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Math1 regulates development of the sensory epithelium in the mammalian cochlea

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The transcription factor Math1 (encoded by the gene *Atoh1*, also called *Math1*) is required for the formation of mechanosensory hair cells in the inner ear; however, its specific molecular role is unknown. Here we show that absence of Math1 in mice results in a complete disruption of formation of the sensory epithelium of the cochlea, including the development of both hair cells and associated supporting cells. In addition, ectopic expression of Math1 in nonsensory regions of the cochlea is sufficient to induce the formation of sensory clusters that contain both hair cells and supporting cells. The formation of these clusters is dependent on inhibitory interactions mediated, most probably, through the Notch pathway, and on inductive interactions that recruit cells to develop as supporting cells through a pathway independent of Math1. These results show that Math1 functions in the developing cochlea to initiate both inductive and inhibitory signals that regulate the overall formation of the sensory epithelia.

Mechanosensory hair cells in the inner ear function as the primary transducers for the perception of sound and balance, and defects in their formation result in profound sensory deficits[1]. All inner ear hair cells are located in sensory epithelia that also contain nonsensory cells, referred to as supporting cells, and defects in the formation of supporting cells also lead to sensory deficits[2]. Despite the importance of both types of cell, our understanding of the factors that regulate the formation of these epithelia remains limited.

Previous studies have shown that the transcription factor Math1 has a crucial role in hair cell formation. In particular, absence of Math1 in mice leads to a complete lack of hair cells[3], whereas its overexpression in a group of epithelial cells located adjacent to the sensory epithelium, termed 'Kölliker's organ', induces nonsensory cells to develop as hair cells[4]. These results show that Math1 has a key role in hair cell formation; however, the specific molecular effects of Math1 remain a matter of debate. Conflicting descriptive results have suggested that Math1 might have a role in the specification of a 'prosensory domain' in which cells become competent to develop as either hair cells or supporting cells[3,5,6], or it might be a factor that functions to regulate only the differentiation of cells that are already committed to develop as hair cells[7]. To determine the specific role of Math1, here we examined its developmental effects and cellular regulation using both gain- and loss-of function models.

RESULTS

Math1 is broadly expressed in cochlear progenitor cells

We analyzed the temporal and spatial distribution of *Math1* expression in the developing sensory epithelium of the cochlea (the organ of Corti) because cellular commitment and differentiation in this organ occur in a highly stereotyped pattern. On the basis of existing data, mice in which the gene encoding β-galactosidase (β-gal) has been introduced into the *Math1* (*Atoh1*) locus (called *Math1*$^{\beta gal/+}$ mice) seem to represent the most sensitive model for assessing *Math1* expression[3].

Cellular commitment and differentiation in the organ of Corti begin in the midbasal region of the cochlear spiral at about embryonic day 13.5 (E13.5) and proceed in a wave that progresses towards both the base and the apex[8]. No *Math1* promoter activity was detected in the developing cochlea at E12.5, although expression was observed in developing vestibular structures. At E13.5, however, *Math1* promoter activity was observed in a broad group of cells that extended along most of the length of the cochlear duct (**Fig. 1a**). Overall, the pattern of staining was broader in the apical part of the cochlea and narrowed towards the base. Cross-sections through the apical region of the cochlea indicated that *Math1* promoter activity was localized to the lateral half of the cochlear duct (the site of the future organ of Corti) but was present in cells throughout the thickness of the epithelium (**Fig. 1b**).

By E15.5, the pattern of *Math1* promoter activity had narrowed along the short axis of the cochlea, although it still persisted from base to apex (**Fig. 1c**). Because the organ of Corti develops in a gradient, it was possible to analyze developmental changes in the pattern of *Math1* expression in a single cochlea. In the least mature (apical) region of the cochlea at E15.5, *Math1* expression was broad and diffuse (**Fig. 1d**). In more mature (basal) regions, some individual cells with noticeably higher *Math1* expression could be identified in the field of diffuse expression (**Fig. 1e**). In the most mature region of the epithelium, individual *Math1*-positive cells were surrounded by *Math1*-negative cells (**Fig. 1f**). As reported previously[3,5–7], expression of *Math1* at postnatal day 0 (P0) was restricted to hair cells (**Fig. 1g–i**).

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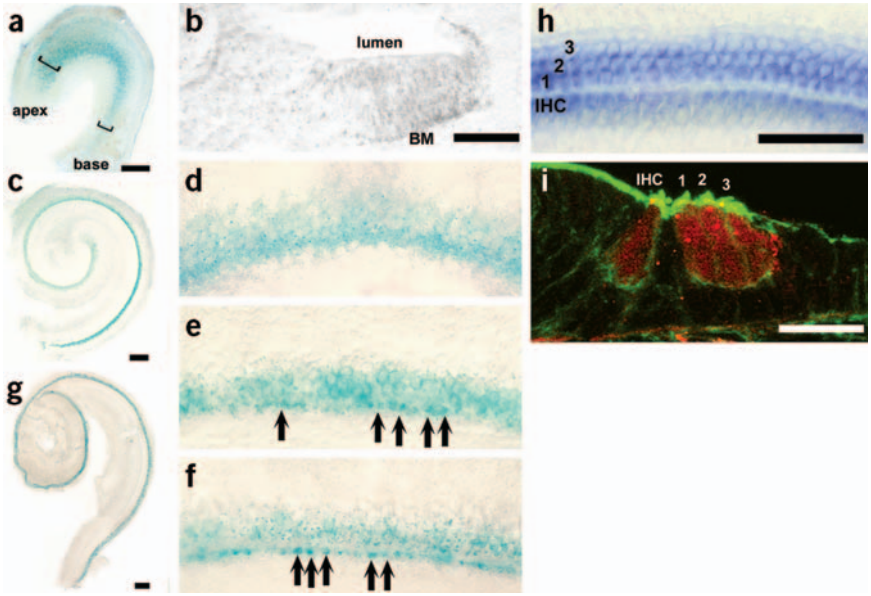

Figure 1 Expression of Math1 in the developing cochlea. (**a**) Whole mount of the developing cochlea at E13.5. Basal (base) and apical (apex) ends of the duct are indicated. The *Math1* promoter (X-gal staining in blue) is expressed in a broad stripe of cells that extends from the base to the apex and is wider at the apex than at the base (compare brackets). (**b**) Cross-section through the apical turn of the cochlear duct at E13.5. Expression of *Math1* (dark stain) is restricted to the lateral half of the duct but spans the epithelium from the basement membrane (BM) to the lumenal surface (lumen). (**c**) Whole mount of an E15.5 cochlea oriented as in **a**. At this stage, expression of *Math1* in the base of the cochlea is fairly restricted, but it becomes more diffuse in more apical regions. (**d**) High-magnification view of the apical region of the cochlea at E15.5, showing that *Math1* expression is broad and diffuse. (**e**) A more basal region of the same E15.5 cochlea in which *Math1* expression is still fairly diffuse, although some individual cells show higher expression (arrows). (**f**) An even more mature region from the same E15.5 cochlea in which individual *Math1*-positive cells (arrows) are clearly separated from one another by unlabeled (*Math1*-negative) cells. (**g**) Whole mount of the cochlea at P0. *Math1* expression is now restricted to a narrow band of cells that extends along the length of the basal-to-apical axis. (**h**) Surface of the organ of Corti at P0. *Math1* expression is restricted to the single row of inner hair cells (IHC) and the three rows of outer hair cells (numbered 1–3). (**i**) Cross-section through the cochlear duct at P0. The section has been double-labeled for the β-gal transgene (red) and for filamentous actin (green). By this stage, expression of *Math1* is completely restricted to the inner and outer hair cells. Scale bars, 100 μm (**a**,**c**,**g**); 50 μm (**b**); 100 μm (**h**; also applies to **d–f**); 20 μm (**i**).

Supporting cell development is disrupted without Math1

The expression pattern determined above showed that *Math1* is initially expressed in progenitor cells that will ultimately develop as either hair cells or supporting cells, which suggests that Math1 might be involved in the specification of a prosensory domain. If so, then absence of Math1 should lead to defects in the formation of both hair cells and supporting cells. A previous study concluded, however, that supporting cells are still present, at least in the vestibular sensory epithelia, in *Math1*-null mice[3]. Because this conclusion was based on a limited morphological analysis, we reexamined supporting cells in the cochleae of *Math1*-null mice according to morphological and molecular criteria. At P0, the cochlear duct was comprised of three regions: Kölliker's organ, the organ of Corti, and the outer sulcus (**Fig. 2a**). In the organ of Corti, specific hair cell and supporting cell types could be identified, including inner and outer hair cells and four unique supporting cell types: the inner phalangeal cells, the inner and outer pillar cells, and Deiter's cells (**Fig. 2a**). In *Math1*-null mice, by contrast, although Kölliker's organ and the outer sulcus appeared normal, the organ of Corti was completely absent (**Fig. 2b**).

To confirm that supporting cell development was disrupted in *Math1*-null mice, we examined the expression of supporting cell–specific markers in cochleae from *Math1*-null and wild-type littermates. Glial fibrillary acidic protein (GFAP) was expressed in inner phalangeal cells and Deiter's cells in the organ of Corti beginning around P0 (ref. 9 and **Fig. 3a**). By comparison, no positive staining for GFAP was observed in any cells located in the cochleae of *Math1*-null mice (**Fig. 3b**). Similarly, the gene encoding fibroblast growth factor receptor 3 (*Fgfr3*), which is expressed strongly in pillar cells and weakly in Deiter's cells in P0 wild-type mice[10,11] (**Fig. 3c**), was completely absent in *Math1*-null mice (**Fig. 3d**).

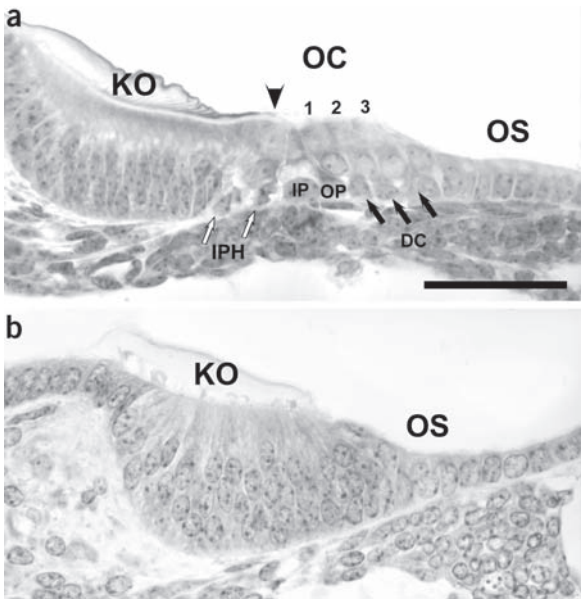

Figure 2 Cross-sections through the cochlear duct in wild-type and *Math1*-null mice at P0. (**a**) Basal turn of the cochlea from a wild-type mouse. Three different regions, Kölliker's organ (KO), the organ of Corti (OC) and the outer sulcus (OS) can be identified. In the OC, individual cell types including the inner (arrowhead) and outer (numbered 1–3) hair cells, and four different types of supporting cell, the inner phalangeal cells (IPH; white arrows), inner pillar cells (IP), outer pillar cells (OP) and Deiter's cells (DC; black arrows), are present. (**b**) Basal turn of the cochlea from a *Math1*-null mouse. KO and OS are present and appear normal; however, the OC seems to be completely absent. No cells with supporting cell morphologies can be identified, and KO and OS appear to be directly adjacent to one another. Scale bar, 30 μm (**a**; also applies to **b**).

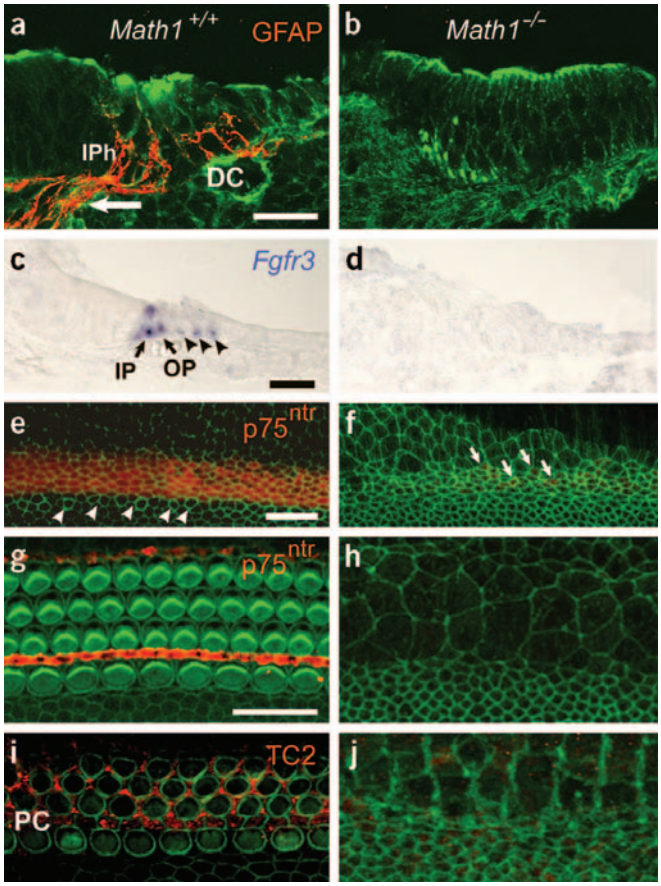

Figure 3 Expression of supporting cell markers in the cochleae of wild-type and *Math1*-null mice. Antibody or *in situ* labeling is indicated in red or blue for wild-type (**a**,**c**,**e**,**g**,**i**) and *Math1*-null (**b**,**d**,**f**,**h**,**j**) mice. Cell boundaries are labeled in green. (**a**,**b**) Cross-sections through the basal turns of the organ of Corti (OC) at P0 labeled with an antibody to GFAP. GFAP is expressed by Deiter's cells (DC), inner phalangeal cells (IPh) and auditory nerve fibers (arrows) in wild-type cochleae, but no signal is present in *Math1*-null cochleae. (**c**,**d**) *In situ* hybridization for *Fgfr3* at P0. Signal is present in the inner (IP) and outer (OP) pillar cells and, more weakly, in Deiter's cells (arrowheads) in the wild-type OC, but no signal is present in the *Math1*-null OC. (**e**,**f**) Expression of $p75^{ntr}$ in a surface view of the developing OC at E16. A band of $p75^{ntr}$ expression is located directly adjacent to developing inner hair cells (arrowheads) in the wild-type OC. Only scattered cells that weakly express $p75^{ntr}$ can be identified (arrows) in the *Math1*-null OC. (**g**,**h**) Surface view of the OC at P0 labeled as in **e**. A row of $p75^{ntr}$-positive pillar cells is located between the inner and outer hair cells in the wild-type OC. By contrast, no $p75^{ntr}$ labeling is present and cellular patterning is completely disrupted in the *Math1*-null OC. (**i**,**j**) Surface view of the OC at P0 labeled for expression of TC2. Pillar cells (PC) and Deiter's cells (between outer hair cells) are labeled in the wild-type OC, but no signal for TC2 is present in the *Math1*-null OC. Scale bars, 20 μm (applies to all images).

Next, we examined two supporting cell markers with onsets of expression that closely follow the presumed timing of supporting cell specification. The low-affinity nerve growth factor receptor, $p75^{ntr}$, is initially expressed in a band of progenitor cells in the organ of Corti between E14.5 and E16 and becomes restricted to pillar cells and Hensen's cells between E17 and P0 (refs. 11,12 and **Fig. 3e**,**g**). In the cochleae of *Math1*-null mice, expression of $p75^{ntr}$ was barely detectable at E16 and was completely lost by P0 (**Fig. 3f**,**h**). TC2, an antibody that recognizes glycosaminoglycans enriched in chondroitin-4-sulfate, labels pillar cells and Deiter's cells specifically in the postnatal gerbil cochlea[13]. In wild-type cochleae, weak staining for TC2 was observed in developing pillar and Deiter's cells at E16 (data not shown), with stronger staining at P0 (**Fig. 3i**). By contrast, no staining was observed for TC2 in the cochleae of *Math1*-null mice at either E16 (data not shown) or P0 (**Fig. 3j**).

We also evaluated the expression of jagged-1, S100A1 and β-tectorin. The onset of expression for each of these molecules precedes that of Math1, but all three ultimately become restricted to supporting cells[14–16]. Normal expression of S100A1 and β-tectorin was observed in cochleae from *Math1*-null mice at earlier time points (E12 and E16; **Fig. 4a**,**b** and data not shown); by P0, however, expression of all three markers was completely absent (**Fig. 4c–h**). On the basis of these results, it seems likely that the expression of each of these genes becomes dependent on the differentiation of cells as supporting cells. Their expression is not maintained in the absence of *Math1*, however, suggesting that the differentiation of supporting cells is disrupted.

Ectopic expression of Math1 induces supporting cell development

The above results showed that, in addition to its role in hair cell formation, Math1 is also necessary for the development of supporting cells. This result is consistent with the idea that Math1 has a role in the specification of a prosensory domain, but it could also be a result of the disruption of potential inductive and/or trophic interactions between developing hair cells and supporting cells.

To examine these hypotheses, we expressed wild-type Math1 (referred to here as $Math1^{ORF}$ to delineate it from endogenous Math1) ectopically (Methods) in cells in Kölliker's organ in cochlear explants established at E13.5 or E14.5 (**Fig. 5a**). The expression vector also contained an internal ribosome entry site (IRES) sequence to generate independent transcripts of enhanced green fluorescent protein (EGFP) under the control of the same promoter (Methods). Explants were maintained for 6 days *in vitro* (DIV) and then labeled with antibodies to EGFP, and either to jagged-1 or otogelin[17] as supporting cell markers (**Fig. 5b** and data not shown), or to myosin VI or myosin VIIa as hair cell markers[18] (**Fig. 5c** and **Supplementary Fig. 1** online). We did not observe cells that were positive for jagged-1, otogelin, myosin VI or myosin VIIa in Kölliker's organ of explants that were not transfected or were transfected with EGFP alone (**Fig. 5d** and data not shown).

As previously reported[4], more than 96% of cells in Kölliker's organ that were transfected with $Math1^{ORF}$ developed as hair cells (**Table 1** and **Supplementary Fig. 1** online). Staining with antibodies to either jagged-1 or otogelin showed, however, that transfection with $Math1^{ORF}$ (**Fig. 5e**) also induced the formation of clusters of jagged-1- or otogelin-positive cells that surrounded the EGFP-positive hair cells in Kölliker's organ (**Fig. 5f**,**g**). Closer analysis of the jagged-1-positive clusters indicated that they typically comprised a few EGFP-positive hair cells (**Fig. 5h**) surrounded by a roughly equivalent or slightly greater number of jagged-1-positive cells (**Fig. 5i**). The morphologies of the jagged-1-positive cells were consistent with those of supporting cells (**Fig. 5i**, inset), and many had apical projections that interdigitated between the EGFP-positive hair cells (**Fig. 5j**). Similar morphologies and cellular arrangements were also observed for otogelin-positive cells in transfected clusters (**Fig. 5k–l**). The morphology of these clusters was reminiscent of more ancestral hair cell structures, such as the lateral-line neuromasts found in fish and amphibians[19].

The observation that the supporting cell–like cells in these clusters were not transfected with $Math1^{ORF}$ suggested that hair cells can induce surrounding Math1-negative cells to develop as supporting cells, and therefore that transient expression of Math1 is not required for the formation of supporting cells. Considering the broad expression of endogenous Math1 at E13.5 (**Fig. 1**), however, it seemed possible that

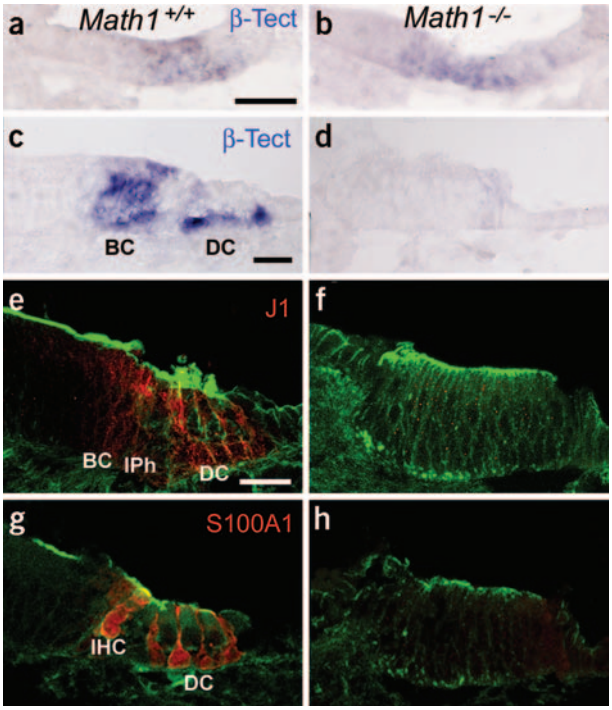

Figure 4 Supporting cell differentiation is disrupted in *Math1*-null mice. *In situ* or antibody labeling is indicated in red or blue for wild-type (**a**,**c**,**e**,**g**) and *Math1*-null (**b**,**d**,**f**,**h**) mice. (**a**,**b**) Cross-section through the cochlear duct at E16, showing a diffuse pattern of β-tectorin expression in both wild-type and *Math1*-null cochleae. (**c**,**d**) By P0, β-tectorin is expressed in border cells (BC) and Deiter's cells (DC) in the wild-type organ of Corti (OC) but no signal is present in the *Math1*-null OC. (**e–h**) Antibody labeling for jagged-1 (J1) and S100A1 in red in cross-sections of the OC at P0. Cell boundaries are labeled in green. Jagged-1 is present in inner phalangeal cells (IPh), Deiter's cells (DC) and border cells (BC) located adjacent to the IPh in the wild-type OC, but no signal is present in the *Math1*-null OC. S100A1 is expressed in inner hair cells (IHC) and Deiter's cells in the wild-type OC but is completely absent in the *Math1*-null OC. Scale bars, 50 μm (**a**; also applies to **b**); 20 μm (**c**; also apples to **d**); 20 μm (**e**; also applies to **f**,**g**,**h**).

the Kölliker's organ cells that were recruited to develop as supporting cells might be, in fact, cells that had expressed small amounts of Math1 at the time of transfection. We therefore transfected *Math1*^{ORF} into Kölliker's organ in explants established from mice at E17.5. By this stage, expression of Math1 was restricted to the sensory epithelium (**Fig. 1**). As observed in earlier explants (E13.5 and E14.5), expression of Math1^{ORF} was sufficient to induce the differentiation of hair cells in Kölliker's organ (data not shown). In addition, many ectopic hair cells were surrounded by cells that were positive for jagged-1 or otogelin (**Fig. 5m** and data not shown), although the overall induction of jagged-1- or otogelin-positive cells was less than that observed when transfections were carried out at earlier time points.

The induction of supporting cell markers in Kölliker's organ of E17.5 explants is consistent with the theory that endogenous expression of Math1 is not required for the development of supporting cells; however, an alternative possibility is that *Math1*^{ORF}-transfected cells induce transient expression of Math1 in neighboring cells as part of the program of supporting cell development. To determine whether any induction of Math1 expression occurs, *Math1*^{ORF} was transfected into explants established from *Math1*$^{\beta gal/+}$ mice and *Math1* promoter activity was assayed by an antibody to β-gal (**Fig. 1** and **Supplementary Fig. 2** online). Expression of β-gal was observed in the organ of Corti in transfected cells, confirming the presence of a positive feedback loop for Math1 expression[20], but not in cells surrounding the transfected cells (**Fig. 5n**,**o**).

As final confirmation that Math1 expression is not directly required for the development of supporting cells, *Math1*^{ORF} was transfected into Kölliker's organ in Math1-null mice. The absence of the sensory epithelium made the identification of Kölliker's organ difficult in *Math1*-null explants; however, clusters of transfected cells were observed to induce the expression of both otogelin and jagged-1 (**Fig. 5p**,**q** and **Supplementary Fig. 4** online), albeit with less efficiency than in wild-type explants. These results show that supporting cells can develop in the complete absence of Math1, and that hair cells generate inductive signals that are sufficient for the development of supporting cells.

Cells that transiently express Math1 can form supporting cells

The above results indicated that, although Math1 is required for the formation of both hair cells and supporting cells, only the formation of hair cells is directly regulated by this transcription factor. Because *Math1* is initially expressed in progenitor cells that ultimately develop as supporting cells (**Fig. 1**), it must be downregulated in those progenitor cells that go on to develop as supporting cells. Although the mechanism for this downregulation is unknown, it seems likely cell-cell interactions between adjacent *Math1*-positive progenitor cells may be involved. Contrary to this, inhibition of Math1 through cell-cell interactions apparently did not occur when *Math1*^{ORF} was transfected into cells in Kolliker's organ, because almost all transfected cells, even those in contact with other transfected cells, developed as hair cells (**Table 1**). We expressed *Math1*^{ORF} under the control of a strong promoter, however, which might inhibit or eliminate any subsequent endogenous regulatory interactions. To examine cell-cell interactions, we therefore generated an inducible form of *Math1* by fusing the *Math1* open reading frame (ORF) to a gene encoding a tamoxifen-specific version of the ligand-binding domain of the estrogen receptor (ER)[21]. The resulting Math1-ER fusion protein (hereafter designated Math1^{ER}) was sequestered in the cytosol in an inactive complex[22,23] but could be activated in the presence of tamoxifen.

Initial results indicated that expression of Math1^{ER} in Kölliker's organ did not generate hair cells or supporting cells in the absence of tamoxifen (**Supplementary Fig. 3** online). By contrast, exposure to 15 nM tamoxifen for 6 DIV resulted in the formation of groups of hair cells and jagged-1-positive cells in Kölliker's organ (**Supplementary Fig. 3**). Quantification of the effects of expressing *Math1*^{ER} in the presence of continuous tamoxifen indicated that more than 76% of transfected cells developed as hair cells. This value is lower than the 96% induction of hair cells observed in cells expressing *Math1*^{ORF}, possibly as a result of either incomplete penetration of tamoxifen or a decrease in the efficacy of Math1 binding to DNA.

To determine whether transient activation of Math1^{ER} would be also sufficient to induce the development of hair cells, explants were treated with tamoxifen for 48 h and then allowed to recover for 96 h. Analysis of isolated transfected cells indicated that 80% developed as hair cells (**Fig. 6a–d** and **Table 1**), showing that transient activation of Math1^{ER} is sufficient to induce hair cell differentiation. In addition, 6.5% of the isolated transfected cells expressed jagged-1. Although these cells were classified as isolated because they were not in contact with other transfected cells, they were always located in clusters of hair cells and jagged-1-positive cells, suggesting that supporting cells may induce the additional development of supporting cells.

These results indicated that transient activation of Math1^{ER} initiates an autonomous Math1 pathway that includes a positive feedback loop for Math1 expression. To determine whether cell-cell contacts are involved in such a pathway, we determined the fates of transfected cells

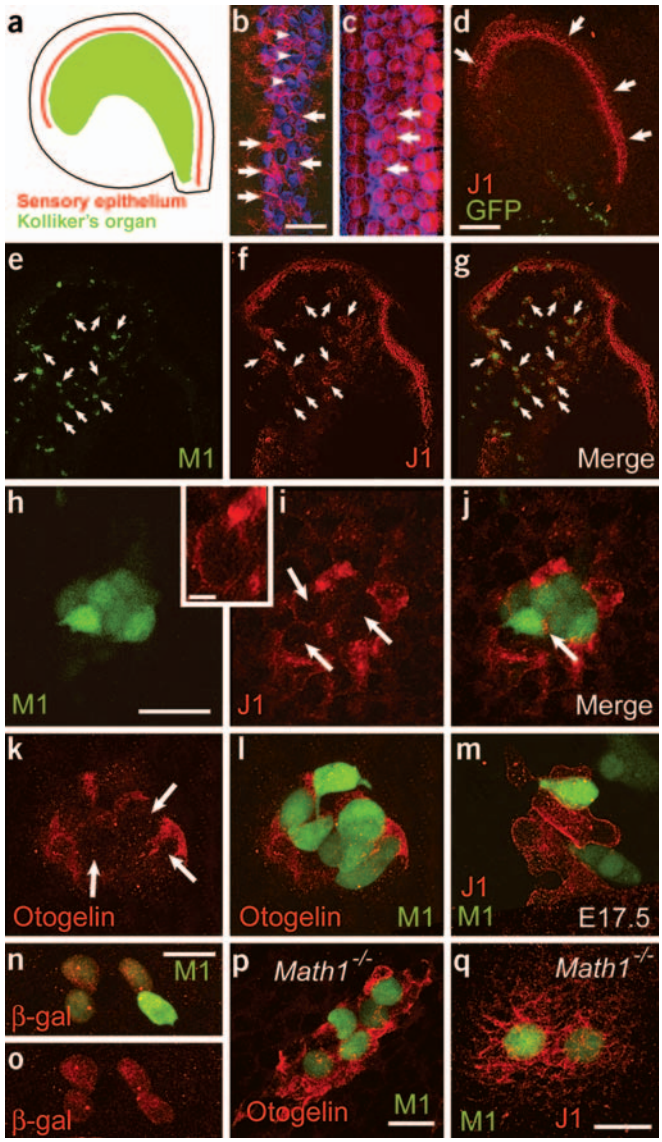

Figure 5 *Math1*^{ORF} expression induces formation of hair cells and supporting cells. (**a**) Representation of an E13 cochlear explant after 6 DIV. The sensory epithelium develops in a band around the periphery of the explant; Kölliker's organ (KO) is located in the center. (**b**) Antibody to jagged-1 (J1, red) labels supporting cells (arrows) in the sensory epithelium of an E13 explant. J1-positive supporting cells interdigitate between the mechanosensory hair cells, as indicated by the blue stereociliary bundles (arrowheads). (**c**) Hair cells express myosin VI (red). Stereociliary bundles are labeled as in **b**, but appear pink because of overlap with myosin VI labeling of hair cells (arrows). (**d**) Low-magnification image of an E13 explant transfected with an EGFP-expressing vector after 6 DIV. Supporting cells are indicated by J1 expression (red); expression of EGFP in transfected cells is shown in green (GFP). Expression of J1 is strong in cells in the sensory epithelium (arrows), but J1 is not expressed in KO in either transfected or untransfected cells. (**e**) Detection of *Math1*^{ORF}-transfected cells (green) in an explant established at E13 and maintained for 6 DIV. Clusters of transfected cells are present in KO (arrows). (**f**) As **e**, showing groups of J1-positive cells (red) in KO (arrows). (**g**) Merged image of **e** and **f**. Each cluster of *Math1*^{ORF}-positive hair cells is surrounded by a group of J1 positive cells (arrows). (**h**) High-magnification image of a cluster of *Math1*^{ORF}-transfected cells (green). (**i**) As **h**, showing expression of J1. Note the J1-negative cells in the center of the cluster (arrows). Inset, *z*-axis view of a single cell from the cluster in **i**, showing the constricted lumenal projection of one of the J1-positive cells. This morphology is consistent with that of supporting cells in the organ of Corti. (**j**) Merged image of **h** and **i**. The transfected hair cells (green) are located in the center of the cluster and are surrounded by J1-positive cells (red). The lumenal projection from one of the J1-positive cells interdigitates between two hair cells (arrow). (**k**) Cluster of otogelin-positive cells (red) in KO of an E13 explant. Note the otogelin-negative cells in the center of the cluster (arrows). (**l**) As **k**, but with *Math1*^{ORF}-transfected hair cells (green) added. Note that the transfected hair cells are located in the center of the cluster. (**m**) Cluster of *Math1*^{ORF}-transfected cells (green) in KO of an explant established at E17.5. Surrounding cells are positive for J1. (**n**) High-magnification image of four *Math1*^{ORF}-transfected cells (green). Expression of β-gal (red) indicates activation of the endogenous *Math1* promoter. (**o**) As **n**, but with the green channel removed. Although the *Math1* promoter has been activated in transfected cells, no activity is detected in the cells surrounding the transfected cells. (**p**) Cluster of *Math1*^{ORF}-transfected cells (green) in KO of an explant established at E13 from a *Math1*-null mouse. Surrounding cells are positive for otogelin. (**q**) Cluster of *Math1*^{ORF}-transfected cells (green) in KO of an explant established at E13 from a *Math1*-null mouse. Surrounding cells are positive for J1. Scale bars, 15 μm (**b**; applies to **c**); 200 μm (**d**; applies to **e**–**g**,**k**–**m**); 20 μm (**h**; applies to **i**–**l**); 20 μm (**m**); 5 μm (inset, **i**); 20 μm (**n**; applies to **o**); 20 μm (**p**,**q**).

in contact with other transfected cells (clusters) after transient activation of Math1^{ER}. Similar to the expression of *Math1*^{ORF} or the continuous activation of Math1^{ER}, transient activation of Math1^{ER} resulted in the formation of clusters of jagged-1-positive cells in Kölliker's organ (**Fig. 6e**,**f**). Examination of these clusters indicated, however, that some of the jagged-1-positive supporting cells were also transfected with *Math1*^{ER} (**Fig. 6g**,**h**).

Triple labeling of the same clusters with phalloidin to identify stereociliary bundles indicated that many of these clusters comprised a mixture of hair cells and supporting cells (**Fig. 6i**,**j**). These clusters appeared similar in morphology to the clusters observed in explants transfected with *Math1*^{ORF}; in contrast to those clusters, however, some of the jagged-1-positive cells in these clusters were also transfected with *Math1*^{ER} (**Fig. 6k–n**). Quantification indicated that the number of transfected cells in clusters that developed as hair cells after transient activation of Math1^{ER} was only 50% (**Table 1**). In addition, under these conditions more than 32% of the transfected cells expressed jagged-1 (as compared with only 6.5% of isolated transfected cells in the same explants). These results suggest that cell-cell interactions can indeed divert the fate of cells from their development as hair cells, probably through the regulation of Math1 expression.

The notch signaling pathway is activated in transfected cells

We considered that the most likely mechanism for inhibitory cell-cell interactions in clusters of *Math1*^{ER}-transfected cells would be activation of the Notch signaling pathway. Notch signaling has been shown to have a role in hair cell specification in the inner ear[24–30], although the occurrence of inhibitory interactions between neighboring cells has not been directly shown.

Notch1 is expressed in Kolliker's organ cells between E13 and E18 (ref. 24), and expression of the Notch ligand Jagged2 by hair cells is necessary for activation of Notch target genes in the organ of Corti[6]. To determine whether ectopic hair cells activate the Notch pathway, we analyzed the expression of Jagged2 by immunocytochemistry. Expression of Jagged2 was observed in hair cells in the sensory epithelium and in *Math1*-transfected cells in Kolliker's organ (**Fig. 7a–c**). To determine whether ectopic hair cells activated Notch signaling, nuclear localization of the intracellular domain (ICD) of Notch1 (Notch-ICD) was determined by immunocytochemistry[31]. Control experiments indicated that an antibody to Notch-ICD localized to cells in the organ of Corti that have been shown[29,30] to express the Notch target genes *Hes1* and *Hes5* (**Fig. 7d**,**e**). As expected, cells with

Table 1 Fates of *Math1*-transfected cells under different conditions

	Clusters			Isolated cells		
Vector	%HC	%SC	%UN	%HC	%SC	%UN
Math1^{ORF} [a]	97.6 (211)	ND	2.4	94.3 (159)	ND	5.7
Math1^{ER}						
Tx 120 h[b]	76.4 (89)	ND	23.6	75.0 (32)	ND	25.0
Tx 48 h[c]	50.0 (110)	32.7	17.3	80.0 (20)	6.5	13.5
Tx 48 h, DAPT 120 h[d]	73.7 (103)	1.2	25.1	82.1 (28)	1.5	16.4

For each condition, fates were determined for transfected cells located either in clusters with other transfected cells or in isolation (that is, not contacting any other transfected cells). Values are a percentage of the total number of cells counted. HC, hair cells; ND, not determined; SC, supporting cells; Tx, tamoxifen; UN, undefined cells.

[a]Expression of *Math1*^{ORF} induced more than 94% of transfected cells to develop as hair cells regardless of cell-cell contacts. [b]A similar, although slightly decreased, result was obtained for cells expressing *Math1*^{ER} in the presence of continuous tamoxifen (see text for details). [c]Transient (48-h) activation of Math1^{ER} resulted in a 34.6% decrease in the number of cells that developed as hair cells as compared with activation of *Math1*^{ER} by continuous tamoxifen, but only in clusters of transfected cells. Nearly 33% of transfected cells in these explants also expressed the supporting cell marker jagged-1, suggesting that they had differentiated as supporting cells. [d]Transient activation of Math1^{ER} followed by treatment with DAPT returned the percentage of transfected cells that developed as hair cells to the value observed with continuous tamoxifen treatment, suggesting that the Notch signaling pathway has a role in the decrease in hair cells observed in explants treated with tamoxifen for 48 h.

nuclear localized Notch-ICD were observed in cells surrounding ectopic hair cells (**Fig. 7f**).

Inhibition of γ-secretase activity inhibits supporting cell formation

To examine whether Notch signaling is involved in cell fate, explants transfected with *Math1*^{ER} and treated with tamoxifen for 48 h were subsequently treated with *N*-*S*-phenyl-glycine-*t*-butyl ester (DAPT), an inhibitor of γ-secretase that inhibits the Notch pathway both *in vivo* and *in vitro*[32–34]. Analysis of cell fates in *Math1*^{ER}-transfected clusters after sequential treatment with tamoxifen and DAPT indicated that inhibition of γ-secretase resulted in a marked increase in the number of transfected cells that developed as hair cells (74%; **Fig. 7g** and **Table 1**). In addition, the number of jagged-1-positive transfected cells in similarly treated explants decreased to about 1% (**Table 1**). These results show that the effects of inhibiting γ-secretase are consistent with the proposed role of Notch signaling in the determination of cell fate in the ear.

DISCUSSION

Math1 is not directly required for supporting cell development

The results presented here, along with previous work, show that in the cochlea *Math1* is initially expressed in a broad group of progenitor cells beginning at about E13. As development continues, expression of *Math1* is downregulated in progenitors that develop as supporting cells, but is maintained in progenitors that develop as hair cells. *Math1* is therefore transiently expressed in cells that develop as supporting cells. Because ectopic hair cells in Kölliker's organ can recruit surrounding naive cells to develop as supporting cells, however, Math1 does not have a direct role in supporting cell development. Instead, our results indicate that the loss of supporting cells in the cochleae of *Math1* mutants is a consequence of the loss of hair cells and that hair cells may generate inductive signals that are required for supporting cell formation. This conclusion is supported, first, by the observation that the expression of early markers of supporting cells is normal but subsequently disappears, presumably as a result of the lack of development of hair cells; and second, by the fact that deletion of *Math1* does not induce an increase in cell death[7], suggesting that hair cells do not generate signals that are required for the survival of supporting cells.

It is important to consider that our analysis was restricted to the cochlea, and that the loss of supporting cells observed in *Math1*-null explants may not occur in the vestibular epithelia. As discussed above, supporting cells in the organ of Corti are highly specialized, suggesting that the role of Math1 may be different in this epithelium. In fact, many important aspects of vestibular supporting cells are present in the utricles of *Math1*-null explants[3], suggesting that supporting cell development may not be affected in this epithelium.

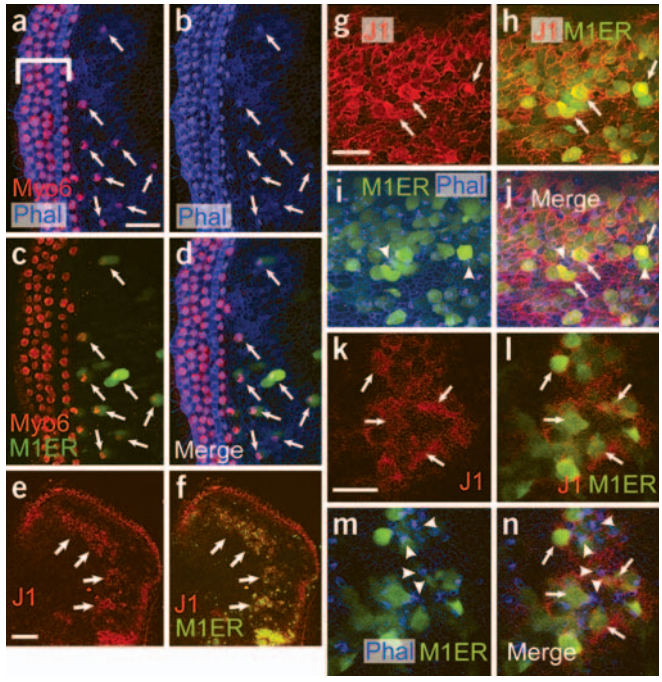

Figure 6 Cells that transiently express *Math1*^{ER} can develop as supporting cells. (**a–d**) An E13 explant was transfected with *Math1*^{ER}, treated for 48 h with tamoxifen and then incubated in normal media for 96 h. (**a**) Hair cells are labeled by staining with myosin VI (Myo6, red) and by the presence of stereociliary bundles (Phal, blue). A normal complement of hair cells is present in the organ of Corti (bracket) and ectopic hair cells are present in Kölliker's organ (KO; arrows). (**b**) As **a**, but with the red channel removed to allow visualization of stereociliary bundles (arrows) on ectopic hair cells. (**c**) As **a**, but with transfected cells shown in green (M1ER). Note that many transfected cells in KO have developed as hair cells. (**d**) As **a**, showing that transient activation of Math1^{ER} is sufficient to induce KO cells to develop as hair cells. (**e**,**f**) Low-magnification image of an E13 explant treated as in **a–d**. (**e**) Jagged-1-positive cells (J1, red) are present in both the sensory epithelium and in clusters in KO (arrows). (**f**) As **e**, but with the transfected cells shown in green. Note the correlation between transfection and J1 expression in the KO (arrows). (**g**) Higher-magnification view of a large region of J1-positive (red) cells in KO of an E13 explant transfected with *Math1*^{ER} after 6 DIV. (**h**) As **g**, but with transfected cells shown in green. Many of the J1-positive cells also express EGFP (arrows). (**i**) As **g**, but with transfected cells (green) and filamentous actin (blue) shown. Some transfected cells have also developed as hair cells (arrowheads). (**j**) As **g**, but with all three labels indicated. Transfected cells in the cluster have developed as either hair cells (arrowheads) or supporting cells (arrows). (**k**) Cluster of J1-positive cells (red) in KO of an explant treated as in **g**. (**l**) As **k**, but with transfected cells shown in green. Transfected cells that are also J1-positive are indicated (arrows). (**m**) As **k**, but with transfected cells (green) and filamentous actin (blue) indicated. Transfected hair cells (arrowheads) are also present in both clusters. (**n**) As **k**, but with all three labels indicated. Both clusters comprise transfected cells that have developed as either hair cells (arrowheads) or supporting cells (arrows). Scale bars, 30 μm (**a**; applies to **b–d**); 200 μm (**e**; applies to f); 20 μm (**g**; applies to **h–j**); 20 μm (**k**; applies to **l–n**).

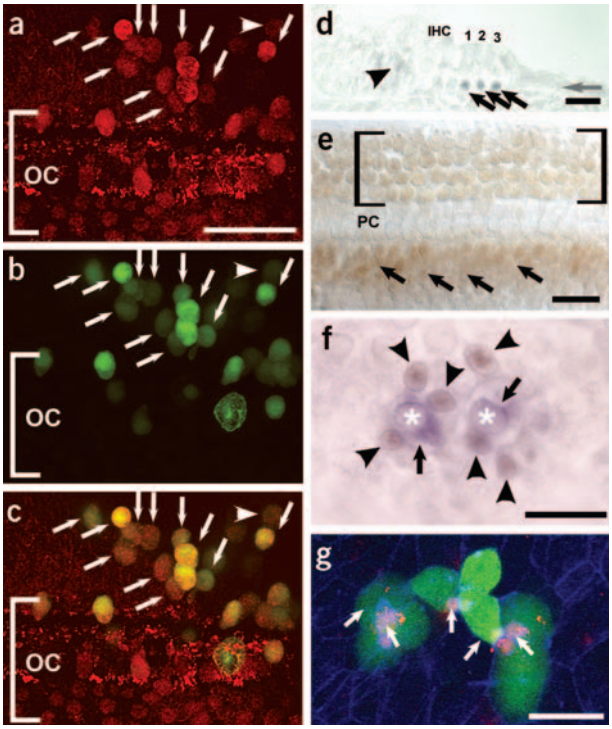

Figure 7 Components of the Notch signaling pathway are expressed in ectopic hair cells and inhibition of γ-secretase influences cell fate decisions by supporting cells. (**a**) High-magnification image of Jagged2-positive cells (red) in an E13 explant after 6 DIV. Cells positive for Jagged2 are located in both the organ of Corti (OC; bracket) and Kölliker's organ (KO; arrows). (**b**) As **a**, but with cells transfected with *Math1*^{ORF} shown in green (arrows). (**c**) Merged image of **a** and **b**. Most transfected cells located in KO also express Jagged2 (arrows), and even cells that are weakly transfected (arrowhead) express Jagged2. (**d**) Cross-section through the cochlear duct at P0 showing localization of Notch-ICD. Nuclear Notch-ICD is present in the three Deiter's cells (arrows) and is also weakly expressed in a group of cells in KO (arrowheads). Inner (IHC) and outer (numbered 1–3) hair cells are negative. Gray arrow indicates the plane of focus in **e**. (**e**) Expression of Notch-ICD in a whole mount of the cochlea at P0. The plane of focus is at the level of the Deiter cell and pillar cell nuclei (see **d**). Notch-ICD is present in the nuclei of Deiter's cells (bracketed area) but is negative in the pillar cells (PC). Notch-ICD is also present in a group of cells in KO (arrows). These cells are slightly out of focus because the nuclei are located at a higher plane of focus (compare with **d**). (**f**) High-magnification image of myosin VI–positive (purple) ectopic hair cells (arrows and white asterisks in nuclei) generated by transfection with *Math1*^{ORF} in KO of an E13 explant after 3 DIV. Cells surrounding the ectopic hair cells show nuclear localization of Notch-ICD (black nuclei, arrowheads), indicating activation of the Notch signaling pathway in response to transfection with *Math1*. (**g**) Three groups of hair cells (labeled with myosin VI in red) in KO of an E13 explant transfected with *Math1*^{ER}, treated with tamoxifen for 48 h and then incubated for 96 h in media containing DAPT. Despite the close proximity of these cells, all transfected (green) cells have developed as hair cells (arrows). Scale bars, 50 μm (**a**; applies to **b**,**c**); 20 μm (**d–g**).

Hair cells can induce supporting cell development

The existence of inductive interactions between hair cells and supporting cells has been suggested previously[35–40], but without experimental proof. In addition, the observation that supporting cells can develop in Kölliker's organ, a nonsensory structure, indicates that all of the signals required for supporting cell development, at least in the Kolliker's organ epithelium, must be produced by hair cells. These results suggest that in the cochlea the presence of hair cells is sufficient for the organization of at least rudimentary sensory epithelia. It is intriguing, however, that only more ancestral supporting cell markers, such as jagged-1 and otogelin were induced in these clusters, whereas markers of more derived supporting cell types, such as p75^{ntr}, were not observed (data not shown). These results suggest that additional factors may be required for formation of the specialized sensory epithelium in the organ of Corti.

Notch signaling regulates supporting-cell fate

Although the Notch signaling pathway was known to be involved in the regulation of hair cell number, its specific role in the ear had not been determined[24–28]. In particular, the previous observation that inhibition of Notch pathway signaling does not consistently disrupt supporting cell development suggested that Notch might not have a role in the final determination of cells as hair cells or supporting cells. Our current results indicate, however, that ectopic expression of Math1 is sufficient to activate the Notch pathway and suggest that Math1 itself is a target of that activation.

It is important to consider that the effects of inhibition of γ-secretase may not be limited to the Notch pathway[41]; however, other studies have indicated that inhibition of γ-secretase leads to a phenocopy of Notch mutation in both *Drosophila* and zebrafish[32,33], strongly suggesting that inhibition of Notch is a primary effect of this treatment. These results, along with previous work showing that activation of Notch1 leads to the expression of *HES* genes and that *HES* genes can inhibit and downregulate Math1 (refs 6,29,30), are consistent with a molecular signaling loop that uses both basic helix–loop–helix and Notch components to regulate the specification of hair cells and supporting cells. At the same time, developing hair cells must express other, as yet unidentified, inductive signals that recruit these 'Notch-diverted' cells to develop as supporting cells.

Math1 defines a 'pro-hair cell' equivalence group

Existing data suggest that hair cell sensory epithelia develop in several steps, including the specification of groups of progenitor cells, termed 'prosensory domains', that become capable of developing as either hair cells or supporting cells, and a subsequent step in which cells in these groups become committed to specific cell fates[42,43]. It had been suggested that Math1 might specify the prosensory domain[6] or, alternatively, might be a differentiation factor for cells that are already committed to develop as hair cells[7].

Our results indicate that Math1 does not function specifically in either of these processes in the cochlea. Although *Math1* is expressed in a population of cells that correlates with the prosensory domain, our finding that *Math1* expression is not required for generating supporting cells suggests that Math1 is not involved in the formation of this domain. However, the relatively broad expression of Math1, coupled with the fact that subsequent cell-cell interactions lead to the down regulation of Math1 in some of these cells, indicates that Math1 is expressed in prosensory cells that have not, and will not, become committed to develop as hair cells. In addition, cells that maintain Math1 expression invariably develop as hair cells. These results are consistent with the formation of a developmental equivalence group in which all of the cells in the prosensory domain initiate a Math1-dependent program, which if unabated will lead to the specification of the hair cell fate. As discussed above, however, an early target of the Math1-dependent program is activation of the Notch pathway, which leads to downregulation of Math1 and a diversion from the hair cell fate.

The concept of the prosensory domain should be reconsidered in light of our finding that ectopic hair cells in Kölliker's organ can induce

surrounding cells to develop as supporting cells. This result suggests either that the formation of a prosensory domain is not required for the development of cochlear sensory epithelia, or that cells in Kölliker's organ should be considered as part of the prosensory domain. At this point, it is not possible to determine which of these possibilities is more likely. With the exception of Math1, the best marker of the putative prosensory domain in the cochlea is the cell cycle inhibitor $p27^{kip1}$, which is expressed in a pattern that roughly overlaps with that of Math1 and is excluded from the Kölliker's organ[7,44]. Because hair cells and supporting cells can develop outside the expression domains of Math1 and $p27^{kip1}$ (ref. 4), however, the prosensory domain in the cochlea may be much larger than was previously thought and may be defined before the expression of either Math1 or $P27^{kip1}$.

In summary, our results show that Math1 specifies the formation of a 'pro-hair cell' equivalence group in the cochlea. Consistent with this molecular effect, Math1 is not directly required for the formation of supporting cells; however, absence of Math1 results in a complete loss of both hair cells and supporting cells in the cochlea as a result of a disruption in interactions between developing hair cells and surrounding uncommitted progenitor cells. Our results also show that developing hair cells produce two signals that influence the fate of surrounding cells: an inhibitory signal that most probably uses the Notch pathway to divert cells from developing as hair cells, and an inductive signal that recruits these same cells to develop as supporting cells.

METHODS

***Math1*-null mice and expression of *Math1*.** *Math1*-null mice were generated by crossing $Math1^{+/\beta gal}$ heterozygotes as described[3]. We obtained cochleae at specific time points between E12 and E18.5. All experiments involving mice were approved by the National Institutes of Health Animal Care and Use Committee. Expression of the gene encoding β-gal was determined by staining whole mounts as described[44].

Immunohistochemistry. Cochleae were dissected from wild-type and mutant littermates at the time points described above. Some cochleae were processed as whole mounts and others were sectioned in a cryostat at a thickness of 12 μm.

Whole-mount immunostaining of whole cochleae was done as described[11] with antibody to $p75^{ntr}$ (Chemicon; diluted 1:1,000) and with TC2 (a gift of A. Capehart, East Carolina University; undiluted). Antibody binding was detected by an Alexa-594 (red fluorophore)-conjugated secondary antibody (Molecular Probes; 1:1,000).

Cryostat sections were incubated overnight in primary antibodies to S100A1 (DAKO; 1:100), GFAP (DAKO; 1:100) and Jagged1 (Santa Cruz; 2 μg/ml). Antibody labeling was visualized as described. We used Alexa-488-conjugated phalloidin (Molecular Probes; 1:1,000) to visualize cellular borders in both whole mounts and cryosections.

***In situ* hybridization.** Cryostat sections of cochleae from *Math1*-null and wild-type littermates were obtained as described and *in situ* hybridization for *Fgfr3* or β-tectorin was done as described[45].

Construction of the $Math1^{ER}$ construct. The cDNA for the tamoxifen-specific form of the ER was a gift of A. McMahon (Harvard University, Boston, MA). This fragment was ligated into the *Not*I site of a pcDNA-Math1 expression vector generated and provided by M. Rivolta (University of Sheffield, Sheffield, UK). Two independent PCR reactions were then done with primers that removed the stop codon from the *Math1* cDNA and generated short complementary overhangs at the end of the Math1 and the beginning of ER cDNAs. We carried out a third PCR with primers from the beginning of Math1 and the end of the ER using the products of the first two PCR reactions as a template to generate the fusion construct. Construction of an in-frame Math1-ER fusion product was confirmed by DNA sequencing and the product was ligated into a pIRES2-EGFP vector (Clontech).

Transfection of cochlear explants. Cochlear explant cultures were established from E13.5 and E14.5 embryos as described[46]. Individual cells in Kolliker's organ were transfected by square-wave electroporation as described[4]. Both the $Math1^{ORF}$ and $Math1^{ER}$ vectors contained an IRES sequence followed by the cDNA for enhanced green fluorescent protein (EGFP).

The $Math1^{ER}$ fusion protein was activated by treating explants with 15 nM tamoxifen in dimethyl sulfoxide (Sigma) for either 48 h (transient activation) or 6 DIV (continuous activation). Preliminary results indicated that long-term exposure to tamoxifen concentrations greater than 15 nM resulted in toxicity. To inhibit Notch signaling, the γ-secretase inhibitor DAPT (Sigma) was added to culture media at a concentration of 2 μM for the duration of the experiment. Control experiments indicated that DAPT treatment of explant cultures transfected with EGFP alone did not induce ectopic hair cells (data not shown).

Transfected cells were identified by an antibody to GFP conjugated to Alexa-488 (Molecular Probes). Supporting cells were labeled with antibodies to either jagged-1 (Santa Cruz) or otogelin (a gift of C. Petit, Pasteur Institute), and hair cells were labeled with antibodies to myosin VI (Sigma) or myosin VIIa (a gift of C. Petit). All antibody labeling was visualized with a secondary antibody conjugated to Alexa-594 (Molecular Probes). Cell boundaries and stereociliary bundles were visualized with phalloidin conjugated to Alexa-633 (Molecular Probes). Expression of β-gal was detected by using an antibody to β-gal as described (Promega).

Expression of Notch pathway components. Expression of Jagged2 was determined by an antibody specific for human Jagged2 protein (Orbigen). Activation of Notch was determined by a Val1744 antibody (Cell Signaling Technology). Samples were fixed in 100% methanol, as described in the manufacturer's protocol. Antibody labeling was detected by a Vector Elite ABC kit (Vector Laboratories) using diaminobenzidine as a chromogen. Because the EGFP epitope could not be detected after methanol fixation, ectopic hair cells were identified by labeling with an antibody to myosin VI followed by an alkaline phosphatase chromogen reaction.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Bryant, J., Goodyear, R.J. & Richardson, G.P. Sensory organ development in the inner ear: molecular and cellular mechanisms. *Br. Med. Bull.* **63**, 39–57 (2002).
2. Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G. & Ornitz, D.M. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.* **12**, 390–397 (1996).
3. Bermingham, N.A. *et al. Math1*: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837–1841 (1999).
4. Zheng, J.L. & Gao, W.Q. Overexpression of *Math1* induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* **3**, 580–586 (2000).
5. Shailam, R. *et al.* Expression of proneural and neurogenic genes in the embryonic mammalian vestibular system. *J. Neurocytol.* **28**, 809–819 (1999).
6. Lanford, P.J., Shailam, R., Norton, C.R., Gridley, T. & Kelley, M.W. Expression of *Math1* and *HES5* in the cochleae of wildtype and Jag2 mutant mice. *J. Assoc. Res. Otolaryngol.* **1**, 161–171 (2000).
7. Chen, P., Johnson, J.E., Zoghbi, H.Y. & Segil, N. The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* **129**, 2495–2505 (2002).
8. Rubel, E.W. Ontogeny of structure and function in the vertebrate auditory system. in *Handbook of Sensory Physiology*, Vol. IX (ed. Jacobson, M.) 135–237 (Springer, New York, 1978).
9. Rio, C., Dikkes, P., Liberman, M.C. & Corfas, G. Glial fibrillary acidic protein expression and promoter activity in the inner ear of developing and adult mice. *J. Comp. Neurol.* **442**, 156–162 (2002).
10. Peters, K., Ornitz, D., Werner, S. & Williams, L. Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev. Biol.* **155**, 423–430 (1993).
11. Mueller, K.L., Jacques, B.E. & Kelley, M.W. Fibroblast growth factor signaling regulates pillar cell development in the organ of Corti. *J. Neurosci.* **22**, 9368–9377 (2002).

12. von Bartheld, C.S. *et al.* Expression of nerve growth factor (NGF) receptors in the developing inner ear of chick and rat. *Development* **11**3, 455–470 (1991).
13. Bianchi, L.M., Liu, H., Krug, E.L. & Capehart, A.A. Selective and transient expression of a native chondroitin sulfate epitope in Deiters' cells, pillar cells, and the developing tectorial membrane. *Anat. Rec.* **256**, 64–71 (1999).
14. Morrison, A., Hodgetts, C., Gossler, A., Hrabe de Angelis, M. & Lewis, J. Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. *Mech. Dev.* **84**, 169–172 (1999).
15. Coppens, A.G., Kiss, R., Heizmann, C.W., Schafer, B.W. & Poncelet, L. Immunolocalization of the calcium binding S100A1, S100A5 and S100A6 proteins in the dog cochlea during postnatal development. *Brain Res. Dev. Brain Res.* **126**, 191–199 (2001).
16. Rau, A., Legan, P.K. & Richardson, G.P. Tectorin mRNA expression is spatially and temporally restricted during mouse inner ear development. *J. Comp. Neurol.* **405**, 271–280 (1999).
17. El-Amraoui, A., Cohen-Salmon, M., Petit, C. & Simmler, M.C. Spatiotemporal expression of Otogelin in the developing and adult mouse inner ear. *Hear. Res.* **158**, 151–159 (2001).
18. Hasson, T. *et al.* Unconventional myosins in inner-ear sensory epithelia. *J. Cell. Biol.* **137**, 1287–1307 (1997).
19. Cernuda-Cernuda, R. & Garcia-Fernandez, J.M. Structural diversity of the ordinary and specialized lateral line organs. *Microsc. Res. Tech.* **34**, 302–312 (1996).
20. Helms, A.D., Abney, A.L., Ben-Arie, N., Zoghbi, H.Y. & Johnson, J.E. Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* **127**, 1185–1196 (2000).
21. Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G. & Evan, G.I. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.* **23**, 1686–1690 (1995).
22. Mattioni, T., Louvion, J.F. & Picard, D. Regulation of protein activities by fusion to steroid binding domains. *Methods Cell Biol.* **43**, 335–352 (1994).
23. Picard, D. Regulation of protein function through expression of chimaeric proteins. *Curr. Opin. Biotechnol.* **5**, 511–515 (1994).
24. Lanford, P.J. *et al.* Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat. Genet.* **21**, 289–292 (1999).
25. Zine, A., Van De Water, T.R. & de Ribaupierre, F. Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* **127**, 3373–3383 (2000).
26. Haddon, C., Jiang, Y.J., Smithers, L. & Lewis, J. Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637–4644 (1998).
27. Haddon, C. *et al.* Hair cells without supporting cells: further studies in the ear of the zebrafish mind bomb mutant. *J. Neurocytol.* **28**, 837–850 (1999).
28. Riley, B.B., Chiang, M., Farmer, L. & Heck, R. The deltaA gene of zebrafish mediates lateral inhibition of hair cells in the inner ear and is regulated by pax2.1. *Development* **126**, 5669–78 (1999).
29. Zheng, J.L., Shou, J., Guillemot, F., Kageyama, R. & Gao, W.Q. Hes1 is a negative regulator of inner ear hair cell differentiation. *Development* **127**, 4551–4560 (2000).
30. Zine, A. *et al.* Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* **21**, 4712–4720 (2001).
31. Lin, M.H. and Kopan, R. Long-range, nonautonomous effects of activated Notch1 on tissue homeostasis in the nail. *Dev. Biol.* **263**, 343–359 (2003).
32. Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. & Haass, C. A γ-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep.* **3**, 688–694 (2002).
33. Micchelli, C.A. *et al.* γ-Secretase/presenilin inhibitors for Alzheimer's disease phenocopy Notch mutations in *Drosophila*. *FASEB J.* **17**, 79–81 (2003).
34. Cheng, H.T. *et al.* γ-Secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. *Development* **130**, 5031–5042 (2003).
35. Lewis, J. Rules for the production of sensory cells. *Ciba Found. Symp.* **160**, 25–39 (1991).
36. Corwin, J.T., Jones, J.E., Katayama, A., Kelley, M.W. & Warchol, M.E. Hair cell regeneration: the identities of progenitor cells, potential triggers and instructive cues. *Ciba Found. Symp.* **160**, 103–120 (1991).
37. Adam, J. *et al.* Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. *Development* **125**, 4645–4654 (1998).
38. Goodyear, R., Holley, M. & Richardson, G. Hair and supporting-cell differentiation during the development of the avian inner ear. *J. Comp. Neurol.* **351**, 81–93 (1995).
39. Kelley, M.W., Talreja, D.R. & Corwin, J.T. Replacement of hair cells after laser microbeam irradiation in cultured organs of corti from embryonic and neonatal mice. *J. Neurosci.* **15**, 3013–3026 (1995).
40. Kelley, M.W., Xu, X.M., Wagner, M.A., Warchol, M.E. & Corwin, J.T. 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 (1993).
41. Kimberly, W.T. & Wolfe, M.S. Identity and function of γ-secretase. *J. Neurosci. Res.* **74**, 353–360 (2003).
42. Cole, L.K. *et al.* Sensory organ generation in the chicken inner ear: contributions of bone morphogenetic protein 4, serrate1, and lunatic fringe. *J. Comp. Neurol.* **424**, 509–520 (2000).
43. Kelley, M.W. & Bianchi, L.M. Development and neuronal innervation of the organ of Corti. in *Handbook of the Mouse Auditory Research: From Behavior to Molecular Biology* (ed. Willott, J.F.) 137–156 (CRC, New York, 2001).
44. Chen, P. & Segil, N. $p27^{Kip1}$ links proliferation to morphogenesis in the developing organ of Corti. *Development* **126**, 1581–1590 (1999).
45. Morsli, H., Choo, D., Ryan, A., Johnson, R. & Wu, D.K. Development of the mouse inner ear and origin of its sensory organs. *J. Neurosci.* **18**, 3327–3335 (1998).
46. Montcouquiol, M. & Kelley, M.W. Planar and vertical signals control cellular differentiation and patterning in the mammalian cochlea. *J. Neurosci.* **23**, 9469–9478 (2003).