

Light Microscopic Evidence That Direct Transdifferentiation Gives Rise to New Hair Cells in Regenerating Avian Auditory Epithelium

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Hair cell regeneration in the avian inner ear is an increasingly well-characterized phenomenon. It is clear that proliferation of supporting cells and their post-mitotic differentiation into new hair cells is one mechanism for new hair cell production. It remains uncertain, however, whether all supporting cells are capable of giving rise to new hair cells and whether supporting cells may directly transdifferentiate into new hair cells without first dividing. To address these questions, we continuously infused a cell division marker, tritiated thymidine, into the labyrinth in animals that sustained near-total hair cell loss to the basal cochlea, and in age-matched controls. Nine- to thirteen-day old chicks were given a single injection of 250 mg/kg of gentamicin, which caused near-total hair cell loss in the basal 500 μm of the cochlea. Tritiated thymidine was then supplied continuously for five or twelve days prior to sacrifice, via an intralabyrinth cannula and an osmotic minipump. After autoradiographic processing of the cochlea, quantitative analyses demonstrated that: (1) the majority of supporting cells did not divide, even after near-total hair cell loss and (2) approximately one-third of the new hair cells were not labeled with tritiated thymidine. These results suggest that all supporting cells may not be competent to enter the mitotic cycle to become hair cell progenitors, and that direct transdifferentiation plays a significant role in hair cell replacement in the damaged chick cochlea.

Key words: Hair cell regeneration; ototoxicity; transdifferentiation; Avian; basilar papilla.

HAIR CELL REGENERATION IN the avian inner ear has now been extensively studied. It is known that there is ongoing production of new hair cells in the normal, undamaged vestibular epithelium and that the rate of production of new hair cells in the vestibular epithelium is upregulated following hair cell loss (Jørgensen and Mathiesen, 1988; Weisleder and Rubel, 1993). In the undamaged auditory epithelium there is an extremely low rate of supporting cell division and no significant production of new hair cells (Oesterle and Rubel, 1993). However, following hair cell loss, the auditory epithelium is capable of producing new hair cells to replace those lost (Cruz *et al.*, 1987; Cotanche, 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Lippe *et al.*, 1991). The new hair cells are at first recognizably immature; later, they differentiate to become indistinguishable from the embryonically produced mature hair cells at the light and electron microscopic levels (Cotanche, 1987; Duckert and Rubel, 1990, 1993; Cotanche and Corwin, 1991). Following aminoglycoside ototoxicity, the regenerated auditory epithelium ultimately has a full complement of mature hair cells in normal orientation and is nearly indistinguishable from undamaged epithelium (Girod *et al.*, 1991; Duckert and Rubel, 1993). The new hair cells have been shown to be functional and contribute to the recovery of behavioral responses to acoustic stimuli (Tucci and Rubel, 1990; Girod *et al.*, 1991; Marean *et al.*, 1994).

The details of the cellular processes that lead to regeneration of the inner ear epithelium are not well known. It has been demonstrated that the supporting cells in the sensory epithelium are a progenitor population for new hair cells in both the auditory and vestibular epithelium (Girod *et al.*, 1989; Tsue *et al.*, 1994). It is not known, however, whether all supporting cells are potential progenitor cells, or whether there are separate subpopulations of supporting cells, some with and some without the capacity to give rise to new hair cells.

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Many of the new hair cells arise via cell division. Supporting cells and possibly hyaline cells undergo mitosis in the first few days following hair cell loss, before new hair cells are present in the damaged region (Girod *et al.*, 1989; Stone and Cotanche, 1994). Some of these cells' daughter cells differentiate into mature hair cells, a process we will refer to as *post-mitotic differentiation* (see Discussion; Nomenclature). It is not known, however, whether some supporting cells in the avian basilar papilla differentiate into hair cells without undergoing mitosis, a process we will refer to as *direct transdifferentiation*.

The experiment presented in this report was designed to address two questions: (1) Are all or most supporting cells in the chick auditory sensory epithelium potential hair cell progenitors? (2) Do some new hair cells arise via direct transdifferentiation? To investigate whether all or the majority of supporting cells are potential hair cell progenitors, we developed an aminoglycoside damage protocol in which a single large injection of gentamicin is used to cause near-total hair cell loss within a specific region (the basal 500 μm) of the auditory epithelium (e.g., Janas *et al.*, 1995). Our assumption was that near-total hair cell loss would provide the maximal stimulus for potential progenitor cells to divide. In order to label all cells that divided following injury, we developed a technique to deliver the cell division marker, tritiated thymidine, continuously to the labyrinth for a prolonged period. By providing the maximal stimulus to new hair cell production, and continuously labeling dividing cells, we hoped to label *all* cells that had undergone mitosis between the time of aminoglycoside injection and sacrifice of the animal.

These same conditions (maximal hair cell loss and continuous presence of cell division label) make it possible to look for evidence of direct transdifferentiation. Since virtually all hair cells in the basal region were killed by the ototoxic agent, the great majority of hair cells present after recovery are new hair cells. Moreover, it is possible to distinguish mature from immature hair cells on the basis of previously defined morphologic criteria (Cotanche, 1987; Duckert and Rubel, 1990, 1993). We reasoned that a significant number of unlabeled new hair cells, despite the continuous presence of tritiated thymidine within the cochlea, would provide strong evidence that some hair cells arose via direct transdifferentiation.

MATERIALS AND METHODS

Nine- to thirteen-day old White Leghorn chicks (*Gallus domesticus*) were anesthetized with 120 mg/kg of intramuscular ketamine and 30 mg/kg of intraperitoneal sodium pentobarbital. In most cases, chicks were deprived of food and water for 1–6 hours preoperatively. Lidocaine was injected in the area of the skin incisions. The lateral ampulla was exposed via a post-auricular approach, a fistula was created in the ampulla with a 28 gauge needle, and a microcannula (Alzet Brain Infusion Kit, Alza Corporation, Palo

Alto, CA) was implanted in the fistula. The area around the catheter tip was gently packed with bone wax and the cannula then stabilized with dental acrylic. The cannula was attached to an osmotic infusion pump (Alzet osmotic pump, model 2002, Alza Corporation) which delivered tritiated thymidine (70–90 Ci/mmol, 1 $\mu\text{Ci}/\text{ml}$) at a rate of 0.5 $\mu\text{l}/\text{hour}$ for two weeks. The osmotic infusion pump was implanted in a subcutaneous pocket on the chick's back. The flexible catheter connecting the pump with the cannula was tunneled through the subcutaneous tissue of the neck. Skin wounds were sutured. This system was well tolerated in over sixty preliminary and experimental animals. Many chicks had a temporary mild head tilt, but most ate well and gained weight normally. Two chicks required sacrifice for intractable vertigo; these animals were excluded from the study.

Control Experiments

The following control experiments were performed to ensure that the pump-cannula system reliably delivered tritiated thymidine for the entire 12-day period between gentamicin injection and sacrifice.

Three control chicks had the pump-cannula system implanted and tritiated thymidine infused for 26 to 30 hours. They were then sacrificed. After autoradiographic processing, these sections were examined for tritiated thymidine labeling of cells directly beneath the basilar membrane (stromal cells). It was presumed that labeled stromal cells at this time would demonstrate the presence of sufficient amounts of tritiated thymidine within the cochlea to ensure supporting and hair cell labeling within 30 hours after pump placement, before the beginning of the experimental period.

One animal received a gentamicin injection 11 days after pump placement and was sacrificed three days later. Since sensory epithelial damage did not occur until 11 days after pump placement, it was presumed that the labeling of sensory epithelial cells would indicate adequate levels of tritiated thymidine during the final three days of the experimental period.

In addition, in all experimental and control groups, perilymph samples were taken from the majority of animals at the time of sacrifice and the presence of tritium confirmed by liquid scintillation counting. Perilymph was not sampled in six animals because of concern that this procedure might adversely affect the histology. Finally, in every section examined, stromal cells were examined for labeling to ensure that tritiated thymidine was present in that tissue during at least some part of the experimental period.

Experimental Groups

There were four experimental groups (Table 1). All chicks had pumps implanted and tritiated thymidine infused. Chicks in groups G-5 ($n = 8$) and G-12 ($n = 13$) received a single injection of gentamicin (250 mg/kg, subcutaneously) two days following the pump place-

TABLE 1
Experimental Groups and Experimental Design

All animals had pumps implanted on day 0. Experimental groups (G-5 and G-12) had gentamicin injections on day 2. Control groups (C-5 and C-12) did not receive gentamicin. Chicks were sacrificed either five days after gentamicin injection (G-5 and C-5) or twelve days after gentamicin injection (G-12 and C-12).

GROUP	G-5	C-5	G-12	C-12
DAY 0		Pump placed in all groups		
DAY 2	GENT	(CONTROL)	GENT	(CONTROL)
DAY 7	SACRIFICE	SACRIFICE		
DAY 14			SACRIFICE	SACRIFICE

ment. Chicks in groups C-5 ($n = 2$) and C-12 ($n = 4$) did not receive gentamicin. Animals were sacrificed at either 5 days after gentamicin injection (7 days after pump placement) to confirm the severity of the hair cell injury, or at 12 days after gentamicin injection (14 days after pump placement), to allow time for new hair cell production and the initial stages of differentiation to occur. The control groups (C-5 and C-12) were used to confirm that, without gentamicin treatment, no tritiated thymidine labeling of supporting or hair cells occurred.

After sacrifice by anesthetic overdose, animals were decapitated and the labyrinth perfused with 3.5% glutaraldehyde. The temporal portion of the skull was then rapidly removed and immersed in the same fixative. After overnight fixation, the membranous cochlea was removed, dehydrated in a graded series of methanols and acetones, embedded in Polybed media (Polysciences, Warrington, Pennsylvania), and serially sectioned at 3 μm in a basal-to-apical progression. Sections were mounted on acid-washed chrome-alum subbed microscope slides. The slides were dipped in Kodak NTB2 Nuclear Track emulsion (1:1 dilution) and allowed to expose at 4°C for 1–7 days. We found that after direct intralabyrinthine infusion of tritiated thymidine, short exposure times yielded robust labeling. The emulsion was developed in D-19 developer for 4 minutes, rinsed in distilled water, and fixed in Kodak fix for 3.5 minutes at 13°C. The slides were then washed, lightly counterstained with toluidine blue and coverslipped with DPX (Gallard-Schlesinger, Carle Place, New York).

Counting Criteria

All sections were examined from each cochlea and ten sections were quantitatively analyzed from each cochlea. Beginning with the basal-most section containing recognizable sensory epithelia, every 17th section was analyzed until 10 sections had been analyzed. The epithelium was thus sampled every 50 μm from 50 μm to 500 μm from the basal end. If a section was lost or uncountable due to artifact, the next serial section was used. A labeled nucleus was defined as one with five or more silver grains

directly above it. The distinction between labeled and unlabeled nuclei was straightforward due to the low background deposition in all sections analyzed. The following criteria were used for cell counts:

1. A cell was counted as present in a section only if its nucleus was clearly demarcated. Cells with only a small nuclear fragment were not counted.
2. Supporting cell nuclei were identified by their location on or near the basilar membrane, by the hypodense cytoplasm surrounding the nucleus and by the absence of morphologic features of hair cells.
3. A cell was counted as a hair cell if it contained a cuticular plate or stereocilia bundle. Cells without cuticular plate or stereocilia were also counted as hair cells if their nuclei were well above the supporting cell layer, and they had the characteristic morphology of new, immature hair cells, and their cytoplasm clearly contacted the luminal surface.
4. A small percentage (2.1%) of cells could not be clearly classified as either supporting cells or hair cells in the damaged epithelium. Almost all of these were cells whose nuclei were located midway between the luminal and the basal aspects of the epithelium and whose cytoplasm did not reach the luminal surface. They could not be counted as hair cells in the absence of distinct morphologic features of new, immature hair cells (see below) or if they did not reach the luminal surface. They could not be counted as supporting cells based on the location of their nuclei, which was well above the basilar membrane. These cells were counted as indeterminate cells; their numbers were too small to affect the results.
5. Mature and immature hair cells were distinguished based on morphologic criteria previously described (Cotanche, 1987; Duckert and Rubel, 1990, 1993). The basal end of the normal avian epithelium is populated almost exclusively by short hair cells which, when mature, have a characteristic "goblet" shape, very basophilic cytoplasm and nucleus, easily recognizable cuticular plate, and a stereocilia bundle with a regular, "stairstep" organization of stereocilia

(Figure 1a). Immature hair cells have a characteristic elongate, fusiform shape, and cytoplasmic and nuclear density intermediate between that of supporting cells and mature hair cells (Figure 1b–1d).

The counts from the ten sections selected from each cochlea were averaged and treated as a single number from each cochlea for statistical purposes. (It was initially planned to analyze the number of cell types as a function of their location along the cochlea. However, the average number of hair cells per section was so low in the damaged animals that analysis as a function of position did not appear to be meaningful.) Results of the counts were arbitrarily expressed as the number of a given cell type per section. Data were analyzed using the Kruskal-Wallis Test and the Mann-Whitney U test using Statview 4.1 (Abacus Concepts, Inc., Berkeley, CA).

All experimental methods and animal care procedures used in this investigation were approved by the University of Washington Animal Care Committee under NIH grant DC00395.

RESULTS

Control Experiments

In the cochleas from control chicks sacrificed 30 hours after pump-cannula implantation, numerous labeled stromal cells were seen throughout the cochleas. No labeling of supporting or hair cells was seen. This demonstrates that a level of tritiated thymidine high enough to label dividing cells was present within the cochleas well before the gentamicin injection at 48 hours.

In the chick which received the gentamicin injection 11 days after pump placement, there were early signs of damage to the sensory epithelium, consistent with recent ototoxic exposure. In this animal, numerous labeled supporting cells were seen. Since placement of the pump-cannula system alone did not result in any labeling of the epithelial cells in control animals (i.e., in groups C-5 and C-12), the labeling of these epithelial cells must have been due to the epithelial damage caused by the injection of gentamicin at day 11. The labeling of support cells, therefore, occurred during the last 2–3 days of the pump life, demonstrating that the pump-cannula system still provided adequate levels of biologically active tritiated thymidine within the cochlea at the end of the experimental period.

In every animal in which perilymph was sampled (21 of 27), the presence of tritium was confirmed by levels at least several times greater than background on scintillation counting. In every section examined in every experimental and control animal, numerous labeled stromal cells were seen throughout the non-sensory tissues of the cochleas, confirming levels of tritiated thymidine adequate to label dividing cells during some period of the experiment.

Animals Not Exposed to Gentamicin

Table 2 shows the average cell counts from all groups for both hair cells and supporting cells. For convenience, the animals which did not receive gentamicin are grouped on the left. In both of these two groups (group C-5 and group C-12), no labeling of sensory epithelial cells was seen. The counts of both hair cells and supporting cells were similar in the two groups.

TABLE 2
Cell Type Distribution

Data are presented as average number of cells/section, averaged over 10 sections from each animal (see text). Variability is indicated by the number following each mean (\pm) and is the SEM of the values between animals. Percentages reflect unrounded data and thus do not correspond exactly to raw data, which was rounded to nearest 0.1.

GROUP	C-5 (n = 2)	C-12 (n = 4)	G-5 (n = 8)	G-12 (n = 13)
Total SCs ¹	13.25 \pm 2.1	9.8 \pm 1.3	10.9 \pm 0.6	12.6 \pm 1.2
Labeled SCs	0	0	1.7 \pm 0.3 (16%)	1.9 \pm 0.4 (14%)
Unlabeled SCs	13.25 \pm 2.1 (100%)	9.8 \pm 1.3 (100%)	9.2 \pm 0.6 (84%)	10.7 \pm 1.2 (86%)
Total HCs	5.4 \pm 0.6	5.4 \pm 0.7	1.8 \pm 0.2	2.3 \pm 0.2
Labeled HCs	0	0	1.2 \pm 0.2 (64%)	1.2 \pm 0.2 (50%)
Unlabeled HCs	5.4 \pm 0.6 (100%)	5.4 \pm 0.7 (100%)	0.6 \pm 0.1 (36%)	1.1 \pm 0.2 (50%)
Immature HCs	0	0	1.7 \pm 0.2	1.6 \pm 0.2
Labeled IHCs	0	0	1.2 \pm 0.2 (68%)	1.1 \pm 0.2 (65%)
Unlabeled IHCs	0	0	0.5 \pm 0.1 (32%)	0.6 \pm 0.1 (35%)
Mature HCs	5.4 \pm 0.6	5.4 \pm 0.7	0.12 \pm 0.07	0.7 \pm 0.3
Labeled MHCs	0	0	0.01 \pm 0.01 (9%)	0.1 \pm 0.04 (14%)
Unlabeled MHCs	5.4 \pm 0.6 (100%)	5.4 \pm 0.7 (100%)	0.11 \pm 0.07 (91%)	0.6 \pm 0.2 (86%)

¹Abbreviations: SC = sensory epithelium supporting cell; HC = hair cell; IHC = immature hair cell; MHC = mature hair cell

Animals Sacrificed Five Days After Gentamicin Injection

Figure 1 shows representative sections through the basal end of the cochlea in a normal animal and in an animal treated with gentamicin. A graphic representation of the distribution of hair cell classifications is shown in Figure 2. In chicks sacrificed five days after gentamicin injection (Group G-5), near-total hair cell loss was seen in the basal 500 μm of the cochlea (Figure 1b–1c). An average of 1.8 hair cells/section were seen. Of these, only 0.12/section were mature, compared to an average of 5.4 mature hair cells/section in the undamaged cochleas

(Table 2). This suggests the gentamicin insult caused the death of 98% of the hair cells in the basal 500 μm of the cochlear epithelium.

The average total supporting cell number seen in group G-5 (10.9/section) was not significantly different from the undamaged animals (13.25 in group C-5 and 9.8 in group C-12). Tritiated thymidine labeling was seen in only 16% of the supporting cells.

Of the immature hair cells, 1.2/section (68%) were labeled, implying that they arose via post-mitotic differentiation and 0.5/section (32%) were unlabeled, implying that they arose via direct transdifferentiation.

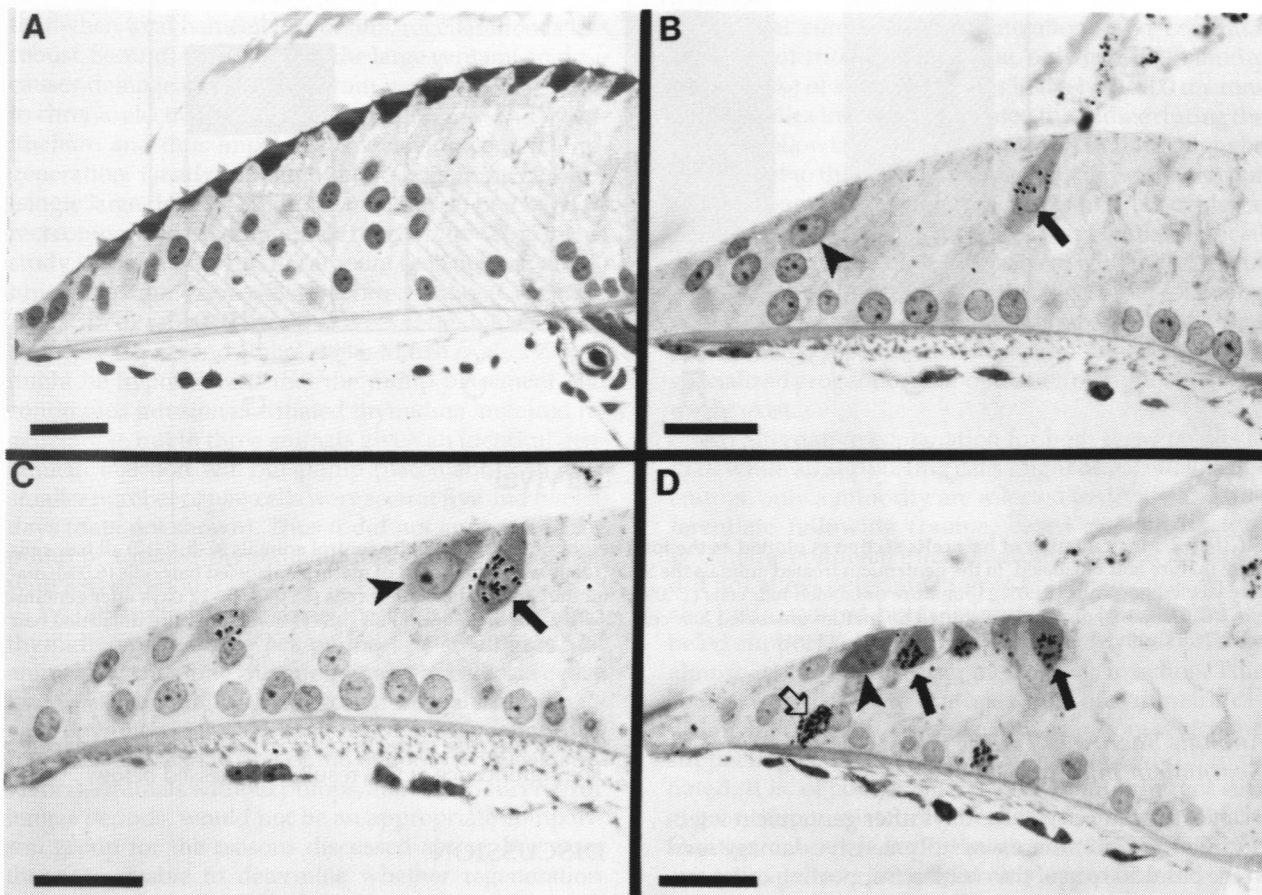


FIGURE 1 A. Tissue section through normal chick cochlea, ~200 μm from the basal end. The basal end is populated almost entirely with short hair cells, which have a characteristic goblet shape and very dense cytoplasm and nucleus. B. Section through a chick cochlea, 144 μm from the basal end of the sensory epithelium, 5 days after gentamicin injection. Two regenerating hair cells are seen, one labeled (long arrow) and one unlabeled (arrowhead). Both have the elongate morphology and intermediate density nucleus and cytoplasm characteristic of regenerating hair cells. C. Section through a chick cochlea 180 μm from the basal end of the sensory epithelium, 5 days after gentamicin injection. As in B, two characteristic regenerating hair cells are seen, one labeled (long arrow) and one unlabeled (arrowhead). At the abneural aspect of the epithelium, another labeled cell is visible. This cell is most likely a regenerating hair cell, but since its nucleus was not centrally sectioned (only a fragment of the nucleus is visible on the section), it was not counted as present on this section (see counting criteria in the text). D. Section through a chick cochlea, 210 μm from the basal end of the sensory epithelium, 12 days after gentamicin injection. Two supporting cells are labeled (open arrow). Three cells are present with the characteristic morphology of regenerating hair cells; two labeled (long arrows) and one unlabeled (arrowhead). Between the two labeled regenerating hair cells are two cellular fragments; both are most likely regenerating, labeled hair cells (note that both have small numbers of silver grains overlying) but neither could be counted because their nuclei were not centrally sectioned. Calibration bars = 20 micrometers.

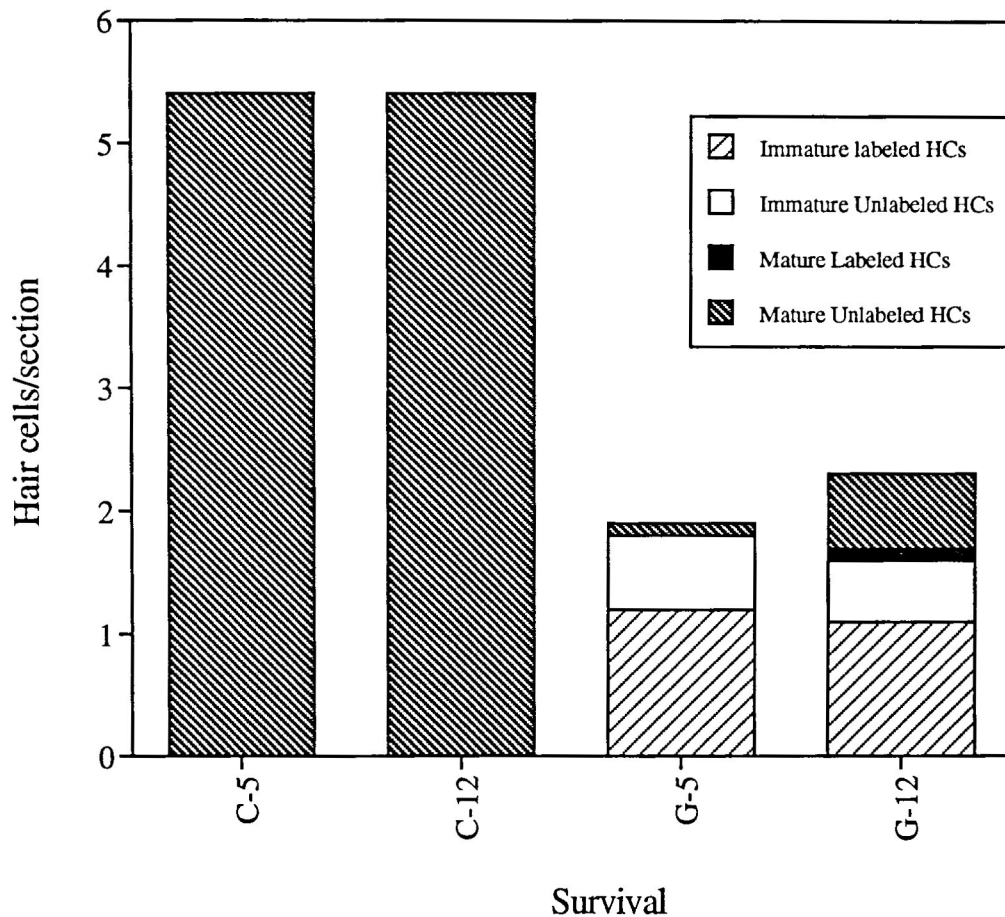


FIGURE 2 Mean number of hair cells/section is plotted as the total height of each bar. In the control animals (C-5, C-12) all hair cells were mature and unlabeled. In the gentamicin treated animals the 5 day totals were composed of mature unlabeled hair cells (6.0%); mature labeled hair cells (<1.0%); immature unlabeled hair cells (27.0%); and immature labeled hair cells (66.0%). By 12 days after gentamicin treatment this distribution shifted to: mature unlabeled hair cells (26.0%); mature labeled hair cells (4.0%); immature unlabeled hair cells (26.0%); and immature labeled hair cells (47.0%).

Animals Sacrificed Twelve Days After Gentamicin Injection

In chicks sacrificed twelve days after gentamicin injection (Group G-12), there was still extensive damage seen in the basal 500 μm of the cochlea. Supporting cell number (12.6/section) was similar to all other groups, and only 14% of supporting cells were labeled. Hair cell number had increased to an average of 2.3/section, of which 1.6 (70%) were immature. Of the immature hair cells, 0.5/section (35%) were unlabeled (Figure 1d).

The most striking result in this group was the much greater number of unlabeled mature hair cells compared to the animals sacrificed five days after gentamicin (0.6/section vs. 0.1/section). The difference in the number of unlabeled mature and total mature hair cells in the G-5 versus G-12 groups approached statistical significance ($p < 0.06$). This is particularly striking in that there was no

similar increase in the number of mature *labeled* hair cells. The significance of this result is addressed below.

DISCUSSION

Regeneration After Near-Total Hair Cell Loss

A model in which nearly all hair cells were killed by a single ototoxic insult was selected for this experiment for two reasons. It was felt that near-total hair cell loss would provide the maximal stimulus for supporting cells to divide and differentiate, thus enabling us to identify the maximum number of potential progenitor cells. Secondly, it would be impossible to identify hair cells which arose via direct transdifferentiation unless all pre-existing unlabeled cells were eliminated. We chose the basal 500 microns of the cochlea because pre-

vious work (Janas *et al.*, 1995) had shown that this area could be completely denuded of hair cells with a single high dose of gentamicin.

Janas *et al.*, (1995) have recently reported complete restoration of hair cell numbers by two weeks after a smaller (100mg/kg vs 250mg/kg) dose of gentamicin. Duckert and Rubel (1993) report return to the full complement of hair cells at 6 weeks after a 10 day course of gentamicin at 50 mg/kg/day. In the present study hair cell number recovered only to 33% of normal at five days and 43% of normal at twelve days. The reasons for the less than complete regeneration at this time in this damage paradigm are unclear, but probably involve multiple factors. First, it has been hypothesized that remaining hair cells serve as a structural aid in regeneration, and that when total hair cell loss occurs, regeneration is less robust. Second, it may be that the large gentamicin dose causes damage to the tegmentum vasculosum, leading to chronic electrolyte imbalances within the sensory epithelium and thus impairing or delaying complete regeneration. Finally, although differences in procedures (single large dose vs multiple small doses) preclude direct comparisons, the amount of regeneration seen in this study is consistent with the amount seen at comparable times after aminoglycosides, reported earlier (Cruz *et al.*, 1987; Girod *et al.*, 1991) or at 1–3 weeks after intense noise exposure (Ryals and Rubel, 1988; Marsh *et al.*, 1990). It might be hypothesized that the pump placement and continuous infusion of tritiated thymidine impaired regeneration, but in three animals given an identical gentamicin injection without pump placement, a slightly smaller number of hair cells were seen at five and twelve days (data not shown). Thus it did not appear that the pump or tritiated thymidine infusion were interfering with regeneration.

We attempted to maintain the infusion of tritiated thymidine over a six week period in a small group of animals by changing the pump under anesthesia every two weeks, but all tissue harvested from these animals was obviously infected and histologically abnormal (this was not seen in any of the one- or two-week survivors). Animals without pumps, allowed to survive for longer periods, would not be an appropriate comparison group for the reasons discussed above. We were therefore unable to determine whether regeneration would have been more complete had a longer time period elapsed.

Our difficulties in establishing an "ideal" damage paradigm for this experiment—that is, one that caused complete, simultaneous hair cell loss and yet allowed complete regeneration during the period of thymidine infusion—illustrate that the process of hair cell regeneration is not identical following every insult, and may proceed differently following different types of damage. The incomplete regeneration seen does not alter the compelling evidence that direct transdifferentiation played a large role in producing new hair cells in this

model. However, it could be a partial explanation for the paucity of labeled supporting cells seen (see below).

Are All Supporting Cells Potential Progenitor Cells?

While avian auditory and vestibular epithelial regeneration has become progressively better described since the first reports (Cruz *et al.*, 1987; Cotanche, 1987; Jørgensen and Mathiesen, 1988), much remains unknown about the cellular events which induce and regulate the processes by which supporting cells give rise to new hair cells. It is clear that some supporting cells are progenitors to new hair cells, but it is not certain whether *all* cells in the supporting cell layer are potential progenitors. Our results show that, following near-total hair cell loss (presumably a maximal stimulus for regeneration), and continual presence of tritiated thymidine, only a small minority (about 15%) of supporting cells in the basal 500 microns of the cochlea incorporate tritiated thymidine during the 12 days following damage. While this finding might be due in part to the incomplete regeneration seen with our damage paradigm, it also represents indirect evidence that all supporting cells may not be potential hair cell progenitors. This would be consistent with the results of Stone and Cotanche (1994), who suggest that following noise-induced damage a limited number of supporting cells undergo repeated divisions, also suggesting that a specialized progenitor subpopulation of supporting cells might exist.

An alternative explanation for both these results is that, while all supporting cells might be *potential* progenitors, only a minority are selected to divide and differentiate following trauma, based on local factors within the epithelial microenvironment. Our methods did not allow determination of how many mitotic cycles had occurred but it is interesting to note that labeled support cells outnumbered labeled hair cells by almost 2:1 by 12 days after the ototoxic injection. This could mean that there was one round of symmetric division, followed by a round of division from which the supporting cell was renewed and a hair cell differentiated. It is, of course, also possible that different sets of progenitors exist for hair cells and supporting cells. In either case, it is important to note that at the time our animals were sacrificed only approximately 43% of the original hair cell number were present. Previous studies using noise or aminoglycoside ototoxicity suggest that eventually the full complement of hair cells will be restored. Thus additional hair cells must be added by one of three processes: additional rounds of mitosis by the precursors identified in this study, proliferation of a new subset of support cells, or delayed differentiation of either labeled or unlabeled support cells. It will be important to determine the relative contributions of each of these processes by combining pulse labeling and cell counting methods at longer survival times.

Direct Transdifferentiation

Although cell division, as identified by a marker of DNA synthesis, clearly plays a major role in the production of new hair cells, it is not known with certainty whether cell division is a necessary event before a supporting cell can give rise to a new hair cell. Unlabeled hair cells in areas of regeneration have been noted in many previous experiments (e.g., Corwin and Cotanche, 1988; Girod *et al.*, 1989), suggesting that in some cases supporting cells may give rise to new hair cells without first dividing. However, most previous *in vivo* work has utilized intermittent injections of DNA synthesis markers. Since the biological half-life of these markers is probably only a few hours in most systems, intermittent injections probably do not maintain a continuous level of label within the inner ear. In these studies, therefore, unlabeled new hair cells could be cells which arose via direct transdifferentiation, but could also be hair cells which arose via mitotic activity and cell division which occurred while insufficient label was present.

Our results provide strong evidence that direct transdifferentiation is one pathway for new hair cell production. Despite the continuous presence of tritiated thymidine in the labyrinth, about one third of all morphologically immature hair cells were unlabeled, implying that they arose through a process not involving DNA synthesis. The presence of tritiated thymidine from the beginning to the end of the experimental period was confirmed by control experiments, including one documenting its biological activity at the end of the two-week experimental period. Furthermore, numerous labeled epithelial cells were seen in every damaged animal, so there is no reason to believe that tritiated thymidine was not able to diffuse freely throughout the epithelium. In many cases, unlabeled immature hair cells were seen in sections side-by-side with heavily labeled immature hair cells at apparently identical stages of maturation, making it improbable that there were temporal gaps in the presence of tritiated thymidine which would explain why some new hair cells were not labeled. Finally, this was a very consistent result—at least one unlabeled immature hair cell was seen in every damaged animal examined (a total of 21 chicks). It should be kept in mind that this report assessed new hair cells in only the proximal (basal) 500 μ m of the cochlea, which is populated mostly by short hair cells in most avian species (e.g., Takasaka and Smith, 1970; Tanaka and Smith, 1975). While we observed unlabeled tall and short hair cells (both immature and mature) in this region, it is entirely possible that at more distal locations the relative numbers of hair cells replaced by mitotic vs non-mitotic processes may be altered.

An unexpected observation was that the number of mature unlabeled hair cells at twelve days was much larger than at five days while the number of mature la-

beled cells did not show a similar change. This could suggest that those hair cells which arise via direct transdifferentiation may mature more rapidly than those which arise via proliferation and post-mitotic differentiation. However, our data are too variable to allow any strong conclusion to be drawn.

A number of other recent reports have suggested the presence of an alternate pathway for hair cell regeneration, not requiring mitosis. Balak *et al.* (1990) showed that under some conditions this process is responsible for replacing lateral line hair cells. Baird *et al.* (1995) have presented a series of experiments suggesting that direct transdifferentiation may occur in an amphibian species, and Adler and Raphael (1995) have presented qualitative evidence for direct transdifferentiation in avian auditory epithelia. Recent work in the mammalian vestibular epithelia (Rubel *et al.*, 1995), although inconclusive, has yielded indirect evidence that direct transdifferentiation may be responsible for the limited regeneration seen in that system by Forge *et al.* (1994).

These data are consistent with data from a number of other biological systems. Although it has traditionally been believed that most or all regeneration of damaged tissues occurred via the proliferation and differentiation of a latent, undifferentiated stem cell population, a number of workers in the past two decades have shown that in many systems mature cells can change their phenotype, often in response to damage (Eguchi and Kodama, 1993). In most cases the mature cells undergo mitosis before redifferentiating into a new phenotype, but in a number of systems there is now convincing evidence that they may do so without first dividing (Beresford, 1990).

The possibility of an alternate, non-mitotic pathway for hair cell production should be considered in the interpretation of many previous and future results in the field. Several studies have shown an alteration in the number of new hair or supporting cells due to various known or unknown diffusible factors (Tsue *et al.*, 1994; Lambert, 1994; Yamashita and Oesterle, 1995). It is unclear, however, if the rate of direct transdifferentiation would be altered by the same factors which alter the rate of proliferation, and most studies have not been designed to address this issue. Experiments designed to determine the identity of factors which regulate hair cell regeneration must be interpreted in light of the possibility that two different pathways, possibly regulated by entirely different factors, are responsible for hair cell regeneration.

Nomenclature

At present, there is no general agreement on the naming of cellular processes involved in generating new hair cells or of the cells involved. In selecting the terms used in this paper, we reviewed the biological literature and

the dictionary definitions of the terms used and attempted to use a nomenclature that is technically correct, intuitively understandable and as compatible as possible with previously used nomenclature.

Regeneration. Regeneration describes "the natural renewal of a structure, as of a lost tissue or part" (Dorland's Illustrated Medical Dictionary, 1981). While it is technically correct to speak of regeneration of a single cell, the term is more commonly used to describe replacement of a tissue or structure. We have used the phrase *hair cell regeneration* to describe the entire set of events leading to the reconstitution of a mature-appearing, functional sensory epithelium following damage. When discussing specific mechanisms by which individual new hair cells arise, we have used more specific terms (see below).

Proliferation and post-mitotic differentiation. Following hair cell loss, some supporting cells divide, presumably undergoing de-differentiation prior to mitosis (Figure 3a). There may be multiple cycles of cell division prior to daughter cells differentiating into mature hair cells or supporting cells (Balak *et al.*, 1990; Stone and Cotanche, 1994). This process has been called *regeneration* by ourselves and others, and *transdifferentiation* by Raphael *et al.* (1994). *Regeneration*, as discussed above, is better used to describe events at the tissue level rather than the cellular level. *Transdifferentiation* is used in the general biological literature to describe both mitotic and non-mitotic pathways (Beresford, 1990; Eguchi and Kodama, 1993). We therefore chose the terms *proliferation* to describe supporting cell division and *post-mitotic differentiation* for the processes by which daughter cells become new mature hair cells or supporting cells. This terminology

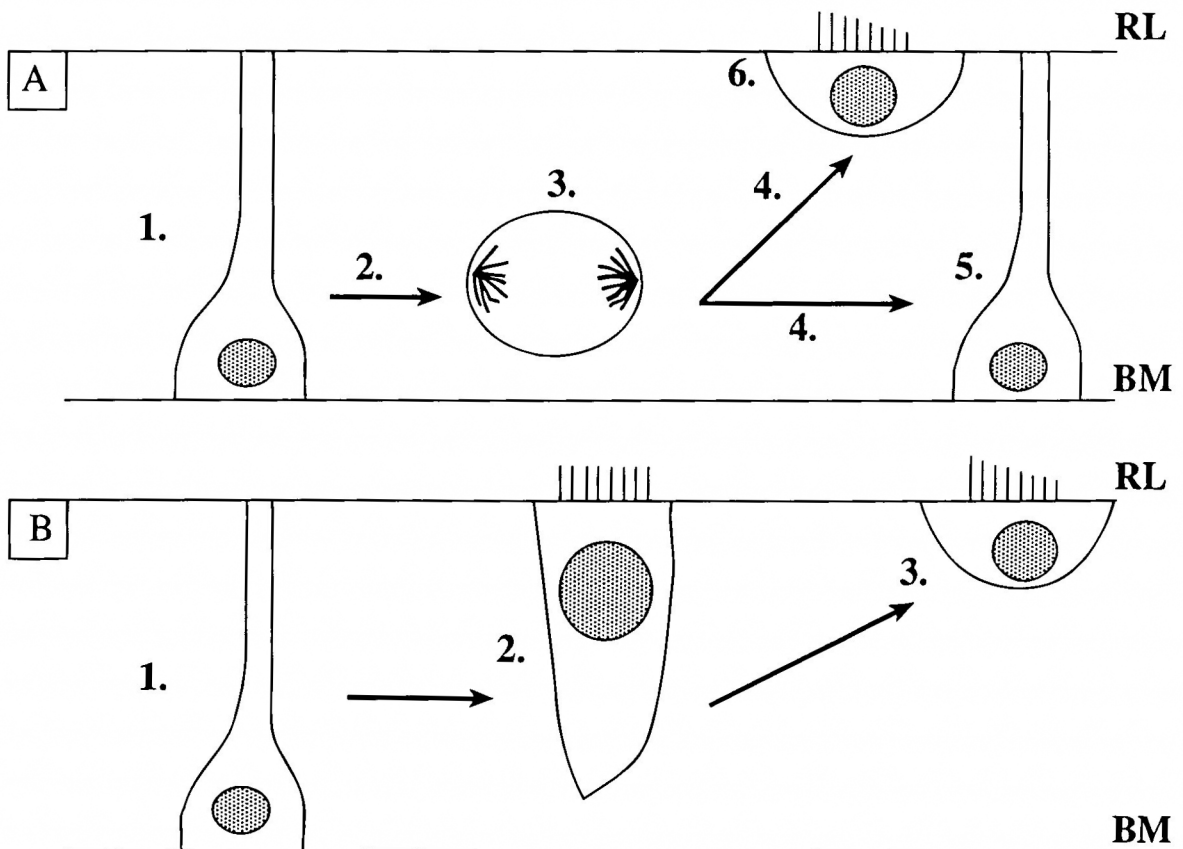


FIGURE 3 Proposed mechanisms of hair cell regeneration and nomenclature. (A) Supporting cell (1) undergoes de-differentiation (2) and proliferation (3), leading to two daughter cells. Daughter cells may undergo post-mitotic differentiation (4) to become mature supporting cells or hair cells (5,6). Only one division is shown for clarity, but there is evidence that there may be repeated cycles of division prior to differentiation (Balak *et al.*, 1990; Stone and Cotanche, 1994). Likewise, for simplicity, an asymmetric division is shown yielding one supporting cell and one hair cell; there is no experimental evidence that indicates this would necessarily be the case. Raphael *et al.* (1994) have presented evidence that, in at least some cases, a single division may yield two daughter cells that both become hair cells. RL = reticular lamina; BM = basement membrane.

(B) Supporting cell (1) becomes a mature hair cell (3) via direct transdifferentiation, transiently having a characteristic immature morphology (2).

follows that of Cotanche and Lee (1994), who used the terms *proliferation*, *differentiation* and *maturation*.

Direct transdifferentiation. The process by which supporting cells become new hair cells without first dividing (Figure 3b) has been described by ourselves and Baird *et al.* (1995) as *transdifferentiation*, and by Adler and Raphael (1995) as *conversion*. As mentioned above, transdifferentiation is used in the biological literature to describe both mitotic and non-mitotic pathways (Beresford, 1990; Eguchi and Kodama, 1993). *Conversion* has acquired numerous specific meanings in a variety of biological fields and, like transdifferentiation, is not generally understood to specifically refer to a non-mitotic process. We therefore adopted from Beresford (1990) the term *direct transdifferentiation* to describe hair cell production *without mitosis*.

Precursor and progenitor. *Precursor* refers to an entity which precedes another in time or space, but not in ancestry; it is also used to describe something (e.g., a chemical compound) which changes its form to become something else (Webster's Dictionary, 1993). *Precursor*, in the sense of a compound which precedes another in a reaction, is the more appropriate term to describe supporting cells which alter their differentiated state without dividing. *Progenitor* describes a parent or other ancestor (Webster's Dictionary, 1993). *Progenitor* is thus the more appropriate term to describe supporting cells which give rise to new hair cells via division and post-mitotic differentiation.

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