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Delta1 expression during avian hair cell regeneration

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SUMMARY

Postembryonic production of hair cells, the highly specialized receptors for hearing, balance and motion detection, occurs in a precisely controlled manner in select species, including avians. *Notch1*, *Delta1* and *Serrate1* mediate cell specification in several tissues and species. We examined expression of the chicken homologs of these genes in the normal and drug-damaged chick inner ear to determine if signaling through this pathway changes during hair cell regeneration. In untreated post-hatch chicks, *Delta1* mRNA is abundant in a subpopulation of cells in the utricle, which undergoes continual postembryonic hair cell production, but it is absent from all cells in the basilar papilla, which is mitotically quiescent. By 3 days after drug-induced hair cell injury, *Delta1* expression is highly upregulated in areas of cell proliferation in both the utricle and basilar papilla. *Delta1* mRNA levels are elevated in progenitor cells during DNA synthesis and/or gap 2 phases of the cell cycle and expression is maintained in both daughter cells immediately after mitosis. *Delta1* expression remains upregulated in cells that differentiate into hair cells and is downregulated in cells that do not acquire the hair cell fate. *Delta1* mRNA levels return to normal by 10 days after hair cell injury. *Serrate1* is expressed in both hair cells and support cells in the utricle and basilar papilla, and its expression does not change during the course of drug-induced hair cell regeneration. In contrast, *Notch1* expression, which is limited to support cells in the quiescent epithelium, is increased in post-M-phase cell pairs during hair cell regeneration. This study provides initial evidence that Delta-Notch signaling may be involved in maintaining the correct cell types and patterns during postembryonic replacement of sensory epithelial cells in the chick inner ear.

Key words: Notch, Delta, Hair cell, Differentiation, Regeneration, Chick

INTRODUCTION

The otocyst, the precursor to the specialized epithelia of the inner ear, arises from a thickened invagination of the ectoderm. In birds, 8 distinct patches of sensory epithelium differentiate within the otocyst. Three cristae (located in the ampullae of the semicircular canals) and four macculae (the utricle, saccule, lagena and maccula neglecta) serve vestibular functions. The elongated basilar papilla is auditory in function. A subset of cells that form within these patches – hair cells – serves to transduce the energy of motion and sound into interpretable neural signals. The other primary cell type – support cells – provides mechanical and physiological support to hair cells and the epithelium. Hair cells and support cells are organized in a precise array of alternating cell types; numerous support cells surround each hair cell and, as a result, hair cells do not contact each other.

Mammals are born with their full compliment of hair cells, but permanent sensory deficits ensue after injury to mature inner ear epithelia, because production of new hair cells does not occur. In contrast, in the vestibular epithelia of postembryonic birds, hair cells are removed from the epithelium via apoptosis and replaced by mitotic regeneration (Jorgensen and Mathiessen, 1988; Roberson et al., 1992; Weisleder and Rubel, 1992; Kil et al., 1997). Further, after hair cells are experimentally damaged in the vestibular epithelia of post-hatch chicks, basal levels of cell proliferation are increased and new hair cells and support cells are produced (reviewed in Oesterle and Rubel, 1996). There is no ongoing mitotic activity or cell production in the mature avian basilar papilla (Oesterle and Rubel, 1993). However, regeneration of new hair cells and support cells is stimulated there following experimental damage (reviewed in Rubel, 1992; Cotanche et al., 1994). During both spontaneous and damage-induced hair cell regeneration in the inner ear, the cellular organization of the sensory epithelium is precisely maintained. Very little is know about the molecules that direct the formation of the correct numbers, types and patterns of cells during hair cell regeneration.

During development, several molecules, such as bone morphogenic proteins, retinoic acid-associated proteins and various transcription factors are expressed differentially across the axis of the otocyst, suggesting they play a role is specifying different regions within the inner ear (reviewed in Corey and

Breakefield, 1994; Fekete, 1996; Whitfield et al., 1997; Fritzsch et al., 1998). It has been proposed that the formation of the precise array of hair cells and support cells is regulated by lateral inhibition, as emerging hair cells prevent the surrounding uncommitted cells from differentiating into hair cells (Lewis, 1991; Goodyear et al., 1995), and that rearrangement of cells after their differentiation works to perfect the pattern (Goodyear and Richardson, 1997).

The initial process – lateral inhibition (Wigglesworth, 1940) – is mediated in several biological systems by the lin-12/Notch family of extracellular receptors (reviewed in Lewis, 1996; Kimble and Simpson, 1997; Weinmaster, 1997). These receptors are activated by members of the Delta-Serrate-Lag2 family of ligands. The ultimate effects of ligand-mediated activation of Notch are to inhibit expression of proneural genes that activate neural differentiation (reviewed in Lee, 1997), and of *Delta* (Haenlin et al., 1994; Hinz et al., 1994; Kunisch et al., 1994; Chitnis and Kintner, 1996; Heitzler et al., 1996; Ma et al., 1996; de la Pompa et al., 1997). Lateral inhibition through Notch signaling drives cell fate decisions during development in a wide variety of tissues across many species, ranging from the central nervous systems of mammals (Bao and Cepko, 1997; de la Pompa et al., 1997), chicks (Austin et al., 1995; Henrique et al., 1997a) and frogs (Coffman et al., 1993; Chitnis et al., 1995; Dorsky et al., 1995, 1997) to feather development in chicks (Crowe et al., 1998). A similar mechanism has been proposed to occur in the otocyst (Lewis, 1991), throughout which patches of precursor cells with the potential to form hair cells and support cells are scattered (Knowlton, 1967). *Notch* and the genes for its ligands, Delta and Serrate, are expressed in the developing otocyst of chicks (Myat et al., 1996; Adam et al., 1998), mammals (Lindsell et al., 1996) and zebrafish (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998a), and Notch signaling appears to be necessary for normal development of hair cells in the inner ear (Haddon et al., 1998b). Notch signaling is also important for cell specification in some adult vertebrate tissues (reviewed in Gridley, 1997; Robey, 1997).

In this study, we examined expression of the chicken homologs of *Delta1, Serrate1* and *Notch1* in the postembryonic inner ear to determine if lateral inhibition is a potential mechanism for regulating cell fate determination during regenerative hair cell production. We performed in situ hybridization with cRNA probes directed against chicken *Delta1*, *Serrate1* and *Notch1*, which were recently cloned (Myat et al., 1996; Henrique et al., 1995). *Serrate1* is expressed throughout the auditory epithelium of untreated chicks, suggesting that Serrate1/Notch1 signaling is independent of new hair cell production. *Delta1* and *Notch1* expression are transiently altered in regenerating epithelia; transcripts for both genes are present at similar levels in post-M-phase pairs of cells, and *Delta1* mRNA levels are elevated and maintained in early differentiating regenerated hair cells. These findings implicate Delta1/Notch1 signaling in mediating lateral inhibition during the re-establishment of the cellular pattern during hair cell regeneration.

MATERIALS AND METHODS

Animal care

Fertilized eggs or 1-day-old hatchlings of White Leghorn chickens were received from H and N International (Redmond, WA). Eggs were placed in a humidified incubator at 37°C, and hatchlings were stored in heated brooders with ample food and water, at the Animal Care Facility at the University of Washington. All procedures were approved by the University's Animal Care Committee.

Gentamicin treatment and BrdU injections

Post-hatch chicks between 6 and 9 days (40-70 grams) received a single subcutaneous injection of gentamicin (400 mg/kg; Lyphomed) and recovered in the Animal Care Facility. At 3 days after gentamicin treatment, chicks received a single intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg) in sterile phosphate-buffered saline (PBS).

Tissue preparation

The following tissues were used in this study: (a) cochlear ducts (containing the basilar papilla and lagena) and utricles from control chicks, (b) cochlear ducts and utricles from chicks euthanized at different times after gentamicin injection, and (c) cochlear ducts and utricles from chicks that received a single BrdU injection at 3 days after gentamicin injection and were euthanized at different times after BrdU injection. Chicks were euthanized by Pentobarbital overdose (intraperitoneal; 100 mg/kg) and decapitated. Cochlear ducts and utricles were dissected and fixed in 3.7% formaldehyde (with 2 mM EGTA in DEPC-PBS). Tissues overlying the sensory epithelia (tegmentum vasculosum, tectorial membrane and otoconia) were removed, and remaining tissues were dehydrated in methanol and stored at –80°C.

In situ hybridization

In situ hybridization with digoxigenin (DIG)-dUTP-labeled cRNA probes (generous gift of Dr Julian Lewis, ICRF, London, UK) was performed on whole-mount cochlear ducts and utricles using alkaline phosphatase-conjugated anti-DIG antibodies (Boehringer Mannheim), according to Henrique et al. (1995). At least two runs per experiment were conducted; approximately 7 organs per experimental variable were included in each run. Sense controls for the chicken *Delta1* and *Notch1* probes were performed in each run. The cRNA probes for chicken *Delta1*, *Serrate1* and *Notch1* have been described (Henrique et al., 1995; Myat et al., 1996).

Immunoreactions

To detect BrdU, calmodulin, calretinin or β-tubulin in whole-mount cochlear ducts or utricles, immunohistochemistry was performed using the avidin-biotin-chromogen reaction (Stone and Cotanche, 1994; Stone et al., 1996). Some reactions were enhanced by NiCl. The following primary antibodies and dilutions were used: mouse anti-BrdU (1/100; Becton Dickenson), mouse anti-calmodulin (1/500; Sigma), rabbit anti-calretinin (1/500, Chemicon) and mouse anti-β-tubulin (1/1000; Anthony Frankfurter, University of Virginia). To double-label utricles for calretinin and BrdU, anti-calretinin was detected with Bodipy-FL-conjugated goat anti-rabbit IgG (1/300, Molecular Probes) and anti-BrdU was detected with Lissamine rhodamine-conjugated goat anti-mouse IgG (1/300, Jackson ImmunoResearch) according to Memberg and Hall (1995). To detect hair cells after *Delta1* in situ hybridization, whole mounts were reacted with anti-calretinin, then Bodipy-FL goat anti-rabbit IgG. To detect BrdU after in situ hybridization, whole-mount utricles were placed in 2 N HCl for 15 minutes at 37°C followed by anti-BrdU. The BrdU antibody was detected with Bodipy-FL goat anti-mouse IgG (1/300, Molecular Probes).

Primary antibody incubations were performed overnight at 4°C, while secondary antibody steps were performed for 1-2 hours at room temperature. Tissue was mounted onto microscope slides and coverslipped with Vectashield mounting medium (Vector Laboratories). Photographic slides were generated with a Leica Aristoplan microscope and digitized with a Nikon LS-1000 Scanner. Fluorescent images were collected using a BioRad MRC 1024

confocal microscope with LaserSharp 3.1 acquisition software. Figures were assembled with NIH Image, Adobe Photoshop software and a Phaser dye sublimation printer (Tektronix).

Quantitative analyses

Utricles that were double-labeled to detect *Delta1* mRNA and BrdU were examined and quantified using confocal microscopy. Transmitted light from the *Delta1*-alkaline phosphatase reaction product and emitted fluorescent light from the BrdU/Bodipy-FL were analyzed. At 60×, three regions (203×203 μm) were chosen randomly in three utricles from each time point, and the following parameters were calculated for each region: the number of BrdU-positive nuclei, the proportion of BrdU-positive nuclei that were present in *Delta1*-positive cells or *Delta1*-negative cells, and the layer in which BrdU-positive/*Delta1*-positive and BrdU-positive/*Delta1*-negative cells resided. For this study, we designated the following layers for our analysis. The support cell layer (SCL) constitutes about 1/4 of the epithelium and resides adjacent to the basal lamina; it contains highly packed support cell nuclei, which are small and round/oval. The hair cell layer (HCL) constitutes the remaining 3/4 of the epithelium; it contains the hair cell nuclei, which are large and round and the lumenal portions of the hair cell and support cell cytoplasm. Cells that were considered 'positive' for *Delta1* mRNA contained robust reaction product within the perinuclear cytoplasm.

Delta1 expression was also examined in closely associated pairs of BrdU-positive cells. Four regions (203×203 μm) were chosen randomly in two utricles from each time point. Within each region, each BrdU-labeled cell in the SCL was centered in the field, its *Delta1* mRNA labeling was noted and the same field in the HCL was scanned. If a BrdU-positive nucleus was located in the HCL above, and within two nuclear widths from, the labeled support cell nucleus, its *Delta1* mRNA labeling was noted. 30 pairs of sister cells were examined at each time point.

Finally, calretinin expression was examined in BrdU-labeled sister pairs, using the same approach as described for *Delta1* /BrdU-labeled cells. A total of 20 pairs of sister cells was analyzed in four utricles at 8-12 days post-gentamicin.

RESULTS

Hair cell production in the avian inner ear

The sensory epithelia of the auditory and vestibular organs of the chick inner ear are composed primarily of two cell types – hair cells and support cells. A single hair cell is surrounded by numerous support cells and does not contact another hair cell (Fig. 1A). Hair cells, the sensory receptors, are flask-shaped and contact the lumenal surface, but not the basal lamina (Fig. 1B). Their distinguishing feature is an apical tuft of actin-filled stereocilia. In vestibular hair cells, the stereocilia are considerably longer than those of most auditory hair cells, but other properties closely resemble auditory hair cells. In both epithelia, hair cells in different regions have specialized functions. In the basilar papilla, hair cells in the basal and apical ends of the organ respond to high and low frequencies, respectively (Ryals and Rubel, 1982). In vestibular epithelia, hair cells with large calyceal afferent innervation (Type I) are concentrated in the striolar region of the macculae and the middle of the cristae, while hair cells with mixed afferent and efferent innervation (Type II) are located throughout the epithelium (Jorgensen, 1991).

Support cells are columnar and contact both the lumenal surface and the basal lamina (Fig. 1B). Although the exact identity of the hair cell progenitor has not been determined, cells in the support cell layer have been shown to divide during hair cell regeneration, suggesting that support cells have such potential (reviewed in Cotanche et al., 1994). In auditory and vestibular epithelia, the nuclei of hair cells and support cells have different sizes, shapes and locations within the epithelium. Hair cell nuclei are larger and round and are located at mid-depth within the epithelium; support cell nuclei are smaller and oval or round and lie deep within the epithelium, near the basal lamina (see Fig. 1B). During hair cell development and regeneration, the nuclei of hair cell progenitors migrate from the deep layer toward the lumen as they progress through the cell cycle (Fig. 1C,D; Katayama and Corwin, 1993; Raphael, 1992; Baird et al., 1993; Raphael et al., 1994; Stone and Cotanche, 1994; Tsue et al., 1994). These movements are similar to the intermitotic nuclear migration during neural tube formation (Fujita, 1962; Sauer, 1935). During S phase, progenitor cell nuclei reside adjacent to the basal lamina. By late G_2/M phase, nuclei have left the basal layer and reside near the lumen. Studies of the developing and regenerating chick cochlea suggest that the length of G_2 phase is between 1 and 6 hours (Katayama and Corwin, 1993; Stone and Cotanche, 1994). After cell division is completed, the two sister cells differentiate into their respective cell types, migrating to the appropriate cell layer, or they go on to divide again.

For this study, we induced hair cell death by administering a single injection of the ototoxic aminoglycoside antibiotic, gentamicin (Janas et al., 1995). This paradigm causes complete

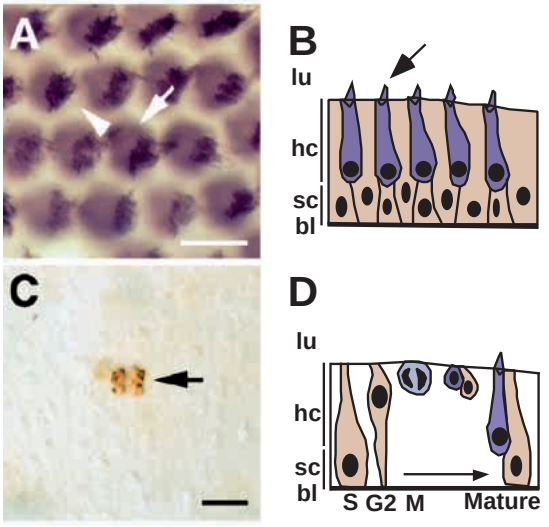

Fig. 1. Anatomy and dynamics of hair cell epithelia in the chick inner ear. (A) A whole-mount of the untreated basilar papilla immunolabeled for the hair cell marker, calmodulin, reveals the alternating pattern of hair cells (purple-black cells, arrow) and support cells (unlabeled cells, arrowhead). (B) Schematic of a transverse section of the inner ear sensory epithelium. Hair cells (purple cells) reside near the lumenal surface (lu), have stereocilia (arrow) and do not contact the basal lamina (bl), while support cells (beige cells) span the depth of the epithelium. (C) During regeneration, mitotic figures (arrow) are evident in the hair cell layer, near the lumen, as seen in a whole-mount control utricle at 6 hours after a single BrdU injection. The location of nuclei at each stage of the cell cycle is illustrated in D. (hc, hair cell layer; sc, support cell layer.) Scale bars: A, 20 μm; C, 10 μm.

hair cell loss in the basal half of the basilar papilla (compare Fig. 2A,E), but leaves the mid-apical and apical portions undamaged (Bhave et al., 1995; Stone et al., 1996). As a result, mitotic activity is selectively elevated in progenitor cells by 3 days post-gentamicin in the basal part of the epithelium (compare Fig. 2C,G), but it remains at baseline levels in undamaged regions. Newly formed hair cells are evident throughout the damaged region by 5 days post-gentamicin and levels of cell division have nearly returned to baseline (zero) by 7 days post-gentamicin (data not shown; Stone et al., 1996).

In the normal, untreated chick utricular epithelium, scattered cells incorporate bromodeoxyuridine (BrdU), a nucleotide analog, within 2 hours after it is injected (Fig. 2D). Similar results were reported by Tsue et al. (1994) and Kil et al. (1997). A single injection of gentamicin causes a small degree of hair cell loss that is restricted to the striolar region (compare Fig. 2B,F). BrdU incorporation is increased in the striola by 3 days after drug treatment, but it is maintained at baseline levels in undamaged, extrastriolar regions (Fig. 2H). By 6 days after gentamicin injection, levels of cell division have approached baseline, and there is morphological evidence for hair cell regeneration in the striola (data not shown). Similar results were recently reported by Bhave et al. (1998).

Delta1 expression is upregulated during hair cell regeneration

We examined *Delta1* expression in inner ear sensory epithelia from untreated chicks and chicks at 3, 5 and 10 days post-gentamicin injection. No *Delta1* mRNA is detected in any region of the basilar papilla of untreated chicks (Fig. 3A,B). In contrast, at 3 days post-gentamicin, *Delta1* expression is markedly upregulated in a subpopulation of cells throughout the basal, damaged end of the basilar papilla (Fig. 3C), but no *Delta1*-expressing cells are detected in the undamaged, apical end (Fig. 3D). Among *Delta1*-expressing cells, isolated cells are most predominant, but closely associated pairs and clusters are also detected. Similar patterns of *Delta1* expression are seen in the basilar papilla at 5 days after gentamicin treatment (data not shown). At both time points, the area of *Delta1* expression ends sharply at the transitional zone between the basal, damaged and the apical, undamaged halves of the epithelium (Fig. 3E). By 10 days post-gentamicin, levels of *Delta1* transcripts in the damaged region of the basilar papilla are noticeably lower than at 3 or 5 days (Fig. 3F,G); only a few scattered *Delta1*-expressing cells are detectable, and they continue to be confined to the damaged region.

Utricles from normal, untreated chicks show significant levels of *Delta1* expression (Fig. 4A,B), as do lagenas (data not shown). In both organs, *Delta1*-expressing cells are distributed uniformly throughout the sensory epithelium; there is no obvious difference in the density of *Delta1*-expressing cells present in the striolar (Fig. 4A) and extrastriolar (Fig. 4B) regions. Cells containing *Delta1* mRNA are predominantly isolated from each other, but some cells are arranged in pairs or clusters. At 3 days post-gentamicin, *Delta1* expression is selectively upregulated relative to controls in the striolar region, where maximal hair cell loss occurs (Fig. 4C), but it does not appear to change in the undamaged, extrastriolar regions (Fig. 4D). Similar patterns of *Delta1* expression are seen in the utricle at 5 days after gentamicin treatment (data not shown). At 10 days post-gentamicin, utricles resemble controls (Fig. 4E,F).

No *Delta1* transcripts are detected in the non-sensory regions of the cochlear duct or utricle of control or drug-treated chicks (data not shown). Further, no signal is seen in untreated

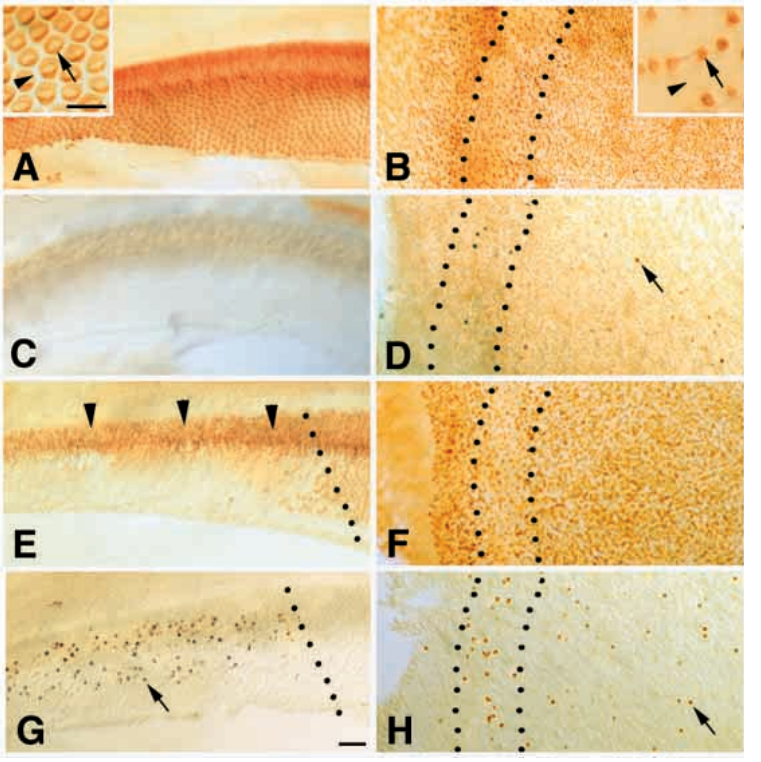

Fig. 2. Hair cell loss and regeneration in the chick inner ear. Patterns of hair cell loss and regeneration in the basilar papilla (A,C,E,G) and utricle (B,D,F,H). Antibody labeling against class III β-tubulin and calretinin reveal the normal hair cell array in the basal half of the basilar papilla (A) and the lateral portion of the utricle (B), respectively. (The striola resides between the dotted lines in B,D,F and H) Insets in A and B show higher magnification of β-tubulin-positive hair cells in the papilla (A, arrow) and calretinin-positive hair cells in the utricle (B, arrow). Both antigens are present in hair cells but are absent from support cells (arrowheads). (C,D) Normal patterns of BrdU incorporation after a 2-hour BrdU pulse/fix. No BrdU-labeled cells are present in the basilar papilla (C), but several BrdU-positive cells (arrow) are scattered uniformly throughout the utricle (D). Antibodies to β-tubulin and calretinin reveal patterns of hair cell loss throughout organs at 3 days post-gentamicin (E). All hair cells in the basal 1/3 to 1/2 of the basilar papilla are killed. (Dotted lines in E,G mark boundary of hair cell loss; basal is toward left and apical is toward right). β-tubulin labeling is absent in the basal basilar papilla, except in nerve terminals (arrowheads). Some hair cells are lost from the utricular striola (F), as demonstrated by the loss of some calretinin immunoreactivity. However, few hair cells die in the extrastriolar regions. By 3 days post-gentamicin, there is significant upregulation of cell division in areas of hair cell loss in both organs. In the basilar papilla (G), BrdU-labeled cells (arrow) are evident throughout the basal region. In the utricle, increased cell division is limited to the striolar region (H, arrow indicates BrdU-labeled cell). Scale bars: insets, 20 μm, A-H, 50 μm.

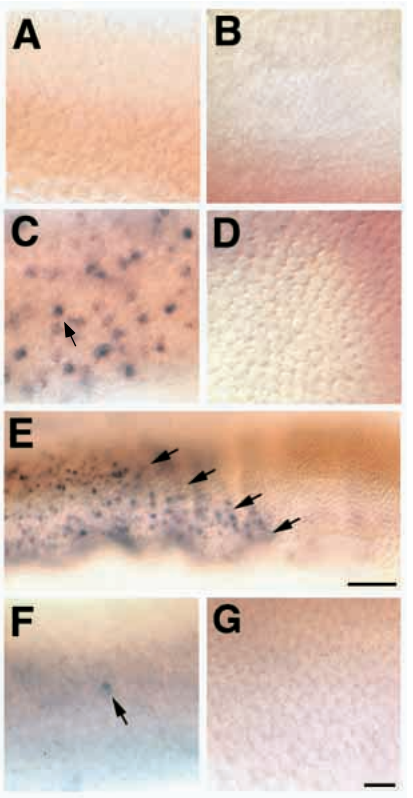

Fig. 3. *Delta1* expression in the normal and damaged basilar papilla. *Delta1* mRNA labeling with in situ hybridization is shown in the basal (A,C,F) and apical (B,D,G) basilar papilla in control tissue (A,B), tissue at 3 days post-gentamicin (C-E), and tissue at 10 days post-gentamicin (F,G). No *Delta1* expression is detected in the basilar papilla in control, undamaged chicks in either the basal (A) or apical (B) regions. By 3 days post-gentamicin, *Delta1* expression is significantly upregulated in cells (arrow) throughout the damaged, basal half of the papilla (C), but it resembles control tissue in the apical half (D). (E) Note the discrete boundary (arrows) of *Delta1* mRNA labeling at the transitional zone between the basal, regenerating cells (left) and the apical, quiescent cells (right). By 10 days post-gentamicin, the number of *Delta1*-expressing cells in the basal basilar papilla has significantly decreased relative to 3 days post-gentamicin (F); only one cell continues to express *Delta1* in this micrograph (arrow). *Delta1* expression continues to be absent from the apical, undamaged basilar papilla (G). Scale bars: A-D,F,G, 20 μm; E, 100 μm.

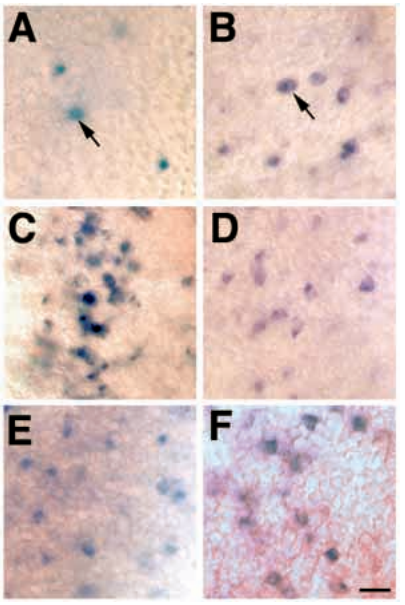

Fig. 4. *Delta1* expression in the normal and damaged utricle. *Delta1* mRNA labeling with in situ hybridization is shown in the striolar (A,C,E) and extrastriolar (B,D,F) regions of the untreated utricle (A,B), tissue at 3 days post-gentamicin (C,D), and tissue at 10 days post-gentamicin (E,F). In control chicks (A,B), *Delta1* expression is detected in cells (arrows) scattered throughout the epithelium. No difference in the density of *Delta1*-expressing cells was detected in the striolar (A) and extrastriolar (B) regions. By 3 days post-gentamicin, the number of *Delta1*-expressing cells is higher in the striola (C), where hair cell regeneration is upregulated, but not in the extrastriolar regions (D), where hair cell production resembles controls. By 10 days post-gentamicin, *Delta1* mRNA levels approach control levels in the striolar (E) and extrastriolar (F) regions. Scale bar, 20 μm.

or treated cochlear ducts or utricles probed with sense *Delta1* cRNA (data not shown).

Delta1 is first expressed in mitotic progenitors and is then upregulated in differentiating hair cells

Delta1 expression, when seen, is limited to a subpopulation of cells in the auditory and vestibular epithelia. The majority of *Delta1*-expressing cells are isolated cells located in the hair cell layer (Fig. 5A,B). In these cells, *Delta1* mRNA is detected in the perinuclear and neck regions, and stereocilia are not evident. In addition, *Delta1* expression is often detected in pairs, and rarely in clusters, of cells that are in direct contact or very closely associated (Fig. 5C,D). Pairs of *Delta1*-expressing cells are round, small, morphologically undifferentiated and located close to the lumenal surface. Based on cellular mechanics during post-hatch avian hair cell production (Fig. 1D), we hypothesized that the isolated cells that we detected in the hair cell layer are hair cells during early stages of differentiation and the pairs of *Delta1*-expressing cells are sister cells, newly generated from one cell division.

To determine how *Delta1* expression changes in cells relative to their stage in the cell cycle, we examined utricles of untreated chicks that received a single injection of the proliferation marker, bromodeoxyuridine (BrdU), and were killed at either 1.5, 3 or 6 hours, or 3 or 8 days after the injection. Whole-mount utricles were reacted to detect *Delta1* mRNA and BrdU and examined with confocal microscopy to determine the layer of the epithelium in which *Delta1*-positive/BrdU-positive cells were located at each time point. This experimental paradigm allowed us to determine when *Delta1* expression is upregulated in cells relative to incorporation of BrdU during S phase and throughout their differentiation. We opted to use control utricles rather than drug-damaged basilar papillas for these experiments because fewer cells incorporate BrdU in the utricle and, therefore, an analysis of BrdU labeling in individual cells and in pairs of post-M-phase sister cells is easier to perform and interpret.

The positions and arrangements of BrdU-labeled nuclei at different times after BrdU injection reflect their different stage of the cell cycle. At 1.5 hours post-BrdU injection, the numbers

Table 1. BrdU/*Delta1* mRNA double labeling in utricles from untreated chicks

	1.5 h post-BrdU	6 h post-BrdU	3 d post-BrdU	8 d post-BrdU
HCL°	50%	100%	63%	69%
SCL	50%	0%	37%	31%
+*Delta1*/SCL*	2% (2/126)	0% (0/56)	1% (3/228)	4% (6/135)
+*Delta1*/HCL	21% (27/126)	55% (31/56)	40% (90/228)	33% (44/135)
–*Delta1*/SCL	48% (60/126)	0% (0/56)	36% (83/228)	27% (36/135)
–*Delta1*/HCL	29% (37/126)	45% (25/56)	23% (52/228)	36% (49/135)

Total no. of cells counted for experiment: 545

Total no. of BrdU+ cells counted per time-point: 1.5 hours=126, 6 hours=56, 3 days=228, 8 days=135.

°Values reflect percentage of BrdU-positive cells present in each layer.

*Values reflect percentage of BrdU-positive cells per category per time point. The categories are as follows: +*Delta1*/SCL=*Delta1*-positive cells located in support cell layer; +*Delta1*/HCL=*Delta1*-positive cells located in hair cell layer; –*Delta1*/SCL=*Delta1*-negative cells located in support cell layer; –*Delta1*/HCL=*Delta1*-negative cells located in hair cell layer. In parentheses, values expressed are the number of cells in each category per time point divided by the number of BrdU-positive cells counted per time point.

of BrdU-labeled nuclei are evenly distributed between the hair cell layer (HCL) and support cell layer (SCL) (Table 1, top). We detected no BrdU-labeled mitotic figures or post-cytokinetic pairs of cells at this time, indicating that cells that had incorporated BrdU had not yet undergone mitosis by 1.5 hours. At 6 hours post-BrdU, 100% of BrdU-positive nuclei are located in the HCL. BrdU-labeled mitotic figures and post-cytokinetic pairs are detected, and some BrdU-labeled nuclei are present in single cells. At 3 days post-BrdU, 63% of BrdU-positive nuclei are located in the HCL and 37% are located in the SCL, reflecting the migration and differentiation of post-M-phase cells. At 8 days post-BrdU, 69% of BrdU-labeled nuclei are located in the HCL and 31% are located in the SCL.

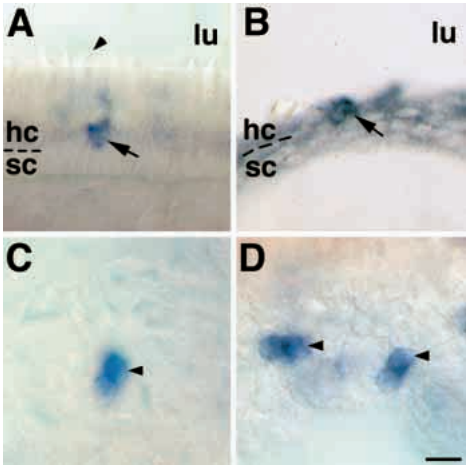

Fig. 5. Cellular patterns of *Delta1* expression. *Delta1* mRNA is detected in two general types of cells in the basilar papilla and utricle. The first cell type (shown in A,B) contains abundant *Delta1* mRNA around the nucleus, which resides in the hair cell (hc) layer. An example of this cell type is shown in A (arrow) in the extrastriolar part of a whole-mount untreated utricle, whose curved edge affords a transverse view of epithelial cells. In vestibular epithelia, *Delta1*-expressing cells are flask-shaped, with necks extending toward the lumen (lu). (The junction of the hair cell and support cell (sc) layers is shown as dashed lines in A and B.) The stereocilia of hair cells are evident (arrowhead). Another example of the first phenotype is shown in B (arrow), in a cryosectioned basilar papilla at 5 days post-gentamicin (B). Due to the drug damage in this sample, all existing hair cells were killed and, as a result, the epithelium is much thinner than in untreated samples. The second *Delta1*-expressing cell type (arrowheads in C,D) contains less *Delta1* mRNA and is rounded, associated in pairs and located near the lumenal surface. Examples of such pairs of cells are shown in whole mounts of the untreated utricle (C) and the basilar papilla at 5 days post-gentamicin (D); the focal plane is the lumenal surface of the epithelium. Scale bar, 5 μm.

Delta1 expression changes during the course of cell proliferation and differentiation (Table 1, bottom; Fig. 6). At 1.5 hours post-BrdU, the majority of BrdU-labeled cells (77% [97/126]; Table 1) do not contain *Delta1* transcripts (Fig. 6A,B). Of the BrdU-labeled cells that do contain *Delta1* mRNA, the vast majority (93% [27/29]) are located in the HCL. At 3 hours post-BrdU, we also detected double-labeled cells in the HCL (Fig. 6C,D). At 6 hours post-BrdU, 100% of double-labeled cells are located in the HCL. Double-labeling is detected in single cells in the HCL and in pairs of closely associated, post-M-phase cells located near the lumenal surface (Fig. 6E,F). At 3 days post-BrdU, 63% (90/142) of BrdU-labeled cells in the HCL express *Delta1*, whereas 97% (83/86) of BrdU-positive cells in the SCL are *Delta1*-negative. At 8 days post-BrdU, the percentage of cells in the HCL that are double-labeled is decreased relative to 3 days (47% [44/93] versus 63%). Still, the majority of BrdU-positive cells in the SCL (86% [36/42]) do not express *Delta1*.

These results show that *Delta1* is first expressed in progenitor cells in either S phase or G_2 phase and that both post-M-phase daughter cells contain similar levels of *Delta1* transcripts. Further, these results suggest that *Delta1* expression is upregulated in cells that acquire the hair cell phenotype, but remains low in cells that do not develop into hair cells.

A previous study suggests that, in the untreated chick utricle, each mitotic event yields a hair cell and a support cell (Roberson et al., 1992). Since the experiments presented in that study were performed in sectioned material using morphological criteria and did not involve a direct analysis of sister pairs, we further tested this hypothesis in whole-mount material using an immunological marker. Control chicks at post-hatch day 7 were given a single injection of BrdU, and utricles were fixed between 8 and 12 days after the injection, when differentiation of the BrdU-labeled cells was estimated to be completed (Roberson et al., 1992). Utricles were reacted to detect calretinin, which is expressed in hair cells and not support cells (e.g. Dechesne et al., 1994; Pack and Slepecky, 1995; Baird et al., 1997; Zheng and Gao, 1997), and BrdU. We identified distinct and closely associated pairs of BrdU-positive

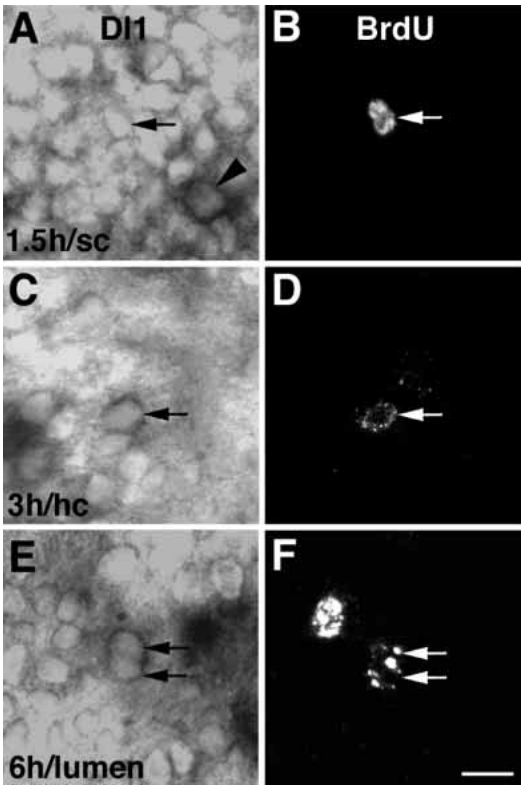

Fig. 6. *Delta1* is expressed in cells at late stages of the cell cycle. *Delta1* expression in double-labeled control utricles is shown in transmitted light slices in the left column, and BrdU labeling is shown in confocal slices in the right column. Each pair of panels (A-B,C-D,E-F) shows the same field of the epithelium. (A,B) Slices through the support cell (sc) layer at 1.5 hours post-BrdU. (C,D, and F,G) Slices through the hair cell (hc) layer at 3 and 6 hours post-BrdU, respectively. Note the higher nuclear density in the support cell layer (A) compared to the hair cell layer (C,E). At 1.5 hours post-BrdU, no *Delta1* mRNA is detected in BrdU-labeled cells, shown here in the support cell layer (A,B; arrows point to same cell in each panel; arrowhead in A points to a *Delta1*+/BrdU– cell for reference). At 3 hours post-BrdU, *Delta1* expression is detected in isolated BrdU-labeled cells in the hair cell layer (C,D; arrows point to same cell in each panel). At 6 hours post-BrdU, *Delta1* mRNA is detected in closely associated pairs of cells near the lumen (E,F; arrows point to same cells in each panel). Scale bar, 10 μm.

cells that were likely to be sister cells from the same mitotic division. We determined in which layer each cell in the pair was located and whether each cell expressed calretinin. In every one of the 20 pairs examined, cell division resulted in the production of a calretinin-positive cell in the HCL and a calretinin-negative cell in the SCL (Fig. 7). Therefore, cell division in the normal, untreated utricle appears to be predominantly asymmetric, producing a hair cell and a non-hair cell. We were unable to confirm that the non-hair cell is a support cell, since we did not have a reliable support cell marker.

These data allowed us to confirm how *Delta1* expression changes in each cell within a post-M-phase pair during differentiation. We examined normal, untreated utricles at 3 and 8 days post-BrdU injection and located pairs of BrdU-positive cells that were closely associated and therefore likely

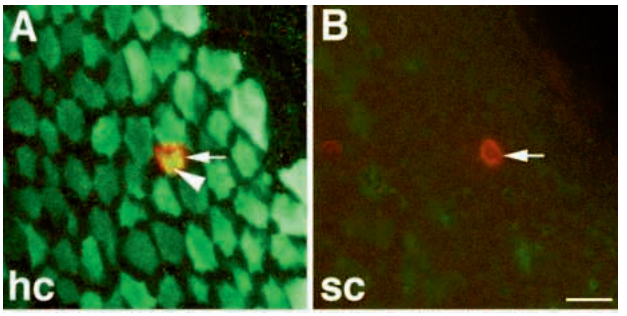

Fig. 7. Hair cell production in the utricle occurs via asymmetric divisions. At 8-12 days after a single BrdU injection, pairs of BrdU-positive nuclei (presumed sister cells) are segregated into the hair cell layer (hc, A) and the support cell layer (sc, B). The cell with the BrdU-labeled nucleus (A, red label, arrow) in the hair cell layer is calretinin-positive (A, green label, arrowhead), whereas the cell with the BrdU-labeled nucleus (B, red label, arrow) in the support cell layer is calretinin-negative. Scale bar, 10 μm.

to be products of the same division. Next, we determined if either cell in the pair expressed *Delta1*. At 3 days post-BrdU, the two sister cell nuclei are segregated into the HCL and SCL in the majority of pairs. Most commonly (70% of pairs), the nucleus that is located in the HCL is surrounded by abundant *Delta1* message, while the nucleus in the SCL does not express detectable levels of *Delta1* (Table 2; Fig. 8A-D). We detected no pairs in which the cell in the SCL was double-labeled. By 8 days post-BrdU, in 72% of pairs examined, the BrdU-labeled cell in the HCL had lost detectable levels of *Delta1* mRNA (Table 2; Fig. 8E-H).

To confirm that *Delta1* is expressed in hair cells at some point during their differentiation, utricles at 5 days post-gentamicin were processed for in situ hybridization to detect *Delta1* mRNA and then reacted for immunofluorescence to detect calretinin. Calretinin-positive cells are exclusively detected in the HCL. A subpopulation of calretinin-positive cells contains *Delta1* mRNA and many *Delta1*-expressing cells located in the hair cell layer are strongly immunoreactive for calretinin (Fig. 9).

Taken together, these findings demonstrate that: (1) during early differentiation, *Delta1* expression is maintained in cells developing a hair cell fate, but it is rapidly downregulated in cells that do not acquire a hair cell fate, and (2) during late differentiation of newly generated hair cells, *Delta1* expression

Table 2. BrdU/*Delta1* mRNA double labeling in sister pairs in utricles from untreated chicks

	3 days post-BrdU	8 days post-BrdU
+hc/+sc°	0	0
+hc/-sc	70%	28%
–hc/+sc	0	0
–hc/-sc	30%	72%

°Values reflect *Delta1* mRNA labeling patterns of pairs of BrdU-labeled cells, expressed as a percentage of the pairs counted. The pairing variables are as follows: +hc/+sc = both cells in the pair express *Delta1*; +hc/–sc = only the hair cell expresses *Delta1*; –hc/+sc = only the support cell expresses *Delta1*; –hc/–sc = neither cell in pair expresses *Delta1*.

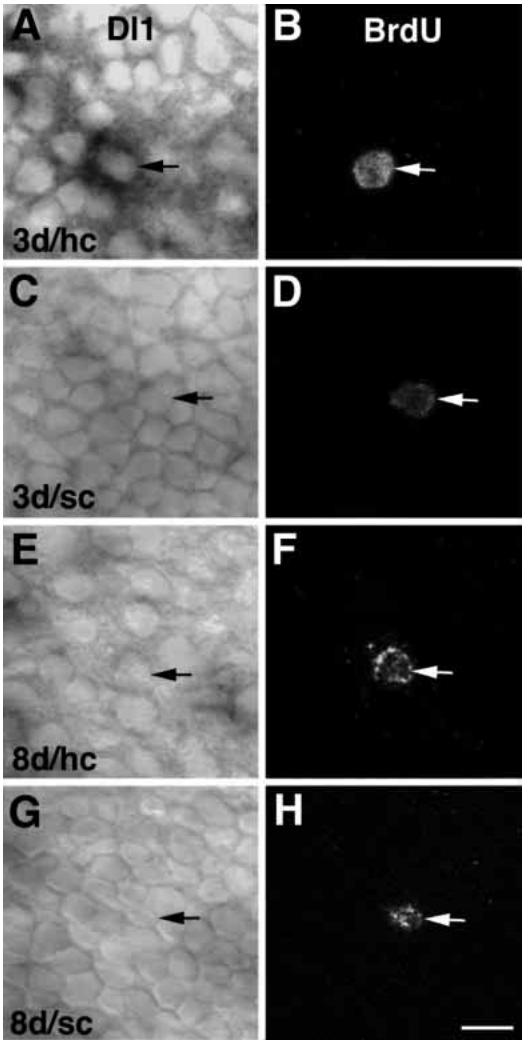

Fig. 8. In regenerated hair cells, *Delta1* is expressed by 3 days post-BrdU and downregulated by 8 days post-BrdU. *Delta1* mRNA and BrdU double-labeling in paired cells in control utricles. *Delta1* expression is shown in transmitted light slices in left column, and BrdU labeling is shown in confocal slices in right column. Each set of four panels (A-D and E-H) shows a pair of presumed sister cells within the same field of the epithelium; panels A-D show tissue at 3 days post-BrdU, and panels E-H show tissue at 8 days post-BrdU. One cell in each pair is located in the hair cell (hc) layer (A,B,E,F) and the other cell is located in the support cell (sc) layer (C,D,G,H). At 3 days post-BrdU, the cell in the hair cell layer (A,B) contains high levels of *Delta1* mRNA (arrows in A and B point to same cell). In contrast, the cell in the support cell layer (C,D) expresses no *Delta1* (arrows in C and D point to the same cell). At 8 days post-BrdU, *Delta1* mRNA levels are downregulated in many cells in the hair cell layer (E,F) and remain very low in the cell in the support cell layer (G,H). Scale bar, 10 μm.

is downregulated such that mature hair cells contain no *Delta1* transcripts.

Serrate1 is expressed in hair cells and support cells

In untreated chicks, *Serrate1* is expressed throughout all of the sensory epithelia that we examined. In the basilar papilla, *Serrate1* expression appears elevated in hair cells relative to

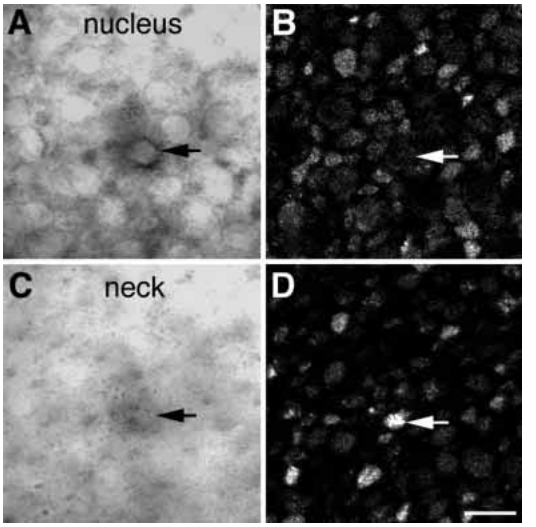

Fig. 9. *Delta1* is co-expressed in some cells with the hair cell-specific antigen, calretinin. Transmitted light microscopy showing *Delta1* expression (A,C) and confocal microscopy showing calretinin immunofluorescence (B,D) in the same field within a utricle at 5 days post-gentamicin. The slices shown in A and B are through the hair cell nuclei, and the slices in C and D are through the hair cell necks, at the lumenal surface. Arrows in all four panels point to the same cell. In the perinuclear region of the cell, there are high levels of *Delta1* mRNA (A, arrow) and low levels of calretinin protein (B, arrow). In the neck region of the cell, no *Delta1* mRNA is present (C, arrow), but there is intense calretinin immunofluorescence (D, arrow). Scale bar, 10 μm.

support cells (Fig. 10A), while in the utricle (Fig. 10B,C) and the lagena (data not shown), *Serrate1* mRNA levels are similar in the support cell and hair cell layers. Some variation in the levels of *Serrate1* expression was seen within both layers of the untreated utricle (Fig. 10B,C). We detected no significant change in support cell *Serrate1* expression relative to controls at 3 days or 10 days post-gentamicin in either the basilar papilla or utricle (data not shown). No *Serrate1* transcripts are detected in non-sensory regions of the cochlear duct or utricle of control or drug-treated chicks (data not shown).

Notch1 expression is upregulated during hair cell regeneration

In untreated chicks, *Notch1* transcripts are detected throughout the length of the sensory epithelium of the basilar papilla (Fig. 11A,C) and in the utricle (Fig. 11B,D). In the auditory organ, *Notch1* expression is limited to the support cell layer (Fig. 11A,C). In the utricle, *Notch1* mRNA is detected in both the hair cell and support cell layers (Fig. 11B,D), but it is unclear whether this labeling is restricted to support cells or includes hair cells. There appear to be higher levels of *Notch1* mRNA in the utricle relative to the basilar papilla. In contrast to the basilar papilla, there are distinct areas of elevated *Notch1* expression in certain regions of the utricle, such as the striola (Fig. 11D). *Notch1* is also expressed in the lagena in patterns similar to the utricular epithelium (data not shown).

A 3 days post-gentamicin, *Notch1* expression is highly elevated in cells located near the lumen in the basal, damaged basilar papilla (Fig. 11E). In most cases, cells with elevated levels of *Notch1* mRNA are organized into discrete pairs or

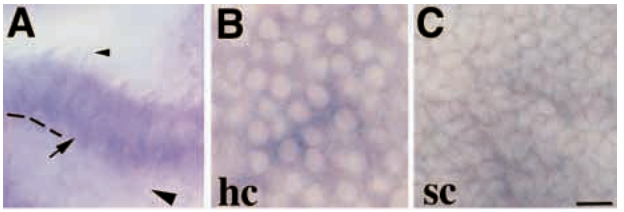

Fig. 10. *Serrate1* expression in inner ear epithelia. *Serrate1* mRNA labeling with in situ hybridization in whole-mount control basilar papilla (A) and utricle (B,C). *Serrate1* is heavily expressed in hair cells of basilar papilla (A, arrow and small arrowhead point to hair cell base and stereocilia, respectively) and mildly expressed in support cells (A, large arrowhead). The curved view of the epithelium was photographed here, allowing a side-view of the cells, similar to a section. The junction of the hair cell (hc) and support cell (sc) layers is shown as dashed lines. In whole mounts of the utricle, *Serrate1* mRNA is detected in both the hair cell (hc, B) and support cell (sc, C) layers. Scale bars, 10 μm.

clusters (Fig. 11E). *Notch1*-expressing pairs of cells are also detected in the hair cell layer of untreated and treated utricles (Fig. 11F). At 3 days post-gentamicin, we detected no significant change in *Notch1* expression in the utricle, except that it appears somewhat stronger in the striolar region (data not shown). By 10 days post-gentamicin, spatial patterns of *Notch1* expression have returned to normal in both organs (data not shown).

No distinct *Notch1* expression is seen outside of the sensory epithelial regions of the organs (data not shown). Further, no signal is seen in either untreated or drug-damaged tissue probed with sense *Notch1* cRNA (data not shown).

DISCUSSION

Cell production in the normal postembryonic chick is predominantly asymmetric, generating a hair cell and a non-hair cell

In mature avian vestibular organs, support cells, which are believed to serve as the progenitor cells to new hair cells and support cells, are mitotically active, and new hair cells and support cells are regenerated at a steady state throughout the life of the animal (reviewed in Oesterle and Rubel, 1996). This cell proliferation is a form of regeneration, not hypertrophy, since a small contingent of cells are continually dying and the overall size of the organ does not change (Jorgensen, 1991; Weisleder and Rubel, 1992; Kil et al., 1997). We have shown that cellular regeneration in the untreated chick utricle occurs mainly by asymmetric cell division: each mitotic event generates a hair cell and a non-hair cell. Evidence for asymmetric division as the primary mode of ongoing hair cell production in the chick utricle has been presented previously (Roberson et al., 1992). In addition, asymmetric division has been reported after damage-induced hair cell regeneration in the chick basilar papilla (Stone and Cotanche, 1994) and the salamander lateral line organ (Jones and Corwin, 1996). This mode of cell division, which generates a terminally differentiated cell (e.g. hair cell) and a progenitor cell, occurs in some vertebrate systems during development (e.g. mouse neocortex [Chenn and McConnell, 1995] and chick inner ear

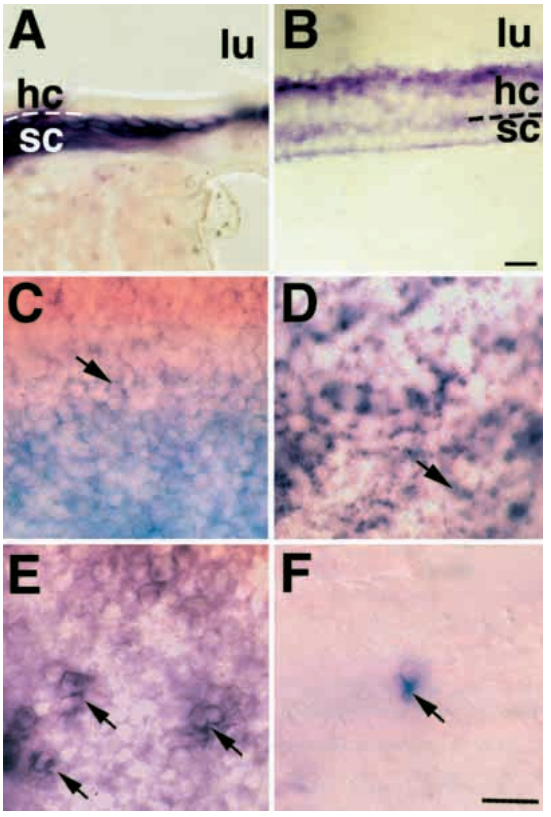

Fig. 11. *Notch1* expression in inner ear epithelia. *Notch1* mRNA labeling with in situ hybridization in sections (A,B) and whole-mounts (C-F) of control and drug-treated basilar papilla and utricle. In untreated chicks, *Notch1* is expressed in the basilar papilla (A,C), as well as the utricle (B,D). The junction of the hair cell (hc) and support cell (sc) layers is shown as dashed lines in A and B. In the basilar papilla, *Notch1* mRNA is confined to the support cell layer (A). *Notch1* expression (arrow) is seen throughout the support cell layer of the basilar papilla (C). In the utricle, *Notch1* mRNA is detected in the support cell layer and near the lumen (B). *Notch1* expression (arrow) is highly variable throughout the hair cell layer in the utricle (D). 3 days post-gentamicin, *Notch1* mRNA levels are elevated in pairs and clusters of cells (arrows) in the hair cell layer of the basilar papilla (E) and the utricle (F). lu, lumen. Scale bars: A,B, 20 μm; C-F, 10 μm.

[Fekete et al., 1998]). One benefit of asymmetric cell production is maintenance of the progenitor population.

Delta1 expression is activated during postembryonic hair cell production in chicks

During ongoing and damage-induced hair cell regeneration, cell proliferation and differentiation lead to the formation of

Table 3. Summary of in situ hybridization patterns

	Control utricle	Control basilar papilla	Damaged basilar papilla
Notch1	+	+	++
Delta1	+	–	++
Serrate1	+	+	+

+ moderate expression
++ high expression
– no expression

the correct numbers and types of cells, thereby re-establishing the highly stereotyped cellular array in the epithelium. Lateral inhibition has been proposed as a mechanism for regulating cell specification and patterning during hair cell regeneration (Cotanche, 1987; Corwin et al., 1991; Lewis, 1991), but it has not been experimentally tested. To begin to explore the role of Notch signaling and lateral inhibition in hair cell regeneration, we examined expression of *Notch1* and genes encoding its ligands, Delta1 and Serrate1, in the mature chick inner ear epithelia in regenerating and quiescent states.

In the mature inner ear, *Notch1* is transcribed throughout the support cell population in both the mitotically active vestibular epithelium and the mitotically quiescent auditory epithelium. In the normal utricle, *Delta1* is expressed in progenitor cells, in presumed sister cells shortly after mitosis, and in scattered hair cells in the early stages of differentiation. No *Delta1* mRNA is detected in the basilar papilla of untreated birds. However, following ototoxic drug treatment, *Delta1* and *Notch1* expression is elevated in the mitotically active regions of both vestibular and auditory epithelia, while cells in the undamaged regions of both epithelia exhibit no change in expression of either gene. After hair cell regeneration is complete and the new compliment of cells is established, *Delta1* and *Notch1* transcript levels return to baseline in auditory and vestibular epithelia. *Serrate1* is expressed uniformly throughout the auditory and vestibular sensory epithelia of the mature inner ear, and no change in *Serrate1* expression is detected after experimentally induced hair cell damage. These results are summarized in Table 3.

For the most part, the patterns of expression of Notch signaling genes in the postembryonic, regenerating inner ear that we report here parallel those described for the developing otocyst (Adam et al., 1998). *Serrate1* and *Notch1* are transcribed in all cells throughout the sensory epithelial anlagen as hair cells and support cells are born, and *Delta1* is expressed in a subpopulation of cells that will ultimately differentiate into hair cells. These findings suggest that similar molecular mechanisms may be employed to regulate cell fate and patterning during pre-embryonic hair cell generation and during ongoing and damage-induced postembryonic hair cell regeneration.

Delta1 is first expressed in mitotic hair cell progenitors

Delta1 mRNA levels are elevated during spontaneous and drug-induced hair cell regeneration in progenitor cells by 1.5 hours after they incorporate BrdU, which corresponds to mid-to-late S phase or early G_2 phase (Katayama and Corwin, 1993; Stone and Cotanche, 1994; Tsue et al., 1994). This finding contrasts with patterns of *Delta1* expression during development of most tetrapod vertebrates, in which *Delta1* transcripts are confined to individual post-M-phase cells (Chitnis et al., 1995; Myat et al., 1996; Henrique et al., 1997a). Further, our results differ from those reported for the developing chick otocyst (Adam et al., 1998), where *Delta1* mRNA is first detected in immature hair cells. The role of Delta1 at a relatively early stage of cell division during hair cell regeneration in the bird remains unclear. A recent study suggests that Notch may mediate arrest of the cell cycle in G_1 or G_2 phase (Johnston and Edgar, 1998). Thus, Delta1 activity in premitotic progenitor cells may inhibit adjacent cells from progressing through the cell cycle, thereby permitting the maintenance of the progenitor population and ensuring production of the proper number of cells (Henrique et al., 1997a). This mechanism clearly does not occur in a steady-state manner in the undamaged basilar papilla, since no *Delta1* transcripts are detected there, but it may be activated once hair cell damage has occurred. Alternatively, early transcription of *Delta1* may occur in preparation for cell fate determination after mitosis (see below).

The exact identity of the hair cell progenitor during avian hair cell regeneration is unknown. Progenitor cells in S phase have nuclei located in the support cell layer (Stone and Cotanche, 1994; Tsue et al., 1994; Warchol and Corwin, 1996) and they have similar ultrastructural features as support cells (Presson et al., 1996). Thus, support cells represent the best candidates for progenitor cells. We found that, at 1.5 hours after BrdU injection, when most proliferative cells incorporating BrdU would be in S phase, the numbers of BrdU-labeled nuclei are evenly distributed between the hair cell and support cell layers (Table 1). This observation is in contrast to the findings of Tsue et al. (1994) in the same tissue – the normal, undamaged chick utricle. They reported that only 5% of cells that incorporated [^{3}H]thymidine after a 1-hour pulse are located in the hair cell layer. It is likely that this discrepancy is attributable to one of two methodological factors: (1) we used a longer nucleotide pulse period (1.5 hours), which may have permitted more lumenal migration of progenitor cell nuclei, and (2) we used whole-mount preparations, whereas Tsue et al. (1994) used sections and, as a result, the boundary between the support cell and hair cell layers may have been designated at different depths relative to the lumen.

An alternative explanation for this discrepancy is that the primary progenitor for new cells during regeneration is not a support cell, but is another type of cell – perhaps a stem cell – whose nucleus resides in the hair cell layer during some stage of the cell cycle. Accordingly, it is possible that the *Delta1*-positive and BrdU-positive cells that we detected in the hair cell layer at 1.5 hours post-BrdU represent such a cell, which we will call an ‘x-cell’. This explanation is supported by our observation that 69% of BrdU-labeled cells at 8 days post-BrdU reside in the hair cell layer and 31% reside in the support cell layer (Table 1). If each asymmetric division always generates a hair cell and a support cell, then one would predict that BrdU-labeled cells would be equally split between the two layers at this time, when differentiation of each cell type has occurred. On the contrary, the ratios that we observed more accurately fit a model in which each division yields either a hair cell and a support cell, or a hair cell and an x-cell (which resides in the hair cell layer). In this case, the likelihood of generating each cell type is: 50% for a hair cell, 25% for a support cell, and 25% for an x-cell.

Notch1 and *Delta1* are equally expressed in sister cells destined to take on separate fates

During hair cell regeneration, we noted expression of *Delta1* and *Notch1* in pairs of closely associated cells that were identified as post-M-phase sister cells. Sister pairs in the normal chick utricle represent an equivalence group, since each cell appears to have equal potential to form a hair cell or a non-hair cell. *Notch1* and *Delta1* transcripts appear to be equally partitioned between the two sister cells during mitosis. Based

on these observations at the mRNA level, both cells generated in a single mitotic event may initially have equal potential to signal through *Notch1* and thereby to inhibit differentiation along the hair cell pathway. We hope to confirm this finding in future studies using antibodies to *Notch1* and *Delta1*. If protein levels are indeed equal in post-M-phase sister cells, then expression of neurogenic genes during hair cell regeneration more closely resembles the uniform levels seen in *Drosophila* proneural clusters (Haenlin et al., 1990; Kooh et al., 1993; Hinz et al., 1994; Kunisch et al., 1994) than the asymmetric levels between sister cells reported during mouse neocortical development (Chenn and McConnell, 1995).

By 3 days after BrdU uptake in the chick utricle, *Delta1* expression is downregulated in one of the sister cells, which will become a non-hair cell, and upregulated in the other sister cell, which will become a hair cell. A similar progression toward asymmetry in *Delta1* expression within an equivalence group occurs in invertebrate systems (Goriely et al., 1991; Heitzler and Simpson, 1991) and is thought to represent a shift in Notch signaling through which individual cells are selected to differentiate. The mechanism by which the initial symmetry in *Delta1* expression is overcome is unknown, but it has been postulated that stochastic changes in relative levels of Delta among equivalent cells are amplified by positive feedback through Notch signaling (Goriely et al., 1991; Heitzler et al., 1991; Collier et al., 1996). Recently, doubt has been shed on the significance of the asymmetry of *Delta1* expression in guiding cell fate decisions in the *Drosophila* nervous system. Studies in which all cells in a proneural equivalence group are induced to express equal levels of *Delta1* demonstrated that asymmetric *Delta1* expression is not necessary to attain appropriate specification of individual cells in an equivalence group (Seugnet et al., 1997). Therefore, it is likely that other cellular mechanisms are involved. For example, individual cells in equivalence groups may be endowed with intrinsic asymmetries at early times. During mitosis, they may inherit relatively different levels of Numb, a membrane-associated protein that is thought to control cell fate by antagonizing Notch activity (Jan and Jan, 1995), or they may possess higher levels of proneural gene activity, which drives differentiation in a cell-autonomous fashion (Cubas et al., 1991; Goriely et al., 1991; Henrique et al., 1997b). These additional mechanisms governing cell fate decisions remain to be examined in the regenerating inner ear.

Is there a role for *Notch1* in mitotically quiescent tissues?

In the normal avian auditory organ (the basilar papilla), *Notch1* is abundantly expressed in the support cell population, which is growth-arrested in the G_1 phase of the cell cycle (reviewed in Cotanche et al., 1994; Bhave et al., 1995). This observation raises the following questions: Is Notch signaling active in the mitotically quiescent epithelium? If so, what is its role there? And, which ligand(s) is stimulating (or blocking) Notch1 activity?

Hair cells are selectively expelled from inner ear sensory epithelia after ototoxic damage. Support cells persist, and some are triggered to advance in the cell cycle and form new hair cells. Several recent studies suggest that hair cells may also be generated by a non-mitotic mechanism, termed direct transdifferentiation, after they are damaged (reviewed in Roberson and Rubel, 1995: Stone et al., 1998). During this process, support cells are thought to convert into hair cells, by transforming their original pattern of gene expression into one of hair cells. It has been hypothesized that progenitors are released from growth arrest due to a change in direct cell-cell signaling after hair cell loss (Cotanche, 1987; Lewis, 1991; Corwin et al., 1991). One can extend this hypothesis to include an inhibitory role of cell-cell signaling on direct transdifferentiation of support cells into hair cells in control tissues. Our observations demonstrate that Delta1 is not responsible for mediating a steady-state block on mitotic activity or direct transdifferentiation of support cells, since it is not expressed in the untreated basilar papilla. On the contrary, it is possible that a Delta subtype other than Delta1 is responsible for this signaling; in other vertebrates, such as zebrafish, multiple Delta subtypes appear to play different roles during development (Haddon et al., 1998a; Dornseifer et al., 1997; Appel and Eisen, 1998). We were unable to explore this possibility, since only *Delta1* has been cloned in the chicken at this time.

Since *Serrate1* is expressed in hair cells in undamaged epithelia, it serves as a more suitable candidate for a steady-state lateral inhibitor of mitosis or transdifferentiation by support cells. This role remains to be tested experimentally. *Serrate1* is expressed in sensory, but not non-sensory, regions of the inner ear organs, in both regenerating and developing animals (Adam et al., 1998). *Notch1* transcripts are similarly restricted. These patterns of gene expression suggest another potential role for Serrate/Notch signaling: to generally maintain the differentiated states of cells within specialized regions of the inner ear.

We have proposed different roles for Delta1 and Serrate1 in the inner ear sensory epithelia of postembryonic chicks, but we have demonstrated the expression of a single receptor gene, *Notch1*. This proposal presents a logistical problem: it is not evident how a single Notch receptor subtype is able to trigger two different responses. There may be an additional Notch subtype present in the inner ear epithelia, and Delta1 may act through one Notch subtype while Serrate1 acts through another. Alternatively, signaling through Notch1 by each ligand may have various effects in different cellular contexts; e.g. the effect of a single ligand may depend on the cell's level of maturation. Cells with different developmental status (mitotic versus terminally differentiated) may have different degrees of responsiveness to Notch activation due to relative variations in intrinsic factors, e.g. expression of relevant transcription factors (see Lee, 1997) and activity of molecules that cleave Notch (see Kopan and Cagan, 1997). Further, Notch activation by each ligand may vary between cells with different cellular contexts because of differences in ligand concentrations or localizations (Weinmaster, 1997). Finally, although *Notch1* transcripts are present in the untreated basilar papilla, it remains to be proven that they are translated and that Notch signaling is, in fact, active.

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