Molecular Neurobiology

ISSN0893-7648/03/27(2): 223–237/$20.00

Molecular Mechanisms that Regulate Auditory Hair-Cell Differentiation in the Mammalian Cochlea

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Abstract

Mechanosensory hair cells of the vertebrate cochlea offer an excellent developmental system to study cell-fate specification, and to gain insight into the many human neurological deficits which result in a hearing loss, by affecting primarily the hair cells. Therefore, there is great interest in studying the molecular mechanisms that regulate their specification and differentiation.

Recent studies, based mostly on loss-of-function experiments that target the role of Notch signaling and basic helix-loop-helix genes in inner-ear development have indicated that they can regulate mechanosensory hair cell-fate specification and their initial differentiation.

Index Entries: Hair cell; supporting cell; inner ear; cochlea; organ of Corti; cell-fate specification; differentiation; Notch signaling; bHLH transcription factor.

Introduction

The mammalian inner ear contains two sensory organs, the cochlea and the vestibule, which are responsible for hearing and balance respectively. Both the auditory and vestibular sense organs develop collectively from a thickening of the epithelial sheet in the dorsolateral region of the head known as the otic placode *(1,2)*. The sensory epithelium of the cochlea, the organ of Corti, comprises a highly ordered cellular mosaic of sensory hair cells and supporting cells, arranged into four highly organized rows. In the mouse organ of Corti, there are a single row of inner hair cells (IHCs) and three parallel rows of outer hair cells (OHCs) extending along the entire length of the cochlear duct. Each hair cell is separated from neighboring hair cells by projections from the underlying supporting cells (e.g., Deiters's cells). During early inner-ear development, the generation of the sensory epithelia requires coordination between the processes of morphogenesis and cell-fate specification *(3–5)*.

Received September 16, 2002; Accepted December 4, 2002.
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Morphogenesis will transform an epithelial sheet, the otic placode at the side of the head into a hollow sphere, the otic vesicle or otocyst. Cell-fate specification will produce several inner-ear specific cell phenotypes, i.e., hair cell, supporting cell, and neuron through a series of cell-fate decisions that follow a precise and tightly regulated series of developmental events. How these events are triggered and regulated developmentally, and how these cell types are specified and committed to particular phenotypes from a common cell progenitor remains an important developmental question in the field of inner ear neurobiology.

In this review, I summarize recent progress in elucidating the involvement of Notch signaling and members of basic helix-loop-helix (bHLH) transcription factors in the molecular control of cell-fate specification and differentiation within the developing organ of Corti, especially the hair and supporting cell phenotypes. A number of excellent reviews have recently covered this subject from different points of view, i.e., to explain the regional compartmentalization of inner ear sensory receptor development *(6–9).* The reader is referred to these articles for information on topics outside the scope of this review.

Cell Cycle and Cellular Patterning

It is becoming increasingly clear that within numerous sensory system models, there are complex molecular mechanisms at several levels involving cross-talk between molecules that regulate the cell cycle and those that promote cell-fate determination *(10,11).* For instance, cyclin-dependent kinases (CdKs) can influence developmental processes beyond their prescribed role in cell-proliferation control. A recent study *(12)* has suggested that FGF signaling through fibroblast growth factor receptor1 (Fgfr1) is required to stimulate proliferation of progenitor cells that will give rise to the mouse auditory sensory epithelium based on inducible, Cre-LoxP-mediated gene targeting of Fgfr. Thymidine incorporation experiments have shown that, in the embryonic mouse cochlea, progenitors of hair cells and supporting cells enter terminal mitoses between E12 and E14 of development in a highly regulated apex-to-base gradient *(13).* The majority of progenitor cells having exited the cell cycle on E12 are located in the apex, while those having exited the cell cycle on E14 are located in the base. The molecular factors that play a role in this highly coordinated exit of cochlear progenitor cells from the cell cycle are largely unknown. Two studies have identified Cdk inhibitor $p27^{Kip1}$, an inhibitor of cell-cycle progression, as a major factor involved in the timing of cell-cycle arrest of cochlear progenitor cells *(14,15).* Between E12 and E14, $p27^{Kip1}$ expression exclusively marked the region of the presumptive sensory epithelium that will develop as the organ of Corti *(14,16).* The absence of $p27^{Kip1}$ expression in cells located outside this organ of Corti presumptive region suggests that different molecular factors may regulate cell-cycle arrest in different regions of the otocyst.

Recent studies suggest the existence of certain parallels between the molecular mechanisms that regulate the cellular patterning of the mechanosensory organs of *Drosophila,* i.e., chordotonal organs and vertebrate inner ear *(17,18).* In the first stage of *Drosophila* sense-organ development, clusters of progenitor cells are specified by the expression of proneural genes. These genes are bHLH transcription factors such as those encoded by *achaete-scute* complex or *atonal.* In the second stage, the sense-organ precursor cell is selected from the cluster and accumulates high levels of proneural protein, which activates the transcription program necessary for sense-organ development. The upregulation of proneural genes in the sense-organ precursor cell and their downregulation in neighboring cells is mediated by the Notch-signaling pathway. Essential components of this pathway are integral membrane proteins, i.e., the *Notch* and its ligands *Delta/Serrate:* Notch transmits a lateral inhibitory signal by binding to *Delta/Serrate,* resulting in a downregulation of proneural genes in Notch-expressing cells. Subsequently,

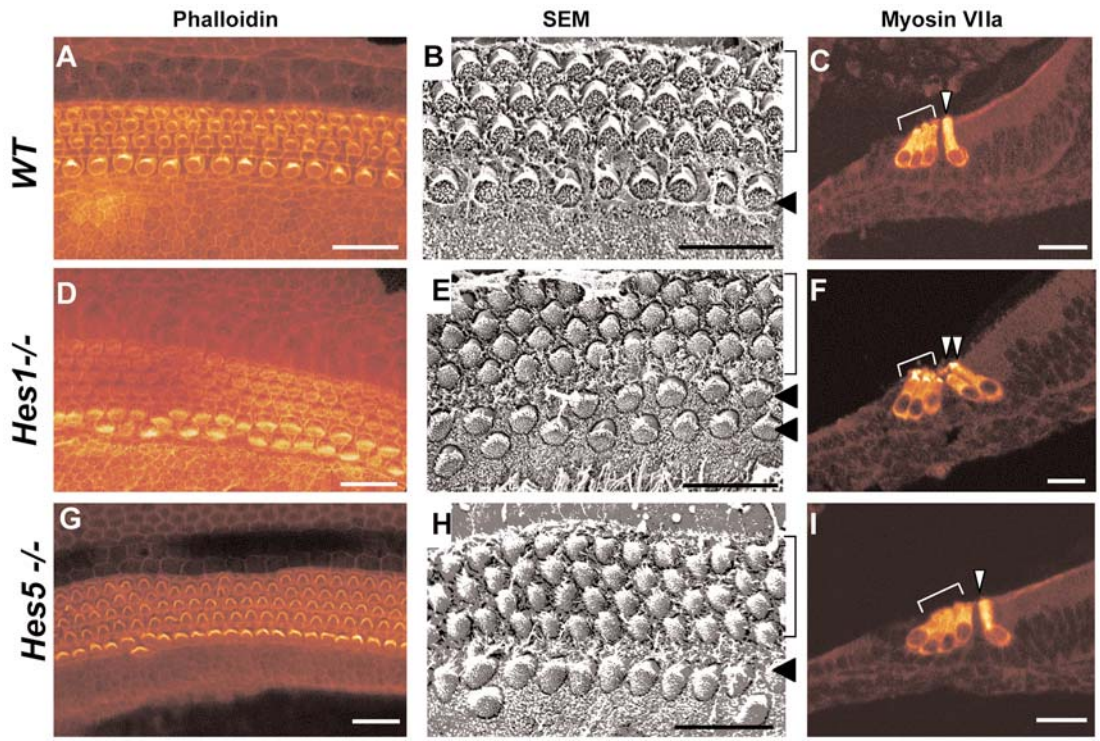

Fig. 1. **(A,B)** Confocal images of phalloidin-stained rat E18 cochlear explants maintained 5 d in vitro in normal medium **(A)** or in medium supplemented with AS-*Jagged1* **(B).** In control untreated explant culture, there is a characteristic single row of IHCs (arrows), and three rows of OHCs (numbered) are present. In AS-*Jagged1* treated explant culture, hair cells are densely packed together and are arranged in two rows of IHCs and six to eight rows of OHCs. *(C,D)* Scanning electron micrographs of E18 cochlear explants maintained 5 d in vitro in either normal medium **(C)** or treated with AS-*Jagged1* **(D).** In control culture, hair cells are arranged in three orderly rows for OHCs and a single row for IHCs, showing uniform orientation of their stereocilia. In AS-*Jagged1* treated explant culture, the regular organization has been lost due to the addition of many extra OHCs. The regular polarity of hair cell stereociliary bundles one to another has been disrupted (arrowheads). Some hair cells are hidden by the regrowth of a tectorial membrane (TM). Scale Bar, 20 μm **(A,B),** 10 μm **(C,D)**.

Notch signaling is used for cell-fate determination leading from a sense-organ precursor cell to the different cell types of the sense organs *(19,20).* The expression of a similar transcription factor family and signaling molecules early during the development of sensory epithelia within the vertebrate inner ear leads to the hypothesis that the cellular patterning of the mammalian mechanosensory hair cells may share similarities with *Drosophila* sense-organ patterning stages. The *atonal* homolog *Math1 (21–24)* and several components of Notch-signaling pathway, in particular, *Notch1* and its ligands *Delta1, Jagged1* and *Jagged2* are expressed within the developing inner ear *(25–29).* Further support comes from the observation that null mutations in components of Notch signaling (Figs. 1–3) lead to perturbation in the cellular patterning of mouse cochlear sensory epithelium *(27,29,30).*

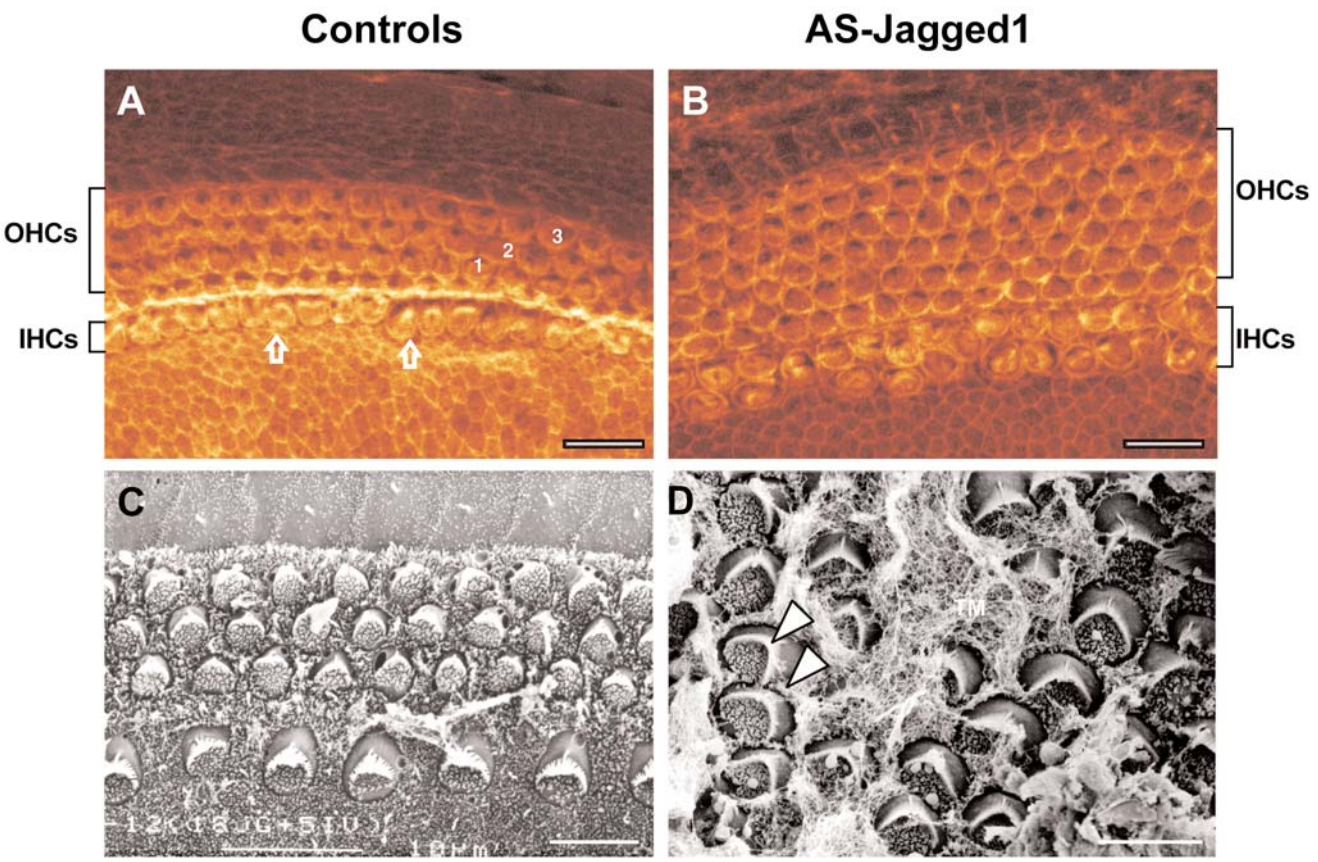

Fig. 2. Hair-cell development in newborn cochleae from wild-type control **(A–C),** *Hes1*$^{-/-}$ **(D–F)** and *Hes5*$^{-/-}$ **(G–I)** mutant mice. **A,D,G)** Confocal images of surface preparations stained with rhodamine phalloidin to visualize the actin-rich stereocilia of the hair cells. **(B,E,H)** Scanning electron microscopy (SEM) of the surface of the organ of Corti in the mid-cochlear turn. **(C,F,I)** Cross-sections through the organ of Corti in the mid-modiolar region immunostained with antibody anti-myosin VIIa. Lack of *Hes1* principally causes the development of supernumerary IHCs. In control cochlea, the normal pattern is well defined, single row of IHCs and three rows of OHCs. In contrast, in *Hes1*$^{-/-}$ cochleae, two rows of IHCs (arrowheads) and three to four rows of OHCs (brackets) are present. A deletion of *Hes5* principally induces the development of region with four rows of OHCs (brackets) instead of three rows along the sensory epithelium; although few IHC pairs are also present. Scale bars: **A,C,D,F,G,I,** 20 μm; **B,E,H** 10 μm. (Copyright 2001, the Society for Neuroscience.)

Specification of the Prosensory Progenitor Domain

The mechanisms coordinating cell-cycle exit and the establishment of the presumptive sensory epithelium within the cochlear duct remain largely unknown. Possible relationships may exist between the specification of prosensory domain and the cell-cycle arrest components, i.e., $p27^{Kip1}$ due to the overlap between the onset of $p27^{Kip1}$ expression and the establishment of this prosensory domain during development *(14,16).* In the embryonic vertebrate nervous system, the crucial step of cell-division arrest has been proposed as a mechanism to insulate already specified prog-

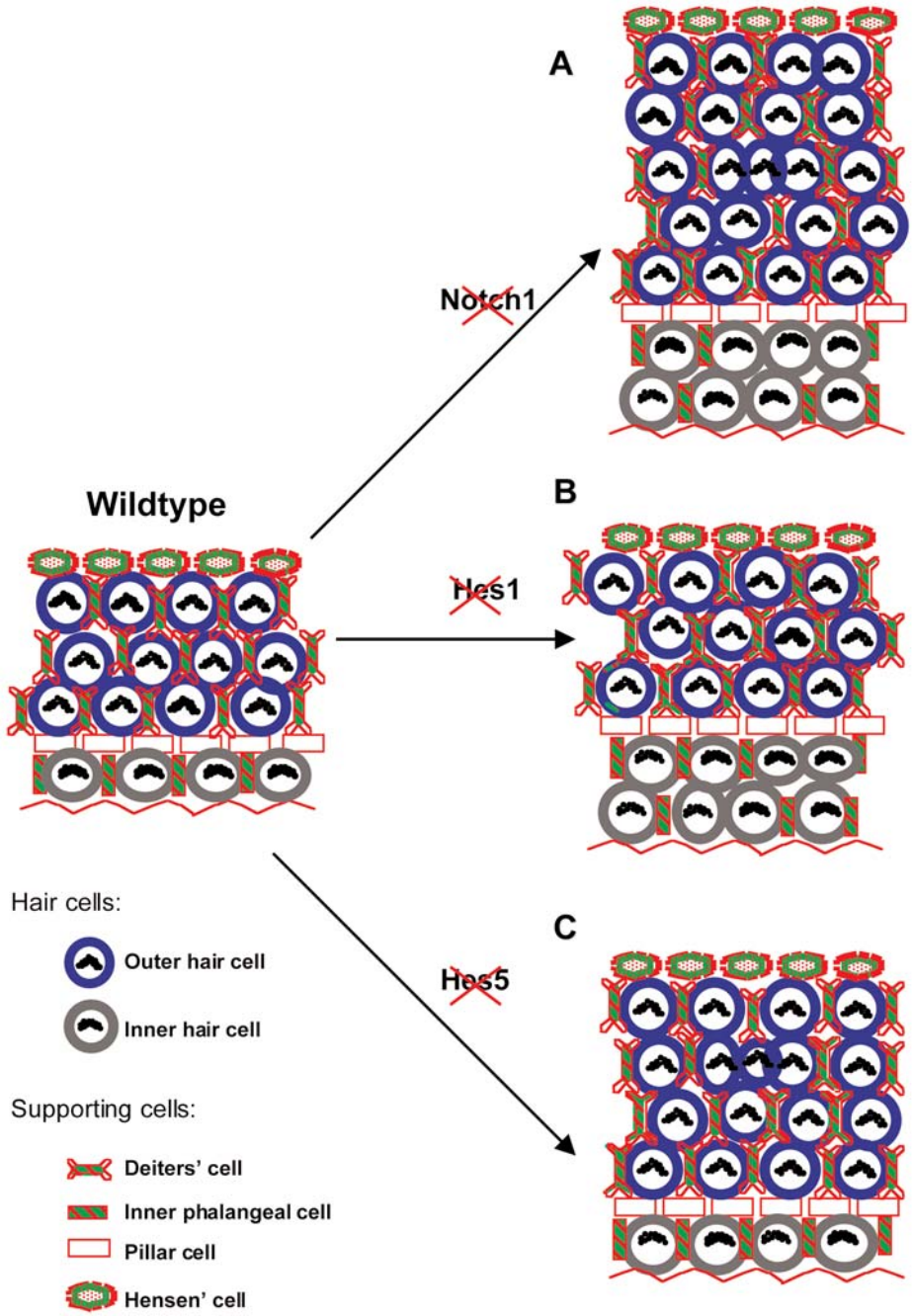

Fig. 3. Schematic diagrams for comparison of the loss-of-function of *Notch1* and *Hes1/Hes5* genes on the cellular arrangement of the developing mammalian organ of Corti. Along its length, the organ of Corti is comprised of a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). Each inner hair cell is separated from adjacent IHCs by a single inner phalangeal cell, and each outer hair cell is separated from adjacent OHCs by Deiters's cells. IHC and OHC regions are separated by a single row of pillar cells. In addition OHC region is laterally delimited by Hensen's cells. **(A)** Phenotype that resulted from antisense-*Notch1* transfected explant cultures. Both IHCs and OHCs are affected by this treatment. Two rows of IHCs instead of one and four to six rows of OHCs instead of three are generated within the sensory epithelium. Some regions displayed hair cells that appear to contact each other without any separation by supporting cells. **(B)** Phenotype that resulted from targeted deletion of *Hes1.* The IHCs are the principal type of sensory hair cells that were affected. They are organized in two rows instead of one. Few IHCs are observed to contact with each other without interposed supporting cells. **(C)** Phenotype that resulted from targeted deletion of *Hes5.* The OHCs are the principal type of sensory hair cells that were affected and found to be often organized in four rows of OHCs instead of three. Few OHCs are in direct contact with each other without any separation by supporting cells.

enitor cells from the influence of extrinsic-fate determining cues *(31).* Numerous lines of evidence indicate that proneural genes not only determine fate of cell progenitors, but may also promote the arrest of their cell division and are therefore involved in coupling these two crucial processes to coordinate cellular patterning *(10).*

The demonstration that *Math1* is necessary and sufficient for the determination of progenitor cells to differentiate into sensory hair cells within the inner ear *(21,32)* made it a logical candidate for playing a similar proneural function as its counterpart *Drosophila atonal.* However, unlike *atonal,* the *Math1* gene doesn't have a true proneural function within the developing sensory epithelium, because its targeted deletion only results in sensory-hair cell loss, but maintains supporting cells *(21),* while mutations in *atonal* result in the loss of the entire cell lineage within the chordotonal organs, due to a failure in progenitor-cell specification *(33,34).* These observations suggest that the ability of *Drosophila atonal* to couple progenitor-cell specification with sensory organ identity is not entirely conserved *(35).* In addition, the establishment of cell types in the inner ear requires supplementary bHLH genes such as the *Ngn1* and *NeuroD*, which are essential for the development of the cochleovestibular ganglion neurons *(36–38). Ngn1* and *NeuroD* are also *Drosophila atonal*-related bHLH factors expressed in sensory neuron progenitors in the peripheral nervous system *(39,40).* The inner ear of *Ngn1* and *NeuroD* knockout mice lack all sensory neurons with morphologically normal hair cells confirming previous in vitro studies *(1,41,42)* and neurotrophin/neurotrophin receptor mutants *(43)* showing that embryonic development of hair cells is independent of innervation. Likewise *Math1, Ngn1* mimicked only partly the proneural function of *Drosophila Atonal.* The inner ear of *Ngn1* null mutants shows a loss of all sensory neurons, with some hair cells located in smaller areas of sensory epithelia *(36).* These data suggest a possible interaction between proneural cells that form sensory neurons and clones that give rise to hair cells and supporting cells. However, other possible interaction cannot be discarded and a direct labeling approach of viral injection into the mouse otocyst in needed to prove that hair cells and neurons have a clonal relationship. Either as yet to be specified proneural gene and/or *Ngn1* could play the role to induce neuroepithelial commitment to the regions of the otocyst that will give rise to the prosensory progenitor domain in which *Math1* will mediate hair-cell differentiation. However, Chen et al., 2002 *(16)* confirmed that *Math1* is not required for the specification of prosensory progenitor domain, but instead acts in terminal cell-fate specification and/or initial differentiation of sensory hair cells. By analyzing the pattern of BrdU incorporation and *Math1*/EGFP transgene expression within the embryonic cochlea, these authors observed that *Math1* is only expressed after the emergence of a zone of nondividing cells between E12.5 and E13.5 that delineates the area of presumptive sensory epithelium. This also suggests that *Math1* is not implicated in the cell-cycle withdrawal of cochlear progenitor cells and the possibility of the existence of other yet to be identified genes with proneural activity in the inner ear sensory epithelia.

Notch signaling through its ligand *Jagged1* has also been suggested to play an earlier role within the inner ear *(25,26).* Expression studies reported *Notch1* and *Jagged1* expression throughout the cochlear duct during embryonic development as early as E12.5 suggesting that Notch signaling could play a role in specifying prosensory domain *(16,24,26,28).* More data are needed on the detailed spatiotemporal expression of *Notch1* and *Jagged1* within the embryonic cochlea in order to support the role of Notch signaling in specifying prosensory domain. Recently, two functional studies provided evidence suggesting that *Jagged1* may play a dual role in the specification of the prosensory progenitor domain within the embryonic cochlea, in addition to its role in the selection between individual progenitor-cell fate (*See* below: cell-fate specification in the organ of Corti). In the dominant mouse mutants *slalom (44)* and *headturner (45),* missense mutations in *Jagged1* lead to

perturbation of the cellular patterning of the organ of Corti characterized by reduced numbers of outer hair cells and a loss of sensory structures in the vestibular system. In addition, the expression of a Notch modulator, i.e., *lunatic fringe (Lfng)* a member of a family of molecules known to be involved in boundary specification in many developmental processes *(46,47),* is also expressed in domains similar to that of *Jagged1 (45),* lending further support to the concept that Notch signaling is involved in boundary formation of the prosensory progenitor domain within the mammalian inner ear. Overall, these results support the role for Notch1 signaling through *Jagged1* in specifying the presumptive sensory epithelium within the embryonic mouse cochlea (Fig. 4). It is possible that this role of the *Jagged1-Notch1* pathway in regulating cell-cycle exit may be accomplished at least in part through the regulation of cell-cycle withdrawal components, i.e., $p27^{Kip1}$. However, Notch is basically known to inhibit differentiation of many systems in vertebrates *(20,49),* but in certain cases such as that of the imaginal wing disk of *Drosophila,* Notch activation causes cell-cycle withdrawal *(50).* However, the role that could play the Notch-signaling pathway in specifying the prosensory progenitor domain in addition to its possible interaction with cell-cycle withdrawal components remains to be directly tested in the case of the developing vertebrate inner ear.

Cell-Fate Specification in the Organ of Corti

Once the specification of the sensory domains within the otic epithelium have been accomplished, the next step is the commitment of sensory progenitor cells to either hair-cell or supporting-cell fates.

Hair-Cell Fate

Our understanding of cell-fate specification in the sensory receptors of the inner ear has advanced considerably in recent years. In vertebrates, retroviral labeling of the embryonic otocyst has identified a common precursor cell for both hair cells and supporting cells *(51,52),* but it is not clear whether this direct lineage relationship extends to the neurons. The molecular mechanisms involved in controlling the pattern of sensory hair cell and supporting cell determination is just beginning to be understood, although there are still several issues that remain to be resolved.

Studies from several laboratories (Table 1) have demonstrated the participation of the Notch-signaling pathway and members of bHLH transcription factors in the molecular basis for both initial selection of hair-cell progenitors and for the subsequent inhibitory interactions that prevent adjacent progenitor cells from developing as hair cells. *Notch1* and its ligands *Delta1, Jagged1* and *Jagged2* are expressed in distinct patterns within the developing inner ear *(22,26,27–29). Notch1* is expressed throughout the ventral wall of the developing cochlea and later become restricted to supporting cells. *Delta1* and *Jagged2* are subsequently expressed in nascent hair cells. Interference with Notch signaling (Fig. 1; Table 1) leads to the production of extra hair cells in zebrafish and in mice *(25,27,30,53,54).* However, unlike zebrafish, in the mouse inner ear, perturbation experiments did not result in the conversion of all cochlear progenitor cells to hair cells, as would be expected in the case of disrupting lateral inhibitory interactions mediated by the *Notch1.* This is probably due to the expression of a variety of *Notch1* ligands (i.e., *Delta1, Jagged1,* and *Jagged2*), of Notch modulators such as *Numb* and *Lnfg (18,48),* or to the presence of another yet unidentified Notch member that might respond selectively to different ligands and exert different downstream effects. Indeed, it has been shown that Notch3 may act as an antagonist for Notch1 activation *(55).*

Recent studies have provided strong evidences that the molecular mechanism for the commitment of progenitor cells to the hair-cell phenotype is positively driven by the induction of the expression of *Math1,* a bHLH transcription factor *(21,32).* Its expression initiates

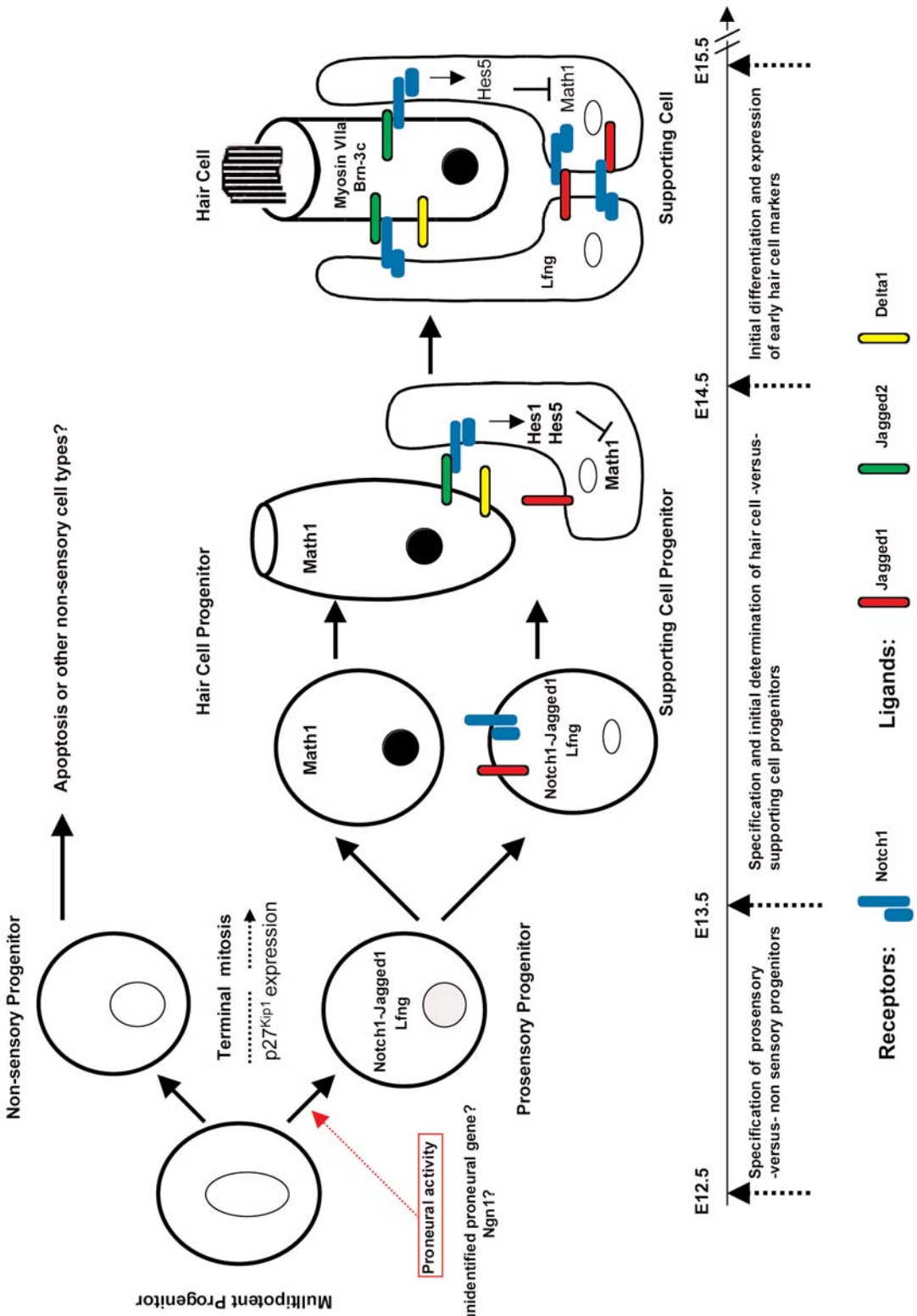
Multipotent Progenitor
Proneural activity
unidentified proneural gene?
Ngn1?
Non-sensory Progenitor
Apoptosis or other non-sensory cell types?
Terminal mitosis
p27Kip1 expression
Prosensory Progenitor
Notch1-Jagged1
Lfng
Hair Cell Progenitor
Math1
Supporting Cell Progenitor
Notch1-Jagged1
Lfng
Hes1
Hes5
Math1
Hair Cell
Myosin VIIa
Brn-3c
Supporting Cell
Hes5
Math1
Lfng
E12.5
E13.5
E14.5
E15.5
Specification of prosensory -versus- non sensory progenitors
Specification and initial determination of hair cell -versus- supporting cell progenitors
Initial differentiation and expression of early hair cell markers
Receptors:
Notch1
Ligands:
Jagged1
Jagged2
Delta1

Fig. 4. *(opposite page)* The possible developmental gene cascades underlying cell-fate decisions in the developing mammalian organ of Corti based on the approximate time at which they are initiated. The figure focuses on the interactions between these genes and extrinsic cues that participate in promoting either hair-cell or supporting-cell fates. Based on analogy with *Drosophila* sense-organ development and given that *Math1* is not a true proneural gene within the mouse embryonic cochlea, an unspecified proneural gene might exist and could play a role in specifying the prosensory progenitor domain by upregulating the entire genes cascades shown here. *Ngn1,* a bHLH gene expressed early in the otocyst could not be totally responsible for the generation of the prosensory domain that will give rise to hair cells and supporting cells, but may participate. This suggestion stems from the fact *Ngn1,* in addition to being essential for sensory neuron development, also shows severe reduction of hair-cell formation in loss-of-function mutants. The combined expression of components of Notch signaling such *Jagged1, Notch1,* and *Lfng* defines the hair-cell generating region molecularly based on their early overlapping spatio-temporal expression within the embryonic cochlear duct. After the establishment of prosensory hair-cell progenitor specification, *Math1* upregulation in a subset of specified hair-cell precursors drives those to differentiate a hair-cell phenotype. At the same time, through a process of local cell–cell interaction, Notch1 signaling upregulates the expression of *Hes1* and *Hes5* genes that leads to the inhibition of *Math1* expression in some of those hair-cell progenitor cells. Because *Math1* is necessary for the differentiation of progenitor cells to hair cells, the inhibition of *Math1* in those progenitor cells diverts them from adopting hair-cell fate and allow them to be committed to supporting cell fate or to return to progenitor cells pool. As differentiation program proceeds, myosin VIIa and Brn3c, two early markers of hair-cell differentiation appear. As supporting cells also contact one another, reciprocal interactions between *Notch1/Jag1* may contribute to keeping them in a state of high-Notch activation that could be reinforced by inhibitory signals from the differentiating hair cells that express both *Jag2* and *Dll1* ligands.

Table 1
Summary of *Notch* and bHLH Genes Affecting Cellular Patterning of the Organ of Corti

Gene	Experiments	Effects on cell fate				Refs.
		IHCs	OHCs	Total HCs	SN	
Notch1	Antisense treatment	(+)	(+)	(+)	nd	*(29)*
Notch1	Heterozygote mutants	nc	(+)	nd	nd	*(30)*
Jag1	Missense mutation					
	Slalom mutant	(+)	–	nd	nd	*(44)*
	Headturner mutant	(+)	–	nd	nd	*(45)*
Jag2	Targeted deletion	(+)	+	(+)	nd	(27)
Jag2/Lnfg	Double knockout	nc	+	nd	nd	*(30)*
DeltaA (zebrafish)	Mutation *(mind bomb)*			(+)	(+)	*(53,54)*
Math1	Targeted deletion	0	0	0	nc	*(21)*
Math1	Ectopic overexpression			+	nc	*(32)*
Hes1	Targeted deletion	(+)	+	(+)	nd	*(58,59)*
Hes5	Targeted deletion	+	(+)	(+)	nd	*(59)*
Ngn1	Targeted deletion	–	–	–	0	*(36)*
NeuroD	Targeted deletion	–	–	–	–	*(37,38)*

(+) significant increase; + weak increase; – significant decrease; 0 total absence; nc no change; nd not determined. IHCs: inner hair cells; OHCs: outer hair cells; SN: sensory neurons.

near the base of the mouse cochlear duct between E13.5 and E14.5 in a limited group of progenitor cells within the sensory domain already specified. The expression of this gene acts to initiate a determinative program that will lead, as differentiation proceeds (E15.5), to the expression of early hair-cell markers such as myosin VI and myosin VIIa. Bermingham et al., *(21)* demonstrated that *Math1* is expressed in the region of the cochlear duct that will develop as the organ of Corti, and that a targeted deletion of the *Math1* gene leads to a failure of hair-cell differentiation. When *Math1* was ectopically overexpressed in rat cochlear duct explants in vitro, certain nonsensory cells within the inner sulcus region adopted a hair-cell fate *(32).* Moreover, two members of a second class of bHLH transcription factors (*Hes1* and *Hes5*), which are downstream effectors of Notch *(56,57),* are also expressed in the supporting cells and in the nonsensory cell types surrounding the organ of Corti *(58,59).* Loss-of-function studies involving *Hes1* and *Hes5* have indicated that these transcription factors have separate and overlapping roles in repressing the commitment of progenitor cells to hair-cell fates *(58,59).* Deletion of *Hes1* and *Hes5* genes leads to a significant increase in the number of IHCs and OHCs respectively (Fig. 2). In the same lines of evidence, Zheng et al., *(58)* showed that transfection of *Hes1*-expressing plasmids in rat cochlear organotypic cultures prevented hair-cell differentiation induced by the ectopic expression of *Math1.* However, this set of experimental data also revealed that the competence to differentiate into hair cells is unlikely to be limited to the prosensory progenitor area since extra hair cells have been observed to develop outside this region, i.e., in the cells of the greater epithelial ridge *(32,59).* The current model for hair-cell-fate specification in the presumptive sensory epithelium holds to both a positive signal inducing *Math1* upregulation in a subset of sensory progenitor cells and Notch signaling that limits the expression of *Math1* in the surrounding progenitor cells.

Supporting Cell Fate

In contrast to the advances recently made concerning the genes that play a role in the determination of progenitor cells to become hair cells, considerably less is known about the factors implicated in the cellular commitment to become one of a variety of supporting cells within the developing organ of Corti. It has recently been demonstrated that the determination of cochlear progenitors as hair cells is mediated through positive stimuli inducing *Math1* expression and, subsequent cell–cell interactions mediated through the Notch-signaling pathway that lead to the expression of *Hes5* and the inhibition of the expression of *Math1* in the surrounding progenitor cells committed to a supporting cell fate *(24).* In addition, *Hes5* expression has been reported to predominate only in the supporting cells in and around the outer hair-cell region *(58,59)* suggesting its possible role in supporting cell determination. Indeed, targeted deletion of *Hes5* principally leads to the generation of supernumerary outer-hair cells *(59).* Moreover, the expression of *Hes5* is downregulated in the supporting cells of mice that have a targeted deletion of *Jagged2,* probably acting through the decrease of Notch activation *(24).* It remains, however, to be experimentally tested whether negative control of *Math1* expression through the *Notch/Hes* pathway occurs during normal development to specify supporting cell fate. Recent studies suggest that *Jagged1* ligand may have a dual role in the mouse embryonic inner ear: an early role, in which *Jagged1* participates in the specification of the prosensory progenitor domain, and a later role in lateral inhibition probably through an increase of Notch activation in the supporting cells *(18).* Indeed, *Jagged1* expression in the mouse inner ear suggests the possibility of these two roles because it changes from being expressed throughout the presumptive sensory region in early development to restricted expression in the supporting cells during hair-cell determination/initial differentiation where it colocalizes with *Notch1 (25,28,29).* In a loss-of-function

approach (Figs.1–3), we transfected cultured rat organ of Corti around the time of differentiation (E16-P3), with antisense oligonucleotides directed against the mRNA for the *Notch1* and *Jagged1 (29).* In these antisense experiments, treatment with either of these oligos induced ectopic differentiation of many rows of extra hair cells. Extra hair cells arose right next to the normal rows of hair cells, and in some cases these might have originated from a conversion of the differentiating supporting cells (i.e., Deiters's cells) that normally separate a hair cell from its neighboring hair cells. The supernumerary hair-cell response was best in explant cultures transfected with *Notch1* oligos indicating the presence of other ligands operating within the developing sensory epithelium that may activate the same receptor, Notch1 to regulate hair-cell differentiation.

On the other hand, it has been suggested, based on the phenotype of mutant mice with targeted deletion of *Jagged2 (27)* and with missense mutations in *Jagged1 (44,45)* genes, that the combinatorial interactions between these two *Notch1* ligands participate to tilt the balance toward either a hair-cell or a supporting-cell fate. However, clear evidence is still lacking whether there are extra supporting cells in the *Jagged1* mutant or reduced number of supporting cells in *Jagged2* mutant mice, perhaps because counting different supporting cell subtypes accurately is difficult and no specific molecular markers for these cells are currently available. Therefore, it is possible that other members of the Notch-signaling pathway could also participate in the control of the specification of the hair cells and supporting cells. In addition, almost nothing is known concerning the specification and the commitment of sensory progenitors to different supporting cell subtypes such as Hensen cell, pillar cell, inner phaleangeal cell phenotypes (Fig. 3). The only gene that has been implicated in the development of cells such as the pillar cells is the Fibroblast growth factor receptor 3 (Fgfr3). Its targeted deletion causes a failure of pillar-cell development because of a defect in either cell-fate determination or differentiation *(60).*

Differentiation of the Organ of Corti

Despite the recent advances in our understanding of the molecular mechanisms governing early development of inner-ear hair cells, very little is known about the molecular bases required for their terminal differentiation, maintenance, and survival. Cellular commitment and differentiation are usually tightly linked because hair-cell differentiation within the organ of Corti proceeds as waves along the base-to-apex axis, as well as along a medial-to-lateral axis *(61,62).* Once inner-ear hair cells have been specified, their terminal differentiation requires the POU domain transcription factor gene *Brn3c (Brn3.1/Pou4f3).* The *Brn3c* gene was found to be fundamental for maturation and survival for cochlear and vestibular hair cells, but not for either the proliferation or the commitment of their progenitor cells to a hair-cell fate *(63–65).* In rat cochlear organotypic cultures, ectopic expression of *Brn3c* was not able to induce the production of supernumerary hair cells *(32).* In the human, a mutation in the *Brn3c*-coding domain has been linked to autosomal dominant progressive hearing loss *(66).* Another gene that has recently been shown to play a role in the differentiation of hair cells is *Barhl1,* a mouse homolog of *Drosophila BarH* homeobox genes. Targeted disruption of this gene caused a progressive degeneration with aging of both IHCs and OHCs within the organ of Corti. In the absence of *Barhl1* gene, cochlear hair cells degenerate over a time of many months, suggesting a role for *Barhl1* in the long-term survival of these sensory cells *(67).* Hair-cell differentiation is also altered by mutations in unconventional myosin genes. In particular, hair-cell stereocilia morphogenesis appears abnormal in mice that carry mutant alleles of genes encoding myosin VIIa *(shaker1),* myosin VI *(snell's waltzer)* and myosin XV *(shaker2) (68).* One other mouse gene encoding for epsin has been identified as required for normal stereocilia morphology. In the mouse mutant *(jerker)* that lacks epsin, hair-cell stereocilia develop but rapidly lose their stiffness, then shorten, and degenerate *(69).*

Conclusions

There has been a great deal of progress in the past five years principally, from loss-of-functions studies that continue to be an invaluable tool in understanding the normal development of the inner ear. The discovery of Notch signaling and bHLH transcription factors in particular, has provided new tools for future dissection of the mechanisms that underlie cell-fate decision processes that lead from pluripotent progenitor cells to terminally differentiated sensory-hair cells. However, we are still a long way from understanding how the multitude of different cell types generated within the organ of Corti from a common pool of ectodermal progenitors particularly, the different supporting cell subtypes (Figs. 3–4). What is becoming clear is that each step of specification of a defined cell type involves not a single molecule but the combinatorial actions of several proteins that may have different functions, as evidenced from expression studies and mutant phenotype analyses. As discussed, *Math1* has been proven to be an intrinsic molecular determinant necessary and sufficient to drive the formation of inner-ear hair cells. However, supplementary efforts must be invested to shed more light on whether *Math1* is functioning in the same manner as proneural genes, by acting also during the earliest developmental steps for the specification of a common sensory progenitor of hair cells and supporting cells. In addition, it remains to be demonstrated whether or not interactions between *Math1* and *Ngn1*-dependent precursors might exist within the mouse otocyst. Mouse mutants that constitutively overexpress *Math1, Ngn1,* or both will help to test this hypothesis. Furthermore, future work intended to unravel the generation of cellular diversity in the organ of Corti should also address the question of how coordinated exit of sensory progenitor cells from cell cycle is controlled, and what are the factors involved in the specification of sensory and nonsensory regions within the embryonic cochlea. The major challenges of the future, with respect to the comprehension of the molecular mechanisms underlying the patterning and cell-fate decision within the inner ear, will be to decipher the genetic regulatory interactions between the Notch-signaling pathway and the bHLH transcription factors. From a clinical standpoint, the factors controlling hair-cell determination and initial differentiation in the mammalian organ of Corti could represent an important type of new experimental tool for initiating the proper regeneration of cells to treat deafness caused by hair cell loss.

Acknowledgments

The author would like to thank Bernd Fritzsch, Francois de Ribaupierre, and Thomas Van De Water for invaluable comments on the manuscript. The author's research was supported by the Swiss National Science Foundation grant 31-56897.99.

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