Hedgehog signaling regulates prosensory cell properties during the basal-to-apical wave of hair cell differentiation in the mammalian cochlea

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SUMMARY
Mechanosensory hair cells and supporting cells develop from common precursors located in the prosensory domain of the developing cochlear epithelium. Prosensory cell differentiation into hair cells or supporting cells proceeds from the basal to the apical region of the cochlea, but the mechanism and significance of this basal-to-apical wave of differentiation remain to be elucidated. Here, we investigated the role of Hedgehog (Hh) signaling in cochlear development by examining the effects of up- and downregulation of Hh signaling \textit{in vivo}. The Hh effector smoothened (Smo) was genetically activated or inactivated specifically in the developing cochlear epithelium after prosensory domain formation. Cochleae expressing a constitutively active allele of Smo showed only one row of inner hair cells with no outer hair cells (OHCs); abnormal undifferentiated prosensory-like cells were present in the lateral compartment instead of OHCs and their adjacent supporting cells. This suggests that Hh signaling inhibits prosensory cell differentiation into hair cells or supporting cells and maintains their properties as prosensory cells. Conversely, in cochleae with the Smo conditional knockout (Smo CKO), hair cell differentiation was preferentially accelerated in the apical region. Smo CKO mice survived after birth, and exhibited hair cell disarrangement in the apical region, a decrease in hair cell number, and hearing impairment. These results indicate that Hh signaling delays hair cell and supporting cell differentiation in the apical region, which forms the basal-to-apical wave of development, and is required for the proper differentiation, arrangement and survival of hair cells and for hearing ability.

KEY WORDS: Hedgehog signaling, Cochlea, Hair cells, Mouse instigate

INTRODUCTION
The perception of sound is mediated through a specialized sensory epithelium in the cochlea, the organ of Corti, which extends along the length of the coiled cochlear duct located in the ventral region of the inner ear. The organ of Corti contains mechanosensory hair cells and non-sensory supporting cells that differentiate from common precursors in the prosensory domain. Following specification of the prosensory domain in the cochlear duct, individual prosensory cells develop as hair cells or supporting cells. Initially, inner hair cells (IHCs) differentiate in the mid-basal region of the cochlea; hair cell differentiation then proceeds towards the apex forming a basal-to-apical wave along the entire length of the sensory epithelium. At each point along the cochlear, differentiation also proceeds from the IHCs towards the outer hair cells (OHCs) in a medial-to-lateral wave orthogonally across the width of the sensory epithelium. The significance of these hair cell differentiation waves is unknown. Atoh1, a key inducer of hair cell formation, is expressed in a basal-to-apical and medial-to-lateral wave in the developing cochlear epithelium, and is negatively regulated by Notch signaling in the formation of the mosaic of hair cells and supporting cells (Lanford et al., 1999; Chen et al., 2002; Woods et al., 2004; Kiernan et al., 2005; Brooker et al., 2006; Kelley, 2007). Although a series of studies confirmed that Notch signaling has a crucial role in lateral inhibition of hair cell and supporting cell differentiation, the factors that regulate the onset of Atoh1 expression in the cochlea have not been fully determined (Driver and Kelley, 2009).

Recently, it was suggested that Hh signaling could be a candidate for the key regulator inhibiting hair cell differentiation and could be involved in the basal-to-apical wave of cochlear hair cell differentiation. Hh signaling is essential for many aspects of vertebrate development (Varjosalo and Taipale, 2008). Hh protein, such as Sonic hedgehog (Shh), is synthesized in and secreted from Hh-producing cells, travels over many cell diameters, and interacts with its receptor patched 1 (Ptc1) on Hh-responding cells, resulting in de-repression of smoothened (Smo). De-repression of Smo activates downstream pathways that culminate in activation of Gli activators and repression of Gli repressors (Wang et al., 2007). It was reported that Shh is expressed in the developing spiral ganglion (Driver et al., 2008; Liu et al., 2010), and that Shh inhibits hair cell formation in embryonic cochlear explants (Driver et al., 2008). In addition, in Shh\textsuperscript{Cre/EGFP+} mice, GFP was observed in all spiral ganglion neurons of embryonic day (E)13.5 embryos, but GFP expression was subsequently lost from the basal turn towards the apex and finally became undetectable in all spiral ganglion neurons at parturition (Liu et al., 2010). Thus, the decline in the basal-to-apical wave of Hh signaling resembles that of hair cell differentiation, suggesting that Shh derived from the spiral ganglion might inhibit hair cell differentiation.

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In this study, we examined the roles of Hh signaling in the developing cochlear sensory epithelium by conditional genetic activation and inactivation of Smo specific to cochlear epithelium in vivo. Smo encodes a membrane protein essential for the transduction of Hh signals into the cytoplasm. We found that Hh signaling inhibited prosensory cell differentiation into hair cells or supporting cells, and delayed differentiation in the apical region. Our results demonstrate the important role of Hh signaling in cochlear sensory epithelium development, and also provide insight into the significance of the temporal basal-to-apical wave of hair cell differentiation for acquisition of normal hearing ability.

MATERIALS AND METHODS

Mouse breeding

Smo floxed (The Jackson Laboratory) (Long et al., 2001; Komada et al., 2008), Rosa26-SmoM2 (The Jackson Laboratory) (Long et al., 2001) and Emx2+/Cre (Kimura et al., 2005) mice were used to produce Emx2+/Cre; Smo floxed/floxed (Smo conditional knockout, Smo CKO) mice and Emx2+/Cre; R26-SmoM2 (SmoM2 conditionally activated, R26-SmoM2) mice. Because R26-SmoM2 pups die immediately after birth, analyses of R26-SmoM2 were made at embryonic stages. RbpJ floxed (Han et al., 2002), Rosa26-SmoM2 and Emx2+/Cre mice were used to produce Emx2+/Cre; RbpJ floxed/floxed and Emx2+/Cre; RbpJ floxed/floxed; R26-SmoM2 mice. For analysis of Cre recombine activity, Emx2+/Cre mice were crossed with Rosa26-CFP mice. These mice were maintained on C57BL/6; ICR mixed background. Date of vaginal plug was defined as E0.5.

Histochemistry and in situ hybridization

Whole heads (E11.5-E15.5) or inner ears (E17.5) and cochlear explant cultures of developing cochleae were mostly active in the epithelium, including the cochlear duct and the surrounding mesenchyme at E11.5 and E13.5 but not at E18.5 (supplementary material Fig. S1E-H, arrows). These differences among groups. Differences at P<0.05 were regarded as statistically significant.

ABR, DPOAEs and endocochlear potential 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., 2009). Thresholds were determined at 4, 10, 20 and 40 kHz frequencies from a set of responses at varying intensities with 5-dB sound pressure level intervals.

Distortion-product otoacoustic emissions (DPOAEs) and endocochlear potential recording was performed as described previously (Hamaguchi et al., 2012).

RESULTS

Hh signaling functions in the prosensory domain of developing cochlea

We examined the expression pattern of Shh and Gli1 in the developing inner ear (supplementary material Fig. S1). Shh was expressed in E13.5 spiral ganglion (supplementary material Fig. S1C, asterisk) but not in the epithelium of the cochlear duct (supplementary material Fig. S1B-D, arrows) or in the spiral ganglion at other ages (supplementary material Fig. S1A,D, asterisks). Gli1 expression occurred in the presumptive cochlear epithelium and surrounding mesenchyme at E11.5 and E13.5, but not at E18.5 (supplementary material Fig. S1E-H, arrows). These observations were compatible with the literature (Driver et al., 2008; Liu et al., 2010) and suggest that Hh signaling functions in both the cochlear epithelium and the surrounding mesenchyme at E11.5 and E13.5 but not at E18.5. As the Cre recombine of Emx2+/Cre cochlea was mostly active in the epithelium, including the prosensory domain, but not in the surrounding mesenchyme until E13.5 (Tateya et al., 2011) (supplementary material Fig. S2), we decided to use the Emx2+/Cre line for the genetic activation and inactivation of Smo in our investigation of the specific roles of Hh signaling in the prosensory domain of cochlea.

Cochlear hair cell differentiation is accelerated in Smo CKO and inhibited in R26-SmoM2 cochlea

By using Emx2+/Cre mice, we generated Smo CKO, in which Smo was inactivated, and R26-SmoM2 mice, in which a constitutively active allele of Smo (SmoM2) was expressed. The cochlear
phenotypes of E18.5 control, Smo CKO and R26-SmoM2 mice were analyzed (Fig. 1). At E18.5 in controls, only IHCs were seen near the apex of the cochleae (Fig. 1A,D, arrowheads). In Smo CKO cochleae, however, both IHCs and OHCs were seen in the most apical region of the cochlear epithelium (Fig. 1B,E, arrowheads); thus, following inhibition of Hh signaling, hair cell differentiation in the apex had been accelerated and completed earlier than in the control. Both IHCs and OHCs were regularly formed in the basal turn of Smo CKO cochleae, similar to the control (Fig. 1G,H). In R26-SmoM2 cochleae, IHCs and OHCs were not formed near the apex (Fig. 1C,F), and only IHCs were observed in the basal region (Fig. 1I), suggesting that hair cell differentiation was inhibited or delayed by excessive Hh signaling.

Early hair cell differentiation in Smo CKO cochleae was more obvious at E16.5 (Fig. 2). Because the cochlea is shorter in the Smo CKO (supplementary material Table S3), we also examined regions located at comparable distances from the base of both Smo CKO and control cochleae. Hair cells in E16.5 Smo CKO cochleae were more differentiated than those in the comparable area of E16.5 control cochleae. Hair cell formation had occurred partially in E16.5 control cochleae, and myosin VI-positive OHCs were seen only in the basal turn (Fig. 2A,C). No IHCs or OHCs were seen in the apex of control cochleae (Fig. 2G), and no myosin VI-positive OHCs were seen in the apical and middle turns (Fig. 2E,I). By contrast, IHCs and OHCs were present in all areas of the E16.5 Smo CKO cochlea (Fig. 2B,D,F,H) and the wave of hair cell differentiation had reached the apex (Fig. 2H,I).

**Hh signaling is required for normal morphogenesis of the cochlea**

The lengths of cochlear epithelium along the basal-to-apical axis of Smo CKO and R26-SmoM2 cochleae were compared with cochleae from control. Smo CKO cochleae were significantly shorter than the controls whereas R26-SmoM2 cochleae were significantly longer (Fig. 1J). The lengths of Smo CKO cochleae were also

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**Fig. 1. Effects of activation and inactivation of Hh signaling on E18.5 cochlear epithelium.** (A-I) Representative examples of cochleae from control (A,D,G), Smo CKO (B,E,H) and R26-SmoM2 (C,F,I) mice visualized by anti-myosin VI (green) and phalloidin (magenta). (A-C) Whole-mount preparations of E18.5 cochlea from control (A), Smo CKO (B) and R26-SmoM2 embryos (C). (D-F) Magnified images of the apices of the cochlear epithelia (indicated by arrows and boxed areas in A-C). Arrowheads in D indicate IHCs; arrowheads in E indicate IHCs and OHCs. (G-I) Magnified images of the basal turn of cochleae (indicated by black asterisks and brackets in A-C). IHCs are indicated by black asterisks. Brackets indicate OHCs (G,H) and the lateral compartment which is supposed to produce OHCs and adjacent supporting cells (I). Scale bars: in A (for A,B), 1 mm; C, 1 mm; D (for D-F), 100 μm; G (for G-I), 10 μm. (J,K) The length of cochlear epithelium along the basal-to-apical axis (J) and the hair cell number per 100 μm (K) in E18.5 cochleae of Smo CKO and R26-SmoM2 embryos compared with control siblings. Hair cell numbers per 100 μm were determined by counting hair cells along a 150-μm cochlear length located at the 50% position. OHCs were not found in R26-SmoM2 cochleae. **P<0.01. Error bars represent standard errors.

**Fig. 2. Hair cell differentiation is accomplished earlier in Smo CKO cochlea.** Whole-mount preparations of E16.5 cochlea from control (A,C,E,G) and Smo CKO (B,D,F,H) embryos visualized by anti-myosin VI (green) and phalloidin (magenta). (A,C,E) Hair cells at positions 20% (A), 40% (C) and 60% (E) from the base along the basal-to-apical axis of the control cochlea. The positions from the base were calculated by using the average cochlear length of three control animals. (B,D,F) Hair cells in the regions located at the equivalent distance from the base as A (B), C (D) and E (F). (G,H) The apices of control (G) and Smo CKO (H) cochlear epithelia. (I) Diagram of E16.5 hair cell differentiation in control and Smo CKO cochleae. The locations of A-H are schematically shown. Scale bars: F (for A-F), 20 μm; H (for G,H), 50 μm.
compared with those of control siblings at E16.5, P7 and 4 weeks after birth (4W). At all ages examined, the Smo CKO cochleae were ~85% of the length of the control (supplementary material Table S3). The number of IHCs and OHCs per length along the basal-to-apical axis of E18.5 Smo CKO and R26-SmoM2 cochleae were compared with control (Fig. 1K). No OHCs were observed in R26-SmoM2 cochleae. The numbers of IHCs and OHCs in Smo CKO cochleae, and of IHCs in R26-SmoM2 cochleae per unit length were smaller than in controls (Fig. 1K). These results suggested that Hh signaling is required for normal morphogenesis of the cochlea.

To examine further the morphogenesis of Smo CKO cochleae, Smo CKO pups and control siblings were analyzed at P7; this developmental stage was selected as hair cells are still differentiating at E18.5 but have finished differentiation by P7. The number of IHCs and OHCs were counted at positions 25%, 50% and 75% from the apex along the basal-to-apical axis (Fig. 3A). IHC numbers at 25% from the apex, and the OHC numbers at 25%, 50% and 75% from the apex were significantly decreased in Smo CKO cochleae compared with control cochleae (*P<0.05). Error bars represent standard errors. (B-I) The surface of control (B,F,H) and P7 Smo CKO (C,E,G) cochlear epithelium, visualized by anti-myosin VI (green) and phalloidin (magenta). (B-E) Apical turn. (F,G) Middle turn. (H,I) Basal turn. IHCs and OHCs are indicated by asterisks and brackets, respectively. B and C show the most apical part of hair cell rows, and arrows indicate the hair cells at the most apical position. D is more basal than C, and E is more basal than D. Arrowheads indicate ectopic hair cells with U-shaped stereocilia in the rows of OHCs in Smo CKO cochleae (D,E,G). (JK) Weak p75NTR expression in supporting cells (arrowheads) surrounding an abnormal hair cell with U-shaped stereocilia in the row of OHCs (asterisks) in P4 Smo CKO cochlea. (J) p75NTR (green) and phalloidin (magenta). (K) p75NTR alone. Scale bars: B (for B-I), 20 μm; K (for J,K), 10 μm.

**Fig. 3. Deformity of hair cell rows in the apical part of Smo CKO cochleae.** (A) Hair cell number per 100 μm determined by counting hair cells along a 200 μm cochlear length located at positions 25%, 50% and 75% of P7 Smo CKO and control cochleae from the apex along the basal-to-apical axis. Three animals per each group were used. The IHC numbers at 25% from the apex, and the OHC numbers at 25%, 50% and 75% from the apex were significantly decreased in Smo CKO cochleae compared with control cochleae (*P<0.05). Error bars represent standard errors. (B-E) Apical turn. (F,G) Middle turn. (H,I) Basal turn. IHCs and OHCs are indicated by asterisks and brackets, respectively. B and C show the most apical part of hair cell rows, and arrows indicate the hair cells at the most apical position. D is more basal than C, and E is more basal than D. Arrowheads indicate ectopic hair cells with U-shaped stereocilia in the rows of OHCs in Smo CKO cochleae (D,E,G). (JK) Weak p75NTR expression in supporting cells (arrowheads) surrounding an abnormal hair cell with U-shaped stereocilia in the row of OHCs (asterisks) in P4 Smo CKO cochlea. (J) p75NTR (green) and phalloidin (magenta). (K) p75NTR alone. Scale bars: B (for B-I), 20 μm; K (for J,K), 10 μm.

Smo CKO cochleae exhibit deformity of hair cell rows especially in the apical region

The surface of the cochlear epithelium from postnatal Smo CKO was compared with that of control cochleae. In the apex of P7 control cochleae, OHCs mostly formed three rows (Fig. 3B). However, in the apical turn of P7 Smo CKO cochleae, the OHCs were not formed in the short distance of the apical end (Fig. 3C) and were decreased in number (Fig. 3C-E); additionally, cells with U-shaped stereocilia, which resembled IHCs, were present in the rows of OHCs that have V-shaped stereocilia (Fig. 3D,E, arrowheads). The ectopic hair cells with U-shaped stereocilia in the OHC rows were occasionally present in the middle turn of Smo CKO cochleae (Fig. 3G, arrowhead) but not in the basal turn (Fig. 3I); they were not observed in any region of the control cochleae (Fig. 3F,H). Similarly, in 4W Smo CKO mice, the cochleae showed a reduction in the OHC rows and ectopic hair cells with U-shaped stereocilia in the OHC rows in the apical turn (supplementary material Fig. S3). Regular hair cell alignment was observed in the basal region of 4W Smo CKO cochleae (data not shown). These results suggest that inactivation of Hh signaling caused deformity of hair cell rows as well as a decrease in hair cell numbers, and that the deformity was more severe in the apical than the basal region and in OHCs than in IHCs.

Inner and outer pillar cells are specific subtypes of supporting cells and are arranged in adjacent rows that form a boundary between rows of IHCs and OHCs. Weak expression of the pillar cell marker p75NTR (Ngfr – Mouse Genome Informatics) was observed in supporting cells between ectopic hair cells with U-shaped stereocilia and OHCs (Fig. 3J,K). These results suggest that ectopic hair cells with U-shaped stereocilia were accompanied by ectopic pillar cells, causing deformity of the Corti tunnel.
Hh signaling maintains prosensory cell properties

To examine further the role of Hh signaling in the developing sensory epithelium, the expression patterns of hair cell markers (myosin VI, Atoh1), supporting cell markers (Prox1, Hes5), and prosensory and supporting cell markers (p27Kip1, Bmp4) were compared in E17.5 control, Smo CKO and R26-SmoM2 cochleae. In the sensory epithelium of the mid-basal turn at E17.5, both hair cells and supporting cells could be identified by their morphologies and through use of specific markers (Fig. 4A,D,G,J). Smo CKO mice showed the same expression patterns as controls, suggesting that hair cells and supporting cells developed normally in the absence of Hh signaling in the mid-basal turn at this stage (Fig. 4B,E,H,K).

In R26-SmoM2 cochleae, the hair cell markers Atoh1 and myosin VI were co-expressed by a single row of hair cells (Fig. 4C,F). These hair cells also expressed Fgf8 (Fig. 6G,H), and therefore they were IHCs. In E17.5 R26-SmoM2 cochleae, the epithelium of the lateral compartment, where OHCs and adjacent supporting cells normally developed, appeared to be thicker than that of control, and the cell nuclei were still stratified as in E13.5-E14.5 prosensory epithelium (Fig. 4C,F). Both the hair cell markers Atoh1 and myosin VI, and the supporting cell markers Prox1 and Hes5 were downregulated in the lateral compartment of R26-SmoM2 cochlear epithelium (Fig. 4C,F). qRT-PCR analysis of E18.5 control and R26-SmoM2 cochleae also revealed that the hair cell markers Atoh1 and myosin VIa (Myo7a) and the supporting cell markers Hes5 and Prox1 were downregulated in R26-SmoM2 cochleae (Fig. 5A). Conversely, E13.5 cochleae cultured with the Hh signaling inhibitor cyclopamine showed upregulation of these genes (Fig. 5B).

Bmp4 is known to be expressed in the prosensory domain but at E17.5 its expression is limited to the lateral non-sensory domains, outer sulcus. Bmp4 is expressed in the outer sulcus of control and Smo CKO cochleae (Fig. 4M,N). Bmp4 is also expressed in R26-SmoM2 cochlear epithelium lateral to the p27Kip1-positive lateral compartment (Fig. 4O,R).

p27Kip1 is known to be expressed in both prosensory cells and supporting cells. In the mid-basal turn of E17.5 control and Smo CKO cochleae, p27Kip1 was limited to supporting cells and was not expressed in hair cells (Fig. 4P,Q). Cells in the lateral compartment of R26-SmoM2 cochlear epithelium expressed p27Kip1, and the nuclei of p27Kip1-positive cells remained stratified, similar to wild-type E13.5-E14.5 prosensory epithelium (Fig. 4R) (Hayashi et al., 2008a; Ohyama et al., 2010). These results suggest that prosensory cell-like cells are maintained in the lateral compartment by excessive Hh signaling in R26-SmoM2 cochlear epithelium.

It was previously shown that inactivation of the Notch mediator Rbpj induces excess hair cell formation (Yamamoto et al., 2006). We therefore examined whether inactivation of Rbpj induces hair cell differentiation of prosensory-like cells produced by elevated SmoM2 expression. The cochlea of R26-SmoM2;Rbpj CKO mice, in which SmoM2 was expressed continuously and Rbpj was conditionally inactivated, showed excessive IHC formation, suggesting fate conversion from adjacent supporting cells; however, the cochlea did not show OHC differentiation in the lateral compartment (supplementary material Fig. S4). These data indicate that downregulation of Hh signaling is necessary for prosensory cells to differentiate into OHCs.

Hh signaling affects Fgf signaling

Fgf signaling has been suggested to function in the prosensory epithelium: Fgf1 CKO cochleae have fewer hair cells and supporting cells (Pirvola et al., 2002), and Fgf20 knockout cochleae have fewer OHCs (Huh et al., 2012). In our study, the rows of OHCs were also reduced especially in the apical region of Smo CKO cochleae (Fig. 3C,D). Therefore, we analyzed the relationship between Hh and Fgf signaling by examining the expression of Fgf20 in E18.5 control and R26-SmoM2 cochleae. Fgf20 expression, which occurs in the prosensory domain but not in developing hair cells, was not detectable in E18.5 control cochleae.
Fig. 5. Effect of Hh activation and inactivation on mRNA levels of prosensory and sensory cell markers in the developing cochlea. (A) Atoh1, myosin VIIA, Hey1 and Hey2 mRNA levels in E18.5 cochleae of R26-SmoM2 and control siblings detected by qRT-PCR. The levels are shown as a ratio to the average of the control. Atoh1, myosin VIIA, Hey1 and Hey2 mRNA levels in E13.5 cochleae cultured for 24 hours with or without 5 μM cyclopamine. **P<0.01. Error bars represent standard errors.

R26-SmoM2 cochleae were found to express Fgf8 (Fig. 6E-H). In E18.5 control cochlea, Fgfr3 expression occurred in a population of developing OHCs and adjacent supporting cells (Fig. 6I,K). In E18.5 R26-SmoM2 cochlea, Fgfr3 expression was found in a progenitor population of the lateral compartment of the mid-basal turn but was not detected in more apical regions (Fig. 6L,J). Hey2 expression in E18.5 R26-SmoM2 cochlea was also seen in a progenitor population of the lateral compartment; in control cochlea, Hey2 expression became restricted to supporting cells (Fig. 6M-P). These results indicate that Fgf20 expression was sustained by activation of Hh signaling, and might delay pillar cell differentiation mediated by Fgf8, Fgfr3 and Hey2 in the lateral compartment. Delay of pillar cell differentiation was also seen in cochleae of E17.5 R26-SmoM2 mice compared with those of E17.5 control (supplementary material Fig. S5).

If the signaling mediated by Fgf20 is downstream of Hh signaling, the inhibition of Fgf20 activities should cancel out the effect of excessive Hh signaling. To test this hypothesis, E18.5 R26-SmoM2 cochleae were cultured with anti-Fgf20 antibody or SU5402 for 48 hours. In negative control cochlear cultures, OHCs were not detected (Fig. 7A,B). By contrast, in cochlear cultures with anti-Fgf20 antibody or SU5402, more than one row of hair cells appeared (Fig. 7C-F), suggesting that Fgf20 was downstream of Hh signaling and was involved in prosensory cell maintenance. Therefore, Fgf20 inhibition normalized the differentiation of prosensory cell-like cells produced by excessive Hh signaling.

The stereocilia of IHCs in cochlear cultures with the anti-Fgf20 antibody appeared to be similar to those in control cultures; however, the morphology of the IHCs in cochlear cultures with SU5402 was altered (Fig. 7A,C,E). This observation suggests that the effect of the anti-Fgf20 antibody was more specific with respect to the function of SmoM2.

**Smo CKO mice exhibit hearing loss**

In Smo CKO mice, although there was some deformity of hair cells, both OHCs and IHCs were almost properly arranged. We therefore investigated next whether Smo CKO mice have any hearing impairment. The ABR thresholds of Smo CKO mice and control siblings were measured from 4 to 40 weeks of age (Fig. 8). Smo CKO mice showed a mild hearing deficit at 4 weeks of age compared with controls, and both Smo CKO and control mice displayed age-related progressive hearing loss (Fig. 8A). The latter change might be related to their C57BL/6 genetic background. The difference in average ABR thresholds between Smo CKO and control mice tended to be larger at lower frequencies (4 kHz and 10 kHz) at all ages (Fig. 8B); this difference is in agreement with our observation that congenital abnormality of the Smo CKO cochlea principally affected the apical region.

Comparison of the temporal changes in 10-Hz ABR thresholds for each animal (ABR thresholds for which are shown in Fig. 8A) showed that progression of hearing loss in Smo CKO mice was more varied than in control mice (Fig. 8C).

After measurement of ABR thresholds at 40 weeks of age, we carried out a histological analysis of the cochleae of Smo CKO mice with residual hearing, deaf Smo CKO mice and their control siblings (supplementary material Fig. S6). Hair cell loss was observed in Smo CKO mice and also, to a lesser extent, in control mice, especially in the basal turn; the loss was most severe in cochleae of deaf mice and mildest in control mice, suggesting that the ABR threshold increase was proportional to hair cell loss (supplementary material Fig. S6A-I). Loss of neurons from the spiral ganglion was observed in Smo CKO and control mice, especially in the basal turn. Neuronal loss...
was more severe in cochleae of deaf mice than in control cochleae or in Smo CKO mice with residual hearing (supplementary material Fig. S6J-R). However, neurons were still present in the cochleae of deaf mice, suggesting that neuronal loss was probably caused by hair cell loss (supplementary material Fig. S6J-R).

After measurement of the ABR threshold at 40 weeks of age, some mice were used for measurement of endocochlear potential. SmoCKO1, SmoCKO2 and SmoCKO5 mice of Fig. 8C showed endocochlear potentials of 86 mV, 70 mV and 110 mV, respectively, suggesting that defects in endolymph homeostasis were unlikely to be the cause of the hearing loss. Moreover, there were no detectable histological abnormalities in the lateral walls in Smo CKO cochleae (data not shown). These results indicated that the hair cell abnormality in Smo CKO cochleae resulted in hearing loss and probably led to hair cell fragility during adulthood.

As Emx2 is robustly expressed throughout the forebrain, the hearing phenotype associated with Smo deletion might have been caused by loss of Smo in the central nervous system rather than in the organ of Corti. To address this issue, we analyzed ABR waveforms and DPOAE data of 6-week-old Smo CKO and control mice. The ABR waveforms of Smo CKO mice indicate that the cause of hearing impairment was in the periphery of the auditory pathway (supplementary material Fig. S7). Moreover, Smo CKO mice showed lower DPOAEs than control (supplementary material Fig. S7). These results suggest that the hearing phenotype associated with Smo deletion is caused by loss of Smo in cochlea.

**DISCUSSION**

**The roles of Hh signaling in cochlear epithelium after prosensory specification**

Previous studies revealed that Hh signaling is needed for cochlear formation and that Shh knockout mice show a complete absence of cochleae (Riccomagno et al., 2002). The ventral part of the inner ear, the cochlea, requires more Hh signaling than the dorsal part, the vestibular organ, and this dorsal-to-ventral Hh signaling gradient is controlled by various Gli activators and repressors (Bok et al., 2007). We found that the roles of Hh signaling in the cochlear epithelium after prosensory specification are inhibition of prosensory cell differentiation into hair cells or supporting cells and maintenance of prosensory cell properties. This is different from the roles of Hh signaling found in the inner ear at an earlier stage and suggests that Hh signaling has multiple roles in inner ear development, similar to other signaling pathways, such as Notch signaling and Fgf signaling (Brooker et al., 2006; Kelley, 2007).

Analysis of a mutant mouse model of Pallister-Hall syndrome (Gli3Δ699/Δ699), in which Hh signaling is only partially lost, showed that Gli3Δ699/Δ699 cochleae were shorter than normal and contained additional rows of hair cells and ectopic hair cells, suggesting that...
Hh signaling regulates prosensory formation (Driver et al., 2008). These phenotypes of Gli3ΔΔ699/Δ699 cochleae differ from that of Smo CKO cochleae. The Gli3 mutant phenotypes seem to be more severe, even though both Gli1 and Gli2 were still functional and were able to respond to Hh signaling in Gli3ΔΔ699/Δ699 cochleae, whereas the cells that lost Smo were unable to respond to Hh signaling. We speculate that the differences in phenotypes between Gli3ΔΔ699/Δ699 and Smo CKO mice are due to differences regarding when and where Hh signaling is inactivated. In Gli3ΔΔ699/Δ699 mutant cochleae, Hh signaling is inhibited from the beginning of development in both the cochlear epithelium and the surrounding mesenchyme, and therefore the defects in the cochlear epithelium might be affected by Hh signaling suppression in the surrounding mesenchyme or Hh signaling suppression before prosensory formation. In Smo CKO cochleae, inhibition of Hh signaling is almost limited to the epithelium after prosensory formation because of the Cre recombinase activity in Emx2Cre+ mice (Tateya et al., 2011) (supplementary material Fig. S2). Possibly, use of genetic inactivation of Smo in the Emx2Cre mouse line might distinguish the roles of Hh signaling more specific to the cochlear epithelium after prosensory specification from other Hh signaling roles during inner ear development.

**Inactivation of Smo affects the apical region of the cochlea more severely**

Smo inactivation affects the hair cell and supporting cell development more severely in the apical region of the cochlea in this study, and the reason for this might be that cochlear sensory epithelium differentiation occurs in a wave. Prosensory cell differentiation has been reported to occur earlier in the basal region than in the apical region and earlier in the IHCs than in the OHCs (Chen et al., 2002). In our study, the apical region was more affected than the basal region, and the lateral compartment (containing OHCs and adjacent supporting cells) was more affected than the medial compartment (containing IHCs and adjacent supporting cells), in both Smo CKO cochleae and R26-SmoM2 cochleae. Thus, the regions in which late differentiation of prosensory cells into hair cells or supporting cells occurred were those that displayed the greatest defects. This might be partly due to insufficient effectiveness of Cre recombinase at early developmental stages: the Cre recombinase in Emx2Cre+ mice begins to be active ~E11.5 and thereafter shows increasing activity. The efficiency of Cre-dependent recombination in Emx2Cre+ mouse was ~90% in E14.5 cochlea (Tateya et al., 2011). However, the first hair cell marker Atoh1 appears in the base of the cochlea ~E12.5 and then later extends towards the apex (Lanford et al., 2000; Woods et al., 2004). Therefore, the Cre recombinase in Emx2Cre+ mice might be too late to affect basal region and medial compartment development. Another possible reason why genetic inactivation of Hh signaling affects a limited region of the cochlea might be the intrinsic function of Hh signaling, which might be more effective for inhibition of prosensory cell differentiation in the apical region. Obviously, there might be other, as yet unknown, factors that influence prosensory differentiation.

**The basal-to-apical wave of hair cell differentiation produced by Hh signaling is needed for normal hearing ability**

It was previously shown that the basal-to-apical wave of Shh declines in spiral ganglion neurons, suggesting that the diffusion of Shh from spiral ganglion neurons inhibits hair cell differentiation and produces the basal-to-apical wave of hair cell differentiation (Liu et al., 2010). In agreement with this suggestion, we found here that hair cell differentiation was accelerated in Smo CKO cochleae, and that the basal-to-apical wave of hair cell differentiation was weakened in the apical region.

The significance of the basal-to-apical wave of hair cell differentiation remains to be determined. We showed that hair cells in the apical region of Smo CKO cochleae had more severe abnormalities than those in other regions, and that acceleration of hair cell differentiation was greatest in the apical region. Moreover, hearing loss in Smo CKO mice tended to be more severe for lower frequencies than higher frequencies; this change in sensitivity is compatible with the fact that the apical region of the cochlea is responsible for the ability to hear lower frequencies. These results...
indicate that Hh signaling is important for normal hair cell development, particularly in the apical region, and for the maintenance of normal hearing ability.

It was reported that six out of 12 Pallister-Hall syndrome cases had sensorineural hearing loss at low frequencies (Driver et al., 2008). However, because the Gli3<sup>Shh/+</sup> genotype (the mouse model for the syndrome) causes neonatal lethality in mice, it was not possible to assess the effect of this mutation on their hearing ability, leaving the role of Hh signaling in hearing unanswered. Thus, the Smo CKO mouse line used in this study is the first model for hearing impairment caused by inhibition of Hh signaling and will be useful for further analysis of the role of Hh signaling in the maintenance of hair cells.

**Fgf20 is downstream of Hh signaling and turned off by withdrawal of Hh signaling before hair cell differentiation**

Our study suggested that Fgf20 was a downstream gene in Hh signaling and that inhibition of Fgf20 normalized hair cell differentiation despite excessive Hh signaling. These findings may conflict with a previous study, which showed that Fgf20 knockout mice were deaf, and had undifferentiated cells instead of mature OHCs and adjacent supporting cells in the lateral compartment of the cochlea (Huh et al., 2012). Inhibition of Fgf20 signaling was also reported to inhibit hair cell and supporting cell development in vitro (Hayashi et al., 2008b). These studies suggest that Fgf20 is needed for specification of hair cells and supporting cells in the prosensory domain, and that lack of Fgf20 causes a decrease in hair cell and supporting cell numbers. However, it is possible that other factors could specify hair cells and supporting cells in the prosensory domain, as there was some formation of OHCs and adjacent supporting cells in Fgf20 knockout cochleae.

It was also shown that expression of Fgf20 appears in the base of the cochlea at E13.5 and then extends towards the apex and disappears from the base. This base-to-apical wave of Fgf20 expression does not overlap with expression of the hair cell marker myosin VI (Hayashi et al., 2008b). The pattern of Fgf20 expression parallels Shh expression in the spiral ganglion and is compatible with our findings that Fgf20 is downstream of Hh signaling and that Fgf20 downregulation promotes hair cell differentiation. It is likely that Fgf20 needs to be turned on and off for hair cell development in the prosensory domain; our study indicates that withdrawal of Hh signaling turns off Fgf20, thereby promoting hair cell differentiation.

**Acknowledgements**

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**Competing interests statement**

The authors declare no competing financial interests.

**Author contributions**

T.T., J.I. and R.K. designed research; T.T. and I.I. performed mouse breeding; I.I. generated in situ hybridization probes; I.T., K.H. and H.T. performed ABR, DPOAEs and endocochlear potential recording; T.T. performed all other experiments; T.T. and R.K. wrote the manuscript.

**Supplemental material**

Supplemental material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.095398/-/DC1

**References**


Ohyama, T., Basch, M. L., Mishina, Y., Lyons, K. M., Segil, N. and Groves, A. K. (2010). BMP signaling is necessary for patterning the sensory and


**Fig. S1. Expression of Shh and Gli1 in the developing otic vesicle and cochlea.** (A-D) *In situ* hybridization showing Shh expression. (E-H) *In situ* hybridization showing Gli1 expression. Arrowheads in A and E indicate the expression of Shh and Gli1 in the neural tube, respectively. Asterisks indicate E11.5 statoacoustic ganglion (ganglion of CNVIII) (A,E) and E13.5 and E18.5 spiral ganglion (C,D,G,H). Arrows indicate E11.5 presumptive cochlear epithelium (B,F), E13.5 prosensory epithelium (C,G) and E18.5 sensory epithelium (D,H). Dorsal is up and lateral is to the right (A-C,E,F). At E11.5, Shh was strongly expressed in the floor plate of the neural tube (A, arrowhead) and Gli1 was also expressed in the neural tube (E, arrowhead). In the inner ear, Shh was expressed in E13.5 spiral ganglion (C) but not in the epithelium of cochlear duct (B-D, arrows) or the spiral ganglion of other ages (A,D, asterisks). Gli1 expression occurred in the presumptive cochlear epithelium and surrounding mesenchyme at E11.5 and E13.5, but not at E18.5 (E-H, arrows). Scale bars: A (for A,E), 200 μm; B (for B,F), 100 μm; C (for C,D,G,H), 50 μm.

**Fig. S2. Cre recombinase activity was examined in the developing cochlea of Emx2^{+/-};Rosa26-CFP mice.** (A) E11.5 section of otic vesicle. CFP expression was sparsely seen in Sox2-positive (red) prosensory domain of the presumptive cochlea (asterisk). (B) E13.5 cochlear section. The prosensory domain was labeled with p27^{kip1} (red) (asterisks). CFP expression was seen in most epithelial cells of the cochlear duct. (C) E18.5 cochlear section. CFP expression was seen in most epithelial cells of the cochlea duct but rarely seen in the spiral ganglion (arrowhead). Both hair cells and supporting cells were CFP-positive in the organ of Corti. (D) Two-month-old adult cochlear section. CFP expression was seen throughout the floor of cochlea duct epithelium. Scale bar: A (for A-D), 100 μm.
Fig. S3. Hair cell abnormalities in the apical turn of cochlea from 4-week-old Smo CKO mice. Reconstruction of sequential images of hair cell rows in 4W Smo CKO cochlear apical turn obtained by confocal microscopy. (A,B) MyosinVI (red), phalloidin (green) and Tuj1(blue). (C,D) MyosinVI, the same region as A (C) and B (D). OHCs formed only two rows (A-D). Asterisks in A and C indicate an ectopic cell with U-shape stereocilia located between the first and second OHC rows. Arrowheads indicate ectopic cells with U-shape stereocilia located between the IHC and OHC rows (B,D). Tuj1-positive fibers were seen around the ectopic IHC-like cells. Scale bar: D (for A-D), 20 μm.
Fig. S4. Prosensory cell-like cells in the lateral compartment produced by Hh activation are maintained despite Notch signaling inhibition. Representative images of E18.5 cochleae from control (A,E,I), R26-SmoM2;RbpJ<sup>1/2</sup> (B,F,J), RbpJ CKO (C,G,K) and R26-SmoM2;RbpJ CKO (D,H,L) embryos. (A-D) Surface preparation of cochlear epithelium visualized by Myosin VI. (E-H) Cross-section of cochlea immunohistochemically stained for MyosinVI (red, F-H) and p27<sup>kip1</sup> (green), and counterstained with DAPI (blue). (I-L) Cross-section of cochlea immunohistochemically stained for Tuj1 (red, J,L) or MyosinVI (red, K), Prox1 (green), and counterstained with DAPI (blue). Asterisks (E-L) indicate IHCs. In E18.5 R26-SmoM2;RbpJ<sup>1/2</sup> cochlea, prosensory-like cells are present in the lateral compartment (F), more lateral to the IHC (F, asterisk). P27<sup>kip1</sup>-positive cells adjacent to IHCs appear to be supporting cells (inner phalangeal cells and pillar cells). Arrowheads indicate Prox1-positive cells that are thought to be pillar cells (J, arrowheads). In R26-SmoM2;RbpJ CKO cochlea, the number of IHCs increases probably due to fate conversion of inner phalangeal cells and pillar cells, but prosensory-like cells in the lateral compartment do not differentiate into hair cells or supporting cells (D,H,L). Scale bar: L (for A-L), 20 μm.

Fig. S5. Pillar cell differentiation is delayed in R26-SmoM2 cochlea. Whole-mount preparations of E17.5 cochlea from control (A,C,E,G) and R26-SmoM2 CKO embryos (B,D,F,H) visualized by anti-p75<sup>NTR</sup> (green in A,B,E,F) or MyosinVI (green in C,D,G,H) and phalloidin (magenta). (A,C,E,G) Hair cells at positions 45% (A,C) and 90% (E,G) from the base along the basal-to-apical axis of the control cochlea. The distances were calculated from the average cochlear length of six control animals. (B,D,F,H) Hair cells in the regions located at the equivalent distance from the base as A (B,D) and E (F,H). Scale bar: H (for A-H), 20 μm.
Fig. S6. Histological analyses of cochleae from adult Smo CKO mice. (A-I) Surface preparation of cochlear epithelium in control mice (A-C), Smo CKO mice with residual hearing (D-F), and deaf Smo CKO mice (G-I), visualized by anti-MyosinVI (Green) and phalloidin (magenta). Hair cell loss is observed not only in Smo CKO but also in control mice at 40 weeks of age and appears to be mildest in the apical turn (A,D,G), more severe in the middle turn (B,E,H) and the most severe in the basal turn (C,F,I). Hearing ability seems to be proportional to the remaining hair cell numbers: the control cochleae (A-C) maintain many hair cells, whereas Smo CKO cochleae with residual hearing have fewer (D-F) and deaf Smo CKO cochleae (G-I) have the least number of hair cells. Hair cells are completely lost in the basal turn of deaf Smo CKO mice. (J-R) Cross-sections of control mice (J-L), Smo CKO mice with residual hearing (M-O), and deaf Smo CKO mice (P-R), showing the spiral ganglia of the middle turn (J,M,P), mid-basal turn (K,N,Q) and basal turn (L,O,R), stained with Cresyl Violet. The loss of neurons in spiral ganglion is observed in Smo CKO and control mice at 40 weeks of age especially in the basal turn (L,O,R). Neuronal loss is more severe in the cochleae of deaf mice than in control and Smo CKO mice with residual hearing, although neurons still remain even in the cochleae of the deaf mice. Scale bars: I (for A-I), 20 μm; R (for J-R), 20 μm.
Fig. S7. ABR waveforms and DPOAE data of 6-week-old Smo CKO mice. (A-H) Representative ABR waveforms of 6-week-old control (A,C,E,G) and Smo CKO (B,D,F,H) mice. All waveforms were 10 kHz and left ears. Waves at 40 dB (A), 20 dB (C), 10 dB (E) and 0 dB (G) are shown in control, and waves at 90 dB (B), 70 dB (D), 50 dB (F) and 40 dB (H) are shown in Smo CKO. X-axis; mS, Y-axis; mV. In A-D, Waves I-VI were apparent. Both of control and Smo CKO ABR waveforms showed a lower peak of Wave I when sound pressure was reduced (E,F), and waves generated by more central portion of auditory pathway (Waves II-V in E and F) were relatively maintained. These ABR waveforms of Smo CKO mice indicate that the cause of hearing impairment was in the periphery of the auditory pathway. (I,J) DP and NF (noise floor) of the same ears in control (I) and Smo CKO (J) mice, measured at 6 weeks of age. Smo CKO mice showed lower DP than control, suggesting that the function of OHCs was diminished in 6-week-old Smo CKO cochleae.
Table S1. Primary antibodies used for immunohistochemistry

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<td>BD Transduction Laboratories</td>
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### Table S2. Primer sets for real time RT-PCR

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Table S3. The ratio of Smo CKO cochlea length to the mean of control siblings

<table>
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