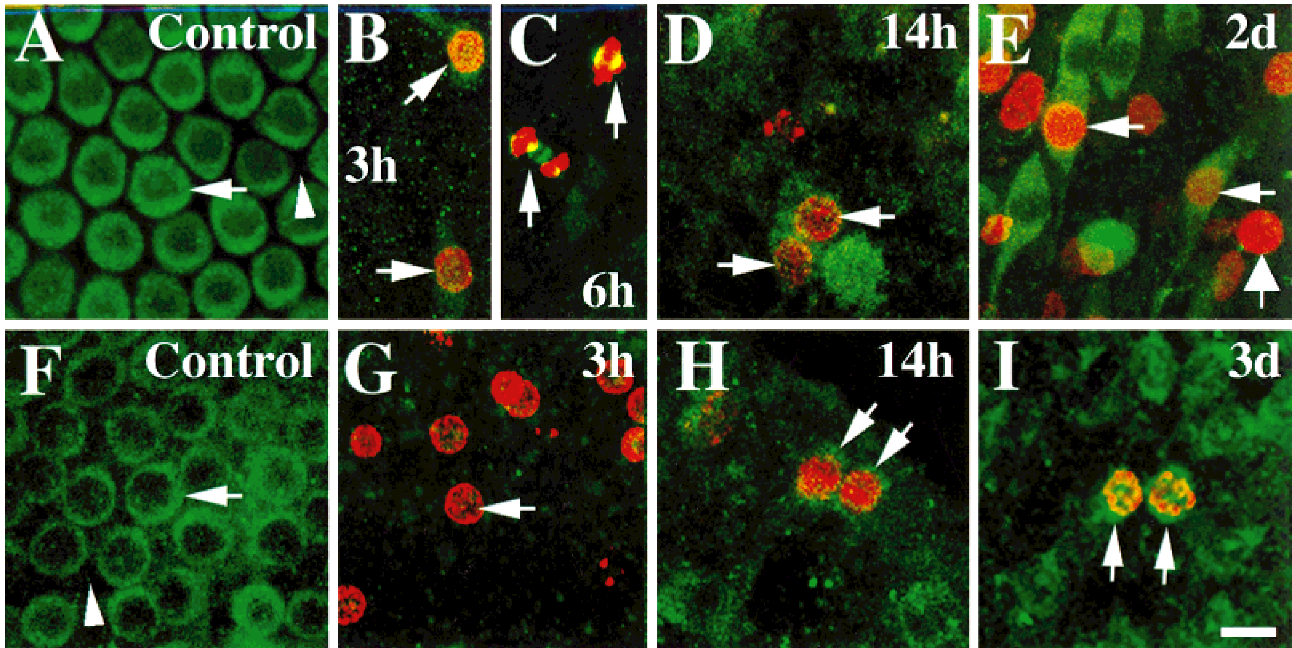

Fig. 6. Calmodulin and ribosomal RNA immunolabeling at different stages of hair cell differentiation. Panels show stacked confocal slices through 66–100% of the depth of the lesioned epithelium of whole-mount immunolabeled basilar papillas. Slices are parallel to the lumenal surface. The relative depths of different structures in the epithelium are not evident. A–E show basilar papillas double labeled with antibodies to bromodeoxyuridine (BrdU) (red) and calmodulin (green). The time after BrdU injection, in hours (h) or days (d), is indicated in the lower left corner of each panel. **A:** In the control basilar papilla, calmodulin is detected in the hair cell cytoplasm (arrow) and stereocilia (not shown because of plane of section), but not in support cells (arrowhead). **B:** At 3 hours post-BrdU, calmodulin is detected in some elongated, BrdU-positive cells (arrows) in the damaged area. **C:** At 6 hours post-BrdU, calmodulin is present in BrdU-labeled mitotic figures (arrows) in the damaged area. **D:** At 14 hours post-BrdU, calmodulin is seen in BrdU-labeled cells (arrows) that are rounded and located in the hair cell layer. A pair of double-labeled cells is indicated. **E:** At 2 days post-BrdU, calmodulin (arrows) is detected in elongated BrdU-positive cells. The thick arrow indicates a BrdU-positive/TuJ1-negative cell. F–I show basilar papillas double-labeled with antibodies to BrdU (red) and rRNA (Y10B; green). **F:** In control basilar papillas, rRNA is abundant in the hair cell cytoplasm (arrow), but not in support cells (arrowhead). **G:** At 3 hours post-BrdU, BrdU-labeled cells (arrow) are Y10B-negative. **H:** At 14 hours post-BrdU, some BrdU-labeled cells (arrows) are Y10B-positive. Such cells are round and located in the hair cell layer. A pair of double-labeled cells is indicated. **I:** At 3 days post-BrdU, Y10B labeling (arrows) in the perinuclear area of BrdU-positive cells persists. Double-labeled cells are round. A pair of double-labeled cells is indicated. Scale bar = 10 μm in I (applies to A–I).

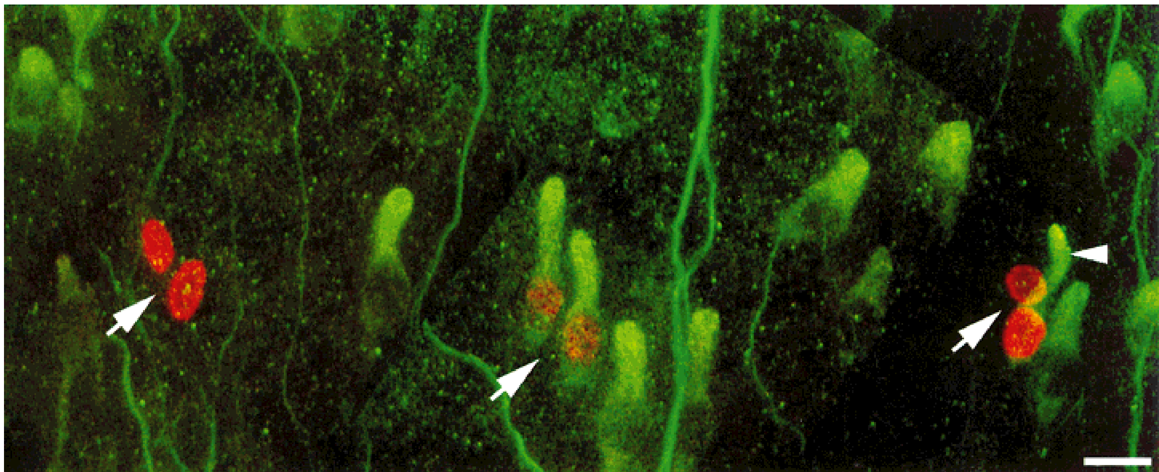

Fig. 7. Hair cell regeneration occurs by both symmetric and asymmetric modes of differentiation. Stacked confocal slices through the entire depth of the lesioned epithelium of a whole-mount immunolabeled basilar papilla. Slices are parallel to the lumenal surface. The relative depths of different structures in the epithelium are not evident. Chicks received a single bromodeoxyuridine (BrdU) injection at 3 days post-gentamicin and recovered for 3 days after the BrdU injection. TuJ1 labeling is green and BrdU labeling is red. Three sets of paired BrdU-positive nuclei (arrows) are evident in this panel. In the left-most pair, both cells are TuJ1-negative. In the middle pair, both cells are TuJ1-positive and appear similar morphologically. In the right-most pair, one cell is TuJ1-positive (arrowhead points to labeled neck region) and one cell is TuJ1-negative. Scale bar = 10 μm.

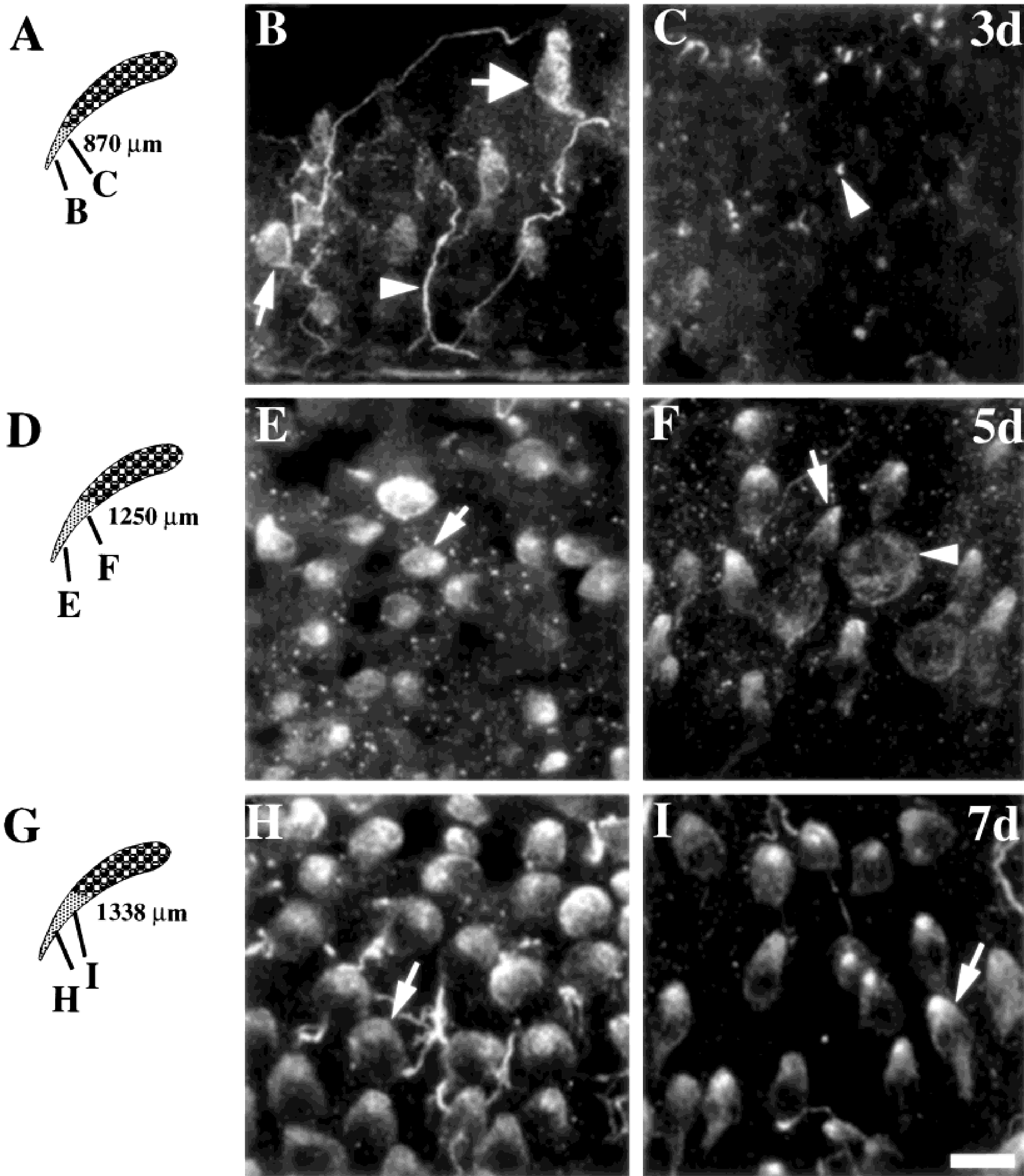

Fig. 8. Hair cell differentiation follows a basal-to-apical progression. **A, D, G** are schematic drawings of the basilar papilla at 3, 5, and 7 days after gentamicin, respectively (base is to the left). The thick stippled areas represent intact epithelium, whereas the lightly stippled areas represent lesioned/regenerating epithelium. The length of the papilla that is spanned by the lesion is indicated. **B, E, H and C, F, I** show high-magnification images of the basal and apical portions of the lesion, respectively, at 3 days (B, C), 5 days (E, F), and 7 days (H, I) post-gentamicin. The time after gentamicin injection, in days (d), is indicated in the upper right corner. The approximate origin along the length of the basilar papilla of the high-magnification images is shown in A, D, G. Images are stacked confocal slices through the lumenal two-thirds of whole-mount basilar papillas immunolabeled with TuJ1. Slices are parallel to the lumenal surface. The relative depths of different structures in the epithelium are not evident. B: At 3 days post-gentamicin, a few TuJ1-positive cells (arrows) and nerves (arrowhead) are present in the basal tip of the lesion. Some labeled cells have elongated cell bodies (thick arrow), while others appear round (thin arrow). C: In the apical part of the lesion, no TuJ1-positive cells are evident, but TuJ1-positive nerves are present (arrowhead). E: At 5 days post-gentamicin, there are numerous small and round TuJ1-positive cells (arrow) in the basal tip of the papilla. F: In the apical part of the lesion, there are TuJ1-positive cells that are large and round (arrowhead) and some that are small and elongated (arrow). H,I: At 7 days post-gentamicin, there are large and round TuJ1-positive cells (H, arrow) in the basal tip of the lesion and elongated TuJ1-positive cells in the apical part of the lesion (I, arrow). Scale bar = 10 μm in I (applies to B, C, E, F, H, I).

tubulin, labels differentiating hair cells during ototoxin-induced cellular regeneration. We also showed that (1) class IIIβ-tubulin distinguishes regenerated hair cells from support cells as early as 14 hours after the progenitor cell is in S phase, (2) regenerated hair cells pass through a stage of differentiation during which they are highly elongated and appear to contact both the lumenal surface and the basal lamina, resembling support cells, (3) regenerated hair cells and support cells are formed by asymmetric and symmetric modes of differentiation, and (4) the appearance and subsequent maturation of regenerated hair cells progress from base to apex over time.

TuJ1 is an early marker for regenerated hair cells

For research on cellular development and regeneration, it is critically important to have markers that label new cells as early as possible after cell fate has been determined. Only a few hair cell-selective antibodies have been shown to label early differentiating hair cells in the chick basilar papilla. During development, an antibody to the 275-kDa HCA exclusively labels hair cells as early as E6.5 (Bartolami et al., 1990), which is approximately when stereocilia are first detectable by scanning electron microscopy (Cotanche and Sulik, 1984). During regeneration, antibodies to the proteins fimbrin (Lee and Cotanche, 1996), calmodulin, and βIII-tubulin (Stone et al., 1996a) selectively label hair cells by 4 days after damage is induced.

To further examine the time course of βIII-tubulin immunoreactivity during hair cell regeneration, we used a single pulse of BrdU to selectively label a subpopulation of mitotically active progenitor cells in the basilar papilla at 3 days post-gentamicin. By double-labeling organs with antibodies to BrdU and βIII-tubulin at different times after the BrdU injection, we temporally tracked the differentiation of cells generated by progenitor cell division. In organs fixed at 3 or 6 hours after BrdU, cells that incorporated BrdU were not immunoreactive for βIII-tubulin. Because it probably takes progenitor cells in the regenerating basilar papilla between 3 and 6 hours to pass from S phase to mitosis (Katayama and Corwin, 1993; Stone and Cotanche, 1994; Tsue et al., 1994), it is likely that these BrdU-positive/TuJ1-negative cells are progenitor cells in S, G2, or M phase. Interestingly, at E4.5, βIII-tubulin appears to be expressed in all cells in the region of the developing otocyst that will form the basilar papilla (Molea et al., 1999). This time corresponds to the onset of terminal mitosis in the basilar papilla anlage (Katayama and Corwin, 1989). This observation suggests that, unlike the regenerating basilar papilla, sensory epithelial progenitors of the embryonic basilar papilla are βIII-tubulin–positive.

βIII-tubulin is first detected in some postmitotic cells at 14 hours after BrdU incorporation. By 2 days post-BrdU, βIII-tubulin–positive cells are also immunoreactive for HCA. Therefore, the βIII-tubulin–positive cells that we observed in the basilar papilla are regenerating hair cells. Interestingly, βIII-tubulin immunoreactivity in newly regenerated hair cells precedes HCA immunoreactivity. In contrast, during development, expression of TuJ1 and HCA in immature hair cells appears to be coincidental, occurring first at E6.5 (Bartolami et al., 1990; Molea et al., 1999). The expression of both antigens relative to the cell's birth date during development has not been studied. In the stereocilia of regenerating hair cells, HCA protein expression precedes phalloidin labeling by approximately 2 days. During development, immunoreactivity for HCA is also achieved well before phalloidin binds the developing hair cell bundle (Bartolami et al., 1990; Goodyear et al., 1995). Thus, the timing of HCA expression relative to phalloidin binding is similar in the developing and regenerating basilar papilla, but the timing of HCA protein expression relative to βIII-tubulin protein expression appears to differ.

Class IIIβ-tubulin immunoreactivity reveals three general periods of hair cell differentiation

Immunolabeling of hair cell-selective antigens in BrdU-positive cells at various times after BrdU incorporation has revealed several general changes in hair cell morphology over the course of regeneration. Although we acknowledge that differentiation of hair cells into mature, functional receptors is a continuous process, we have simplified this morphologic progression into three general periods to facilitate this discussion (Fig. 9). We provide this overview as a guideline and not as a summary of distinct stages of hair cell maturation.

Early hair cell differentiation. During the early period of hair cell differentiation during regeneration (14 hours to 1 day after BrdU), βIII-tubulin levels are elevated in cells that reside very close to the lumen. This observation suggests that these cells had not migrated vertically from the lumenal surface of the basilar papilla, which is the site of mitosis (Raphael, 1992; Katayama and Corwin, 1993; Stone and Cotanche, 1994). During this period, labeling with TuJ1 suggests that differentiating hair cells are round or slightly fusiform. βIII-Tubulin is concentrated in the hair cell cytoplasm lumenal to the nucleus, and little βIII-tubulin immunoreactivity is seen below the level of the nucleus. TuJ1 does not label a descending process on hair cells during this period of differentiation, unlike at later times (see below). Because this observation does not disprove that such a process is present in early differentiating hair cells, additional anatomic studies are needed to make this assessment. HCA is not detectable in regenerating hair cells during this period, suggesting that stereocilia formation is not yet under way.

During this period, some βIII-tubulin–positive cells seem to be in contact with neural elements in the epithelium. Previous papers have reported synapses on regenerated hair cells at early stages of differentiation (Duckert and Rubel, 1990; Wang and Raphael, 1997). Because we did not perform any additional labeling experiments or immunologic transmission electron microscopy, we could not assess whether nerve processes are in direct contact with and/or form synapses on immature, regenerating hair cells.

Intermediate hair cell differentiation. During the intermediate period of hair cell differentiation (2–4 days after BrdU incorporation), the nuclei of βIII-tubulin–positive/BrdU-positive cells migrate ablumenally into the support cell layer. Many such cells are highly elongated and have ascending and descending processes that contain βIII-tubulin. The ascending process contacts the lumen, whereas the descending process of many cells approaches, and may contact, the basal lamina. By 2 days

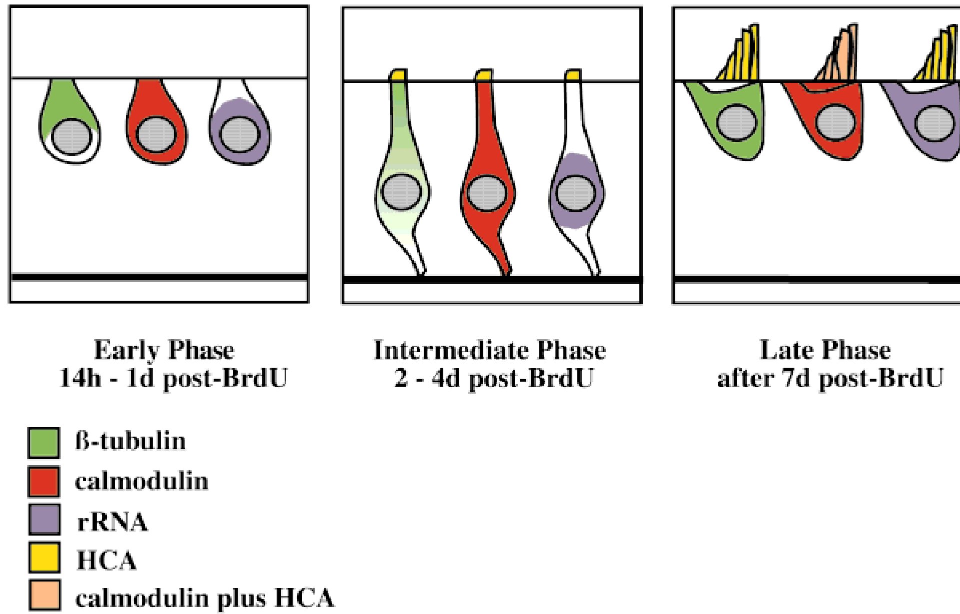

Fig. 9. Schematic illustration of βIII-tubulin, calmodulin, rRNA, and hair-cell specific antigen (HCA) immunolabeling during different stages of hair cell differentiation during regeneration. BrdU, bromodeoxyuridine.

after BrdU incorporation, βIII-tubulin–positive/BrdU-positive cells also begin to be HCA-positive. Approximately 1 day later, the actin-rich precursors to the stereocilia are detectable with phalloidin labeling.

Our findings demonstrate that postmitotic cells in the intermediate period of hair cell differentiation have a phenotype characteristic of both hair cells and support cells. These cells have stereocilia, which are specific for hair cells, and an elongated soma and apparent contact with both the lumenal surface and the basal lamina, features that are thought to be specific for support cells. Similar morphology has been noted among developing hair cells in the chick otocyst (Ginzberg and Gilula, 1979; Whitehead and Morest, 1985a,b; Molea et al., 1999). Cells with the hair cell-support cell phenotype have also been reported in postembryonic, regenerating inner ear tissues. Steyger et al. (1997) examined drug-damaged frog vestibular end organs with antibodies to calcium-binding proteins, which selectively label mature hair cells. They noted that many labeled cells contact the basal lamina and are shaped like support cells. Adler and Raphael (1996) and Westrum et al. (1998) used transmission electron microscopy to examine regenerated cells in the noise-damaged and drug-damaged chick basilar papilla, respectively. They describe cells having some features of hair cells (i.e., dense cytoplasm and immature stereocilia) that also contact the basal lamina, like support cells. These observations are relevant to the suggestion that mitotic activity may not be necessary for the regenerative process (Baird et al., 1993; Adler and Raphael, 1996; Baird et al., 1996; Roberson et al., 1996; Forge et al., 1998). Collectively, these studies led to the hypothesis that some cells can be regenerated through a phenotypic conversion from a support cell to a hair cell without an intervening mitosis, a process termed direct transdifferention (Roberson et al., 1996). We show here that the hair cell-support cell phenotype is a normal stage of mitotic regeneration. Because several previous studies did not determine whether the cells with the hair cell-support cell phenotype were products of a recent cell division, it is possible that those cells were also regenerated mitotically. Thus, one must be cautious when using solely morphological criteria as evidence for direct transdifferentiation.

Late hair cell differentiation. During the late period of hair cell differentiation (7 days post-BrdU and later), hair cells are rounded, their nuclei are close to the lumen, and βIII-tubulin levels are strong throughout the perinuclear cytoplasm. Thus, in most respects, regenerated hair cells resemble mature hair cells (Stone et al., 1996a) at this stage of differentiation.

The temporal and morphologic progression of hair cell differentiation that we observed with TuJ1 labeling was confirmed with antibodies to two other hair cell–selective antigens, calmodulin and rRNA. Like TuJ1, antibodies to calmodulin also label postmitotic cells by 14 hours post-BrdU. During most stages of differentiation, the cellular distribution of calmodulin closely resembles that of βIII-tubulin (Fig. 9). This observation supports our previous study of calmodulin immunoreactivity in differentiating hair cells in the drug-damaged basilar papilla (Stone et al., 1996a). However, in the current study, we also detected low levels of calmodulin in premitotic support cells, at 3 hours after BrdU injection, and in mitotic support cells, at 6 hours post-BrdU injection. Our finding of calmodulin staining in cycling cells is in agreement with previous reports in other tissues (e.g., Henry et al., 1997; Moser et al., 1997) and with calmodulin's known role in the cell cycle (for review, see Santella, 1998). However, this finding contradicts data from our previous study (Stone et al., 1996a), in which no calmodulin labeling in cycling progenitor cells was detected. The most likely reason for this disparity is the different methods used in the

two studies. For this study, we used immunofluorescence to detect calmodulin in whole-mounts, fixed the tissue, and then immunofluorescently detected BrdU. These methods were required for our analysis with confocal microscopy. In the previous study, we used immunohistochemistry with horseradish peroxidase to detect BrdU and then calmodulin in whole-mounts. The tissue was then embedded in plastic and sectioned. In additional experiments, [^{3}H]thymidine replaced BrdU. It is likely that the alcohol washes used during plastic embedding stripped some of the calmodulin immunoreactivity from the tissues. In addition, the order in which the calmodulin and BrdU antigens were tagged was reversed for the two studies. In the previous study, tissue was treated with HCl before detecting both BrdU and calmodulin. Because HCl degrades proteins, it is likely that this method caused some deterioration of the calmodulin antigen. This potential pitfall was avoided in the current study, in which antibodies to calmodulin were added before HCl treatment and BrdU detection (Memberg and Hall, 1995).

Similar to TuJ1, Y10B, which labels ribosomal RNA, did not label progenitor cells in the cell cycle, but it did label early differentiating hair cells by 14 hours after BrdU incorporation. However, Y10B immunoreactivity is apparent in support cells in regenerating tissue; thus, it is not by itself a reliable hair cell marker. The intracellular distribution of the Y10B antigen in regenerated hair cells differed significantly from that of βIII-tubulin and calmodulin during the intermediate stage of differentiation (Fig. 9). Rather than being present throughout the entire extent of the cell, including the cytoplasmic processes, it was confined to the perinuclear cytoplasm.

Hair cell production results from both symmetric and asymmetric differentiation

Regenerated cell in the posthatch chick basilar papilla at 3 days after gentamicin differentiate as either hair cells or as nonhair cells, as disclosed by positive or negative labeling with one or more hair cell markers, respectively. We have assumed that most nonhair cells are support cells, which are the most numerous cells in the epithelium. However, in the absence of a support cell marker, we cannot be certain that this is true. Only a few cell types other than hair cells and support cells have been characterized in the basilar papilla; they are microglia-like cells and macrophages (Bhave et al., 1998). These cells are present in small numbers relative to hair cells and support cells. Therefore, it is unlikely that they compose a large percentage of the cells that are born after a single gentamicin injection. The support cell population itself has been poorly characterized. Support cells serve many functions in mature chicks: hair cell progenitors (Girod et al., 1989; Raphael, 1992; Stone and Cotanche, 1994), tectorial membrane regenerators (Cotanche, 1987b, 1992), sustainers of the structural integrity of the epithelium, etc. Although there is no morphologic evidence that there are distinct support cell subtypes, it is possible that the nonhair cells we have examined in this study represent only a subset of the support cell population, e.g., progenitor cells only.

Avian support cells may divide more than once during regeneration. After a single BrdU injection after noise damage, the number of BrdU-positive cells in the basilar papilla increases significantly over time (Stone and Cotanche, 1994). Also, some BrdU-positive cells form clusters over time (Stone and Cotanche, 1994). Furthermore, we have found a small number of cells in the basilar papilla that incorporate BrdU at 3 days post-gentamicin injection also incorporate tritiated thymidine at 4 days post-gentamicin (Stone et al., 1999). These studies suggest that some support cells possess the ability to reenter the cell cycle after having completed at least one round of division. We must consider, therefore, that postmitotic cells that do not label for hair cell markers may differentiate into support cells, reenter the cell cycle and divide again, or die.

We noted the preponderance of closely associated pairs of BrdU-positive nuclei with similar BrdU patterning as early as 14 hours and as late as 7 days post-BrdU injection, which we assumed were sibling cells generated from a single mitotic event. This observation suggests that most sibling cells do not migrate far from their site of genesis. During development of the chick basilar papilla, cells also appear to remain close to their birth site (Katayama and Corwin, 1993; Fekete et al., 1998). However, avian hair cells and support cells may become reorganized during later stages of development (Goodyear and Richardson, 1997).

Postmitotic sister cells that are born in the posthatch chick basilar papilla at 3 days post-gentamicin can differentiate asymmetrically, forming one hair cell and one support cell, or symmetrically, forming either two hair cells or two support cells. At this stage of regeneration, mitotically generated cells appear to have equal likelihood of differentiating into either a hair cell or a support cell, and symmetric differentiation is two times as likely to occur as asymmetric differentiation. Because we only examined the fate of cells generated at 3 days post-gentamicin, we do not know whether particular modes of cell production are more likely at different times during the regenerative process. However, it is clear from these findings that auditory hair cells and support cells share a common progenitor during regeneration. Experimental evidence in support of a bipotential progenitor cell during development of the basilar papilla has been presented (Katayama and Corwin, 1989; Fekete et al., 1998).

In contrast to regeneration in the damaged basilar papilla, asymmetric differentiation appears to predominate during hair cell production in the developing chick basilar papilla (Fekete et al., 1998), in the undamaged utricle in the posthatch chick (Stone and Rubel, 1999), and in the damaged salamander lateral line (Jones and Corwin, 1996). Why do such different modes of cell production occur in generally similar epithelia? Variation in the mode of cell differentiation may be one manner in which production of the appropriate numbers and types of cells is regulated in inner ear organs. Such a strategy appears to mediate neurogenesis in the developing mammalian cerebral cortex; symmetric divisions that generate two progenitor cells are most common during early neurogenesis, whereas asymmetric divisions, forming a neuron and a progenitor cell, predominate during late neurogenesis (Chenn and McConnell, 1995).

Modes of cell production are controlled by both extrinsic and intrinsic factors. The interplay of both mechanisms is beginning to be understood in the developing *Drosophila* nervous system (reviewed in Lin and Schagat, 1997). For example, signaling between adjacent cells by means of the Notch receptor leads to lateral inhibition of neural differentiation in postmitotic cells (reviewed in Simpson, 1997).

Asymmetric inheritance of the membrane-bound protein, Numb, during cytokinesis can interfere with Notch signaling and affect cell fate (reviewed in Campos-Ortega, 1996). It is not known whether these molecules regulate the mode of cell production during development and regeneration of the sensory epithelia in the avian inner ear. However, some recent studies suggest that Notch signaling may be important for these processes. Genes for *Notch1* and its ligands, Delta1 and Serrate1, are expressed in the sensory epithelia of the otocyst (Adam et al., 1998) and the regenerating inner ear (Stone and Rubel, 1999) in chickens when hair cell specification is occurring. A role for Notch signaling in developmental hair cell specification is supported by a study by Haddon et al. (1998), who showed that supernumerary hair cells are generated at the expense of support cells in otocysts of zebrafish lacking the *mind bomb* gene, which misexpress genes encoding the Notch ligands, Delta, and SerrateB. Furthermore, mice that lack the Notch ligand, Jagged2, also generate an extra complement of hair cells (Lanford et al., 1999).

Class IIIβ-tubulin immunoreactivity discloses a basal-to-apical progression of hair cell differentiation

Aminoglycoside antibiotics selectively injure or kill hair cells in the basal, high-frequency end of auditory organs in many species (reviewed in Cotanche et al., 1994; Garetz and Schacht, 1996). With repeated or higher doses of the drug, hair cell loss spreads apically. Longer and higher dose regimens lead to successively more apical spread of the hair cell lesion. In chicks, the spatial patterns of BrdU incorporation and hair cell repopulation after multiple injections of aminoglycoside mirror the basal-to-apical progression of hair cell loss (Cruz et al., 1987; Girod et al., 1990; Hashino et al., 1995).

In the current study, we use TuJ1 labeling to demonstrate that the appearance and differentiation of new hair cells starts in the basal tip and spreads apically after a single high dose of gentamicin. It is likely that this progression does not represent acceleration in the rate of differentiation among cells born in the base relative to those born in the apex. Rather, this progression is probably because hair cell death is initiated in the base, and consequently, the signals that stimulate or permit cell regeneration are initiated there as well.

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