In vitro growth and differentiation of mammalian sensory hair cell progenitors: a requirement for EGF and periotic mesenchyme

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Abstract

The sensory hair cells and supporting cells of the organ of Corti are generated by a precise program of coordinated cell division and differentiation. Since no regeneration occurs in the mature organ of Corti, loss of hair cells leads to deafness. To investigate the molecular basis of hair cell differentiation and their lack of regeneration, we have established a dissociated cell culture system in which sensory hair cells and supporting cells can be generated from mitotic precursors. By incorporating a Math1-GFP transgene expressed exclusively in hair cells, we have used this system to characterize the conditions required for the growth and differentiation of hair cells in culture. These conditions include a requirement for epidermal growth factor, as well as the presence of periotic mesenchymal cells. Lastly, we show that early postnatal cochlear tissue also contains cells that can divide and generate new sensory hair cells in vitro.

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Introduction

Little is known concerning the progenitors of mammalian sensory hair cells, or of the factors and cell–cell interactions that control their differentiation. In birds, hair cells and their surrounding supporting cells have been shown to have a common progenitor, both in development (Fekete et al., 1998; Lang and Fekete, 2001) and in regeneration (Warchol and Corwin, 1996). Birds and other nonmammalian vertebrates are able to regenerate hair cells by mitotic stimulation of quiescent progenitor cells, probably the supporting cells themselves, which persist into adult life (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). In mammals, however, no regeneration is observed following destruction of cochlear hair cells. It is not known whether the lack of hair cell regeneration in mammals is caused by a depletion of hair cell progenitors during embryogenesis or by a lack of appropriate stimuli in the adult.

In the mouse cochlea, proliferation, mitotic exit, and cell-type specification are all tightly coordinated to generate a functional organ. The cochlea is evident as an outgrowth of the ventral otic vesicle by E11.5 (Fig. 1); at this time, the progenitors of hair cells and supporting cells are still dividing (Ruben, 1967). These progenitors exit the cell cycle between E12.5 and E13.5 (Fig. 1) (Ruben, 1967) to form a zone of nonproliferating cells within the lateral wall of the partially elongated cochlear duct (Chen et al., 2002). Synchronization of cell cycle exit is dependent on the expression of the cell cycle inhibitor p27Kip1, which remains highly expressed in differentiated supporting cells (Chen and Segil, 1999; Lowenstein et al., 1999). After E14.5, a wave of differentiation begins in the basal portion of the cochlea that patterns the postmitotic prosensory domain into the stereotyped mosaic of intercalating hair cells and supporting cells (Lim and Lane, 1969). Sensory hair cell differentiation requires Math1, the mammalian atonal homologue (Fig. 1) (Bermingham et al., 1999), and the proper patterning of the cochlea is thought to be governed in part by Notch-dependent interactions (Lanford
et al., 1999; Zine et al., 2000). Subsequent to Math1 expression, sensory hair cells induce myosin-VIIa (Fig. 1) (Sahly et al., 1997) and calretinin (Fig. 1) (Zheng and Gao, 1997). By E16.5, the organ of Corti has assumed the gross appearance of the postnatal organ (Fig. 1, cf. E16.5 with P2) although hair cell differentiation has not yet extended into the apical tip of the cochlea. As the patterning of the cochlea nears completion at E17.5 (Sher, 1971), sensory hair cells begin to elaborate their characteristic stereocilia (Lim and Anniko, 1985).

To better understand the molecular control of progenitor cell fates within the developing cochlea, we have established an in vitro system that supports the growth and differentiation of dissociated mitotic progenitors of the embryonic cochlea into hair cells and supporting cells. Using a transgenic fluorescent marker to monitor hair cell differentiation in real time, we have defined two requirements for the growth and differentiation of the embryonic hair cell progenitor. These are the presence of factors derived from the periotic mesenchyme, which surrounds the inner ear, and the presence of epidermal growth factor. In this culture system, dissociated epithelial cells form islands in which hair cells arise from mitotic progenitors and display biochemical and morphological characteristics of differentiated hair cells. In addition, we show that postnatal cochlear cells can also generate new hair cells through a process of cell division, although at a reduced frequency.

**Materials and methods**

**Experimental animals**

The Math1-GFP transgenic mouse strain used in this study was previously described (Chen et al., 2002). The promoter contains about 1.4 kb of the Math1 enhancer including the positive autoregulatory elements (transgene #15 plus 150 bp of 3' sequence as described in; Helms et al., 2000), the β-actin promoter, and a nuclear localization signal for GFP. Male mice homozygous or heterozygous for the Math1-GFP transgene were bred with CD-1 female mice. Transgenic mice were identified by direct observation of GFP-mediated fluorescence. Animals for timed mating were put together in the evening, and the next morning was designated as embryonic day 0.5 (E0.5). Animal care was in accordance with institutional (AALARAC) guidelines.

**Tissue isolation**

Timed mated pregnant mice at E13.5 were euthanized with CO₂ followed by cervical dislocation, and the embryos were isolated and placed in PBS (Gibco). Each embryo was then observed under green fluorescence and separated into transgenic or nontransgenic pools. At E13.5, all transgenic embryos strongly express Math1-GFP in the neural tube and can be readily distinguished from their nontransgenic littermates. Math1-GFP transgenic embryos were used as a source for cochlea epithelial cells; nontransgenic littermates were the source for inner ear mesenchyme.

To isolate inner ear epithelial cells, whole inner ears were dissected out of embryos and placed in PBS (Gibco). The inner ears were incubated in calcium–magnesium-free PBS (CMF–PBS; Gibco) containing dispase (1 mg/ml; Gibco) and collagenase (1 mg/ml; Worthington) to free the epithelium from surrounding mesenchyme. After 8 min, the tissue was transferred to a well with DMEM containing 5% fetal calf serum (FCS). The inner ear epithelium was dissected out using 30-gauge needles (Becton-Dickinson). The vestibular sensory organs and the cochlea duct were carefully
separated, and the cochlea ducts were examined under green fluorescence to verify that no vestibular tissue was present. The cochlea ducts were washed with CMF–PBS and dissociated in either 1 mM EDTA (Gibco) in CMF–PBS for 15 min at 37°C, or in 0.05% trypsin (Sigma) in CMF–PBS for 2 min at 37°C. Similar results were obtained with both methods. After this incubation, the tissue was resuspended in 5% FCS in DMEM and triturated for several minutes to achieve full dissociation.

For the isolation of cochlear tissue from postnatal animals, P1–P2 pups were sacrificed by decapitation and screened under green fluorescence for expression of the Math1-GFP transgene. Inner ears, and subsequently cochlear tissue, were isolated from transgenic pups. Reissner’s membrane and the spiral ganglia were both removed from each cochlea (note the preparation in Fig. 1). Postnatal cochleae were dissociated with 1 mg/ml elastase (Sigma), 1 mg/ml collagenase (Worthington), and 0.5 mg/ml trypsin in CMF–PBS for 15 min at 37°C, followed by several minutes of trituration. Dissociated cells were sorted on a Cytomation Mo-Flo, and the Math1-GFP negative fraction was collected in DMEM + 5% FCS.

Cell culture

Substrates and media

Cells were plated on poly-D-lysine (0.5 mg/ml; Sigma) and fibronectin (25 μg/ml; Gibco)-coated glass coverslips (12 mm; Fisher) in 24-well culture dishes (0.3 ml/well; Corning). In some experiments, cultures were plated into poly-D-lysine- and fibronectin-coated CC2 Lab-Tek II chamber slides (Nunc). Sterile coverslips and chamber slides were first briefly rinsed with poly-D-lysine, allowed to dry, washed with sterile water, incubated with fibronectin for 2 h, allowed to dry, and washed with sterile water. Cultures were maintained in a defined medium: DMEM-F12 (Gibco) with B27 supplement (Gibco), 1 mM n-acetyl-L-cysteine (Sigma), penicillin–streptomycin (Gibco), and 20 ng/ml epidermal growth factor (Sigma), in a 5% CO2/5% O2 humidified incubator (Forma Scientific). FGF2 (NIH), heregulin (Sigma), and TGFα (Accurate Chemical and Scientific) were used at 25, 30, and 20 ng/ml, respectively. Cultures were fed weekly.

Plating

Cell number and viability of the dissociated cells were assessed on a hemacytometer using Trypan Blue exclusion. Math1-GFP transgenic epithelial cells were mixed with nontransgenic mesenchymal cells at a 1:1 ratio, and 16,000 cells were plated in a four-microliter drop into coated wells (typically covering a 4–5 mm² area). For sorted postnatal tissue, 10,000 sorted cells were cultured with 8000 non-transgenic mesenchymal cells. The cultures were placed in the incubator for 20 min to allow the cells to adhere, and then fed with culture medium. That same day, the cultures were examined on an inverted fluorescence Axiosvert 135 microscope (Zeiss) to document any Math1-GFP+ cells present at plating; images were collected with a Spot camera (Diagnostic Instruments, Inc.). This equipment was also used for documentation of Math1-GFP expression in living cultures.

In two experiments, the number of surviving epithelial cells was determined by quantifying Hoechst-stained nuclei in E-cadherin-stained epithelial islands at 1 day in vitro (DIV) and at several later time points. In these instances, Math1-GFP+ cells were first counted in the living cultures. The 1 DIV cultures were fixed, stained with E-cadherin and Hoechst, and quantified. The later time points were trypsinized, and the cells were then fixed in solution with 4% paraformaldehyde and stained in solution with E-cadherin and Hoechst. The cells were then smeared onto a microscope slide and the number of E-cadherin+ cells and Math1-GFP+ cells were determined. These numbers were used to estimate the total number of epithelial cells in cultures.

BrdU pulse

5-Bromodeoxyuridine (BrdU, final concentration, 1.0 μM; Sigma) was added to the culture medium to label dividing cells after the wells had been documented for Math1-GFP expression. Use of the standard concentrations of BrdU, such as 10 μM, led to a total lack of Math1-GFP+ cells, suggesting a dose-dependent BrdU toxicity not unusual in developmental systems (Kolb et al., 1999; Stockdale et al., 1964; Webster et al., 1973). Plates containing BrdU-labeled cultures were not examined for Math1-GFP fluorescence until after fixation.

Immunocytochemical staining

Antibodies

The following antibodies were used in this study: two anti-BrdU antibodies (Chemicon, mouse monoclonal, dilution, 1:200; and Chemicon, sheep polyclonal, 1:200), anti-E-cadherin (Sigma DECMA1 rat monoclonal, 1:2000), anti-calretinin (Chemicon, goat polyclonal, 1:2400), anti-GFP (Molecular Probes, rabbit polyclonal, 1:1000), anti-p27Kip1 (NeoMarker, mouse monoclonal, 1:200), anti-Math1 (rabbit polyclonal, 1:100; Helms et al., 2000), anti-myosin-VIa (rabbit polyclonal, courtesy of Christine Petit, Pasteur Institute, 1:3000), pan-cytokeratin antibody (Sigma, mouse monoclonal, 1:200), and anti-S100A1 (DAKO, 1:200). All secondary antibodies were purchased from Jackson Immunochemicals.

Inner ear sections

Cryostat sections (14 micron) were washed three times in PBS and incubated in 0.1% Triton for 15 min. To visualize
F-actin, the sections were incubated with phalloidin–rhodamine (concentrations as directed, Molecular Probes) diluted in PBS for 15 min.

For immunostaining, sections were blocked in 10% normal donkey serum for 30 min and then incubated with various primary antibodies diluted in PBS + 1% BSA overnight at 4°C. Sections were then washed three times in PBS and incubated with appropriate secondary antibodies diluted in PBS at room temperature for 60 min. Sections were mounted in Fluoromount-G (Southern Biotech). Sections used for anti-p27kip1 staining were first boiled in a microwave oven for 10 min in 10 mM citric acid buffer, pH 6.0.

Cultures

Cultures on coverslips were fixed in 4% paraformaldehyde in PBS for at least 15 min, washed in PBS, and then permeabilized in 0.2% Triton X-100 for 5 min in PBS on ice. Cultures were then blocked with 10% normal donkey serum in PBS for 30 min at room temperature, and incubated with primary antibody in PBS + 1% BSA for 1 h at room temperature (or alternatively, overnight at 4°C). Dilutions for cultures were the same as those for sections. The cultures were then washed and incubated in secondary antibody diluted in PBS for 1 h at room temperature. The coverslips were mounted on microscope slides with Fluoromount-G.

For BrdU staining, after permeabilization, the cells were incubated in 2 N HCl for 10 min and neutralized with 0.1 M boric acid (pH 8.5) for 10 min. This treatment typically preserved the native GFP fluorescence. After BrdU staining, some cultures were triple labeled with other antibodies, such as myosin-VIIa, using the same procedure above. If triple labeling was not required, the cultures were incubated for 5 min with a 1:50,000 dilution of saturated Hoechst-33258 (Sigma) solution to visualize the nuclear morphology of labeled cells. For p27kip1 staining, cells were boiled in citric acid buffer, similarly to sections, before blocking. For p27kip1/BrdU double labeling, cells were both treated with 2 N HCl and boiled in citric acid before blocking and primary antibody incubation steps. For this procedure, the sheep polyclonal BrdU antibody was used instead of the mouse monoclonal.

Microscopy and image processing

Three-color immunolabeled cultures were imaged using a Zeiss Axiocam digital camera on a Zeiss Axioplan 2 upright microscope. Two-color immunolabeled cultures and sections were imaged using a Zeiss LSM 410 inverted laser scanning confocal microscope. Images were processed using Photoshop 6.0.

Transmission electron microscopy

Three-week-old cochlear cultures were carefully scraped from their coverslips with 30-gauge needles, incubated in Karnovsky’s fixative for 1 h, washed in cacodylate buffer, and postfixed in 1% osmium. They were then dehydrated in a graded series of ethanol and embedded in Spurr resin. Sections (80 nm) were collected and stained with aqueous uranyl acetate, followed by Reynold’s lead. Sections were observed and photographed with a JEOL 100CX Transmission Electron Microscope at 80 kV.

Results

Detecting newly differentiated hair cells using a Math1-GFP transgenic mouse

To identify living hair cells early in the process of differentiation, we used a transgenic mouse line in which GFP is expressed from a portion of the Math1 enhancer that includes the positive autoregulatory element (Chen et al., 2002; Helms et al., 2000; Lumpkin et al., 2003). The transgene faithfully recapitulates the expression pattern of Math1 protein at E14.5 (Chen et al., 2002; Lumpkin et al., 2003) and E16.5 (Figs. 2A–C). Math1-GFP is expressed in young hair cells identified by the marker myosin-VIIa (Chen et al., 2002; Lumpkin et al., 2003) (Figs. 2D–F). Neither Math1 nor myosin-VIIa are expressed in E13.5 cochlear epithelium (Bermingham et al., 1999; Chen et al., 2002), correspondingly, Math1-GFP is also absent (see micrographs of transgenic cochlear in Figs. 3B, C). Math1-GFP persists in cochlear hair cells as they continue to mature (Figs. 2H, K). Both calretinin antibody and phalloidin, the latter of which binds to the polymerized actin of hair cell stereocilia (Estes et al., 1981), colocalize with Math1-GFP+ cells in postnatal (P1) cochlea sections (Figs. 2G–L). These results confirm that hair cells express Math1-GFP correctly at both early and late stages of cochlear differentiation, and that this transgene can serve as a useful marker of living hair cells.

Math1-GFP+ cells are produced from mitotic progenitors in culture

At E13.5, approximately 85% of the cells fated to become hair cells have exited the cell cycle (Ruben, 1967), even though no markers of hair cell differentiation are detectable, including Math1-GFP (Bermingham et al., 1999). We dissociated cochlear epithelia from E13.5 Math1-GFP transgenic embryos (Figs. 3A–C), into single cells (Fig. 3D), and plated them at high-density in adherent cultures along with dissociated periotic mesenchymal cells from nontransgenic mice (Fig. 3E, and see below). Previous studies have implicated EGFR ligands, such as EGF and TGF-α, in proliferation (Yamashita and Oesterle, 1995) and hair cell survival (Romand and Chardin, 1999) in cultures of older tissue. We therefore supplemented the cultures with 20 ng/ml EGF. At the time of plating, visual inspection of the dissociated cells confirmed that the cultures contained no Math1-GFP+ hair cells (Fig. 4B).
Starting between 1 and 2 days in vitro (DIV), epithelial islands formed as distinct populations within a surrounding population of mesenchymal cells. Approximately 35% of these islands contained Math1-GFP+ cells (Fig. 4). Since the mesenchymal tissue was taken from nontransgenic mice (Fig. 3), all Math1-GFP+ cells derived from the cochlear epithelium. By 6 DIV, the islands had enlarged and taken on a three-dimensional character. We confirmed the epithelial nature of the islands by staining with pan-cytokeratin antibodies (Fig. 4G). After 13 days, the size of the epithelial islands increased, as did the total number of Math1-GFP+ cells. A quantification of island growth and Math1-GFP+ cell production is described further below.

To determine whether the Math1-GFP+ cells produced in culture derive from mitotic progenitors, we incubated cochlear cultures with bromodeoxyuridine (BrdU). At E13.5 in vivo, approximately 85% of cochlear sensory cells fated to be hair cells have withdrawn from mitosis (Ruben, 1967); a subset of these cells will subsequently begin to express Math1 after an additional 1–3 days (Chen et al., 2002). It is likely that some or most of the differentiation observed in vitro at these initial time points is due to the presence of already-specified sensory cells. We reasoned that if the culture conditions were inducing proliferation of sensory precursors, it should be observable after any such specified cells differentiate. Accordingly, we photographed 3-day-old cultures to record the number and position of Math1-GFP+ cells already present in the cultures; consequently, 1.0 μM BrdU was then added. Three days later, we fixed the cultures, stained them for BrdU, and rephotographed them. We used these data to determine if newly generated Math1-GFP+ cells were differentiating from cells that had proliferated in vitro. New Math1-GFP+ cells (25 ± 5%) had incorporated BrdU, indicating that the progenitors of these cells divided, exited the cell cycle, and differentiated into Math1-GFP+ cells during this 3-day period (n = 3 experiments). BrdU+/Math1-GFP+ cells were observed in 20–30% of Math1-GFP cell clusters. To confirm that the BrdU+/Math1-GFP+ cells expressed other hair cell markers, we labeled some cultures with BrdU for 9 days to allow ample time for expression of myosin-VIIa. We then triple labeled the cultures for GFP, BrdU, and myosin-VIIa.
Fig. 5A–D shows a representative field from a cochlear culture containing a cluster of hair cells, including triple-labeled cells, in an epithelial island at 12 DIV. These results show that a significant number of newly expressing Math1-GFP+ cells derive from cells which divided in vitro.

To better characterize the differentiation of Math1-GFP+ cells in culture, we quantified the appearance of Math1-GFP+ cells and of epithelial cells in islands over time. Typically, 16–25% of epithelial cells, identified by E-cadherin staining and Hoechst, survived 1 DIV (Table 1). Between 1 and 7 DIV, the total number of epithelial cells in
indicates its epithelial origin. Scale bar: 50 μm. No Math1-GFP fluorescence is observed (B). Scale bar: 50 μm. 

Brightfield and fluorescence views of a freshly plated cochlear cell culture. The right corner is a small piece of debris, which marks the field. (A, B) Co cultured with mesenchymal cells for 2 weeks. The black line in the lower right corner has enlarged and flattened out (cf. E and C) and the number of Math1-GFP+ cells within it has increased. (G, H) Pan-cytokeratin staining of a 6 DIV island (G, red) containing numerous Math1-GFP+ cells (H, green), indicating its epithelial origin. Scale bar: 50 μm.

A culture increased approximately threefold, but then remained constant between 7 and 14 DIV (Table 1). Similarly, between 7 and 14 DIV, the number of epithelial islands and the percentage of total islands containing Math1-GFP+ cells do not increase (Table 1). In contrast, new Math1-GFP+ cells continue to be added after 7 DIV (Table 1). Taken together, these data indicate that after 7 DIV, new Math1-GFP+ cells are only or primarily added to existing Math1-GFP+ clusters.

This observation was confirmed by categorizing Math1-GFP+ clusters by size and plotting changes in cluster size distribution over time in a single experiment (Fig. 6). The total number of Math1-GFP+ cells increased for nearly 3 weeks, but the number of Math1-GFP+ islands remained constant after 6 DIV. Analysis of cluster to cluster differences in hair cell generation capacity showed that some clusters added almost no Math1-GFP+ cells during the culture period, whereas others added many cells. At 6 DIV, clusters of Math1-GFP+ cells range in size from 1 to 16 cells (Fig. 6A). By 19 DIV, cluster size ranges from 1 to 82 cells with a relative decrease in the number of small clusters and an increase in the number of larger clusters (Fig. 6G). Taken together, these data indicate that the total number of epithelial islands established by 6 DIV does not change in the subsequent weeks of culture. Likewise, the percentage of islands that contain Math1-GFP+ clusters of hair cells appears to be established in the first week of culture, but once established, those that contain Math1-GFP+ cells can continue to add additional Math1-GFP+ cells for up to 3 weeks (Fig. 6). Moreover, we cannot predict, based on morphological criteria or differentiation at 1 week in vitro, which islands will exhibit robust differentiation at 19 DIV (cf. Fig. 6B, C with H, I).

Newly differentiated Math1-GFP+ cells express mature hair cell markers

We immunolabeled representative cochlear cultures containing Math1-GFP+ cells with the hair cell marker myosin-VIIa. The presence of Math1-GFP+ nuclei colocalizing with myosin-VIIa+ cell bodies (Figs. 7A, B, D) confirmed their hair cell identity. Math1-GFP+ cells also expressed a second hair cell marker, calretinin (Figs. 7E–G). In vivo, cochlear hair cells develop characteristically shaped stereocilia starting around E18.5 (Lim and Anniko, 1985), which can be observed by staining with fluorescent phalloidin. We observed stereocilia-like projections on in vitro-generated hair cells from cochlear epithelia (Figs. 7C, D, white).

Ultrastructural analysis of cochlear cultures confirmed that in vitro-differentiated cochlear hair cells possessed stereotypical hair cell morphology (Fig. 8). Thin sections of two different cultures revealed the epithelial islands to be hollow domes consisting of polarized epithelial cells surrounded by a band of fibrocyte-like cells. Stereocilia-like bundles project into the lumen of the island from these polarized cells (Fig. 8A). At their base, these bundles (Figs.

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<td>na*: not applicable. Epithelial islands are not morphologically distinct at 1 DIV. Data from a representative experiment in which 8000 cochlear epithelial cells were plated in high-density culture with 8000 mesenchymal cells. Math1-GFP+ cells and epithelial islands were counted before the cultures were fixed (four cultures each, ± SEM); epithelial cells were quantified after staining with E-cadherin and Hoechst, either in situ within the culture (1 DIV; two cultures, ± SEM) or after dissociation (7 DIV and 14 DIV; four cultures each, ± SEM).</td>
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Fig. 5. Progenitors divide in vitro to produce differentiated hair cells. Cochlear cultures were incubated with 1 μM BrdU from days 3 to 12. Each image is a projection of eight confocal optical sections, each 1 μm, from the same field. (A) Math1-GFP fluorescence in an epithelial island. (B) BrdU staining, marking cells that descended from progenitors that divided in vitro. (C) Myosin-VIIa staining, indicating further differentiation of the Math1-GFP+ cells. Note the typical polarized sensory hair cell morphology of the cells. (D) Merged image. Approximately 40% of the Math1-GFP+/myosin-VIIa+ cells in this field are also BrdU+. Yellow arrows indicate triple-labeled cells. Scale bar: 25 μm.

Fig. 6. Variability in the size of clusters of Math1-GFP+ cells within epithelial islands. (A, D, G) Distribution of Math1-GFP+ clusters within epithelial islands over time as indicated. (B, C, E, F, H, I) Sequential observations of two clusters of Math1-GFP+ cells. (A–C) Differentiation of Math1-GFP+ cells at 6 DIV. (A) Histogram of cluster size (x axis) versus number of clusters (y axis), with a mean of 5.1 (red arrow) and a maximum of 15. (B) Brightfield image of two adjacent epithelial islands. Each island contains a cluster of Math1-GFP+ cells (C). The size of each cluster is indicated on the histogram (A, C, yellow, cyan stars). (D–F) Same experiment at 11 DIV. Histogram of cluster size (D) shows that most, but not all, have an increase in Math1-GFP+ cell number: the mean is now 13.5 (red arrow) and the maximum 51. Both epithelial islands have enlarged in size (E) and increased comparably in Math1-GFP+ cell number (F, note yellow and cyan stars in D). (G–I) Same experiment at 19 DIV. Histogram of cluster size (G) shows a mostly flattened distribution between 1 (minimum) and 82 (maximum), with a mean of 21.1 (red arrow). Although the two islands depicted began with similar numbers of Math1-GFP+ cells, the top cluster has nearly twice as many (42, yellow star) as the bottom cluster (23, cyan star).
8B, D, black arrows) insert into an apparent cuticular plate (Figs. 8B, D, white arrows) and tight junctions between the bundle-bearing cell and its neighbor were readily seen at higher magnification (Fig. 8C, white arrow). The projections observed in culture had typical stereocilia morphology (Friedmann and Ballantyne, 1984): they were large, straight, had narrowed bases, and contained dense filaments (Fig. 8C, black arrow and inset). In some sections (Fig. 8E, arrows), the projections appeared to possess rootlets (Friedmann and Ballantyne, 1984). Taken together with the immunocytochemical data, this strongly suggests that in vitro-differentiated hair cells express elements of a mature morphological phenotype.

The in vitro differentiation and survival of Math1-GFP+ cells requires the presence of mesenchymal cells and epidermal growth factor

To better understand the requirements of sensory hair cell progenitors for survival and differentiation in vitro, we tested the necessity of having periotic mesenchymal cells and EGF in culture. We plated E13.5 dissociated cochlear epithelial cells in the absence (Figs. 9A, B, E, F) and presence (Figs. 9C, D, G, H) of periotic mesenchymal cells. At 1 DIV, both conditions contained a small number of Math1-GFP+ cells (data not shown). After 6 DIV, the epithelial cells plated in the absence of mesenchyme had formed a sheet devoid of Math1-GFP+ cells (Fig. 9E, F). Pan-cytokeratin staining confirmed their identity as epithelial cells with a large, flat morphology (Fig. 9E, insert). In contrast, when we plated the same cochlear epithelial cells with mesenchymal cells, Math1-GFP+ cells are observed in epithelial islands (Figs. 9G, H, arrows). While a 1:1 ratio of the two cell types has been used throughout the described experiments, the ratio of epithelial cells to mesenchymal cells was manipulated in two early experiments to characterize the optimal plating conditions for Math1-GFP differentiation. Math1-GFP differentiation in epithelial islands was observed when the epithelial cell to mesenchymal cell ratio was between 2:1 and 1:3; outside this range differentiation was much reduced (data not shown).

EGF was also required for the differentiation of hair cells in cochlear cultures. In cultures containing at least 40 epithelial islands, more than 100 Math1-GFP+ cells could be observed after 2 weeks (Fig. 9I, blue squares). In contrast, sister cultures maintained in the absence of EGF had virtually no Math1-GFP+ cells (Fig. 9I, red squares), suggesting that EGF is necessary for the production and/or survival of Math1-GFP+ cells. We observed optimal differentiation with EGF concentrations of 1–20 ng/ml (Fig. 9J). We obtained similar results with cultures supplemented with 20 ng/ml TGFα; these factors have been reported to promote proliferation in sensory regions of cultured utricles (Yamashita and Oesterle, 1995). In contrast, neither FGF2 (25 ng/ml) nor heregulin (30 ng/ml), two factors also reported to promote proliferation in utricular supporting cells (Zheng et al., 1997, 1999), supported the production of Math1-GFP+ cells in vitro (data not shown).
To test whether the effect of EGF was due to an indirect action of EGF on mesenchymal cells, we cultured periotic mesenchymal cells alone for 1 day in EGF-containing medium, and then replated with fresh cochlear epithelial cells in the presence or absence of EGF. Math1-GFP+ cells were only observed in the presence of EGF, regardless of whether the mesenchymal cells were pretreated with EGF (data not shown). This suggests that EGF is not exerting its effect solely through an indirect action on mesenchymal cells.

Supporting cell markers are expressed in cochlear cultures in vitro

The appearance of hair cells in vivo is accompanied by the differentiation of adjacent supporting cells, which derive from a common progenitor in birds (Fekete et al., 1998). To determine whether the cells within the epithelial islands adjacent to the hair cells expressed a supporting cell phenotype, we examined the expression patterns of two supporting cell markers, p27Kip1 and S100A1 (Chen and Segil, 1999; Coppens et al., 2001; Lowenheim et al., 1999). p27Kip1 is initially expressed throughout the sensory patch starting at E13.5, but becomes restricted to supporting cells at later stages (Fig. 10A). In adult mice, S100 calcium binding proteins are expressed in Deiter’s and Henson’s cells, in some spiral ganglion cells, in the spiral ligament, and in some cells of the stria vascularis (Foster et al., 1994). In the neonatal dog cochlea, S100 isoform A1 is specifically expressed in Deiter’s cells and in the pillar cells; during the second postnatal week, it is observed in all the supporting cells of the organ of Corti as well as in the reticular membrane (Coppens et al., 2001). We observed S100A1 staining in Deiter’s cells and the pillar cells at E17.5 in the mouse cochlea (Fig. 10B), but no staining before E15.5 (data not shown). In sections through the middle turn of the E16.5 cochlea, S100A1 staining was also observed in the inner hair cells (data not shown).

To determine whether the cells within the epithelial islands adjacent to the hair cells expressed a supporting cell phenotype, we immunolabeled 14-day cochlear cultures for p27Kip1 and S100A1, and also for Math1-GFP (Figs. 10C–H). Both p27Kip1+ and S100A1 labeled a subset of Math1-GFP/C0 epithelial cells (Figs. 10C–H). These data suggest that supporting cells, like hair cells, also differentiate in culture.

To determine whether the p27Kip1+ cells also derived from mitotic precursors in vitro, we added BrdU to cochlear cultures at 3 DIV, and the cultures were fixed at 12 DIV. A significant fraction (30.2 ± 5.0%) of p27Kip1+ nuclei also labeled for BrdU (Figs. 10I–K). These data indicate that like hair cells, some of these supporting cells derive from cochlear epithelial cells that divided in culture.

Differentiation of new Math1-GFP+ cells from older tissue in vitro

The mammalian cochlea, unlike those of birds, does not regenerate new sensory hair cells in vivo if the existing hair cells are killed, indicating that any endogenous progenitors for sensory hair cells have either disappeared or...
have become inhibited from regeneration (reviewed in Stone et al., 1998). As a preliminary test to distinguish between these possibilities, we assayed cochlear tissue from postnatal day 2 (P2) Math1-GFP transgenic mouse pups for its capacity to generate sensory hair cells. To ensure that any sensory hair cells observed at the end of the culture period differentiated in vitro, we used fluorescence-activated cell sorting (FACS) to obtain a cell suspension that was verifiably free of Math1-GFP+ sensory hair cells (Fig. 11A). In addition, we added BrdU after 1 DIV to determine whether any new Math1-GFP+ cells derived from a cell that divided in culture.

New Math1-GFP+ cells arose in culture from dissociated P2 cochleae at a low but reproducible rate (Figs. 11B–E, F–I). In a representative experiment (Fig. 11), we observed an average of 3.9 ± 1.7 new Math1-GFP+ cells after 7 DIV in each of 40 cultures generated from the sort. Importantly, we only observed single Math1-GFP+ cells, such as the ones depicted (Figs. 10B–E, F–I), or occasionally doublets, from sorted P2 cochlea cells. This is significantly lower than that of embryonic tissue, which generates clusters of up to 14 Math1-GFP+ cells (cf. Figs. 5A–C) and 50–100 Math1-GFP+ cells per culture after 1 week (cf. Figs. 9I, J). New Math1-GFP+ cells (25.9%) were labeled with BrdU (Figs. 11C, G). Taken together, these results indicate that the postnatal mammalian cochlea may contain a low level of sensory hair cell progenitor capacity, but in comparison to embryonic epithelium, both the size and frequency of clusters are much reduced (cf. Table 1, Figs. 5A–C and 9I, J). Moreover, EGF did not significantly stimulate sensory hair cell development from postnatal tissue compared to control medium (data not shown).

Discussion

The high incidence of hearing loss in humans (Gates et al., 1990; Moscicki et al., 1985), combined with the lack of cochlear sensory hair cell regeneration in mammals (Chardin and Romand, 1995; Rubel et al., 1995), has fueled an interest in the possibility of identifying progenitors or stem cells that may be manipulated to participate in regenerative events. Here we have defined culture conditions that allow proliferation and differentiation of cochlear sensory progen-
itors, derived from both embryonic and early postnatal cochlear tissues, into hair cells and supporting cells. This process occurs continuously over a period of up to 2 weeks in culture and requires the presence of periotic mesenchyme and EGF. In vitro-derived hair cells express aspects of a mature phenotype by both immunocytological and ultrastructural criteria. Finally, we show that sensory hair cell progenitor activity in the cochlea is still detectable, but significantly diminished at birth, when tested in vitro. Our results form the basis for future investigations into the capacity of various otic epithelial subpopulations to act as hair cell and supporting cell progenitors.

Mammalian sensory progenitors and their relevance to regeneration

In birds and many cold-blooded vertebrates, damage to hair cells induces mature, normally quiescent supporting cells to act as progenitors: they reenter the cell cycle and divide asymmetrically to give a hair cell and a supporting cell (Corwin and Cotanche, 1988; Cotanche, 1987; Cruz et al., 1987; Ryals and Rubel, 1988). In contrast, the death of hair cells in the mammalian organ of Corti does not lead to supporting cell reentry into the cell cycle, and regeneration does not occur (reviewed in Stone et al., 1998). However, in
the mammalian vestibular system, very limited hair cell regeneration from mitotic precursors following hair cell damage has been reported (Forge et al., 1993; Oesterle et al., 2003; Warchol et al., 1993). These data suggest that in the vestibular epithelium of mature mammals, a very small number of cells retains the capacity to reenter the cell cycle and cause new hair cells. This supposition has been confirmed recently by a report of self-renewing sensory hair cell progenitors from adult utricle (Li et al., 2003). It is not clear whether these cells are quiescent supporting cells, or a specialized progenitor in the vestibular sensory epithelium. However, the lack of any detectable regeneration from mitotic precursors in the organ of Corti, either in vivo (Daudet et al., 1998; Faddis et al., 1998; Roberson and Rubel, 1994; Rubel et al., 1995) or in vitro (Chardin and Romand, 1995; Romand and Chardin, 1999; Zine and deRibaupierre, 1998), suggests either that these cells do not exist in the postnatal cochlea, or that the regenerative stimulus is lost or actively blocked. To distinguish between these possibilities, it is necessary to challenge cochlear cells with an environment able to support the proliferation and differentiation of precursor cells into sensory hair cells.

The generation of hair cells from embryonic cochlear epithelium in vitro

Our experiments demonstrate that cochlear hair cells can differentiate from mitotic progenitors in dissociated cell
culture (Fig. 5). At the stage at which we isolate cochlear tissue (E13.5), approximately 85% of sensory hair cell progenitors have exited the cell cycle (Ruben, 1967). In culture, these cochlear epithelial cells aggregate into islands in the presence of mesenchyme, and approximately 35% of these islands accumulate sensory hair cells over time (Table 1) as they increase in size (Figs. 4 and 6). Individual islands can contain dozens of sensory hair cells (Figs. 4 and 6). A minority of these sensory hair cells (25%) differentiating between 3 and 6 DIV can be labeled with BrdU, indicating that their precursors divided in culture and differentiated during that period. One possible explanation for the observation that fewer than 100% of the hair cells produced in culture derive from dividing progenitors is that only those cells still capable of dividing in vivo at the time of isolation are able to respond to the established culture conditions. Alternatively, the postmitotic precursors known to be present in the organ of Corti at the time of isolation differentiate along with those cells that are still dividing and in some way limit the further proliferation of the mitotic precursors. Such a scenario would be consistent with the current model of organ of Corti development that involves Notch pathway signaling and lateral inhibition (Lanford et al., 1999; Zine et al., 2000). These mechanisms are not mutually exclusive.

A requirement for mesenchyme and EGF in the differentiation of sensory hair cell progenitors

We find that both mesenchyme and EGF are necessary for the differentiation of sensory hair cells in dissociated culture (Fig. 9). In the presence of EGF but not mesenchymal cells, cochlear epithelial cells form sheetlets of large, flat, cuboidal cells, which have a strikingly different morphology compared to the epithelial cells present in epithelial–mesenchymal cocultures (cf. Figs. 4G and 9E). In contrast, in the presence of mesenchyme but without EGF, epithelial islands form, but hair cells do not arise. Only when both mesenchyme and EGF are present do we see robust production of hair cells.

Reciprocal epithelial–mesenchymal interactions are important in many developing systems, including other sensory systems (Araki et al., 2002; Fuhrmann et al., 2000). Historically, however, studies on epithelial–mesenchymal interactions in the inner ear have tended to focus on the role of inner ear epithelium in signaling to the surrounding mesenchyme to promote chondrogenesis of the otic capsule (Frenz and Van De Water, 1991; Legan and Richardson, 1997). Our observations are consistent with earlier reports by Anniko, which suggested that sensory hair cell development is promoted by the inclusion of small amounts of periotic mesenchyme in organotypic cultures of E12.5 mouse otocysts (Anniko, 1985; Anniko and Schacht, 1984). A requirement for mesenchyme in the development of hair cells in isolated explants of cochlea tissue has also been recently reported (Montcouquiol and Kelley, 2003).

At present, we do not know the mechanism by which EGF promotes the production of hair cells. A number of studies have implicated members of the EGF and TGF-α families in the proliferation of mammalian inner ear epithelium (Kuntz and Oesterle, 1998; Oesterle and Hum, 1999; Yamashita and Oesterle, 1995). EGF may promote the survival and/or proliferation of hair cell progenitors in our cultures directly, or may act indirectly on the mesenchyme to produce other factors. These questions are currently under investigation. A recent study (Bianchi et al., 2002) established high density cultures of dissociated inner ear epithelium and mesenchyme from E13.5 and E14.5 mouse embryos in the absence of EGF, when vestibular hair cells are already present. They observed maturation of existing hair cells in culture, but did not see de novo production of hair cells from dividing progenitors. These results support our current observations that EGF is required for the production of cochlear hair cells from dividing progenitor cells.

Maturation of cochlear hair cells in vitro

In vitro, Math1-GFP+ hair cells increase in number for over 2 weeks, while they continue to mature and develop some characteristics that are similar to those elaborated by hair cells in vivo. These characteristics include the expression of myosin-VIIa (Sahly et al., 1997) and calretinin (Dechesne et al., 1994), as well as an elongated, polarized appearance. Moreover, like hair cells in vivo, in vitro-differentiated hair cells also lack p27Kip1 immunoreactivity (Fig. 10). Phalloidin staining reveals the presence of numerous, actin-rich projections reminiscent of stereocilia (Fig. 7), whose presence was confirmed by electron microscopy (Fig. 8). Electron microscopy also confirms the presence of a lumen in the center of the epithelial islands. Strikingly, the stereocilia-like structures are oriented towards this lumen, suggesting that the forces that control cellular polarity and other aspects of inner ear morphogenesis are at least partially recapitulated in vitro. We do not know whether these in vitro-differentiated hair cells express other characteristics of mature hair cells in vivo, for example, an appropriate repertoire of ion channels and differential expression of outer and inner hair cell characteristics such as prestin (Zheng et al., 2000; reviewed in Ashmore and Mammano, 2001).

Epithelial islands containing sensory hair cells also contain cells immunoreactive for p27Kip1 and S100A1, indicative of supporting cells (Fig. 10). Addition of BrdU in vitro labels approximately 30% of the cells that subsequently express p27Kip1, indicating that their progenitors cycled during the culture period. Induction of S100A1 by cultured epithelial cells suggests that supporting cells differentiate in vitro along with sensory hair cells.

Generation of hair cells from postnatal organ of Corti

Our experiments show that the postnatal organ of Corti contains cells that are able to generate new myosin-VIIa+ cells in vitro after removing all the existing sensory hair
cells by cell sorting against Math1-GFP. The origin of these sensory hair cell precursors is not known. These new hair cells are only found as singlets or doublets, suggesting that their progenitors may have reduced proliferative capacity compared to embryonic progenitors. However, as in the embryonic situation, several scenarios limiting proliferation of these progenitor cells are possible, such as the intrinsic capacity of the progenitors and/or limitations imposed by the ongoing differentiation that occurs within the epithelial islands. In any case, by using the Math1-GFP transgenic system, we are able to conclude by direct observation that the hair cells in our cultures do not represent a contaminating fraction of postmitotic hair cells that survived isolation, but rather the de novo differentiation of hair cells from mitotic precursors.

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