Development 129, 2495-2505 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1807

The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination

Ping Chen[1,*], Jane E. Johnson[2], Huda Y. Zoghbi[3] and Neil Segil[1,4,*]

[1]Gonda Department of Cell and Molecular Biology, House Ear Institute, Los Angeles, CA 90057, USA
[2]Center for Basic Neuroscience, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, USA
[3]Department of Molecular and Human Genetics, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA
[4]Department of Cell and Neurobiology, University of Southern California Medical School, Los Angeles, CA 90033, USA
*Authors for correspondence (e-mail: pchen@hei.org and nsegil@hei.org)

Accepted 5 March 2002

SUMMARY

During embryonic development of the inner ear, the sensory primordium that gives rise to the organ of Corti from within the cochlear epithelium is patterned into a stereotyped array of inner and outer sensory hair cells separated from each other by non-sensory supporting cells. *Math1*, a close homolog of the *Drosophila* proneural gene *atonal*, has been found to be both necessary and sufficient for the production of hair cells in the mouse inner ear. Our results indicate that *Math1* is not required to establish the postmitotic sensory primordium from which the cells of the organ of Corti arise, but instead is limited to a role in the selection and/or differentiation of sensory hair cells from within the established primordium. This is based on the observation that Math1 is only expressed after the appearance of a zone of non-proliferating cells that delineates the sensory primordium within the cochlear anlage. The expression of Math1 is limited to a subpopulation of cells within the sensory primordium that appear to differentiate exclusively into hair cells as the sensory epithelium matures and elongates through a process that probably involves radial intercalation of cells. Furthermore, mutation of *Math1* does not affect the establishment of this postmitotic sensory primordium, even though the subsequent generation of hair cells is blocked in these mutants. Finally, in *Math1* mutant embryos, a subpopulation of the cells within the sensory epithelium undergo apoptosis in a temporal gradient similar to the basal-to-apical gradient of hair cell differentiation that occurs in the cochlea of wild-type animals.

Key words: Inner ear development, Organ of Corti, Hair cell, Prosensory domain, Math1, $p27^{Kip1}$, Mouse

INTRODUCTION

The organ of Corti, the sensory organ responsible for hearing in mammals, differentiates within the cochlear epithelium to produce a complex array of sensory hair cells and non-sensory supporting cells whose organization is crucial for normal ear function (Rueda et al., 1996). Remarkably, many of the molecular mechanisms involved in the development of these sensory arrays appear to be conserved during the development of functionally related and highly patterned sensory organs in invertebrates (Eddison et al., 2000; Hassan and Bellen, 2000). In particular, these studies have indicated the importance of homologs of proneural and neurogenic genes involved in developmental patterning of sensory structures in *Drosophila* (Hassan and Bellen, 2000). These include members of the proneural group of basic helix-loop-helix (bHLH) transcription factors such as *Achaete-scute* and *atonal* (Bermingham et al., 1999; Zheng and Gao, 2000; Itoh and Chitnis, 2001), as well as several elements in the Notch signaling pathway (Eddison et al., 2000; Haddon et al., 1998; Lanford, 1999; Lanford et al., 2000; Lewis et al., 1998; Shailam et al., 1999; Zhang et al., 2000; Zheng et al., 2000; Zine et al., 2000; Zine et al., 2001).

Proneural genes in *Drosophila* function in at least two steps in the development of the sensory elements of the *Drosophila* nervous system. First, they specify a cluster of cells in the naïve ectoderm of the embryo, endowing these cells with the competence to become neural/sensory precursors (reviewed by Jarman and Ahmed, 1998; Lewis, 1996). Proneural genes function a second time during the selection of a specific cell within the proneural field, the sensory organ precursor (SOP), which will go on to divide through a fixed lineage and differentiate into supporting cells and neurons of the *Drosophila* sensory organ. Once selected, under the influence of the relevant proneural gene, the SOP actively inhibits the differentiation of other cells in the prosensory domain through Notch-dependent lateral inhibition. Loss of the proneural gene leads to the loss of the entire lineage associated with the sensory structure in question (Jarman and Ahmed, 1998).

Similar to the development of sensory arrays in *Drosophila*, development of the organ of Corti appears to be separated into

a 'proneural-like phase', during which a field of cells are specified within the cochlear epithelium to give rise to sensory structures, followed by a 'neurogenic-like phase', during which this field is patterned into sensory hair cells and supporting cells. The presence of proneural and neurogenic phases is supported by the observation that mutations in the elements of the Notch-DSL (Delta/Serrate/Lag-2) pathway have been shown to lead to perturbations in cellular patterning of the mouse cochlear epithelium, causing defects in the characteristic rows of inner and outer hair cells (Lanford et al., 1999; Zhang et al., 2000; Zheng et al., 2000; Zine et al., 2000). Further support comes from the observation that *Math1* (*Atoh1* – Mouse Genome Informatics), a member of the bHLH family of transcription factors and a close homolog of the *Drosophila* proneural gene *atonal*, has been found to be required for the differentiation of sensory hair cells within the developing cochlea (Bermingham et al., 1999; Ben-Arie et al., 2000).

In spite of the fact that *Math1* can complement the proneural function of *atonal* in *Drosophila* (Ben-Arie et al., 2000), the proneural nature of the role of *Math1* in mammalian development is unclear. In addition to its role in inner ear development, *Math1* is required for the formation of several neuronal cell types, including the granule cells of the cerebellum (Ben-Arie et al., 1997; Helms and Johnson, 1998), pontine nuclei (Ben-Arie et al., 2000), proprioceptive interneurons whose axons form the spino- and cuneo-cerebellar tracts (Bermingham et al., 2001; Gowan et al., 2001) and secretory cells in the small intestinal epithelium (Yang et al., 2001). In the absence of *Math1*, development of all these cell types is compromised. Nonetheless, in the cerebellum, for example, it remains unknown whether the lack of granule cell development results from a lack of progenitors specified by *Math1*, or from a defect in proliferation or differentiation of these precursors (Ben-Arie et al., 1997; Helms et al., 2000). Similarly, in the developing inner ear of *Math1*-mutant mice, supporting cells, which (based on work in birds and fish) are believed to share a common precursor with the sensory hair cells (Fekete et al., 1998; Haddon et al., 1998; Haddon et al., 1999; Jones and Corwin, 1996; Riley et al., 1999; Stone et al., 1999), appear to be able to differentiate and survive even though hair cells fail to differentiate (Bermingham et al., 1999). This result suggests that the specification of the common precursor of hair cells and supporting cells may not be strictly dependent on the presence of *Math1*, and thus *Math1* may not be functioning in the same manner as proneural genes in *Drosophila*.

Formation of the cochlear epithelium (Fig. 1A-D), within which the organ of Corti will differentiate, begins as an out-pocketing of the ventromedial otocyst prior to E12 (Fig. 1A) (Morsli et al., 1998). Growth of this cochlear out-pocketing will continue until the cochlea has reached its mature length at approximately E18 (Fig. 1D). Between E12 and E14, cells in a region of cochlear epithelium representing the primordial organ of Corti exit the cell cycle (Ruben, 1967). This region is marked by the expression of $p27^{Kip1}$ (*Cdkn1b* – Mouse Genome Informatics), a cyclin-dependent kinase inhibitor involved in timing cell cycle exit in the sensory epithelium (Chen and Segil, 1999).

Starting between E14.5 and E15.5 (Fig. 1C), when the cochlear duct has made three-quarters of its final one and a half turns (Fig. 1D), a temporal gradient of hair cell and supporting cell differentiation initiates in the mid-basal region of the cochlea [visualized in our work by the expression of enhanced green fluorescent protein (EGFP) under the control of a Math1 enhancer, see Results for details]. Over the next 3 days, hair cell differentiation proceeds until the entire length of the sensory epithelium is patterned into one inner row and three outer rows of hair cells, finishing between E17 and E18 (Sher, 1972; Li and Ruben, 1979; Lim and Anniko, 1985; Chen and Segil, 1999). Simultaneous with the leading edge of the basal-to-apical gradient, a second gradient of differentiation advances orthogonally across the width of the sensory epithelia leading to the sequential appearance first of inner hair cells, then of each row of outer hair cells (Sher, 1972; Li and Ruben, 1979; Lim and Anniko, 1985; Chen and Segil, 1999).

We have examined the role of *Math1* in the formation of the sensory primordium and subsequent differentiation of sensory cells both along and across the developing organ of Corti. We report three pieces of evidence indicating that *Math1* is not involved in the specification of the sensory primordium within the cochlea. First, Math1 is expressed only in cells after they have exited the cell cycle to form a zone of non-proliferating cells (ZNPC) along the length of the cochlear duct at the site of the developing organ of Corti. The expression of Math1 within the ZNPC is limited to a subpopulation of cells that appear to differentiate exclusively into hair cells as the sensory epithelium matures and elongates through a process that probably involves radial intercalation of cells. Second, in *Math1*-null mutants, development of the prosensory domain, as assayed by the appearance of the ZNPC and expression of $p27^{Kip1}$, occurs normally, even though hair cells fail to differentiate. Finally, we observe that in *Math1*-null animals, a population of cells within the sensory epithelium dies by apoptosis in a basal-to-apical gradient, similar in timing to that normally observed for hair cell differentiation. Together, these results show that although *Math1* is required for the selection and/or differentiation of hair cells, it is not required for the establishment of the ZNPC, the operationally defined prosensory domain within the developing cochlear anlage. Thus, *Math1* does not play a role in the specification of the common hair cell and supporting cell lineage in the organ of Corti, and appears to play a more restricted prosensory role than its *Drosophila* homolog *atonal*.

MATERIALS AND METHODS

Generation of transgenic mice

Transgenic mice carrying EGFP under the control of an enhancer from the *Math1* gene (*Math1*/EGFP) were made as previously described (Helms et al., 2000). The *Math1*/EGFP transgene contains an ~1.4 kb sequence from the *Math1* enhancer (Helms et al., 2000) (Tg15 plus 150 bp on the 3′ end) fused to the reporter gene BGnEGFP. BGnEGFP contains the β-globin basal promoter, a nuclear localization signal and EGFP (Clontech). Genotyping was carried out by PCR using oligos 5′ CGA AGG CTA CGT CCA GGA GCG CAC CAT 3′ and 5′ GCA CGG GGC CGT CGC CGA TGG GGG TGT TCT GC 3′, and by direct observation of EGFP-mediated fluorescence. Animals for timed mating were put together in the evening, and the next morning was designated as gestation day 0.5 (E0.5). The day of birth is designated P0. Animal care was in accordance with institutional guidelines.

The *Math1*-null mice used in this study (Ben-Arie et al., 1997) were outcrossed to CD1 wild-type mice. Genotyping was carried out by

PCR using oligos 5′ TCT GCT GCA TTC TCC CGA GC 3′ and 5′ GCA CCG AGT AAC CCC CAG AG 3′ for the wild-type *Math1* allele, and oligos 5′ GAA CCC AAA GAC CTT TTG CAC 3′ and 5′ CAC GAG ACT AGT GAG ACG TG 3′ for the *Math1*-null allele, and confirmed by immunohistochemistry for Math1 protein.

BrdU injections

BrdU was dissolved at 5 mg/ml in 7 mM NaOH in PBS and injected at 50 μg BrdU per gram of body weight. Mice were injected intraperitoneally at 2 hour intervals and sacrificed at times specified in the text. Cochlear tissues were dissected and fixed in 4% paraformaldehyde for 1-4 hours and then sectioned for immunodetection of incorporated BrdU.

Cochlear wholemounts

To visualize the expression of *Math1*/EGFP in the developing cochlea (Fig. 1), unfixed bulla from embryos aged E12.5, E13.5 and E14.5 were dissected in Dulbecco's phosphate-buffered saline (PBS) (GibcoBRL), then incubated in Dulbecco's PBS containing 1 mg/ml of collagenase (Worthington Biochemical Corporation) and 1 mg/ml of dispase (GibcoBRL) for 10-15 minutes. The enzyme solution was replaced with fresh Dulbecco's PBS and surrounding non-epithelial tissues were removed to expose the inner ear epithelium. The cochlear ducts from embryos older than E15 were dissected without enzyme treatment. The cochleae from *Math1*/EGFP transgenic animals were photographed under low magnification on a Zeiss inverted compound microscope and photographed digitally with a Spot camera (Diagnostic Instruments).

Immunohistochemistry

Cochleae were dissected in Hanks balanced saline solution (GibcoBRL) and fixed in 4% paraformaldehyde in PBS. Immunohistochemistry was as previously described (Chen and Segil, 1999). The following antibodies were used in these studies: anti-$p27^{Kip1}$ (NeoMarker, mouse monoclonal, dilution 1:100); anti-Jagged1 (Santa Cruz, goat polyclonal, dilution 1:50- 1:100); anti-BrdU (Chemicon, mouse monoclonal, dilution 1:100); anti-activated Caspase 3 (R&D Systems, rabbit polyclonal, dilution1:200); anti-EGFP (Molecular Probes, rabbit polyclonal, dilution 1:1000); anti-Math1 (rabbit polyclonal, dilution 1:100)) (Helms and Johnson, 1998); anti-MyosinVIIa (rabbit polyclonal, courtesy of Christine Petit, Pasteur Institute, dilution 1:1500). Sections stained with anti-$p27^{Kip1}$ were first treated in a microwave oven for 10 minutes in 10 mM citric acid buffer, pH 6.0. For BrdU staining, sections were incubated in 50% formamide/2×SSC at 65°C for 2 hours and then washed with 2×SSC/2N HCl at 37ºC for 30 minutes. After neutralization with 0.1 M boric acid (pH 8.5) for 10 minutes, the sections were stained with an antibody against BrdU.

RESULTS

A zone of non-proliferating cells (ZNPC) forms at the site of the developing organ of Corti

In mice, the progenitors of hair cells and supporting cells become postmitotic between E12 and E14 of development and remain that way for the life of the animal (Ruben, 1967). We have previously reported that the expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ increases at this time along the length of the cochlear anlage in a narrow zone of cells within which hair cells and supporting cells differentiate (Chen and Segil, 1999). Accurate timing of cell cycle exit of this population is dependent on the expression of $p27^{Kip1}$ (Chen and Segil, 1999). In order to assess the pattern of Math1 expression in relation to the landmarks of cell cycle exit and $p27^{Kip1}$ upregulation, we have more carefully analyzed these events in the primordial cochlear duct.

Timed-mated pregnant female mice were injected with BrdU on either E12.5, E13.5 or E14.5 (three injections, 2 hours apart) and the animals were killed 8 hours after the first injection. The pattern of BrdU incorporation into the nuclei of replicating cells within the entire cochlear duct was compared with the onset of $p27^{Kip1}$ expression by immunohistochemistry (Fig. 2). Adjacent sections through the basal region of the cochlea are shown. After administration of an 8 hour pulse of BrdU at E12.5, cells throughout the cochlear epithelium have incorporated BrdU, indicating that they are still in the cell cycle (Fig. 2A). This includes the site of the future organ of Corti, as ascertained by the presence of spiral ganglion neurons that were stained with antibody against neurofilament and neuronal β-tubulin (data not shown, see arrow in Fig. 2A). $p27^{Kip1}$ is not yet expressed in the cochlea at this stage (Fig. 2B). By contrast, following BrdU administration on E13.5, a distinct ZNPC is formed, as seen by the lack of BrdU incorporation in this region (Fig. 2C, bracket). The establishment of this ZNPC between E12.5 and E13.5 correlates with the onset of $p27^{Kip1}$ expression both temporally and spatially (Fig. 2C,D). At E13.5, the ZNPC runs the apical to basal extent of the cochlear anlage (Fig. 3C), and is indicative of a population wide cell cycle exit that occurs within a narrow band of cells, delimited by $p27^{Kip1}$ expression. $p27^{Kip1}$ protein continues to be expressed on E14.5 (Fig. 2F), prior to the onset of hair cell and supporting cell differentiation, as cells in the regions on either side of the nascent organ of Corti continue to divide and incorporate BrdU (Fig. 2E, bracket). In summary, a zone of non-proliferating cells (ZNPC) is formed at the site of the future organ of Corti by E13.5, coinciding temporally and spatially with the expression domain of $p27^{Kip1}$.

The onset of *Math1* expression follows the establishment of the ZNPC

We have previously shown that differentiation of hair cells and supporting cells of the organ of Corti takes place strictly within the limits defined by the expression of $p27^{Kip1}$ (Chen and Segil, 1999), which also reflects the boundaries of the ZNPC (Fig. 2C-F). A previous report also indicates that for a short time after the onset of hair cell differentiation, the cells surrounding the newly formed rows of inner and outer hair cells can differentiate into hair cells, should existing hair cells be ablated (Kelley et al., 1995). Thus, it is likely that the cells within the ZNPC are competent to differentiate into sensory hair cells. For Math1 to play a role in the establishment of this competence, it would have to be expressed before or concomitant with the establishment of the ZNPC.

To compare more easily the temporal and spatial pattern of Math1 expression with that of $p27^{Kip1}$ and the establishment of the ZNPC, we made use of a transgenic mouse harboring a cDNA coding for EGFP under the direct control of a *Math1* enhancer (Helms et al., 2000). The expression of the transgene, *Math1*/EGFP, in the developing inner ear is illustrated in Fig. 1. Comparison between the pattern of *Math1*/EGFP expression and that of endogenous Math1 protein detected by immunohistochemistry, indicates that the onset of *Math1*/EGFP transgene expression matches the expression of the endogenous Math1 (compare Fig. 3B with 3C; compare 3D with 3E; data not shown). A previous report has indicated that

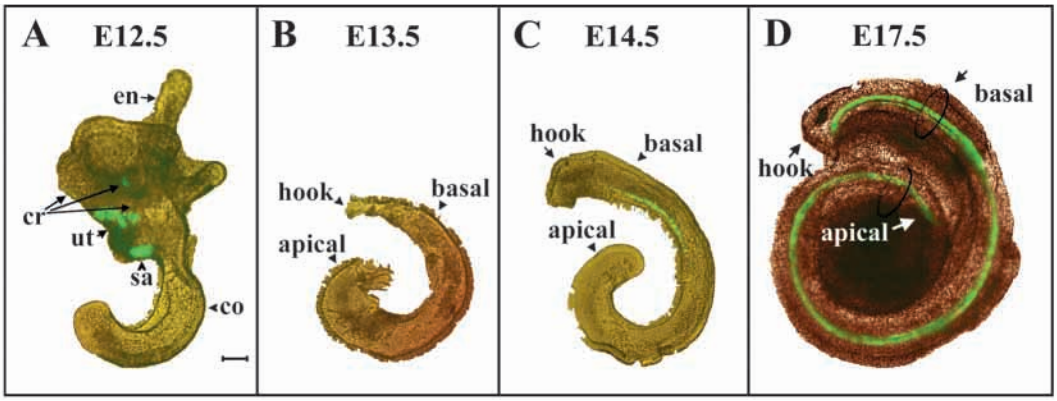

Fig. 1. Embryonic development of the cochlear epithelium. Isolated epithelial wholemounts from E12.5 to E17.5 embryos, which carry an EGFP transgene (green) under the control of a *Math1* enhancer. EGFP expression (green) marks the sites of the developing sensory regions in the inner ear. (A) The entire E12.5 otic epithelium is shown. *Math1*/EGFP is expressed in the vestibular sensory primordia, utriclus (ut), sacculus (sa) and three cristae (cr), but not in the nascent cochlear duct (co). en, endolymphatic sac. (B-D) *Math1*/EGFP expression begins in the mid-basal region of the cochlear duct sometime between E13.5 (B) and E14.5 (C). From this region, expression of *Math1*/EGFP progresses in a gradient that spreads longitudinally towards both the hook region at the extreme base of the cochlea and the apical tip of the cochlear duct, as well as from medial to lateral across the developing organ of Corti. The process of patterning the cochlea is complete between E17.5 (D) and E18.5. Scale bar: 200 μm.

Math1 is expressed as early as E12 in the inner ear (Bermingham et al., 1999). However, at this time both Math1 immunoreactivity (Fig. 3A) and *Math1*/EGFP expression (Fig. 1A) are limited to the sensory regions of the vestibular portion of the inner ear, consistent with the earlier onset of differentiation observed in this part of the inner ear (Ruben, 1967). The expression of Math1 is not observed in the cochlear duct on either E12.5 (Fig. 3A, arrow; Fig. 1A) or E13.5 (Fig. 3B; Fig. 1B), at which time the ZNPC has already been established. The presence of the ZNPC in the absence of *Math1*/EGFP expression is dramatically demonstrated in a horizontal section through the developing cochlear duct of an E13.5 *Math1*/EGFP transgenic embryo injected with BrdU (Fig. 3C, ZNPC in brackets).

The first cells to express Math1 in the cochlea appear between E13.5 and E14.5 as a discrete column of cells that spans the entire depth of the sensory epithelium near the base of the cochlear duct, as assayed both by immunohistochemistry (Fig. 3D) and by Math1/EGFP (Fig. 3E,F). These cells appear near the medial border of the ZNPC within the domain of $p27^{Kip1}$ expression (Fig. 3E,F). At this time, Math1 has not yet appeared in the middle, apical or the hook regions of the still elongating cochlea (Fig. 1C).

The observation that the onset of Math1 expression takes place within the boundaries of the ZNPC suggests that Math1 is expressed only in cells that have exited the cell cycle. To determine whether the appearance of Math1 in the nascent organ of Corti is strictly tied to the postmitotic state, timed-mated pregnant mice were injected with BrdU at E12.5 (Fig. 4A) and E13.5 (Fig. 4B), and allowed to survive until E14.5, when $Math1^+$ cells first appear in the base of the cochlea (Fig. 3D). *Math1*/EGFP transgenic mice were used to identify the Math1-expressing cells. The presence of BrdU and Math1/EGFP double-labeled cells is indicative of the time of cessation of cell division of the Math1/$EGFP^+$ cells. In mice injected at E12.5 and sacrificed on E14.5, numerous double-positive (yellow) cells were observed (Fig. 4A, sacrificed 48-54 hours after the initial BrdU injection), indicating that at the time of injection of BrdU, these cells were still in the cell cycle. However, BrdU injection 1 day later (on E13.5) yielded no

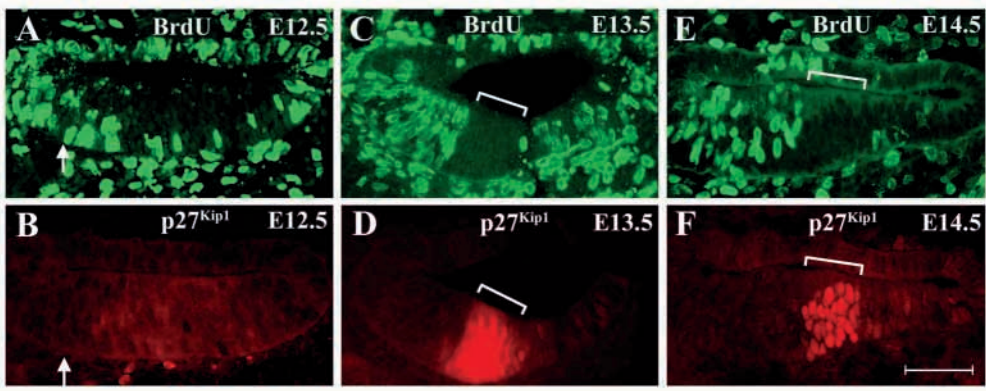

Fig. 2. The sensory primordium in the cochlea is marked by $p27^{Kip1}$ and the zone of non-proliferating cells (ZNPC). Timed-mated pregnant animals were injected with BrdU at E12.5 (A,B), E13.5 (C,D) and E14.5 (E,F), and sacrificed 8 hours later. Adjacent sections from each stage were stained with antibody to either BrdU (A,C,E; green) or $p27^{Kip1}$ (B,D,F; red). (A,B) At E12.5, BrdU stained nuclei are seen throughout the cochlear epithelium, including at the site of future organ of Corti formation (A). $p27^{Kip1}$ is not expressed at this time (B). The site of future organ of Corti formation (arrows) was determined by staining for spiral ganglion cell axons containing βIII-tubulin or neurofilament (data not shown). (C,D) At E13.5, BrdU-stained nuclei are absent from a region of the cochlear epithelium (brackets), here termed the zone of non-proliferating cells (ZNPC) (C). In an adjacent section, the ZNPC can be seen to correspond precisely with the expression domain of $p27^{Kip1}$ (D). (E,F) At E14.5, the ZNPC is still present (E) and the $p27^{Kip1}$ staining persists (F). Scale bar: 50 μm.

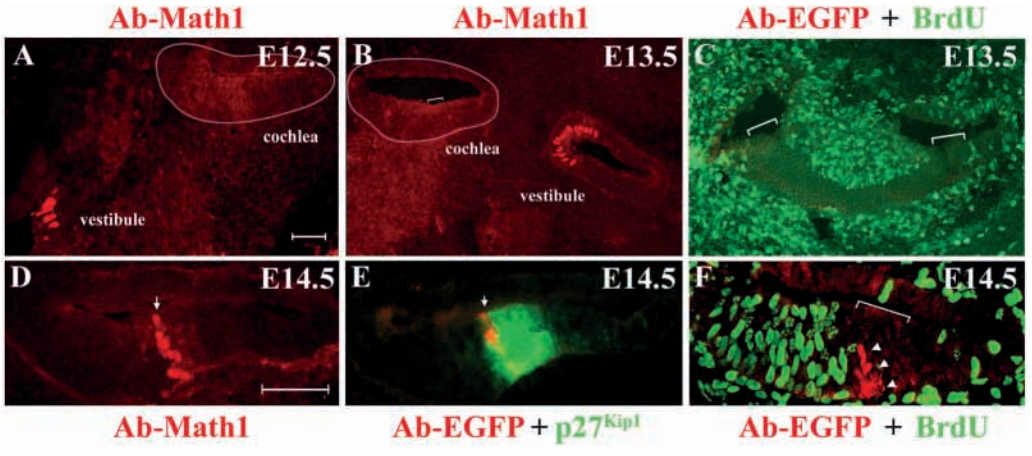

Fig. 3. The onset of Math1 expression in the cochlea. (A-C) Sections from E12.5 (A) and E13.5 (B) were stained with antibody to Math1 (Ab-Math1). No Math1 expression was visible in the cochlear epithelium (outlined in white) before E13.5, although cells in the vestibular system were stained by E12.5 (A,B). A section through the E13.5 cochlea, parallel to the cochlear duct was double labeled with antibodies against BrdU (green) and EGFP (red) (C). The apical to basal extent of the ZNPC is revealed by the BrdU-negative staining region (brackets) where no *Math1*/EGFP expression is detected, indicating the ZNPC is established prior to the onset of *Math1*/EGFP expression. (D-F) Sections through E14.5 cochlear epithelium. Sections were labeled with antibody to Math1 (D, red), double-labeled with antibodies to EGFP (E, red) and $p27^{Kip1}$ (E, green), or double-labeled with antibodies to EGFP (F, red) and BrdU (F, green). Math1 staining first appears on E14.5 as a column of cells spanning the cochlear epithelium (D, arrow). *Math1*/EGFP cells (arrows in E,F) appear at the medial border of the $p27^{Kip1}$-stained domain (E, bracket) or the ZNPC (F, bracket). Scale bars: in A, 50 μm in A-C; in D, 50 μm in D-F.

double-positive cells when sacrificed at E14.5 and the ZNPC is clearly visible (Fig. 4B, bracket). Together, these data indicate that sensory epithelial cells in the primordial organ of Corti become postmitotic between E12.5 and E13.5, prior to the expression of Math1, and that Math1 expression initiates at the medial border of the ZNPC where inner hair cells first begin to appear.

Math1 expression foreshadows the appearance of MyosinVIIa in a gradient of differentiation within the nascent organ of Corti

Starting at E15.5, expression of the early hair cell-specific marker MyosinVIIa (Hasson et al., 1995; Sahly et al., 1997) can be detected in inner hair cells at the medial border of the $p27^{Kip1}$-expressing domain near the base of the cochlea (Chen and Segil, 1999). From this point of origin, differentiation spreads longitudinally towards both the apex and the hook region of the cochlea (Fig. 1), as well as laterally from inner to outer hair cells (Chen and Segil, 1999). As differentiation proceeds within the developing organ of Corti, $p27^{Kip1}$ expression is downregulated in nascent hair cells and maintained in the supporting cells (Chen and Segil, 1999). To ascertain the relationship of Math1 expression to the gradient of hair cell differentiation, we compared the expression of Math1 and MyosinVIIa in sections through the developing cochlear duct (Fig. 5).

Math1 expression initiates near the base of the cochlea between E13.5 and E14.5 in a single column of cells at the medial border of the ZNPC (Fig. 3E,F), where inner hair cells will appear (Chen and Segil, 1999). At this time, the epithelium within which the ZNPC forms is approximately four to five cells deep (Fig. 2D,F; Fig. 3D,E) (Sher, 1972; Lim and Anniko, 1985; Kelley and Bianchi, 2001). By E14.5, we have observed the beginnings of a second discrete column of $Math1^+$ cells near the base of the cochlear duct (Fig. 5B, arrow) separated from the first by $Math1^-$ cells. In more apical turns of the cochlea in the same section, only a single column of $Math1^+$ cells is visible (compare Fig. 5A with 5B), suggesting that the gradient of Math1 expression proceeds medially to laterally, as well as longitudinally (Fig. 5A,B; Fig. 1C,D). This pattern of expression is recapitulated as the temporal gradient of Math1 expression progresses from base to apex as development proceeds (Fig. 5C).

By E15.5, the sensory epithelium in the base of the cochlea has matured into a bilayered structure (Fig. 5D,F,H) and a

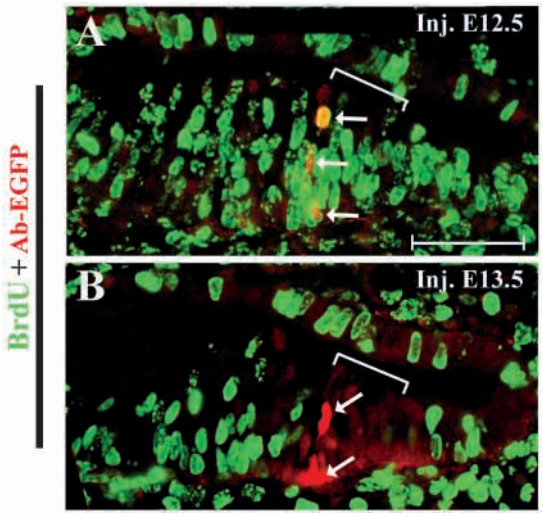

Fig. 4. Math1expression is restricted to postmitotic cells in the cochlea. Timed-mated pregnant *Math1*/EGFP transgenic animals were injected with BrdU at either E12.5 (A) or E13.5 (B) and then sacrificed at E14.5. Sections through the cochlear epithelium were double labeled with antibodies against BrdU (green) and EGFP (red). Cells double labeled with both antibodies are yellow. Brackets indicate the sensory primordium. (A) Section through an E14.5 cochlea exposed to BrdU on E12.5. *Math1*/EGFP cells (arrowheads) are double labeled with BrdU (yellow) indicating that on E12.5, cells that are destined to become *Math1*/EGFP positive are still in the cell cycle and incorporating BrdU. (B) Section through an E14.5 cochlea exposed to BrdU at E13.5. No double-labeled cells are present, indicating that at E13.5, cells that are destined to become Math1-positive (red, indicated by arrowheads) at E14.5 have exited the cell cycle and do not incorporate BrdU. Scale bar: 50 μm.

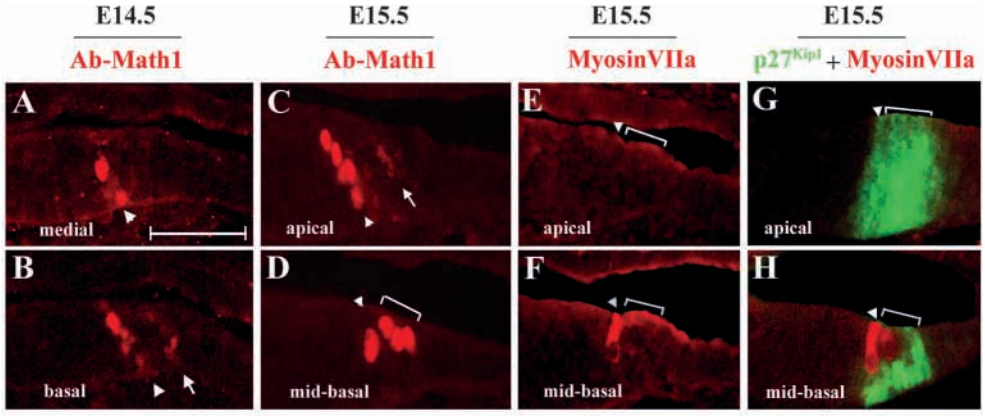

Fig. 5. A gradient of Math1 expression precedes the appearance of the early hair cell differentiation marker, MyosinVIIa. (A-D) Sections through the developing cochlear duct stained with antibody to Math1. (E,F) Sections stained with antibody to MyosinVIIa. (G,H) Sections double-labeled with antibodies to MyosinVIIa (red) and $p27^{Kip1}$ (green). (A,B) At E14.5, Math1 expression has spread to the medial region of the cochlear duct (A) from its site of initiation in the base (B, see Fig. 1 for reference). In the medial region (A), a single column of cells spanning the cochlear epithelium is seen, while in the base (B) two columns of Math1 stained cells are observed (arrowhead, medial; arrow, lateral). The $Math1^{+}$ columns are separated by a row(s) of $Math1^{-}$ cells. (C-H) One day later, at E15.5, the gradient of Math1 expression has progressed and two columns of $Math1^{+}$ cells are seen in the apical region of the cochlear duct (C, arrowhead, medial; arrow, lateral), and $Math1^{+}$ cells in the mid-basal region (D) have begun to resolve into the stereotyped pattern of one row of inner (arrowhead) and three rows of outer (bracket) hair cells. In contrast to the Math1 staining observed in the base (C,D), no MyosinVIIa staining is observed in adjacent sections in the apex (E), while only a single inner hair cell is stained with MyosinVIIa in the base (F, arrowhead), indicating that Math1 expression precedes that of MyosinVIIa. Sections double-labeled with antibody to $p27^{Kip1}$ (green) and MyosinVIIa (red) reveal the change from the thickened epithelium that is present in the undifferentiated apical region (G) compared with the bilayered epithelium in the differentiated organ of Corti (H). Scale bar: 50 μm.

single row of inner hair cells and three rows of outer hair cells are visible with Math1 labeling (Fig. 5D). In an adjacent section stained for MyosinVIIa (Fig. 5F), only the inner hair cell (Fig. 5F, arrowhead) and the innermost outer hair cell (Fig. 5F, the outer hair cell region is indicated by a bracket) are labeled by MyosinVIIa. The lack of staining in the two outermost rows of outer hair cells (Fig. 5F, bracket) is indicative of an inner to outer (medial to lateral) gradient of differentiation. The location of the developing organ of Corti is shown in an adjacent section by double-labeling for $p27^{Kip1}$ and MyosinVIIa (Fig. 5H). Math1 expression clearly precedes MyosinVIIa expression and $p27^{Kip1}$ downregulation in nascent hair cells in this medial to lateral gradient (compare Fig. 5D with 5F,H).

Math1 expression also precedes MyosinVIIa expression in the basal-to-apical gradient (compare Fig. 5C with 5E and 5G). At the apical end of the gradient on E15.5, the same columnar pattern of $Math1^{+}$ cells (Fig. 5C) that were seen a day earlier in the base (Fig. 5B), are now visible (compare Fig. 5B with 5C), and the sensory epithelium has not begun to thin to the mature state. MyosinVIIa expression has not begun at this apical level (Fig. 5E), nor has downregulation of $p27^{Kip1}$ occurred (Fig. 5G).

Math1 is not required for the establishment of the ZNPC

We have shown that the onset of Math1 expression occurs in a limited subset of cells confined to the previously established ZNPC (Fig. 3), suggesting that the sensory primordium is established without the help of *Math1*. However, because these determinations rely on the early detection of Math1 immunoreactivity or transgene expression, it is possible that

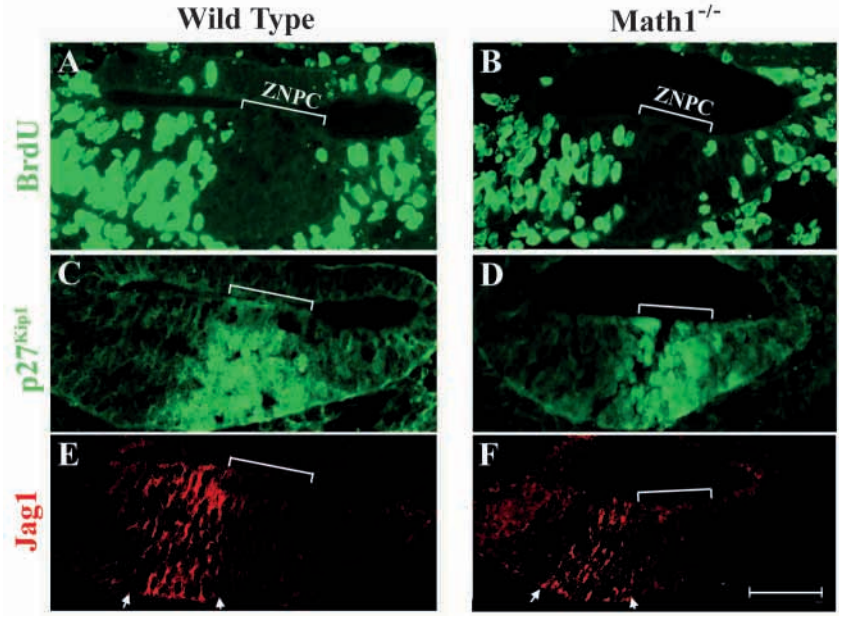

Fig. 6. The ZNPC forms normally in wild-type and *Math1*$^{-/-}$ embryos. Adjacent sections from E14.5 embryos were stained with antibody to BrdU (A,B), $p27^{Kip1}$ (C,D) or Jagged1 (E,F) to reveal the formation of the zone of non-proliferating cells within the cochlear duct of wild-type (A,C,E) and *Math1*$^{-/-}$ (B,D,F) animals. BrdU was injected into timed-mated pregnant females 8 hours prior to sacrifice. Brackets indicate the ZNPC seen as the absence of BrdU staining in cells that have withdrawn from the cell cycle (A,B), and the site of $p27^{Kip1}$ staining (C,D). Brackets in E,F were placed after overlaying sections A-D above. The Jagged1-positive domain is largely medial (left) to the ZNPC, but appears to overlap the ZNPC slightly at its lateral edge. Arrows in E,F indicate the boundaries of the Jagged1 expression domain. Scale bar: 50 μm.

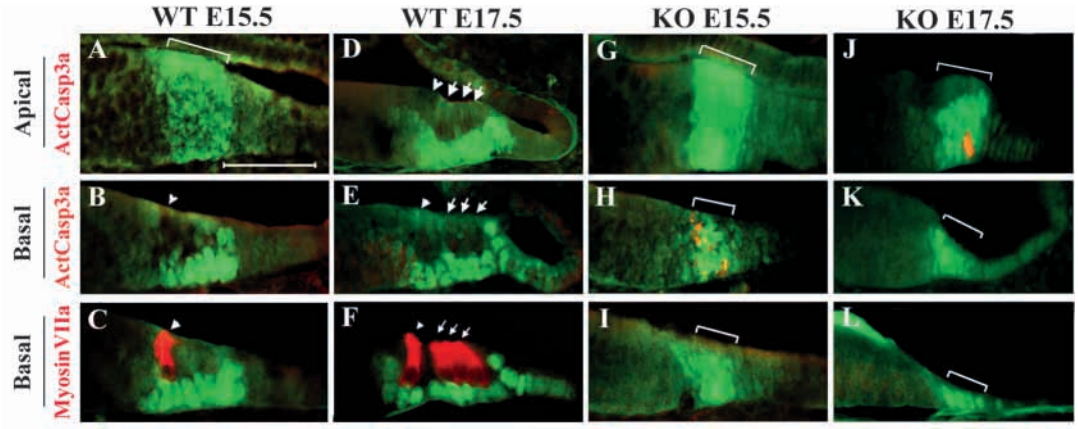

Fig. 7. A gradient of apoptosis in *Math1*$^{-/-}$ organ of Corti parallels the normal base-to-apex gradient of hair cell differentiation. Sections were prepared from wild type [A-F, (WT)] and *Math1*$^{-/-}$ [G-L (KO)] littermates at E15.5 (A-C,G-I) and E17.5 (D-F,J-L). Apical regions (A,D,G,J) of the developing organ of Corti were compared with basal regions (B,C,E,F,H,I,K,L) in order to observe the gradient of differentiation. All sections were stained with antibody to p27^{Kip1} to reveal the sensory epithelium (green). In addition, sections were double-labeled with antibody to either Activated Caspase 3 (ActCasp3) (red; A,B,D,E,G,H,J,K) to reveal apoptotic cells, or MyosinVIIa to reveal the state of hair cell differentiation (red; C,F,I,L). Brackets indicate the extent of the p27^{Kip1}-containing region, arrowheads indicate inner hair cells and arrows indicate outer hair cells. Scale bar: 50 μm.

the *Math1* gene functions prior to our ability to detect its presence. As a functional test of the expression data presented so far, we have compared the development of the sensory primordium in wild-type and *Math1*-null embryos.

Timed-mated pregnant mice were given an 8 hour pulse of BrdU starting on E14.5 (three injections, one every 2 hours and then sacrificed 2 hours after the last injection). The pattern of BrdU incorporation indicated that the ZNPC had formed prior to this time in both wild-type and *Math1*$^{-/-}$ littermates (Fig. 6A,B, brackets). Likewise, the pattern of p27^{Kip1} expression is the same in wild-type and *Math1*$^{-/-}$ littermates (Fig. 6C,D, brackets). Thus, according to these criteria, *Math1* activity is not needed for the establishment of the sensory primordium.

The developmental mechanism(s) that specify the borders of the sensory primordium are currently unknown. Recent evidence suggests that several Notch ligands may play a role in the patterning of the early sensory primordium of the cochlea, in addition to its role in the selection of hair cell versus supporting cell fate (Eddison et al., 2000; Kelley and Bianchi, 2001). In particular, Jagged1, a member of the DSL family of Notch ligands, is expressed in the cochlear duct during embryonic development (Morrison et al., 1999) and has been implicated in the establishment of the borders of the sensory

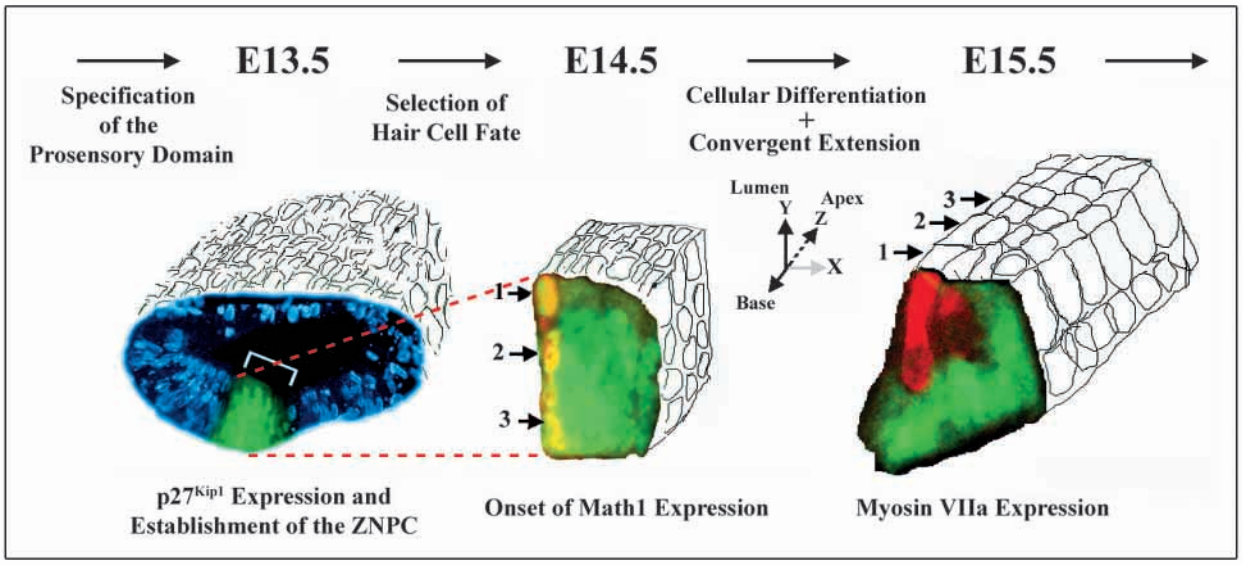

Fig. 8. A model of organ of Corti development. Starting between E12.5 and E13.5, a prosensory domain can be recognized in the cochlear duct as a zone of non-proliferating cells (ZNPC, bracket) that fail to incorporate BrdU (blue) into their nuclei and which express the CDK inhibitor p27^{Kip1} (green). Between E13.5 and E14.5, the cells of the ZNPC continue to express p27^{Kip1} and Math1 expression begins in the base of the cochlea in cells at the medial edge of the ZNPC (yellow). Math1-positive cells appear as columns of nascent hair cells (Cells 1-3) that span the depth of the sensory epithelium. As differentiation proceeds between E14.5 and E15.5, MyosinVIIa (red), an early marker of hair cell differentiation appears in the base of the cochlea, and the hair cells are displaced in a longitudinal direction by a process of radial intercalation as the columns of nascent hair cells take their place in the mature bilayer of the organ of Corti (Cells 1-3). It is this process of convergent extension that is proposed to partly underlie the postmitotic longitudinal (Z axis) growth of the organ of Corti between E13.5 and E17.5 (see Fig. 1).

primordium (Kiernan et al., 2001; Tsai et al., 2001; Kelley and Bianchi, 2001). We sought to use the appearance of Jagged1 as an additional marker for the establishment of the sensory primordium and assayed whether its spatial and temporal expression was affected by the presence or absence of Math1 activity. Immunohistochemical staining of alternate sections through the same E14.5 cochlea that were stained for BrdU (Fig. 6A,B) and $p27^{Kip1}$ (Fig. 6C,D) indicated that Jagged1 appears in a domain medial to, but slightly overlapping, the $p27^{Kip1}$-expressing ZNPC in both wild-type and *Math1* mutant embryos (compare Fig. 6E,F with 6A-D). The normal appearance of Jagged1 immunoreactivity in *Math1*-mutants is a further indication that Math1 is not involved in the establishment of the sensory primordium of the cochlea.

A gradient of apoptosis in the Math1$^{-/-}$ organ of Corti parallels the basal-to-apical gradient of hair cell differentiation in the wild-type organ of Corti

Hair cells in the *Math1*-null organ of Corti fail to differentiate (Bermingham et al., 1999). Both the organ of Corti and the vestibular system from these animals appear as a single layer of cells, which (according to morphological criteria) appear to be entirely supporting cells (Bermingham et al., 1999). To analyze the failure of hair cell differentiation and the reduction in the thickness of the sensory epithelium that accompanies the maturation of the organ of Corti, we compared wild-type and *Math1*$^{-/-}$ embryos for the presence of apoptotic cells.

Adjacent sections through the organ of Corti of wild-type and mutant embryos from E15.5-E17.5 were stained with anti-$p27^{Kip1}$ to reveal the location of the developing sensory epithelium. These same sections were then double-labeled either with antibody against Activated Caspase 3 (ActCasp3), a marker of apoptotic cells, or against MyosinVIIa, in order to compare the state of hair cell differentiation in the organ of Corti in the presence and absence of Math1 (Fig. 7).

At E15.5 in wild-type embryos, the apical regions of the developing organ of Corti had yet to show signs of differentiation. MyosinVIIa was not expressed (data not shown) and $p27^{Kip1}$ downregulation had not occurred (Fig. 7A). By contrast, MyosinVIIa expression and $p27^{Kip1}$ downregulation could be observed at this same time in the base of the cochlea (Fig. 7B,C), indicative of the initiation of the gradient of hair cell differentiation. The appearance of MyosinVIIa in only the inner hair cells at this time is also indicative of the medial-to-lateral gradient of differentiation previously described (Fig. 5). By E17, the wild-type organ of Corti is patterned along its entire length into one row of inner and three rows of outer hair cells (Fig. 7D-F). The expression of $p27^{Kip1}$ continues in supporting cells surrounding hair cells and has also spread to Claudius' cells that are lateral to the hair cell region (Fig. 7D-F). Between E14 and E18, the sensory epithelium thins from a depth of four to five cells (Fig. 5B,C; Fig. 7A, apex), to a bilayer of hair cells and supporting cells that is characteristic of the mature organ of Corti (Fig. 5D; Fig. 7F, base). However, during this period, antibody staining of ActCasp3, which is indicative of apoptotic cells, is not detected within the organ of Corti (Fig. 7A,B,D,E).

By contrast, in *Math1*-null embryos, a gradient of apoptosis is observed (Fig. 7G,H,J,K), and, as previously reported (Bermingham et al., 1999), hair cells fail to differentiate, as indicated by the lack of MyosinVIIa staining (Fig. 7I,L). The gradient of apoptosis parallels the normal base-to-apex pattern of maturation, with apoptotic cells first appearing in the base of the cochlear duct on E15.5 (Fig. 7H), while no apoptotic cells are seen in the apex in the mutant organ of Corti at this time (Fig. 7G). Only later, at E17.5, do apoptotic cells appear in the apex (Fig. 7J), just at the time when the wave of hair cell differentiation would normally arrive (Fig. 7D). Prior to the onset of hair cell differentiation, the sensory epithelium of the mutant organ of Corti appears normal (four to five cells deep) (compare Fig. 7A with 7G). At E17, in the base of the cochlea of wild-type animals, the organ of Corti has differentiated into three rows of outer hair cells and one row of inner hair cells (Fig. 7F), while comparable sections from the mutant embryo indicate that apoptosis, present at E15, is no longer ongoing (Fig. 7K), but that the sensory epithelium has thinned to one layer of $p27^{Kip1}$-positive cells (Fig. 7L). Despite their reported morphological resemblance to supporting cells (Bermingham et al., 1999), it is noteworthy that we failed to detect a marker of supporting cells, Jagged1 (Morrison et al., 1999), in the surviving cells of the Math1$^{-/-}$ organ of Corti (data not shown).

Aberrant apoptotic activity is also observed in the sensory epithelia of the vestibular system in the absence of *Math1*, and the sensory epithelia of the vestibular system thins to a single layer of cells (data not shown). Together, these observations indicate that a population of cells in the developing sensory epithelium of the inner ear die in the absence of hair cell differentiation that is normally activated by Math1.

DISCUSSION

Previous reports have shown that Math1 is both necessary and sufficient for the differentiation of sensory hair cells in the inner ear (Bermingham et al., 1999; Zheng and Gao, 2000). We have observed that Math1 is expressed only after the progenitors of hair cells and supporting cells have exited the cell cycle and formed a postmitotic sensory primordium, here termed the ZNPC. In addition, Math1 expression is limited to a subpopulation of cells within the ZNPC that appear as a column of cells spanning the multi-layered sensory primordium, and then go on to differentiate exclusively into hair cells as the sensory epithelium matures and elongates. These observations confirm the hypothesis that *Math1*, unlike its *Drosophila* homolog *atonal*, is not involved in hair cell precursor selection during development of the organ of Corti (Hassan and Bellen, 2000). Further confirmation of this hypothesis comes from our observation that the ZNPC forms normally in the absence of *Math1* and that a subpopulation of cells, probably those destined to become hair cells, undergo apoptosis in *Math1*-mutant animals.

The establishment of the ZNPC

Cells in the developing cochlea that are destined to form the organ of Corti have previously been reported to exit the cell cycle in a relatively synchronous wave between E12 and E14 (Ruben, 1967). We have used both the cell cycle exit and the expression of $p27^{Kip1}$ as markers with which to assay the establishment of the ZNPC. Our previous observations on the function of $p27^{Kip1}$ during organ of Corti development emphasized the importance of coordinating cell cycle control with cellular differentiation (Chen and Segil, 1999). We have

extended these earlier observations by narrowing the developmental window during which the signal for cell cycle withdrawal occurs, to between E12.5 and E13.5, and by identifying an additional marker, Jagged1, whose pattern of expression suggests a role in the establishment of the medial border of the ZNPC (Fig. 6E). However, the mechanism coordinating cell cycle exit and differentiation remains obscure. Although Math1 does not regulate cell cycle exit within the sensory primordium, the possibility remains that another, yet to be discovered, proneural gene is responsible for the specification of the sensory primordium and the timing of cell cycle exit.

The relatively synchronous cell cycle exit within the ZNPC differentiates these cells from the surrounding cochlear epithelium, which continues to divide after E13.5 (Fig. 2). In *p27^{Kip1}*-knockout embryos, the synchronicity of cell cycle exit is disturbed and the ZNPC cannot be recognized as a discrete entity (data not shown). Nonetheless, Math1 is expressed only after cells within the *p27^{Kip1}*-mutant sensory primordium do exit the cell cycle, as in the wild-type animal (data not shown). Among the tissues where *Math1* plays a role, the strict coupling of the postmitotic state to the induction of Math1 expression appears to be unique to the organ of Corti. In other systems, such as the cerebellum, the spinal cord and the gut (Ben-Arie et al., 1997; Helms et al., 2000; Yang et al., 2001), Math1 is expressed in the proliferating precursor cells. The common theme that connects Math1 expression in various cell types may be its expression only at the time of, or after, the selection of a particular cell fate within a group of multipotent cells, regardless of the cell cycle state. In the cochlea, the inductive signal(s) for the expression of Math1 and the selection/differentiation of hair cells within the sensory primordium appears to be postmitotic.

Uncoupling the specification of the sensory primordium from selection of hair cell fate

During the development of the CNS and PNS in *Drosophila*, various bHLH proteins function as proneural genes, required for the specification of a so-called 'equivalence group', of cells that have become competent to form neuronal precursors. Typically, the same bHLH gene that imparts competence to the equivalence group is then required in a second step, in which single cells from within this group are selected to go on and become neurons or sensory cells through a tightly regulated series of asymmetric cell divisions coupled to differentiation of specific cell types (Jarman and Ahmed, 1998). Upregulation of the proneural gene in the selected cell is not only necessary for the differentiation of that cell, but also for the subsequent downregulation of the same gene in adjacent cells through a process of lateral inhibition mediated by Notch signaling (for a review, see Lewis, 1996).

Earlier reports, based on in situ hybridization evidence, suggest that prior to hair cell differentiation, Math1 is first expressed in a horizontal band three to four cells wide running the length of the cochlea and then, as the organ of Corti matures, expression becomes limited to hair cells (Lanford et al., 2000). This observation, together with the demonstration that Notch signaling plays an important role in patterning hair cell differentiation (Lanford et al., 1999; Lewis et al., 1998; Zheng et al., 2000; Zine et al., 2000; Zine et al., 2001), suggested a model in which Math1 may specify an equivalence group, analogous to a *Drosophila* proneural cluster, which is subsequently refined by lateral inhibition via Notch signaling and results in the limited expression of Math1 in hair cells. Our observations differ, in that at the earliest times of Math1 expression in the developing cochlea, we observe discrete vertical columns of Math1$^+$ cells spanning the four to five cell deep sensory epithelium (Fig. 3). Circumstantial evidence in the form of labeling with anti-MyosinVIIa and anti-Math1 (Fig. 5) indicates that Math1 expression is probably limited to nascent hair cells as the differentiation of the organ of Corti progresses from base to apex in the cochlea (Fig. 5). This suggests that while *Math1* plays a crucial role in hair cell differentiation, it is unlikely to play a role in defining a competence domain within the cochlear epithelium and, in turn, suggests a role for *Math1* that differs somewhat from that outlined above.

In our model, the ZNPC, which arises between E12 and E13 (Fig. 2), before the expression of Math1, represents an operationally defined prosensory domain by virtue of the fact that the entire complement of hair cells and supporting cells in the mature organ of Corti arise from this postmitotic cell population. Because we lack prospective markers for an earlier definition of the prosensory domain, we are not able to establish when the prosensory domain first forms or its size at the time when it first arises. At E13, however, the ZNPC probably represents an equivalence group, in that hair cell and supporting cell differentiation has not yet occurred within its borders, and competence to differentiate into hair cell appears to persist for some time even after the differentiation of hair cells is well under way (Kelley et al., 1995). However, competence to differentiate into hair cells is unlikely to be limited to the ZNPC. Several groups have demonstrated that abnormal perturbations of Notch pathway signaling may lead to hair cell differentiation outside this region in nearby cochlear epithelia (Kelley et al., 1993; Zine et al., 2001). In addition, ectopic expression of Math1 can lead to hair cell differentiation within the greater epithelial ridge of postnatal animals (Zheng and Gao, 2000).

Shortly after the establishment of the ZNPC, a signal(s) of unknown origin stimulates the initial expression of Math1 near the base of the cochlea. Expression initiates in a series of discrete columns of cells that span the depth of the sensory epithelium (Fig. 8). We believe these cells represent nascent hair cells, which by a process of convergent intercalation will form the inner hair cells of the organ of Corti. This pattern of Math1 induction is then propagated in the basal-to-apical direction, as well as in a medial-to-lateral direction, in order to give rise to the rows of outer hair cells (Fig. 8). The basis of this propagated patterning event is currently unknown, but is likely to depend both on positive stimuli inducing Math1 expression, and lateral inhibition that limits the expression of Math1 in the surrounding supporting cell precursors. In this model, cells within the prosensory domain would receive a positive stimulus to express Math1, which in genetically *Math1*-null animals leads to apoptosis in a pattern of cell death that parallels the normal process of hair cell differentiation.

Interestingly, cell death is also observed in mice lacking *Brn3.1* (*Pou4f3* – Mouse Genome Informatics) (Xiang et al., 1997; Xiang et al., 1998), a member of the POU-domain family of transcription factors (Erkman et al., 1996). In *Brn3.1*-null animals, hair cells initiate their program of differentiation, as

evidenced by their changing morphology and the expression of several early hair cell markers, including MyosinVIIa, but then abruptly die (Xiang et al., 1997; Xiang et al., 1998). It is likely that cell death in *Math1*-null animals is caused by a failure of differentiation, possibly involving a failure to activate the transcription of *Brn3.1*.

While homologs of bHLH genes are involved in the development of the nervous system in many species, including mammals (Lee, 1997), it appears that the two functions ascribed to these factors in *Drosophila*, equivalence group specification and SOP selection, have been uncoupled evolutionarily. Evidence for this includes the observation that mutation of other members of the family of vertebrate bHLH proteins appears to cause the loss of a single cell type in the different tissues within which they are expressed (Hassan and Bellen, 2000), rather than the failure of the entire sensory lineage, as occurs in *Drosophila* mutants. Similarly, in the mouse inner ear, loss of *Math1* leads to the death of only a subpopulation of cells within the prosensory domain, probably cells that are otherwise destined to become hair cells.

The molecular pathways that specify the boundaries of the ZNPC and coordinate its appearance with the cellular differentiation of the organ of Corti remain elusive. In addition to its role in the selection of hair cell versus supporting cell fate (Haddon et al., 1998; Eddison et al., 2000; Lanford et al., 1999), the role of the Notch signaling pathway in the formation of the boundaries for the sensory primordium remains to be tested directly. Our demonstration that Jagged1 expression appears to share a border with the prosensory domain defined by the ZNPC is suggestive of such a role.

A model of organ of Corti growth by radial extension

Radial and mediolateral intercalation of cells is the basis for the movements of convergence and extension that function in gastrulation, neurulation and formation of the vertebrate body axis (Keller et al., 2000; Zajac et al., 2000). The morphological changes that occur in the absence of significant cell birth and cell death during maturation and elongation of the organ of Corti from E13, when the cochlear duct is three-quarters of a turn long, to E18, when the cochlear duct has achieved its mature one and a half turns (Ruben, 1967) (Figs 1, 2, 5, 7), suggest that a similar process involving radial intercalation may be at work in the sensory epithelia of the cochlear duct. This model is derived from three observations. First, few new cells are added to the organ of Corti following E13.5, so the elongation process has to be accounted for by non-mitotic growth. Second, that the sensory epithelium thins from four to five cells deep at E13, to two cells deep in its mature state (Figs 5 and 7). Third, that staining with antibody to ActCasp3 reveals no apoptotic cells within the developing organ during this period of development (Fig. 7 and data not shown). Thus, radial intercalation of cells (movement along the *y*-axis in Fig. 8) leads to the thinning and the longitudinal extension (along the *z*-axis in Fig. 8) of the developing sensory organ during the elongation of the cochlear duct. In this process, hair cell progenitors, in the form of $Math1^+$ cells that were aligned in the same location along the *y*-axis, are displaced along the *z*-axis as they differentiate (Fig. 8). At the same time, new columns of $Math1^+$ cells that form lateral to this initial column (see Fig. 5B,C) recapitulate the process to form the rows of outer hair cells. A similar scenario of cellular rearrangement and elongation within the basilar papilla has been observed in the inner ear of birds (Goodyear and Richardson, 1997), though direct proof of this hypothesis in either species awaits direct observation of the process using marked cells.

We thank Andres Collazo and Andrew Groves for invaluable comments on the manuscript. Robert Rainey, XianKui Li and Sandy Oba provided assistance in animal care and genotyping. We also thank Christine Petit (Pasteur Institute) who supplied the anti-MyosinVIIa antibody used in this study. This work is supported in part by grants from NIDCD RO3 DC04237 (P. C.) and RO1 DC04189 (N. S.), and the Oberkotter Foundation.

REFERENCES

Ben-Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M. and Zoghbi, H. Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. *Nature* **390**, 169-172.

Ben-Arie, N., Hassan, B. A., Bermingham, N. A., Malicki, D. M., Armstrong, D., Matzuk, M., Bellen, H. J. and Zoghbi, H. Y. (2000). Functional conservation of atonal and Math1 in the CNS and PNS. *Development* **127**, 1039-1048.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-1841.

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B. and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on Math1. *Neuron* **30**, 411-422.

Chen, P. and Segil, N. (1999). $p27^{Kip1}$ links cell proliferation to morphogenesis in the developing organ of Corti. *Development* **126**, 1581-1590.

Eddison, M., Le Roux, I. and Lewis, J. (2000). Notch signaling in the development of the inner ear: lessons from drosophila. *Proc. Natl. Acad. Sci. USA* **97**, 11692-11699.

Erkman, L., McEvilly, R. J., Luo, L., Ryan, A. K., Hooshmand, F., O'Connell, S. M., Keithley, E. M., Rapaport, D. H., Ryan, A. F. and Rosenfeld, M. G. (1996). Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. *Nature* **381**, 603-606.

Fekete, D. M., Muthukumar, S. and Karagogeos, D. (1998). Hair cells and supporting cells share a common progenitor in the avian inner ear. *J. Neurosci.* **18**, 7811-7821.

Goodyear, R. and Richardson, G. (1997). Pattern formation in the basilar papilla: evidence for cell rearrangement. *J. Neurosci.* **17**, 6289-6301.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activity of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-232.

Haddon, C., Jiang, Y. J., Smithers, L. and Lewis, J. (1998). Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637-4644.

Haddon, C., Mowbray, C., Whitfield, T., Jones, D., Gschmeissner, S. and Lewis, J. (1999). Hair cells without supporting cells: further studies in the ear of the zebrafish mind bomb mutant. *J. Neurocytol.* **28**, 837-850.

Hassan, B. A. and Bellen, H. J. (2000). Doing the MATH: is the mouse a good model for fly development? *Genes Dev.* **14**, 1852-1865.

Hasson, T., Heintzelman, M. B., Santos-Sacchi, J., Corey, D. P. and Mooseker, M. S. (1995). Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. *Proc. Natl. Acad. Sci. USA* **92**, 9815-9819.

Helms, A. W. and Johnson, J. E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* **125**, 919-928.

Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y. and Johnson, J. E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* **127**, 1185-1196.

Itoh, M. and Chitnis, A. B. (2001). Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. *Mech. Dev.* **102**, 263-266.

Jarman, A. P. and Ahmed, I. (1998). The specificity of proneural genes in determining Drosophila sense organ identity. *Mech. Dev.* **76**, 117-125.

Jones, J. E. and Corwin, J. T. (1996). Regeneration of sensory cells after laser ablation in the lateral line system: hair cell lineage and macrophage behavior revealed by time-lapse video microscopy. *J. Neurosci.* **16**, 649-662.

Keller, R., Davidson, L., Edlund, A., Elul, T., Ezin, M., Shook, D. and Skoglund, P. (2000). Mechanisms of convergence and extension by cell intercalation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 897-922.

Kelley, M. W., Xu, X. M., Wagner, M. A., Warchol, M. E. and Corwin, J. T. (1993). The developing organ of Corti contains retinoic acid and forms supernumerary hair cells in response to exogenous retinoic acid in culture. *Development* **119**, 1041-1053.

Kelley, M. W., Talreja, D. R. and Corwin, J. T. (1995). Replacement of hair cells after laser microbeam irradiation in cultured organs of corti from embryonic and neonatal mice. *J. Neurosci.* **15**, 3013-3026.

Kelley, M. W. and Bianchi, L. M. (2001). *Handbook of Mouse Auditory Research, From Behavior to Molecular Biology* (ed. J. F. Willott). Boca Raton: CRC Press.

Kiernan, A. E., Ahituv, N., Fuchs, H., Balling, R., Avraham, K. B., Steel, K. P. and Hrabe de Angelis, M. (2001). The Notch ligand Jagged1 is required for inner ear sensory development. *Proc. Natl. Acad. Sci. USA* **98**, 3873-3878.

Lanford, P. J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T. and Kelley, M. W. (1999). Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat. Genet.* **21**, 289-292.

Lanford, P. J., Shailam, R., Norton, C. R., Gridley, T. and Kelley, M. W. (2000). Expression of Math1 and Hes5 in the cochlea of wildtype and Jag2 mutant mice. *J. Assoc. Res. Otolaryngol.* **1**, 161-171.

Lee, J. E. (1997). Basic helix-loop-helix genes in neural development. *Curr. Opin. Neurobiol.* **7**, 13-20.

Lewis, A. K., Frantz, G. D., Carpenter, D. A., de Sauvage, F. J. and Gao, W. Q. (1998). Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. *Mech. Dev.* **78**, 159-163.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **6**, 3-10.

Li, C. W. and Ruben, R. J. (1979). Further study of the surface morphology of the embryonic mouse cochlear sensory epithelia. *Otolaryngol. Head Neck Surg.* **87**, 479-485.

Lim, D. J. and Anniko, M. (1985). Developmental morphology of the mouse inner ear. *Acta Otolaryngology* **422 Suppl.**, 1-69.

Morrison, A., Hodgetts, C., Gossler, A., Hrabe de Angelis, M. and Lewis, J. (1999). Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. *Mech. Dev.* **84**, 169-172.

Morsli, H., Choo, D., Ryan, A., Johnson, R. and Wu, D. K. (1998). Development of the mouse inner ear and origin of its sensory organs. *J. Neurosci.* **18**, 3327-3335.

Riley, B. B., Chiang, M., Farmer, L. and Heck, R. (1999). The deltaA gene of zebrafish mediates lateral inhibition of hair cells in the inner ear and is regulated by pax2.1. *Development* **126**, 5669-5678.

Ruben, R. J. (1967). Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Oto-laryngologica* **220 Suppl.**, 1-44.

Rueda, J., Cantos, R. and Lim, D. J. (1996). Tectorial membrane-organ of Corti relationship during cochlear development. *Anat. Embryol. (Berl)* **194**, 501-514.

Sahly, I., El-Amraoui, A., Abitbol, M., Petit, C. and Dufier, J. L. (1997). Expression of myosin VIIa during mouse embryogenesis. *Anat. Embryol.* **196**, 159-170.

Shailam, R., Lanford, P. J., Dolinsky, C. M., Norton, C. R., Gridley, T. and Kelley, M. W. (1999). Expression of proneural and neurogenic genes in the embryonic mammalian vestibular system. *J. Neurocytol.* **28**, 809-819.

Sher, A. E. (1972). The embryonic and postnatal development of the inner ear of the mouse. *Acta Oto-laryngologica*, **285 Suppl.**, 1-77.

Stone, J. S., Choi, Y. S., Woolley, S. M., Yamashita, H. and Rubel, E. W. (1999). Progenitor cell cycling during hair cell regeneration in the vestibular and auditory epithelia of the chick. *J. Neurocytol.* **28**, 863-876.

Tsai, H., Hardisty, R. E., Rhodes, C., Kiernan, A. E., Roby, P., Tymowska-Lalanne, Z., Mburu, P., Rastan, S., Hunter, A. J., Brown, S. D. and Steel, K. P. (2001). The mouse slalom mutant demonstrates a role for Jagged1 in neuroepithelial patterning in the organ of Corti. *Hum. Mol. Genet.* **10**, 507-512.

Xiang, M., Gan, L., Li, D., Chen, Z., Zhou, L., O'Malley., B. W., Jr., Klein, W. and Nathans, J. (1997). Essential role of POU-domain factor Brn3c in auditory and vestibular hair cell development. *Proc. Natl. Acad. Sci. USA* **94**, 9445-9450.

Xiang, M., Gao, W., Hasson, T. and Shin, J. J. (1998). Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. *Development* **125**, 3935-3946.

Yang, Q., Bermingham, N. A., Finegold, M. J. and Zoghbi, H. Y. (2001). Requirement of Math1 for Secretory Cell Lineage Commitment in the Mouse Intestine. *Science* **294**, 2155-2158.

Zajac, M., Jones, G. L. and Glazier, J. A. (2000). Model of convergent extension in animal morphogenesis. *Phys. Rev. Lett.* **85**, 2022-2025.

Zhang, N., Martin, G. V., Kelley, M. W. and Gridley, T. (2000). A mutation in the Lunatic fringe gene suppresses the effects of a Jagged2 mutation on inner hair cell development in the cochlea. *Curr. Biol.* **10**, 659-662.

Zheng, J. L. and Gao, W. Q. (2000). Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* **3**, 580-586.

Zheng, J. L., Shou, J., Guillemot, F., Kageyama, R. and Gao, W. (2000). Hes1 is a negative regulator of inner ear hair cell differentiation. *Development* **127**, 4551-4560.

Zine, A., Van De Water, T. R. and de Ribaupierre, F. (2000). Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* **127**, 3373-3383.

Zine, A., Aubert, A., Qiu, J., Therianos, S., Guillemot, F., Kageyama, R. and de Ribaupierre, F. (2001). Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* **21**, 4712-4720.