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Research Article

Epithelial supporting cells can differentiate into outer hair cells and Deiters' cells in the cultured organ of Corti

B. Malgrange[a,*], M. Thiry[b], T. R. Van De Water[c], L. Nguyen[a], G. Moonen[a] and P. P. Lefebvre[a]

[a] Center for Molecular and Cellular Neuroscience, Place Delcour, 17, 4020 Liège (Belgium), Fax +32 43 66 59 12, e-mail: bmalgrange@ulg.ac.be
[b] Laboratory of Tissue and Cell Biology, University of Liège, Liège (Belgium)
[c] Department of Otolaryngology, University of Miami, Miami, Florida (USA)

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Abstract. The organ of Corti is a complex structure containing a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs), supported respectively by one row of inner phalangeal cells and three rows of Deiters' cells. When fetal rat organ of Corti explants are cultured, supernumerary OHCs and supernumerary Deiters' cells are produced, without any additional cell proliferation. Analysis of semi- and ultrathin sections revealed that supernumerary OHCs are produced at the distal edge of the organ of Corti. Quantitative analysis of cell types present in the organ of Corti demonstrates that when the number of OHCs increases: (i) the total number of cells remains constant; (ii) the number of Deiters' cells increases; (iii) the number of tectal cells decreases and of Hensen's cells decreases. Using specific HC markers, i.e. jagged2 (Jag2) and Math1, we showed that in addition to existing OHCs, supernumerary OHCs, tectal cells and Hensen's cells expressed these markers in embryonic day 19 organ of Corti explants after 5 days in vitro. The results of this study suggest that Hensen's cells retain the capacity to differentiate into either tectal cells, which differentiate into OHCs, or into undertectal cells which differentiate into Deiters' cells.

Key words. Jagged2; Math1; organ of Corti; cochlea; rat; tectal cell.

The organ of Corti contains four rows of mechanosensory hair cells (HCs), i.e. a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). Within each row, OHCs are separated from one another by an interceding non-sensory supporting cell (SC), named the Deiters' cell, forming a mosaic that extends along the cochlear duct [1]. The complex morphogenesis of the inner ear is directed by a series of cell fate specifications. A number of genes are implicated in inner ear morphogenesis and HC differentiation [2–4]. These include genes for transcription factors, secreted factors, receptor/tyrosine kinases, cyclin-dependent kinase inhibitors and membrane-bound signalling proteins [5–9]. Several studies, in particular, have indicated that the Notch pathway is involved in the development of the vertebrate inner ear [9–15]. In the mammalian cochlea, Notch1 and its ligands Jag1 and Jag2 are expressed in a manner consistent with lateral inhibition [13, 14, 16]. In addition, supernumerary HCs differentiate in both the inner and outer hair cell rows in neonatal mice containing a targeted deletion of the *Jag2* gene ($Jag2^{\Delta DSL}$) [13]. Recent studies have also demonstrated that mutation in the *Lunatic fringe* (*Lfng*) gene, another component of the Notch pathway, suppresses the effect of *Jag2* mutation on HC development [17]. When combined, the results of these studies support a role for Notch-mediated lateral inhibition in determining of the number of progenitor cells that will de-

* Corresponding author.

velop as HCs and SCs. The specific downstream mediators and targets of the Notch pathway in the inner ear have not yet been fully determined, but include genes encoding the proneural gene *Math1* as well as the *HES* (Hairy and Enhancer of Split) family of inhibitory bHLH proteins [18, 19]. *Math1* has been shown to be a positive regulator for differentiation of inner ear HCs as a targeted deletion of the *Math1* gene leads to a failure of HC differentiation [20] and overexpression of *Math1* in postnatal rat cochlear explants induces production of extra HCs, in the region of the greater epithelial ridge [8]. In addition, temporal expression of *Math1* correlates well with the time period of HC differentiation. Transcripts for *Math1* can be first detected in the basal turn of the cochlea beginning on embryonic day 13 (E13) in mice in a relatively narrow band of cells in the region of the cochlear duct that will develop as the sensory epithelium. By E17, *Math1* transcripts are clearly restricted to inner and outer HCs along the full length of the sensory epithelium [21].

The capacity to produce new SCs and to repair the auditory epithelium are limited to the embryonic through neonatal period of maturation. Kelley and colleagues have reported that retinoic acid treatment on E13–E15 mouse organ of Corti explants induces the formation of supernumerary HCs [22]. Two rows of IHCs and up to seven rows of OHCs with accompanying extra Deiters' cells are observed in response to this treatment. These authors have proposed that retinoic acid induces the expansion of a 'prosensory cell' population, which is competent to commit to develop as either HCs or SCs. Results from a study in which developing HCs are ablated between E15 and postnatal day 0 by a laser-induced microlesion indicated that at least some progenitor cells located in the OHC region had the potential to develop as new HCs [23]. Supernumerary HCs also occur in vivo during the normal development of the organ of Corti, in particular in the human foetus [24–26]. The phenomena of overproduction of HCs and Deiters' cells has also been reported to occur spontaneously in cultures of embryonic and neonatal rat organ of Corti explants and can be significantly enhanced by treatment with either transforming growth factor β1 (TGFβ1) or epidermal growth factor (EGF) [27, 28]. The ability to produce supernumerary HCs and Deiters' cells in vitro persisted even when cell division was blocked by an antimitotic agent [28]. Formation of supernumerary HCs and Deiters' cells in vitro suggests that cells located in the area where HCs and SCs form retain the potential to differentiate into these cell types during early cochlear development. Identification of the precursor cells of either supernumerary HCs or Deiters' cells is an important step to elaborate a regeneration/repair strategy in the adult animal [29].

In this work, we have used the capacity of E19 rat organ of Corti to produce spontaneously supernumerary OHCs and Deiters' cells in culture after 5 days in vitro (5DIV) as a model for the identification of OHCs and Deiters' cells precursors. In the region of the organ of Corti where supernumerary OHCs and Deiters' cells arise, we have analysed the cell population to identify cells that show similarities with OHCs and Deiters' cells using morphological techniques, because no specific antigen for either supporting cell type has been characterized. We have also evaluated the possible correlation between the occurrence of supernumerary OHCs and Deiters' cells, and the disappearance of other cells located in the organ of Corti, since the production of supernumerary cells seems to involve the direct differentiation of other cells. Particular attention is focussed on the external edge of the organ of Corti since new OHCs appear at this location. The first cells bordering the most external OHC are the tectal cells [30]. These cells were initially described by several authors as similar to Hensen's cells [31, 32]. However, tectal cells in fact constitute a separate population with a number of morphological features different from those found in Hensen's cells, including (i) the lack of contact with the basilar membrane, (ii) a sparse population of short microvilli on their endolymphatic surface and (iii) a cytoplasm that is more electrodense than that of the adjacent Hensen's cells and which contains more organelles [33, 34]. Hensen's cells located laterally to tectal cells are still considered as a part of the organ of Corti and form a layer of tall cells arranged in several rows which contact the basilar membrane of the cochlea [35]. Lateral to the Hensen's cells are the Claudius cells which differ from Hensen's cells in that they are shorter and flatter, possess short apical microvilli and have a straight lateral cell border [36, 37]. These cells do not belong to the organ of Corti. Another cell type is present in the organ of Corti, beneath the tectal cells, and are called undertectal cells. They are morphologically similar to Deiters' cells or Hensen's cells and can be identified by their location: they have no contact with OHCs, while Deiters' cells do, and their apical portion never reaches the lumen of the scala media, as opposed to Hensen's and Deiters' cells.

Finally, we evaluated the possible expression of specific HC markers, i.e. myosin VIIa [38], Jag2 [9], and *Math1* [21], in cells which are candidates as the precursors of supernumerary OHCs.

Materials and Methods

Dissection and culture of the organ of Corti

Timed-mated pregnant Wistar rats were euthanised at E19 with CO_2. Embryos were removed from the uterus and transferred into a glass Petri dish containing Dulbecco's phosphate-buffered saline (PBS). Care and use of the animals reported in this study were approved by the Belgian National Fund for Scientific Research in accordance with NIH guidelines. Both cochleae were dissected

free from the calvaria with watchmaker forceps and the aid of a stereomicroscope. Each organ of Corti was freed from surrounding tissues and explanted intact in one piece onto the surface of a sterile membrane (Millicell™, 12 mm; Millipore, Bedford, MA, USA) in minimum essential medium (MEM; Gibco, Gent, Belgium) into a 24-well culture plate (Nunc). Cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Medium was renewed every 2 days.

Semithin sections and electron microscopy

Explants were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 45 min. Specimens were then postfixed in 1% OsO_4 in the same cacodylate buffer for 30 min, dehydrated in graded ethanols and propylene oxide and embedded in epoxy resin (Agar Scientific, UK). Organ of Corti explants were sectioned parallel to the longitudinal axis of the HCs. Serial semithin sections, 1 µm thick, were cut with a diamond knife, stained with toluidine blue (0.5%) and observed by light microscopy. Ultrathin sections, 80 nm thick, were contrasted with a 2.5% uranyl acetate stain (10 min at 20 °C) followed by staining in a 2.6% citrate lead solution (10 min at 20 °C) and examined using a Jeol CX-100II electron microscope at 60 kV. Some of the ultrathin sections were acetylated as previously described [39].

Quantification of cell populations within the sensory epithelium

To rule out a rearrangement of the HCs in the organ of Corti due to the compression of the explant or to migration of the cells in the sensory epithelium, quantitative analysis of supernumerary OHC production was obtained by counting the number of OHCs per length (10 µm) and surface (100 µm^2) of sensory epithelium regions from the middle turn. Rectangles with defined size were traced in control and supernumerary OHC conditions as illustrated in figure 1A and B, respectively, and all the OHCs present in this surface were counted. To quantify OHCs per length unit, a double arrow was drawn for each culture condition and all the OHCs present in the field were counted. If supernumerary cells were actually produced, the number of OHCs per unit of length should increase while the number of cells per surface unit should remain the same. In this case, the number of OHCs per unit of width increases, which can only be explained by an apposition of supernumerary OHCs as extrarows. After showing that new cells effectively arose in our culture conditions, the extent of supernumerary OHCs was determined by measuring the length of the supernumerary zone on images obtained from the confocal microscope. The total length of each organ of Corti explant (n = 12) was measured and the extent of the region with supernumerary OHCs was expressed as percent of total length. These supernumerary regions are defined by the presence of more than three rows of OHCs. Results were expressed as the mean ± SD (n = 6) and significance was determined using the Student t-test.

Defined cell populations that compose the organ of Corti were evaluated by cell counts of semithin sections selected exclusively in the midportion of the explants in order to rule out the biases of randomly selected locations of the organ of Corti, as the morphological differentiation of the organ of Corti is known to occur in a basal-to-apical gradient [40, 41]. Only cells with identifiable nuclei are counted. OHCs are easily identified by their cuticular plates and stereocilia bundles. Supernumerary hair cells are usually less mature and very often lack stereocilia and a complete cuticular plate. However, at the magnification used to count the cells, at least a portion of cuticular plate was observed, thus allowing the identification of the cell as an OHC. Deiters' cells are recognised by their location under OHCs and their extensions between OHCs which reach and form part of the reticula lamina. Below IHCs are located the inner phalangeal cells that correspond to the Deiters' cells that are located under OHCs. Between IHCs and OHCs, pillar cells extend from the basilar membrane to the endolymphatic lumen. Tectal cells have a triangular morphology, their cytoplasm is darker than the surrounding cells and their nuclei are located at approximately the same level as the OHCs. Undertectal cells are cuboidal in shape and are located just under the tectal cells, with their basal somas resting directly on the basilar membrane. Hensen's cells are also in contact with the basilar membrane, organised as a single layer of cells and containing dense aggregates in the supranuclear region of their cytoplasm (i.e. the large glycogen aggregates observed at the ultrastructural level). Seven to 18 semithin toluidine-blue-stained sections from the middle turn of E19 rat organ of Corti explants after 5DIV from three different cultures were analysed for each condition and defined by the number of supernumerary row(s) of OHCs or Deiters'cells, i.e. zero, one, two or three extra rows of supernumerary cells (corresponding to four, five or six rows of cells) as compared to control organ of Corti which contains three rows of OHCs and Deiters' cells (i.e. no extra row of supernumerary cells). The sections on which cell counts were performed were separated by at least 100 µm. Furthermore, to clearly identify the cells, serial sections were evaluated allowing, for example, assessment of the contact with the lumen, with the basilar membrane or with other cell types. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post-test.

Whole-mount phalloidin and antibody staining

Whole-mount preparations were fixed with either 4% paraformaldehyde (10 min at 20 °C) or methanol (5 min at –20 °C). The following primary antibodies were used: rabbit anti-myosin VIIa (1/200; Tama Hasson, University

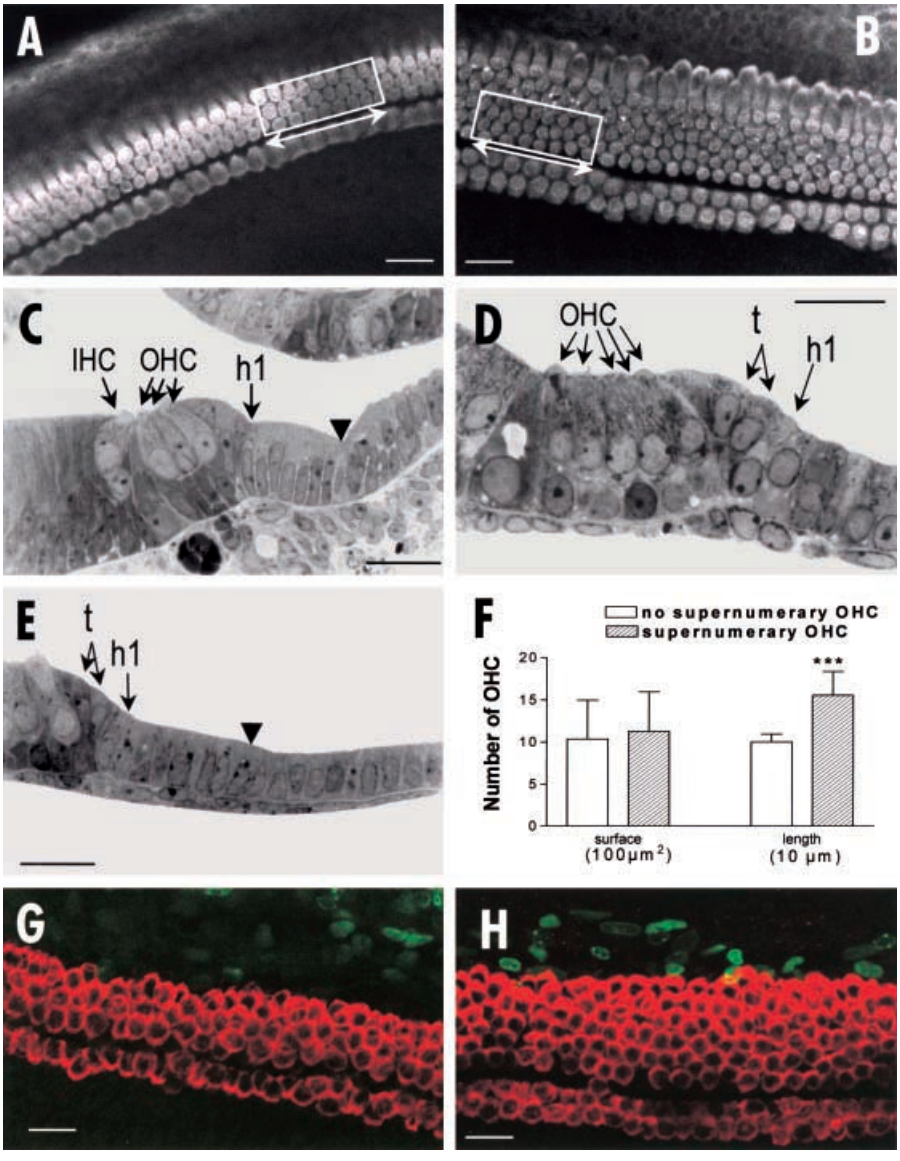

Figure 1. Both supernumerary OHCs and Deiters' cells form in response to organ culture conditions. Confocal micrographs of myosin-VIIa-stained HCs of a normal and a supernumerary HC middle turn segment of E19 explants after 5DIV (*A*, *B*). Semithin sections of corresponding regions stained with toluidine blue (*C, E*). *A*, *C*, *E* Normal segment of an explant showing a single row of IHCs and three rows of OHCs. *B*, *D* Segment of an E19 explant showing two rows of supernumerary OHCs. How cells are counted is illustrated in *C*, *D* and *E*. Externally to the tectal cells (t), a long Hensen's cell with dark-coloured cytoplasm (h1) is observed. The last Hensen's cell (arrowhead in *E*) at the external edge of the organ of Corti is easily identified by the presence of dense aggregates (glycogen) situated above the nucleus, which is located in the basal half of the cell. A notch is often identified between the Hensen's cells and the Claudius cells (arrowhead in *C*). (*F*) Counts of the number of myosin VIIa positive OHCs per surface unit (not significantly different between control and supernumerary zone) and per length unit (significantly increased in the supernumerary zone compared to the control) which demonstrate that new HCs are produced. *G, H* Confocal micrographs of myosin VIIa (red)/BrdU (green) double-stained E19 rat organ of Corti explants selected in their middle turn portion in the control (*G*) and supernumerary (*H*) zone showing the absence of incorporation of BrdU in the sensory epithelium (bar, 15 µm).

of California at San Diego, San Diego, CA, USA), rabbit anti-Math1 (1/200; CeMines, USA), goat anti-Jag2 (1/100; Santa Cruz Inc., Santa Cruz, CA, USA). These polyclonal antibodies have been previously used for specific identification of hair cells [9, 42]. Hair bundles are visualised after phalloidin staining (Alexa488 labelled phalloidin, 1/200; Molecular Probes, Leiden, Netherlands; or TRITC-labelled phalloidin, 1/100; Sigma, Bornem, Belgium). The secondary antibodies used are Alexa488-conjugated donkey anti-goat antibody (1/500; Molecular Probes) and FITC- or TRITC-conjugated donkey anti-rabbit antibodies (1/500; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Primary antibodies incubations were performed overnight at 4 °C,

while secondary antibodies incubations were performed for 1 h at 20 °C. Sequential double-labelling experiments were done with a 4% paraformaldehyde fixation step in between each immunoreaction. Preparations were mounted on microscope slides and coverslipped with Fluoprep mounting medium (BioMérieux, Marcy l'Etoile, France). To label cells in S phase in vitro, bromodeoxyuridine (BrdU, 10 µM; Sigma) was added to the medium for either 2 or 5DIV. Fixation of explants with 4% paraformaldehyde for 5 min at 20 °C, was followed by a 5-min incubation in 1% Triton-X100 in PBS. After a 30-min incubation in blocking buffer, cells were stained with anti-myosin VIIa, followed by Alexa568 conjugated anti-rabbit antibody. Cells were postfixed in 4% paraformaldehyde at 20 °C for 10 min, incubated in 2 N HCl for 10 min and then neutralised with 0.1 M sodium tetraborate, pH 8.5. Cells were incubated for 45 min with anti-BrdU FITC-conjugated antibody (1:3; Becton-Dickinson, Heidelberg, Germany).

Confocal laser scanning microscopy

Observations were performed on a BioRad MRC1024 confocal microscope with LaserSharp 3.1 acquisition software. Digital images of a compressed Z-series of scans were made using either ×40, ×63 or ×100 oil objectives lenses. Continuous series of optical sections in the Z-plane were saved and recombined to produce a single reconstruction of the entire thickness of the epithelium selected in the midportion of the explant of organ of Corti. Images used for the figures were processed with confocal assistant software (CAS, version 4.0; Todd Clarke Brelje).

Results

Supernumerary OHCs arise distal to the third row of OHCs

E19 organ of Corti explants cultured for 5 DIV routinely produce supernumerary OHCs detected using an anti-myosin VIIa antibody which specifically stains HCs [38] (compare fig. 1B to 1A). These supernumerary OHCs arose as extra rows, respecting the architecture of the existing sensory epithelium (fig. 1A–D). The total length of the supernumerary OHC regions which are never located at the apical turn was measured after 5DIV and represented 16 ± 4% of the total length of the organ of Corti explant in unsupplemented MEM. The number of extra rows of OHCs and size of the region of supernumerary OHCs reached a maximum after 5DIV and did not increase even after an additional 5DIV (data not shown). The cell types in toluidine blue-stained semithin sections from both normal and supernumerary OHC regions of the 5DIV organ of Corti explants were examined by light microscopy. In the region of the normal organ of Corti, cuticular plates and stereociliary bundles could be identified in the three rows of OHCs (fig. 1C). In the region of supernumerary OHCs, four to five rows of OHCs and corresponding Deiters' cells were usually identified (fig. 1D). In some cases, a sixth row of the two cell types was observed. The most external row of supernumerary OHCs often appeared shorter than OHCs in the other rows.

To rule out a rearrangement of the OHCs in the organ of Corti due to compression of the explant or to migration of the sensory cells, the number of OHCs per unit surface area (100 µm^2) and length unit (10 µm) of the explant was compared. The results show that the number of OHCs counted per surface unit remained identical to the control when supernumerary OHCs were observed, while the number of OHCs per length unit increased significantly in a comparable condition (fig. 1F). These data clearly demonstrated that supernumerary OHCs arise in the organ of Corti and that there was no rearrangement of the existing HCs.

Production of supernumerary OHCs and Deiters' cells results from the differentiation of existing epithelial cells located within the sensory epithelium

The production of supernumerary OHCs and Deiters' cells can result either from the differentiation of an existing cell type (i.e. a prosensory cell) or from the division of progenitor cells and subsequent differentiation of a newly produced daughter cell into either an OHC or a Deiters' cell. In a previous study, we demonstrated that the production of supernumerary OHCs induced by the treatment of explants with EGF is not altered by the incubation of the organ of Corti with the antimitotic drug, cytosine arabinoside, favouring the hypothesis of differentiation of an existing cell type into an OHC [28]. In this study, we looked for the presence of dividing precursor cells in the E19 rat organ of Corti explants. The explants were incubated for either 2 or 5DIV in the presence of 10 µM BrdU. In the area of the HCs and SCs, no epithelial cells were labelled with BrdU (fig. 1G, H). BrdU-positive nuclei (green) are present in the explants, however, these cells are located at the level of the connective tissue below the epithelium and never colocalized with myosin VIIA-positive HCs. Furthermore, careful examination of semithin sections of E19 organ of Corti explants after 1, 2, 3 and 5DIV was undertaken to detect the presence of mitotic figures in epithelial cells of these organotypic cultures. With over 60 non-consecutive sections examined per culture condition, not one mitotic figure was observed within the sensory epithelium, in particular in the region of the SC types, in areas containing either a normal number of OHCs and Deiters' cells or in those areas containing numerous rows of supernumerary OHCs and Deiters' cells. Mitotic figures were, however, frequently observed in adjacent connective tissues that are of mesenchymal origin confirming the BrdU labelling. Produc-

tion of supernumerary OHCs and Deiters' cells in the absence of any evidence of cell division argues in favour of direct transdifferentiation of existing cell types into both OHCs and Deiters' cells.

Tectal cells and OHCs share morphologic characteristics

Figure 2A shows a region of the normal E19 organ of Corti after 5DIV. In this section, the two major types of classically described cells are identified: sensory HCs and non-sensory SCs. The HCs include both IHCs and OHCs, while the SCs consist of pillar cells, inner phalangeal cells, Deiters' cells, Hensen's cells, tectal cells and undertectal cells. As the supernumerary OHCs occurred in the same area as the existing OHCs, we hypothesised that these newly generated supernumerary OHCs differentiated from closely situated cells. As shown in figure 2B, in a supernumerary OHCs region, an immature OHC (O4) is identified between the third row of OHCs (O3) and the first tectal cell (t1). The immaturity of this new OHC is essentially characterised by an immature cuticular plate and the absence of stereocilia. This hypothesis is reinforced by our previous observation that supernumerary OHCs arose distally to the third row of OHCs [43]. To locate and identify cells within the sensory epithelium that have retained the capacity to differ-

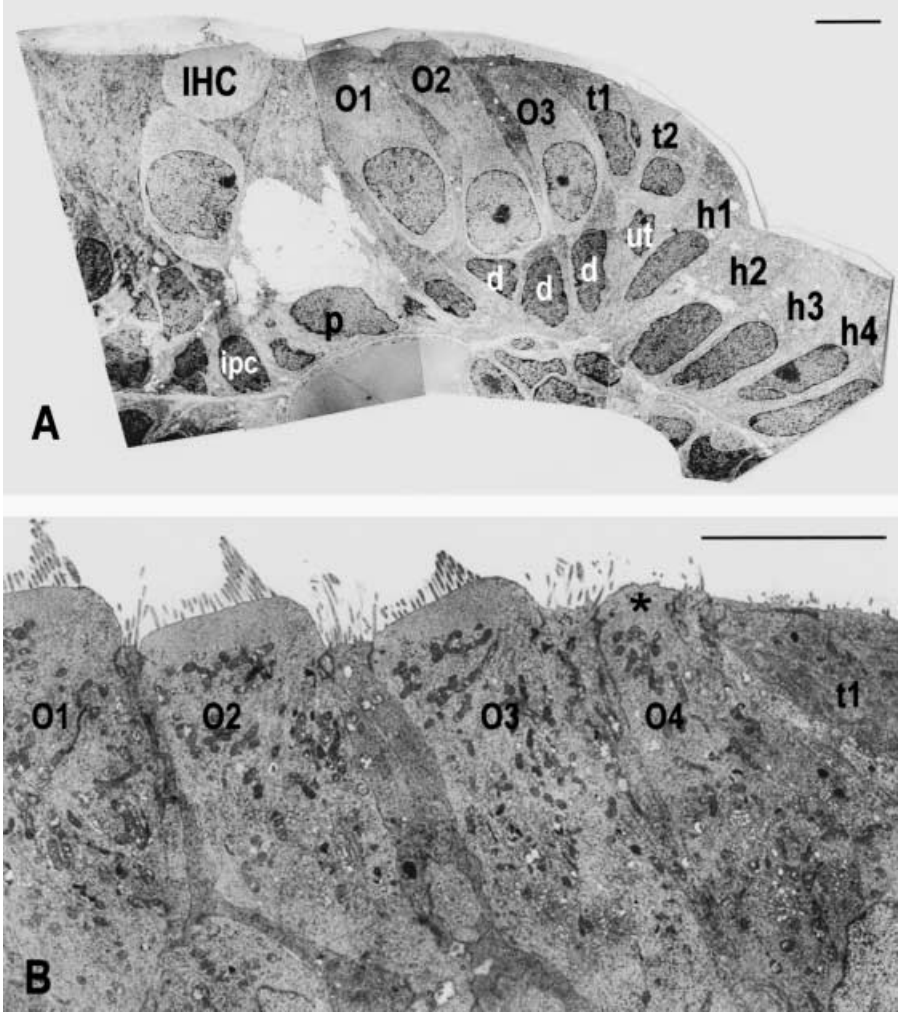

Figure 2. (*A*) Organ of Corti explants have regions of normal OHC and Deiters' cell configurations. Ultrathin cross section through the E19 rat organ of Corti explant after 5DIV without supernumerary OHCs or Deiters'cells. Outer hair cells (O) and inner hair cells (IHC) are easily identified. They are separated by pillar cells (p). IHCs and OHCs are supported by inner phalangeal cells (ipc) or Deiters' cells (d), respectively. These SCs extended from the basilar membrane to the lumen of the scala media. On the outer side of OHCs, there was also a segment of simple epithelium formed by cells extended from the basilar membrane to the lumen of the scala media: Hensen's cells (h). In contrast, the upper or tectal cells (t), which are located between the first Hensen's cell (h1) and the third row of OHCs, does not come into contact with the basilar membrane. The undertectal cell (ut) is located under the tectal cell (bar, 5 µm). (*B*) Organ of Corti explants have regions with supernumerary OHCs and Deiters' cells. Ultrathin cross-section showing an immature OHC with an incomplete cuticular plate (*) located between a normal OHC (O3) and a tectal cell (t1). (bar, 5 µm).

entiate into OHCs, we examined and compared the ultrastructure of five distinct cell types present in the organ of Corti explants after 5DIV: OHCs, Hensen's cells, tectal cells, undertectal cells and Deiters' cells. In cross sections through areas of the E19 rat organ of Corti explants that do not contain supernumerary IHCs and OHCs, OHCs were easily identified with their cuticular plates and stereociliary bundles protruding into the lumen of the scala media (fig. 2A). Nuclei of OHCs are situated in the basal portion of these sensory cells, have a single prominent nucleolus and exhibit a thin edge of condensed chromatin associated with the nuclear envelope. Cytoplasm of the maturing OHCs is enriched with organelles such as mitochondria, Golgi complexes, free ribosomes and rough endoplasmic reticulum (RER). Mitochondria of the OHCs are preferentially found under the cuticular plates (fig. 3A) and the Golgi apparatus is located between the cuticular plate and the nucleus. RER is observed predominantly in supranuclear areas and on the inner side under the cuticular plates. In the portion of the organ of Corti explant containing supernumerary OHCs, the fine structure of most OHCs is identical to that described above for the sections of these explants containing a normal configuration of HCs. One exception is that the cuticular plate of the supernumerary OHCs, located in the most distal row adjacent to the tectal cell, appears incomplete (fig. 3B).

All OHCs, including rows of supernumerary OHCs, are supported by a Deiters' cell (figs. 1C, D, E, 2) whose cell soma extends from the basilar membrane to the lumen of the scala media. At its apical pole, the Deiters' cell exhibits a tuft of microvilli. Deiters' cell nuclei are situated in the basal portion of the cell and contain a perinuclear layer of condensed chromatin. Cytoplasm of Deiters' cells is characterised by the presence of an abundant RER that has a distended lumen filled with fibrillar material (fig. 3A, lower left). Large aggregates of glycogen are present in the vicinity of the nuclei of the Deiters' cells (data not shown).

Distal to the last row of OHCs is a simple epithelium formed by Hensen's cells whose somas extended from the basilar membrane to the lumen of the scala media (fig. 2A). The apical pole of Hensen's cells contains only a few microvilli and the elongated nucleus of Hensen's cells is located in the basal portion of its soma. Cytoplasm of the Hensen's cell contains the same organelles present in the Deiters' cell. In particular, an abundant and distended RER and aggregates of glycogen are observed between the nucleus of the Hensen's cell and its apical cell membrane (fig. 3C). The Hensen's cell (h1) located nearest to the last row of OHCs differs from the other Hensen's cells in that its elongated nucleus is more centrally located within its soma (fig. 2).

A fourth type of supporting cell, the tectal cell (figs. 2, 3D), is characterised by a sparse population of short microvilli present on its apical surface, a cytoplasm containing abundant RER that is either slightly or not distended and that is preferentially localised at the inner side of the cell between the nucleus and the apical cell membrane, and only a few small aggregates of glycogen. Beneath the tectal cells lie cells that we have named 'undertectal cells' (ut, fig. 2). These cells belong to the family of the Hensen's cells. They contact the basal lamina but do not extend to the lumen of the scala media and present an ultrastructural morphology that is similar to that of both Deiters' cells and Hensen's cells.

Table 1 presents a summary of the principal ultrastructural features of these five cell types, indicating that tectal cells and OHCs share morphological characteristics.

Number of tectal cells and Hensen's cells decreases when the number of OHCs and Deiters' cells increases

Considering the absence of any detectable mitotic activity in the neurosensory epithelium of the organ of Corti, the production of supernumerary OHCs and Deiters' cells must result from the differentiation of cells located within the sensory epithelium. If this is true, the total number of cells in the organ of Corti has to remain constant, even when the number of OHCs and Deiters' cells increases. Cell counts were performed on toluidine blue-stained semithin sections from areas of a normal configuration of the organ of Corti and from areas that contained supernumerary OHCs and Deiters' cells. Figures 1C–E and 2 illustrate the various cell types that were counted: HCs, the SCs which include Deiters' cells and inner phalangeal cells located beneath OHCs and IHCs, respectively, tectal cells, undertectal cells, pillar cells and Hensen's cells. Supernumerary hair cells are usually less mature and very often lack stereocilia and a complete cuticular plate.

Table 1. Summary of the principal morphological features of cells present in E19 rat organ of Corti explants cultured for 5DIV.

	OHC	Deiters' cell	Tectal cell	Undertectal cell	Hensen's cell
Basilar membrane contact	no	yes	no	yes	yes
RER form	not distended	distended	not or little distended	distended	distended
Glycogen aggregates	absent	large	small	large	large
Cuticular plate	present	absent	absent but presence of a thin layer of fibrillary material	absent	absent

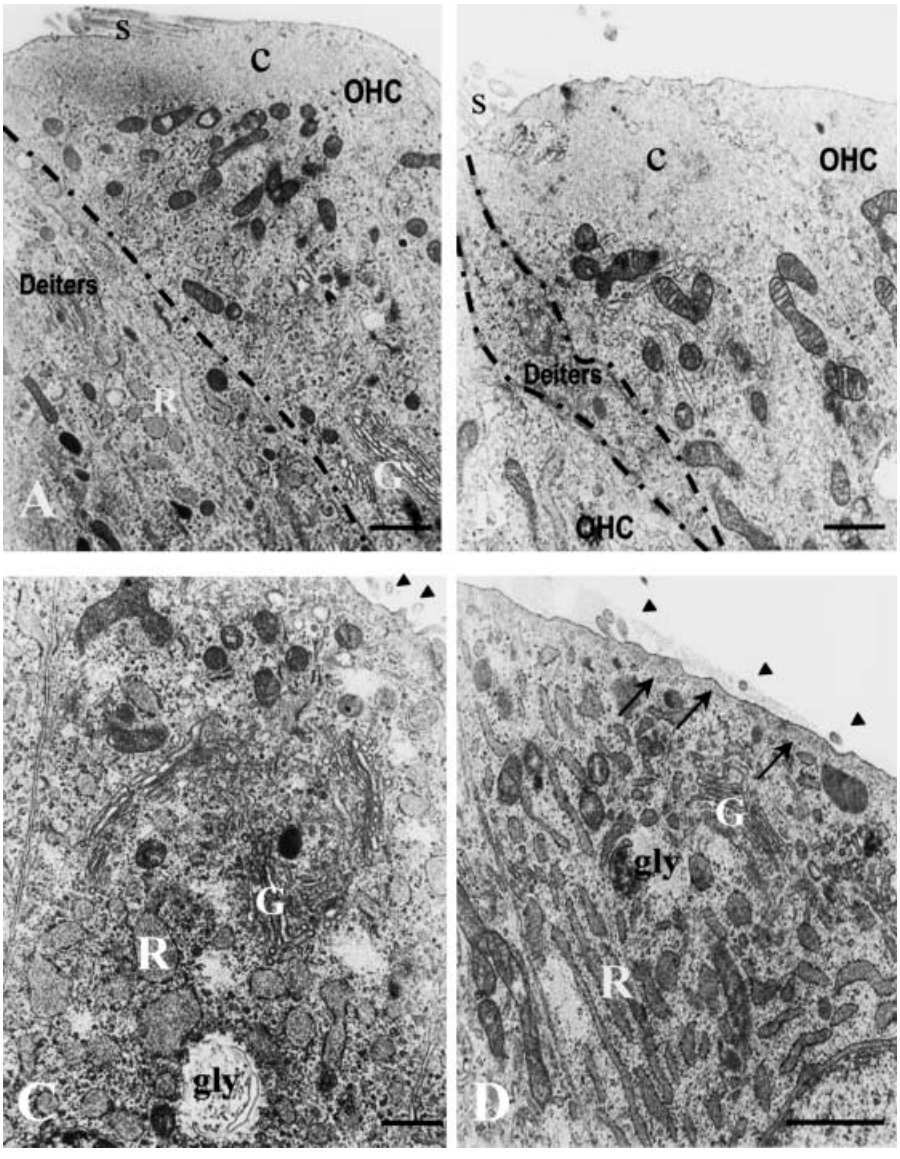

Figure 3. Photomicrograph of ultrathin sections through a segment of Corti's organ containing supernumerary HCs. (*A*) An outer hair cell (upper right, OHC) and its corresponding Deiters' cell (lower left, Deiters). The OHC has a well-developed cuticular plate with anchored stereocilia. The cytoplasm is enriched with different organelles such as mitochondria, Golgi complexes, free ribosomes and RER. Mitochondria are preferentially found under the cuticular plate (c). RER is predominantly present in the supranuclear area and on the inner side under the cuticular plate. Stereocilia (s) are observed at the apical portion of the cell. (*B*) An immature hair cell. In this cell, the cuticular plate (c) appears incomplete. The fine structure of this cell is identical to that observed in explants containing a normal number of OHCs. Stereocilia (s) are already present. (*C*) A Hensen's cell. These cells form a simple epithelium. At their apical pole, only a few microvilli are obvious (arrowheads). The elongated nucleus is located in the basal portion of the cells. Their cytoplasm contains the same organelles (see Golgi apparatus, G) as those present in Deiters' cells. In particular, an abundant and distended RER (R) and aggregates of glycogen (gly) are found between the nucleus and the apical cell membrane. (*D*) A tectal cell. The tectal cells do not come into contact with the basilar membrane. RER (R) is rarely or not distended and only a few small aggregates of glycogen (gly) are encountered. RER is also preferentially formed on the inner side of the cell between the nucleus and the apical cell membrane. Mitochondria are further predominantly detected under the apical cell membrane. Microvilli (arrowheads) and a thin layer of material (arrows) are observed at the apical portion of the cell.

However, at least a portion of the cuticular plate was easily identified, thus allowing the identification of the cell as an OHC. Tectal cells are easily identified because of (i) the location of their nuclei, situated at approximately the same level as the nuclei of the OHCs (ii) dense coloration of their cytoplasm in response to toluidine blue staining an (iii) the triangular shape of their somas (fig. 1C–E). Tectal cells are immediately adjacent to the last row of OHCs (see O3 and t1, t2, fig. 2) and are bordered on their distal side by a long Hensen's cell (h1), whose soma both contacts the basilar membrane and borders the endolymphatic lumen, and contains a more centrally located nucleus. Hensen's cells are identified as cells located on the basilar membrane and showing at least two of the three following morphological criteria to differentiate them from the Claudius cells: (i) the presence of large aggregates of glycogen, which are characterized by an accumulation of dense materials above the nucleus when stained with toluidine blue (fig. 1C–E), (ii) the height of the supranuclear cytoplasm region which is more important in Hensen's cells than in Claudius cells and (iii) the identification of a notch between the Hensen's cells and Claudius cells (fig. 1C).

Seven to 18 sections were evaluated for the number of supernumerary OHCs or supernumerary Deiters' cells (i.e. zero, one, two or three supernumerary cells). Figure 4 summarises the data obtained from these cell counts. The total number of cells per section (i.e. IHCs + OHCs + Deiters' cells + inner phalangeal cells + pillar cells + tectal cells + undertectal cells + Hensen's cells) remained constant in organ of Corti sections even when the number of supernumerary OHCs or supernumerary Deiters' cells per section increased (fig. 4A). The number of Deiters' cells on cross-sections of organ of Corti explants correlated with the

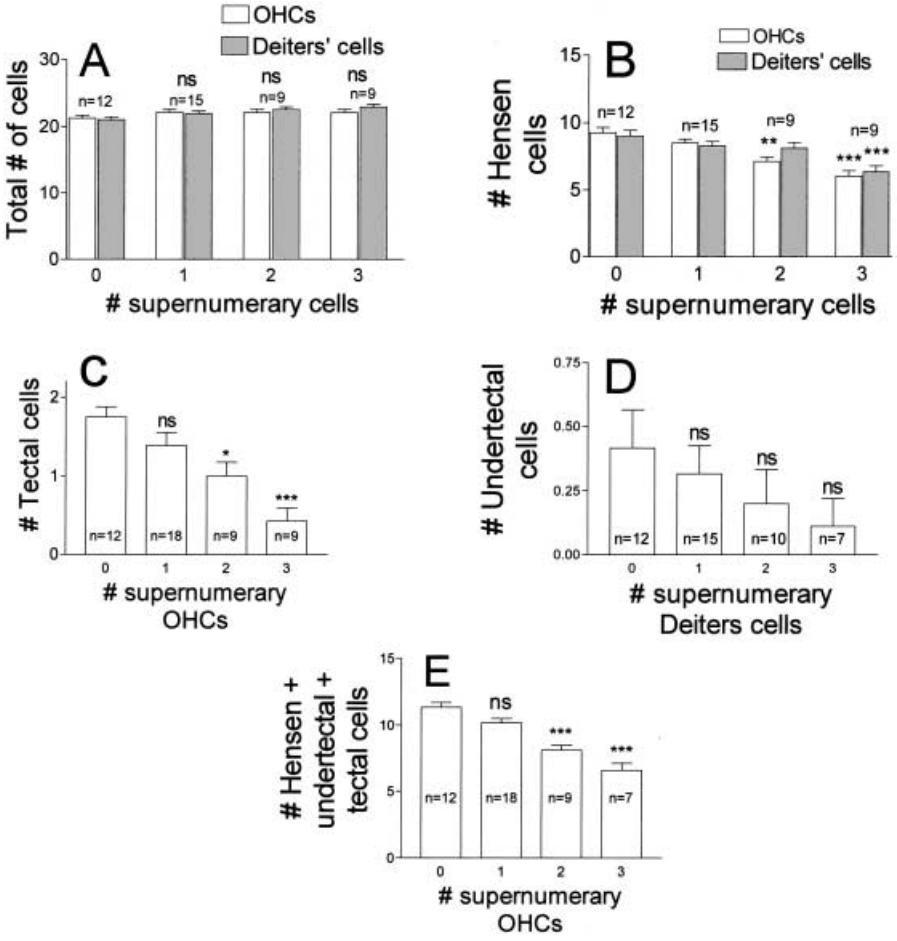

Figure 4. Quantitative analysis of each cell population within E19 rat organs of Corti in culture for 5DIV. (*A*) Total number of cells observed on cross-sections of organ of Corti explants cultured for 5DIV as a function of the number of supernumerary cells (OHCs or Deiters' cells). (*B*) Number of Hensen's cells as a function of the number of supernumerary cells (OHCs or Deiters' cells). (*C*) Number of tectal cells as a function of the number of supernumerary OHCs. (*D*) Number of undertectal cells as a function of the number of supernumerary Deiters' cells. (*E*) Number of Hensen's + tectal + undertectal cells as a function of the number of supernumerary OHCs. Cell counts were performed on semithin sections stained with toluidine blue (n = 7–18). Results are expressed as mean ± SD (* $p < 0.05$,** $p < 0.01$ and *** $p < 0.001$).

number of OHCs. In areas of explants devoid of supernumerary OHCs (i.e. containing three OHCs), 3.25 ± 0.45 Deiters' cells were counted. For the gain of each supernumerary OHC, a gain of one supernumerary Deiters' cell was observed. When five OHCs were present, we counted 4.9 ± 0.78 Deiters' cells. In the areas of organ of Corti explants without supernumerary OHCs, the number of Hensen's cells was 9.2 ± 1.4 per section. When the number of rows of OHCs or Deiters' cells increased in cross sections of the organ of Corti explant, the number of Hensen's cells decreased significantly (fig. 4B).

As the number of supernumerary OHCs increased, the number of tectal cells significantly decreased when compared to the normal organ of Corti (fig. 4C). Counts of the undertectal cells, the cells in contact with the basilar membrane and located under the tectal cells, showed a decreasing trend as the number of supernumerary OHCs increased (data not shown) or as the number of Deiters' cells increased (fig. 4D).

Furthermore, for three OHCs and the three corresponding Deiters' cells, we counted 11.4 ± 1.2 Hensen's cells + undertectal cells + tectal cells; in the presence of six OHCs (i.e. three supernumerary OHCs), there were 6.5 ± 1.4 Hensen's cells + undertectal cells + tectal cells (fig. 4E) which approximately corresponded to a decrease of two cells of the pool of cells composed of Hensen's cells + undertectal cells + tectal cells per OHC added. These data show that, when supernumerary OHCs and their corresponding Deiters' cells arise in the organ of Corti, the pool of cells composed of Hensen's cells + undertectal cells + tectal cells degreases, suggesting that these cells might be the precursors of either OHCs and their associated Deiters' cells.

Hensen's cells express the HC markers Jag2 and Math1 when the organ of Corti is cultured

Myosin VIIa has been reported to initiate its expression in HCs of E14 mouse otocysts [44], corresponding to rat E16.5 inner ears. In E19 rat organ of Corti explants, either at the time of excision or after 5DIV, myosin VIIa immunolabelling is highly specific to HCs including the supernumerary OHCs that differentiate in the explants (fig.

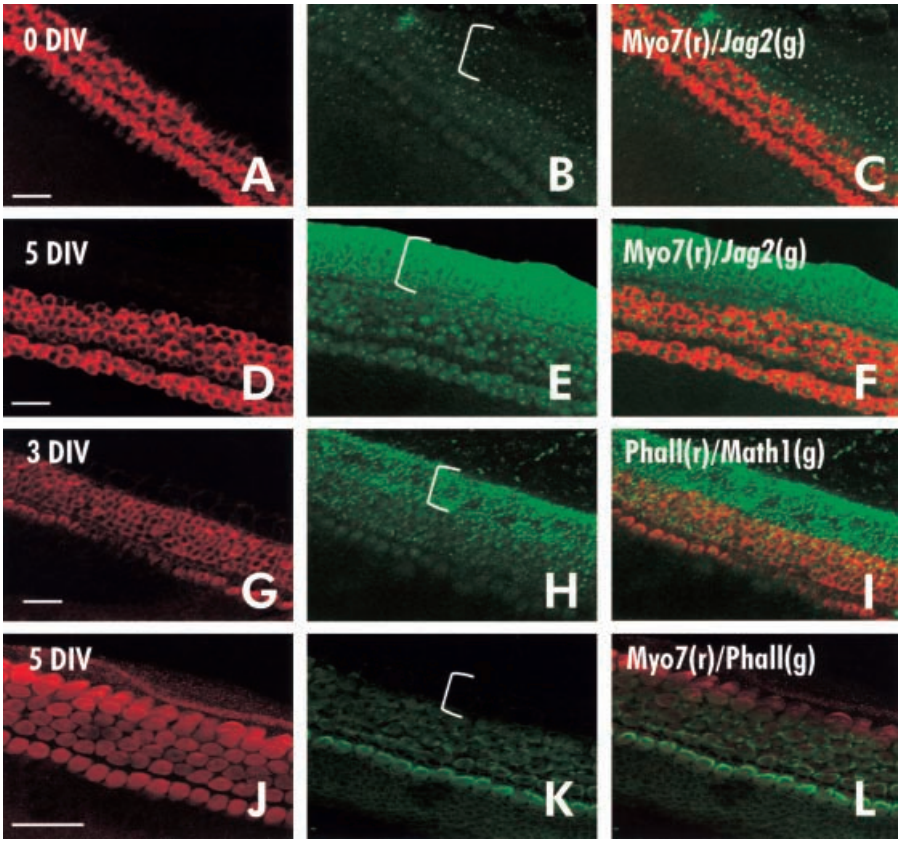

Figure 5. Confocal micrographs of myosin VIIa/Jag2 (*A–F*), phalloidin/Math1 (*G–I*) and myosin VIIa/phalloidin (*J–L*) double-stained E19 rat organ of Corti explants selected in their middle turn portion. (*A–C*) E19 organ of Corti at the time of explantation, showing weak labelling for Jag2 (green) in HCs and strong labelling for myosin VIIa (red). (*C*, *F*, *I*, *L*) Superposition of, images *A*+*B*, *D*+*E*, *G*+*H* and *J*+*K*, respectively. Jag2 after 5DIV and Math1 after 3DIV are strongly expressed in HCs (including supernumerary OHCs), tectal cells and Hensen's cells (*E*, *F* and *H*, *I*, respectively). Phalloidin staining shows that the apical portion of the cells is more differentiated (organised and strongly labelled stereocilia) in HCs located medially (*K*, *L*). The tectal cell and Hensen's cell area is indicated by brackets (bar, 15 µm).

5A, D, and J). The other markers, i.e. Jag2, Math1 and actin, have been shown to be expressed in inner ear HCs as early as E17 in mice which corresponds to E20 in the rat inner ear [21]. Double immunolabelling for myosinVIIA/Jag2 (fig. 5A–C), myosinVIIA/Math1 and myosin VIIA/phalloidin (results not shown) of E19 rat organ of Corti at the time of excision revealed that myosin VIIa is present in all auditory HCs. Weak expression for Jag2 was observed and restricted to HCs (fig. 5B, C), while Math1 did not immunostain the sensory epithelium (not shown). All HCs, including supernumerary OHCs, were strongly immunopositive for Jag2 (fig. 5D–F) and Math1 (data not shown) in the E19 explants after 5DIV. In these 5DIV explants, the Jag2 and Math1 antibodies also immunostained both tectal cells and Hensen's cells. Math1 immunopositivity (fig. 5H, I) was detected earlier than Jag2 immunolabelling in these cells, after 3 and 5DIV, respectively Math1 immunoreactivity was decreased but still present in Hensen's cells after 5DIV (result not shown). Omission of primary antibodies resulted in a complete loss of immunostaining (data not shown). Tectal cells and Hensen's cells were never immunopositive for myosin VIIa. Staining of actin by FITC-phalloidin did not clearly identify OHCs in E19 explants at the time of excision. When phalloidin labelling of actin was performed on E19 explants after 5DIV, both the normal configuration of HCs and all rows of supernumerary OHCs were stained, with the exception of the most distal row of supernumerary OHCs. This pattern of phalloidin labelling suggests that the last row of supernumerary OHCs comprises the most immature of these newly differentiated cells (fig. 5K, L).

Discussion

Supernumerary HCs arose by a non-mitotic differentiation pathway

In rodents, auditory HC and Deiters' cell precursors have their terminal mitosis between E12–E16 [45]. Recently, the organ of Corti was shown to retain the capacity to produce HCs after this initial period of HC genesis [46]. In particular, supernumerary HC production has been reported when organ of Corti explants are cultured in vitro [47]. This overproduction of auditory HCs can be induced by the addition of growth factors such as TGFβ1 or EGF to the culture medium [27, 28]. Our current results show that, under unsupplemented culture conditions, i.e. in the absence of growth or differentiating factors, the production of supernumerary OHCs occurs in E19 rat organs of Corti. We observed up to six rows of OHCs in some regions of the organ of Corti explants. Indeed, we clearly demonstrate using HC counts per surface and per length unit that supernumerary HCs arise in the organ of Corti and not from a rearrangement of existing HCs. Two mechanisms can be conceptually proposed to explain the appearance of supernumerary OHCs and Deiters' cells in the auditory neuroepithelium. Progenitor cells undergo mitosis and the resultant daughter cells become both OHCs and Deiters' cells. A second mechanism of OHC and Deiters' cell production is the differentiation of a prosensory cell which is located in the vicinity of these cells and which retains the capacity to differentiate into either an HC or a Deiters' cell. BrdU labelling of explants producing supernumerary OHCs and Deiters' cells fails to reveal the incorporation of this thymidine analogue by supernumerary OHCs or in any of the SCs including Deiters' cells. Furthermore, mitotic figures in the sensory epithelium are never observed in any sections of the explants. Finally, the total number of cells in the organ of Corti does not change, even when the number of OHCs and Deiters' cells increases. Based on these results and our previous observation of the development of supernumerary OHCs in the presence of a mitotic blocker [28], we propose that the production of both supernumerary OHCs and Deiters' cells in the late embryonic or early postnatal organ of Corti explants is the consequence of the differentiation of precursor cells located within the sensory epithelium. This hypothesis is in accordance with the theory that replacement HCs might arise directly from SCs, without an intervening mitotic event, by 'phenotypic conversion' or 'transdifferentiation' of SCs. This 'cell conversion' is observed in the amphibian lateral line organ after laser ablation of HCs [48], in amphibian vestibular sensory epithelia after aminoglycoside-induced HC damage [49, 50], in avian basilar papilla after noise trauma [51] or aminoglycoside damage [52] and in utricular maculae of mature guinea pigs following gentamicin-induced HC death [53–55].

Tectal and Hensen's cells serve as OHCs precursors

The precursor or prosensory cell that will become a supernumerary OHC should be located at the outer edge of the organ of Corti because OHCs displaying the greatest degree of immaturity (i.e. immature stereocilia and incomplete cuticular plates) are observed at the level of the external row of OHCs in supernumerary OHC portions of the organ of Corti. Phalloidin labelling of f-actin provides an additional proof, in that the cuticular plate is less formed in the most external myosin VIIa-immunopositive OHCs, in accordance with our ultrastructural findings showing an incompletely formed cuticular plate under the apical cell membrane of these most distal OHCs. The ultrastructural study of the organ of Corti that compares supernumerary areas to zones of normal OHC production reveals that cells adjacent to the last row of OHCs, named tectal cells and belonging to the family of SCs, share some similarities with both OHCs and Hensen's cells (see table 1) [30]. These observations suggest that tectal cells might be prosensory cells which can

differentiate into OHCs. Math1 is a bHLH transcription factor whose expression has been described exclusively in differentiating HCs and clearly precedes myosin VIIa expression in a basal-to-apical gradient [8, 21, 42] in the in vivo organ of Corti. When the organ of Corti is cultured, the expression of this HC marker is observed at the level of HCs, supernumerary OHCs, tectal cells and Hensen's cells, reinforcing their potential role in the production of supernumerary OHCs. When the rat organ of Corti is explanted at E19, Jag2 immunolabelling is exclusively localised in the HCs. This expression, which is very weak, increases with time in culture and corresponds to the immunolabel pattern reported during normal embryonic development of HCs [13]. After 2DIV, Jag2 immunoreactivity is clearly observed in HCs, but not in SCs. After 5DIV, the expression of Jag2 is not restricted to HCs, but extends to tectal cells and Hensen's cells, where a strong Jag2 immunoreactivity is observed. The occurrence of this HC marker in these SCs further strengthens their hypothesised role as precursors of supernumerary OHCs when stimulated by explantation and culture. A wave of Math1 immunolabelling closely followed by the expression of Jag2 observed in Hensen's cells in cultured E19 organ of Corti explants is similar to the sequence of events observed during the normal development of the mouse organ of Corti [21]. As a result of the expression of Math1, Hensen's cells present within the epithelium become competent to develop as OHCs. Next, a subset of these cells upregulate expression of the Notch ligand Jag2. The presence of Jag2 results in activation of the Notch receptors on adjacent cells. Notch is expressed in all SCs, but not in HCs in E19 explant of organ of Corti, either at the time of explantation or after 5DIV (result not shown). Interestingly, inhibition of Notch expression using antisense oligonucleotides leads to the production of supernumerary HCs in E19 rat explants of organ of Corti in culture. But in these conditions, HC-HC contacts have been reported, suggesting that sufficient numbers of supernumerary Deiters' cells are not produced [9].

HCs and Deiters' cells may share a common precursor

The only way to identify the fate of cells located in the sensory epithelium is to count the cells on histological sections of organs of Corti, since no specific cell marker exists for each supporting cell type. Analysis of the semithin sections in the explant portion including supernumerary OHCs has shown that each time a supernumerary OHC is observed, a supernumerary Deiters' cell also arises. In over 40 sections and numerous surface preparations of organ of Corti immunostained for myosin VIIa, the classically described mosaic with an invariant alternation of OHCs and Deiters' cells is present and contact between two OHCs is never observed. In addition, quantitative data presented in this paper show that the number of tectal cells does not decrease in strict correlation with the increases in the number of supernumerary OHCs. Therefore, a constant replacement of tectal cells should occur within the sensory epithelium. Hensen's cell counts reveal that when a supernumerary OHC and a Deiters' cell are produced, the number of Hensen's + tectal + undertectal cells decreases by two, demonstrating that these cells can act as precursors for both OHCs and Deiters' cells (fig. 6). The data presented in this paper suggest the following sequence of events to explain the production of supernumerary HCs and Deiters' cells in the developing organ of Corti. The tectal cells differentiate into OHCs and are subsequently replaced by the differentiation of Hensen's cells. A similar mechanism can be proposed for the supernumerary Deiters' cells which derived from the differentiation of undertectal cells which themselves are replaced by Hensen's cells. Hensen's cells constitute in this model the population of precursors of both cell lineages. Indeed, cell lineage studies in chick show that HCs and SCs share a common progenitor [56] and a conditionally immortalised cell line from mammalian sensory epithelium can acquire some characteristics of both HCs and SCs [57]. Another argument in favour of a common precursor is the fact that cell fate determination in the inner ear has been proposed to occur through a lateral inhibition model whereby a cell that is becoming committed to a particular pathway of differentiation inhibits its immediate neighbours from doing likewise [58, 59]. A de facto requirement of this model is that no intrinsic bias in cell fates will exist among the precursor cells giving rise to HCs and SCs. This means that a situation in which HCs and SCs would arise from separate populations of precursor cells would rule out the involvement of a lateral specification model in cell fate choices.

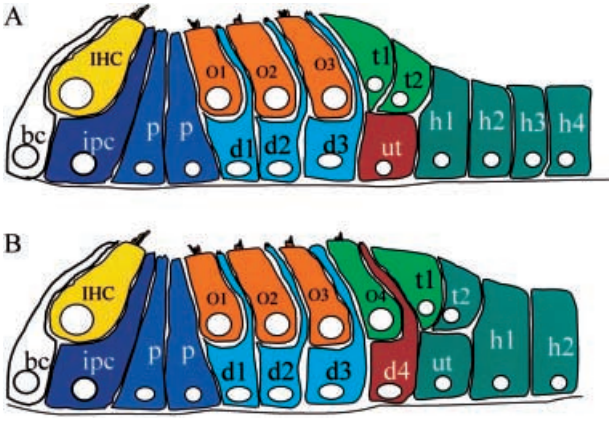

Figure 6. Schematic diagram showing the origin of supernumerary OHCs. Hensen's cells (h) retain the capacity to differentiate into (i) tectal cells (t) which subsequently differentiate into OHCs (O) or (ii) into undertectal cells (ut), which differentiate into Deiters' cells (d). IHC, inner hair cells; ipc, inner phalangeal cells; p, pillar cells; b, border cells.

Is there a role for Hensen's and tectal cells in HC regeneration?

The results described in this paper lend further credence to the hypothesis that a single precursor cell type can give rise to both HCs and SCs during HC regeneration. However, it is important to note that there are differences between regeneration and development. In our cell culture model, two Hensen's cells give rise to one HC and one Deiters' cell. In contrast, many of the regenerative paradigms cause a selective HC loss, leaving a population of differentiated SCs undamaged. In ototoxin-damaged postnatal rat organ of Corti explants, exogenous trophic factors were previously shown to enhance the ability of undifferentiated cells and/or SCs to produce new immature HCs [60–62]. Further studies will be necessary to identify the role of tectal and Hensen's cells in HC replenishment within the postnatal organ of Corti after aminoglycoside treatment and whether this capacity persists in the cochleae of adult animals. The presence of tectal cells and Hensen's cells in the adult organ of Corti is encouraging [30]. Their potential to differentiate into HCs opens the prospect of the replacement of lost auditory HCs, once factors controlling their differentiation and suppressive mechanisms of lateral inhibition have been identified.

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