

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 *in vivo*. The Hh effector *smoothed* (*Smo*) was genetically activated or inactivated specifically in the developing cochlear epithelium after prosensory domain formation. Cochlea 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 cochlea 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 cochlea, 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 (Ptch1) on Hh-responding cells, resulting in de-repression of *smoothed* (*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*^{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^{flox^{ed}} (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*^{flox^{ed}/flox^{ed}} (*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*^{flox^{ed}} (Han et al., 2002), *Rosa26-SmoM2* and *Emx2*^{+/^{Cre}} mice were used to produce *Emx2*^{+/^{Cre}}; *Rbpj*^{flox^{ed}/flox^{ed}} and *Emx2*^{+/^{Cre}}; *Rbpj*^{flox^{ed}/flox^{ed}}; *R26-SmoM2* mice. For analysis of Cre recombinase 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-adult) were fixed immediately in 4% paraformaldehyde in 0.1 M PBS pH 7.4, then 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). The primary antibodies used in this study are listed in supplementary material Table S1. Goat or donkey anti-species IgG conjugated with Alexa 488 or Alexa 594 were used as secondary antibodies. Nuclei were stained with 4,6'-diamidino-2-phenylindole (DAPI). For p27^{Kip1} (Cdkn1b – Mouse Genome Informatics) and Prox1 staining, samples were heated in 10 mM sodium citrate at 90°C for 10 minutes prior to the staining procedure (Tateya et al., 2011). For staining of whole-mount preparations of the cochleae, cochlear ducts were opened to expose the developing sensory epithelia prior to the staining procedure (Yamamoto et al., 2009). Actin filaments were visualized with Alexa488-conjugated phalloidin (1:200) (Invitrogen-Molecular Probes).

In situ hybridization was carried out using mouse *Shh*, *Gli1*, *Hes5*, *Hey2*, *Bmp4*, *Atoh1*, *Fgf8*, *Fgf20* and *Fgfr3* probes, as described previously (Imayoshi et al., 2008).

Determination of cochlear length and cell density

Cochleae were dissected from embryos or pups at specific developmental time points. The developing cochlear epithelium was exposed, labeled with phalloidin and anti-myosin VI, and flat-mounted as previously described (Yamamoto et al., 2009). Fluorescent samples were imaged using a Zeiss LSM510 confocal microscope, and the entire length of the cochlear duct was measured using LSM510 operating software to identify the 25%, 50% and 75% positions along the basal-to-apical axis of the cochlear duct. At each position, the number of hair cells within 150 μm (E18.5) or 200 μm [post-natal day (P)7] parallel to the basal-to-apical axis of the cochlea was counted.

Cochlear explant cultures

Cochleae were dissected from mouse embryos, placed in PBS and the cochlear epithelium was removed mechanically from the cochlear bone and cut at the hook region. Spiral ganglion neurons remained attached to the cochlear epithelium. The entire cochlear epithelium with attached spiral ganglion neurons was placed onto culture inserts (Millipore) and cultured in DMEM/F-12 supplemented with 3 mg/ml glucose and 0.1 mg/ml

ampicillin (Ono et al., 2009). Cyclopamine (Enzo Life Sciences; 5 μM) was used for Hh signaling inhibition, and DMSO was used for the negative control of Hh signaling inhibition. For Fgf20 inhibition, 60 μg/ml anti-Fgf20 antibody (R&D Systems) was used, and 60 μg/ml of goat IgG (R&D Systems) was used for the negative control of Fgf signaling inhibition (Hayashi et al., 2008b). SU5402 (Calbiochem; 10 μg/ml) was also used for Fgf signaling inhibition.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA from membranous cochleae was reverse-transcribed using Rever-Tra Ace (TOYOBO) and Random Primer (TOYOBO). Real-time PCR was performed using Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and THUNDERBIRD SYBR qPCR Mix (TOYOBO), according to the manufacturer's protocols. *Gapdh* was used as a control. Primers are listed in supplementary material Table S2.

Statistical analysis

Three or more mice from each group were analyzed in all experiments. Control animals were the siblings of *Smo* CKO mice and included both *Emx2*^{+/⁺} and *Smo* heterozygote (*Emx2*^{+/^{Cre}} and *Smo*^{+/^{flox^{ed}}}, respectively) mice because *Smo* heterozygote cochleae did not show any significant differences from *Emx2*^{+/⁺} cochleae (data not shown). 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, 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 cochleae

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 recombinase of *Emx2*^{+/^{Cre}} cochleae 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 cochleae.

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

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

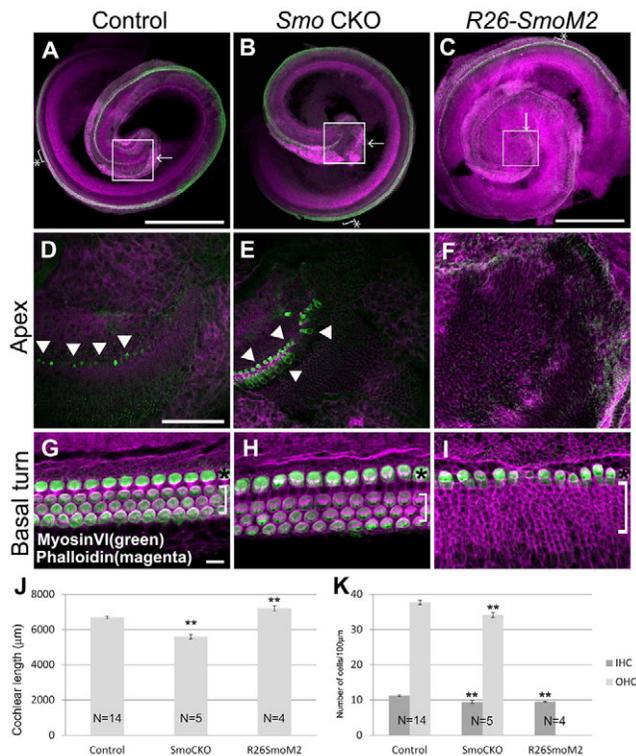


Fig. 1. Effects of activation and inactivation of Hh signaling on E18.5 cochlear epithelium. (A-I) Representative examples of cochleae from control (A,D,G), *Smo* CKO (B,E,H) and *R26-SmoM2* (C,F,I) mice visualized by anti-myosin VI (green) and phalloidin (magenta).

(A-C) Whole-mount preparations of E18.5 cochleae 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 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.

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*

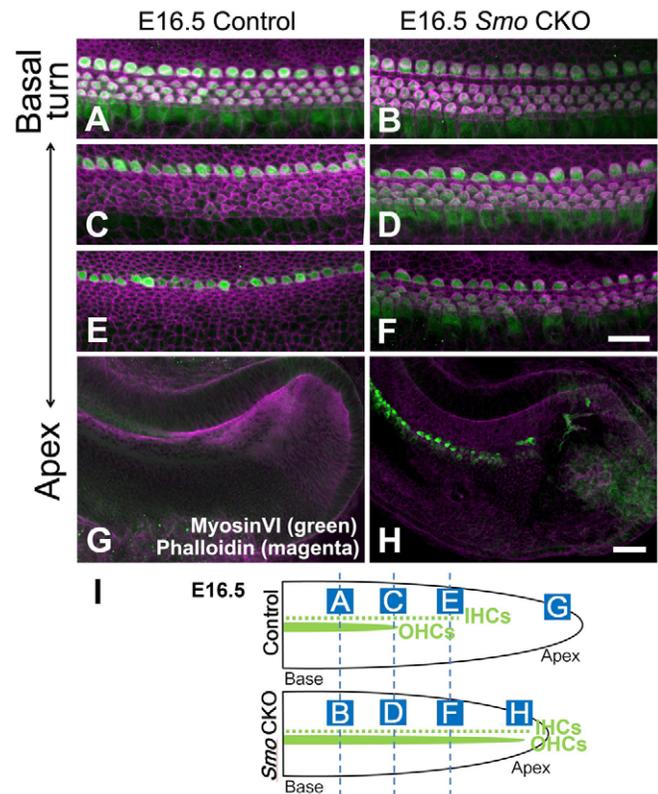


Fig. 2. Hair cell differentiation is accomplished earlier in *Smo* CKO cochlea.

Whole-mount preparations of E16.5 cochleae 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.

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

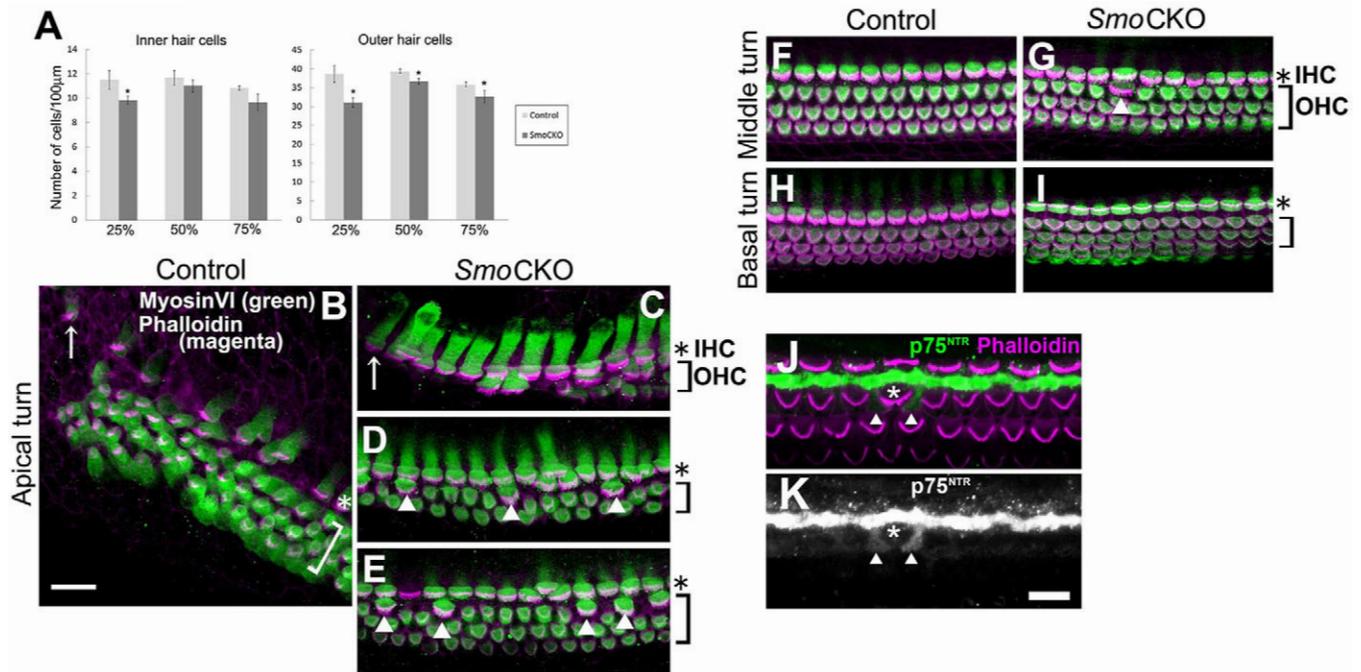


Fig. 3. Deformity of hair cell rows in the apical part of *Smo* CKO cochleae. (A) Hair cell number per 100 μm determined by counting hair cells along a 200 μm cochlear length located at positions 25%, 50% and 75% of P7 *Smo* CKO and control cochleae from the apex along the basal-to-apical axis. Three 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–I) The surface of control (B,F,H) and P7 *Smo* CKO (C–E,G,I) 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). (J,K) Weak $p75^{\text{NTR}}$ 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) $p75^{\text{NTR}}$ (green) and phalloidin (magenta). (K) $p75^{\text{NTR}}$ alone. Scale bars: B (for B–I), 20 μm ; K (for J,K), 10 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 OHC numbers at 25%, 50% and 75% from the apex were significantly decreased in *Smo* CKO cochleae compared with control cochleae. These results indicated that inactivation of Hh signaling led to a decrease in the total hair cell number with a greater reduction in the apical than the basal region and a larger decrease in OHCs than in IHCs.

***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 cochlea 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 $p75^{\text{NTR}}$ (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.

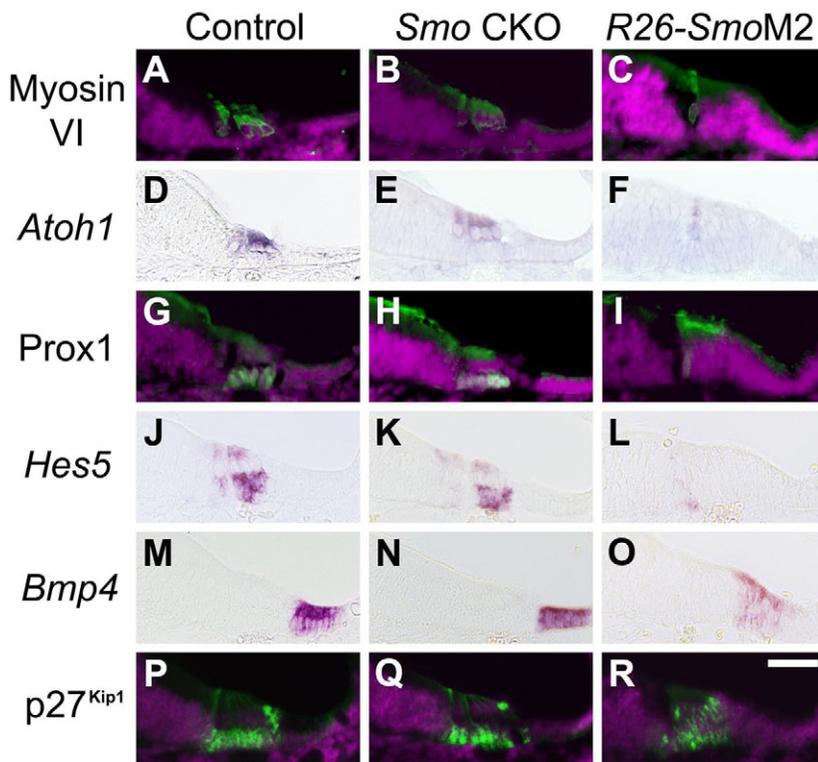


Fig. 4. Expression of markers for prosensory and sensory cells in E17.5 cochleae. Expression patterns of the hair cell markers myosin VI (A-C, green) and *Atoh1* (D-F); the supporting cell markers Prox1 (G-I, green) and *Hes5* (J-L); and the prosensory and supporting cell markers *Bmp4* (M-O) and $p27^{Kip1}$ (P-R, green). Representative examples of cross-sections through the mid-basal turn of E17.5 control, *Smo* CKO and *R26-SmoM2* cochleae are shown. *Atoh1*, *Hes5* and *Bmp4* were detected by *in situ* hybridization. Myosin VI, Prox1 and $p27^{Kip1}$ were labeled by immunohistochemistry and the nuclei were counterstained with DAPI (magenta). Scale bar: R (for A-R), 20 μ m

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 ($p27^{Kip1}$, *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,I,L). qRT-PCR analysis of E18.5 control and *R26-SmoM2* cochleae also revealed that the hair cell markers *Atoh1* and myosin VIIa (*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 $p27^{Kip1}$ -positive lateral compartment (Fig. 4O,R).

$p27^{Kip1}$ 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, $p27^{Kip1}$ 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 $p27^{Kip1}$, and the nuclei of $p27^{Kip1}$ -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

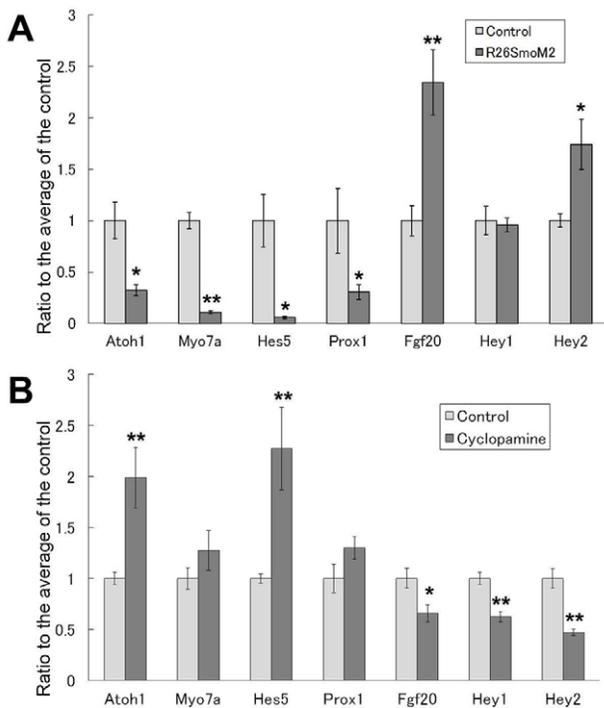


Fig. 5. Effect of Hh activation and inactivation on mRNA levels of prosensory and sensory cell markers in the developing cochleae.

(A) *Atoh1*, myosin VIIA, *Hes5*, *Prox1*, *Fgf20*, *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, *Hes5* and *Prox1* were significantly downregulated, and *Fgf20* and *Hey2* were significantly upregulated in *R26-SmoM2* cochleae. (B) mRNA levels of *Atoh1*, myosin VIIA, *Hes5*, *Prox1*, *Fgf20*, *Hey1* and *Hey2* in E13.5 cochleae cultured for 24 hours with or without 5 μ M cyclopamine. The levels are shown as a ratio to the average of the control without 5 μ M cyclopamine. *Atoh1* and *Hes5* were significantly upregulated, and *Fgf20*, *Hey1* and *Hey2* were significantly downregulated. * $P < 0.05$; ** $P < 0.01$. Error bars represent standard errors.

(Fig. 6A,C). However, *Fgf20* was upregulated in the lateral compartment of *R26-SmoM2* cochleae compared with control cochleae (Fig. 6A-D). qRT-PCR analysis also showed that *Fgf20* was upregulated in E18.5 *R26-SmoM2* cochleae (Fig. 5A) but downregulated in E13.5 cochleae cultured with cyclopamine (Fig. 5B). These results indicated that *Fgf20* was upregulated when Hh signaling was abnormally activated.

Fgf8, *Fgfr3* and *Hey2* are known to be involved in the differentiation of pillar cells, a subtype of supporting cells that are located between IHCs and OHCs (Mueller et al., 2002; Jacques et al., 2007; Puligilla et al., 2007; Doetzlhofer et al., 2009). Beginning at \sim E16.5, *Fgfr3* is expressed in a population of progenitor cells that will develop as OHCs and adjacent supporting cells including pillar cells and Deiters cells; as development proceeds, expression of *Fgfr3* becomes restricted to developing pillar cells (Mueller et al., 2002; Hayashi et al., 2007; Jacques et al., 2007). Expression of *Hey2*, a downstream effector of Fgf signaling, occurs in the prosensory domain from E12.5; as development continues, it becomes restricted to developing pillar cells, similar to *Fgfr3* (Hayashi et al., 2008a; Doetzlhofer et al., 2009). We examined the expression of *Fgf8*, *Fgfr3* and *Hey2* in E18.5 control and *R26-SmoM2* cochleae (Fig. 6). IHCs of both E18.5 control and E18.5

R26-SmoM2 cochleae were found to express *Fgf8* (Fig. 6E-H). In E18.5 control cochleae, *Fgfr3* expression occurred in a population of developing OHCs and adjacent supporting cells (Fig. 6I,K). In E18.5 *R26-SmoM2* cochleae, *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. 6J,L). *Hey2* expression in E18.5 *R26-SmoM2* cochleae was also seen in a progenitor population of the lateral compartment; in control cochleae, *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 found (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 cochleae 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

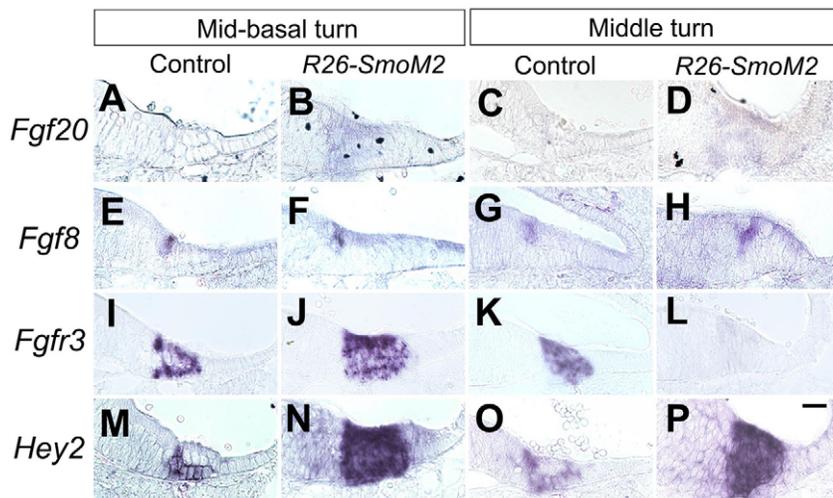


Fig. 6. Activation of Hh signaling affects Fgf signaling. Expression of *Fgf20* (A-D), *Fgf8* (E-H), *Fgfr3* (I-L) and *Hey2* (M-P) detected by *in situ* hybridization. Representative cross-sections of the mid-basal turn and middle turn in E18.5 control and *R26-SmoM2* cochleae are shown. Scale bar: P (for A-P), 20 μ m.

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. *Smo*CKO1, *Smo*CKO2 and *Smo*CKO5 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 cochleae.

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 (*Gli3* ^{Δ 699/ Δ 699}), in which Hh signaling is only partially lost, showed that *Gli3* ^{Δ 699/ Δ 699} cochleae were shorter than normal and contained additional rows of hair cells and ectopic hair cells, suggesting that

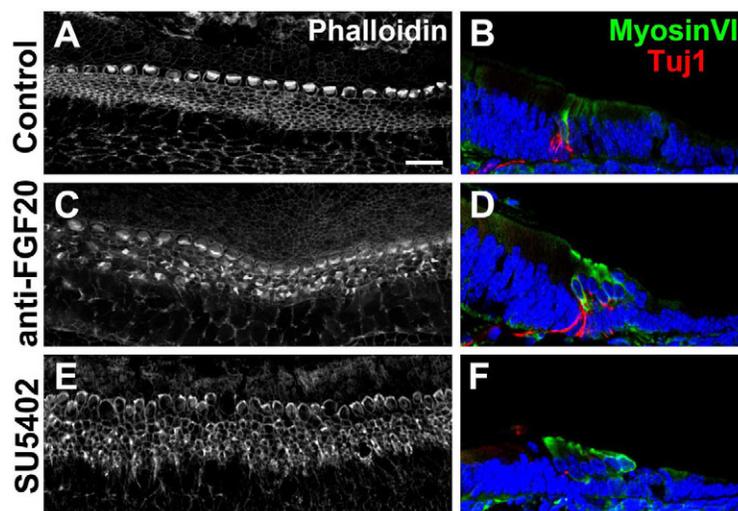


Fig. 7. Fgf20 inhibition promotes OHC differentiation in cultured *R26-SmoM2* cochleae. E18.5 *R26-SmoM2* cochleae cultured with control 60 μ g/ml goat IgG (A,B), 60 μ g/ml anti-Fgf20 antibody (C,D) or 10 μ M FGF signaling inhibitor SU5402 (E,F) for 48 hours. (A,C,E) Cochlear epithelial surface visualized with phalloidin. (B,D,F) Cross-section of cultured cochleae labeled immunohistochemically for myosin VI (green) and Tuj1 (red), and counterstained with DAPI (blue). Scale bar: A (for A-F), 20 μ m.

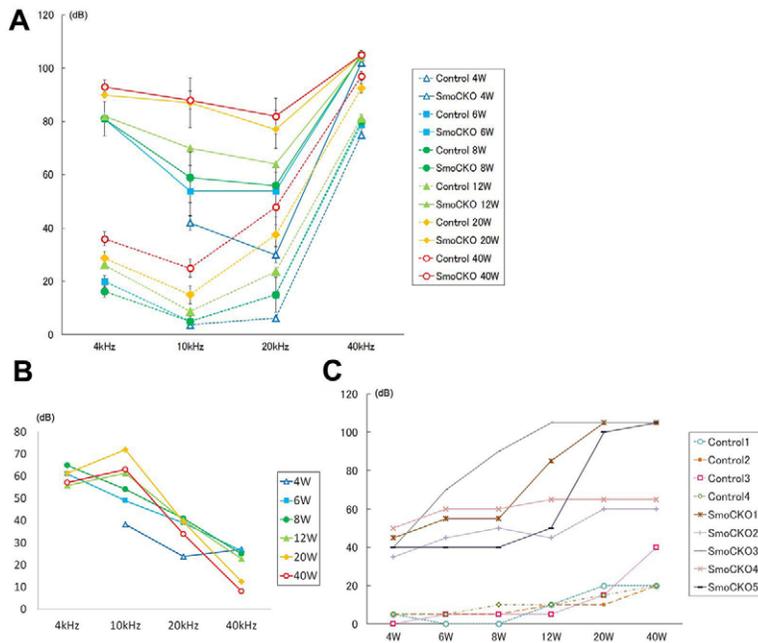


Fig. 8. ABR thresholds of *Smo* CKO mice. ABR thresholds of *Smo* CKO mice and control siblings from 4 to 40 weeks of age. (A) Average ABR thresholds of *Smo* CKO mice and control siblings. Error bars represent xxx. (B) Differences in average ABR thresholds between *Smo* CKO and control mice. The average threshold at each frequency of *Smo* CKO mice was subtracted from that of control mice. (C) Temporal change of 10 kHz in ABR threshold for each animal for which ABR threshold is shown in A. These data show progressive hearing loss in *Smo* CKO mice compared with control mice. Error bars represent standard errors.

Hh signaling regulates prosensory cell formation (Driver et al., 2008). These phenotypes of *Gli3*^{Δ699/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 *Gli3*^{Δ699/Δ699} cochleae, whereas the cells that lost *Smo* were unable to respond to Hh signaling. We speculate that the differences in phenotypes between *Gli3*^{Δ699/Δ699} and *Smo* CKO mice are due to differences regarding when and where Hh signaling is inactivated. In *Gli3*^{Δ699/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 cell formation. In *Smo* CKO cochleae, inhibition of Hh signaling is almost limited to the epithelium after prosensory cell formation because of the Cre recombinase activity in *Emx2*^{+Cre} mice (Tateya et al., 2011) (supplementary material Fig. S2). Possibly, use of genetic inactivation of *Smo* in the *Emx2*^{+Cre} 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 cochleae 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 recombination at early developmental stages: the Cre recombinase in *Emx2*^{+Cre} mice begins to be active ~E11.5 and thereafter shows increasing activity. The efficiency of Cre-dependent recombination in *Emx2*^{+Cre} mice was ~90% in E14.5 cochleae (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 recombination in *Emx2*^{+Cre} 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 cochleae 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*^{Δ699/Δ699} 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.

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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.

Supplementary material

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

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