

Hes1 and Hes5 Activities Are Required for the Normal Development of the Hair Cells in the Mammalian Inner Ear

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The mammalian inner ear contains two sensory organs, the cochlea and vestibule. Their sensory neuroepithelia are characterized by a mosaic of hair cells and supporting cells. Cochlear hair cells differentiate in four rows: a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). Recent studies have shown that *Math1*, a mammalian homolog of *Drosophila atonal* is a positive regulator of hair cell differentiation. The basic helix–loop–helix (bHLH) genes *Hes1* and *Hes5* (mammalian *hairy* and *Enhancer-of-split* homologs) can influence cell fate determination by acting as negative regulators to inhibit the action of bHLH-positive regulators. We show by using reverse transcription-PCR analysis that *Hes1*, *Hes5*, and *Math1* are expressed in the developing mouse cochlea. *In situ* hybridization revealed a widespread expression of *Hes1* in the greater epithelial ridge (GER) and in lesser epithelial ridge (LER)

regions. *Hes5* is predominantly expressed in the LER, in supporting cells, and in a narrow band of cells within the GER.

Examination of cochlea from *Hes1*^{-/-} mice showed a significant increase in the number of IHCs, whereas cochlea from *Hes5*^{-/-} mice showed a significant increase in the number of OHCs. In the vestibular system, targeted deletion of *Hes1* and to a lesser extent *Hes5* lead to formation of supernumerary hair cells in the saccule and utricle.

The supernumerary hair cells in the mutant mice showed an upregulation of *Math1*. These data indicate that *Hes1* and *Hes5* participate together for the control of inner ear hair cell production, likely through the negative regulation of *Math1*.

Key words: *Hes1*; *Hes5*; *Math1*; basic helix–loop–helix (bHLH) transcription factors; mouse mutant; cochlea; utricle; saccule; hair cell differentiation

Regulatory cascades of positive and negative basic helix–loop–helix (bHLH) transcription factors play essential roles in the generation of numerous types of neurons from a homogenous population of ectodermal progenitor cells in the mammalian nervous system (Kageyama and Nakanishi, 1997; Lee, 1997). The inner ear initially forms as a thickening of the ectoderm, termed the otic placode in the hindbrain. The otic placode gives rise to neurons of the VIIIth cranial nerve and invaginates to become the otocyst, from which the organ of Corti and vestibular organs will develop (Van de Water, 1983; Torres and Giraldez, 1998). The sensory epithelia of these organs consist of mechanoreceptive hair cells, supporting cells, and nerve endings. In the mouse cochlea, terminal mitoses occur between embryonic day 13 (E13) and E14 (Ruben, 1967), and differentiating hair cells can first be identified on E15 in the base (Anniko, 1983; Lim and Anniko, 1985). Histological studies suggest that, during cochlea morphogenesis, inner hair cells (IHCs) derive from progenitor cells located in the greater epithelial ridge (GER) that is medial to the future organ of Corti, whereas outer hair cells (OHCs) derive from the distal progenitor cells in the lesser epithelial ridge

(LER) (Lim and Rueda, 1992). The time course of hair cell development includes cell fate commitment–determination, initial differentiation, maturation, and acquisition of stereociliary bundles (Kelley et al., 1993; Fekete, 1996; Torres and Giraldez, 1998). Recently, two evidences regarding initial determination–differentiation of mammals cochlear hair cells have been established. First, the involvement of the Notch1 signaling pathway in regulating the number of progenitor cells that develop as hair cells, contributing to the generation of the regular cochlear mosaic (Lanford et al., 1999; Zine et al., 2000a). Second, the requirement of the bHLH transcription factor *Math1* as a positive regulator of the specification of hair cells in the mouse inner ear (Bermingham et al., 1999; Zheng and Gao, 2000).

The negative bHLH transcription factors *Hes1* and *Hes5* homologs to the products of *Drosophila hairy* and *Enhancer-of-split* [*E(spl)*] (Akazawa et al., 1992; Sasai et al., 1992) have been demonstrated to affect cell fate determination by inhibiting the action of bHLH-positive regulators during the development of the CNS (Kageyama and Nakanishi, 1997).

In addition, previous studies reported that both *Hes1* and *Hes5* expression is induced by Notch activation (Jarriault et al., 1995, 1998; Ohtsuka et al., 1999). Although a recent study demonstrated that *Hes1* acts as a negative regulator of IHC differentiation (Zheng et al., 2000), none have been shown to be required for the control of OHC differentiation. The accurate control of both IHCs and OHCs is necessary for the generation of the regular mosaic of hair cells and supporting cells within the mammalian cochlea. Here we examined by, using reverse transcription (RT)-PCR analysis, the expression of *Hes1*, *Hes5*, and *Math1*

Received Nov. 27, 2000; revised April 2, 2001; accepted April 4, 2001.

This research was supported by Swiss National Science Foundation Grant 31-56897.99. We thank Drs. Mark Mooseker for his gift of anti-myosinVIIa, Jane Johnson for anti-*Math1* antibody, Thomas Van de Water for comments and suggestions, and Luc Pellerin for laboratory facilities.

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genes in the developing mouse cochlea. Using *in situ* hybridization, *Hes1* and *Hes5* mRNA expression patterns were examined in E18 and newborn cochleae.

The roles of *Hes1* and *Hes5* in hair cell differentiation were investigated by analyzing the developing inner ear from mice that lack both *Hes1* and *Hes5* genes.

MATERIALS AND METHODS

Animals. Compound *Hes1* and *Hes5* embryos and early neonatal [postnatal day 0 (P0)] mutants were obtained from intercrosses of *Hes1*^{+/-}; *Hes5*^{+/-} or *Hes1*^{+/-}; *Hes5*^{-/-} mice. These mutant mouse lines have been described previously (Ishibashi et al., 1995; Ohtsuka et al., 1999; Cau et al., 2000). E0.5 was defined as noon of the day that vaginal plug was observed. Inner ear tissues were collected from E16–E18 embryos or immediately after birth, because *Hes1*^{-/-} mice die during gestation or within 1 d after birth (Ishibashi et al., 1995). Genotyping of the embryos and P0 mice was performed by PCR with tail DNA.

RT-PCR. Sensory epithelia were dissected out from 10–14 cochleae of five developmental stages from E12.5 to newborn (P0). RT-PCR was performed and controlled as described previously (Zine et al., 2000b) with few modifications. Total RNA was isolated using TRIZOL (Life Technologies, Gaithersburg, MD). Polyadenylated RNA was purified from total RNA through oligo-dT cellulose separation (Amersham Pharmacia Biotech, Uppsala, Sweden). mRNA was reverse transcribed using Superscript II RNase free reverse transcriptase (Life Technologies).

For PCR amplification, the typical thermocycle profile was as follows: 5 min at 95°C, 45 sec at 58°C, and 1.5 min at 72°C for 40 cycles. The PCR products were separated on a 1% agarose gel, stained with ethidium bromide, purified, and sequenced. The primers for RT-PCR analysis are as follows: for *Hes1*, upstream CAGCCAGTGTCAACACGACAC and downstream TCGTTCATGCACTCGCTGAG; for *Hes5*, upstream CGCATCAA CAGCAGCATAGAG and downstream TGGAAAGTGGT AAA GCAGCTTC; for *Math1*, upstream AGTGACGGAGAGTTTTC-CCC and downstream CTGCAGCCGTCGGAAGTCAA; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), upstream GT-CATCATCTCCGCCCTTCTGC and downstream GATGCCTGCTTCACCACCTTCTTG.

In situ hybridization. Cochleae were dissected from E18 and P0 inner ears and fixed in 4% paraformaldehyde for 3 hr in phosphate buffer, pH 7.4, overnight at 4°C.

For cryostat sections, cochleae were cryoprotected in 20% sucrose in PBS, embedded in OCT compound (Tissue Tek; Miles, Elkhart, IN), and mounted for sectioning. For whole-mount surface preparation, cochleae were dissected from all of the surrounding tissue to expose the developing sensory epithelia. RNA probes were synthesized from cDNA for *Hes1* (Tomita et al., 1996) and *Hes5* (Akazawa et al., 1992). *Hes1* was linearized by *Xho*I and transcribed by T3, and *Hes5* was linearized by *Hind*III and transcribed by T3 RNA polymerase.

Both whole-mount surface preparations and cryostat sections (10 μm) were processed with a digoxigenin (DIG)-labeled cRNA probes as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). In brief, after fixation, the samples were incubated in 1% Triton X-100 and digested with Proteinase K. They were incubated in a prehybridization solution for 2 hr at 50°C and then exposed to cRNA probes overnight at 50°C. The probes were revealed by alkaline-phosphatase-coupled anti-DIG antibodies (Boehringer Mannheim, Mannheim, Germany), which were reacted with nitroblue-tetrazolium-chloride and 5-bromo-4-chlor-indolyl-phosphate substrates for color reaction. Some of the whole-mount cochlear preparations were cryosectioned after the *in situ* hybridization procedures.

Histology and immunocytochemistry. Cochleae from embryos (E16–E18) and P0 mice were fixed overnight in 4% paraformaldehyde in PBS at 4°C. We performed immunofluorescence analysis on whole-mount surface preparations and paraffin sections of the cochleae as described previously (Zine et al., 2000a). Both cochlea paraffin sections and surface preparations were preincubated in PBS containing 5% normal donkey serum for 2 hr and then incubated with polyclonal antibodies against myosin VIIa (Hasson et al., 1995) or against *Math1* (Helms and Johnson, 1998). Antibody incubations were done overnight at 4°C, and binding was visualized with rhodamine or Cy3-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Other cochlear surface preparations were stained with rhodamine-conjugated phalloidin (5 μg/ml) for 45 min to visualize the pattern of hair cell

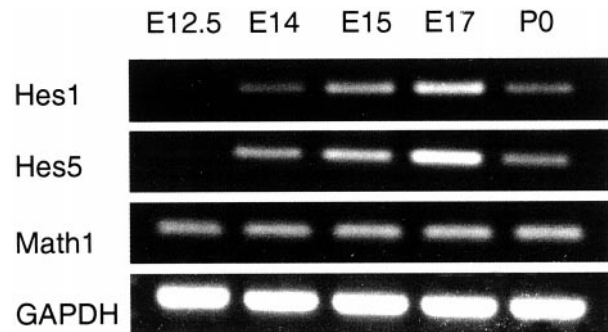


Figure 1. RT-PCR analysis of the temporal expression of *Hes1*, *Hes5*, and *Math1* in the sensory epithelia dissected from the developing cochleae. All cDNA was coamplified with GAPDH as shown. *Math1* transcripts were detected early at E12.5, whereas transcripts for *Hes1* and *Hes5* were detected by E14. This expression of *Hes1*, *Hes5*, and *Math1* was maintained in the developing sensory epithelium until P0.

differentiation in the organ of Corti, in particular the actin-rich stereocilia.

Confocal and scanning electron microscopy. Digital images were captured on a Leica (Nussloch, Germany) TCS-NT confocal laser scanning microscope. Images used for the figures were processed with either NIH Image or Photoshop (Adobe Systems, Mountain View, CA) software programs.

For scanning electron microscopy, cochleae were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr and then post-fixed for 1 hr with 1% osmium tetroxide. After washing in cacodylate buffer, cochleae were dehydrated in ascending concentrations of ethanol, dried at the critical point, and sputter coated with gold. All material was examined in a Jeol (Peabody, MA) 630F scanning electron microscope operating at 5 kV.

Quantification of hair cells. We quantified the number of cochlear hair cells in early neonatal (P0) mutant mice versus wild-type littermates. Count was made on cochlear surface preparations processed with rhodamine-conjugated phalloidin. We made analysis on a region that covers a 1600 μm length of the organ of Corti, beginning near the base of cochlea and extending toward the middle turn.

In the vestibular sensory epithelia, we counted, using the 40× objective lens, the number of hair cells of P0 utricle and saccule maculae of mutants versus those of wild-type mice on transverse sections immunostained with myosin VIIa across the entire section length of the sensory epithelium. Only segments in which the section was perpendicular to the surface of the neuroepithelium were counted. Hair cell counts were made from three to five distant sections for each macula, and two to five animals were counted for each genotype.

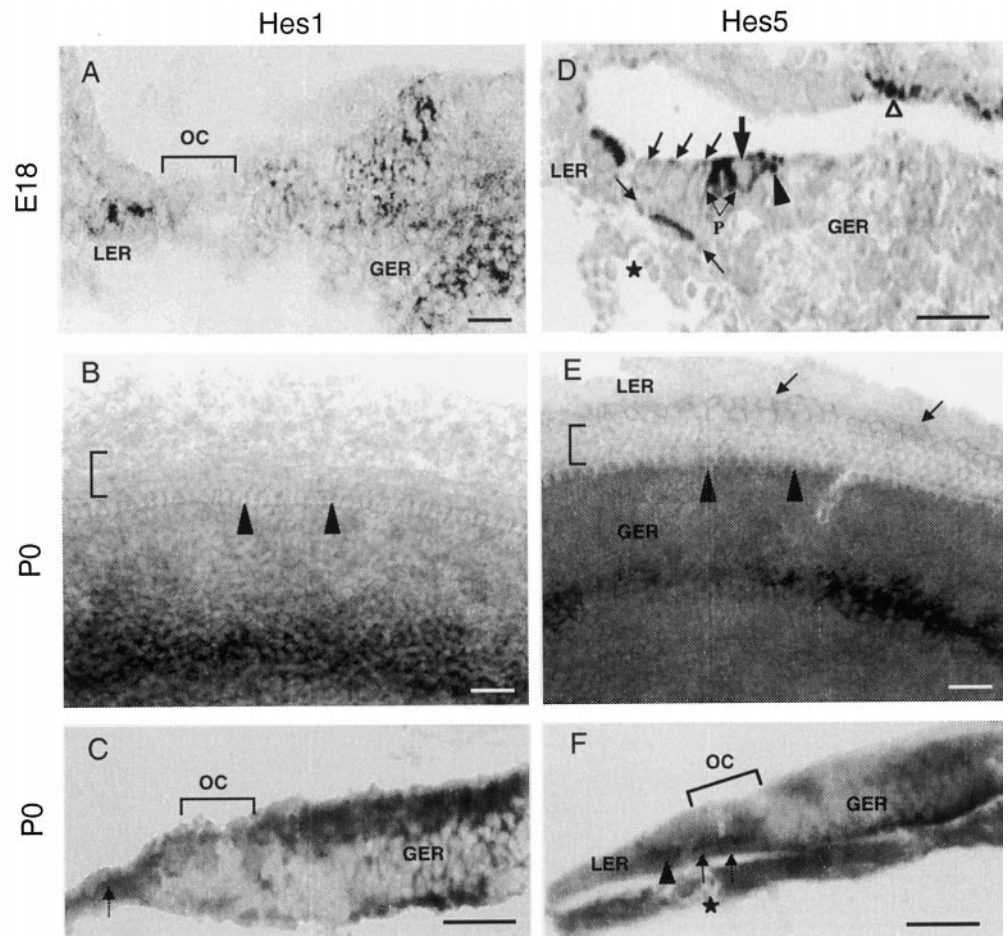
RESULTS

Hes1, *Hes5*, and *Math1* are expressed in the developing cochlea

RT-PCR analyses (Fig. 1) were performed with dissected sensory epithelia from the developing cochleae that principally consist of the organ of Corti, a part of the basilar membrane, GER, and LER, as described previously (Zine and de Ribaupierre, 1999). *Hes1* and *Hes5* transcripts became detected by E14, a stage just before the initiation of the cytological differentiation of hair cells at E15 (Lim and Anniko, 1985). *Math1* was expressed as early as E12.5, consistent with a study reporting the initiation of *Math1*/LacZ expression in the embryonic organ of Corti by E12.5 (Birmingham et al., 1999). The expression of *Hes1*, *Hes5*, and *Math1* between E14 and P0, a developmental period that is critical for initial hair cell differentiation, suggests the possible functional relationship between these genes.

To determine the cellular expression patterns of *Hes1* and *Hes5* genes, we used nonradioactive *in situ* hybridization on cryosections and whole-mount surface preparations from E18–P0 co-

Figure 2. Nonradioactive *Hes1* (A–C) and *Hes5* (D–F) RNA *in situ* hybridization in the developing mouse cochlea at E18 and P0. A, D, *In situ* hybridization on transverse sections through the base of E18 cochlea. *Hes1* mRNA (A) is expressed in the LER cells and in a large area of GER, except in the organ of Corti (OC). *Hes5* mRNA (D) is expressed in the LER, pillar cells (P), and Deiter's cells at the base of OHCs (area bracketed by arrows). *Hes5* is also found in the inner phalangeal cells (arrowhead), which are adjacent to the inner hair cell region. Hair cells (large arrow indicates IHC; oblique arrows indicate the three rows of OHCs) are lacking *Hes5* hybridization signal. Note the *Hes5* expression in the marginal cells of the stria vascularis (open arrowhead). Asterisk indicates the spiral vessel, a landmark for the location of the organ of Corti at this stage of maturation. B, E, *In situ* hybridization on whole-mount surface preparations from the middle turn of the cochlear duct at P0. Expression of *Hes1* (B) spans a large area of the GER, as observed in a cochlea section (A). Expression of *Hes5* (E) is observed in a relatively narrow band of cells of the GER. There is a faint but specific *Hes5* expression in the cells within the LER (arrows). Note the absence of both *Hes1* and *Hes5* hybridization signal from the hair cells (arrowheads indicate the location of IHC row; bracket indicates the OHC rows). C, F, Transverse sections through the whole-mount cochlear surface preparations presented in B and E, respectively. C, This section confirms *Hes1* expression in the GER cells and its lack from the cells of the organ of Corti (bracket). Hybridization label for *Hes1* is also located in the LER (arrow). F, *Hes5* expression is restricted to the nonsensory supporting cells (Deiter's cells; arrows) within the organ of Corti. This section also confirms the specific expression of *Hes5* is the LER cells (arrowhead). A weak label for *Hes5* is observed in the GER region and in cells located near the basilar membrane. Asterisk indicates the spiral vessel. Scale bars, 20 μ m.



chleae (Fig. 2). *In situ* hybridization on E18 cochlea sections revealed *Hes1* expression principally in two areas, which include the GER and LER. The organ of Corti, which contains the hair cells and supporting cells, was devoid from *Hes1* signal at this stage of maturation (Fig. 2A), although a weak hybridization signal for *Hes1* was observed in the supporting cells region in the basal cochlear turn of E15 mice, in addition to the GER and LER (data not shown).

Whole-mount cochlear surface preparations at P0 (Fig. 2B) also showed a widespread expression of *Hes1* in the GER area and its lack from the organ of Corti. A section (Fig. 2C) through the whole-mount surface preparation presented in Fig. 2B confirmed *Hes1* expression in the GER and LER and the absence of *Hes1* hybridization signal from the organ of Corti. *Hes5 in situ* hybridization (Fig. 2D–F) performed on E18–P0 cochleae indicated both overlapping and distinct expression patterns compared with that of *Hes1*. In the organ of Corti (Fig. 2D,F), *Hes5* is expressed in the pillar cells, Deiter's cells, and the inner phalangeal cells. Like *Hes1*, the expression of *Hes5* was not detected in the hair cells. *Hes5* was also heavily expressed in the cells of the LER (Fig. 2D), although its expression in the GER was confined to a narrow band of cells (Fig. 2E). Marginal cells of the stria vascularis showed a specific *Hes5* expression.

To define the roles of *Hes1* and *Hes5* genes in hair cell devel-

opment, we analyzed cochlear surface preparations and inner ear sections of different genotypes that resulted from *Hes1*^{+/-}; *Hes5*^{+/-} or *Hes1*^{+/-}; *Hes5*^{-/-} intercrosses. We obtained eight possible genotypes (Cau et al., 200) and performed hair cell counts on six of them in the case of cochlea (as summarized in Table 1) and four of them in the case of the vestibule (see Fig. 6).

Deletion of *Hes1* gene leads to a significant increase in the number of IHCs

Whole-mount surface preparation of the cochleae from *Hes1*^{-/-} mice stained with rhodamine phalloidin revealed many regions (38% of the relative length of the basilar membrane; Table 1) with a nearly complete row of supernumerary IHCs (Fig. 3D–F) compared with wild-types (<1% of basilar membrane; Table 1), which have only a single row of IHCs (Fig. 3A–C). In addition, a few regions (5% of basilar membrane with a fourth row of OHCs; Table 1) of *Hes1*^{-/-} mutant cochleae presented four instead of three rows of OHCs (Fig. 3D,F). The presence of the supernumerary hair cells was confirmed by immunostaining cross-sections of cochleae with anti-myosin VIIa, a hair cell-specific marker (Fig. 3E) and by scanning electron microscopy (Fig. 3F). There was a significant increase in the total number of hair cells in cochleae of *Hes1*^{-/-} mice compared with wild types. This was principally attributable to a significant increase in the number of

Table 1. Effects of deletion of *Hes1* and *Hes5* on auditory hair cell differentiation

	Cochlea #	IHCs	OHCs	Total HCs	IHC pairs	Relative length of BM with:	
						IHC pairs	Fourth row OHCs
WT	a	208	619	827	1		
	b	189	581	770	0		
	c	202	604	806	1		
Mean		200 ± 10	601 ± 19	801 ± 29	0.66	<1%	<1%
<i>Hes1</i> ^{-/-}	a	291	640	931	NC		
	b	261	583	844	NC		
	c	275	615	890	NC		
Mean		276 ± 15**	612 ± 28	888 ± 43*		38%	5%
<i>p</i> value		0.0018	0.5987	0.0442			
<i>Hes1</i> ^{-/-} ; <i>Hes5</i> ^{+/-}	a	338	645	983	NC		
	b	315	609	924	NC		
Mean		326 ± 16**	627 ± 25	953 ± 42*		63%	12%
<i>p</i> value		0.0015	0.2831	0.0052			
<i>Hes5</i> ^{-/-}	a	201	663	864	7		
	b	230	713	943	11		
	c	219	674	893	15		
Mean		217 ± 14	683 ± 26*	900 ± 40*	11	5%	45%
<i>p</i> value		0.1691	0.0120	0.0254			
<i>Hes1</i> ^{+/-} ; <i>Hes5</i> ^{-/-}	a	227	750	977	6		
	b	219	723	942	14		
	c	197	701	898	23		
Mean		214 ± 15	725 ± 25**	939 ± 40*	14	7%	62%
<i>p</i> value		0.2379	0.0024	0.0082			
<i>Hes1</i> ^{+/-} ; <i>Hes5</i> ^{+/-}	a	221	645	866	10		
	b	207	633	840	5		
	c	198	597	795	6		
Mean		209 ± 12	625 ± 25	834 ± 36	9	4%	12%
<i>p</i> value		0.3609	0.2626	0.2866			

Hair cells were quantitated after labeling with rhodamine phalloidin of cochleae isolated at P0 from mutant, heterozygote, and wild-type mice. One cochlea was counted for each animal [3 animals (a–c) were counted for each genotype], except for *Hes1*^{-/-};*Hes5*^{+/-} (*n* = 2). The length counted for mutant, heterozygote, and wild type was 1.6 mm for each cochlea and included a sector that extends from the basal toward the midturn. Data are expressed as mean ± SD, and significance was determined using a Student's two-tailed, *p* value *t* test. **p* < 0.05 and ***p* < 0.005 indicate significant values from wild type. Relative length of the basilar membrane with IHC pairs was computed as follows: $IHC_{mutant} - IHC_{wt}/IHC_{wt}$. Relative length of the basilar membrane with fourth row OHCs was computed as follows: $OHC_{mutant} - OHC_{wt}/\frac{1}{2} OHC_{wt}$. NC, Not counted; BM, basilar membrane; WT, wild type.

IHCs (***p* < 0.005), because the number of OHCs in *Hes1*^{-/-} cochleae was just slightly higher than wild types (Table 1). The *Hes1*^{-/-} mice of this study showed a severe cochlea phenotype, with respect to the production of total cochlear hair cells when compared with the results of a recent study (Zheng et al., 2000). This could be attributable to differences in the developmental stage at which hair cell counts were made or to the length of cochlea covered by hair cell counts. In this study, we quantified the number of hair cells in cochleae collected from newborn (P0) mice for either mutant and wild types and along a 1.6 mm length of the sensory epithelium, i.e., nearly 70% of the total length.

Deletion of *Hes5* gene leads to a significant increase in the number of OHCs

Cochleae from *Hes5*^{-/-} mice revealed many regions (45% of the relative length of the basilar membrane with a fourth row of OHCs; Table 1) that principally contained four rows of OHCs rather than three (Fig. 3*G–I*, Table 1), although, in these mutants, doublets of IHCs (5% of basilar membrane; Table 1) were interspersed within regions of a single row of IHCs (Fig. 3*G,I*). Moreover, sometimes cochleae of *Hes5*^{-/-} mice exhibited a few

supernumerary hair cells scattered outside the sensory epithelium in the GER (these extra hair cells at this location were not included in hair cells counts), as defined by phalloidin (Fig. 4*A*) and myosin VIIa (Fig. 4*B–D*) labeling. In contrast to *Hes1*^{-/-} mice, the significant increase in the total number of hair cells in the cochleae of *Hes5*^{-/-} mice was attributable to the significant increase in the number OHCs (**p* < 0.05) compared with wild types (Fig. 3*G–I*, Table 1).

We also examined the cochleae of *Hes5*^{-/-} mice during the postnatal development because, *Hes5* single mutants were viable. We observed that the supernumerary hair cells survived until P10 (the latest stage we examined) and also developed morphological characteristics of the normal hair cells (data not shown).

Enhanced increase of hair cell production in *Hes1*^{-/-};*Hes5*^{+/-} and *Hes1*^{+/-};*Hes5*^{-/-} mutants

Cochleae from double mutant that were homozygous for *Hes1* and heterozygous for *Hes5* (*Hes1*^{-/-};*Hes5*^{+/-}) and those heterozygous for *Hes1* and homozygous for *Hes5* (*Hes1*^{+/-};*Hes5*^{-/-}) mice showed a more significant increase in the total number of

Figure 3. A comparison of hair cell development in P0 cochleae from wild-type control (*A–C*), *Hes1*^{-/-} (*D–F*), *Hes5*^{-/-} (*G–I*), and *Hes1*^{-/-};*Hes5*^{+/-} (*J*) mutants and *Hes1*^{+/-};*Hes5*^{+/-} heterozygous mice (*K*). Lack of *Hes1* and *Hes5* causes the development of supernumerary hair cells. *A, D, G, J, K*, Confocal images of surface preparations stained with rhodamine phalloidin to visualize the actin-rich stereocilia of the hair cells. *B, E, H*, Cross-sections through the organ of Corti in the midmodiolar region immunostained with antibody anti-myosin VIIa. *C, F, I*, Scanning electron microscopy of the surface of the organ of Corti in the midcochlear turn. In control cochlea, the normal pattern is well defined, with a single row of IHCs and three rows of OHCs. In contrast, in *Hes1*^{-/-} cochleae, two rows of IHCs and three to four rows of OHCs are present. In *Hes5*^{-/-} cochleae, four rows of OHCs are often present, in addition to dispersed regions along the sensory epithelium that contain few IHC pairs. *J*, *Hes1*^{-/-};*Hes5*^{+/-} cochleae revealed the same phenotype as *Hes1*^{-/-}, but the effect on the number of supernumerary hair cells was more important in *Hes1*^{-/-};*Hes5*^{+/-} cochleae (see Table 1). *K*, Cochlear surface preparation from double heterozygous mouse cochlea indicating regions with extra OHC rows and a few pairs of IHCs. Brackets mark the OHCs rows, and arrowheads point to the IHC row. Scale bars: *A, B, D, E, G, H, J, K*, 20 μ m; *C, F, I*, 10 μ m.

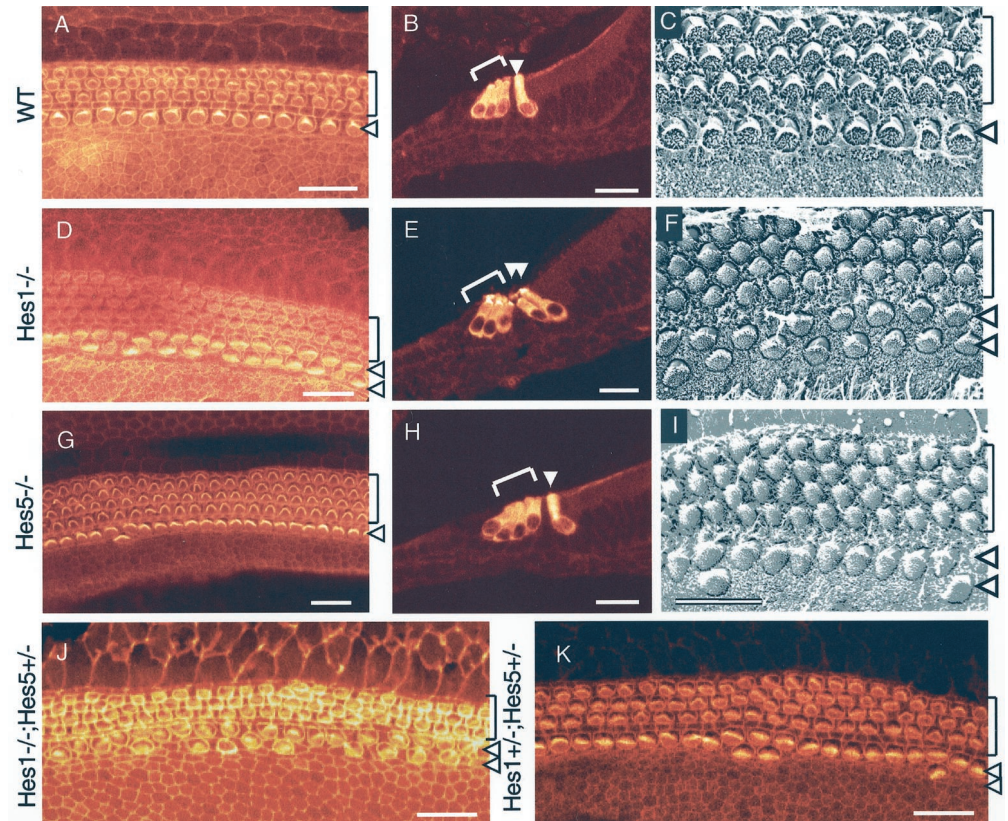
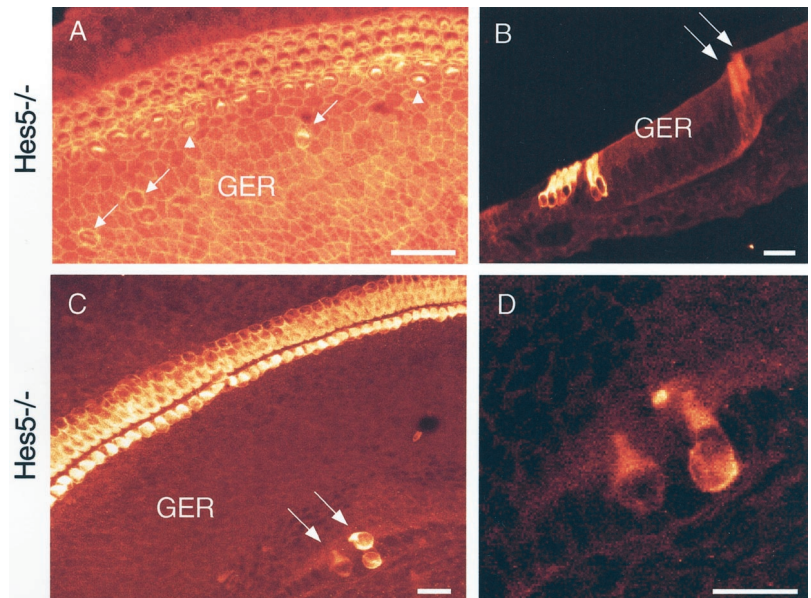


Figure 4. Formation of supernumerary hair cells in both the organ of Corti and the GER in P0 cochlea of *Hes5*-deficient mice. *A*, Cochlear surface preparation stained with phalloidin, revealing pairs of IHCs in the organ of Corti (arrowheads), in addition to some extra differentiating hair cells with immature stereocilia scattered in the GER (arrows). *B*, Cross-section through the midmodiolar region of the organ of Corti immunostained with anti-myosin VIIa. Arrows indicate the two differentiating extra hair cells outside the organ of Corti, in the GER area. Note also the extra hair cells in the organ of Corti. *C*, Cochlear surface preparation immunostained with myosin VIIa, indicating the presence of differentiating extra hair cells in the GER (arrows). *D*, High-magnification of the extra hair cells seen in the GER in *C* showing their characteristic shape and basal nuclei. Scale bars, 20 μ m.



hair cells than *Hes1* and *Hes5* single mutants when compared with the values of wild types (Table 1). This increase in the number of hair cells was attributable to a significant increase in the number of IHCs (***p* < 0.005) in *Hes1*^{-/-};*Hes5*^{+/-} mice and in the number of OHCs (***p* < 0.005) in *Hes1*^{+/-};*Hes5*^{-/-} mice compared with wild types (for IHCs: wild type, 200 ± 10, mean ± SD; *Hes1*^{-/-}, 276 ± 15; *Hes1*^{-/-};*Hes5*^{+/-}, 326 ± 16) (for OHCs: wild type, 601 ± 19; *Hes5*^{-/-}, 683 ± 26; *Hes1*^{+/-};*Hes5*^{-/-}, 725 ±

25). In addition, the length of the basilar membrane (Table 1) with IHC pairs increased from 38% in *Hes1*^{-/-} to 63% in *Hes1*^{-/-};*Hes5*^{+/-} mice and that with a fourth row of OHCs increased from 45% in *Hes5*^{-/-} to 62% in *Hes1*^{+/-};*Hes5*^{-/-}. This pronounced defect in *Hes1*^{-/-};*Hes5*^{+/-} and *Hes1*^{+/-};*Hes5*^{-/-} mutant cochleae suggests that the dosage of both *Hes1* and *Hes5* gene products is important for the control of hair cell production. There was a mild increase, statistically insignificant,

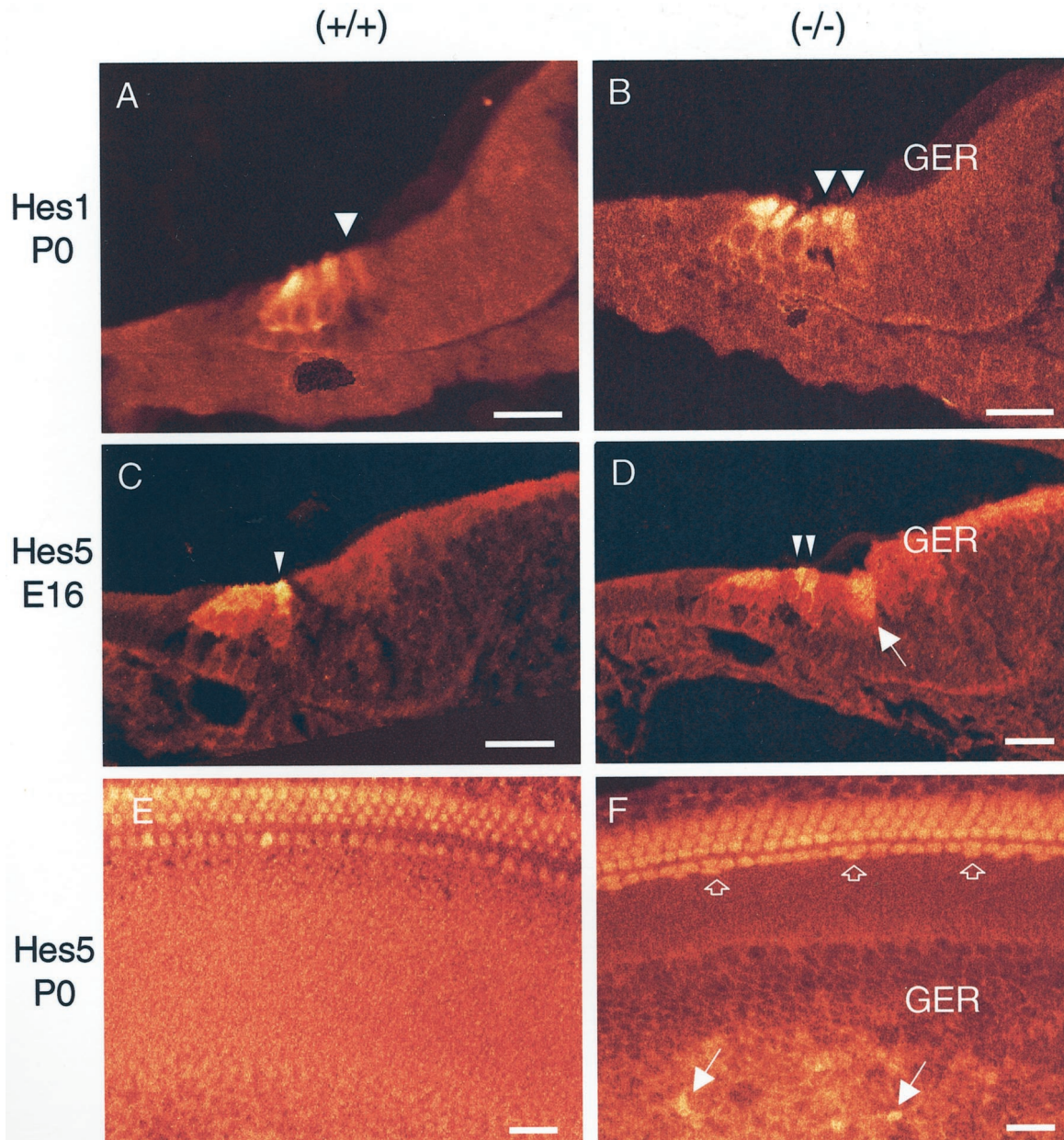


Figure 5. Expression patterns of Math1 protein in the developing cochleae of wild-type (control) (*A*, *C*, *E*), *Hes1*-deficient (*B*), and *Hes5*-deficient (*D*, *F*) mice. *A*, *B*, Cross-sections through the midturn of P0 cochlea from control and *Hes1*^{-/-} mice. In control, a single inner hair cell (arrowhead) and three outer hair cells express Math1. In *Hes1*^{-/-}, Math1 expression also included the extra IHCs (arrowheads). *C*, *D*, Cross-sections of the midturn E16 cochleae from control and *Hes5*^{-/-} mice showing the differentiating extra hair cells (arrowheads). Note a diffuse expression of Math1 in the apical region of some cells in the GER of *Hes5*^{-/-} mice (arrow). *E*, *F*, Cochlear surface preparations obtained from P0 control and *Hes5*^{-/-} mice. In control, one row of IHCs and three rows of OHCs express Math1. In *Hes5*^{-/-}, Math1 expression highlights the differentiating extra hair cells in both the organ of Corti (open arrows) and the GER (arrows). Scale bars, 20 μ m.

in the number of hair cells in the double heterozygote (*Hes1*^{+/-}; *Hes5*^{+/-}) mice compared with wild types (Fig. 3*K*, Table 1). Because double homozygous embryos for *Hes1* and *Hes5* die before E11.5 (Ohtsuka et al., 1999), well before the formation of the cochlea, we were unable to assess hair cell differentiation in double null mutant mice.

Analysis of Math1 expression in the cochleae of *Hes1*- and *Hes5*-deficient mice

Because Math1 has been shown (Bermingham et al., 1999) to be required for the genesis of hair cells, we examined by immunohistochemistry whether the supernumerary hair cells in *Hes1* and

Hes5 mutant mice also expressed Math1 (Fig. 5). Antibody anti-Math1 (Helms and Johnson, 1998) was used to detect Math1 expression in wild-type, *Hes1*, and *Hes5* mutant cochleae. In wild-types, Math1 expression was restricted to the differentiating hair cells at E16 (Fig. 5*C*) and by P0 (Fig. 5*A*, *E*) was but absent in the supporting cells and in cells outside the sensory epithelium. Interestingly, in *Hes1*^{-/-} (Fig. 5*B*) and *Hes5*^{-/-} (Fig. 5*D*) mutant cochleae, Math1 expression also included the supernumerary hair cells that developed within the sensory epithelium. The presence of supernumerary hair cells in the sensory epithelium of E16 *Hes5*^{-/-} (Fig. 5*D*) and E16 *Hes1*^{-/-} mice (data not shown) at the

earliest developmental time points at which these cells can be distinguished suggests that *Hes1* and *Hes5* genes may have roles in initial steps of hair cell determination–differentiation. In addition, *Math1* expression was seen in a subset of extra hair cells that developed outside the organ of Corti, in the GER of *Hes5*^{-/-} cochlea (Fig. 5F). This upregulation of *Math1* in differentiating extra hair cells of *Hes1*^{-/-} and *Hes5*^{-/-} mutant cochleae is consistent with a study demonstrating that *Math1* is necessary for the genesis of hair cells in the mice inner ear (Bermingham et al., 1999). Moreover, it was demonstrated in a recent gain-of-function study that overexpression of *Math1* in postnatal cochlear cultures resulted in extra hair cells observed in the GER (Zheng and Gao, 2000).

Hes1 and *Hes5* genes are also involved in hair cell differentiation in the utricle and saccule sensory epithelia

To analyze the effects of targeted deletion of *Hes1* and *Hes5* on hair cell differentiation within the mouse vestibular organs, we counted the number of cells within the luminal hair cell layer of P0 utricle and saccule on serial sections, immunolabeled with anti-myosin VIIa (Fig. 6A–D). This quantitation (Fig. 6E,F) showed a significant increase in the mean number of hair cells per unit length of sensory epithelium in the utricle (Fig. 6F) of *Hes1*^{-/-} mice (18.7 ± 4.4 , mean \pm SD; $p < 0.0001$) compared with the wild-type control mice (12.8 ± 1.2). The number of hair cells in the utricle of *Hes5*^{-/-} mice (16.2 ± 3), although lower than that in the utricle of *Hes1*^{-/-} mice, represents a significant increase over the value of wild-type control mice. The number of hair cells in the double heterozygote (*Hes1*^{+/-};*Hes5*^{+/-}) utricles was below that of *Hes1* and *Hes5* single mutants and slightly higher than that of the wild-type utricle mice. In the saccule (Fig. 6E), hair cell differentiation is similarly affected by *Hes1* and *Hes5* deletions as in the utricle. We found a significant, although reduced relative to the value in the utricle, increase in the number of hair cells in *Hes1*^{-/-} (16.5 ± 1.7) and *Hes5*^{-/-} (14.9 ± 1.9) mice when compared with the wild-type (11.1 ± 1.3) saccule mice.

Our quantitative data from the utricle of *Hes1*^{-/-} mice are in the same range as those reported by Zheng et al. (2000). Their total hair cells counts showed an increase of 36% in the *Hes1*^{-/-} utricles compared with those from the wild types. This value is very close to the increase in hair cell density that we found for the utricle (38% increase for *Hes1*^{-/-}; 20% for *Hes5*^{-/-}). In the case of the saccule, our counts indicate an increase of 34% for *Hes1*^{-/-} and of 21% for *Hes5*^{-/-} when compared with the wild types.

DISCUSSION

Our experiments demonstrate that *Hes1* and *Hes5* have separate and overlapping roles in regulating the differentiation of hair cells in the mammalian inner ear. In the cochlea, *Hes1* has a significant influence on the production of IHCs, whereas *Hes5* has a significant influence on the production of OHCs, although a few differentiating extra hair cells were seen in the GER of some cochleae of *Hes5*^{-/-} mice. In the vestibular organs, targeted deletion of either *Hes1* or *Hes5* leads to the formation of supernumerary hair cells in both the utricle and saccule sensory epithelia.

Our RT-PCR analysis with isolated cochlear sensory epithelia indicates that *Hes1*, *Hes5*, and *Math1* genes are expressed during a developmental period that is critical for initial hair cell differ-

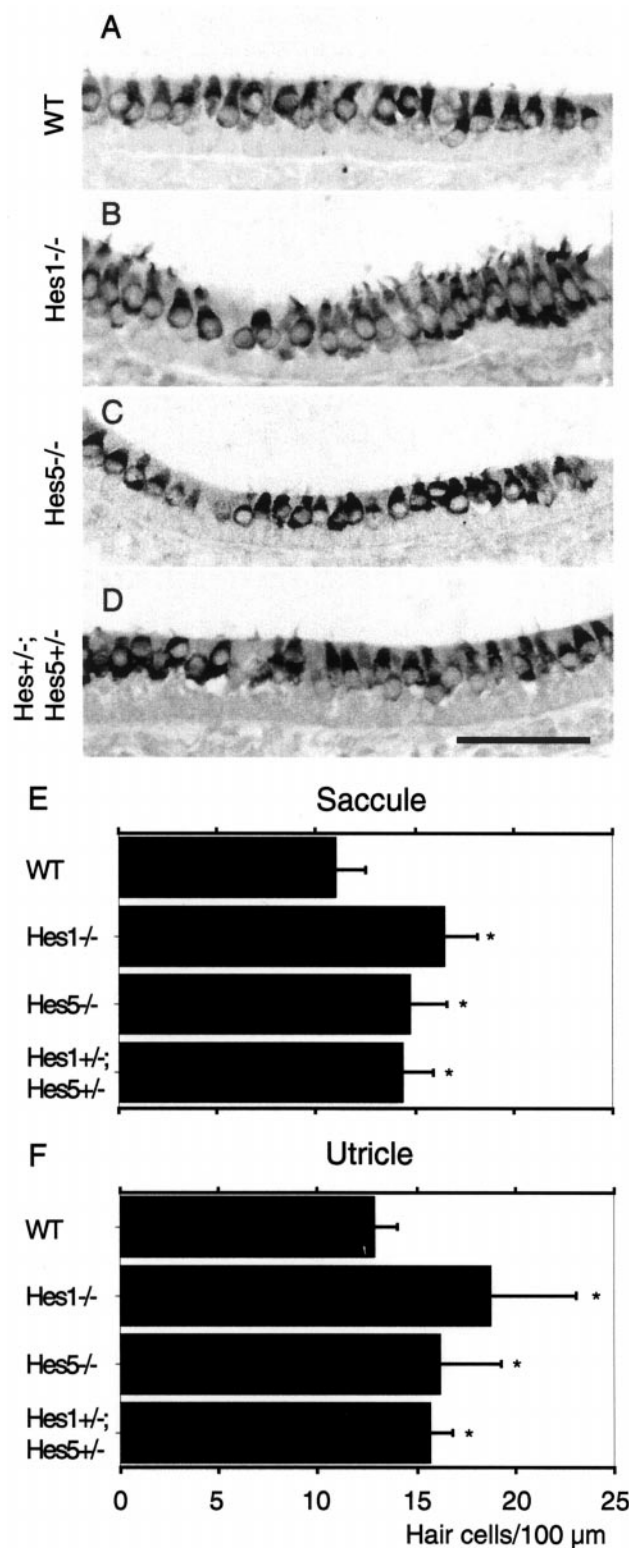


Figure 6. Formation of supernumerary hair cells in the utricle and saccule of *Hes1*- and *Hes5*-deficient mice. Myosin VIIa immunolabeling of P0 saccular section from wild-type (WT) control (A), *Hes1*^{-/-} (B), *Hes5*^{-/-} (C), and *Hes1*^{+/-};*Hes5*^{+/-} heterozygous mice (D). E, F, Hair cell counts from *Hes1*^{-/-}, *Hes5*^{-/-} and *Hes1*^{+/-};*Hes5*^{+/-} mouse saccules and utricles, respectively. Hair cell densities are expressed as a mean number per 100 μ m length of the sensory epithelium cut perpendicular to the epithelial surface. Data are expressed as mean \pm SD, and significance was determined using a Student's *t* test. * $p < 0.0001$ indicates significant values from wild type. Scale bar: A–D, 50 μ m.

entiation. Our results using *in situ* hybridization with E18–P0 mouse cochleae revealed *Hes1* expression in the GER and LER regions, whereas undetectable to a low level of *Hes1* expression was seen in the supporting cells of the basal turn of E15 cochlea. *Hes5* expression predominated in the LER and in supporting cells of the sensory epithelium of E18–P0 cochleae. In addition, *in situ* hybridization with whole-mount preparations of the developing cochlea showed a narrow band of *Hes5*-expressing cells in the GER area (Fig. 2E). These expression data are consistent to some extent with the phenotypic characterization of *Hes1* and *Hes5* mutant cochleae. Indeed, during embryonic development, IHCs derive from progenitor cells located in the most distal domain of the GER, whereas OHCs derive from progenitor cells located in the proximal domain of LER (Lim and Rueda, 1992).

It is interesting that *Hes5* was also expressed in a narrow band of cells within the GER, as in some of the *Hes5*^{-/-} cochleae in which a few ectopic hair cells were observed in this region, in addition to differentiation of the fourth row of OHCs that arose right next to the normal rows. Within the vestibular system, either *Hes1* or *Hes5* deletions induced the formation of supernumerary hair cells in both the utricle and saccule epithelia. This suggests an overlapping role *Hes1* and *Hes5* genes in preventing the formation of supernumerary utricular and saccular hair cells during the normal development of the inner ear. Our quantitative data of the vestibular system indicated that, in *Hes5* mutant mice, the formation of extra hair cells, although significant compared with the wild-type mice, was less important than that of *Hes1* mutant mice. This could fit with the expression study in the utricular epithelium of the rat (Zheng et al., 2000) demonstrating that, unlike *Hes1*, which is expressed in the supporting cells throughout the sensory epithelium, *Hes5* expression was confined to the supporting cells in the central area of the utricle (striola). In contrast to the vestibular system, *Hes1* expression in E18–P0 mouse cochlea was undetectable in the supporting cells. This suggests that *Hes1* may act differently to regulate hair cell differentiation in the inner ear neuroepithelia.

The upregulation of *Math1* in the sensory epithelia of *Hes1* and *Hes5* mutant cochleae (Fig. 5) suggests that *Hes* genes regulate hair cell differentiation, possibly by antagonizing *Math1*. This may also occur within the vestibular system because it has been shown that *Hes1* and *Hes5* are expressed in the supporting cell layer of the developing utricle (Zheng et al., 2000). In addition, *Math1* is expressed in the vestibular hair cells and is also required for their differentiation (Bermingham et al., 1999; Shailam et al., 1999).

These results are consistent with previous studies demonstrating that the transcription of *Math1* is repressed by the transcriptional activities of *Hes* genes (Takebayashi et al., 1994; Akazawa et al., 1995), although the function of *Hes5* as a repressor of *Math1* transcription remains speculative. The increase in cochlear hair cell production in *Hes1*^{-/-};*Hes5*^{+/-} and *Hes1*^{+/-};*Hes5*^{-/-} mutant mice compared with *Hes1* and *Hes5* single mutant mice suggests that *Hes1* and *Hes5* genes operate in a common signaling pathway and may functionally compensate for each other. Recent studies have demonstrated that activation of Notch results in the subsequent activation of *Hes1* and *Hes5* genes (Jarriault et al., 1995, 1998; Ohtsuka et al., 1999). Notch proteins are ligands-activated transmembrane receptors involved in cell fate selection throughout development of both *Drosophila* and vertebrates (Artavanis-Tsakonas et al., 1999).

Studies from several laboratories have reported that the Notch pathway is involved in the development of the vertebrate inner ear (Adam et al., 1998; Haddon et al., 1998; Lanford et al., 1999;

Morrison et al., 1999; Eddison et al., 2000; Zhang et al., 2000; Zine et al., 2000a). In the mammalian cochlea, targeted deletion of *Jagged2* gene, which encodes one of the Notch ligands, resulted in supernumerary hair cells (Lanford et al., 1999). Additionally, alteration of Notch signaling by either *Notch1* or *Jagged1* antisense oligonucleotides resulted in an increase in the number of hair cells in organotypic cultures of the developing cochlea (Zine et al., 2000a). These similarities in phenotypes when *Notch1* and *Hes1/Hes5* functions are altered suggest that Notch1 and *Hes*-type factors act in the same pathway during cochlea development.

In comparison, the expression of *Hes5* and to a lesser extent that of *Hes1* in the developing cochlea is quite similar to that of *Notch1* and *Jagged1* in terms of their spatial distribution. Previous studies have shown that both *Notch1* and its ligand *Jagged1* were expressed throughout the prospective cochlear sensory epithelium before hair cell differentiation (Morrison et al., 1999; Zine et al., 2000a). Their expression was downregulated in the differentiating hair cells and persisted in supporting cells and in undifferentiated cells within the GER and LER of the developing cochlea. Altogether, these results may support the role of *Hes1* and *Hes5* as downstream mediators of Notch1 signaling pathway in the cochlea and a model in which Notch1-mediated lateral inhibition participates in the regulation of hair cell differentiation. However, phenotypic characterization of both *Hes1* and *Hes5* null mutant cochleae suggests that the function of these *Hes* genes is not restricted to a lateral inhibitory mechanism between neighboring cells. We did not observe regions of the organ of Corti displaying only hair cells without interposed supporting cells in *Hes1*^{-/-} and *Hes5*^{-/-} mice, as would be expected if the *Hes1* and *Hes5* genes operate only in a lateral inhibitory manner. According to the early expression of *Hes1* in the embryonic mouse cochlea at E14 (Fig. 1), in combination to its broad expression throughout cells of the GER and LER (Fig. 2), *Hes1* might also serve as a pre patterning gene, acting to demarcate sensory epithelium versus nonsensory epithelium progenitor cell domains against which determination–differentiation cues are directed. In contrast to *Hes1*, *Hes5* expression was observed, in addition to GER and LER, in the supporting cells, which includes Deiter's cells and pillar cells within the sensory epithelium of E18 cochlea (Fig. 2D). This spatial distribution of *Hes5* in the cell types that are closely apposed to the differentiating OHCs may support a role of *Hes5* in Notch-mediated lateral inhibition because Deiter's cells and pillar cells also express Notch1 receptor at this stage of maturation (Zine et al., 2000a).

Such a system parallels to some extent a role of their *Drosophila* homolog genes, the *hairy* and [*E(spl)*] complex in the generation of mechanosensory bristles that resemble the basic feature of vertebrate mechanosensory hair cells (Chan and Yan, 1999). In *Drosophila*, the *hairy* gene participates in proneural cluster positioning as a pre patterning gene and the [*E(spl)*] genes act at a later stage in the repression of local neuronal fates within the proneural cluster. This later function is dependent on Notch activation of the genes of the [*E(spl)*] complex. In both cases, the effect of *hairy* and [*E(spl)*] complex genes is the negative regulation of neurogenesis through repression of *bHLH* genes, such as *atonal* (a *Drosophila* homolog of *Math1*) and the *achaete–scute* complex (Jennings et al., 1994; Fisher and Caudy, 1998). Therefore, *Hes1* and *Hes5* may have Notch-dependent and/or Notch-independent activities in the developing mammalian inner ear, as is possible with their *Drosophila* homologs, and raise the hypothesis that the function of Notch–*Hes*–*Math1* pathway has been evolutionarily conserved.

Our findings demonstrate that *Hes1* and *Hes5* activities are important for repressing the commitment of progenitor cells to IHCs and OHCs fates, respectively, likely by antagonizing *Math1*. This negative regulation is critical for the correct number of hair cells to be produced and for the establishment of the normal cochlear mosaic of a single row of IHCs and three rows of OHCs. In the vestibular system, *Hes1* and *Hes5* also act as negative regulators of hair cell differentiation within the utricle and saccule epithelia.

A complete understanding of the role of the Notch–Hes–*Math1* pathway directing hair cell fate in the mammalian inner ear may help to develop strategies to solve the problem of auditory hair cells loss. It is possible that simultaneous downregulation of both of *Hes1* and *Hes5* in the cochlea might be used to stimulate the replacement of lost auditory hair cells. Such studies may have a significant therapeutic value, because loss of auditory hair cells through disease, trauma, and aging is a common cause of hearing loss and/or deafness.

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