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Cooperative functions of *Hes/Hey* genes in auditory hair cell and supporting cell development

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Abstract

Notch-mediated lateral inhibition has been reported to regulate auditory hair cell and supporting cell development from common precursors. While the Notch effector genes Hes1, Hes5 and Hey1 are expressed in the developing cochlea, inactivation of either of them causes only mild abnormality, suggesting their functional redundancy. To explore the roles of Hes/Hey genes in cochlear development, we examined compound heterozygous or homozygous mutant mice that lacked Hes1, Hes5 and Hey1 alleles. We found that a reduction in Hes/Hey gene dosage led to graded increase of hair cell formation. However, if at least one allele of Hes1, Hes5 or Hey1 was intact, excessive hair cells were accompanied by overproduction of supporting cells, suggesting that the hair cell increase does not occur at the expense of supporting cells, and that each Hes/Hey gene functions to induce supporting cells. By contrast, when all alleles of Hes1, Hes5 and Hey1 were inactivated, the number of hair cells increased more drastically, whereas that of supporting cells was unchanged compared with control, suggesting that supporting cell formation was balanced by their overproduction and fate conversion into hair cells. The increase of the cell numbers seemed to occur after the prosensory domain formation in the mutants because the proliferation state and the size of the prosensory domain were not affected. Thus, Hes1, Hes5 and Hey1 cooperatively inhibit hair cell formation, and one allele of either Hes1, Hes5 or Hey1 is sufficient for supporting cell production probably by lateral inhibition in the sensory epithelium. Strikingly, Hes/Hey mutations lead to disorganized cell alignment and polarity and to hearing loss despite hair cell overproduction. These results suggest that Hes/Hey gene dosage is essential not only for generation of appropriate numbers of hair cells and supporting cells by controlling cell proliferation and lateral inhibition but also for the hearing ability by regulating the cell alignment and polarity.

Key words: hair cell; supporting cell; Hes1; Hes5; Hey1; developing cochlea
Introduction

A specialized sensory epithelium of the cochlea of the mammalian inner ear, called the organ of Corti, contains sensory hair cells and non-sensory supporting cells. These cells are aligned in rows: one row of inner hair cells (IHC), three rows of outer hair cells (OHC), and supporting cells that surround each row of hair cells. During development, the prosensory domain is formed in the floor of the cochlear duct, and then hair cells and supporting cells differentiate from common precursors in the prosensory domain. The mechanism of the alternate hair cell and supporting cell formation has been extensively analyzed, and it has been shown that Notch-mediated lateral inhibition plays an important role in this process (Kelly, 2006). Notch-mediated lateral inhibition is known to generate two cell types: Notch ligands activate Notch signaling in neighboring cells, while activation of Notch signaling leads to expression of repressor genes such as Hes1, thereby inducing adoption of a fate different from Notch ligand-expressing cells (Kageyama et al., 2007).

Hair cells express the Notch ligands Deltalike1 (Dll1), Deltalike3 (Dll3) and Jagged2 (Jag2) (Lanford et al., 1999; Morrison et al., 1999; Hartman et al., 2007), while supporting cells express the Notch effector genes Hes1, Hes5, Hey1, Hey2 and HeyL (Hayashi et al. 2008; Li et al. 2008; Doetzlhofer et al., 2008), suggesting that hair cells activate Notch signaling in the neighboring supporting cells. Genetic ablation of Notch ligand or effector genes leads to overproduction of hair cells (Kiernan et al., 2005), and treatment of a γ-secretase inhibitor, which blocks Notch signaling, increases the number of hair cells at the expense of supporting cells in the cochlear explant culture (Takebayashi et al., 2007). Thus, it is likely that hair cells inhibit the neighboring cells from differentiating into the same cell type by activation of Notch signaling, and that the latter cells adopt the alternative fate, supporting cells. In agreement with this idea, hair cells can induce surrounding cells to develop as supporting cells (Woods et al., 2004). These results suggest that Notch-mediated lateral inhibition regulates hair cell versus supporting cell specification.

In spite of extensive studies, the role of Notch signaling in lateral inhibition is still obscure. In Dll1; Jag2 double mutant cochlear, hair cells increased in number, but the concomitant decrease of the supporting cells was much milder (Kiernan et al., 2005). Furthermore, in compound mutant mice that lacked the Notch effectors Hes and Hey genes, excessive hair cell formation was accompanied by overproduction of supporting cells (Li et al., 2008). Thus, the fate change from supporting cells to hair cells does not account for overproduction of hair cells, and it has been suggested that Notch
signaling has an additional role in cell proliferation (Kiernan et al., 2005; Murata et al., 2009).

To investigate the pleiotropic roles of Notch signaling, we examined the cochlea of compound heterozygous or homozygous mutant mice that lacked Hes1, Hes5 and Hey1 alleles. We found that a reduction in Hes/Hey gene dosage led to graded increase in hair cell formation accompanied by overproduction of supporting cells, and that only when all of Hes1, Hes5 and Hey1 were inactivated, the number of hair cells increased at the expense of supporting cells. These results suggest that Notch signaling is essential for generation of appropriate numbers of hair cells and supporting cells by regulating cell proliferation and lateral inhibition. We also found that Hes/Hey mutations lead to hearing loss despite hair cell overproduction.

Materials and methods

Mice breeding
Hes1 floxed, Hes3;Hes5 knockout (Hatakeyama et al., 2004; Imayoshi et al., 2008), Hey1(Hesrl) knockout (Kokubo et al., 2005) and Emx2^Cre mice (Kimura et al., 2005) were used to produce various compound heterozygous or homozygous mutant mice. The loci of Hes3 and Hes5 are close to each other, and Hes5 deficiency was accompanied by Hes3 deficiency in this study. However, because there was no Hes3 expression in the cochlea (see Supplemental Fig. S5C), Hes3 was not mentioned in this study. Hes1^[Δ];Hes5^[Δ];Hey1^[Δ] mice were crossed with Emx2^Cre;Hes1^[Δ];Hes5^[Δ];Hey1^[Δ] mice, Emx2^Cre;Hes1^[Δ];Hes5^[Δ];Hey1^[Δ] mice, Emx2^Cre;Hes1^[Δ];Hes5^[Δ];Hey1^[Δ] mice, or Emx2^Cre;Hes1^[Δ];Hes5^[Δ];Hey1^[Δ] mice. In the following, Hes1^[Δ] and Hes1^[Δ] are the abridgements of Emx2^Cre;Hes1^[Δ] and Emx2^Cre;Hes1^[Δ], respectively. For analysis of Cre recombinase activity, Emx2^Cre mice were crossed with ROSA26-CFP mice (Srinivas et al., 2001). These mice were maintained on C57BL/6; ICR mixed background. Plug date was defined as embryonic day 0.5 (E0.5).

Histochemistry and in situ hybridization
Whole heads (E10.5–E14.5) or inner ears (E17.5-adult) were immediately fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline pH7.4 (PBS), cryoprotected in 30% sucrose in PBS and embedded in OCT for cryostat sectioning.

Immunostaining of cochlear sections was performed, as described previously (Imayoshi et al., 2008). Primary antibodies used for this study were: anti-BrdU
(Oxford Biotechnology, rat monoclonal, 1:100 dilution), anti-p27Kip1 (BD Transduction Laboratories, mouse monoclonal, 1:200), anti-MyosinVI (Proteus Bio-Sciences, rabbit polyclonal, 1:200), and anti-Prox1 (Chemicon, rabbit polyclonal, 1:1000). Secondary antibodies were goat or donkey anti-species IgG conjugated with Alexa 405, Alexa 488 or Alexa 594. Nuclei were labeled by 4,6-diamidino-2-phenylindole (DAPI). For p27^{Kip1}, Prox1 and BrdU staining, samples were heated in 10mM sodium citrate at 90°C for 10 min (p27^{Kip1} and Prox1) or 30 min (BrdU) prior to staining procedure. For staining of whole mount preparations of the cochlea, cochlear ducts were opened to expose the developing sensory epithelia prior to staining procedure (Yamamoto et al., 2009). Apoptotic cells were detected using Aoptag® Fluorescein In Situ Apoptosis Detection Kit (Millipore), following the manufacturer's instruction.

In situ hybridization was carried out using mouse *Hes1*, *Hes3*, *Hes5*, *Hey1*, *Hey2*, *HeyL* and *Hes3* and *Atoh1* probes, as described previously (Imayoshi et al., 2008). Immunolabeling of MyosinVI was performed after in situ hybridization as described above.

**Quantification of hair cells**
The number of hair cells in E18.5 mice was counted on cochlear surface preparations processed with MyosinVI immunohistochemistry, as described previously (Zine et al., 2001). We analyzed a region that covered a 1.6-mm length of the organ of Corti including the hook region, beginning at the basal end of cochlea and extending toward the middle turn.

**Cell counts of supporting cell marker- and MyosinVI-immunolabeled sections**
Every 2 sections of all mid-modiolar serial sections were labeled for DAPI, Prox1 and p27^{Kip1} or for DAPI and MyosinVI. Sections were analyzed with LSM510 confocal microscopy (Zeiss). Inner hair cells, outer hair cells, Prox1^+ cells, and p27^{Kip1}-positive and Prox1-negative cells in the basal and middle turns of the organ of Corti were counted. Prox1^+ cells include Pillar cells and Deiters’ cells. p27^{Kip1}-positive and Prox1-negative cells in the greater epithelial ridge were regarded as inner phalangeal cells. The total number of each cell type per cochlea duct section was calculated. At least 25 sections per cochlea and at least 3 animals per genotype were used.

**Bromodeoxyuridine (BrdU) administration**
BrdU (Sigma) was dissolved in PBS. 50 μg/g body weight of BrdU was given to the
pregnant mice at E13.5, E14.5 or E17.5 by a single intraperitoneal injection at a concentration of 10 mg/ml. Every 2 sections of all mid-molar serial sections were labeled for DAPI, BrdU and MyosinVI, or Ki67, BrdU and MyosinVI. The number of BrdU+ cells and MyosinVI+ cells, or Ki67+ cells and BrdU+ cells were counted.

**Quantitative reverse transcription PCR (qRT-PCR)**
E14.5 cochlear epithelia were dissected and mesenchyme of cochlear tissue was removed using a thermolysin treatment as previously described (Yamamoto et al. 2009). Total RNA was reverse-transcribed by using Rever-Tra Ace (TOYBO) and Random Primer (TOYBO). Real-time PCR was done by using Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) and THUNDERBIRD SYBR qPCR Mix (TOYBO), according to the manufacturer’s protocols. GAPDH was used as a control. The following primers were used for real-time PCR: Hes1 forward, tgaaggattcacaataaaattctctggg; Hes1 reverse, cgctcttctcatgtagctctttgtagac; GAPDH forward, atctctttgctgactcagccttgtcccg; and GAPDH reverse, agttgagtcattgaaggggtcgttgatgg.

**Statistical analysis**
Three or more embryos for each genotype group were analyzed in all experiments. A repeated-measures analysis of variance and a Student-Newman-Keuls test were used to detect differences among groups. Differences at p < 0.05 were regarded as statistically significant.

**ABR recording**
An Auditory Brainstem Response (ABR) recording was used to monitor the auditory function of the experimental animals. Under general anesthesia, ABR measurements were performed as previously described (Kada et al., 2008). Thresholds were determined for the frequencies of 10, 20, and 40 kHz from a set of responses at varying intensities with 5-dB Sound Pressure Level intervals.

**Results**

**The expression of Hes1, Hes5 and Hey1 in the developing auditory sensory epithelium**
We first examined expression of Hes1, Hes5 and Hey1 in the developing inner ear. Hes1
expression detected by in situ hybridization was faint, and therefore Venus-Hes1 mice were used; Venus (a YFP variant) fragment was knocked in at the Hes1 locus so that the Venus-Hes1 fusion protein was expressed under the control of the endogenous Hes1 promoter (It will be reported elsewhere). At E11.5, Venus-Hes1 was slightly expressed in the epithelium of presumptive cochlea (Fig. 1A arrow, and B), but more abundant in mesenchyme around the epithelium. Hey1 was broadly expressed in the prosensory domain, while Hes5 was not detected in the epithelium of otocyst (Fig. 1D-F,H-J). At E13.5, Hey1 was expressed in the prosensory domain, whereas Hes1 and Hes5 expression were not seen in the cochlea duct (Fig. 1C,G,K). In agreement with the previous data (Hayashi et al., 2008; Li et al., 2008), Hes1 and Hey1 expression were seen in the E14.5 cochlea duct (Fig. 1R,S,U,V). Hes5 was also expressed near hair cells (Atoh1+) (Fig. 1M,P). At E17.5, Hes1 was expressed by Hensen cells, while Hes5 was expressed by inner pharangeal cells and Deiters’ cells, and Hey1 expression was seen in Kollikers organ, Deiters’ cells and Hensen cells (Fig. 1Q,T,W), similar to the previous data (Zheng et al., 2000; Zine et al., 2001; Hayashi et al., 2008; Li et al., 2008; Doetzlhofer et al., 2008). Thus, Hes/Hey genes were continuously expressed both in the early presumptive cochlea epithelium and in the organ of Corti at a lateral inhibition phase.

**Cre recombinase activity in the developing cochleae of Emx2^{+\text{Cre}} mice**

To determine the Cre recombinase activity in the developing cochleae of Emx2^{+\text{Cre}} mice, we crossed these mice with ROSA26-CFP reporter mice. At E11.5, CFP expression was observed only in subsets of otocyst epithelial cells (Supplemental Fig. S1A-E). However, at E13.5, CFP expression occurred in most epithelial cells of the cochlea duct including the prosensory domain, which was labeled with anti-p27^{kip1} antibody (Supplemental Fig. S1F,J, arrows), and of the organ of Corti at E18.5 and P1 (Supplemental Fig. S1K,L,P,Q,R). CFP expression also occurred in most cells of both the greater epithelial ledge and lesser epithelial ledge (Supplemental Fig. S1L) as well as in most hair cells (Supplemental Fig. S1Q) and supporting cells (Supplemental Fig. S1R). CFP expression was limited to the epithelial cells in the cochleae, and only several CFP$^+$ cells were observed in the spiral ganglion (Supplemental Fig. S1K,P, arrowheads) and the maculae of sacculus and utricle (Supplemental Fig.S1M,N). No CFP$^+$ cells were seen in the crista ampullaris of the semicircular canals (Supplemental Fig. S1O). These reporter expression patterns were consistent with Emx2 expression in the inner ears (Holley et al., 2010).

The above results indicated that the Cre recombinase became fully active by
E13.5 in the cochlea of Emx2^{Cre} mice. In agreement with this idea, Hes1 mRNA levels were reduced to 64% and 9% in the cochlea epithelium of Emx2^{Cre};Hes1^{fl/fl} (designated Hes1^{fl/fl}) and Emx2^{Cre};Hes1^{l/s} (designated Hes1^ΔΔ) mice, respectively, at E14.5 (Supplemental Fig. S1S), suggesting that Hes1 expression was effectively lost by E14.5 in Hes1 conditional knock out mice.

It was previously reported that extra inner hair cells and outer hair cells were found in Emx2 heterozygous cochlea, although no quantification analysis was performed (Rhodes et al., 2003). It was also reported that Emx2 knock out mice have shortened cochlea with effects on both proliferation and differentiation of hair cells whereas Emx2 heterozygous mice have no phenotypes in the cochlea (Holley et al. 2010). We compared the inner and outer hair cell numbers of Emx2^{Cre} cochlea with those of wild type animals, but there was no significant difference (Fig. 2I). Furthermore, we performed Auditory Brainstem Response (ABR) recording of adult Emx2^{Cre} mice, and these mice were found to have no hearing impairment (data not shown). Thus, we decided to use Emx2^{Cre} mice as a Cre driver to inactivate Hes1 in the developing cochlea.

**Graded increase in hair cell formation with a reduction in Hes/Hey gene dosage**

The gross appearance of Hes/Hey compound mutant cochlea appeared to be normal (Fig. 2A,B), but a reduction in Hes/Hey gene dosage led to graded increase in hair cell formation (Fig. 2C-H). To compare the number of hair cells, we counted hair cell marker-positive cells over the length of 1.6 mm from the basal end of E18.5 cochlea, corresponding to approximately one quarter of the cochlea length. Wild type, Emx2^{Cre} and Hes1^ΔΔ;Hes5^{l/s};Hey1^{l/s} mice were not significantly different from one another in both the inner and outer hair cell numbers (Fig. 2C,I). Mice that lacked either Hes5 (Hes5^{l/s};Hey1^{l/s}) or Hey1 (Hes5^{l/s};Hey1^{l/s}) or both (Hes5^{l/s};Hey1^{l/s}, Fig. 2D) but retained one Hes1 allele (Hes1^ΔΔ) exhibited mild increase of both inner and outer hair cells (Fig. 2D, red- and green-labeled, and Fig. 2I), although this increase was not significant (Supplemental Table S1). However, in mice that lacked Hes1 (Hes1^ΔΔ;Hes5^{l/s};Hey1^{l/s}), the numbers of both inner and outer hair cells were significantly increased (p<0.01) from the former groups (Fig. 2E,I, Supplemental Table S1). Additional deletion of Hes5 (Hes1^ΔΔ;Hes5^{l/s};Hey1^{l/s}) or Hey1 (Hes1^ΔΔ;Hes5^{l/s};Hey1^{l/s}) did not significantly change the inner and outer hair cell numbers compared with Hes1^ΔΔ;Hes5^{l/s};Hey1^{l/s} (Fig. 2F,G,I, Supplemental Table S1). When all genes were inactivated (Hes1^ΔΔ;Hes5^{l/s};Hey1^{l/s}), both inner and outer hair cells were further increased in number (Fig. 2H,I, and Supplemental Table S1). These
results indicate that Hes1, Hes5 and Hey1 cooperatively inhibit hair cell formation, while Hes1 is the most effective.

The cochleae of Hes1<sup>+/−</sup>;Hes5<sup>+/−</sup>;Hey1<sup>−/−</sup> mice were shorter than those of the other genotypes (p<0.01), but the difference was less than 10% (Supplemental Fig. S2), suggesting that not only the numbers per length but also the total numbers of both inner and outer hair cells were significantly increased in Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> mice. The average numbers of hair cell rows in the cochlear displayed 1.5-fold to 2-fold increase in Hes/Hey compound mutant cochlea (Figs. 2I, 3Q, 4J, and Supplemental Fig. S3), indicating that the total number of hair cells indeed increased in the absence of Hes/Hey genes. Hair cell overproduction occurred similarly in the regions of 25%, 50% and 75% from the basal end of the E18.5 mutant cochlea, suggesting that there is no apparent regional difference in the phenotypes (Supplemental Fig. S4).

**Changes of supporting cells that surround outer hair cells in Hes/Hey mutants**

We next examined how supporting cell formation is affected by hair cell increase in the mutant cochlear. We first analyzed supporting cells that surround outer hair cells. Prox1 was expressed in the nuclei of specific subtypes of supporting cells, pillar cells (Fig. 3D, yellow arrows) and Deiters’ cells (Fig. 3D, yellow arrowheads) at E18.5. Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea showed normal 5 rows of Prox1<sup>+</sup> cells (Fig. 3D,G) and 3 rows of outer hair cells (Fig. 3A,H). In Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea, excessive hair cells were formed (Fig. 3J, arrows), and excessive Prox1<sup>+</sup> cells were also present at the same place (Fig. 3I, arrowheads), indicating that an extra row of outer hair cells were accompanied by an extra row of Deiters’ cells. Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea (Fig. 3B,L) and Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea (Fig. 3N) had more outer hair cells, and these excessive hair cells were accompanied by excessive Prox1<sup>+</sup> cells, which mostly formed 6 rows (Fig. 3E,K,M). These results suggest that outer hair cells and supporting cells were overproduced in pairs in these compound mutant cochleae. However, in Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea, much more outer hair cells were formed (Fig. 3C,P), whereas Prox1<sup>+</sup> cells were disarranged or missing at the same place (Fig. 3F,O, white asterisks). Thus, when all Hes1, Hes5 and Hey1 were inactivated, outer hair cell increase was accompanied by relative decrease of Deiters’ cells (Fig. 3Q and Supplemental Fig. S3).

Quantification of outer hair cells and Prox1<sup>+</sup> pillar and Deiters’ cells in the basal and middle turn on serial sections of E18.5 cochleae showed that Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea had on average 3.1 outer hair cells and 5.2 Prox1<sup>+</sup> pillar and Deiters’ cells (Fig. 3Q). In Hes1<sup>+/−</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea, both outer hair cells
and Prox1+ cells were significantly increased in number to 3.9 and 5.8, respectively (P<0.01, Supplemental Table S2). If the remaining Hey allele was further deleted (Hes1ΔA;Hes5+/−;Heyl−/−), there were even more outer hair cells (4.7, P<0.01, Supplemental Table S2A), but less Prox1+ cells (5.1, P<0.01, Supplemental Table S2B) than Hes1ΔA;Hes5−/−;Heyl−/− cochlea, although the number of Prox1+ cell rows varied between 4 to 6. When all Hes1, Hes5 and Heyl were inactivated (Hes1ΔA;Hes5−/−;Heyl−/−), excessive hair cell formation occurred in the same plane as decreased Deiters’ cell formation (Fig. 3C,F), suggesting that excessive hair cells are formed at the expense of supporting cells, and that this fate conversion balanced supporting cell overproduction.

**Changes of supporting cells that surround inner hair cells in Hes/Hey mutants**

We also analyzed inner phalangeal cells, supporting cells that surround inner hair cells. Inner phalangeal cells are negative for pillar cell markers such as p75NTR and Prox1 but express p27Kip1, which can label all subtypes of supporting cells at E18.5 cochlea. In Hes1ΔA;Hes5−/−;Heyl−/− cochlea, which had excessive inner hair cells, inner phalangeal cells increased in number (Fig. 4D,E, brackets, F, asterisks, J, and Supplemental Table S2B), compared with Hes1+Δ;Hes5+/−;Heyl+/− and Hes1−/Δ;Hes5−/−;Heyl−/− cochlea (Fig. 4A,B, brackets, C, asterisks, J, and Supplemental Table S2B). These results indicated that excessive inner hair cell formation was accompanied by excessive supporting cell formation (Fig. 4J and Supplemental Fig. S3). However, when all Hes1, Hes5 and Heyl were inactivated (Hes1ΔA;Hes5−/−;Heyl−/−), inner hair cells increased further, whereas phalangeal cells relatively decreased in number to the level that was not significantly different from Hes1+Δ;Hes5+/−;Heyl+/− cochlea (Fig. 4G,H, brackets, I, asterisks, J, Supplemental Fig. S3 and Supplemental Table S2B), suggesting that further increase of the inner hair cell number occurred at the expense of supporting cells, and that this fate conversion balanced supporting cell overproduction.

These results together suggest that the overproduction of hair cells by reduction of Hes/Hey gene dosage was accompanied by supporting cell overproduction if at least one allele of either Hes1, Hes5 or Heyl was intact. However, if all alleles of Hes1, Hes5 and Heyl were inactivated, it is likely that the number of hair cells increased at the expense of supporting cells. In Hes1ΔA;Hes5−/−;Heyl−/− cochlea, however, there were still many supporting cells, suggesting that the fate conversion was not complete. This was probably due to compensation by Hey2 and HeyL, which were expressed similarly in both the control and Hes1ΔA;Hes5−/−;Heyl−/− cochlea (Supplemental Fig. S5).
Cell alignment and polarity were disturbed by Hes/Hey deficiency

In the developing cochleae, each hair cell gradually forms both stereocilia and kinocilium, which have a unique polarity: a bundle of stereocilia, visualized by phalloidin, forms a V shape directing outward, and a kinocilium (acetylated α-tubulin*) is located at the tip of the V shape. We next assessed the alignment and polarity of hair cells in Hes/Hey compound mutant cochlea by examining the stereocilia and kinocilium. In Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea, hair cells were properly aligned, and the polarity of stereocilia and kinocilium was mostly normal (Fig. 5A,B). In Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea, most outer hair cells were properly aligned, and the polarity of stereocilia and kinocilium was mostly normal (Fig. 5C,D). However, although excessive inner hair cells were aligned in two rows, the polarity of the stereocilia and kinocilium of inner hair cells was disorganized in Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea (Fig. 5C,D). When all Hes1, Hes5 and Hey1 were inactivated (Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup>), the alignment of both inner and outer hair cells was severely disorganized and hair cell rows were wavy and disconnected (Fig. 5E,F). Furthermore, the polarity of stereocilia and kinocilium of inner and outer hair cells was abnormal, pointing rather randomly (Fig. 5E,F). These results indicated that the cell alignment and polarity were more severely disorganized as Hes/Hey gene dosage decreased.

Abnormal cell proliferation in the epithelium by Hes/Hey deficiency

If at least one allele of either Hes1, Hes5 or Hey1 was intact, excessive hair cells were accompanied by excessive formation of supporting cells, suggesting that the cell proliferation is enhanced by decrease of Hes/Hey gene dosage. We thus examined the cell proliferation of these mutant cochleae. At E18.5, the proliferation marker Ki67 was not detectable in hair cells or their neighboring supporting cells of Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> and Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea as well as in the control (Fig. 6A-D and data not shown) except for Kolliker’s organ. In contrast, in Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea, Ki67 expression was observed in some cells next to hair cells (Fig. 6E, F, arrows), suggesting that cell proliferation was prolonged in the absence of Hes/Hey genes.

We also administered BrdU at E14.5 and examined BrdU uptake at E18.5. There were no BrdU<sup>+</sup> hair cells or supporting cells in the control and Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea (Fig. 6G,H,M), suggesting that all hair cell/supporting cell precursors exited the cell cycle by E14.5. However, BrdU<sup>+</sup> supporting cells were observed in Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> and Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea (Fig. 6I-L, arrows). Moreover, some hair cells incorporated BrdU in Hes1<sup>Δ/Δ</sup>;Hes5<sup>−/−</sup>;Hey1<sup>−/−</sup> cochlea.
cochleae (Fig. 6K,L, arrows). The proportions of BrdU+ hair cells were calculated in the basal and middle turn on serial sections of E18.5 cochleae. No BrdU+ hair cells were found in Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlea (Fig. 6M). In contrast, the proportion of BrdU incorporation in hair cells of Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlea was about 2.7%, which was significantly higher than that of Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} (p<0.01), Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} (p<0.05), and Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlea (p<0.05) (Fig. 6M). These proportions could be underestimated because BrdU was administered only once (at E14.5) to the mother mice in this experiment. These results together suggest that cell proliferation is prolonged by decrease of Hes/Hey gene dosage, and that particularly in Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlear, a substantial number of hair cell/supporting cell precursors are proliferating at E14.5, when normal precursors have already exited the cell cycle.

We further evaluated the increased cell proliferation of Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlear. BrdU was administered at E17.5 and the cell proliferation in cochlea sensory epithelium was analyzed at E18.5 (Supplemental Fig. S6). The proportion of Ki67\textsuperscript{−}BrdU\textsuperscript{+} cells (Supplemental Fig. S6A,B, arrows) to Ki67\textsuperscript{+} cells was only 9.2%, suggesting that the majority of dividing cells (Ki67+) did not enter the S phase during the past day. Similarly, the proportion of Ki67\textsuperscript{−}BrdU\textsuperscript{+} cells to BrdU\textsuperscript{+} cells was only 6.6%, suggesting that the majority of the cells that entered the S phase during the past day exited the cell cycle. Furthermore, only a few Ki67\textsuperscript{−}BrdU\textsuperscript{+} and BrdU\textsuperscript{+} cells were found in pairs, suggesting that continuously dividing cells are rare during E17.5-18.5 in Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlear. BrdU\textsuperscript{+} or Ki67\textsuperscript{+} hair cells were occasionally detectable (Supplemental Fig. S6C,D), suggesting that some dividing cells can give rise to hair cells. Nevertheless, the number of dividing cells in the prosensory domain seemed to be too small to account for the overall increase of the cell number in the mutant cochlear (IHC:OHC = 1:3 in the wild type and 2:5 in Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−}). Because there were many Ki67\textsuperscript{+} and BrdU\textsuperscript{+} cells near the border of the prosensory domain (Fig. 6), some excessive hair and supporting cells could derive from the outside of the prosensory domain, although definitive evidence for this possibility remains to be demonstrated.

**Prosensory domain appears to be unaffected in Hes/Hey mutants**

We next examined whether increased cell proliferation is involved in expansion of the prosensory domain. In the control cochleae, p27\textsuperscript{Kip1} and Sox2 expression occurred at both E14.5 and E15.5 (Fig. 7C,D,K,L), and similar expression was observed in Hes1\textsuperscript{AA}:Hes5\textsuperscript{+/−}:Hey1\textsuperscript{+/−} cochlea (Fig. 7G,H,O,P). The size of the p27\textsuperscript{Kip1} and Sox2
expression region was similar between the control and $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ cochlea, suggesting that the prosensory domain did not expand in $Hes/Hey$ mutants. MyosinVI+ hair cells and Prox1+ supporting cells were mostly absent at E14.5 in the control and $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ cochlea (Fig. 7A,B,E,F). At E15.5, there were some MyosinVI+ outer hair cells and Prox1+ supporting cells in both the control and $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ cochlea (Fig. 7I,J,M,N). Thus, the onset of hair cell and supporting cell differentiation in the prosensory domain was not affected by $Hes/Hey$ deficiency.

The cell proliferation feature of the prosensory domain of the control siblings and $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ mutants was further examined. BrdU was administered at E13.5 and the cell proliferation was analyzed at E14.5. The majority of BrdU+ cells were negative for Ki67 in both cochleae at E14.5, indicating that most proliferating prosensory cells at E13.5 became quiescent at E14.5 (Supplemental Fig. S7). These results suggest that $Hes/Hey$ deficiency did not affect the timing of cell cycle exit.

**Hearing loss by $Hes/Hey$ deficiency**

Some of the $Hes/Hey$ compound mutant mice, which exhibited excessive hair cell and supporting cell formation, survived more than 2 months. We next recorded auditory brainstem responses of these mutant mice to determine whether excessive hair cells affect the hearing ability. Both control and mutant mice showed hearing loss at a high frequency (40kHz) probably due to the background containing C57BL/6. The thresholds of the lower frequencies were not significantly affected in $Hes5$-null (#566) and $Hey1$-null (#495) mice (Table 1), while $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ mice (#692) exhibited some mild hearing defect at 20kHz (Table 1). By contrast, in $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ (#562) and $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ mice (#923), the thresholds of the lower frequencies were significantly increased, indicating severe hearing loss (Table 1).

We next examined the histology of the cochlea of adult mutant mice. The cochlea of $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ mice had a mild increase in the hair cell number at E18.5 (Fig. 2I) but seemed to be normal at 9 months of age (Supplemental Fig. S8A), suggesting that excessive hair cells were eliminated after birth. $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ cochlea had nearly two rows of inner hair cells (Fig. 2F,I) and the polarity of the stereocilia and kinocilium of inner hair cells was disorganized at E18.5 (Fig. 5C,D). Notably, histological analysis of $Hes1^{+/A};Hes5^{1/2};Hey1^{1/-}$ cochlea at six months of age showed that inner hair cells formed into almost one row (Supplemental Fig. S8B,C), indicating that the number of excessive inner hair cells decreased after birth. There
were still excessive outer hair cells in the adult *Hes1*^ΔA;*Hes5^-/-;Hey1^+/−* cochlea at six months of age (Supplemental Fig. S8B,C), but they seemed to be decreased compared with E18.5 cochlea. Indeed, many extra hair cells underwent apoptosis in *Hes*/Hey mutant cochlear at P3 (Supplemental Fig. S9). The polarity of outer hair cells was maintained almost normally, but the alignment of outer hair cells was disturbed (Supplemental Fig. S8B,C). Neurons of spiral ganglion were maintained in the adult *Hes1*^ΔA;*Hes5^-/-;Hey1^+/−* cochlea, suggesting that the hearing loss was not due to the defect of spiral ganglion (Supplemental Fig. S8D,E). These data together suggest that excessive hair cells do not improve the hearing ability, and that the polarity and alignment of these cells are important for hearing. Moreover, many excessive hair cells died rather than became integrated into the hair cell rows to function as normal hair cells.

**Discussion**

**Reduced Hes/Hey gene dosage leads to graded phenotypes in hair and supporting cell formation**

It has been shown that Notch signaling inhibits hair cell formation, and that Notch-mediated lateral inhibition regulates hair cell versus supporting cell development from common precursors (Lanford et al., 1999; Kienan et al., 2005; Brooker et al., 2006; Takebayashi et al., 2007). Several Notch target genes, including *Hes1*, *Hes5*, *Hey1*, *Hey2* and *HeyL*, have been reported to be expressed in supporting cells (Lanford et al., 2000; Zheng et al., 2000; Zine et al., 2001; Murata et al., 2006; Hayashi et al., 2008; Li et al., 2008; Hartman et al., 2009; Doetzlhofer et al., 2009). The expression of *Hes1*, *Hes5* and *Hey1* occurred in different but overlapping subtypes of supporting cells. *Hey1* mRNA was expressed broadly and continuously from E11.5 to E17.5, whereas *Hes1* expression was faint at all ages. *Hes5* expression was limited to differentiating cells after E14.5. These expression patterns do not necessarily reflect the activities of endogenous Hes/Hey proteins. It has been reported that *Hes1* deficiency leads to predominant increase of the inner hair cell number, whereas *Hes5* deficiency causes predominant increase of the outer hair cell number (Zheng et al., 2000; Zine et al., 2001). Such tendency was not observed in *Hes1*, *Hes5* and *Hey1* compound hetero- or homozygous mutant mice. Mice that lacked either *Hes5* or *Hey1* or both in *Hes1*^ΔA* background exhibited mild increase of inner and outer hair cell formation, while mice that lacked *Hes1* in *Hes5^-/-;Hey1^+/−* background showed more significant increase of
both inner and outer hair cell formation. When all Hes1, Hes5 and Hey1 were inactivated, both inner and outer hair cells were further increased in number. These results indicate that all Hes1, Hes5 and Hey1 are involved in inhibition of both inner and outer hair cell formation, although Hes1 seems to be the most effective. In addition, hair cell overproduction was always accompanied by supporting cell overproduction if at least one allele of either Hes1, Hes5 or Hey1 was intact. Thus, all Hes1, Hes5 and Hey1 have a similar activity to promote supporting cell versus hair cell specification.

When all Hes1, Hes5 and Hey1 genes were inactivated, even more hair cells were produced without excessive formation of supporting cells. This defect is similar to the one caused by treatment with a γ-secretase inhibitor (Takebayashi et al., 2007) and to the one of Dll1; Jag2 double mutant cochlea (Kiernan et al., 2005). Furthermore, death of supporting cells was not increased in Hes1ΔN;Hes5Δν;Hey1Δν cochlea at E14.5 (data not shown), suggesting that hair cell overproduction without excessive formation of supporting cells is due to fate conversion of supporting cells to hair cells in addition to increased supporting cell formation. Therefore, Hes/Hey genes may cooperatively hold the supporting cell property and prevent the fate conversion from supporting cells into hair cells. Our results suggest that Hes1, Hes5 and Hey1 are the main Notch effectors in the developing cochlea, cooperatively determine the strength of Notch signaling, and maintain the supporting cell property.

**Hes/Hey genes function to induce supporting cells**

It has been reported that ectopic hair cells were surrounded by ectopic supporting cells (Woods et al., 2004; Driver et al., 2008; Li et al., 2008). Hair cells are thought to use specific inductive mechanisms to recruit surrounding cells to develop as supporting cells, although the precise mechanisms for this induction are unknown (Driver et al., 2009). Our results suggest that Hes/Hey genes are needed for induction of supporting cells.

We observed that supporting cells still remained in Hes1ΔN;Hes5Δν;Hey1Δν cochlea, and that Hey2 and HeyL were expressed in these supporting cells, suggesting that Hey2 and HeyL might compensate the deficiency of Hes1, Hes5 and Hey1. It is known that Hey2 is controlled by the Fgf pathway, and that this pathway specifically regulates pillar cell development (Doetzlhofer et al. 2009), suggesting that Hey2 is involved in supporting cell formation. HeyL may also have an activity to induce supporting cells, although the specific function of HeyL in cochlea remains to be determined.
What contributes excessive hair and supporting cell production

We found that if at least one allele of Hes1, Hes5, or Hey1 was intact, both hair cells and supporting cells were formed excessively in the mutant cochlea. The total numbers of hair cells and supporting cells increased in Hes1^{Δ};Hes5^{+/-};Hey1^{+/-}, Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} and Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} cochlea compared with Hes1^{+/-};Hes5^{+/-};Hey1^{+/-} cochlea. Among the former three genotypes, the total cell number seemed to be unchanged. The precise mechanism of the increase of the total cell number in these mutants remained to be determined, but our results suggest that abnormal Ki67+ cells and BrdU-incorporating cells remained at a later stage in the sensory epithelium of Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} cochlea, indicating that prolonged proliferation contributes to excessive hair cell and supporting cell formation. The cell types that are inhibited from proliferation by Hes/Hey genes are not known, but these cells could be common precursors for hair cells and supporting cells because excessive hair cells and supporting cells are often found in pairs in the mutant cochlea (Figs. 3 and 4). However, there were much fewer Ki67+ or BrdU+ hair cells in Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} and Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} cochlea than in Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} cochlea (Fig. 6M), although the total cell numbers were similar in these mutants (Supplementary Fig. S3), suggesting that prolonged proliferation alone cannot explain the defects.

Alternatively, excessive cells could be provided by reserved cells in the prosensory domain that are not supposed to differentiate into hair cells or adjacent supporting cells if Hes/Hey genes properly function. The total number of hair and supporting cells seemed to reach the plateau in Hes1^{Δ};Hes5^{+/-};Hey1^{+/-}, Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} and Hes1^{Δ};Hes5^{+/-};Hey1^{+/-} cochlea (Supplementary Fig. S3), and it is possible that such reserved cells had been consumed in these mutants. Proliferating cells near the border of the prosensory domain also could contribute to hair and supporting cell increase.

The mechanism of Notch-mediated regulation of cell proliferation

The mechanism of how Notch signaling inhibits cell proliferation in the developing cochlea is not known. Previous studies showed that sustained Hes1 expression leads to G1 arrest of neural progenitor cells by repressing the expression of cell cycle regulators such as cyclin D1, whereas oscillating Hes1 expression promotes cell cycle progression (Baek et al., 2006; Shimojo et al., 2008). Hes1 expression could be sustained in supporting cells, and without sustained Hes1 expression, these cells could
undergo extra cell divisions, although the dynamics of Hes1 expression remains to be
determined. It was reported that the CDK inhibitor p27^Kipl regulates the cell cycle exit, and
that deletion of p27^Kipl results in extension of cell proliferation in the auditory sensory
epithelium (Chen et al., 1999; Löwenheim et al., 1999., Lee et al., 2006). In the
developing cochlear epithelium of Hes1-null mice, p27^Kipl expression occurred
prematurely, and BrdU-incorporating dividing cells were reduced in number, suggesting
that the Notch-Hes1 pathway may promote proliferation of sensory precursor cells via
down-regulation of p27^Kipl expression (Murata et al., 2009). This phenotype is
opposite to our present results of Hes1 conditional knock out cochlea. This difference
could be due to the different timing of Hes1 inactivation. In the cochlea of Emt2^{+/Cre}
mice, Cre activity is weak in the prosensory domain at E11.5 but fully active by E13.5,
suggesting that Cre-induced Hes1 inactivation mostly occurred after specification of
the prosensory domain. By contrast, in conventional Hes1-null mice, Hes1 is inactive
before the prosensory domain formation. The mechanism of how Notch signaling
differentially regulates cell proliferation depending on developmental stages remains to
be determined.

**Notch signaling regulates the cell alignment and polarity**

Both inner and outer hair cells are aligned in rows (one and three rows, respectively),
and each hair cell has a unique polarity of stereociliary bundles. If at least one allele of
Hes1, Hes5, or Hey1 was intact, the hair cell rows including extra ones were relatively
well maintained in the Hes1/Hey compound mutant cochlea. However, when all three
genes were inactivated, hair cell rows became wavy and sometimes disconnected. In
the former case, excessive hair cells were accompanied by excessive supporting cells,
whereas in the latter case, hair cells were overproduced at the expense of supporting
cells. These results suggest that proper ratios of hair cells and supporting cells may be
important for organization of the cell alignment. In E18.5 Hes1^{+/Δ};Hes5^{−/−};Hey1^{−/−}
cochlea, the polarity of stereocilia and kinocilium was also severely disorganized,
suggesting that supporting cells may help hair cells keep the normal polarity. An
increase in cell numbers in the organ of Corti could also affect the mechanical
properties of the tissue, thereby affecting the cell alignment. Further studies will be
required to understand the role of supporting cells in maintenance of the alignment and
polarity of hair cells.

Some of the compound mutant mice survived more than two months, and we
were able to assess their hearing ability. Both Hes1^{+/Δ};Hes5^{−/−};Hey1^{+/−} and
Hes1⁺⁺⁺⁺;Hes5⁺⁺⁺⁺;Hey1⁻⁻⁻⁻ mice exhibited normal hearing ability. In contrast, Hes1⁺⁺⁺⁺;Hes5⁺⁺⁺⁺;Hey1⁻⁻⁻⁻ and Hes1⁺⁺⁺⁺;Hes5⁻⁻⁻⁻;Hey1⁻⁻⁻⁻ mice exhibited severe hearing impairment. Histological analysis showed that Hes1⁺⁺⁺⁺;Hes5⁻⁻⁻⁻;Hey1⁻⁻⁻⁻ cochlea had less excessive inner hair cells in the adult than at E18.5. It suggests that excessive inner hair cells died after birth probably because they could be non-functional. There were still excessive outer hair cells in the adult Hes1⁺⁺⁺⁺;Hes5⁻⁻⁻⁻;Hey1⁻⁻⁻⁻ cochlea but notably, these excessive hair cells were not helpful to the hearing ability. The polarity of outer hair cells was maintained almost normally, but the alignment of outer hair cells seemed to be disturbed. It is not clear whether the disturbance of outer hair cell alignment can be the only reason for hearing loss or not, and further analysis will be needed to know the effects of Hes/Hey deficiency on hearing function. These results imply that excessive hair cells are not useful for hearing, and that the polarity and alignment may be important for the survival and the function of hair cells. Thus, for the purpose of regenerative medicine, not only the number but also the polarity and alignment of hair cells might be important for recovery of the hearing ability.

Conclusion

Genetic inactivation of Hes1, Hes5 and Hey1 was achieved in the cochlea epithelium after prosensory domain formation. These results suggest that Hes1, Hes5 and Hey1 cooperatively inhibit hair cell formation and abnormal proliferation, and that at least one allele of either Hes1, Hes5 or Hey1 is sufficient for supporting cell production and inhibition of fate conversion from supporting cells to hair cells. Abnormal hair cell and supporting cell formation leads to disorganized cell alignment and polarity, leading to hearing loss despite hair cell overproduction.

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References

Baek JH, Hatakeyama J, Sakamoto S, Ohtsuka T, Kageyama R (2006) Persistent and high levels of
Hes1 expression regulate boundary formation in the developing central nervous system. Development 133:2467-76.


**Figure legends**

Figure 1. Expression of Hes1, Hes5 and Hey1 in the developing otic vesicle and cochlea. **A-B**, Venus-Hes1 was detected by anti-GFP antibody at E11.5 (green, arrow). Venus (a YFP variant) fragment was knocked in at the Hes1 locus so that the Venus-Hes1 fusion protein was expressed under the control of the endogenous Hes1 promoter. Hes1 was slightly expressed in the epithelium of presumptive cochlea at E11.5. Co-labeling is a prosensory marker, Sox2 (red), and DAPI (blue). **C-W**, in-situ hybridization of Hes1 (C, R-T), Hes5 (D-G,O-Q) and Hey1 (H-K,U-W). At E11.5, Hey1 was broadly expressed in the prosensory domain, while Hes5 was not detected in the epithelium of otocyst (D-F, H-J, arrows indicate the presumptive cochlea). At E13.5, Hey1 was expressed in the prosensory domain, whereas Hes1 and Hes5 expression were not seen in the cochlea duct (C,G,K, asterisks indicate the prosensory domain). At E14.5, Hes1 expression was faint, but occurred at a higher level in the greater epithelial ledge (S). Hes5 was expressed around hair cells. Hey1 expression was broad in the apical turn (U) and more limited to the prosensory domain in the mid-basal turn (V). At E17.5, Hes1 was expressed in Hensen cells (T), while Hes5 was expressed in inner pharangeal cells and Deiters’s cells (Q). Hey1 expression occurred in Kollikers organ, Deiters’ cells and Hensen cells (W). Atoh1 expression was also shown for the landmark of inner (i) and outer (o) hair cells (L-N). Scale bars: A, 500µm; B, 100µm; C (for C-K), 200µm; L (for L,M,O,P,R,S,U,V), 100µm; N (for N,Q,T,W), 50µm.
Figure 2. Graded increase in hair cell formation with a reduction in Hes/Hey gene dosage. A,B, E18.5 cochleae of Emx2\textsuperscript{Cre} and Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} mice. The gross appearance of the cochlea epithelia in Hes/Hey mutants seemed to be normal. Otic capsules and stria vascularis were removed. C-H, Whole mount immunolabeling of MyosinVI. A reduction in Hes/Hey gene dosage led to graded increase in hair cell formation. Red and green asterisks indicate excessive inner and outer hair cells, respectively. Hes1 conditional knockout cochlea (E-H) had more inner and outer hair cells than Hes1 heterozygous mutants (C,D). I, The inner (IHC) and outer hair cell (OHC) numbers per 100\textmu m were shown. Hair cells were counted over the length of 1600\textmu m from the basal end of E18.5 cochlea, corresponding to approximately one quarter of the cochlea length. Wild type (WT), Emx2\textsuperscript{Cre} and Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} mice were not significantly different from one another in both inner and outer hair cell numbers. In Hes1 conditional knockout mice, the numbers of both inner and outer hair cells were significantly increased (p<0.01) from the other groups. The effect of Hes5 and Hey1 was limited compared with Hes1, but Hes5 and Hey1 deficiency strengthened the phenotype of Hes1 conditional knockout. Error bars indicate standard deviation. Scale bars: A (for A, B), 100\textmu m; C (for C-H), 100\textmu m.

Figure 3. Defects of supporting cells surrounding outer hair cells in Hes/Hey mutants. A-F, Representatives of MyosinVI' hair cells and Prox1' supporting cells in E18.5 cochlea sections. At this stage, Prox1 was expressed in the nuclei of pillar cells, supporting cells between inner and outer hair cells (D, yellow arrows), and Deiters’ cells, supporting cells surrounding outer hair cells (D, yellow arrowheads). Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} cochlea sections showed a normal pattern; 1 inner hair cell, 3 outer hair cells and 5 Prox1' cells (A,D). In Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} cochlea sections, there were 2 inner hair cells, 4 outer hair cells and 6 Prox1'cells (B,E), suggesting that the outer hair cell increase was accompanied by Deiters’ cell increase (see yellow asterisks in K). In Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} cochlea sections, there were 2 inner hair cells, 5 outer hair cells and 4-6 Prox1' cells in most regions (C,F). There were 4 Prox1' cells in F. G-P, Whole mount staining of Hes/Hey mutant cochleae. Hair cells and supporting cells were visualized by Phalloidin and anti-Prox1 antibody, respectively. In Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} cochlea, there were normal 1 inner hair cell, 3 outer hair cells and 5 rows of Prox1' cells (G,H). In Hes1\textsuperscript{+\textminus};Hes5\textsuperscript{−\textminus};Hey1\textsuperscript{−\textminus} cochlea, excessive hair cells (J, white arrows) and excessive Prox1' cells (I, white arrowheads) were present at the same site, indicating that the fourth row of outer hair cells were surrounded by the
fourth row of Deiters’ cells. More hair cells were seen in $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/--};\text{Hey1}^{+/--}$ cochlea and $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, and Prox1$^+$ cells also increased in number at the same place, forming mostly 6 rows (K-N). In $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, even more hair cells were formed (P), and at the same site, Prox1$^+$ cells were disarranged or missing (O, asterisks). **Q**, Quantification of outer hair cells (OHC) and Prox1$^+$ pillar and Deiters’ cells (PC + DC) in the basal and middle turn on serial sections of E18.5 cochleae. The total number of each cell type per cochlea duct section was calculated. Error bars indicate standard deviation. Scale bars: C (for A-F), 20µm; H (for G-P), 20µm

Figure 4. Defects of supporting cells surrounding inner hair cells in $\text{Hes/Hey}$ mutants. **A-I**, Whole mount immunostaining of p27$^{kip1}$ and p75$^{NTR}$ (A,B,D,E,G,H) and section immunohistochemistry of p27$^{kip1}$ and Prox1 (C,F,I). Brackets indicate inner phalangeal cells (A,D,G) and hair cells (B,E,H, shadow of p27$^{kip1}$). Inner phalangeal cells and inner hair cells are indicated by asterisks and arrowheads, respectively (C,F,I). In $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, an inner hair cell was surrounded by two inner phalangeal cells (A-C). In $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, two inner hair cells were surrounded by three inner phalangeal cells (D-F). By contrast, in $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, two inner hair cells were surrounded by two inner phalangeal cells (G-I). Thus, overproduction of hair cells by reduction of $\text{Hes/Hey}$ gene dosage was accompanied by supporting cell overproduction in $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, but when all of $\text{Hes1}$, $\text{Hes5}$ and $\text{Hey1}$ were inactivated, the number of hair cells increased without excess supporting cells. **J**, Quantification of inner hair cells (IHC) and inner phalangeal cells (IPHC) in the basal and middle turn on serial sections of E18.5 cochleae. p27$^{kip1}$-positive and Prox1-negative cells were inner phalangeal cells in the greater epithelial ridge. The total number of each cell type per cochlea duct section was calculated. Error bars indicate standard deviation. Scale bar: 10µm.

Figure 5. Disturbed cell alignment and polarity by $\text{Hes/Hey}$ deficiency. Hair cell stereocilia, kinocilium and pillar cells were labeled with phalloidin (red), anti-acetylated α-tubulin antibody (green), and anti-p75$^{NTR}$ antibody (blue), respectively. In the control cochlea, a bundle of stereocilia (phalloidin$^+$) forms a V shape directing outward, and a kinocilium is located at the tip of the V shape (anti-acetylated α-tubulin$^+$). The cell alignment and polarity were mostly normal in $\text{Hes1}^{+/-};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ (A,B). In $\text{Hes1}^{\Delta \lambda};\text{Hes5}^{+/-};\text{Hey1}^{+/-}$ cochlea, most outer hair cells were properly aligned, and their polarity of stereocilia and kinocilium was mostly normal (C,D). However, although
excessive inner hair cells were aligned in two rows, the polarity of the stereocilia and
kinocilium of inner hair cells was somewhat disorganized in $Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$
cochlea (C,D). In $Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea, the alignment of outer hair cells
was severely disorganized, while excessive inner hair cells were also aligned in a
disorganized manner (E,F). Furthermore, the polarity of stereocilia and kinocilium of
outer and inner hair cells was abnormal, pointing rather randomly (E,F). Scale bar:
10μm.

Figure 6. Abnormal proliferation in the epithelium by $Hes/Hey$ deficiency. E18.5
cochlea sections of $Hes/Hey$ mutants were examined. Hair cells were labeled by anti-
MyosinVI antibody. Sections from apical to middle turn (A,C,E,G,I,K) and from mid-
basal to basal turn (B,D,F,H,J,L) of cochleae were analyzed. A-F, Ki67⁺ proliferating
cells. Proliferating cells were occasionally observed next to hair cells in
$Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea (E,F, arrows). G-L, BrdU⁺ cells. 50 μg/g body weight
of BrdU was given to pregnant mice at E14.5 by a single i.p. injection. BrdU⁺ hair
cells were occasionally found in $Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea (K,L, arrows). Scale
bars: B (for A-F), 20μm; H (for G-L), 20μm. M, Proportion of BrdU⁺ MyosinVI⁺ cells
to total MyosinVI⁺ cells. Error bars indicate standard errors.

Figure 7. The prosensory domain appeared to be unaffected in $Hes/Hey$ mutants.
Immunohistochemistry of E14.5 and E15.5 cochlea sections. A-H, At E14.5,
MyosinVI⁺ hair cells and Prox1⁺ supporting cells did not appear in both the control and
$Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea (A,B,E,F). p27Kip1 and Sox2 were expressed similarly
in the control and $Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea at this stage (C,D,G,H). I-P, At
E15.5, there were some MyosinVI⁺ hair cells and Prox1⁺ supporting cells in both the
control and $Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea (I,J,M,N). Thus, the onset of hair cell and
supporting cell differentiation in the prosensory domain was not affected by $Hes/Hey$
deficiency. p27Kip1 and Sox2 were expressed similarly in the control and
$Hes1^{ΔA};Hes5^{−/−};Hey1^{−/−}$ cochlea (K,L,O,P). Scale bars: A (for A-H), 20μm; I (for I-P),
20μm.
Figure 1
Figure 2

E18.5

Emx2<sup>Cre</sup> / Hes1<sup>−/−</sup>; Hes5<sup>−/−</sup>; Hey1<sup>−/−</sup>
Figure 3

**A** MyosinVI

**B** Prox1

**C** Phalloidin

**D** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**E** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**F** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**G** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**H** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**I** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**J** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**K** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**L** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**M** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**N** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**O** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**P** Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)

**Q**

Cell number per coelomic duct section

- Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)
- Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)
- Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)
- Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)
- Hes1\(^{+/-}\);Hes5\(^{+/-}\);Hey1\(^{-/-}\)
Figure 5

Phalloidin
Acetylated α-Tubulin
p75

Hes1^{−/−};Hes5^{−/−};Hey1^{−/−}

IHC
OHC

Hes1^{+/−};Hes5^{−/−};Hey1^{−/−}

IHC
OHC

Hes1^{+/−};Hes5^{−/−};Hey1^{−/−}

IHC
OHC
Figure 6

E18.5

Ki67 MyosinVI

Hes1<sup>-/-</sup>,Hes5<sup>+/+</sup>,Hey1<sup>+/+</sup>

Hes1<sup>+/+</sup>,Hes5<sup>-/-</sup>,Hey1<sup>+/+</sup>

Hes1<sup>Δ/Δ</sup>,Hes5<sup>-/-</sup>,Hey1<sup>+/+</sup>

BrdU MyosinVI

Hes1<sup>-/-</sup>,Hes5<sup>-/-</sup>,Hey1<sup>+/+</sup>

Hes1<sup>Δ/Δ</sup>,Hes5<sup>-/-</sup>,Hey1<sup>+/+</sup>

Hes1<sup>Δ/Δ</sup>,Hes5<sup>-/-</sup>,Hey1<sup>+/+</sup>

M

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Graph:

- Hes1<sup>(+/+)</sup>, Hes5<sup>(+/+)</sup>, Hey1<sup>(+/+)</sup>
- Hes1<sup>(+/+)</sup>, Hes5<sup>(Δ/Δ)</sup>, Hey1<sup>(+/+)</sup>
- Hes1<sup>(Δ/Δ)</sup>, Hes5<sup>(+/+)</sup>, Hey1<sup>(+/+)</sup>
- Hes1<sup>(Δ/Δ)</sup>, Hes5<sup>(Δ/Δ)</sup>, Hey1<sup>(+/+)</sup>
Figure 7

E14.5
Cre(-) control
Hes1^{Δ/Δ}, Hes5^{Δ/Δ}, Hey1^{Δ/Δ}

MyosinVI Ki67
Prox1 Ki67
p27 Ki67
Sox2 Tuj1

E15.5
Cre(-) control
Hes1^{Δ/Δ}, Hes5^{Δ/Δ}, Hey1^{Δ/Δ}
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**Table 1. Auditory brainstem responses of Hes/Hey mutant mice**

Auditory brainstem responses (ABR) were recorded with *Hes/Hey* mutant mice and their siblings. All mice showed hearing loss at a high frequency (40kHz), and this was probably due to the background containing C57BL/6. The thresholds of the lower frequencies were increased when both alleles of *Hes1* were lost. SO: scale out.
Supplementary Information

Figure S1. Cre recombinase activity was examined in the developing cochlea of Emx2\textsuperscript{Cres}\textsuperscript{+}ROSA26-CFP mice. A-E, E11.5 otic vesicle, sectioned from rostral to caudal. The prosensory domain was labeled with Sox2 (red). The cochlea develops from a tubular outpocketing at the ventral-medial region of the otic vesicle (asterisks in D and E). CFP expression was sparsely seen in the prosensory domain (Sox2\textsuperscript{+}) of the presumptive cochlea. F-J, E13.5 otic vesicle, sectioned from rostral to cordal. The cochlea appears from G to J (arrows). The prosensory domain was labeled with p27\textsuperscript{Kip1} (red). CFP expression was seen in most epithelial cells of the cochlea duct and some cells of the vestibular system. K-O, E18.5 inner ear sections. CFP expression was seen in most epithelial cells of the cochlea duct and some cells of the spiral ganglion (K,
arrowheads). Both hair cells and supporting cells were CFP$^+$ in the organ of Corti (L). Approximately a half of hair cells in the maculae of the vestibular system were CFP$^+$ (M, N). There were no CFP$^+$ cells in the ampullae of semicircular canals (O). P-R, Whole-mount immunostaining of P1 cochleae. CFP expression was seen throughout the cochlea epithelium (P) and hardly found in the spiral ganglion (P, arrowheads). Almost all hair cells (Q) and Prox1$^+$ cells (supporting cells surrounding hair cells, R) expressed CFP. S, Hes1 mRNA level in E14.5 cochlea epithelia was reduced to 64% and 9% in Emx2$^{Cre}:Hes1^{+/}$(Hes1$^{+/}$) and Emx2$^{Cre}:Hes1^{+/}$ (Hes1$^{+/}$) mice, respectively, compared to the control. Thus, Hes1 expression was effectively lost by E14.5 in the cochlear of Hes1 conditional knock out mice. The average mRNA level of the control, Cre(-), was taken as 1. Scale bars: E (for A-E), 100μm; J (for F-J), 100μm; K, 100μm; L, 20μm; M (for M-O), 100μm; P, 200μm; Q (for Q, R), 20μm.

![E18.5 cochlea length](image)

Figure S2. E18.5 cochlea length. The cochleae of Hes1$^{+/}$;Hes5$^{+/}$;Hey1$^{+/}$ mice were significantly shorter than the other genotypes (* p<0.01), but the difference was less than 10%. The cochlea lengths of other genotypes were not significantly different from the wild type (WT).
Figure S3. The average numbers of hair and supporting cells per cochlear duct section. IHC, inner hair cells; IPhC, inner phalangeal cells; OHC, outer hair cells; PC+DC, pillar cells and Deiters’ cells.
Figure S4. E18.5 cochlea surface at length of 25%, 50% and 75% from the apex. Excessive hair cells were similarly observed in any part of the mutant cochlea ducts.

Figure S5. Hey2, HeyL and Hey3 expression in E18.5 cochlea. In situ hybridization of Hey2, HeyL and Hey3 was performed with E18.5 cochlea sections of Hes1+/Δ;Hes5−/−;Hey1+/− and Hes1+/Δ;Hes5−/−;Hey1+/− mice. Immunostaining of MyosinVI was shown in yellow. Hey2 was expressed at a similar level in the pillar cells of Hes1+/Δ;Hes5−/−;Hey1+/− and Hes1+/Δ;Hes5−/−;Hey1+/− cochlea (A and D, arrows). Hey2 expression occurred in Hensen cells of Hes1+/Δ;Hes5−/−;Hey1+/− cochlea (D, arrowhead) at a higher level than those of Hes1+/Δ;Hes5−/−;Hey1+/− cochlea. HeyL was expressed at a similar level in Kollikers organ of Hes1+/Δ;Hes5−/−;Hey1+/− and Hes1+/Δ;Hes5−/−;Hey1+/− cochlea (B and E, asterisks). Hey3 was not expressed in Hes1+/Δ;Hes5−/−;Hey1+/− cochlea (C).
Figure S6. E18.5 cochlea sections of $\text{Hes}^{1\Delta A}\cdot\text{Hes}^{5-/-}\cdot\text{Hey}^{1-/-}$ mutants. BrdU was administered at E17.5, and immunohistochemistry of Ki67 (red), BrdU (green) and MyosinVI (blue) was performed at E18.5. A,B, Arrows indicate Ki67$^+$BrdU$^+$ cells. Ki67$^+$BrdU$^+$ cells were rather rare in the organ of Corti and did not accompany adjacent BrdU$^+$ cells. C,D, There were occasionally BrdU$^+$ hair cells (C, arrow) and Ki67$^+$ hair cells (D, arrow).
Figure S7. E14.5 cochlea sections of *Hes/Hey* mutants. 50 µg/g body weight of BrdU was administered at E13.5, and immunohistochemistry of Ki67 (red), BrdU (green) and p27Kip1 (blue) was performed at E14.5. p27Kip1*I-BrdU* cells were seen in the prosensory domain. There were more in the basal turn (B,D) than in the apical turn (A,C), but BrdU*Ki67* positive cells were not seen in the prosensory domain of control (A,B) and *Hes1*Δ/Δ;*Hes5*Δ/Δ;*Hey1*Δ/Δ cochleae (C,D). This result suggests that proliferating prosensory cells at E13.5 became quiescent at E14.5, and that *Hes/Hey* deficiency did not affect the timing of cell cycle exit.
Figure S8. Adult *Hes*/Hey mutant cochleae. **A-C**, Immunostaining of whole-mount cochleae of *Hes1<sup>+/Δ;Hes5<sup>−/−;Hey1<sup>+/−** (A, #566 in Table 1) and *Hes1<sup>Δ/Δ;Hes5<sup>−/−;Hey1<sup>+/−** (B,C, #923 in Table 1). Hair cells of *Hes1<sup>+/Δ;Hes5<sup>−/−;Hey1<sup>+/−** appeared normal (A). In *Hes1<sup>Δ/Δ;Hes5<sup>−/−;Hey1<sup>+/−** cochlea, inner hair cells formed into almost one row at 6 months of age (B,C), although the mutant cochlea had two rows of inner hair cells at E18.5. Therefore, excessive inner hair cells decreased in number. Arrow indicates paired inner hair cells (B,C). There were still excessive outer hair cells in the adult *Hes1<sup>Δ/Δ;Hes5<sup>−/−;Hey1<sup>+/−** cochlea (B). The polarity and alignment of outer hair cells seemed to be disturbed (B,C). **D,E**, Neurons in spiral ganglion were normally maintained in the cochleae of *Hes1<sup>+/Δ;Hes5<sup>−/−;Hey1<sup>+/−** (D) and *Hes1<sup>Δ/Δ;Hes5<sup>−/−;Hey1<sup>+/−** (E). Scale bars: A (for A,B,D,E), 100µm; C, 10µm.
Figure S9. Postnatal day 3 Hes/Hey mutant cochleae.  

A,B, Immunohistochemistry of MyosinVI (red), TUNEL (green) and DAPI (blue) was performed.  

A’,B’, TUNEL (gray scale). Hair cell loss was not observed in Hes1<sup>+/Δ</sup>;Hes5<sup>+/−;</sup>Hey1<sup>−/−</sup> cochlea (A and A’), whereas outer hair cells were TUNEL<sup>+</sup> in Hes1<sup>Δ/Δ</sup>;Hes5<sup>+/−;</sup>Hey1<sup>−/−</sup> cochlea (B and B’, arrows), suggesting that extra hair cells died rather than became integrated into the normal network.
Table S1. Statistical analysis of the result shown in Fig. 2I.
IHC, inner hair cells (pink); OHC, outer hair cells (light blue). *: p<0.05, **: p<0.01, NS, not significant.
Table S2. Statistical analysis of the results shown in Figs. 3Q and 4J.  *A*, Inner hair cells (IHC, pink) and outer hair cells (OHC, light blue).  *B*, Inner phalangeal cells (IPhC, yellow) and Prox1-positive pillar cells and Deiters’ cells (PC+DC, light green).

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*, p<0.05; **, p<0.01; NS, not significant.