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Expression of *Math1* and *HES5* in the Cochleae of Wildtype and Jag2 Mutant Mice

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ABSTRACT

The sensory epithelium within the mammalian cochlea (the organ of Corti) is a strictly ordered cellular array consisting of sensory hair cells and nonsensory supporting cells. Previous research has demonstrated that Notch-mediated lateral inhibition plays a key role in the determination of cell types within this array. Specifically, genetic deletion of the Notch ligand, Jagged2, results in a significant increase in the number of hair cells that develop within the sensory epithelium, presumably as a result of a decrease in Notch activation. In contrast, the downstream mediators and targets of the Notch pathway in the inner ear have not been determined but they may include genes encoding the proneural gene *Math1* as well as the *HES* family of inhibitory bHLH proteins. To determine the potential roles of these genes in cochlear development, *in situ* hybridization for *Math1* and *HES5* was performed on the cochleae of wildtype vs. *Jagged2* mutants ($Jag2^{\Delta DSL}$). Results in wild-type cochleae show that expression of *Math1* transcripts in the duct begins on E13 and ultimately becomes restricted to hair cells in the sensory epithelium. In contrast, expression of *HES5* begins on E15 and becomes restricted to supporting cells in the epithelium. Results in *Jag2* mutant cochleae suggest that *Math1* transcripts are ultimately maintained in a larger number of cells as compared with wildtype, while transcripts for *HES5* are dramatically reduced throughout the epithelium. These results are consistent with the hypothesis that activation of Notch via Jagged2 acts to inhibit expression of *Math1* in cochlear progenitor cells, possibly through the activity of *HES5*.

Keywords: Notch, Jagged2, lateral inhibition, inner ear, hair cell

INTRODUCTION

The restriction of multipotent embryonic progenitor cells to specific cell fates is a complex and highly regulated developmental process. The molecular mechanisms that drive this process have been characterized most successfully in invertebrate model systems where structure and development are strictly ordered and perturbations of the system are readily detectable (e.g., *Drosophila* retina) (reviewed in Wolff and Ready 1993). In vertebrate species, similar highly structured systems are rare. However, the sensory epithelium within the mammalian cochlea (the organ of Corti) is one example of a vertebrate system in which the physical arrangement of cell types and the spatiotemporal sequence of their development are highly invariant. The organ of Corti contains four rows of mechanosensory hair cells, including a single row of inner hair cells and three rows of outer hair cells. Within each row, hair cells are separated from one another by an interceding nonsensory supporting cell, forming a mosaic that extends along the length of the cochlear duct (reviewed in Lim and Rueda 1992). During embryogenesis, the development of this mosaic begins

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near the base of the cochlea and proceeds toward the apex in a wave of differentiation (Lim and Anniko 1985) that is reminiscent of development in the compound eye of *Drosophila* (Wolff and Ready 1993).

The results of previous studies have led to the suggestion that cell fates within the developing organ of Corti may be determined in part through intercellular inhibitory interactions (lateral inhibition) (Corwin et al. 1991; Lewis 1991). In particular, laser microbeam ablation of individual differentiating hair cells within the embryonic cochlea demonstrated that removal of these cells allows adjacent progenitor cells to alter their fates and to develop as replacement hair cells (Kelley et al. 1995). This result suggests that newly developing hair cells produce an inhibitory signal that prevents the differentiation of the hair cell phenotype in their immediate neighbors.

Similar lateral inhibitory signals play a role in the determination of cell types in a variety of invertebrates and nonplacental vertebrates (reviewed in Muskavitch 1994; Lewis 1996). In addition, these signals appear to be one component of a larger series of determining steps that progressively restricts the number of progenitor cells that may become competent to develop as a given cell type (reviewed in Campos–Ortega 1995; Modollel 1997). Specifically, lateral inhibition diverts a subset of cells within the progenitor pool from advancing from one level of competency to the next by repressing the activity of a default developmental program (Dorsky et al. 1997). The subset of cells that are diverted by lateral inhibition are subsequently returned to the progenitor pool.

This mechanism plays a key role in the determination of cell fates in the developing nervous system in both vertebrates and invertebrates (reviewed in Anderson and Jan 1997). For example, during development of *Drosophila*, neural competency is established in groups of progenitor cells through the activity of "proneural" basic helix–loop–helix (bHLH) genes such as *atonal* (*ato*) and the *achaete-scute* (*AS-C*) complex (Jarmen et al. 1993, 1994; Modellel 1997). All progenitors that express these proneural genes have the potential to develop as neural cell types and, therefore, constitute a proneural "equivalence group" (Doe and Goodman 1985). As development proceeds, a subset of cells within this equivalence group is diverted from the neural fate through lateral inhibition, mediated through the activity of "neurogenic" genes such as *Notch, Delta,* and the *enhancer of split* complex [*E*(*spl*)] (reviewed in Artavanis–Tsakonis et al. 1995). Binding of the receptor, Notch, to its ligand, Delta, results in the activation of a downstream pathway that results in the increased expression of *E*(*spl*) (reviewed in Bray 1997). The bHLH proteins encoded by *E*(*spl*) negatively regulate the expression of neural phenotypes through repression of proneural genes (Fig. 1) (Nakao and Campos–Ortega 1996; Giebel and Campos–Ortega 1997; Jimenez and Ish–Horowicz 1997; Bray 1997).

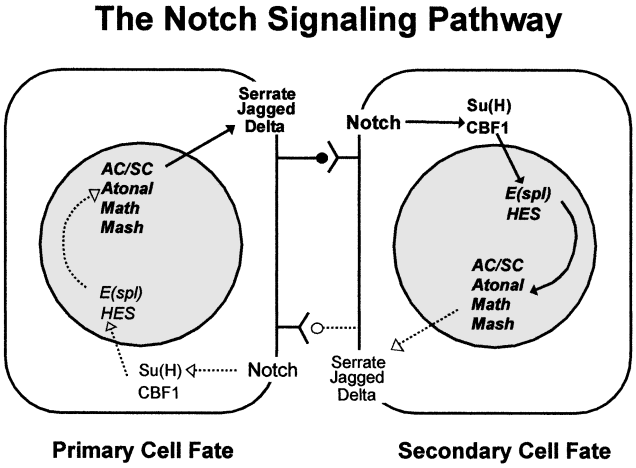

FIG. 1. Diagram of the Notch pathway. Research in vertebrates and invertebrates indicates that the Notch signaling pathway may function in the following manner: During embryonic development, a population of progenitor cells expresses the Notch receptor (N). Within this progenitor pool, specific cells begin to express proneural genes (*atonal, acheate-scute, Math*, and *MASH*). One effect of proneural gene expression is the upregulation of genes encoding the Notch ligands (Delta, Serrate, and Jagged). In addition, a single progenitor cell may produce more of the ligand than its neighbors, via random fluctuations in gene expression or through some additional extrinsic or intrinsic biasing mechanism. Increased levels of ligand expression in a given progenitor result in a subsequent increase in the activation of Notch in adjacent cells. Increased activation of Notch in turn results in the upregulation of downstream effector molecules (*enhancer of split, HES*) that act as negative regulators of proneural gene expression. Ultimately, the regulatory feeback loop between proneural gene expression and activation of the Notch pathway leads to the establishment of two stable populations of progenitor cells. One population continues to express proneural genes and the Notch ligand and will develop as the primary cell type. The second population does not express proneural genes and the Notch ligand and will develop as a secondary or tertiary cell type.

Mammalian homologs for many of the neurogenic and proneural genes have now been identified, including *HES* [*E*(*spl*)], *Mash* (*AS-C*), and *Math* (*ato*) (Akazawa et al. 1992, 1995; Sasai et al. 1992; Sommer et al. 1996; Ma et al. 1996). Recent studies have demonstrated that activation of Notch results in the subsequent activation of specific *HES* genes (*HES1* and *HES5*) (Tomita et al. 1996; Kageyama and Nakanishi 1997; Ohtsuka et al. 1999). In addition, mutations in these and other Notch pathway-related genes results in the upregulation of mammalian proneural genes and neural markers (Ishibashi et al. 1995; Pompa et al. 1997; Ohtsuka et al. 1999). Until recently, however, a role for any of the neurogenic genes in lateral inhibition had not been demonstrated directly in mammals. Studies from several laboratories have indi-

cated that the Notch pathway is involved in the development of the vertebrate inner ear (Lindsell et al. 1996; Luo et al. 1997; Adam et al. 1998; Haddon et al. 1998; Lanford et al. 1999; Morrison et al. 1999). In the mammalian cochlea, *Notch1* and its ligands, encoded by *Jag2* and *Delta1* (*Dll1*), are expressed in a manner consistent with a role in lateral inhibition (Lanford et al. 1999; Morrison et al. 1999). In addition, cochleae from embryonic mice containing a targeted deletion of the *Jag2* gene ($Jag2^{\Delta DSL}$)(Jiang et al. 1998) contain a greater number of hair cells in the sensory epithelium compared with wildtype (Lanford et al. 1999). Combined, these results support a role for Notch-mediated lateral inhibition in the determination of the number of progenitor cells that will develop as hair cells.

The specific genes that are regulated by activation of the Notch pathway and the downstream molecules that mediate this regulation have not yet been determined in the inner ear; however, the results of a recent study indicate that the proneural gene *Math1* may be one candidate. Bermingham and colleagues (1999) demonstrated the presence of *Math1* promoter activity in the developing organ of Corti at developmental time points consistent with the determination of sensory cell fates. Their study also demonstrated that genetic deletion of *Math1* results in the complete absence of hair cells in the sensory epithelium. While a specific regulatory relationship between the Notch pathway and the expression of *Math1* has not been demonstrated in the inner ear, the results of previous studies indicate that the activity of mammalian proneural proteins can be repressed by HES proteins (Sasai et al. 1992; Akazawa et al. 1995). Consequently, it seems possible that Notch-dependent bHLH genes such as *HES5* are expressed in the developing cochlea and that these genes are responsible for the diversion of sensory progenitor cells from the hair cell fate, via repression of *Math1*. The first aim of the present study was to confirm the expression pattern of *Math1* in the developing organ of Corti and to determine whether *HES5* is expressed in the cochlear duct in a manner consistent with the activation of Notch. The second aim of the study was to determine whether the deletion of *Jag2* alters the expression patterns of these genes in a manner consistent with a decrease in Notch activation. Specifically, we hypothesized that the Notch pathway inhibits sensory progenitor cells from differentiating as hair cells through the activation of *HES* genes and the subsequent repression of *Math1*. Deletion of *Jag2* should result in a decrease in expression of *HES* genes and an increase in the number of cells that maintain *Math1* expression and differentiate as hair cells.

MATERIALS AND METHODS

In situ hybridization

Timed-mated pregnant ICR mice were euthanized with CO_2 and embryos were obtained on gestational days 12–18 (E12–18) or postnatal days 0–3 (P0–3) (staging according to Kaufman, 1992). The temporal bones were dissected out of the cranium, and an opening was made into the cochlear region to ensure adequate fixation. Tissues were fixed in 4% paraformaldehyde, dehydrated to 100% methanol, and stored at –20°C. Prior to processing for *in situ* hybridization, the cochleae were rehydrated and the cochlear capsule and roof of the cochlear duct were removed to expose the developing sensory epithelium.

Synthesis of digoxygenin-labeled cRNA probes was performed according to Wilkinson and Nieto (1993). RNA probes were synthesized from cDNAs for *HES5* (Akazawa et al. 1992) and *Math1* (Akazawa et al. 1995). *In situ* hybridization procedures were modified from Wilkinson and Nieto (1993). Briefly, cochleae were rinsed in phosphate buffered saline (PBS) plus 0.1% Tween 20, treated with 6% H_2O_2, and digested in Proteinase K. The tissues were incubated in prehybridization solution for 2 hours at 70°C, then exposed to RNA probes overnight at 70°C. Bound probes were detected via a standard alkaline phosphatase immunohistochemical reaction. A minimum of two cochleae were processed per time point per probe. Selected tissues were embedded in Tissue-Tek O.C.T. embedding compound and sectioned at 8–10 μm.

In situ hybridization and morphological analyses of cochleae from *Jag2* mutant mice

Animals homozygous for a targeted deletion of *Jag2* (referred to in this text as "*Jag2* mutants") die at birth as a result of craniofacial defects that are not related to the development of the ear (Jiang et al. 1998). Therefore, embryos were obtained at developmental time points between E14.5 and E17.5 as described above. Genotypes were determined initially on the basis of morphological characteristics and subsequently confirmed by polymerase chain reaction (PCR) (Jiang et al. 1998). Cochleae were dissected, fixed, and prepared for *in situ* hybridization as described. Mutant cochleae were processed with probes against *Math1* and *HES5*, as well as *Brn3.1* (kindly provided by E. Huang and L. Reichardt), an early marker for hair cell differentiation (Xiang, et al. 1998). Control cochleae (ICR strain for *Brn 3.1* cochleae; *Jag2* wildtype for *Math1 and HES5*) were also processed for *in situ* hybridization.

To examine the morphological effects of deletion

of *Jag2*, cochleae were fixed in gluteraldehyde, dehydrated, and embedded in methacrylate (Immunobed, Polysciences, Inc., Warrington, PA). Tissues were sectioned at 3–5 μm on a rotary microtome, mounted on glass slides, and stained with thionine.

RESULTS

The results of previous studies in the mammalian cochlea suggest that the determination of hair cell fates in the organ of Corti begins in the base of the cochlea at about E13 and extends along the length of the cochlea as development progresses. Early markers of hair cell differentiation such as the Notch ligands *Jag2* and *Delta1*, as well as MyoVI, MyoVIIA and *Brn3.1*, are detected in the base of the cochlea beginning at E13 and extend to the apex of the cochlea by the time of birth (Hasson et al. 1995, 1997; Erkman et al. 1996; Xiang et al. 1997; Lanford et al. 1999; Morrison et al. 1999). In addition, the morphological characteristics of hair cells (e.g., stereociliary bundles) first become identifiable in the base of the cochlea at about E15, but not in the apical regions of the epithelium until late gestational or early postnatal time points (Lim and Anniko 1985; Lim and Rueda 1992). Based on these results, the developmental time frame between E13 and E17 appears to be critical for the determination of cell fates along the length of the organ of Corti. Consequently, *in situ* hybridization for proneural and neurogenic gene expression in the cochlear duct was performed at time points that bracket this critical developmental period (E12–P3).

Expression of *Math1* in the developing cochlea

The expression pattern of *Math1* mRNA in the developing organ of Corti is consistent with the results of a recent study demonstrating promoter activity for this gene in the developing sensory epithelium (Bermingham et al. 1999) (Fig. 2). Transcripts for *Math1* can first be detected in the basal turn of the cochlea beginning on E13 in a relatively narrow band of cells (Fig. 2A–C). Examination of sectioned cochleae indicates that the location of this band correlates with the region of the cochlear duct that will develop as the sensory epithelium. In addition, the band of *Math1* expression at E13 spans the thickness of the ventral floor of the duct, from the basement membrane to the lumenal surface (Fig. 2B,C).

By E15, the band of *Math1* expression appears to have broadened slightly and has extended to nearly the full length of the cochlear duct (Fig. 2D). Tissue sections demonstrate that *Math1* transcripts are restricted to the region of the cochlear duct overlying the spiral vessel, a transient structure that serves as a landmark for the developing sensory epithelium (Fig. 2E). While the expression of *Math1* still spans the thickness of the membrane at this time point, transcripts appear to be becoming restricted to cells in the lumenal half of the epithelium (Fig. 2E,F). By E17, *Math1* transcripts are clearly restricted to inner and outer hair cells along the full length of the sensory epithelium (Fig. 2G–I). Qualitative assessment of *Math1* expression in tissue sections suggests that the number of cells that express this proneural gene is reduced at E17.5 by comparison with a comparable region of the duct at earlier developmental time points. Finally, by P3 *Math1* expression begins to decrease in the base of the cochlea but remains expressed in the apex, suggesting that this gene is expressed transiently within the cochlear duct and that both upregulation and downregulation of *Math1* occur in basal-to-apical gradients (not shown).

Expression of *HES5* in the developing cochlea

Initiation of *HES5* expression begins at E15 in a narrow band of cells originating in the base of the cochlea and extending toward the apex (Fig. 3A). At this time point, the band of *HES5* expression spans the thickness of the ventral floor of the duct (Fig. 3B,C) and is located in the region of the duct overlying the spiral vessel (Fig. 3B,C). In addition, the band of *HES5* expression appears to occur within the somewhat broader domain of *Math1* expression, based on the positions of the expression bands relative to the spiral vessel (compare Figs. 2E,F and 3B,C). At E16, the band of *HES5* expression has expanded in both the neural and abneural directions and along the basal-to-apical axis (Fig. 3D–F). Transcripts for *HES5* appear to be more intense in cells adjacent to the basement membrane (Fig. 3E,F). In addition, the band of *HES5* expression appears to become divided into two distinct regions by interceding *HES5*-negative cells. This division begins in the base of the cochlea and is still evident in the apical region of the epithelium at E17 (Fig. 3G). Sections through the apical turn of an E17 cochlea indicate that, between E15 and E17, the domain of *HES5* expression includes both supporting cells within the sensory epithelium and cells in the inner sulcus region (Fig. 3H). As development continues, the expression of *HES5* transcripts becomes progressively downregulated from the inner sulcus region of the duct through the sensory epithelium. This downregulation is demonstrated in sections from the basal turn of an E17 cochlea, which indicates expression of *HES5* restricted to Dieter's cells located in the abneural half of the sensory epithelium (Fig. 3I,J). By P0, *HES5* expression is absent in the basal turn of the cochlea but persists in the apical turns, suggesting that tran-

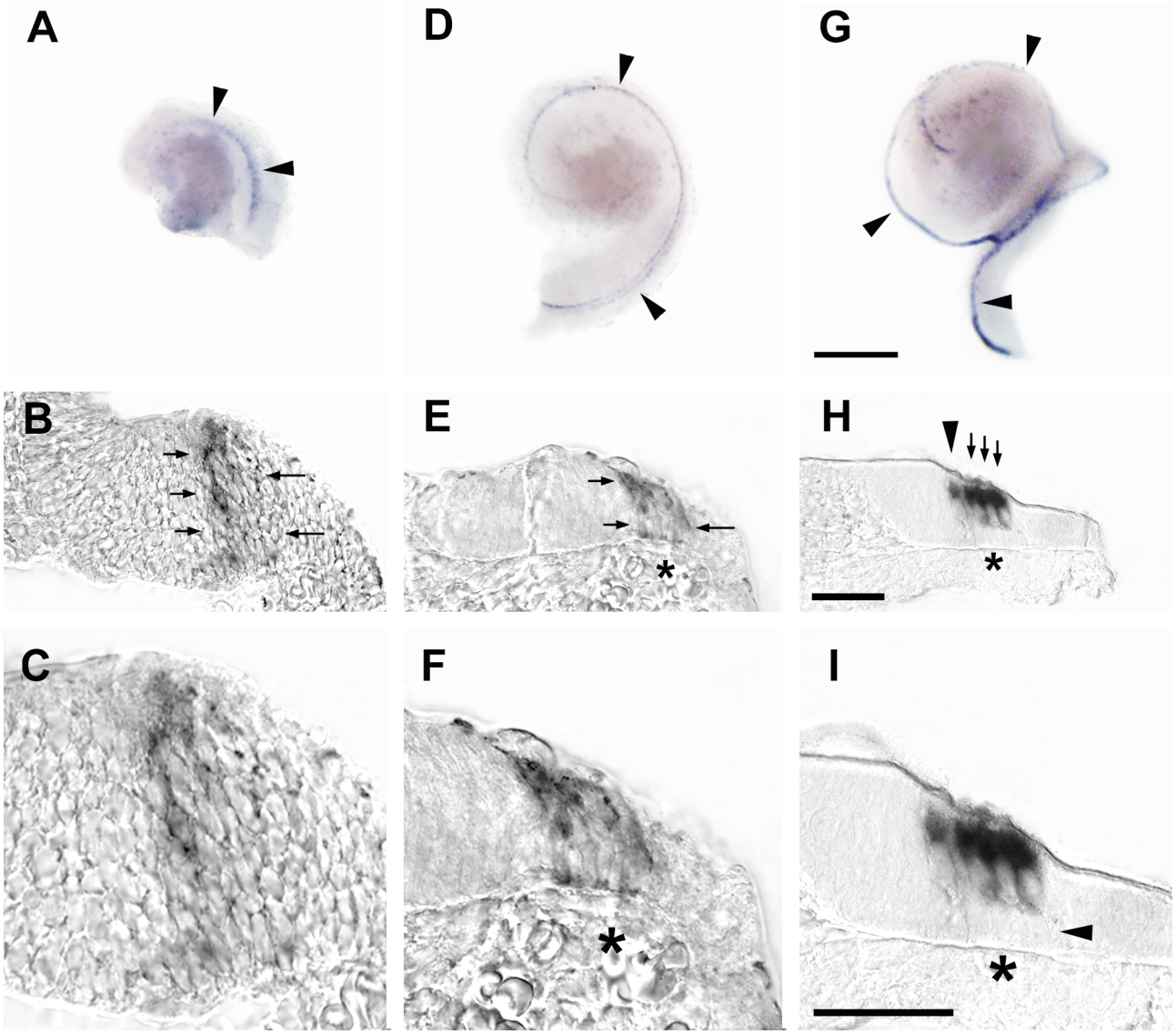

FIG. 2. *In situ* hybridization for *Math1* in cochleae from E13, E15, and E17. The roof of the cochlear duct has been removed. **A**. Whole mount of the cochlear duct at E13. *Math1* transcripts are expressed in a relatively narrow band of cells that does not yet extend the full length of the epithelium (arrowheads). **B**. Cryosection through an E13 cochlea demonstrates that the expression domain for *Math1* extends from the lumenal to basal surfaces (arrows). **C**. Higher magnification view of B. Individual cells expressing *Math1* are clearly labeled at multiple levels within the thickness of the duct. **D**. By E15, the *Math1* expression domain has expanded along the full length of the duct. **E**. Cryosection from the middle turn of the cochlea demonstrates *Math1* expression in cells located throughout the region of the duct that will develop as the sensory epithelium (arrows; asterisk indicates position of the spiral vessel). **F**. Higher magnification of E. *Math1* expression appears to be more intense in cells located in the lumenal half of the epithelium (asterisk indicates spiral vessel). **G**. At E17, transcripts for *Math1* persist along the entire length of the cochlear duct (arrowheads). Scale bar equals 250 μm (same in A and D). **H**. Sections through the base of the cochlea demonstrate that expression of *Math1* is restricted to developing hair cells (arrowhead indicates inner hair cell, arrows indicate outer hair cells, asterisk indicates the spiral vessel). Scale bar equals 25 μm (same in B and E). **I**. Higher magnification of H. Nuclei of Deiter's cells can be distinguished in this section (asterisk indicates the spiral vessel). Expression continues through P3, but appears to become downregulated in a basal-to-apical gradient (not shown). Scale bar equals 25 μm (same in C and F).

scription of this gene is progressively downregulated in a basal-to-apical gradient (not shown).

Morphology and gene expression patterns within the ears of *Jag2* mutant mice

The results of a recent study have demonstrated that targeted deletion of the Notch ligand, Jagged2, results in a significant increase in the number of cells that differentiate as hair cells in the organ of Corti (Lanford et al. 1999). Presumably, this is because of a decrease in the levels of Notch activation, which allows more progenitor cells to become committed to the hair cell fate. Since mutant pups die at birth, our previous study examined cochleae from *Jag2* mutant animals at the latest possible embryonic time point

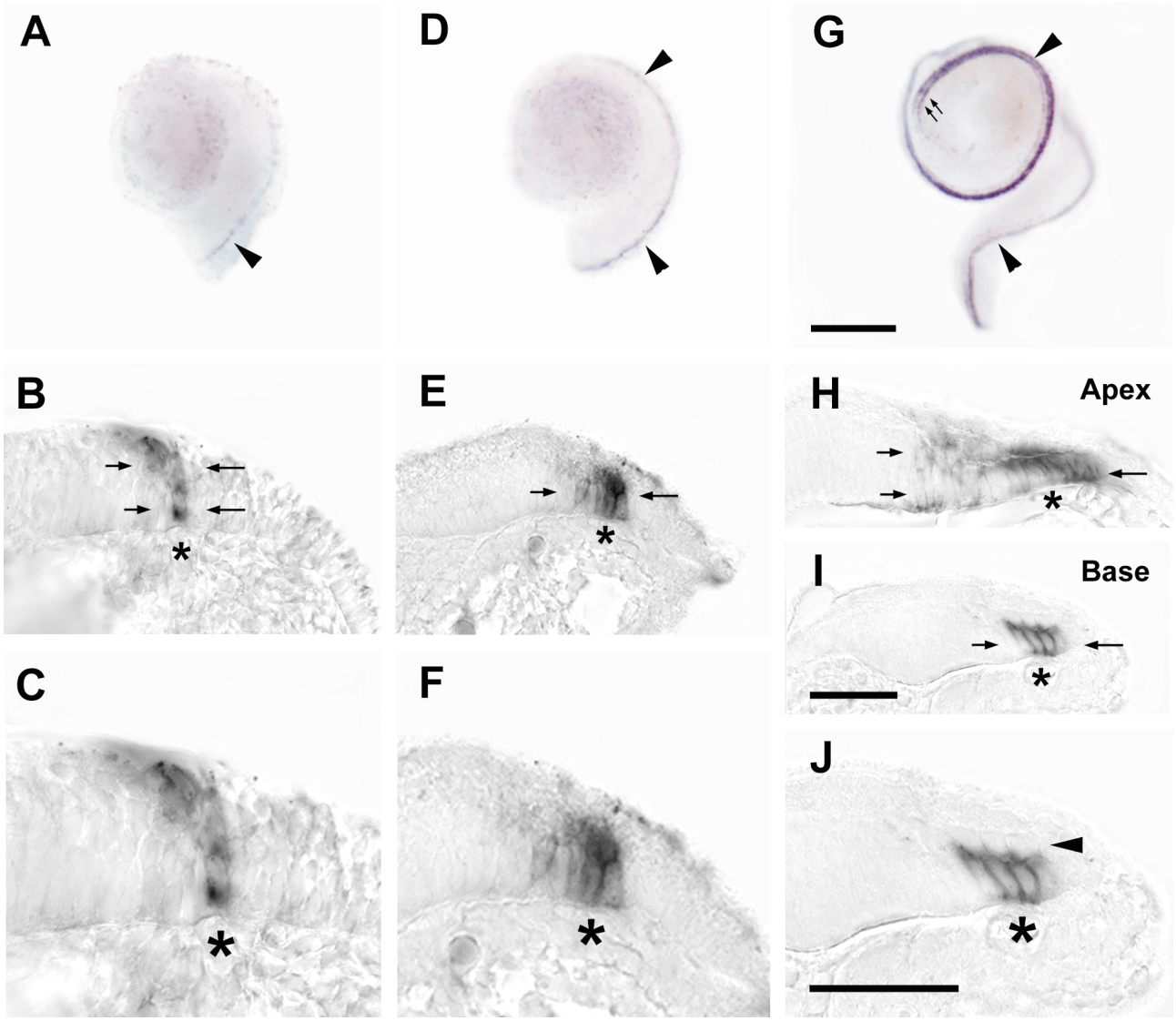

FIG. 3. *In situ* hybridization for *HES5* in cochleae from E15, E16, and E17. The roof of the cochlear duct has been removed. **A**. At E15, *HES5* expression begins in the cochlear duct in a highly restricted band of cells that extends from the base of the cochlea toward its apex (arrowhead). **B**. Cryosections of E15 cochleae demonstrate that this expression is located in the developing sensory region of the duct (arrows; asterisk indicates position of the spiral vessel). This image is a composite of two photographs of the same section, taken at slightly different planes of focus. **C**. Higher magnification of B. Note that the width of *HES5* expression is relatively narrow compared with the expression band of *Math1* at the same time point (see Fig2E,F) (asterisk indicates spiral vessel). **D**. As development proceeds through E16, expression of *HES5* extends along the basal-to-apical axis of the duct (arrowheads). **E**. The band of *HES5* expression has broadened relative to its width at E15 (arrows; asterisk indicates the spiral vessel). **F**. Higher magnification of E. *HES5* expression appears to be more intense in cells located in the basal half of the epithelium (asterisk indicates the spiral vessel). **G**. At E17, expression of *HES5* extends along the entire length of the epithelium (arrowheads). As *HES5* transcripts are expressed along the cochlear duct, subdivisions within the band of expression are distinguishable. These subdivisions are still clearly visible in the apex of the cochlea at E17 (double arrows) Scale bar equals 250 μm (same in A and D). **H**. Cryosection through the apical turn of the E17 cochlea shows the relatively broad band of *HES5* expression that appears to include inner phalangeal cells as well as developing Deiter's cells (arrows; asterisks indicates spiral vessel). This section is slightly oblique. **I**. In contrast to the apical section, a section through the base of the E17 cochlea shows *HES5* expression restricted to Deiter's cells located in the abneural region of the epithelium (arrows; asterisk indicates spiral vessel). This result suggests that *HES5* expression is progressively downregulated from the neural side of the developing epithelium to the abneural side. Scale bar equals 25 μm (same in B, E, H). **J.** Higher magnification of I. Nuclei of hair cells can be distinguished in this section (asterisk indicates the spiral vessel). As development continues, expression of *HES5* appears to become completely downregulated in an apical-to-basal gradient, but persists in the apex of the cochlea through at least P5 (not shown). Scale bar equals 25 μm (same in C and F).

(E17), to ensure the greatest degree of cellular differentiation within the epithelium. The apical one-third of the cochlear duct was omitted from the analysis, since hair cells in this region of the epithelium are still not fully differentiated at E17 and could not be reliably counted. In the present study, we have specifi-

cally examined sections from both the apical and basal regions of E17 mutant cochleae in order to compare the morphology of the duct at early versus late points in hair cell development. Results in *Jag2* mutant cochleae indicate that additional developing hair cells are present along the length of the epithelium at the earliest points at which these cells become morphologically identifiable (Fig. 4A–D). The identity of these additional cells was confirmed by expression of an early marker of hair cell differentiation, *Brn 3.1* (Fig. 4E,F). These results support the hypothesis that Notch signaling acts at an early time point to regulate the number of cells that differentiate as hair cells. The results also suggest that the number of cells within the initial pool of sensory progenitor cells is larger number than is required to form the final population of hair cells in the epithelium.

Expression of *Math1* and *HES5* in *Jag2* mutant cochleae

Results from normal cochleae (see above) indicate that transcripts for *Math1* are expressed in the cochlea at least one day prior to the onset of *Jag2* expression (*Math1* at E13, *Jag2* at E14.5). Consequently, the initiation of *Math1* expression appears to be independent of Jag2-dependent Notch signaling. Results from *Jag2* mutant cochleae indicate, however, that the continued expression of *Math1* in sensory progenitor cells is regulated by the Notch pathway. In *Jag2* mutant cochleae, *Math1* transcripts were consistently detected in the additional inner hair cells present in the epithelium (Fig. 4G,H). Our previous research has demonstrated that the deletion of *Jag2* results in a statistically significant increase in the number of inner hair cells (and total hair cells) that develop in the organ of Corti (Lanford et al. 1999). Since *Math1* expression is maintained only in cells that differentiate as hair cells, the number of *Math1*-positive cells that are ultimately present in *Jag2* mutant cochleae must be greater than in wildtype.

In contrast to *Math1*, both the initiation and maintenance of *HES5* transcripts is dramatically altered in cochleae from *Jag2* mutants. In normal cochleae, transcripts for *HES5* are detectable in the developing sensory epithelium by E15 and continue to be expressed through at least P0 (see above). By comparison, the initial expression of *HES5* in cochleae from *Jag2* mutant animals is markedly decreased, and the overall level of *HES5* expression remains low or undetectable through E17.5 (Fig. 5A–D). A similar downregulation of *HES5* was also present in the sensory epithelia of the semicircular canals (canal cristae) which also express *HES5* (Shailam et al. 1999). These results are consistent with the role of *HES5* as a downstream mediator of Notch signaling and support the hypothesis that

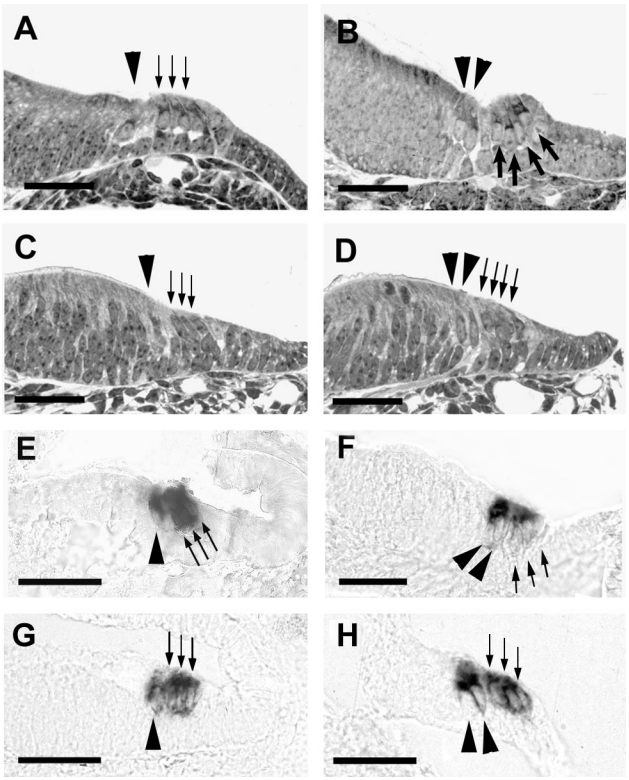

FIG. 4. Effects of deletion of *Jag2* on the development of hair cells in the organ of Corti. **A**–**D**. Morphological analysis of the apex versus the base of cochlea from E17.5 mutants demonstrates that additional developing hair cells are present throughout the epithelium at the earliest developmental time points that these cells can be identified. **A**. Cross section through the basal turn of the cochlear duct from an E17.5 wildtype animal. A single inner hair cell (arrowhead) and three outer hair cells (arrows) are present within the developing organ of Corti. **B**. Cross section through the basal turn of the cochlear duct from an E17.5 *Jag2* mutant. Two inner hair cells (arrowheads) and four outer hair cells (arrows) are present. **C**. Cross section through the apical turn of the cochlear duct from an E17.5 wildtype animal. At this stage in development, the organ of Corti in the apical turn is still relatively undifferentiated. However, the precursors of a single inner hair cell (arrowhead) and three outer hair cells (arrows) can be identified within the ventral epithelium. **D**. Cross section through the apical turn of the cochlear duct from an E17.5 *Jag2* mutant animal. Analysis of this region of the duct reveals that two inner hair cells are identifiable even at this early stage in apical development. **E**. *In situ* hybridization for *Brn 3.1* in a cross section of the basal turn of the cochlear duct from an E17.5 control cochlea (ICR strain). A single inner hair cell (arrowhead) and three outer hair cells (arrows) express *Brn 3.1*. **F**, *In situ* hybridization for *Brn 3.1* in a cross section of the basal turn of the cochlear duct from an E17.5 *Jag2* mutant cochlea. Two inner hair cells (arrowhead) and three outer hair cells (arrows) express *Brn 3.1*. **G**. *In situ* hybridization for *Math1* in a cross section of the basal turn of the cochlear duct from an E17.5 wildtype cochlea. A single inner hair cell (arrowhead) and three outer hair cells (arrows) express *Math1*. This image is a composite of two photographs of the same section, taken at slightly different planes of focus. **H** *In situ* hybridization for *Math1* in a cross section of the basal turn of the cochlear duct from an E17.5 $Jag2^{\Delta DSL}$ cochlea. Two inner hair cells (arrowhead) and three outer hair cells (arrows) express *Math1*. All scale bars equal 25 μm.

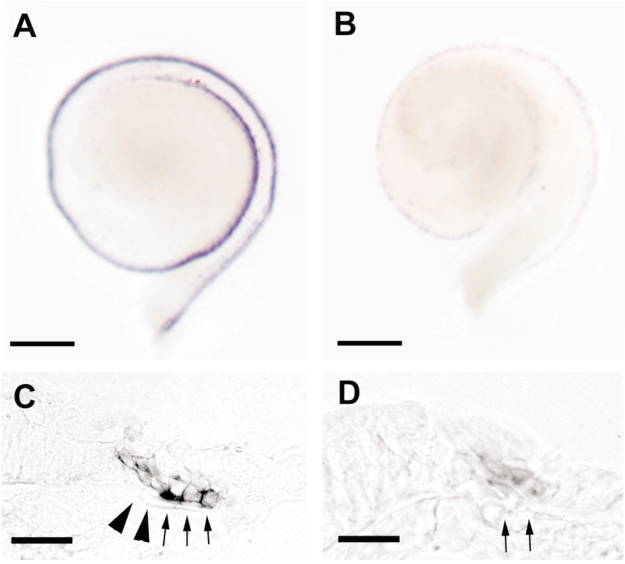

FIG. 5. Effects of deletion of *Jag2* on the expression of *HES5* in the developing organ of Corti. (**A**, **B**). Whole-mount *in situ* hybridization for *HES5* in wildtype (**A**) vs. *Jag2* mutant (**B**) cochleae at E17. In mutant cochleae, the expression of *HES5* is significantly reduced and appears diffuse. Scale bars equal 250 μm. **C**. Cross section of the basal turn of the cochlear duct from an E17.5 wildtype cochlea. Expression of *HES5* is restricted to cells located in the lower (nonhair cell) region of the developing organ of Corti. Arrows indicate three Deiter's cells; arrowheads indicate developing inner phalangeal cells. Scale bar equals 20 μm. **D**. Cross section of the basal turn of the cochlear duct from an E17.5 *Jag2* mutant cochlea. Expression of *HES5* is dramatically reduced in the sensory epithelium. Scale bar equal 20 μm.

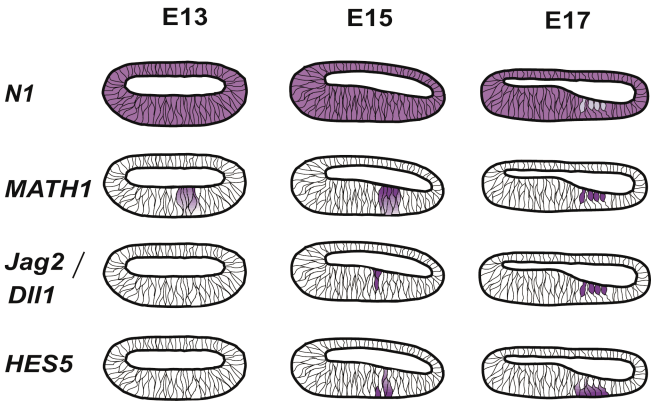

FIG. 6. Expression of proneural and neurogenic genes in the developing cochlea. Summary diagram shows the timing and relative positions of *Notch1, Math1, Jag2, Dll1*, and *HES5* in cross sections through the base of the cochlea at E13, E15, and E17. At E13, *Notch1* is expressed throughout the cochlear duct, while *Math1* is expressed only in a subset of cells within the *Notch1* domain. Transcripts for *Jag2, Dll1*, and *HES5* are not present in the epithelium at this time frame. Transcripts for the two ligands (*Jag2* and *Dll1*) begin to be expressed at E14.5 in a small subset of cells that appears to be located within the *Math1* expression domain (not shown). Transcripts for *Jag2* and *Dll1* continue to be present in the cochlear duct at E15, when *HES5* expression begins. Presumably, the expression of *HES5* is as a result of ligand-dependent *Notch1* activation. Finally, by E17, *Math1, Jag2,* and *Dll1* are localized to hair cells, while *Notch1* and *HES5* are expressed in nonsensory cells.

the deletion of *Jag2* results in a significant reduction in the activation of the Notch pathway in the developing organ of Corti.

DISCUSSION

Functional roles of proneural and neurogenic genes during cochlear development

Previous studies have demonstrated that proneural genes such as the *Drosophila* gene *ato* are initially expressed in a group of equivalent progenitor cells within the developing embryo but become progressively restricted to specific neural cell types (Akazawa et al. 1995; Jarmen et al. 1993, 1994; Ben-Arie et al. 1997; Gupta and Rodrigues 1997; Kim et al. 1997; Helms and Johnson 1998). Similarly, the expression of *Math1* in the mammalian nervous system is relatively broad initially but ultimately becomes restricted to subtypes of neurons in the dorsal neural tube and cerebellum (Ben-Arie et al. 1997; Helms and Johnson 1998). The results of the present study and others (Lanford et al. 1999; Bermingham et al. 1999) indicate that a similar restriction occurs in the developing cochlear duct. Specifically, the number of cells that maintain *Math1* expression and differentiate as hair cells is greater in *Jag2* mutant cochleae than in wildtype, suggesting that *Jag2* plays a role in limiting the number of cells that differentiate as hair cells. While this result does not provide a direct link between *Jag2* and the inhibition of *Math1*, the data are consistent with previous studies demonstrating that *Jag2* activates the Notch pathway (Shawber et al. 1996; Jiang et al. 1998) and that activation of the pathway results in the repression of proneural genes and the restriction of progenitor cells to a particular fate (Anderson and Jan 1997). It is also possible that the deletion of *Jag2* alters the expression of *Math1* via another mechanism, however, such a mechanism has not been demonstrated.

Results from the present study also suggest that transcription of *HES5* in the organ of Corti may be activated via Jag2–Notch binding. First, the spatiotemporal distribution of *HES5* transcripts is consistent with the hypothesis that binding of *Jag2* leads to activation of Notch and expression of *HES5*. Expression of *HES5* begins approximately 24 hours after the onset of *Jag2* expression in the cochlear duct, and transcripts for *HES5* are distributed in a relatively broad pattern that appears to encompass the *Jag2* expression domain. Second, the deletion of *Jag2* results in a dramatic downregulation of *HES5*. This downregulation is consistent

with previous studies demonstrating that the transcription of *HES* genes is dependent upon Notch activation (Sasai et al. 1992; Jarriault et al. 1995, 1998; Hsieh et al. 1997; Nishimura et al. 1998; Ohtsuka et al. 1999). Low levels of *HES5* expression are present in *Jag2* mutant cochleae; however, it is possible that this residual expression may be the result of activation via a second ligand, (e.g., *Delta1*; Morrison et al. 1999), or the activation of *HES5* via proneural genes (Singson et al. 1994; Nellesen et al. 1999). This low level of activity may be responsible for the maintained development of supporting cells in *Jag2* mutant cochleae (Lanford et al. 1999).

The manner in which the inhibitory proteins encoded by *HES5* interact with *Math1* is uncertain. A specific molecular relationship between *HES5* and *Math1* has not been demonstrated. However, Akazawa at al. (1995) presented evidence that indicates that the bHLH proteins encoded by *Math1* mediate E-box-dependent transcription through binding with a second bHLH protein, E47. That study also showed that the transcriptional activity of Math1 proteins is completely antagonized by the presence of bHLH proteins encoded by another *HES* gene, *HES1* (Tomita et al. 1996; Ohtsuka et al. 1999). In a separate study, HES5 proteins were shown to also form nonfunctional heterodimers with E47 and to repress E47-induced transcription (Akazawa et al. 1992). Consequently, expression of HES5 proteins may downregulate the activity of Math1 proteins by competitively binding with E47.

Interestingly, the complementary patterns of *HES5* vs. *Math1* expression in the later stages of cochlear development (e.g., E17) suggest that *HES5* may also regulate the transcription of the *Math1* gene itself. DNA footprinting analysis has shown that HES5 proteins bind to the CACNAG consensus sequence (N-box) and, therefore, may be capable of repressing transcription directly (Akazawa et al. 1995). However, it is not known whether the *Math1* promoter contains an N-box, or whether binding of HES5 is sufficient to downregulate the expression of *Math1*. Consequently, the function of HES5 as are pressor of *Math1* transcription remains speculative.

Role of proneural and neurogenic genes in the development of the organ of Corti; summary and model

Figure 6 provides a summary of neurogenic and proneural gene expression in the developing cochlea based on the results presented here and in a number of other studies. *Notch1* is expressed throughout the cochlear duct during the time frame in which cochlear progenitor cells become committed to sensory vs. nonsensory cell fates (E12–E17) (Lindsell et al. 1996; Lewis et al. 1998; Lanford et al. 1999). Beginning on E13, transcripts for *Math1* can be detected within the ventral floor of the cochlear duct, in a band of expression near the center of the epithelium. Analysis of later time points (E15, E17) indicates that the band of expression of *Math1* correlates with the developing sensory region of the duct. Beginning on E14, transcripts for *Jag2* and *Dll1* can be detected in the base of the cochlea in a band of cells only 1–2 cell diameters in width (Lanford et al. 1999; Morrison et al. 1999). The position of these cells within the epithelium appears to be coincident with the neural edge of the *Math1* expression domain. Expression of these ligands is followed closely by the expression of *HES5* transcripts at E15, which also appears to be coincident with the domain of *Math1* expression. In addition, the *HES5* expression band appears to be subdivided by interceding *HES5*-negative cells. Finally, as the sensory epithelium matures through E17, transcripts for *Jag2*, *Dll1*, and *Math1* are localized to hair cells within the organ of Corti, while transcripts for *Notch1* and *HES5* are restricted to supporting cells.

Based on the combined results of this and other studies, we propose the following working model for the molecular interactions that play a role in the development of the cochlear mosaic. Initially, as a result of the expression of *Math1*, a subset of cells within the cochlear duct become competent to develop as hair cells. Next, a subset of these cells begin to upregulate expression of the Notch ligands, *Jag2* and *Dll1* (*Jag2/Dll1*). Expression of these ligands results in the activation of Notch in adjacent cells and the upregulation of *HES5*. Ultimately, expression of *HES5* leads to the repression of *Math1* and the diversion of progenitors from the hair cell fate.

The mechanisms that regulate the expression of many of these genes within individual cells are still uncertain. In particular, it is not clear how the expression of *Jag2/Dll1* is restricted to a subset of *Math1*-positive progenitor cells. One possible explanation might be that expression of these ligands is dependent upon a critical level of *Math1*. Therefore, as the level of *Math1* increases within a single cell, that cell would become competent to express *Jag2/Dll1*. Since the expression of *Math1* occurs in a gradient along the basal-to-apical axis (and perhaps along the neural-to-abneural axis as well) the number of cells that become competent to express *Jag2/Dll1* at any given moment in time will be extremely limited. In addition, once an individual cell becomes competent to express these ligands, that cell would then activate Notch in its immediate neighbors, thus greatly reducing the number of cells that ultimately express *Jag2/Dll1*.

Clearly, further studies are necessary to clarify the

role of *Math1* in the development of the sensory epithelium, as well as additional aspects of Notch signaling in this system. For example, while deletion of *Jag2* results in the downregulation of *HES5* expression and an increased production of hair cells in the epithelium, it is not clear that expression of *HES5* alone would be sufficient to eliminate the overproduction of hair cells. In addition, deletion of *Jag2* does not result in a significant disruption of the cellular pattern within the organ of Corti, suggesting that other factors contribute to the formation of the cellular mosaic. Clearly, the roles of other Notch ligands, such as *Dll1*, and other *HES*-related genes, such as *HES1* and the recently cloned *Hey1*, *HESr1*, and *Hey2* (Tomita et al. 1996; Kokubo et al. 1999; Leimeister et al. 1999), should be investigated to determine the role that these factors might play in Notch signaling during cochlear development. In addition, other factors unrelated to the Notch pathway probably play a role in the specification of cell types in the sensory epithelium.

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