Developmental Biology 352 (2011) 329–340

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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

Tomoko Tateya [a], Itaru Imayoshi [a,b], Ichiro Tateya [c], Juichi Ito [c], Ryoichiro Kageyama [a,d,*]

[a] Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
[b] Japan Science and Technology Agency, PRESTO, Kyoto 606-8507, Japan
[c] Department of Otolaryngology—Head and Neck Surgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan
[d] Japan Science and Technology Agency, CREST, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received for publication 19 August 2010
Revised 16 December 2010
Accepted 28 January 2011
Available online 16 February 2011

Keywords:
Hair cell
Supporting cell
Hes1
Hes5
Hey1
Developing cochlea

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 *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.

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 (Kelley, 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., 2009), 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 cochlea, hair cells

* Corresponding author at: Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81 75 751 4807.
E-mail address: rkageyam@virus.kyoto-u.ac.jp (R. Kageyama).

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.01.038

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*(*Hesr1*) 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. $Hes1^{f/f}$;$Hes5^{-/-}$;$Hey1^{-/-}$ mice were crossed with $Emx2^{+/Cre}$;$Hes1^{+/f}$;$Hes5^{+/-}$;$Hey1^{+/-}$ mice, $Emx2^{+/Cre}$;$Hes1^{+/f}$;$Hes5^{+/-}$;$Hey1^{-/-}$ mice, $Emx2^{+/Cre}$;$Hes1^{+/f}$;$Hes5^{-/-}$;$Hey1^{+/-}$ mice, or $Emx2^{+/Cre}$;$Hes1^{+/f}$;$Hes5^{-/-}$;$Hey1^{-/-}$ mice. In the following, $Hes1^{+/\Delta}$ and $Hes1^{\Delta/\Delta}$ are the abridgements of $Emx2^{+/Cre}$;$Hes1^{+/f}$ and $Emx2^{+/Cre}$;$Hes1^{f/f}$, 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 0.1 M 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 10 mM 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 Apoptag® 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 *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 cochlear 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-modiolar 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 (TOYOBO) and Random Primer (TOYOBO). Real-time PCR was done by using Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) and THUNDERBIRD SYBR qPCR Mix (TOYOBO), according to the manufacturer's protocols. GAPDH was used as a control. The following primers were used for real-time PCR: Hes1 forward, tgaaggattccaaaaataaaattctctggg; Hes1 reverse, cgcctcttctccatgataggctttgatgac; GAPDH forward, atcttcttgtgcagtgccagcctcgtcccg; and GAPDH reverse, agttgaggtcaatgaaggggtcgttgatgg.

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.