Hair cell replacement in avian vestibular epithelium: Supporting cell to Type I hair cell

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Received 29 April 1994; revised 30 September 1994; accepted 12 October 1994

Abstract

Previous investigations have demonstrated that the sensory epithelium of the avian vestibular system possesses the capacity to replace hair cells both on an ongoing basis and following severe damage. Supporting cells, within the sensory epithelium, are believed to be the progenitors of the regenerated hair cells. In the present study we describe the series of events leading to the formation of a regenerated vestibular hair cell in post-hatched birds. Young chickens received injections of streptomycin sulfate in order to damage the sensory epithelium of the vestibular system. These injections were followed by injections of the cell proliferation marker thymidine. At predetermined intervals, the animals were killed, and the vestibular organs were processed for autoradiography. Our results confirm that hair cells originate from supporting cells. The data also indicate that postmitotic cells migrate towards the lumen of the epithelium where they differentiate into Type II hair cells. At a later time, some of the newly formed hair cells further differentiate into Type I hair cells. These results suggest that both types of avian vestibular hair cells have a common ancestor. The data also provide evidence in support of the hypothesis that calyx enclosed Type I hair cells, only present in birds and mammals, are a more differentiated stage of Type II hair cells.

Keywords: Vestibular system; Chicken; Inner ear; Regeneration

1. Introduction

Research into the regenerative capacity of vertebrate sensory systems has demonstrated that new post-embryonic sensory cells are derived from populations of less differentiated cells from within the sensory epithelium. In the olfactory system of amphibians and mammals, undifferentiated basal cells give rise to new neuroepithelial cells (Graziadei, 1973; Graziadei and Monti Graziadei, 1985; Constanzo and Graziadei, 1987). In the gustatory epithelium, stem and suprabasal cells respond to the signals from sensory axons and produce new taste cells (Oakley, 1991). In the visual system of fish, populations of rod precursors give rise to new rod photoreceptors in the intact adult retina. In the damaged retina, rod precursors stop producing rods and give rise to neuroepithelial cells which will, in turn, be the progenitors of all other retinal cell types (Johns and Fernald, 1981; Raymond et al., 1988; Raymond, 1991). Studies of the lateral line organs of amphibians indicate that supporting cells in the sensory epithelium are the progenitors of the regenerated hair cells (Corwin, 1986; Balak et al., 1990; Jones and Corwin, 1993). In electroreceptor organs of weakly-electric fish, Zakon (1991) reported that basal cells give rise to new organs during regeneration. In the vestibular sensory epithelium of teleosts, Presson and Popper (1990) suggested that a population of embryonic-like neuroepithelial cells are the source of new hair cells and supporting cells. Finally, Girod et al. (1989), Oesterle et al. (1992), Raphael (1992), Hashino and Salvi (1993), and Stone and Cotanche (1994) have shown that the precursors of regenerated hair cells in the avian basilar papilla are less specialized cells from within the papilla.

The capacity of the avian vestibular sensory epithelium to produce hair cells on an ongoing basis (Jørgen-
sen and Mathiesen, 1988; Roberson et al., 1992), and to repair itself following aminoglycoside toxicity (Weisleder and Rubel, 1992, 1993) has been well documented. Repaired vestibular sensory epithelia have been shown to contain hair cells and supporting cells labeled by cell proliferation markers, indicating that these cells originated from post-embryonic (and post-injury) cell divisions. Utilizing two different cell proliferation marker techniques, tritiated-thymidine autoradiography and proliferating cell nuclear antigen immunocytochemistry, Tsue et al. (1994) recently demonstrated that cells within the supporting cell layer are those which proliferate, thus pointing to the supporting cells as precursors of regenerated hair cells. In order to trace the fate of the precursor cells identified by Tsue et al., as hair cell precursors, streptomycin-treated birds were given injections of tritiated-thymidine. Following the radionucleotide injections, animals were allowed to survive for one of the following times: 1, 3, or 12 h, or 5, 10, 20, or 60 days. The vestibular organs of these birds were harvested and processed for autoradiography. Our results confirm that supporting cells are progenitors of regenerated hair cells. In addition, we provide evidence to support the hypothesis that the cellular events leading to an anatomically intact vestibular sensory epithelium in an aminoglycoside-damaged bird are: supporting cell → Type II hair cell → Type I hair cell.

2. Material and methods

2.1. Short term survival

To determine which cell types proliferate in response to damage, 1-week old chicks (Gallus domesticus) received injections of 600 mg/kg streptomycin sulfate for either 5 or 2 days (Table 1). Twenty-four hours after the last aminoglycoside injection, streptomycin-treated animals and an additional control bird at each survival time were injected once with the cell proliferation marker tritiated-thymidine (10 μCi/g body weight). In groups of three (two streptomycin-treated and one control), animals were sacrificed 1 h, 3 h, 12 h, 5 days, or 10 days after the proliferation marker injection. Twelve hours after the injection of tritiated-thymidine, all birds in the 5- and 10-day survival groups received an injection of concentrated ‘cold’ thymidine (100 × the amount of tritiated-thymidine) every 12 h for the next three days. This pulse-chase procedure was done to make it more likely for cells proliferating after the first 12-h period to incorporate non-radioactive thymidine than residual tritiatedthymidine into their nuclear DNA. Thus, labeled cells seen after the five or ten days were considered to be derived from cells that entered s-phase during the 12 h following the 3H-thymidine injection.

2.2. Long term survival

To assess the long term fate of cells that proliferate soon after aminoglycoside damage four 2-week old chickens received daily intramuscular injections of streptomycin sulfate (600 mg/kg) for seven days. Starting on the fifth day of streptomycin injections, the birds received twice-daily injections of tritiated-thymidine (10 μCi/g body weight) for three days. The animals were then allowed to survive twenty or sixty days (N = 2/survival time).

At the appropriate times, all animals were over-anesthetized with Nembutal, perfused transcardially with 0.9% saline solution containing 20,000 IU heparin/L, and fixed with 3.6% paraformaldehyde/0.3% glutaraldehyde fixative in 0.1 M phosphate buffer (pH 7.4). The vestibular organs were dissected, post-fixed in osmium (1% OsO4 in 0.1 M NaPO4 buffer) for 1 h, and embedded in Spurr’s plastic. The tissue was sectioned at 2.5–3 μm, and processed for autoradiography (Sidman, 1970).

2.3. Quantitative analyses

Superior cristae ampullaris of two experimental animals and one age-matched control animal at each survival time were chosen for quantitative analyses. To assess the relative numbers of Type I hair cells, Type II hair cells, and supporting cells in this tissue, an alternating series of 40 tissue sections beginning at the lateral edge of the crista was analyzed. This sampling allowed us to systematically examine approximately 50% of the length of the sensory epithelium since the two halves of each organ are separated by the septum cruciatum which is free of sensory cells.

The sensory epithelium at each section was analyzed with a computer-aided image analysis system which included a DAGE Model 72 video camera, Macintosh IIx computer, Data Translation frame grabber board, and NIH Image software. The sensory epithelium from each tissue section was digitized using a 100 × planapochromatic objective, and the depth of the sensory epithelium was trisected into 3 equivalent zones.

Table 1

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Streptomycin injections</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5 days</td>
<td>1 h</td>
</tr>
<tr>
<td>2</td>
<td>5 days</td>
<td>3 h</td>
</tr>
<tr>
<td>2</td>
<td>2 days</td>
<td>12 h</td>
</tr>
<tr>
<td>2</td>
<td>2 days</td>
<td>5 days</td>
</tr>
<tr>
<td>2</td>
<td>2 days</td>
<td>10 days</td>
</tr>
</tbody>
</table>
The basal third contains nuclei of supporting cells; the middle third contains nuclei of hair cells and an occasional proliferative cell involved in nuclear interkinetic migration; the lumenal third contains apical processes of supporting and sensory cells, and in regenerating tissue, mitotic nuclei (Tsue et al., 1994).

Finally, in animals allowed to survive for 60 days we compared grain density of labeled supporting cells and Type I and Type II hair cells. The area occupied by silver grains was estimated in 180 cells, 60 cells for each cell type, in ampullae of two streptomycin-injected animals. Density estimations were made with the aid of the digital image processing and analysis software package NIH Image. A threshold of 200 on the 256-point gray scale was established. We then encircled the contour of the nucleus of cells that were identified as labeled by light microscopy, and had the computer calculate the area occupied by particles darker than the set threshold.

The care and use of animals reported on in this study were approved by the University of Washington Animal Care and Use Committee, approved by the NIH (grant No. DC00395: Ontogeny of Vertebrate Sensory Processes), and in strict accordance with the guidelines of the Declaration of Helsinki.

3. Results

3.1. Streptomycin-damaged tissue: short term survival

Fig. 1 shows a low power transmission electron micrograph section through a superior crista ampullaris of an untreated bird. The thickness of the

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Fig. 1. Low magnification transmission electron micrograph illustrates the organization of the avian vestibular sensory epithelium. The curved arrows delineate three equivalent sectors that were examined in this investigation. Located in the lower one third of the epithelium, adjacent to the basement membrane (BM), are the nuclei of the supporting cells (open arrows). The nuclei of Type I hair cells (arrowheads), and Type II hair cells (arrows), are situated in the middle one third of the epithelium. The luminal one third of the epithelium is occupied by the apical cytoplasm of all 3 cell types indigenous to the parenchyma. Cross-section of superior crista ampullaris. Bar represents 10 μm.
Table 2
Percentages of labeled cells/region in superior cristae ampullaris of short term survival streptomycin-injected birds and total counts of labeled cells in control animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Labeled cells N</th>
<th>Basal third third</th>
<th>Middle third</th>
<th>Lumenal third</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>41</td>
<td>90.2%</td>
<td>9.8%</td>
<td>-</td>
<td>85.7% (12)</td>
</tr>
<tr>
<td>3 h</td>
<td>43</td>
<td>72.1%</td>
<td>25.5%</td>
<td>2.4%</td>
<td>70% (7)</td>
</tr>
<tr>
<td>12 h</td>
<td>53</td>
<td>64.2%</td>
<td>22.6%</td>
<td>13.2%</td>
<td>71.4% (5)</td>
</tr>
<tr>
<td>5 days</td>
<td>111</td>
<td>45.1%</td>
<td>54.9%</td>
<td>-</td>
<td>81.8% (9)</td>
</tr>
<tr>
<td>10 days</td>
<td>119</td>
<td>45.3%</td>
<td>54.7%</td>
<td>-</td>
<td>54.5% (6)</td>
</tr>
</tbody>
</table>

(≈ 50% of the length of the sensory epithelium was measured).

Fig. 2. Photomicrographs of the crista ampullaris from animals at different stages of hair cell regeneration. A) In the early stages of regeneration (3 h of survival), supporting cells along the basement membrane are labeled (dotted arrows). B) As time progresses (1 day), the nucleus of proliferating supporting cells migrates towards the middle third of the epithelium (curved arrows), and at this location, or above, the cell divides. C) In the early phases of hair cell differentiation (20 days), Type II hair cells are the hair-cell Type most frequently seen (open arrow). D) At longer time intervals (e.g., 60 days), Type I (solid arrow) and Type II hair cells are labeled. Bar in D represents 20 μm for all panels.
Avian vestibular sensory epithelium can be divided into three equivalent parts. Supporting cells nuclei are found on the lower third, hair cells nuclei are located in the middle third, and cell bodies of supporting cells and hair cells compose the uppermost third. Labeled cells were assigned a position in the sensory epithelium based on the aforementioned division. Table 2 presents the percentages of labeled cells, according to position in the vestibular epithelium of streptomycin-treated and control animals at survival periods ranging from 1 h to 10 days after the single tritiated-thymidine injection. Given that the number of labeled cells in control animals was very small, total counts of labeled cells, in addition to percentages, are presented in Table 2. Fig. 2 (A and B) presents photomicrographs of representative sections showing labeling patterns at 3 h and 1 day survival times.

One hour after the cell proliferation marker injection, the majority of labeled cells in ampullae of both treated and control animals are located in the basal third of the sensory epithelium. These labeled nuclei are located just above or within one nuclear diameter of the basement membrane. Three hours after the thymidine injection, the majority of labeled nuclei are still located next to the basement membrane as shown in Fig. 2A, but the number of labeled cells in the middle third has increased (Table 2). By 12 h, the ratio of labeled cells in the basal third to labeled cells in the middle third was 3:1, as contrasted with the observation made at 1 h when the ratio was 9:1. Finally, in tissue from animals allowed to survive five or ten days the relationship changes, slightly more labeled cells are seen in the mid-portions of the sensory epithelium than in the lower portion.

At all survival times, the tissue of control birds contains fewer labeled cells than that of streptomycin-injected animals (Table 2). As previously demonstrated (Weisleder and Rubel, 1993; Tsue et al., 1994), this disparity is a consequence of the increased mitotic activity in the damaged epithelia. Independent of the smaller number of labeled cells, however, the same trend seen in treated animals is repeated in control animals: initially the majority of labeled nuclei are found in the basal third of the epithelium; as survival time increases more labeled nuclei are found in the mid-portion of the epithelium.

Ampullae of treated animals in the 3- and 12-h survival groups display an event not seen in the tissue of other treated animals nor in any control bird. Namely, a small percentage of labeled nuclei are found in the luminal third of the epithelium. As can be appreciated from Fig. 1, the luminal third of the intact sensory epithelium is occupied by cell bodies only, cell nuclei are restricted to the lower two-thirds. Qualitative observations of the labeled nuclei found in the upper third of the epithelium revealed that they bear fewer silver grains than those of cells in the lower portions of the epithelium, and that the label is not a well-organized aggregate on top of the nuclei. While it is possible that some of those labeled nuclei belong to damaged cells being extruded (Balak et al., 1990; Cotanche and Dopyera, 1990), it is more likely that they belong to cells that have migrated to the luminal surface and are at the early stages of mitosis (Tsue et al., 1994). This conclusion is supported by the work of Stone and Cotanche (1994) who found that by 6–12 h after noise damage, mitotic figures or pairs of labeled cells can be seen in the avian cochlea.

Labeled cells in the tissue from 5 and 10 day survival animals are of two kinds: supporting cells and Type II hair cells. Labeled supporting cells are easily identified by the fact that their nucleus is located next to the basement membrane. The nucleus of cells identified as Type II hair cells is found in the mid-portions of the epithelium, and the cells contain identifiable cuticular plate and stereocilia bundle. These cells were classified as Type II because they are cylindrically-shaped and are not enclosed by a nerve calyx. Labeled, nerve-enclosed hair cells (Type I) were not found in the tissue from the 5 or 10 day survival animals. No labeled Type I hair cells were seen in either normal or aminoglycoside-treated animals that survived five or ten days. Weisleder and Rubel (1993) also reported that the first hair cells found in the regenerating epithelia of streptomycin treated chickens meet the criteria of Type II hair cells.

3.2. Streptomycin damaged tissue: long term survival

In ampullae obtained from animals allowed to survive twenty days, labeled nuclei are found in the lower portions of the epithelium and in the middle portion of the sensory epithelium. Most of these nuclei belong to cells that can be identified as Type II hair cells, although a few of the nuclei belong to cells that can be recognized as Type I hair cells. By sixty days, the tissue of streptomycin-treated animals has an abundance of labeled nuclei in both the lower and middle thirds of the sensory epithelium. Supporting cells, Type II hair cells, and Type I hair cells bear labeled nuclei.

To examine cellular dynamics in the vestibular parenchyma, we counted the number of labeled supporting cells, and Type I and Type II hair cells in ampullae of streptomycin-injected animals from the twenty- and sixty-day survival groups. Separate counts by hair cell type were not done at earlier survival times because, as stated above, all identifiable hair cells had characteristics of Type II. Individual data of four streptomycin-treated animals are presented in Table 3. Representative photomicrographs are provided in Fig. 2 (C and D). The population of labeled supporting cells remains relatively stable at both survival times suggest-
Table 3
Total numbers and percentages of labeled supporting cells and Type II and Type I hair cells in superior cristae ampullaris of streptomycin-injected birds in the twenty and sixty days of survival groups

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Survival time</th>
<th>Supporting cells (N) &amp; %</th>
<th>Type II hair cells (N) &amp; %</th>
<th>Type I hair cells (N) &amp; %</th>
</tr>
</thead>
<tbody>
<tr>
<td>907525</td>
<td>20 days</td>
<td>(155) 55.95%</td>
<td>(93) 33.57%</td>
<td>(29) 10.46%</td>
</tr>
<tr>
<td>907527</td>
<td>20 days</td>
<td>(173) 58.44%</td>
<td>(81) 27.36%</td>
<td>(42) 14.71%</td>
</tr>
<tr>
<td>907528</td>
<td>60 days</td>
<td>(135) 53.35%</td>
<td>(51) 20.15%</td>
<td>(67) 26.48%</td>
</tr>
<tr>
<td>907529</td>
<td>60 days</td>
<td>(157) 50.32%</td>
<td>(70) 22.43%</td>
<td>(85) 27.24%</td>
</tr>
<tr>
<td>Average</td>
<td>20 days</td>
<td>(328) 57.24%</td>
<td>(174) 30.36%</td>
<td>(71) 12.39%</td>
</tr>
<tr>
<td>Average</td>
<td>60 days</td>
<td>(292) 51.68%</td>
<td>(121) 21.41%</td>
<td>(152) 26.90%</td>
</tr>
</tbody>
</table>

(~ 50% of the length of the sensory epithelium was measured).

ing that no net addition of this cell type is occurring. The number of Type I and Type II hair cells, however, shows variation. In animals from the twenty day survival group (Fig. 2C), the percentage of labeled Type II hair cells is larger than the percentage of labeled Type I hair cells. This relation changes by sixty days (Fig. 2D), at which time there are more labeled Type I hair cells in the sensory epithelium. A chi square analysis (Edwards, 1967) performed on the total numbers of labeled Type I and Type II hair cells from Table 3 shows that this change in proportions is highly significant ($\chi^2 = 34.9; P < 0.01$). We believe that these percentage changes between Type I and Type II hair cells suggest that new Type I hair cells are being added to the parenchyma through the transformation of the already labeled Type II hair cells.

Finally, we compared the radioactive thymidine content in nuclei of labeled cells by measuring the density of silver grains over the nucleus of labeled supporting cells, and Type II and Type I hair cells. The average nuclear area occupied by silver grains over supporting cells was 12.8 $\mu m^2$ (SEM = 1.1), over Type II hair cells 14.8 $\mu m^2$ (SEM = 1.3), and over Type I hair cells 12.5 $\mu m^2$ (SEM = 1.2). A one factor repeated measures ANOVA did not reveal significant differences among the area of the nucleus occupied by silver grains in supporting cells, Type II, and Type I hair cells [$F (2, 118) = 1.9$]. Given that the content of radioactive thymidine should decrease as the number of cell divisions increases, the similarities in silver grain density among the three cell types is consistent with the hypothesis is that a single supporting cell mitosis can give rise to all cell varieties of the vestibular parenchyma (see Discussion).

4. Discussion

4.1. Supporting cells are progenitors of regenerated hair cells

The data from Tsue et al. (1994) in conjunction with that collected in this investigation indicate that supporting cells, or a yet to be identified subpopulation of supporting cells, are the progenitors of regenerated avian vestibular hair cells. Supporting cells are the predominant cell type remaining in the sensory epithelium following aminoglycoside toxicity. Cells within the supporting cell layer are the first cell type to incorporate proliferation markers. Also, the number of labeled supporting cells remains fairly stable in all survival-time groups in both control and treatment animals (Weisleder and Rubel, 1993), such that the increase in the total number of labeled cells in the sensory epithelium with progressive survival time can be attributed to an increment in the number of labeled cells in the middle portion of the epithelium. Finally, Roberson et al. (1992) also suggested that supporting cells are the progenitors of new hair cells in the avian vestibular epithelium due to frequent pairings of labeled Type II hair cells and labeled supporting cells.

Findings similar to those reported here have been documented during development and in other octovalvularis organs during regeneration (Stone, 1937; Van de Water et al., 1978, Ginsberg and Gilula, 1979; Anniko, 1983; Zakon, 1984; Cotanche, 1987; Girod et al., 1989; Balak et al., 1990; Vischer, 1990; Oesterle et al., 1991; Raphael, 1992; and Stone and Cotanche, 1994), where supporting cells or subpopulations of supporting cells have been shown to be progenitors of regenerated hair cells. Work in teleost vestibular epithelium (Presson and Popper, 1990) has also suggested that a population of less differentiated cells, embryonic-like neuroepithelial cells, are the immediate source of new hair cells. These cells may be housed by the basement membrane to the lumen of the epithelium, and incorporate proliferation markers within 30 min of injections. Although Presson and Popper stress that these cells are different from supporting cells, they also state that embryonic-like neuroepithelial cells and supporting cells share morphological and cytochemical characteristics. As suggested by Corwin et al. (1991) embryonic-like neuroepithelial cells may not be a different population of cells, but may actually be supporting cells that differ from other supporting cells only in that they have entered the mitotic cycle and exhibit the corresponding changes.

4.2. Type II hair cells differentiate into Type I hair cells

Several investigators have suggested that Type I hair cells, as defined in this investigation (i.e., morphology and innervation pattern), are phylogenetically younger than Type II hair cells (Wersäll, 1956, 1960; Flock, 1964; Iurato, 1967; Wersäll and Bagger-Slørbäck, 1974; Anniko, 1983). A question that remains to be answered is whether Type I hair cells develop from Type II hair cells. The results from our experiments and data cur-
rently available suggest that such is the case. Iurato (1967) described cells in the vestibular sensory epithelium which displayed both types of innervation, boutonatal and calyxal, suggesting that a transition from Type II to Type I hair cell could be taking place. In developing cat and mouse, Favre and Sans (1979a,b) report that Type II hair cells are the first recognizable receptor cell of the sensory epithelium, and that Type I hair cells differentiate from Type II cells. Similar results were reported by Nordemar (1983). Finally, in avian otocyst cultures, Friedman (1959) noted that nerve calyces, the trademark of Type I hair cells, could not be identified early in development. Our data also suggest that Type II hair cells differentiate into Type I hair cells. In the tissue of streptomycin-treated animals, the appearance of regenerated Type II hair cells preceded the appearance of regenerated Type I hair cells. In the early stages of regeneration Type II hair cells occupy areas in the epithelium predominantly occupied by Type I hair cells in normal animals. During hair cell regeneration, the number of Type II hair cells is initially larger than that of Type I hair cells. This relation reverses at longer survival times, conceivably as a consequence of the former cell-type being transformed into the latter. In the normal, intact, avian vestibular epithelium and following regeneration, the number of Type I hair cells is almost three times larger than that of Type II hair cells (Weisleder and Rubel, 1993). Finally, the comparable content of radioactive thymidine in the nuclei of Type I and Type II hair cells at the longest survival time (60 days) suggests that Type I hair cells appearance is due to Type II hair cell transformation. If Type I hair cells had been generated by later mitotic activity of either support cells or Type II hair cells, the Type I cells would be expected to have half (or less) radioactive DNA, due to successive cell divisions. The findings that Type I hair cells, Type II hair cells, and support cells have equivalent levels of labeling, and that the density of support cells remains stable while the densities of Type II and Type I hair cells fall and rise, respectively, provide strong support for the transformation hypothesis.

4.3. From supporting cell to Type I hair cell

What is then the sequence of events that leads to hair cell regeneration in the avian vestibular epithelium? The information currently available, and the

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**Fig. 3.** Representation of the sequence of events that lead to hair cell regeneration in the avian vestibular epithelium. A) In the early stages of regeneration (1–3 h of survival), supporting cells along the basement membrane come out of dormancy and begin to synthesize DNA. B) As time progresses (12 h – 5 days), the nucleus of proliferating supporting cells migrates towards the middle third of the epithelium; at this location the cell divides. C) One daughter cell is destined to remain a supporting cell and thus returns to lower portions of the epithelium, the other daughter cell remains in the middle third of the epithelium where it presumably begins to differentiate into a hair cell. In the early phases of hair cell differentiation (five to twenty days of survival), Type II hair cells are the hair-cell type most frequently seen. D) Some Type II hair cells further differentiate into Type I hair cells as revealed by a decrease in the number of cells of the former type with a concomitant increase in the number of Type I hair cells (20 days of survival or longer). These changes occur without significant variation in the absolute number of hair cells.
data from this investigation support the following suc-
cession of events.

First, at the level of the basement membrane, pro-
genitor cells come out of dormancy and begin to syn-
thesize DNA (Fig. 3A). Utilizing immunolabeling tech-
niques for PCNA-cyclin and tissue autoradiography, Tsue et al. (1994) demonstrated that the supporting
cell layer is the sector of the epithelium where DNA
replication occurs, an observation replicated by this
study. Second, proliferating cells migrate from the lower
portions of the epithelium to more apical regions where
they divide (intermitotic nuclear migration) (Fig. 3B).
Tsue et al. (1994) demonstrated that although DNA
replication occurs next to the basement membrane, cell
division actually takes place in the upper regions of
the vestibular parenchyma, after the proliferating cells mi-
grate. Intermitotic nuclear migration has been reported
by Katayama and Corwin (1993) for the embryonic
chick cochlea, and by Raphael (1992) for the damaged
posthatch chick avian cochlea. Third, one daughter cell
returns to the lower portions of the epithelium to main-
tain a stable population of supporting cells while
the other daughter cell differentiates into a Type II
hair cell (Fig. 3C). In our investigation, the total num-
ber of labeled cells along the basement membrane did
not decrease with time, indicating that a stable number
of supporting cells was being maintained. Our observa-
tions also revealed that the first hair cells identified in
the parenchyma were devoid of a nerve calyx, giving
them the criterion identity of Type II hair cells. The
comparable contents of radioactive thymidine in sup-
porting cells, and Type I and Type II hair cells also
suggests that the three cell types have common ances-
tors. Finally, some of the new Type II hair cells further
differentiate into Type I hair cells as defined by cell
shape and the presence of a nerve calyx (Fig. 3D). Hair
cell counts revealed that as survival time increased, so
did the percentage of labeled Type I hair cells, while
the percentage of labeled Type II hair cells decreased
over the same period. These changes, which occurred
without an increase in the total number of labeled hair
cells or a decrease in the nuclear contents of radioac-
tive thymidine, suggest that the latter cell type is trans-
formed into the former. Whether Type II hair cells go
through some transformation to become Type I hair
cells and attract a nerve fiber capable of forming a
calyceal ending, or whether the kind of nerve fiber that
innervates the new hair cell determines its final pheno-
type remains to be determined. This question might be
answered by ultrastructural studies of the regenerating
vestibular epithelium, by cytochemical investigations
of the regenerated cells within the sensory epithelium
when cell-specific markers become available, or by
electrophysiological studies of the regenerated hair cells
and eighth nerve axons.

Acknowledgments

Supported by the Deafness Research Foundation,
and PHS grants DC00395 and NS09256, the Oberkot-
ter Foundation and the National Organization for
Hearing Research. The authors wish to thank Dale
Cunningham for outstanding technical contributions,
Karen Canady, Thom Park, and Harold Zaks for
helpful comments, Janet Clardy and Paul Schwartz
for expert photographic assistance, Mark Walter for edi-
torial assistance, and Gwenn Gage for production of Fig.
3.

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