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Hair Cell Differentiation in Chick Cochlear Epithelium after Aminoglycoside Toxicity: *In Vivo* and *In Vitro* Observations

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Inner ear epithelia of mature birds regenerate hair cells after ototoxic or acoustic insult. The lack of markers that selectively label cells in regenerating epithelia and of culture systems composed primarily of progenitor cells has hampered the identification of cellular and molecular interactions that regulate hair cell regeneration. In control basilar papillae, we identified two markers that selectively label hair cells (calmodulin and TUJ1 β tubulin antibodies) and one marker unique for support cells (cytokeratin antibodies). Examination of regenerating epithelia demonstrated that calmodulin and β tubulin are also expressed in early differentiating hair cells, and cytokeratins are retained in proliferative support cells. Enzymatic and mechanical methods were used to isolate sensory epithelia from mature chick basilar papillae, and epithelia were cultured in different conditions. In control cultures, hair cells are morphologically stable for up to 6 d, because calmodulin immunoreactivity and phalloidin labeling of filamentous actin are retained. The addition of an ototoxic antibiotic to cultures, however, causes complete hair cell loss by 2 d *in vitro* and generates cultures composed of calmodulin-negative, cytokeratin-positive support cells. These cells are highly proliferative for the first 2–7 d after plating, but stop dividing by 9 d. Calmodulin- or TUJ1-positive cells reemerge in cultures treated with antibiotic for 5 d and maintained for an additional 5 d without antibiotic. A subset of calmodulin-positive cells was also labeled with BrdU when it was continuously present in cultures, suggesting that some cells generated in culture begin to differentiate into hair cells.

Key words: hair cells; regeneration; chick; basilar papilla; cell culture; differentiation

Hair cells are sensory receptors for hearing, equilibrium, and motion detection. Some animals demonstrate the capacity to generate hair cells throughout their lifetime (Popper and Hoxter, 1984; Corwin, 1985; Jörgenson and Mathiessen, 1989; Roberson et al., 1992) or to initiate hair cell regeneration in the event of their loss (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). The progenitors of hair cells seem to be a subset of support cells that reside adjacent to hair cells in the sensory epithelia (Girod et al., 1989; Balak et al., 1990; Raphael, 1992; Hashino and Salvi, 1993; Weisleder and Rubel, 1993; Stone and Cotanche, 1994; Tsue et al., 1994a; Roberson et al., 1996). Although mature mammals normally do not generate new hair cells, recent *in vivo* and *in vitro* studies have documented mitotic activity and immature-looking hair cells in mammalian vestibular epithelia after exposure to ototoxic drugs (Forge et al., 1993; Warchol et al., 1993; Rubel et al., 1995), suggesting that hair cell regeneration in mammals may be inducible. The development of culture methods for mature cochlear and vestibular end organs has been initiated to identify molecules that regulate cell proliferation and differentiation in avian and mammalian hair cell epithelia. Co-culture experiments suggest that a diffusible molecule is generated in drug-damaged chick utricles that can stimulate support cell mitosis in undamaged organs (Tsue et al., 1994b). Also, insulin growth factor-1 and transforming growth factor α upregulate cell proliferation in cultured vestibular organs of birds (Oesterle and Rubel, 1996) and mammals (Lambert, 1994; Yamashita and Oesterle, 1995), respectively. Similar progress has been made in identifying factors that regulate cell fate in inner ear epithelia using embryonic organ culture. *In vitro* studies of the prenatal mouse organ of Corti have shown that retinoic acid stimulates cells to differentiate into hair cells (Kelley et al., 1993). Despite these advances, the numerous heterogeneous tissue types present in organ cultures complicate the identification of specific molecular interactions that regulate hair cell regeneration. This problem would be eliminated in cell cultures composed of hair cell progenitors.

The posthatch chick cochlear epithelium is an excellent model for studying hair cell regeneration. Like the organ of Corti, there are normally no mitotically active cells, but in contrast to mammals, the process of hair cell loss stimulates progenitor cells to generate new hair cells. The present study uses a newly developed technique for generating cultures composed primarily of support cells, the presumed hair cell progenitors, from the chick cochlear epithelium. In addition, we characterize immunochemical markers for progenitor cells and early differentiating hair cells during regeneration *in vivo* and apply them to study hair cell loss and regeneration *in vivo* and in cultures. Immunoreactivity for the hair cell marker calmodulin disappears in cultures when hair cell loss is induced but reappears in postmitotic, presumptive differentiating hair cells in cultures several days later. Thus, hair cell differentiation seems to be initiated in cultures of chick cochlear support cells.

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MATERIALS AND METHODS

Animal housing

White Leghorn chicks (*Gallus domesticus*) were purchased from H & N International (Redmond, WA) and housed in heated brooders with ample food and water in the animal care facility of the University of Washington. More than 300 chicks were used for this study, including pilot work for the *in vivo* and *in vitro* studies. All procedures described below were approved by the Animal Care Committee of the University of Washington.

Aminoglycoside treatment in vivo

Chicks between 5 and 7 d posthatch (40–55 gm) were given a single intraperitoneal injection of gentamicin (400 mg/kg; Lyphomed, Deerfield, IL). Age-matched control chicks did not receive a gentamicin injection. Chicks were allowed to recover from the transient systemic toxicity induced by gentamicin in a heated chamber for 3–5 hr and were then returned to the Animal Care Facility where they were provided with ample food and water until euthanasia. Approximately 25% of the injected chicks died as a result of gentamicin toxicity. At 2, 4, 7, or 10 d after gentamicin injection, chicks were euthanized by an intraperitoneal injection of sodium pentobarbital and then decapitated. Cochlear ducts were removed and placed in ice-cold oxygenated HBSS (Life Technologies, Grand Island, NY). After dissection of the tegmentum vasculosum with fine microforceps, the remaining tissue was placed in 0.01% Type I collagenase (Sigma, St. Louis, MO) in HBSS for 3 min. The tectorial membrane was subsequently removed by grabbing it at the apical end of the basilar papilla (the avian equivalent of the organ of Corti) with microforceps and pulling it off the entire length of the sensory epithelium. Basilar papillae were fixed in 4% paraformaldehyde for 30 min, rinsed in PBS, and stored at 4°C until immunohistochemistry was performed.

Immunohistochemistry of cochlear whole mounts

Whole mounts of the basilar papilla were treated with 0.5% H_2O_2 in PBS for 15 min to block endogenous peroxidase activity. After rinses with PBS, 10% normal horse serum in 0.05% Triton X-100/PBS was added for 20 min to block nonspecific immunoglobulin (IgG) binding. For single-labeling experiments, whole mounts were reacted with one of the following monoclonal antibodies for 2 hr at room temperature or overnight at 4°C: anti-*Dictyostelium discoideum* calmodulin (diluted 1:500, Sigma clone 6D4); TUJ1 (diluted 1:1000, a gift from T. Frankfurter, University of Virginia), which detects a neuron-specific class III β tubulin (Lee et al., 1990; Easter et al., 1993); anti-cytokeratins (diluted 1:200; clone 8.13, Sigma) raised against acidic and basic bovine epidermal keratins; or anti-cytokeratins (diluted 1:200; clones AE1 and AE3, Boehringer Mannheim, Indianapolis, IN) raised against acidic and basic human epidermal keratins. Tissue was treated with biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) for 30 min followed by the avidin–biotin–horseradish peroxidase (HRP) reagent (ABC kit BA-2000; Vector Laboratories). Up to this point, all rinse steps were performed in PBS. The tissue was transferred to 50 mM Tris/HCl buffer (Tris, pH 7.6) and treated with 0.04% 3,3′-diaminobenzidine (DAB) and 0.05% H_2O_2 diluted in Tris for 3–10 min. The tissue was then rinsed once in Tris and left in Tris until embedding. For all immunoreactions, the primary antibody was omitted from the reactions as a negative control. Some immunoreacted whole mounts were mounted onto microscope slides coated with 9:1 glycerol/PBS, coverslipped, and examined with a Leitz Aristoplan microscope. Additional whole mounts were embedded in plastic as follows. Specimens were dehydrated through a graded series of ethanol washes (10 min each step) and placed in propylene oxide for two 10 min intervals. Subsequently, whole mounts were placed in a 1:1 mixture of propylene oxide and soft Spurr's plastic, followed by two changes of 100% soft Spurr's plastic (20 min each). Finally, whole mounts were positioned in a cassette mold overnight at 60°C to allow polymerization of the plastic. After they were embedded, immunoreacted whole mounts were sectioned at 3 μM in a basal-to-apical manner. Every fifth and sixth section throughout the basilar papilla was mounted onto a set of two chrome–alum subbed microscope slides. One set of sections was counterstained with toluidine blue. Both sets of sections were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ) and examined with a Leitz Aristoplan microscope.

For TUJ1/calmodulin co-labeling, the TUJ1 antibody was detected in whole mounts using Bodipy/fluorescein isothiocyanate (FITC)-conjugated IgG (1:300; Molecular Probes, Eugene, OR), and the calmodulin antibody was detected in the same tissue with lissamine rhodamine-conjugated IgG (1:300; Jackson Immunoresearch Labs, Westgrove, PA). Whole mounts were mounted with Vectashield (Vector Labs) and examined with a BioRad MRC-1000 confocal laser scanning microscope. Images were digitized using Comos Version 7 software (BioRad, Richmond, CA), imported into Photoshop (Adobe, Mountain View, CA), and printed with a Phaser IISDX dye-sublimation printer (Tektronix, Beaverton, OR).

Differentiation of hair cells in vivo. To assess the temporal and spatial progression of differentiation of regenerated hair cells, chicks were administered a single intramuscular injection of tritiated thymidine (^{3}H-thymidine; 10 μCi/gm; 70–90 Ci/mmol) or an intraperitoneal injection of bromodeoxyuridine (BrdU; 100 mg/kg) at 3 d after gentamicin injection, when cell proliferation is maximal (Bhave et al., 1995). Chicks were euthanized at 2, 24, 48, 72, or 240 hr (10 d) after injection of the S phase marker by an intraperitoneal injection of sodium pentobarbital, and whole mounts of the basilar papillae were removed and fixed. For chicks that received a BrdU injection, whole mounts were treated with 2N HCl in 0.05% Triton X-100/PBS for 15 min at 37°C. After several rinses in PBS, whole mounts were treated with 0.5% H_2O_2 in PBS for 15 min followed by 10% normal horse serum in 0.05% Triton X-100/PBS for 20 min. Anti-BrdU monoclonal antibody (1:100; Becton Dickinson, San Jose, CA) was added for 1 hr, and the antibody was detected via the ABC/HRP reaction described above. BrdU-labeled whole mounts were then immunoreacted to detect calmodulin using the ABC/HRP reaction described above. For chicks that received a ^{3}H-thymidine injection, whole mounts were immunoreacted for calmodulin only. All basilar papillae were embedded in soft Spurr's resin and sectioned at 3 μM in a basal-to-apical manner, and serial sections were mounted onto chrome-alum subbed microscope slides. To detect ^{3}H-thymidine, slides were dipped in NTB2 emulsion (1:1 dilution; Kodak), exposed at 4°C for 5–14 d, developed in D-19, fixed, rinsed, and dried. For both proliferation labels, one set of sections was counterstained with toluidine blue and coverslipped with Permount.

Quantification of ^{3}H-thymidine/calmodulin labeling. Basilar papillae were chosen for quantitative analysis of calmodulin and ^{3}H-thymidine double labeling on the basis of their integrity after processing. Specifically, basilar papillae were included if they had intact cells in all regions and no overlying tectorial membrane, which may hamper penetration of the antibodies. Two to four basilar papillae were analyzed for each experimental treatment using a Leitz Aristoplan microscope.

To identify temporal and spatial patterns of cell proliferation and differentiation in basilar papillae damaged *in vivo*, we performed two analyses on calmodulin-immunoreacted basilar papillae from chicks that received a single ^{3}H-thymidine injection and were euthanized at different time intervals, as described above. First, we counted the number of calmodulin-positive cells with nuclei in either the lumenal compartment (corresponding to the lumenal one third of the epithelium) or the adlumenal compartment (corresponding to the adlumenal two thirds of the epithelium) from chicks at each survival time. The nuclear location and number of calmodulin-positive cells were determined in every section from one series of sections. This analysis was performed only on sections of the basal-most 300 μM of the epithelium. This analysis generated information about the rate of hair cell differentiation in aminoglycoside-damaged regions. Second, we counted ^{3}H-thymidine-positive/calmodulin-negative cells and ^{3}H-thymidine-positive/calmodulin-positive cells in the region. This analysis provided data about the timing of support cell proliferation and support cell and hair cell differentiation in the basal region.

Calmodulin antibody specificity

To test the specificity of the calmodulin immunoreactivity in the chick basilar papilla, we analyzed Western blots of chick cochlear tissue with the original anti-calmodulin antibody and examined the cochlear immunoreactivity of two additional anti-calmodulin antibodies.

Western blot. For Western blots, whole cochlear ducts from six to eight untreated chicks were used. Cochlear ducts were dissected and solubilized in a PAGE sample buffer that contained 5 mM EGTA but did not include SDS or dithiothreitol. Heating of samples was not necessary during solubilization. Samples were sonicated and frozen at −20°C to aid in tissue disruption. The sample concentration was 3 mg/ml as determined by absorbance at 280 nm. A purified calmodulin standard from bovine brain (Sigma) was treated with the same sample buffer. Samples were subjected to native PAGE according to Laemmli (1970), with modifications according to Geiser et al. (1991). The cochlear sample and the calmodulin standard were run on 14% gels (acrylamide/bis-

acrylamide ratio of 38:2) with no stacking gel. All solutions were prepared without SDS. Molecular weight standards were obtained from BioRad.

Proteins were transblotted to polyvinyldine fluoride (PVDF) membrane (Millipore, Bedford, MA) at 50 mV for 2 hr. Proteins were cross-linked to PVDF according to Hulen et al. (1991). PVDF membrane was treated with 0.2% v/v glutaraldehyde for 45 min, followed by two 10 min rinses in 0.5 mg/ml sodium borohydride. The membrane was allowed to air-dry, and immunoblotting was carried out without subsequent methanol wetting according to instructions of the manufacturer. The mouse monoclonal anti-calmodulin antibody (clone 6D4, Sigma) was used at 1:2000, followed by an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:1000; Cappel, Durham, NC). The reaction was visualized using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate alkaline phosphatase kit (BioRad).

Immunohistochemistry. For each antibody reaction, cochlear ducts from two chicks at posthatch day 7 were dissected and fixed as described above for calmodulin immunohistochemistry. All steps for these immunoreactions were identical to those described above, except that the primary antibodies used were from UBI (Lake Placid, NY) (mouse monoclonal antibody directed against synthetic peptide corresponding to the 21 C-terminal amino acids of bovine calmodulin, diluted 1:500) and ICN (Costa Mesa, CA) (goat polyclonal antibody raised against bovine thymus calmodulin, diluted 1:500).

Cochlear epithelial cultures

For each experiment, eight chicks between 4 and 12 d posthatch were decapitated, and the cochlear ducts were removed through the round window and placed in ice-cold dissection medium composed of DMEM plus F12 (DMEM/F12; Life Technologies) and 1% bovine serum albumin (Sigma). The tegmentum vasculosum was removed, and the cochlear duct was placed in room temperature dissection medium with 0.01% collagenase Type I (Sigma) for 5 min. The tectorial membrane was dissected off the sensory epithelium, and the cochlear duct was left in collagenase for an additional 10 min. The needle of a tuberculin syringe was used to loosen the sensory epithelium from the basal lamina, generating large pieces of epithelium and leaving the underlying stroma, border cells, hyaline cells, and clear cells *in situ*. The isolated sensory epithelial cells, composed of hair cells and support cells, were transferred to culture medium composed of DMEM/F12, 5% fetal bovine serum (Sigma), 2 mM sodium bicarbonate, 5 mM HEPES, 0.6% glucose, and a hormone supplement [6 mM putrescine, 0.25 mg/ml insulin, 1 mg/ml transferrin, 400 nM progesterone, and 3 μM sodium selenite (all from Sigma)], mildly triturated with a plastic 1 ml pipette, and spun at 500 rpm for 2 min. The resulting pellets were resuspended in ~0.5 ml of culture medium and plated into one of the following: four wells of a 96-well plastic tissue culture plate (Nunc), four wells of a 16-well tissue culture chamber slide (Nunc), or two wells of a four-well plastic tissue culture plate containing 13 mm^2 glass coverslips. Coverslips and culture plates were either uncoated or coated with 3.0 μg/cm^2 laminin (Life Technologies) or 5 μg/cm^2 fibronectin (Boehringer Mannheim). All media were replenished every 3–4 d.

Hair cells in cultures were killed by adding 65 μM streptomycin (Life Technologies) to culture media at the time of plating. Some cultures were fixed after 2 d with paraformaldehyde for 15 min, and cell types in these cultures were characterized immunocytochemically using antibodies to calmodulin and cytokeratins (see below). Additional cultures were treated with 65 μM streptomycin for 5 d to eliminate all hair cells and were maintained an additional 5 d in control media. In some of these cultures, cells that began to acquire a hair cell phenotype were detected using antibodies to calmodulin or TUJ1 (see below). To determine whether cells with a hair cell phenotype were generated in culture, additional cultures were provided with BrdU (1 μM) for the entire culture period, fixed with paraformaldehyde, and immunoreacted to detect BrdU and calmodulin (see below).

The number of mitotic support cells after different periods of culture was estimated using BrdU pulse/fix labeling. BrdU (10 μM; Sigma) was added to culture media for 2 hr at 2, 3, 5, 7, 9, or 11 d after plating. (BrdU labeling was not performed at earlier culture time points because epithelial attachment was not complete.) Immediately after the 2 hr BrdU pulse, cultures were fixed with paraformaldehyde and immunoreacted to detect BrdU (see below). Two culture wells per time point were analyzed.

The cell types that remained in the cochlear duct after sensory epithelia isolation and culturing were characterized as follows. After the sensory epithelium was dissected, the remaining tissue was fixed in 4% paraformaldehyde/0.1% glutaraldehyde overnight at 4°C, embedded in Spurr's plastic, and sectioned as described above for whole mounts. Sections were counterstained with toluidine blue.

Immunohistochemistry of epithelial explants. The types and dilutions of all primary antibodies used to characterize cultures were the same as those described above for whole mounts. The ABC/HRP method was used in all antibody reactions, except for BrdU/calmodulin double labeling (described below). For all immunoreactions, the primary antibody was omitted from some cultures as a negative control.

Long-term cultures supplemented with BrdU were double-labeled with antibodies to BrdU and calmodulin. First, BrdU was detected using a rat monoclonal antibody (Sera-Lab, Sussex, England) diluted at 1:200 overnight at 4°C followed by FITC-conjugated goat anti-rat antibody (Cappel) diluted at 1:400 for 45 min. Second, calmodulin was labeled in the same tissue using the HRP method described above for whole mounts.

Before microscopic analysis, the culture chambers were removed from slides, and explants were coverslipped with 9:1 glycerol/PBS. Cultures that were grown on coverslips were placed face up on top of a drop of glycerol on a microscope slide and coverslipped with additional glycerol. Labeled tissues were examined with a Leitz Aristoplan microscope. Cultures that were double-labeled for BrdU and calmodulin were examined using confocal microscopy.

Quantification of pulse/fix BrdU labeling. BrdU labeling was quantified in cultures as follows. The number of BrdU-labeled cells per square millimeter was determined using a 10 × 10 eyepiece reticule mounted into a Nikon NMS inverted microscope. The culture dish was arranged on the stage such that the top left boundary of the well was aligned with the top left reticule square. Using a 10× objective, the number of BrdU-labeled cells per reticule square was counted and recorded. The plate was then moved to the left until the reticule lined up with the next region to be analyzed. At the right-hand edge of the well, the plate was shifted upward, and counting proceeded from right to left across the well in the next row. Once counting had proceeded to the left-hand edge of the well, the plate was shifted upward again, and the process was restarted. The total number of labeled cells per well was then converted to the number of labeled cells per square millimeter. BrdU-labeled cells in at least three wells were counted for each time point.

Filamentous actin labeling of hair cells. Cultures or whole mounts of the basilar papilla were fixed with paraformaldehyde and labeled with rhodamine phalloidin (Molecular Probes) diluted 1:50 in 0.05% Triton X-100 in PBS for 1.5 hr. Cultures were coverslipped as described above, except with Vectashield (Vector Laboratories) rather than glycerol, and examined using epifluorescence microscopy.

RESULTS

Histology of the normal and drug-damaged cochlear epithelium

The anatomy of the posthatch chick cochlear duct has been well characterized by numerous investigators (Retzius, 1884; Tanaka and Smith, 1978; Tilney and Saunders, 1983; Tilney et al., 1987; Manley, 1990). The duct is a 2- to 3-mm-long sickle-shaped organ containing the sensory epithelium (or basilar papilla) and other cell types. The sensory epithelium is composed of two morphologically distinct cell types: hair cells and support cells (Fig. 1*A*). Avian hair cells have been classified into three types, which vary systematically across the width of the epithelium (Takasaka and Smith, 1971; Manley, 1990). Tall hair cells are columnar and located toward the superior region of the epithelium, whereas short hair cells are bucket-shaped (Fig. 1*B*) and located toward the inferior (abneural) epithelium. Intermediate hair cells are interposed between the two types and resemble the short hair cells in shape.

Hair cell nuclei and cell bodies are confined to the lumenal surface of the epithelium (Fig. 1*B*). In contrast, support cells span the entire depth of the epithelium, and their nuclei are located below the hair cell nuclei. All hair cells have two prominent lumenal specializations: a bundle of actin-filled stereocilia and a subjacent cuticular plate, which is composed of a meshwork of cytoskeletal proteins. The hair cell cytoplasm is packed with polyribosomes and thus has a very dark appearance with Nissl

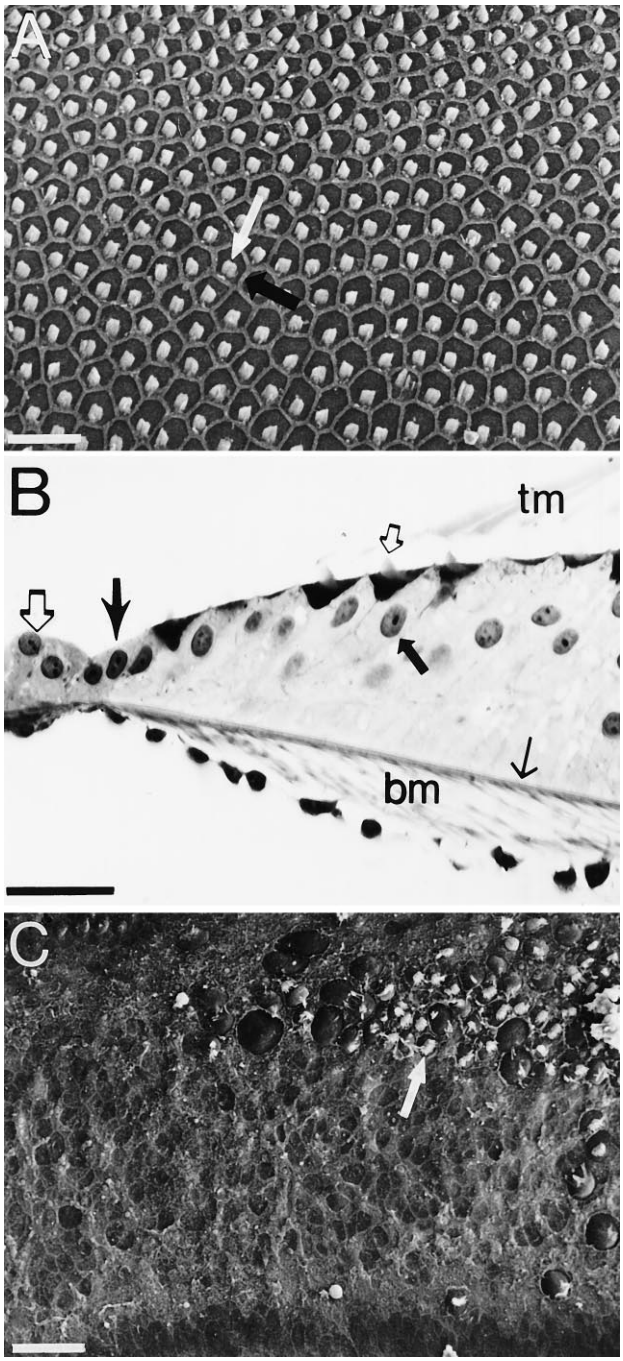

Figure 1. Anatomy of the chick cochlear epithelium. *A*, The highly organized array of hair cells spans the width of the chick cochlear epithelium, as shown in this scanning electron micrograph of the lumenal surface of the basal, high-frequency region of the epithelium. Each hair cell has a bundle of stereocilia (*white arrow*) protruding from its round surface and is surrounded by the lumenal portion of support cells, whose surfaces are rectangular and highly constricted (*black arrow*). *B*, The cochlear epithelium is separated from the underlying stroma, or basilar membrane (*bm*), by a thin basal lamina (*thin arrow*), as shown in this toluidine blue-stained transverse section of the inferior portion of the epithelium. The tectorial membrane (*tm*) occupies the lumen above the epithelium. Because of fixation shrinkage of the tectorial membrane, it no longer contacts the hair cells and support cells, as it does *in vivo*. The stereocilia of hair cells (*small shadowed arrow*) protrude into the lumen. Only short hair cells are shown here, because they predominate in the basal epithelium. The cell bodies of hair cells are confined to the lumenal surface of the epithelium, whereas support cells span the entire depth of the epithelium. In control basilar papillae, support cell nuclei (*straight black arrow*) reside below the hair cell nuclei at different levels relative to the basal lamina. Border cells (*flared black arrow*) and hyaline cells (*large shadowed arrow*) lie outside the sensory epithelium. *C*, Three days after a single injection of gentamicin, hair cells in the basal third of the epithelium have been killed, and the support cells expand to occupy the hair cell-free regions. The zone of transition between the basal, damaged region (toward the *left*) and the apical, undamaged region (toward the *right*) is shown in this scanning electron micrograph. A swollen, surviving hair cell is indicated by a *white arrow*. Scale bar, 25 μm.

stains. The lumenal surfaces of support cells are covered with microvilli, and these cell bodies have relatively low levels of polyribosomes. There are several additional types of cells located adjacent to the sensory epithelium (Fig. 1*B*). Along the inferior edge, there are one to two rows of border cells and several rows of hyaline cells (Cotanche et al., 1992; Oesterle et al., 1992). On the superior edge, homogene cells and clear cells border the sensory epithelial cells.

The ototoxic drug gentamicin, when delivered as a single intraperitoneal injection at 100–400 mg/kg, causes a basal-to-apical progression of hair cell loss throughout the basal, high frequency portion of the basilar papilla (Bhave et al., 1995; Janas et al., 1995). Hair cell loss is detectable by 1 d after injection in the basal tip of the basilar papilla, and it spreads to include the basal one third (~ 800 μM) of the epithelium by 3–5 d after injection (Fig. 1*C*). At this time, all native hair cells are lost from the basal 300–500 μM. Damaged hair cells can be detected at the apical border of the lesion (the transitional zone) between 1 and 5 d after injection, but hair cell morphology in the apical two thirds of the basilar papilla remains normal after the injection. At the basal end, support cells persist after hair cell loss, and their surfaces expand to fill in the areas previously occupied by hair cells. Support cell proliferation occurs between 1 and 7 d after gentamicin injection, with a peak in nucleotide incorporation occurring at 3 d (Bhave et al., 1995). By 5 d after injection, hair cell regeneration is apparent, because small cells with immature stereocilia bundles are detectable by scanning electron microscopy (Bhave et al., 1995; Janas et al., 1995), and these cells label with tritiated thymidine when it is continually provided after damage (Roberson et al., 1996).

Immunolabeling of hair cells in normal and drug-damaged cochlear epithelium

We reacted whole mounts of the basilar papilla with antibodies to calmodulin, which have been shown to label hair cells selectively in the mature mammalian organ of Corti (Flock et al., 1986; Slepecky et al., 1988; Bauwens et al., 1991; Slepecky and Ulfendahl, 1993), and with the TUJ1 antibody, which labels class III β tubulin in early differentiating neurons (Moody et al., 1989; Lee et al., 1990; Easter et al., 1993). In addition to examining the pattern of antibody labeling in the intact whole mounts, we embedded immunoreacted cochleae in plastic and sectioned them so that the cellular distribution of each antigen could be determined.

The antibody raised against calmodulin (Sigma) strongly labeled the cytoplasm of intermediate and short hair cells in all frequency regions of the sensory epithelium, but only lightly labeled the cytoplasm of tall hair cells (Fig. 2*A,B*). The stereocilia and cuticular plates of all hair cells were labeled strongly, but nuclei were not labeled. No support cells were immunoreactive with the calmodulin antibodies; however, hyaline cells displayed light calmodulin labeling throughout their cytoplasm (data not shown). Omission of the primary antibody resulted in complete loss of immunoreaction (data not shown). When we gave chicks a

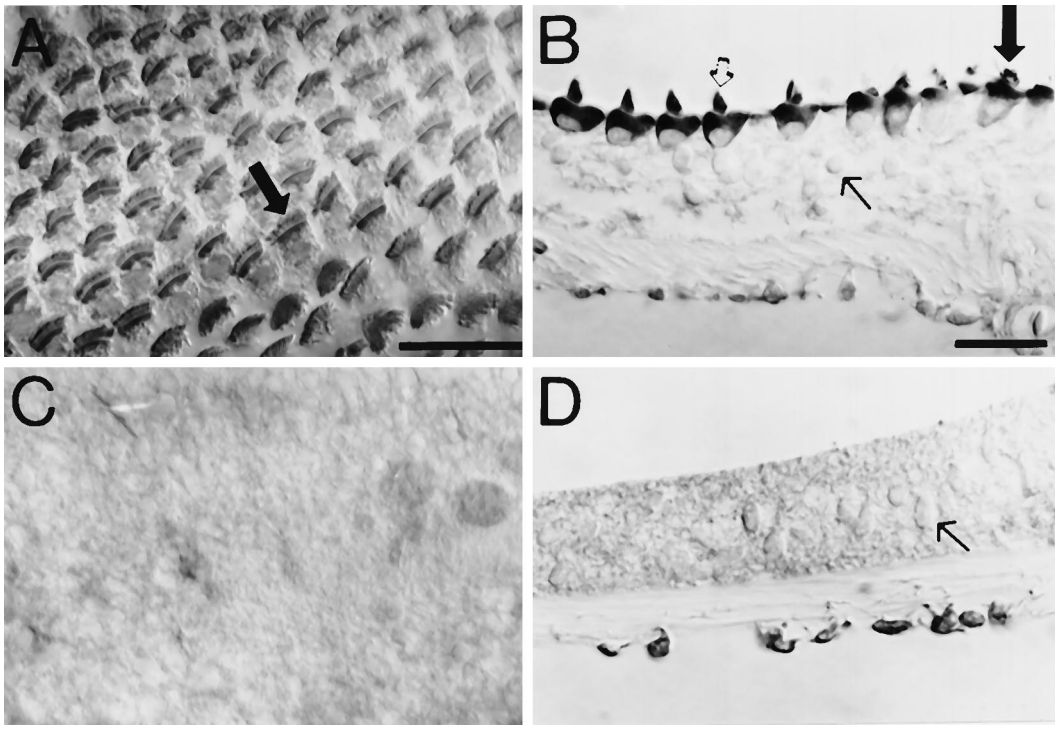

Figure 2. Calmodulin immunolabeling of hair cells in control and drug-damaged chick basilar papillae. *A* and *C* are micrographs of the lumenal surfaces of whole mounts of the midbasal region of the basilar papilla; the inferior edge is toward the *bottom* of the micrograph. *B* and *D* are micrographs of transverse sections of a region similar to that in *A* and *C*; the inferior edge of the epithelium is toward the *left*. *A*, Stereocilia (*arrow*) and cytoplasm of short hair cells in control basilar papillae strongly labeled with antibodies to calmodulin, but the lumenal surfaces of intervening support cells were unlabeled. *B*, Antibodies to calmodulin strongly labeled the cytoplasm and stereocilia (*shadowed arrow*) of short hair cells in control basilar papillae but did not label the nuclei. Tall hair cells (*thick black arrow*) were labeled more lightly than short hair cells. There was no detectable anti-calmodulin labeling in support cells (support cell nucleus indicated by *thin arrow*). *C*, Two days after gentamicin treatment, very few cells were strongly labeled with antibodies to calmodulin in the basal epithelium, where hair cell loss is nearly complete. In this region, the lumenal surface of the epithelium is composed predominantly of support cells and was only lightly labeled with antibodies to calmodulin. *D*, Sections of the damaged region confirmed that there was no strong anti-calmodulin labeling in any cells that remained after hair cell loss (*arrow* indicates support cell nucleus). Scale bars: 25 μm for *A*, *C* (shown in *A*); 25 μm for *B*, *D* (shown in *B*).

single injection of gentamicin (400 mg/kg), calmodulin immunoreactivity disappeared in regions in which hair cell loss occurred. Two days after gentamicin injection, basilar papillae showed very little calmodulin immunoreactivity in the basal third of the epithelium (Fig. 2*C,D*). This result is consistent with the observation that hair cells were completely lost from the basal epithelium and demonstrated that calmodulin immunoreactivity was not upregulated in support cells after aminoglycoside treatment. At the apical border of the drug-induced lesion, the transitional zone, calmodulin-positive hair cells displayed various morphologies characteristic of degenerating hair cells. Some cells were disk-shaped and highly compacted and seemed to be in the process of being lumenally expelled (data not shown). Additional calmodulin-positive hair cells seemed to undergo intraepithelial degeneration, a process that has been reported to occur in the mammalian organ of Corti (Forge, 1995). The cell bodies of such cells were highly distorted, in some cases extending well below the hair cell layer, and were vacuolated (data not shown). Hair cells in the apical, undamaged portion of the basilar papilla retained their normal levels and subcellular distribution of calmodulin immunoreactivity (data not shown).

We examined the specificity of the calmodulin antibody using Western blots. In SDS-PAGE denaturing gels, calmodulin typically runs at 17 kDa. In our nondenaturing gels, both purified calmodulin and calmodulin from the chick basilar papilla appeared as a single band at ~12 kDa (Fig. 3*A*). We believe that this 5 kDa difference is attributable to the fact that we used denatured rather than nondenatured standards, which generated the illusion that calmodulin ran at only 12 kDa. We interpret these findings to confirm that the Sigma antiserum is specific for calmodulin. Two different anti-calmodulin antibodies generated the same labeling pattern in whole mounts of control basilar papillae as did the initial antibody (one is shown in Fig. 3*B*). These findings confirm the specificity of the Sigma anti-calmodulin antibody for calmodulin in chicken. All subsequent immunoreactions for calmodulin discussed here were conducted with the Sigma anti-calmodulin antibody.

The TUJ1 antibody labeled all mature hair cells in the control basilar papilla (Figs. 4*A,B*). Short hair cells were labeled more darkly than tall hair cells (data not shown). In all hair cells, the immunoreaction was present in the cytoplasm, but not the nucleus, cuticular plate, or stereocilia (Fig. 4*B*). The TUJ1 antibody also labeled neural processes throughout the epithelium (Fig. 4*B*). No labeling in support cells (Fig. 4*B*) or hyaline cells (data not shown) was detected. Two days after gentamicin injection, the neural elements remained strongly labeled, but we detected no cytoplasmic TUJ1 immunoreactivity among cell bodies in the basal, damaged portion of the epithelium (Figs. 4*C,D*). Therefore, TUJ1 immunoreactivity was not upregulated in support cells after drug damage. TUJ1 labeling in the undamaged, apical epithelium resembled untreated controls (data not shown).

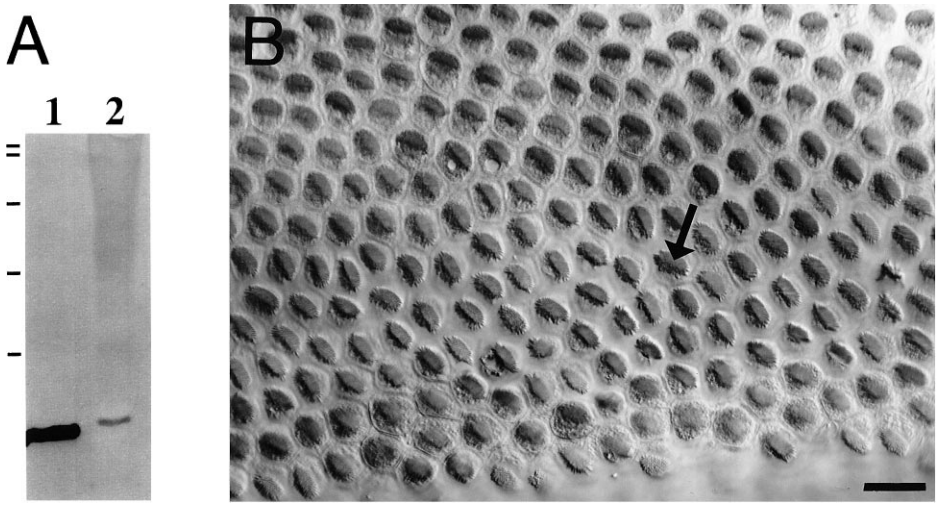

Figure 3. Specificity of anti-calmodulin antibodies. *A*, The specificity of the Sigma anti-calmodulin antibody was confirmed by Western blot analysis. Homogenates of the mature chick cochlear duct were processed through SDS-PAGE, blotted onto PVDF membrane, and probed with the anti-calmodulin antibody. *Lane 1* contained purified bovine calmodulin as a standard, and *lane 2* contained chick cochlear duct samples. Prestained molecular weight standards: 112, 84, 53.2, 34.9, and 20.5 kDa. The 28.7 kDa standard ran indistinguishably as a smear with the 34.9 kDa standard; we arbitrarily designated the smear as 34.9 kDa. *B*, Cochleae immunolabeled with the UBI anti-calmodulin antibody displayed a pattern identical to that of the Sigma primary antibody (Fig. 2*A*), strongly labeling the cytoplasm and stereocilia (*black arrow*) of hair cells. Scale bar, 25 μm.

Immunolabeling of support cells in normal and drug-damaged cochlear epithelium

We reacted whole mounts of the chick basilar papilla with antibodies to cytokeratin, which have been shown to label support cells selectively in the mature mammalian organ of Corti (Schrott et al., 1988; Anniko et al., 1989, 1990; Bauwens et al., 1991; Raphael and Altschuler, 1991). Two antibodies to the intermediate filament cytokeratin labeled support cells: AE1/AE3 (Boehringer Mannheim) and 8.13 (Sigma). In the basal end of the basilar papilla, cytokeratin labeling was strong in support cells located in the inferior third of the epithelium, but support cells in more superior regions were unlabeled or only lightly labeled (Fig. 5*A*). In contrast, in the apical end, support cells throughout the entire width of the epithelium reacted with anti-cytokeratin antibodies (data not shown). The immunoreaction was most intense in the lumenal portion of support cell cytoplasm (Fig. 5*B*). Occasionally,

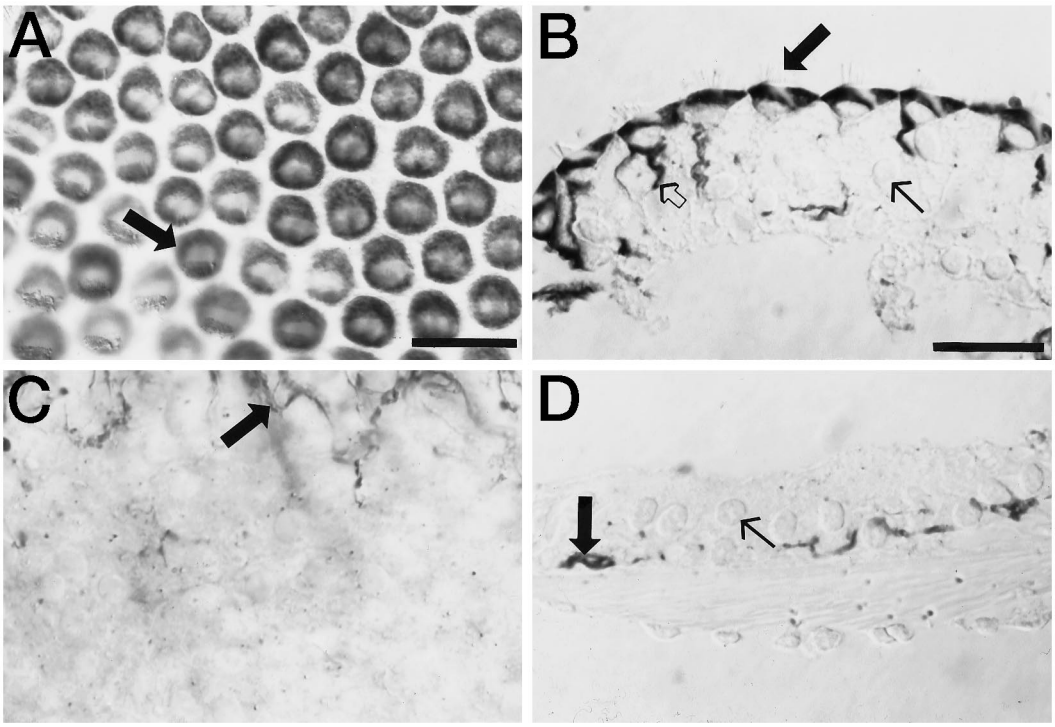

Figure 4. TUJ1 immunolabeling of hair cells in control and drug-damaged chick basilar papillae. *A* and *C* are micrographs of the lumenal surfaces of whole mounts of the midbasal region of the basilar papilla; the inferior edge is toward the *bottom* of the micrograph. *B* and *D* are micrographs of transverse sections of a region similar to that in *A* and *C*; the inferior edge of the epithelium is toward the *left*. *A*, The lumenal surfaces of short hair cells (*arrow*), but not the intervening support cells, in control basilar papillae labeled with the TUJ1 antibody. *B*, The cytoplasm of short hair cells (stereocilia indicated by *thick black arrow*) in control basilar papillae was strongly labeled with the TUJ1 antibody. Tall hair cells were more lightly labeled than short hair cells (data not shown). There was no detectable labeling with TUJ1 in the nucleus, cuticular plate, or stereocilia of any hair cells. Neural elements (*small shadowed arrow*) were labeled, but support cells were not (support cell nucleus is indicated by *thin black arrow*). *C*, Two days after gentamicin treatment, there were no TUJ1-labeled cells present in the basal epithelium, where hair cell loss is nearly complete. The only TUJ1 labeling was detected in the neural elements (*arrow*) that persist after hair cell loss. *D*, Sections of the damaged region confirmed that TUJ1 labeling was confined to the neural elements (*thick arrow*) in the region of complete hair cell loss (*thin arrow* indicates support cell nucleus). Scale bars: 25 μm for *A*, *C* (shown in *A*); 25 μm for *B*, *D* (shown in *B*).

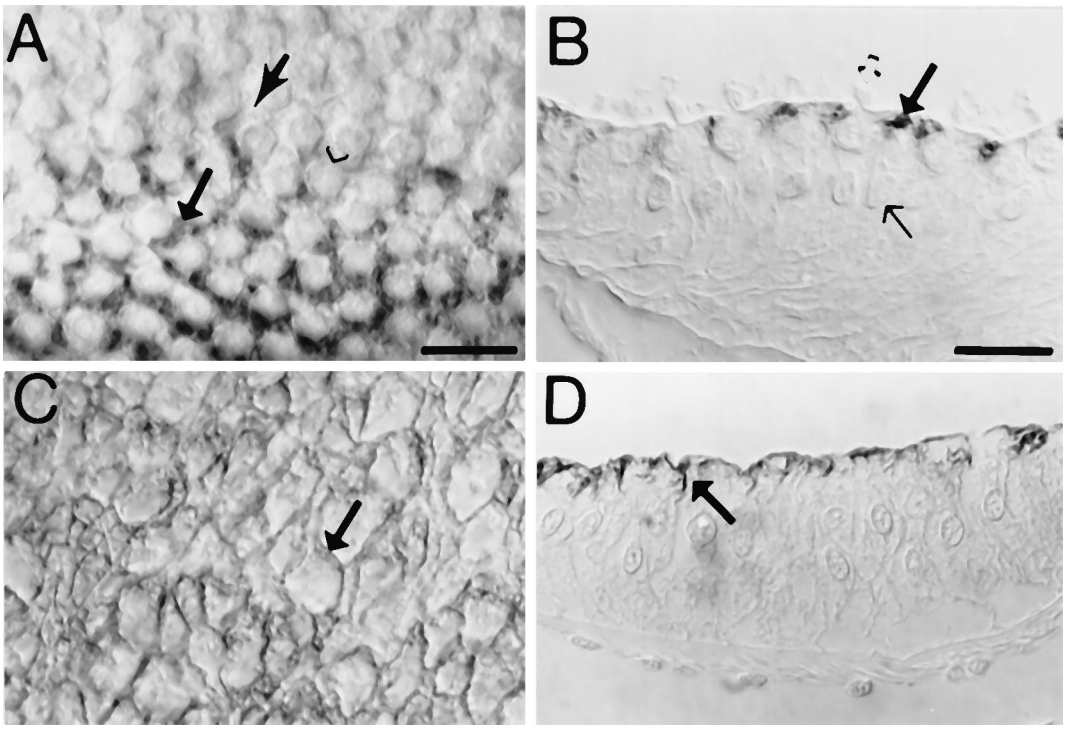

Figure 5. Cytokeratin immunolabeling of support cells in control and drug-damaged chick basilar papillae. *A* and *C* are micrographs of the lumenal surfaces of whole mounts of the midbasal region of the basilar papilla; the inferior edge is toward the *bottom* of the micrograph. *B* and *D* are micrographs of transverse sections of a region similar to that in *A* and *C*; the inferior edge of the epithelium is toward the *left*. *A*, In control cochleae, antibodies to cytokeratins intensely labeled the lumenal surfaces of support cells (*straight black arrow*) but not hair cells (*shadowed arrow* points to hair cell nucleus). In the basal end, strong cytokeratin immunolabeling was restricted to the lumenal surfaces of support cells along the inferior edge of the epithelium (*straight black arrow*); support cells in the middle and superior regions demonstrated weaker or no labeling (*flared black arrow*). In the apical end, support cells throughout the entire width of the epithelium were strongly cytokeratin-positive (data not shown). *B*, Intense cytokeratin labeling was present along the lumenal surfaces of support cells in the basal region of control sensory epithelia (*thick black arrow*). Very little labeling occurred in the cytoplasm of support cells (*thin black arrow* points to support cell nucleus). No labeling occurred in hair cells (*shadowed arrow* indicates hair cell stereocilia). *C*, Two days after gentamicin treatment, the lateral edges of support cells (*arrow*) across the entire width of the damaged epithelium labeled with anti-cytokeratin antibodies. Cytokeratin labeling in the undamaged, apical end of drug-treated cochleae resembled controls (data not shown). *D*, At 2 d after gentamicin treatment, cytokeratin labeling in the damaged sensory epithelium was heaviest along the apicolateral borders of support cells (*arrow*). Scale bars: 25 μm for *A*, *C* (shown in *A*); 25 μm for *B*, *D* (shown in *B*).

some perinuclear reaction was also present (data not shown). We also detected cytokeratin immunoreactivity in border cells and hyaline cells but not in the clear cells (data not shown). Cytokeratin immunoreactivity was never detected in hair cells. Omission of the primary antibody resulted in complete loss of immunoreaction (data not shown).

At 3 d after gentamicin injection, support cells across the entire width of the basal, damaged portion of the cochlear epithelium were labeled with anti-cytokeratin antibodies (Fig. 5*C*). This pattern of labeling differs from that seen in controls, where strong labeling was confined to the abneural edge of the epithelium (Fig. 5*A*). In addition, cytokeratin immunolabeling appeared heavier at the lateral surfaces than the lumenal surfaces in support cells in the region of hair cell loss (Figs. 5*C,D*). This labeling pattern was different from controls, for which the labeling appeared concentrated at the lumenal surface (Fig. 5*A,B*). No change in cytokeratin labeling was detected in the apical, undamaged region (data not shown).

Calmodulin and TUJ1 labeling during hair cell regeneration

We examined the reappearance of hair cell markers in the basal tip of the regenerating epithelium between 4 and 10 d after gentamicin treatment. By 4 d, calmodulin immunoreactivity was present throughout the cytoplasm of a small subset of cells in the basal, damaged region of the epithelium. The nuclei of these calmodulin-positive cells were located at different depths with respect to the lumen. In some cases, nuclei were located close to the lumen, in the hair cell layer (Fig. 6*A*). Many of these cells had small but distinct bundles of stereocilia on their apical surfaces (Fig. 6*A*). We also detected calmodulin-positive cells with nuclei located below the hair cell layer, nearly halfway between the basal lamina and the lumen (Fig. 6*A*). These cells typically spanned the entire depth of the epithelium, contacting the lumen and the basal lamina, and had a relatively narrow lumenal surface with no stereocilia. Cells with both morphological profiles were distinct from drug-damaged cells seen in the transitional zone, because they had no vacuoles or blebbed cytoplasm.

At 7 d after gentamicin injection, immature hair cells with descending tails of cytoplasm were detected again (Fig. 6*B*). Labeled cells had nuclei located predominantly near the lumen, and many had a discernible stereocilia bundle, demonstrating that hair cells had matured relative to 4 d after gentamicin treatment. Stereocilia were never detected on calmodulin-negative cells. At 10 d after injection, most calmodulin-positive cells had bucket-shaped cell bodies, lumenal nuclei, and distinct stereocilia, resembling mature hair cells (Fig. 6*C*). In whole mounts viewed *en face*, calmodulin labeling demonstrated that the mosaic of the regenerating hair cells was nearly reestablished by 10 d after gentamicin

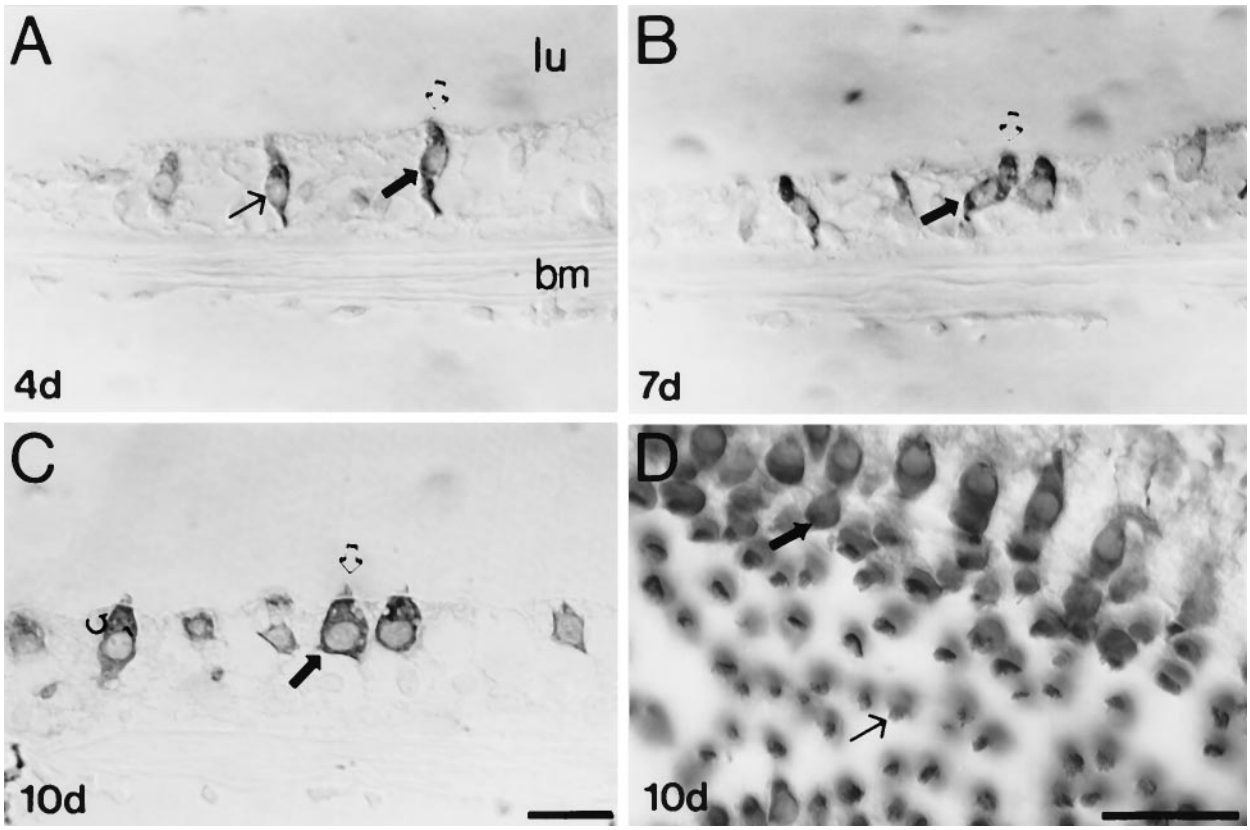

Figure 6. Calmodulin is expressed in early differentiating hair cells *in vivo*. *A–C*, Micrographs of transverse sections of the midbasal region of the basilar papilla (the inferior edge is toward the *left*). *D* shows a whole mount of the midbasal region of the basilar papilla; the neural, superior edge of the epithelium is toward the *top*. *A*, Calmodulin immunoreactivity reappeared in the previously damaged region by 4 d after gentamicin injection. All calmodulin-positive cells seemed to extend from the basilar membrane (*bm*) to the lumen (*lu*) or to have processes that extended adlumenally toward the basilar membrane. Labeled cells had nuclei in either the adlumenal, support cell layer (*thin black arrow*) or the lumenal, hair cell layer (*thick black arrow*). Many of the cells with lumenal nuclei had short immature stereocilia (*shadowed arrow*). *B*, By 7 d after gentamicin injection, most calmodulin-positive cells (*black arrow*) in the damaged region had lumenal nuclei and bundles of short stereocilia (*shadowed arrow*). *C*, By 10 d after gentamicin injection, all calmodulin-positive cells in the recovered epithelium seemed to be relatively mature hair cells with columnar or bucket-shaped cell bodies (*black arrow*) and staircase-shaped bundles of stereocilia (*shadowed arrow*). *D*, The organization of the basal, previously damaged region was nearly restored at 10 d. Calmodulin antibodies labeled the cytoplasm (*thick black arrow*) and stereocilia (*thin black arrow*) of regenerated hair cells. Because of the curvature of the epithelium, some hair cells are seen directly from above, whereas others are viewed from the side. Scale bars: 25 μm for *A–C* (shown in *C*); 50 μm for *D*. All immunoreactions shown were conducted with the Sigma anti-calmodulin antibody.

treatment (Fig. 6*D*). The relative morphological homogeneity of calmodulin-positive cells at each time point suggests that in a given region of the basilar papilla, hair cells differentiate at approximately the same time and rate.

For numerous reasons, we believe that the calmodulin-labeled cells detected at 4 d after gentamicin treatment are early differentiating hair cells. First, the labeled cells were detected in a region where no hair cells or calmodulin immunoreactivity were present in samples examined at 2 d after gentamicin injection. Second, the labeled cells morphologically resembled hair cells during early differentiation. Labeled cells were elongated and fusiform rather than bucket-shaped or columnar, like mature hair cells. Some cells had bundles of short stereocilia, characteristic of embryonic and regenerating hair cells (Cotanche and Sulik, 1984; Cotanche, 1987). In addition, a subset of cells with lumenal nuclei possessed a descending process that seemed to contact the basal lamina, which has been described as a property of early differentiating hair cells during both embryogenesis and regeneration (Whitehead and Morest, 1985a; Duckert and Rubel, 1990). The calmodulin-positive cells with adlumenal nuclei are interpreted to be hair cells at a more immature stage of differentiation than those with more lumenal nuclei and are thought to be in the process of migrating from the basal lamina to the lumen, where they will ultimately reside as mature hair cells. This interpretation is consistent with the electron microscopic observations of Duckert and Rubel (1990).

To examine the identity of these calmodulin-positive cells further, basilar papillae at 4 d after gentamicin treatment were double-labeled with TUJ1 and anti-calmodulin antibodies. TUJ1 and calmodulin immunolabeling occurred in the same subset of cells in the damaged region, and these cells were typically fusiform, with descending processes (Figs. 7*A,B*).

We verified the temporal progression of calmodulin immunoreactivity in differentiating hair cells in the following manner. Chicks were given a single injection of ^{3}H-thymidine or BrdU at 3 d after gentamicin injection, when numerous support cells enter the DNA synthesis (S) phase (Bhave et al., 1995). Because 90% of ^{3}H-thymidine seems to be cleared from chick serum by 2 hr after injection (Katayama and Corwin, 1993), this injection should have labeled only the subset of support cells that were synthesizing DNA at approximately the time of injection and their progeny. Cochleae were fixed at different periods ranging from 2 hr to 10 d

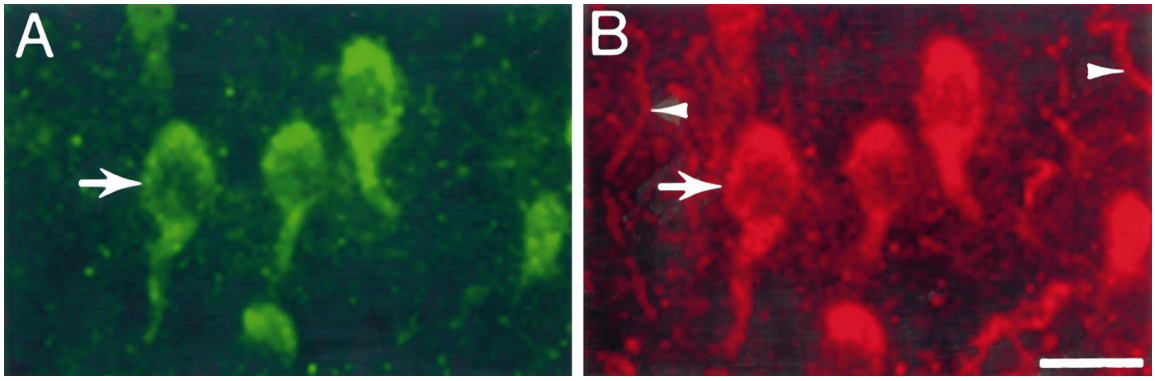

Figure 7. Calmodulin and TUJ1 antibodies co-label early regenerating hair cells. Confocal image of double-labeled cells in the basal region of the basilar papilla at 4 d after gentamicin. *A*, Numerous cells in the regenerating region were labeled with antibodies to calmodulin. Most of the labeled cells had morphological profiles characteristic of early differentiating hair cells (*arrow* indicates one example). *B*, All of the cells that were calmodulin-immunoreactive also labeled with the TUJ1 antibody. One double-labeled cell is indicated by the *arrow*. Neural elements were labeled with the TUJ1 antibody (*arrowheads*) but not with the anti-calmodulin antibody. Scale bar, 10 μm.

after the BrdU or ^{3}H-thymidine injection, and the proliferation marker and calmodulin were detected in the same tissue. This procedure allowed us to observe changes in cellular morphology and calmodulin immunoreactivity in a synchronized group of support cell progeny during the course of differentiation.

In chicks euthanized 2 hr after injection of the proliferation marker (equivalent to 3 d after gentamicin injection), numerous ^{3}H-thymidine/BrdU-positive and calmodulin-negative cells were present in the drug-damaged region of the sensory epithelium (Fig. 8*A*). At this time, the nuclei of these cells were located primarily in the adlumenal layer (Fig. 9). This cellular profile is characteristic of support cells that are in S phase or gap 2 phase of the cycle; by the time of mitosis, support cell nuclei have migrated into the lumenal layer (Raphael, 1992; Katayama and Corwin, 1993; Stone and Cotanche, 1994; Tsue et al., 1994a). No cells were double-labeled in the damaged region at this time (Fig. 9). On the other hand, a small number of double-labeled cells (one to five per cochlea) were detected in the transitional zone between the basal lamina and the lumenal surface. They had morphological features that were unique to degenerating hair cells, such as lumenally displaced cell bodies or vacuolated cytoplasm (data not shown). Such cells could be readily distinguished from differentiating hair cells and therefore were excluded from the quantitative analysis for Figure 9.

Numerous ^{3}H-thymidine/BrdU-positive and calmodulin-negative cells were present 24 hr after injection of the proliferation marker (equivalent to 4 d after gentamicin treatment) (Figs. 8*B*, 9). The nuclei of these cells were nearly evenly distributed between the lumenal and adlumenal compartments of the epithelium (Fig. 9). This finding is in contrast to basilar papillae at 2 hr, when the nuclei of ^{3}H-thymidine/BrdU-positive and calmodulin-negative cells were located primarily within the adlumenal compartment. This pattern of labeling demonstrates that support cells had progressed beyond S phase and were either differentiating into hair cells or support cells or going through a second round of cell division (Raphael, 1992; Katayama and Corwin, 1993; Stone and Cotanche, 1994; Tsue et al., 1994a). It is unlikely that support cells were poised in the first round of division at this time, because it takes ~6 hr for them to progress from S phase (at the time of injection) to M phase (Stone and Cotanche, 1994). At 24 hr after injection, a few ^{3}H-thymidine/BrdU-positive and calmodulin-positive cells were present in the regenerating region (Figs. 8*C*, 9). Double-labeled cells had constricted lumenal surfaces and adlumenally directed tails of cytoplasm (Fig. 8*C*), resembling immature hair cells seen at 4 d and 7 d after gentamicin (see Fig. 6*A,B*).

At 48 hr after injection of the proliferation marker (equivalent to 5 d after gentamicin), there were more double-labeled cells present in the basal, damaged epithelium than at 24 hr (Figs. 8*D*, 9). The majority of the double-labeled cells had nuclei in the lumenal compartment and immature bundles of stereocilia, typical of early differentiating hair cells. Among ^{3}H-thymidine/BrdU-positive and calmodulin-negative cells, there were many more adlumenal cells than lumenal cells at 48 hr (Fig. 9). This finding suggested that support cell mitosis had decreased relative to 24 hr. At 72 hr after injection of the proliferation marker (equivalent to 6 d after gentamicin), the mean number of double-labeled cells increased relative to 48 hr (Fig. 9). Most double-labeled cells had nuclei in the lumenal compartment and distinct stereocilia bundles (Figs. 8*E*, 9), which is characteristic of mature hair cells. There were very few ^{3}H-thymidine/BrdU-positive and calmodulin-negative cells in the lumenal compartments of the epithelium (Fig. 9). This finding suggested that support cell mitosis had nearly ceased by 6 d after gentamicin injection and that the postmitotic cells that were labeled by the ^{3}H-thymidine injection had differentiated into support cells or hair cells, segregating to the appropriate layers of the epithelium.

At 240 hr after injection of the proliferation marker, more double-labeled cells were present than at 72 hr (Figs. 8*F*, 9). All of the double-labeled cells had nuclei located in the lumenal compartment and were morphologically similar to mature hair cells from control basilar papillae (compare with Fig. 2*B*). Cells that were ^{3}H-thymidine/BrdU-positive and calmodulin-negative were also present; their nuclei were located only in the adlumenal compartment (Fig. 9). This finding suggests that the support cells that became labeled with the proliferation marker had ceased to divide by 6 d after the single gentamicin injection. This interpretation is supported by a previous study that found that BrdU incorporation in the damaged region was very low at 7 d after a single gentamicin injection (Bhave et al., 1995). Between 2 and 72 hr after ^{3}H-thymidine injection, the percentage of all ^{3}H-thymidine-positive cells that were calmodulin-positive increased (Fig. 10); however, we detected a small decrease in the percentage of all ^{3}H-thymidine-positive cells that were calmodulin-positive between 72 hr and 240 hr. The reason for this decrease is unknown. One possibility is that cell division continues between 72 and 240 hr, but progeny differentiate into support cells rather than hair cells. Altogether, these findings suggest that the cell progeny

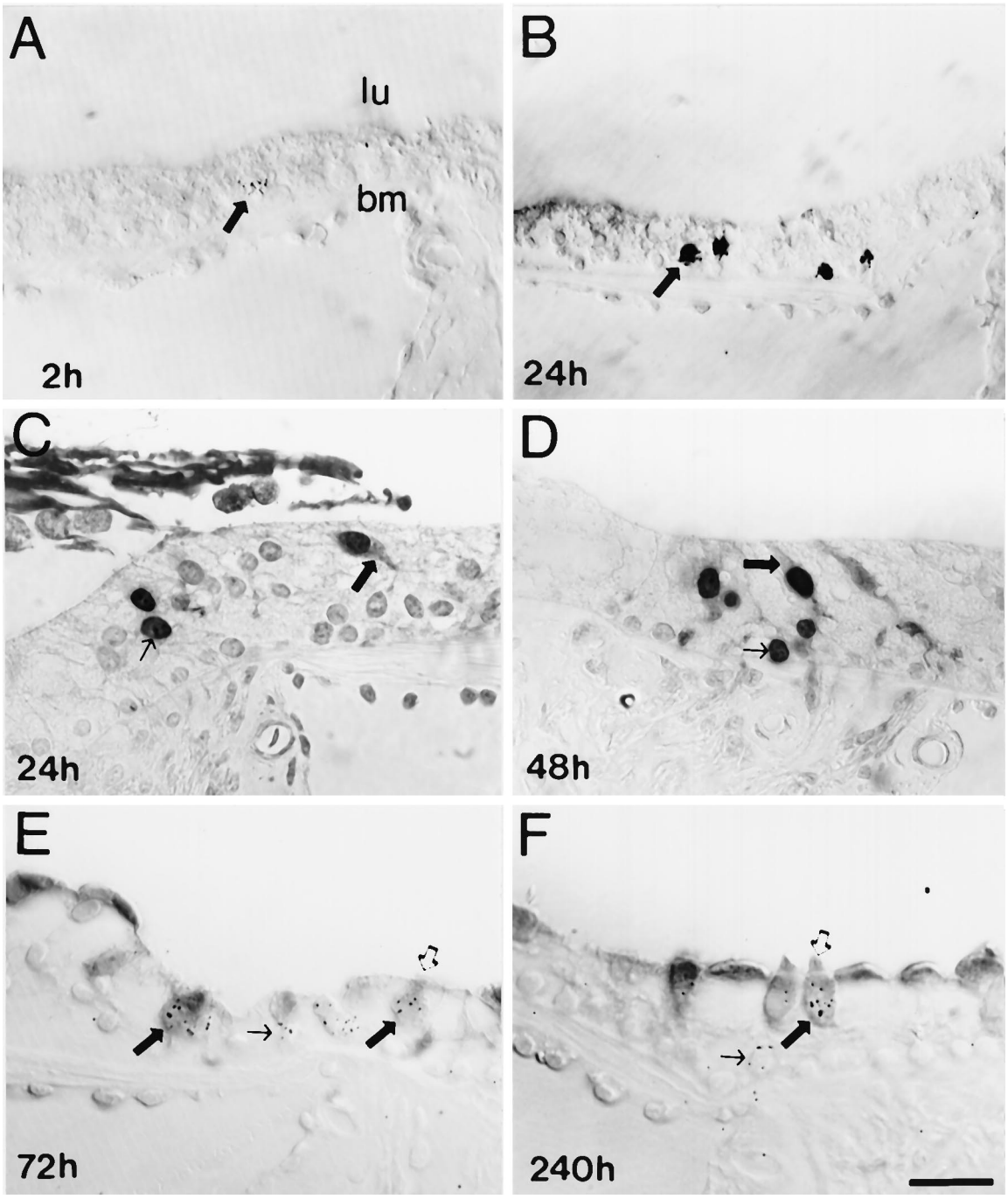

Figure 8. Time course of calmodulin immunoreactivity in differentiating hair cells. *A*, Two hours after ^{3}H-thymidine injection, ^{3}H-thymidine incorporation occurred in calmodulin-negative cells (*arrow*) located in the damaged region. Most ^{3}H-thymidine-positive cells had nuclei in the support cell (adlumenal) layer. The lumen (*lu*) and basilar membrane (*bm*) are shown. No double-labeled cells were detected at this time. *B*, Twenty-four hours after ^{3}H-thymidine injection, numerous calmodulin-negative/^{3}H-thymidine-positive cells (*arrow*) were again detected in the damaged region, but at this time their nuclei were located in both the adlumenal and lumenal layers. *C*, At 24 hr after BrdU injection, a few cells that were labeled for calmodulin and BrdU (*thick arrow*) were detected in the damaged region. Such cells resembled early differentiating hair cells, because they had a process that descended toward the basal lamina. *Thin arrow* points to BrdU-labeled cell that is *not* labeled with antibodies to calmodulin. *D*, At 48 hr after BrdU injection, cells with calmodulin-positive cytoplasm and BrdU-labeled nuclei (*thick arrow*) still seemed relatively immature, because they were elongated and possessed no detectable stereocilia. *Thin arrow* points to BrdU-labeled cell that is *not* labeled with antibodies to calmodulin. *E*, At 72 hr after ^{3}H-thymidine injection, all double-labeled cells seemed more mature than at 48 hr. Most double-labeled cells had rounded cell bodies (*thick arrows*), and some had short immature stereocilia (*shadowed arrow*). *Thin arrow* points to ^{3}H-thymidine-labeled cell that is *not* labeled with antibodies to calmodulin. *F*, By 240 hr after ^{3}H-thymidine injection, most double-labeled cells (*thick arrow*) had the shape of mature hair cells and distinct stereocilia bundles (*shadowed arrow*). *Thin arrow* points to ^{3}H-thymidine-labeled cell that is *not* labeled with antibodies to calmodulin. Scale bar (shown if *F*): 25 μm. All immunoreactions shown were conducted with the Sigma anti-calmodulin antibody.

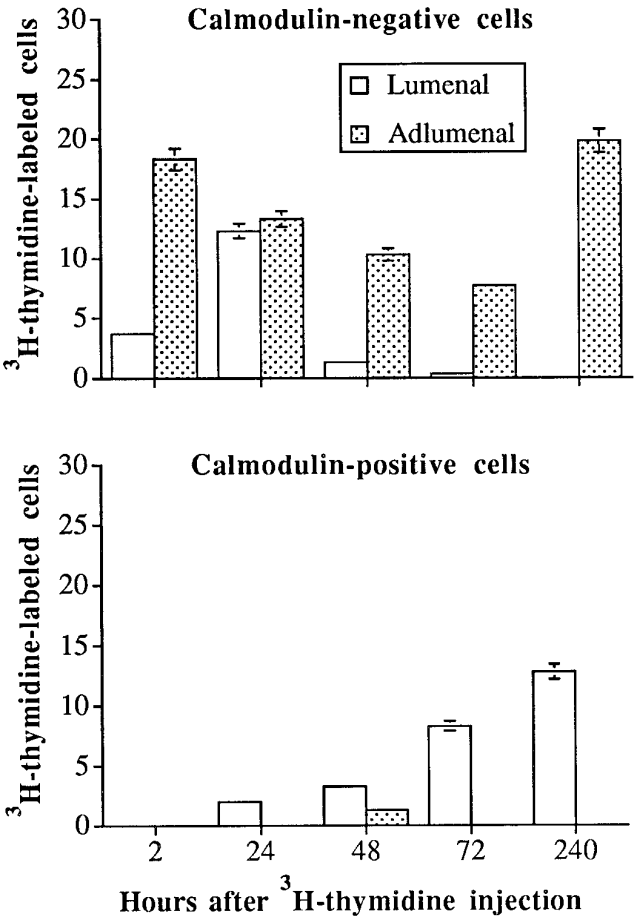

Figure 9. ^{3}H-thymidine labeling in the regenerating cochlear epithelium. The number of thymidine-labeled cells in the basal 300 μM of the cochlea that were calmodulin-positive or calmodulin-negative was counted, and the location of the nucleus of each cell in either the lumenal or adlumenal layer was determined, at 2, 24, 48, 72, and 240 hr after gentamicin treatment *in vivo*. Error bars represent SEM. $n = 3$ basilar papillae per time point.

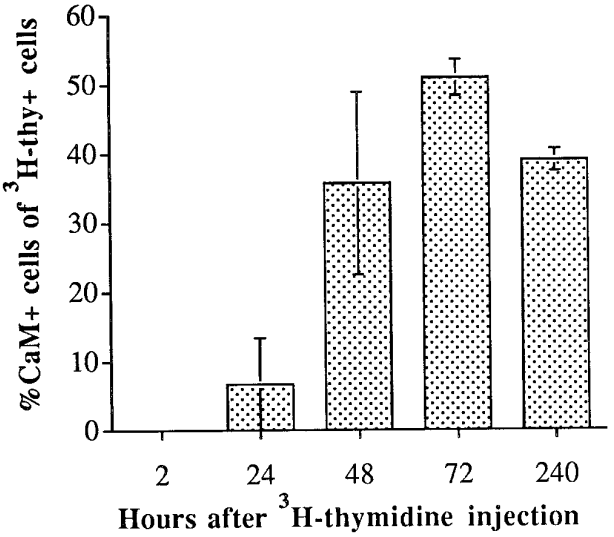

Figure 10. Regeneration of calmodulin-positive cells. The percentage of the total ^{3}H-thymidine-labeled cells that were calmodulin-positive was determined at 2, 24, 48, 72, and 240 hr after gentamicin treatment *in vivo*. Error bars represent SEM. $n = 3$ basilar papillae per time point.

that became labeled with ^{3}H-thymidine were nearly completely differentiated into hair cells or support cells by 240 hr, or 10 d, after gentamicin injection.

Cochlear epithelial cultures

Explants of the chick cochlear epithelium were isolated by enzymatic and mechanical dissociative methods. Before plating, the explanted sheets of cells were recognizable as sensory epithelia because of the hair cell stereocilia that protruded at regular intervals from their surfaces (Fig. 11*A*). To investigate the selectivity of our dissection techniques, we sectioned the cochlear tissue that *remained* after removal of the sensory epithelium and determined which cells were *not* isolated and subsequently cultured. In nearly all of the sections that we examined, the sensory epithelial cells (hair cells and support cells) were completely removed from the basal lamina, but the basal lamina and underlying stroma of the basilar membrane remained intact (Fig. 11*B*). Occasionally, some tall hair cells remained with the cochlear duct after the dissection (data not shown). Typically, the hyaline cells and clear cells were retained in the regions that originally flanked the sensory epithelium. It is highly unlikely that homogene cells were isolated with the sensory epithelia, because they were completely removed during the tectorial membrane dissection. Altogether, these findings suggest that the predominant cells in our cultures were sensory epithelial cells.

Explants were plated on the following surfaces: uncoated glass, uncoated plastic, laminin-coated glass or plastic, and fibronectin-coated glass or plastic. Explants became fully attached to the substratum or uncoated culturing surface by 1–2 d *in vitro*. Attachments seem to be mediated by extension of support cell processes away from the explant (Fig. 11*C*), and neither the timing nor degree of attachment seemed to be affected by the culture surface (data not shown). Some cells migrated away from explants into explant-free regions of the culture dish. These cells had epithelial morphology, with rounded cell bodies and multipolar processes. By 11 d *in vitro*, most of the surface of the culture plate was occupied by epithelial cells (Fig. 11*D*).

Cells with fibroblast-like morphology were detected in very few cultures, including after short periods in culture (2–4 d) and after relatively long periods (10 d), when fibroblast proliferation should have been substantial even if only a few fibroblasts had been isolated originally. The paucity of fibroblasts in these cultures supports our earlier interpretation that our method of removing the sensory epithelium does not disrupt the underlying stroma of the basilar membrane, which would be a primary source of fibroblasts from the cochlear duct.

To define further the cellular composition of control explants, cultures were labeled with antibodies to calmodulin, β tubulin, or cytokeratin, or with rhodamine phalloidin (a fluorescent label for filamentous actin). At 2 d *in vitro*, calmodulin-labeled cells were present in every explant. Calmodulin-positive cells morphologically resembled hair cells, because they had richly labeled cytoplasm and stereocilia, and round unlabeled nuclei (Fig. 12*A*). Calmodulin-negative cells were interposed between the calmodulin-positive cells. These unlabeled cells had oval nuclei and narrow surfaces compared with the calmodulin-positive cells and were presumed to be support cells. The original hexagonal organization of hair cells was maintained at 2 d *in vitro* (Fig. 12*A*). This observation is reinforced by rhodamine phalloidin labeling, which revealed

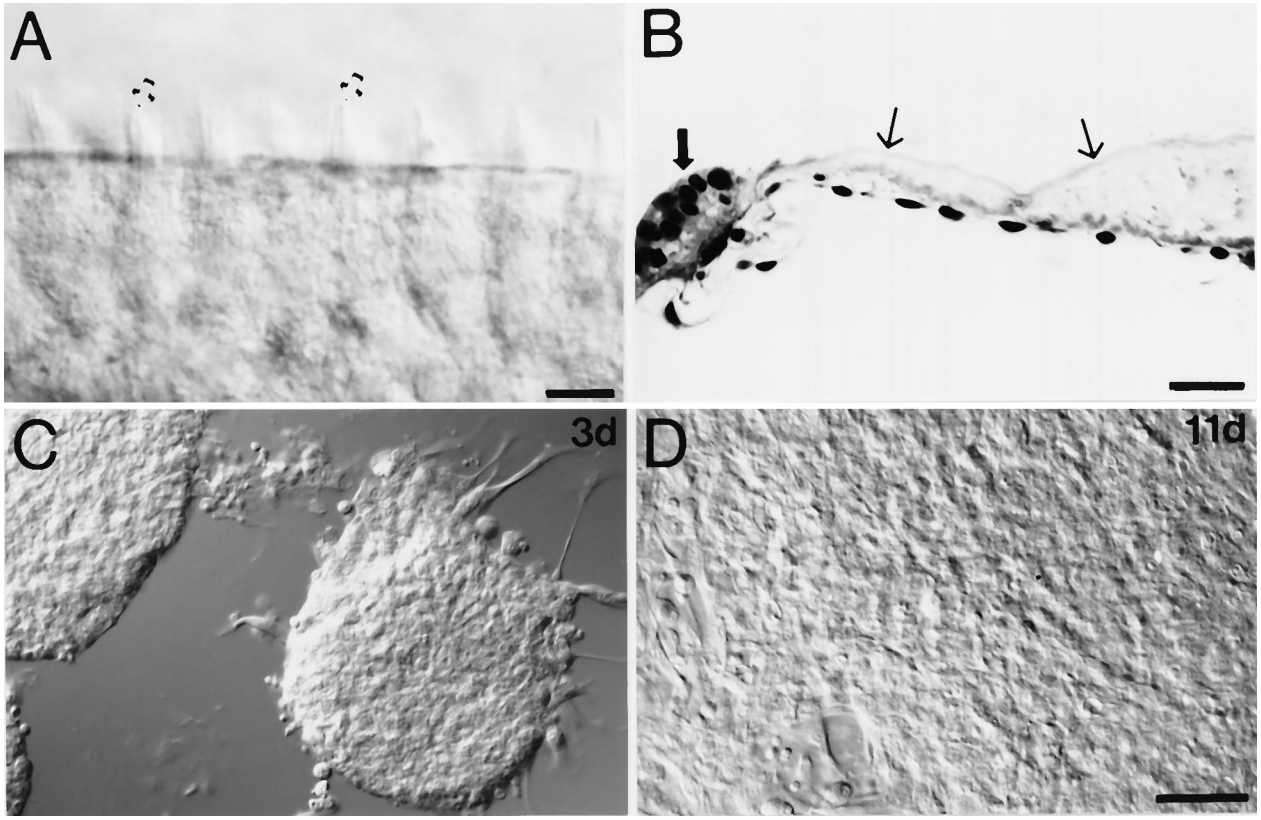

Figure 11. Cultures of the chick cochlear sensory epithelium. *A*, Preplating epithelial explants were recognizable as being derived from the cochlear epithelium, because hair cell stereocilia (*shadowed arrows*) projected from their surfaces. *B*, Sections of the tissue that remained in the cochlear duct after removal of the epithelium, counterstained with toluidine blue, revealed that all sensory epithelial cells had been removed, and the dissection process did not disrupt the basal lamina (*thin black arrows*). Hyaline cells (*thick black arrow*) also remained with the cochlear duct and are not isolated with this technique. *C*, Explants of the cochlear epithelium attach and spread on the coverslips by 2 d after plating. A 3 d culture is shown here. *D*, By 11 d after plating, support cells have proliferated and spread to cover most of the culture well. Scale bars: 25 μm for *A, B*; 100 μm for *C*, *D* (shown in *D*).

the presence of hexagonally arranged stereocilia bundles throughout the explants (Fig. 12*C*). By 6 d *in vitro*, there was extensive loss of calmodulin- and rhodamine phalloidin-labeled cells in control cultures, and this loss led to disruption of the hair cell mosaic (data not shown). Antibodies to cytokeratin labeled the entire surfaces of a subset of cells in 2 d control cultures (Fig. 12*E*). Observation with Nomarski optics demonstrated that cytokeratin-positive cells did not possess stereocilia, but they surrounded cytokeratin-negative cells that did have stereocilia (Fig. 12*E*), suggesting that the cytokeratin-positive cells were support cells. These findings demonstrate that the cell-specific antibodies we defined *in vivo* continued to label the appropriate cell types in our culture system.

In vitro aminoglycoside treatment

To induce hair cell loss and thereby generate an enriched preparation of support cells, a subset of explants was treated for 2 d starting at the time of plating with 65 μM streptomycin, an aminoglycoside antibiotic similar to gentamicin. After 2 d *in vitro* with streptomycin, no calmodulin-positive cells were present in any of the explants we examined (Fig. 12*B*). Similarly, rhodamine phalloidin did not label any stereocilia bundles in cultures treated with the antibiotic (Fig. 12*D*). The cells that remained after this treatment were most likely support cells. They were calmodulin-negative, and their nuclei were round or oval, resembling support cells *in vivo.* In addition, they labeled with rhodamine phalloidin in a manner that resembled support-cell labeling, as described by Raphael (1993) in regions with complete hair cell loss after noise exposure *in vivo*. Antibodies to cytokeratins reacted strongly with all cells that remained in cultures treated for 2 d with streptomycin (Fig. 12*F*). Cytokeratin labeling was present in the lateral borders and cytoplasm of support cells. It is not surprising that support cells survived the *in vitro* streptomycin treatment, because they are not visibly damaged by aminoglycosides *in vivo*.

Support cell proliferation *in vitro*

We examined temporal patterns of support cell proliferation in control and streptomycin-treated cultures by applying a 2 hr BrdU pulse/fix labeling paradigm at different periods after plating. Next, we counted the number of support cells that incorporated BrdU. BrdU-labeled support cell nuclei were round or oval (Fig. 13*A*). In control and antibiotic-treated cultures, numerous BrdU-labeled cells were present at 2 and 3 d *in vitro* (Fig. 13*B*). At 2 d *in vitro*, there were more BrdU-labeled cells present in control cultures than in drug-treated cultures, whereas by 3 d, this relationship was reversed. Between 3 and 11 d *in vitro*, support cells continued to incorporate BrdU, although the number of labeled cells decreased over time in both treated and untreated cultures. In contrast to 2 d, there were consistently more BrdU-labeled cells present in drug-damaged explants than in control explants at all subsequent times examined. By 9 d *in vitro*, very few BrdU-labeled cells were detected in treated or untreated cultures.

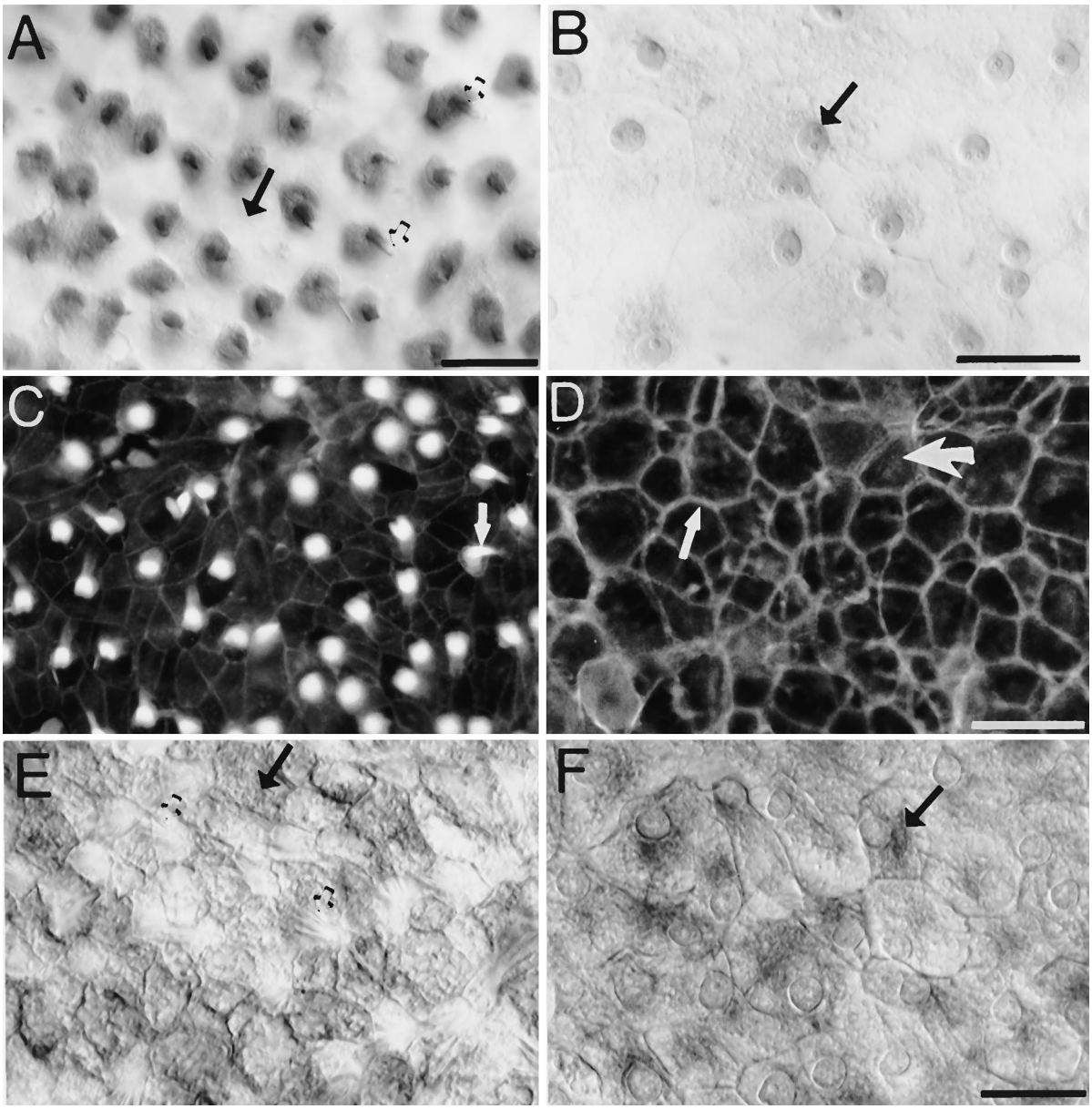

Figure 12. Complete hair cell loss is triggered in epithelial cultures by aminoglycoside antibiotics. Explants of the cochlear epithelium were labeled with cell-specific antibodies after 2 d of culturing with or without streptomycin. *A, C*, and *E* show control cultures, and *B, D*, and *F* show drug-treated cultures. *A*, In control cultures, anti-calmodulin antibodies labeled the cytoplasm and stereocilia (*shadowed arrows*) of hair cells but not support cells (*black arrow*). *B*, In streptomycin-treated cultures, calmodulin immunoreactivity was completely lost (*arrow* points to a support cell nucleus). *C*, In control cultures, rhodamine phalloidin labeled hair cell stereocilia (*white arrow*), the intercellular junctions between hair cells and support cells, and support cell microvilli (not evident in this figure). *D*, In drug-treated cultures, no stereocilia were labeled with rhodamine phalloidin, suggesting that all hair cells were killed. The cells that remained after hair cell loss were labeled with rhodamine phalloidin only at their cell/cell junctions (*straight white arrow*) and microvilli (fuzzy white material at end of *curved white arrow*), which is characteristic of support cells. *E*, In control cultures, antibodies to cytokeratins labeled the lumenal surfaces of support cells (*black arrow*) but not hair cells (stereocilia are indicated by *shadowed arrows*). *F*, In drug-treated cultures, antibodies to cytokeratin labeled the lateral borders and cytoplasm (*black arrow*) of support cells. Scale bars, 50 μm. All immunoreactions shown were conducted with Sigma anti-cytokeratin or anti-calmodulin antibodies.

Reappearance of hair cell phenotypes *in vitro*

To examine the cellular composition of long-term cultures, epithelial explants were treated with streptomycin for 5 d to ensure complete hair cell loss, washed several times with control media, and maintained for an additional 5 d in control media. Similar to cultures after 2 d of streptomycin treatment, many cells in these long-term cultures labeled with antibodies to cytokeratin and with rhodamine phalloidin around their lateral borders (Fig. 14, *B* and *A*, respectively; compare with Fig. 12). This finding suggested that the support cell phenotype was maintained in long-term cultures.

Phalloidin labeling revealed no evidence of stereocilia formation on any cells in explants at this time (Fig. 14*A*). Thus, it was evident that complete hair cell differentiation did not proceed in culture. To determine whether the progeny of cells that proliferated *in vitro* begin to differentiate into a hair cell-specific phenotype, we labeled these cultures with antibodies to calmodulin and β tubulin. Unlike

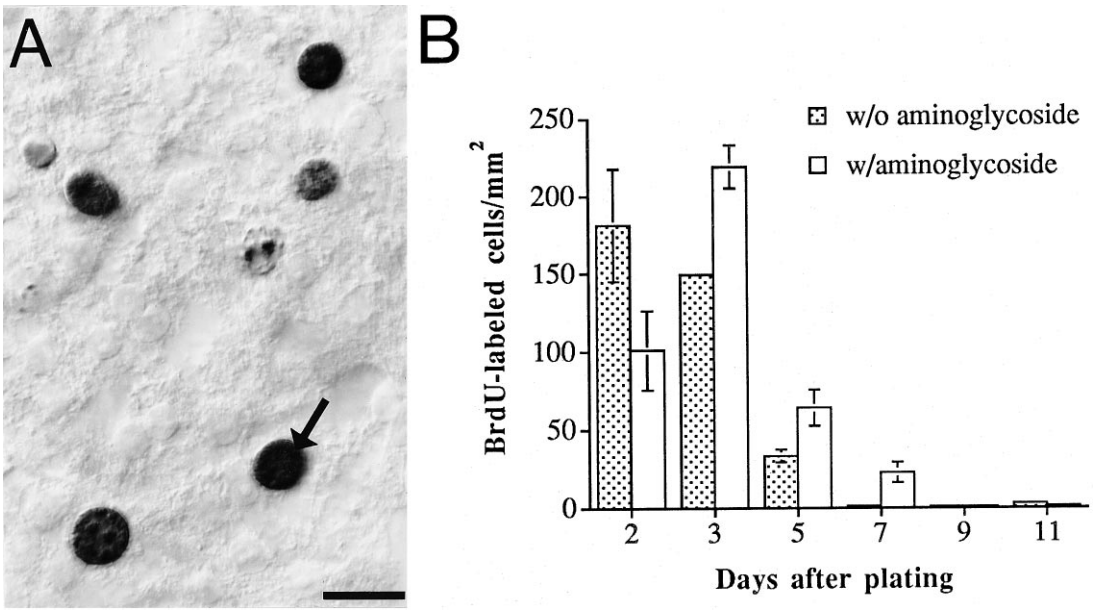

Figure 13. Cell proliferation in epithelial cultures. BrdU incorporation into support cells in control and streptomycin-treated cultures was studied at different times after plating following a pulse/fix BrdU-labeling paradigm. *A*, BrdU labeling (*black arrow*) in a drug-treated culture at 3 d *in vitro*. Scale bar, 25 μm. *B*, BrdU-labeled support cells were quantified at 2, 3, 5, 7, 9, and 11 d after plating. Error bars represent SEM. $n = 2$ culture wells per time point.

cultures immediately after streptomycin treatment, these cultures contained numerous calmodulin-positive cells (Fig. 14*C*). There were also numerous TUJ1-positive cells (Fig. 14*D*), which we did not detect in cultures treated for 2 d with streptomycin (data not shown). These calmodulin-positive or TUJ1-positive cells were round or fusiform, had no apparent organization, and were smaller and much fewer in number than the unlabeled support cells. Thus, the calmodulin-positive or TUJ1-positive cells resembled differentiating or mature hair cells seen *in vivo*.

Several lines of evidence indicate that the cells that labeled with hair cell markers were differentiating hair cells that were generated in culture rather than hair cells that survived streptomycin treatment. First, after 2 d of streptomycin treatment, no calmodulin-positive or TUJ1-positive cells remained in any of the control cultures (Fig. 12*B*). Second, many calmodulin-positive cells were double-labeled for calmodulin and BrdU (Fig. 14*E,F*), demonstrating that they were generated in culture. It is unclear why all calmodulin-positive cells present in the long-term cultures were not labeled with BrdU, because BrdU was continuously present in culture and any cells generated by cell division should possess strong BrdU labeling. One possible explanation is that the same calmodulin-positive cells were generated by a nonmitotic process, such as direct transdifferentiation of support cells to hair cells, which has been suggested to occur *in vivo* during hair cell regeneration in the chick basilar papilla (Adler and Raphael, 1996; Roberson et al., 1996).

DISCUSSION

The goals of the present study were to develop a culture system for the sensory epithelium of the chick basilar papilla that stimulates support cell proliferation and hair cell differentiation and to identify markers that can be used to label mature and differentiating hair cells and support cells in the intact epithelium and in culture. Sensory cells and support cells were isolated from the epithelium and cultured for several days. Although mature hair cells are maintained for 2–4 d in control cultures, most hair cells die spontaneously by 6 d. Complete hair cell death occurs within 2 d when the ototoxin streptomycin is added to cultures. As a result, cultures are composed entirely of support cells, some of which have the potential to serve as progenitors for new hair cells. Support cells proliferate rapidly for 2–7 d after plating and subsequently decrease their mitotic activity. Expression of the hair cell marker calmodulin disappears after *in vitro* aminoglycoside exposure, but reappears after several days in control media. A second hair cell marker, TUJ1, also labels cells in long-term cultures. A subset of calmodulin-positive cells incorporates BrdU during the culture period; however, stereocilia do not seem to form on any cultured cells. These findings suggest that some support cell progeny generated in culture begin to differentiate as hair cells, but new hair cells do not mature completely in these culture conditions.

Cell-selective markers in the normal and regenerating cochlear epithelium *in vivo*

We identified antibodies that selectively distinguish hair cells from support cells in control and regenerating cochlear epithelia. Two separate antibodies to cytokeratins label the lumenal extensions of mature support cells in an increasing gradient from the cochlear base to its apex. A similar pattern of cytokeratin expression in support cells has been demonstrated in normal mammalian cochlea and vestibular organs (Raphael et al., 1987; Schrott et al., 1988; Anniko et al., 1989, 1990; Anniko and Arnold, 1990; Bauwens et al., 1991; Kuipers et al., 1991) but has not been reported previously in the chick inner ear. Cytokeratin labeling is pronounced in only a subset of support cells along the inferior, abneural edge of control basilar papillae. Such specificity of localization does not seem to occur in the mammalian organ of Corti. The role of this distribution of cytokeratins in the chick basilar papilla is unclear. In general, cytokeratins seem to provide rigidity to tissue (Lazarides, 1982). Thus, it is possible that cytokeratin lends mechanical stability to the inferior portion of the basilar papilla, which is not structurally supported by a cartilaginous plate, as is the superior portion. Cytokeratin expression

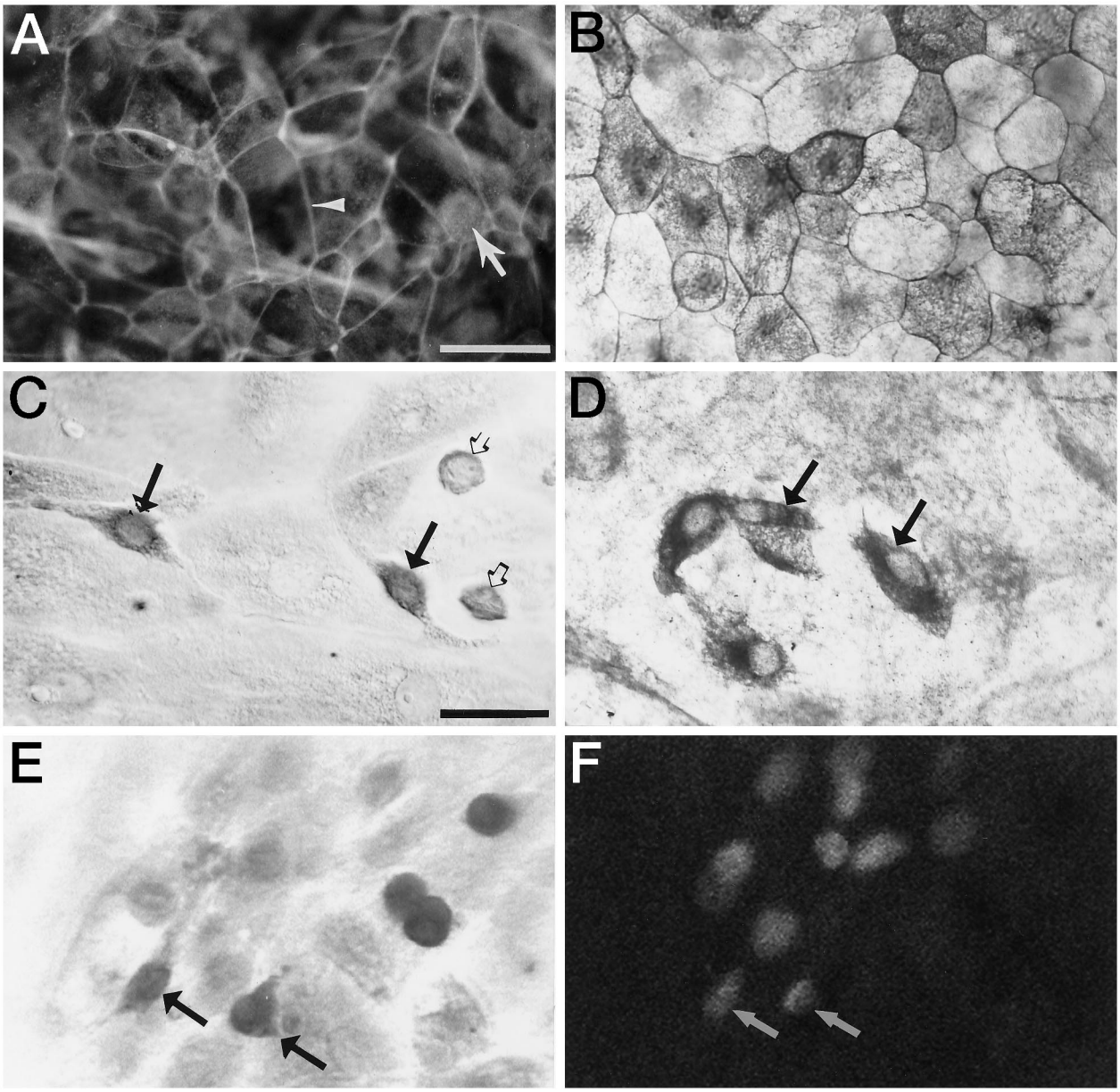

Figure 14. Calmodulin-positive and TUJ1-positive cells reappear in long-term cultures of the cochlear epithelium. *A*, Rhodamine phalloidin labeled the lateral borders (*white arrowhead*) and microvilli (fuzzy material at end of *curved white arrow*) of cells in long-term cultures. No stereociliary bundles were detectable with rhodamine phalloidin labeling. *B*, Cells in long-term cultures label with cytokeratin antibodies. *C*, Calmodulin-positive cells with fusiform cell bodies (*black arrows*) or round cell bodies (*shadowed arrows*) were present. *D*, TUJ1-positive cells (*black arrows*) with shape and size similar to the calmodulin-positive cells were detected. *E*, Digitized image showing calmodulin-positive cells in 10 d culture; *arrows* indicate cells also labeled with BrdU (shown in *F*). *F*, Digitized image derived from fluorescence confocal microscopy of BrdU-labeling from the same field as *E*; *arrows* point to cells that are double-labeled. Scale bars: 100 μm for *A*, *B* (shown in *A*); 50 μm for *C–F* (shown in *C*). In all examples, the Sigma anti-calmodulin antibody was used to detect calmodulin.

changes in response to hair cell loss in the basal third of the epithelium; all support cells appear immunoreactive, and the intracellular distribution changes so that label is concentrated at the lateral borders of support cells. It is likely that this change occurs as a result of support cell expansion, which occurs in conjunction with hair cell loss (Cotanche and Dopyera, 1990) rather than increased mitotic activity, because only a small subset of support cells divides after maximal hair cell loss (Roberson et al., 1996).

The cytoplasm, stereocilia, and cuticular plates of mature and differentiating hair cells are labeled strongly with antibodies to calmodulin, but mitotic and quiescent support cells remain unlabeled. The presence of calmodulin in bullfrog hair cell stereocilia has been demonstrated previously using "bundle blot" protein analysis (Shephard et al., 1989; Gillespie and Hudspeth, 1991). Calmodulin has been immunohistochemically detected in mammalian hair cells (Flock et al., 1986; Slepecky et al., 1988; Slepecky and Ulfendahl, 1993). Calmodulin expression has not been reported previously in developing or regenerating hair cells. The TUJ1 antibody also labels mature and differentiating hair cells but not support cells. This antibody recognizes a form of β tubulin, which is an early marker of differentiating neurons (Moody et al.,

1989; Lee et al., 1990; Easter et al., 1993). Our findings indicate that the TUJ1 antibody is an early marker of differentiating hair cells as well. Additional study is required to determine exactly how early the calmodulin and β tubulin antigens are detectable in regenerating hair cells.

Mechanisms of hair cell differentiation during regeneration *in vivo*

By double labeling for ^{3}H-thymidine (or BrdU) and calmodulin, we have shown that calmodulin is immunodetectable first in a subset of thymidine-labeled cells by 24 hr after thymidine injection (or S phase). We are certain that these double-labeled cells are postmitotic hair cell precursors, rather than support cells, for the following reasons. First, no double-labeled cells are present in the basal, damaged region at 2 hr after thymidine injection, when almost all cells remaining in that region are support cells. Second, the time of appearance of double-labeled cells (at 24 hr after thymidine/BrdU injection or 4 d after gentamicin injection) corresponds to the time when stereocilia bundles of regenerating hair cells first emerge in the damaged region after a single gentamicin injection (Bhave et al., 1995; Janas et al., 1995; W. R. Lippe, personal communication). A previous study has shown that support cells in the chick basilar papilla take as few as 6 hr to progress from S phase to mitosis (Stone and Cotanche, 1994). Thus, it is likely that calmodulin serves as a marker for differentiating hair cells as early as 18 hr after mitosis.

In inner ear epithelia, progenitor cell nuclei migrate from the basal lamina to the lumen during progression through the cell cycle (Ruben et al., 1971; Raphael, 1992; Katayama and Corwin, 1993; Stone and Cotanche, 1994; Tsue et al., 1994a). The temporal and spatial patterns of calmodulin expression in our study suggest that postmitotic cells also migrate from the lumen to a region below the hair cell layer before establishing their permanent location at the lumen. Many calmodulin-positive hair cell precursors seem to possess a physical connection with both the basal lamina and the lumenal surface. Furthermore, some early differentiating, calmodulin-positive hair cells have nuclei located well below the lumenal surface. These observations suggest two possible mechanisms of cell differentiation in the chick basilar papilla during regeneration. First, progenitor cells maintain a physical connection with the basal lamina during mitosis, and postmitotic cells retain a connection with the basal lamina that is permanent in the case of support cells and transient among hair cells. This hypothesis is not consistent with studies of the chick basilar papilla during development (Katayama and Corwin, 1993) and regeneration (Raphael et al., 1994; Tsue et al., 1994a), which have shown that mitotic cells are round and have no apparent connection with the basal lamina. An alternative possibility is that support cells lose contact with the basal lamina during mitosis and daughter cells migrate to more basal positions in the epithelium after mitosis. This descent may be necessary for daughter cells to obtain positional cues to continue dividing or to follow a particular differentiation pathway. For example, cell fate may be influenced by contact with other cells or the extracellular matrix of the basal lamina. Along these lines, Whitehead and Morest (1985b) have suggested that contact with neural elements may guide the establishment of cell polarity among hair cells in the developing chick basilar papilla. Cells that become committed to the hair cell fate would subsequently ascend to attain their lumenal position, whereas committed support cells would remain in an adlumenal location. This scenario is also consistent with electron microscopic observations of Duckert and Rubel (1990).

Progenitor cells in cultures

The identity of hair cell progenitors is poorly defined in avian inner ear epithelia. Although support cells are the most likely candidate (Girod et al., 1989; Raphael, 1992; Stone and Cotanche, 1994; Tsue et al., 1994a), only a small subset (~15%) of support cells completes the cell cycle after elimination of hair cells in a region of the basilar papilla (Roberson et al., 1996). It is unclear in those experiments whether only a subset of support cells is *capable* of dividing or is *stimulated* to divide. Additional studies suggest that some support cells in the chick basilar papilla can nonmitotically transdifferentiate into hair cells (Adler and Raphael, 1996; Roberson et al., 1996).

Aminoglycoside-treated cultures eventually consist primarily of support cells, as demonstrated by their immunoreactivity for cytokeratins and their pattern of rhodamine phalloidin labeling. Because there are no markers that selectively label hair cell progenitors, we cannot be certain what proportion of the cells in culture are progenitor cells; however, because there is significant cell proliferation between 2 and 7 d *in vitro*, it is likely that the proportion of support cells with mitotic potential, i.e., the progenitor cells, is largely amplified during the first week *in vitro*. Therefore, the cell culture method we describe may be an effective system for identifying factors that directly regulate support cell division and for characterizing distinct subsets of support cells on the basis of their molecular profiles or behavior in culture.

There are numerous potential stimuli of mitosis in our cultures. First, the culture media contained serum, which has growth factors, and hormones, many of which are potential mitogens. Second, support cells undergo significant morphological changes by 2 d *in vitro*, including attachment to a laminin substratum, and flattening and spreading, which may also stimulate support cell division. Third, dying hair cells *in vitro* may release factors that are mitogenic for support cells (Balak et al., 1990; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Bhave et al., 1995).

BrdU incorporation decreases in our cultures after 7 d *in vitro*. The cause of this decrease was probably not depletion of media-supplemented mitogens, because culture media were replenished every 3 d. Rather, the decrease in DNA synthesis may be a result of cell confluence and density-dependent inhibition of proliferation. By this time, support cells from individual explants have spread and merged with cells from other explants, generating a continuous sheet of epithelial cells. Alternatively, support cells in the chick basilar papilla may be capable of undergoing a limited number of cell divisions. This hypothesis is supported by the fact that the timing and degree of cell division we detected in our cultures closely resemble those seen *in vivo* after a single injection of gentamicin or noise damage (Stone and Cotanche, 1994; Bhave et al., 1995). Although the proliferative capacity of support cells has not been determined, studies have shown that support cells are able to divide more than once after hair cell loss (Jones and Corwin, 1993; Stone and Cotanche, 1994; Presson, 1995).

Hair cell loss and regeneration in cultures

In our culture system, immunoreactivity for calmodulin or β tubulin serves as an excellent marker for mature hair cells. Labeled hair cells are maintained in control cultures for up to 6 d, but are completely absent in cultures that have been treated with streptomycin for 2 d. These findings were confirmed by phalloidin labeling of stereocilia bundles in control cultures, but not in drug-treated cultures. Calmodulin immunoreactivity and phalloidin labeling of stereocilia are also sparse in control explants cultured for >6 d, suggesting that hair cells die spontaneously in

our culture media. The reason for this spontaneous hair cell loss is not known, but it is likely that the cultures lack factors that are necessary for hair cell survival. This culture system may be an effective tool for identifying factors that protect hair cells from cell death. It is also possible that hair cell death occurs in response to the great physical distortion of epithelial cells once they are plated onto the flat surface of the culture dish.

We detected a small subset of calmodulin-positive or TUJ1-positive cells in mitotically active cultures that were treated previously with streptomycin to eliminate completely the original set of hair cells. These cells morphologically resembled early differentiating hair cells *in vivo*, but they lacked bundles of stereocilia characteristic of mature hair cells. The reappearance of two markers of early differentiating hair cells in these long-term cultures suggests strongly that some cells that were generated in our culture system begin to differentiate as hair cells but are unable to proceed to a mature phenotype. This failure to fully differentiate may occur for several reasons. First, the cultures may lack molecules required for cell polarization and cytoskeletal assembly. Second, the physical nature of this type of culture, a flattened epithelium, may not permit the establishment of the normal hair cell cytoskeleton. Third, differentiating hair cells may not have been cultured long enough for stereocilia formation to occur.

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