Otic ablation of smoothened reveals direct and indirect requirements for Hedgehog signaling in inner ear development

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SUMMARY
In mouse embryos lacking sonic hedgehog (Shh), dorsoventral polarity within the otic vesicle is disrupted. Consequently, ventral otic derivatives, including the cochlear duct and saccule, fail to form, and dorsal otic derivatives, including the semicircular canals, endolympathic duct and utricle, are malformed or absent. Since inner ear patterning and morphogenesis are heavily dependent on extracellular signals derived from tissues that are also compromised by the loss of Shh, the extent to which Shh signaling acts directly on the inner ear for its development is unclear. To address this question, we generated embryos in which smoothened (Smoecko), an essential transducer of Hedgehog (Hh) signaling, was conditionally inactivated in the otic epithelium (Smoecko/). Ventral otic derivatives failed to form in Smoecko/ embryos, whereas vestibular structures developed properly. Consistent with these findings, we demonstrate that ventral, but not dorsal, otic identity is directly dependent on Hh. The role of Hh in cochlear-vestibular ganglion (cvg) formation is more complex, as both direct and indirect signaling mechanisms are implicated. Our data suggest that the loss of cvg neurons in Shh+/− animals is due, in part, to an increase in Wnt responsiveness in the otic vesicle, resulting in the ectopic expression of Tbx1 in the neurogenic domain and subsequent repression of Ngn1 transcription. A mitogenic role for Shh in cvg progenitor proliferation was also revealed in our analysis of Smoecko embryos. Taken together, these data contribute to a better understanding of the intrinsic and extrinsic signaling properties of Shh during inner ear development.

KEY WORDS: Inner ear, Sonic hedgehog, Otic vesicle, Cochlear-vestibular ganglia, Neurogenesis, Mouse

INTRODUCTION
The mammalian inner ear is a sensory organ with dual roles in sound and motion detection. The partitioning of these functions within the inner ear to auditory and vestibular components occurs early in embryonic development, allowing each of these senses to operate independently (Bok et al., 2007a). The auditory portion of the inner ear, the cochlea, derives from the ventral outgrowth of the otic vesicle, which progressively extends and coils as it matures. Mechanosensory hair cells lining the cochlear duct from base to apex respond to sound waves in a tonotopic manner, and transmit information along auditory (spiral) neurons to sound-processing centers in the brain (Rubel and Fritzsch, 2002; Schawander et al., 2010). Vestibular structures, by contrast, mostly derive from dorsal outpockets of the otic vesicle and through incompletely understood mechanisms are sculpted into the three semicircular canals, utricle and saccule (Martin and Swanson, 1993; Bok et al., 2007a). Sensory patches associated with each of these structures detect angular movements of the head (semicircular canals) and linear acceleration along the horizontal (utricle) and vertical (saccule) planes. Vestibular neurons innervating each of these sensory patches transmit sensory information to visual, vestibular and proprioceptive centers to coordinate balance (Straka et al., 2005).

The hindbrain is a crucial source of signals necessary for dorsoventral patterning of the otic vesicle and subsequent morphogenesis into auditory and vestibular components (Giraldez, 1998; Bok et al., 2005; Kil et al., 2005; Riccomagno et al., 2005; Schneider-Maunoury and Pujades, 2007; Liang et al., 2010). Members of the Wnt and Hedgehog (Hh) families play prominent roles in establishing dorsoventral identity within the otic epithelium. Wnt1 and Wnt3a secreted from the dorsal hindbrain regulate the expression of dorsal otic determinants, such as the homeodomain transcription factors Dlx5 and Dlx6 (Riccomagno et al., 2005; Robledo and Lufkin, 2006). Consequently, vestibular morphogenesis fails in Wnt1−/−;Wnt3a−/− mutants (Riccomagno et al., 2005). Sonic hedgehog (Shh), secreted from the floor plate of the hindbrain and notochord, opposes the dorsalizing effects of Wnts by repressing Dlx5 and activating ventral otic genes, including the transcriptional regulators Otx2 and Pax2 (Riccomagno et al., 2002; Riccomagno et al., 2005). The failure to regulate the ventral otic program in Shh−/− embryos results in cochlear agenesis (Favor et al., 1996; Torres et al., 1996; Morsli et al., 1999; Riccomagno et al., 2002; Burton et al., 2004). Interestingly, Shh−/− embryos also display profound deficits in vestibular development, including malformations of the semicircular canals, utricle, saccule and endolympathic duct. Each of these morphological defects can be traced back to alterations in the expression of otic vesicle patterning genes (Riccomagno et al., 2002). For example, the misexpression of Otx1 and of Gbx2 in the Shh mutant otocyst are likely to explain the absence of the lateral semicircular canal and endolympathic duct, respectively (Acampora et al., 1996; Lin et al., 2005).

Shh also functions in inner ear neurogenesis. The cochlear and vestibular neurons that make up the eighth cranial nerve originate from progenitors in the anteroventral region of the otic vesicle that express Ngn1 (Neurog1), a neural determinant required for their specification (Ma et al., 1998). The establishment of the neurogenic domain is one of the earliest signs of asymmetry along the anteroposterior axis of the otic vesicle. The T-box-containing transcription factor Tbx1 is expressed in a complementary pattern

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Accepted 1 July 2011
to Ngn1 and is required to restrict the neurogenic domain to the anterior portion of the otocyst (Raft et al., 2004). Shh\(^{-/-}\) embryos show a significant reduction in Ngn1 expression, suggesting a possible involvement in the regulation of anteroposterior identity within the otic vesicle, although the underlying mechanism has not been elucidated (Riccomagno et al., 2002).

What remains uncertain from these previous studies is the extent to which the inner ear phenotype in Shh\(^{-/-}\) embryos can be attributed to a direct loss of Shh signaling within the otic epithelium versus an indirect consequence of the absence of Shh for tissues surrounding the inner ear. The hindbrain and periotic mesenchyme are sources of other signals essential for inner ear development that are also disrupted in Shh\(^{-/-}\) embryos (Phippard et al., 1999; Riccomagno et al., 2002; Yamagishi et al., 2003; Bok et al., 2005; Riccomagno et al., 2005; Xu et al., 2007; Braunstein et al., 2009; Liang et al., 2010). Thus, their misregulation could also explain the inner ear defects observed in Shh\(^{-/-}\) mutants.

The best evidence in support of Shh acting directly on the otic epithelium comes from the observation that Gli1, a transcriptional target of the Shh pathway, is expressed in a graded manner along the dorsoventral axis of the otocyst, with higher levels detected ventrally, closer to the source of Shh, and lower levels tapering off dorsally (Bok et al., 2007b). Although suggestive, this result does not resolve the functional significance of this signaling gradient.

The analysis of single and compound mutants in Gli2 and Gli3, the transcriptional mediators of Shh signaling, support a model whereby reciprocal gradients of Gli activator and Gli repressor function are required to shape inner ear morphology along the entire dorsoventral axis in response to Shh (Bok et al., 2007b). Of particular interest is the finding that vestibular, but not auditory, defects can be prevented in Shh\(^{-/-}\) mutants by removing a wild-type allele of Gli3 (Shh\(^{-/-}\);Gli3\(^{+/-}\)). This suggests that Shh promotes vestibular morphogenesis by reducing Gli3 repressor function (Bok et al., 2007b). However, it does not address the tissue specificity of this action. Recovery from the vestibular defects in Shh\(^{-/-}\);Gli3\(^{+/-}\) embryos could equally be explained by the reduction of Gli3 repression in the inner ear as it could the reduction of Gli3 in the neural tube, which also shows improvements in patterning and morphology compared with Shh\(^{-/-}\) embryos (Litingung and Chiang, 2000; Bok et al., 2007b).

In order to distinguish between the primary requirements for Shh in inner ear development from its secondary roles in surrounding tissues, we generated conditional mutants in which smoothed (Smoo), an essential Hh signal transduction component, was selectively inactivated in the otic epithelium (Smoo\(^{cKO}\)). Our results demonstrate that Shh acts directly on the otic epithelium to regulate ventral target genes that are necessary for the outgrowth of the cochlear duct and sacculle. The development of dorsal otic derivatives is indirectly dependent on Shh, as these vestibular structures were absent or malformed in Shh\(^{-/-}\) mutants but maintained in the ears of Smoo\(^{cKO}\) embryos. The role of Hh signaling in cochlear-vestibular ganglion (cvg) formation is more complex, as it is dependent on both direct and indirect signaling mechanisms. Our data suggest that the loss of cvg neurons in Shh\(^{-/-}\) animals is partly due to an increase in Wnt responsiveness in the otic vesicle (indirect signaling), resulting in the ectopic expression of Tbx1 in the neurogenic domain and in the subsequent repression of Ngn1 transcription. An unanticipated role for Shh as a mitogen for cvg progenitors was also revealed in our analysis of Smoo\(^{cKO}\) embryos (direct signaling). These data contribute to a better understanding of the intrinsic and extrinsic signaling properties of Shh during inner ear development.

### MATERIALS AND METHODS

#### Animals

**Foxg1\(^{Flox}\)** and Smoo\(^{cKO/loxp}\) mouse lines are described elsewhere (Hebert and McConnell, 2000; Long et al., 2001). Smoo\(^{cKO/loxp}\) mice were maintained on a mixed Swiss-Webster, C57BL6/J background. Shh\(^{-/-}\) (Chiang et al., 1999) and Rosa\(^{Gfp}\)/(Madisen et al., 2010) mice were obtained from Jackson Labs (Bar Harbor, ME, USA). Tbx1\(^{+/-}\) mice were provided by J. Epstein (Liao et al., 2004). Toppal mice were provided by E. Fuchs (DasGupta and Fuchs, 1999).

#### Immunohistochemistry

For immunohistochemistry, embryos were fixed in 4% paraformaldehyde for 1 hour, cryoprotected in 30% sucrose overnight, mounted in OCT embedding medium (Sakura Finetek, Torrance, CA, USA) and snap frozen. Embryos were sectioned at 14 μm and stained with DAPI and the following antibodies: mouse anti-islet1 (Is11; DSHB) 1:100, rabbit anti-phospho-histone H3 (Cell Signaling Technology, Danvers, MA, USA) and mouse anti-α-tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) 1:1000, mouse anti-α-neurofilament (DSHB) 1:200, chicken anti-GFP (Aves Labs, West Grove, PA, USA) or Alexa 488 (Molecular Probes Eugene, OR, USA), donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch West Grove, PA, USA) or Alexa 488 (Molecular Probes Eugene, OR, USA), donkey anti-rabbit IgG conjugated to Cy3 or Alexa 488, or goat anti-chicken IgG conjugated to Alexa 488.

#### In situ hybridization

For section in situ hybridization, embryos were processed in the same manner as for immunohistochemistry. Sections were rehydrated in PBS containing 0.1% Tween 20 (PBS-Tween), and hybridization was performed as described (Nissim et al., 2007). For antibody detection after in situ hybridization, the following modifications were made: Proteinase K treatment was omitted. After completion of the BM purple reaction, slides were washed three times in PBS-Tween, fixed for 10 minutes with 4% paraformaldehyde then washed three times in PBS-Tween. Slides were then incubated in primary antibody and the immunohistochemistry protocol was followed. Whole-mount in situ hybridization was carried out as described (Matise et al., 1998) using digoxigenin-UTP-labeled riboprobes.

#### Embryo culture

Embryo roller culture was performed as described (Martin and Cockcroft, 2008). Briefly, E9.5 embryos were collected in ice-cold L-15 medium without damaging the yolk sac. Embryos were grown under 95% O\(_2\) and 5% CO\(_2\) at 37°C in 100% rat serum (Gemini Bio-Products, West Sacramento, CA, USA) supplemented with 0.175 mg/ml glucose, 2 mM glutamine, 1× Pen-Strep. Embryos were regassed every 12 hours. For LcI treatment, embryos carrying a Toppal transgene were cultured with increasing amounts of LcI to determine an optimal concentration (50 mM) that maximized Wnt reporter activity without excessive toxicity (data not shown). The Fgf inhibitor EMD 341608 (Hamby et al., 1997) was dissolved in DMSO at 3 mg/ml and added to culture media to a final concentration of 25 μM.

#### Inner ear paint fill

Inner ear paint fills were performed essentially as described (Martin and Swanson, 1993), with the exception that Wite-Out Plus (Bic, Milford, CT, USA) was used to fill the inner ears instead of latex paint.

#### Cell counts

The total number of cells in the cvg was determined by counting Isl1\(^{+}\) cRet\(^{-}\) cells in each sequential section through the entire otic vesicle. Brightfield images of section in situ hybridizations were inverted, assigned a color and merged with DAPI and antibody channels in ImageJ. Cells were manually counted using the cell counter plug-in in ImageJ.

#### Area measurements

To determine the percentage of otic vesicle expressing Tbx1, the area of positive staining in lateral whole-mount views was traced in ImageJ and measured, and then divided by the total area of the otic vesicle.
RESULTS

Inactivation of Hedgehog signaling in the otic epithelium

To determine the specific requirements of Hedgehog (Hh) signaling in the inner ear, we generated embryos in which a floxed allele of Smo (Smo\textsuperscript{flox}) was selectively inactivated in the otic epithelium using the Foxg1\textsuperscript{cre} mouse line (Hebert and McConnell, 2000; Long et al., 2001). The Foxg1\textsuperscript{cre}\textsuperscript{rev} line was particularly advantageous for our studies because it is active in all otic progenitors well in advance of when Shh signaling is known to be required in the otic vesicle (Hebert and McConnell, 2000; Riccomagno et al., 2002). Moreover, cre showed minimal expression in tissues surrounding the otic vesicle, including the neural tube and periotic mesenchyme (see Fig. S1 in the supplementary material). In all experiments described below, at least three to five Foxg1\textsuperscript{cre}\textsuperscript{rev};Smoecko\textsuperscript{loxp/} embryos (referred to as Smoecko\textsuperscript{cko} for ear conditional knockout of Smo) were compared with an equal number of control littermates (Foxg1\textsuperscript{cre/}+;Smoloxp/–) genotypes. No differences were seen in ear morphology or vesicle patterning between Foxg1\textsuperscript{cre/}+ and Foxg1\textsuperscript{cre+} genotypes.

We first assessed the effect of deleting Smo in the inner ear by examining the expression of Gli1 and Ptcl1 (Ptch1), two transcriptional targets of Hh signaling. In control embryos, Gli1 expression initiated weakly at E9.5 in the ventral-most region of the otic vesicle (Fig. 1A; n=4). At this stage, Ptcl1 was not yet detected in the otic epithelium despite its strong expression in other Shh-responsive cell types, including the ventral neural tube and periotic mesenchyme (Fig. 1C; n=3). By E10.5 Shh signaling intensified, resulting in robust Gli1 and Ptcl1 staining in ventral regions of the otic vesicle and along the medial wall in a ventral (high) to dorsal (low) gradient (Fig. 1E,G; n=3) (Bok et al., 2007b). Smoecko\textsuperscript{cko} embryos consistently failed to express Gli1 and Ptcl1 in the otic epithelium at both stages analyzed, yet robust expression of these markers was observed in the neural tube and periotic mesenchyme (Fig. 1B,D,F,H). Therefore, the disruption to Hh signaling was both specific and complete in the inner ears of Smoecko\textsuperscript{cko} embryos.

![Fig. 1. Inactivation of Hedgehog signaling in Smoecko otic vesicles.](image1)

In situ hybridization for Gli1 (A,B,E,F) and Ptcl1 (C,D,G,H) on transverse sections through the otic vesicle of control and Smoecko mouse embryos. Arrowheads indicate staining within the otic epithelium. (A,B) At E9.5, Gli1 was detected in the otic epithelium of control (A) but not Smoecko (B) embryos. (C,D) Ptcl1 was not detected in the otocyst of either control (C) or Smoecko (D) embryos at this stage. (E-H) By E10.5, both Ptcl1 and Gli1 were detected in the otic epithelium of control (E,G) but not Smoecko (F,H) embryos. Ptcl1 and Gli1 were also detected in the neural tube and periotic mesenchyme of control and Smoecko embryos. D, dorsal; M, medial; nt, neural tube; ov, otic vesicle; pom, peri-otic mesenchyme.

Cochlear, but not vestibular, morphogenesis is dependent on direct Hh signaling within the otic epithelium

Shh\textsuperscript{−/−} embryos show profound vestibular and auditory defects, including cochlear agenesis, missing or malformed semicircular canals and absence of the utricle, saccule and endolymphatic duct (Fig. 2A,C). If these defects are wholly attributed to the loss of Shh signaling in the otic epithelium, then they should be recapitulated in Smoecko\textsuperscript{cko} embryos. However, if some, or all, of these phenotypes result from secondary consequences of perturbing Shh signaling in tissues surrounding the inner ear, then they should be milder in Smoecko\textsuperscript{cko} embryos.

We visualized the gross anatomy of Smoecko and control inner ears by paint fill at E15.5 (Fig. 2A,B). At this stage, the morphology of the inner ear is near full maturity in wild-type embryos. The vestibulum, which comprises the three semicircular canals, utricle, saccule, endolymphatic duct and common crus, was readily discernible and the cochlear duct had elongated and coiled 1.5 turns (Fig. 2A). Ventral ear structures, namely the cochlear duct and saccule, were entirely absent in Smoecko\textsuperscript{cko} embryos (n=14 ears), a phenotype similar to that observed in Shh\textsuperscript{−/−} mutants (Fig. 2B,C). Remarkably, all dorsal otic derivatives, including the semicircular canals, utricle and endolymphatic duct, were present in Smoecko\textsuperscript{cko} embryos (Fig. 2B). The appearance of dorsal vestibular structures in Smoecko\textsuperscript{cko} embryos contrasts with the pronounced vestibular dysmorphology observed in Shh\textsuperscript{−/−} mutants and suggests that dorsal otic derivatives are not directly dependent on Shh for their development. Conversely, the consistent loss of ventral inner ear structures in Smoecko\textsuperscript{cko} and Shh\textsuperscript{−/−} embryos suggests that Shh signaling, acting directly on the otic epithelium, is required for cochlear duct outgrowth and saccule formation.

![Fig. 2. Cochlear, but not vestibular, morphogenesis is directly dependent on Hh signaling within the otic epithelium.](image2)

Medial view of mouse inner ear paint fills at E15.5. (A) Control inner ears reveal the morphology of the anterior, posterior and lateral semicircular canals (asc, psc, lsc, respectively), endolymphatic duct (ed), common crus (cc), utricle (u), saccule (s) and cochlear duct (cd). (B) Smoecko\textsuperscript{cko} inner ears lacked a cochlear duct and saccule, but all other structures were present. (C) Shh\textsuperscript{−/−} inner ears possessed an anterior semicircular canal, but all other structures were missing. The asterisk marks a large cystic structure.

Direct Hh signaling within the otic epithelium establishes ventral otic identity

At E10.5, the otic vesicle displays regionalized patterns of gene expression that mark competency domains for subsequent development into distinct adult structures (Fekete and Wu, 2002; Bok et al., 2007a). Several of these otic patterning genes are misexpressed in Shh\textsuperscript{−/−} embryos (Riccomagno et al., 2002). In order to distinguish the genes that are dependent on Hh signaling within the otic epithelium from those that are misregulated owing to...
secondary effects of disrupting Shh in neighboring tissues, we surveyed their expression by in situ hybridization in Smo\textsuperscript{cko} embryos.

The transcription factors Pax2, Otx2 and Gata3 are expressed in partially overlapping domains in the ventral otocyst and are necessary for cochlear duct development (Favor et al., 1996; Torres et al., 1996; Morsli et al., 1999; Lawoko-Keräli et al., 2002; Burton et al., 2004; Lillevali et al., 2006). Pax2 is broadly expressed throughout the otic placode before becoming restricted to the ventromedial wall of the otic vesicle (Fig. 3A; data not shown). Otx2 is also expressed in the ventral region of the otocyst (Fig. 3B). The pattern of Gata3 expression at early stages of otic development is dynamic, but is then localized to the elongating cochlear duct and spiral ganglion (Fig. 3C) (Lawoko-Keräli et al., 2002). Each of these genes was downregulated in Shh\textsuperscript{−/−} embryos (Fig. 3M-O) (Riccomagno et al., 2002). A comparable reduction in the expression of Pax2, Otx2 and Gata3 was observed in Smo\textsuperscript{cko} embryos (Fig. 3G-I; n=3), suggesting that Shh signaling within the otic vesicle is required for ventral otic identity and subsequent cochlear duct morphogenesis.

Otx1 is expressed in the lateral wall of the otic vesicle at E10.5 and is required for lateral semicircular canal formation (Fig. 3D) (Acampora et al., 1996; Fritzsch et al., 2001; Hammond and Whitfield, 2006). A significant ventral shift in the expression of Otx1 was observed in the otic vesicle of Shh\textsuperscript{−/−} embryos (Fig. 3P), which is likely to explain the absence of the lateral semicircular canal in these mutants (Riccomagno et al., 2002). In Smo\textsuperscript{cko} embryos, Otx1 was properly localized to the lateral wall of the otocyst, indicating that Hh signaling within the otic epithelium is not required for lateral otic identity (Fig. 3J; n=3). This result also suggests that the lateral semicircular canal defect in Shh\textsuperscript{−/−} embryos is an indirect consequence of perturbing Shh signaling in tissues adjacent to the inner ear.

The additional vestibular dysmorphologies observed in the ears of Shh\textsuperscript{−/−} mutants can also be explained by patterning changes in the otic vesicle. For example, the expression of Gbx2, a homeodomain-containing transcription factor required for endolymphatic duct formation (Lin et al., 2005), is not maintained in the dorsomedial otocyst of Shh\textsuperscript{−/−} mutants (Fig. 3Q) (Riccomagno et al., 2002). Moreover, the dorsal otic expression of Dlx5, a homeodomain-containing transcription factor required for semicircular canal development (Acampora et al., 1999; Depew et al., 1999; Merlo et al., 2002) is expanded ventrally in Shh\textsuperscript{−/−} embryos in a Wnt-dependent manner (Fig. 3R) (Riccomagno et al., 2005). These observations suggested that Shh is necessary for the expression of certain dorsal otic genes (Gbx2), while antagonizing the expression of others (Dlx5, Topgal). However, the regulation of these dorsal otic genes by Shh appears to be indirect as neither is misexpressed in Smo\textsuperscript{cko} embryos (Fig. 3E,F,K,L; n=3). These data also indicate that the antagonistic interaction between the Shh and Wnt signaling pathways that is responsible for setting up the dorsoventral axis of the otocyst does not stem from a cell-intrinsic mechanism within the otic epithelium, but must arise from interplay between these pathways outside of the ear.

**Ngn1 expression is not directly dependent on Hh signaling**

The neurons that make up the eighth cranial nerve and innervate the sensory patches within the inner ear originate from a common progenitor pool in the anteroventral region of the otic vesicle. The bHLH transcription factor Ngn1 is expressed in these neuroblast progenitors (Fig. 4A) and is required for their specification (Ma et al., 1998). The spatial restriction of Ngn1 to the anteroventral otic domain is mediated, in part, by the repressive action of Tbx1, a T-box-containing transcription factor that is expressed in a complementary pattern to Ngn1 (Fig. 4E) (Raft et al., 2004). In Tbx1\textsuperscript{−/−} embryos, Ngn1 is ectopically expressed, resulting in the posterior expansion of neuroblast progenitors (Raft et al., 2004).

Previous studies demonstrated that Ngn1 expression is greatly reduced in Shh\textsuperscript{−/−} embryos, causing a significant reduction in the size of the cvg (Fig. 4B; n=3) (Riccomagno et al., 2002). However, the mechanism underlying the regulation of Ngn1 transcription by Shh was unclear. Novel insight into this issue came from our observation that Tbx1 expression expanded into the neurogenic domain of Shh\textsuperscript{−/−} mutant otic vesicles (Fig. 4E-G; n=5). This raised two possibilities: either the loss of Ngn1 in Shh\textsuperscript{−/−} embryos caused the expansion of Tbx1, or the expansion of Tbx1 resulted in the downregulation of Ngn1 transcription. Since Tbx1 was not expanded in the otic vesicles of Ngn1\textsuperscript{−/−} mutants, we ruled out the former possibility (data not shown). To address the latter prospect, we examined embryos lacking a wild-type allele of Tbx1 on an Shh\textsuperscript{−/−} mutant background. We reasoned that if Tbx1 were responsible for the repression of Ngn1 in Shh\textsuperscript{−/−} embryos, then reducing its dosage should restore Ngn1 transcription. Notably, the pattern of Ngn1 expression in Shh\textsuperscript{−/−};Tbx1\textsuperscript{+/−} embryos was greatly enhanced compared with Shh\textsuperscript{−/−} mutants and closely resembled that of controls (Fig. 4C; n=3). Thus, Shh indirectly regulates Ngn1 by excluding Tbx1 from the neurogenic domain.

We next determined whether the exclusion of Tbx1 from the neurogenic domain was a direct or indirect action of Shh on the otic epithelium. Both Tbx1 and Ngn1 were properly localized to their respective otic territories in Smo\textsuperscript{cko} embryos (Fig. 4D,H; n=3 and n=6, respectively), arguing that their misregulation in Shh\textsuperscript{−/−} mutants was a secondary consequence of disrupting Shh signaling in tissues extrinsic to the inner ear.
Opposing roles for the Wnt and Fgf signaling pathways in cvg neurogenesis

If Shh is not acting directly on the otic epithelium to regulate the anteroposterior positioning of the neurogenic lineage, then what is the responsible signal(s)? Certain members of the Wnt and Fgf families appear to be excellent candidates based on previous studies. For example, Wnts secreted from the dorsal hindbrain have been shown to partially suppress the neurogenic lineage (Riccomagno et al., 2005), whereas Fgfs have been shown to both repress and activate neuronal determinants in the otic epithelium (Alsina et al., 2004; Vazquez-Echeverria et al., 2008; Abello et al., 2010). In Fgf3−/−;Fgf10−/− mouse embryos, neuroblast progenitors are ectopically expressed in the posterior otocyst (Vazquez-Echeverria et al., 2008). Conversely, the pharmacological inhibition of Fgf signaling in the chick otic vesicle causes a dramatic reduction in the expression of Ngn1 and NeuroD and a corresponding loss of cvg neurons (Alsina et al., 2004). These seemingly contradictory results might be attributed to species-specific differences in Fgf signaling activity and/or temporal differences in Fgf ligand utilization.

To investigate whether modulation of the Wnt or Fgf signaling pathways could mimic aspects of the Shh−/− neurogenic phenotype, we cultured wild-type mouse embryos in the presence or absence of the canonical Wnt signaling agonist LiCl (Klein and Melton, 1996) or the Fgf signaling antagonist EMD 341608 (Hamby et al., 1997) and assayed the expression of Ngn1 and Tbx1. Wild-type embryos harvested at E9.5 and cultured in control media for 18 hours showed proper anterior and posterior expression of Ngn1 and Tbx1, respectively (Fig. 5A,D; n=8/9 and n=17/20, respectively). However, when embryos were cultured in LiCl (n=5/5) or EMD 341608 (n=6/8), they showed a consistent and profound downregulation of Ngn1 in the anterior otocyst (Fig. 5B,C). These results confirm that Wnt signaling antagonizes, while Fgf signaling is necessary for, Ngn1 expression in the mouse otocyst. Interestingly, the anterior otic expansion of Tbx1 was only observed in embryos cultured in LiCl (n=6/8) and not EMD 341608 (n=0/15) (Fig. 5E-G; P<0.001, unpaired t-test). Thus, heightened Wnt signaling better recapitulated the anteroposterior polarity defects observed in Shh−/− embryos than did Fgf inhibition.

Shh is a mitogen for cvg progenitors

To determine whether Shh has other functions in cvg formation we quantified the number of Isl1+ neurons in Smocko embryos at E10.5 (36–38 somites), which corresponds to the midway point of cvg neurogenesis (Fig. 6). Double-labeling studies were performed with cRet to distinguish the cvg (Isl1+cRet+) from the nearby neural crest-derived geniculate ganglia (Isl1+cRet−) (Pachnis et al., 1993; Radde-Gallwitz et al., 2004). Smocko embryos displayed a 47% reduction in the number of Isl1+ cvg neurons (1661±51; n=3) compared with control littermates (3529±86; n=4), indicating that Hh signaling has an additional function within the otic epithelium to regulate an aspect of cvg neurogenesis that is distinct from its role in controlling Ngn1 expression (Fig. 6A,B,E). Interestingly, the number of cvg neurons was not significantly different (Tukey-Kramer multiple comparisons test) between Shh−/−;Tbx1−/−; (2039±146; n=3) and Smocko embryos (Fig. 6B,D,E), implying that the further reduction in cvg neurons in Shh−/− (899±95; n=3) compared with Smocko embryos was indeed due to the decrease in Ngn1 expression (Fig. 6B,E,C).

Shh signaling is essential for the proliferation and survival of several populations of neurogenic progenitors (Rowitch et al., 1999; Kenney and Rowitch, 2000; Cayuso et al., 2006). To determine whether Shh functions as a mitogen or survival factor for cvg neuroblasts, we compared the number of mitotically active (phospho-histone H3+) and apoptotic (activated caspase 3+) cells in distinct otic regions of Smocko and control embryos at E9.5 (23 somite stage), shortly after otic vesicle closure. Whereas the total number of mitotically active cells throughout the otic epithelium was not statistically different between Smocko (96±7 cells) and control (112±7 cells) embryos, a significant reduction in the number of phospho-histone H3+ cells was observed in Smocko embryos when only the anteroventral domain was considered (43±4 versus 69±5 cells; P<0.05, unpaired t-test) (Fig. 7A–C; n=4). The reduction in proliferating otic neuroblasts in Smocko embryos
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than Smoecko staining. Control embryos (A) showed a greater number of cvg neurons that the fate of these inner ear neurons is decided early, possibly prior to their delamination from the otic vesicle (Koundakjian et al., 2007). Shh plays a prominent role in assigning identity to neuronal progenitors in the ventral neural tube (Dessaud et al., 2007). Shh is necessary for the proliferation of neurogenic progenitors in the inner ear. 

Fig. 7. A mitogenic role for Hh in cvg progenitors. (A,B) Transverse sections through control (A) and Smoecko (B) mouse embryos at the 23 somite stage stained for Isl1 (red) and phospho-histone H3 (green). (C) The numbers of Isl+ cvg neurons and proliferating neuroblasts were significantly reduced in Smoecko embryos compared with control littermates, whereas the total number of phospho-histone H3+ cells throughout the otic vesicle was unchanged. Error bars indicate s.e.m. NS, not statistically significant by unpaired t-test.

DISCUSSION

Ventral, but not dorsal, otic identity is dependent on direct Hh signaling in the otic epithelium

The cvg comprises a heterogeneous population of presumptive auditory and vestibular neurons. Lineage-tracing studies suggest that the fate of these inner ear neurons is decided early, possibly prior to their delamination from the otic vesicle (Koundakjian et al., 2007). Shh plays a prominent role in assigning identity to neuronal progenitors in the ventral neural tube (Dessaud et al., 2008). To address whether Shh functions in a similar capacity to promote the fate of vestibular and auditory neurons, we evaluated cell type-specific properties of the remaining neurons in Smoecko embryos. Unfortunately, no molecular markers have been described that distinguish auditory from vestibular neurons at progenitor stages of development. We therefore examined unique aspects of their identity after their physical separation into spiral (auditory) and vestibular ganglia. At E13.5, spiral neurons can be identified by expression of the Gata3 transcription factor, which also marks the prosensory domain of the cochlear duct (Fig. 8A,E) (Lawoko-Kerali et al., 2002; Lawoko-Kerali et al., 2004). A population of cells expressing Gata3 was observed in Smoecko embryos, despite the absence of a cochlear duct (Fig. 8C,G). Unlike control embryos, however, the Gata3+ cells from Smoecko mutants also stained positively for activated caspase 3 (Fig. 8C,G), suggesting that spiral neurons were specified in the absence of Hh signaling but subsequently underwent apoptosis. By E15.5, the Gata3+ caspase 3+ cell population was dramatically reduced compared with control littermates (Fig. 8B,F,D,H), and, by E18.5, Gata3+ cells could no longer be detected in Smoecko embryos (data not shown). The timing of the death of spiral neurons in Smoecko ears coincides with when they normally become dependent on Bdnf and NT-3 (Ntf3), two neurotrophins that are secreted from cochlear hair cells and required for spiral neuron survival (Ernfors et al., 1995).

The innervation of vestibular hair cells by vestibular neurons was evaluated by immunostaining with antibodies against myosin VIIa and neurofilament, respectively, at E18.5. Vestibular hair cells in all three cristae and the utricular macula showed proper patterns of innervation in Smoecko embryos as compared with control littermates (Fig. 8I-L). These data indicate that Hh signaling is not required for the specification of vestibular hair cells or the neurons that innervate them.

Auditory and vestibular neurons are specified in Smoecko embryos

Previous work described an essential role for Shh, secreted from the notochord and floor plate of the hindbrain, in shaping inner ear development along its dorsoventral axis (Riccomagno et al., 2002; Bok et al., 2005). However, given the pleiotropic nature of Shh function, it was unclear from these studies whether Shh-dependent phenotypes were directly attributed to a blockade in Shh signaling within the otic epithelium or were due to the loss of secondary signals from Shh-responsive tissues surrounding the inner ear.

To determine which aspects of otic development are dependent on direct Hh signaling we generated Smoecko embryos, in which only the otic epithelium was prevented from responding to Hh, and compared them with Shh+/- mutants. The occurrence of cochlear and saccular agenesis in both Smoecko and Shh+/- embryos indicated that these otic phenotypes were directly attributable to impaired

Correlated with a deficit in cvg neurons, which was readily apparent at E9.5 (Fig. 7A-C). The number of apoptotic cells did not differ between Smoecko and control embryos (data not shown). Taken together, these results indicate that Shh is necessary for the proliferation of neurogenic progenitors in the inner ear.
Shh signaling within the ventral otocyst. Our data further showed that Shh promotes ventral otic identity by regulating the expression of Pax2, Otx2 and Gata3, which are downregulated in both Shh/−/− and Smoecko embryos (Fig. 9).

Our unanticipated finding that Smoecko embryos do not exhibit any of the other vestibular defects observed in Shh/−/− mutants (absence or malformation of semicircular canals, utricle and endolympathic duct) suggests that Shh signaling in the dorsal otocyst is dispensable for vestibular morphogenesis. In keeping with these findings was our observation that a select number of dorsal otic genes (e.g. Dlx5, Gbx2), which were misregulated in Shh/−/− embryos, were appropriately expressed in Smoecko mutants (Figs 3 and 9). Given these results, the validity of the prevailing model that a graded distribution of Shh signaling activity patterns the extent of the dorsoventral axis of the otic vesicle is drawn into question (see below).

**Dorsal otic patterning does not require discrete levels of Gli-R**

The observation that Gli1 is expressed in a graded manner along the dorsoventral axis of the otic vesicle raised the possibility that Shh functions as a morphogen to pattern distinct structures in the ear (Bok et al., 2007b). The strength and duration of Shh signaling at a given position along the dorsoventral axis was postulated to regulate the balance between Gli repressor (Gli-R) and Gli activator (Gli-A) levels, thus influencing the developmental fate of cell types emerging from a specific otic territory. For instance, ventral otic progenitors closest to the source of Shh are dependent on the highest levels of signaling activity (low ratio of Gli-R to Gli-A) and give rise to the cochlea, whereas dorsal otic progenitors that develop furthest from the source of Shh are dependent on lower signaling activity (high ratio of Gli-R to Gli-A) and contribute to vestibular structures (Bok et al., 2007b).

The failure of cochlear duct outgrowth in Shh/−/− and Smoecko embryos, which are predicted to have high ratios of Gli3-R to Gli-A in ventral otic regions, is in agreement with this model. Moreover, the restoration of vestibular development in Shh/−/−;Gli3+/− embryos was taken as evidence in favor of a higher ratio of Gli3-R to Gli-A being required for the patterning of dorsal, as compared with ventral, otic structures (Bok et al., 2007b).
However, the absence of vestibular defects in Smo\textsuperscript{cko} embryos does not fit the hypothesis that graded Hh signaling is responsible for patterning the entirety of the dorsoventral otocyst. The presence of a well-formed vestibulum in Smo\textsuperscript{cko} embryos indicates that discrete levels of Gli3-R are not required to pattern the dorsal otocyst. Instead, we attribute the cause of the vestibular dysmorphologies displayed by Shh\textsuperscript{−/−} embryos to secondary consequences of disrupting Shh signaling in periotic tissues. Similarly, the rescue of dorsal otic structures in Shh\textsuperscript{−/−};Gli3\textsuperscript{−/−} embryos can be explained by the partial restoration of secondary signals in tissues extrinsic to the ear. Therefore, if graded Hh signaling is acting on the otic epithelium, it is only necessary for patterning ventral otic structures.

Cvg neuroblasts are directly dependent on Shh for their proliferation

Our study unmasked a previously unappreciated role for Shh in regulating the expansion of cvg progenitors. This mitogenic role for Shh is similar to that demonstrated for other neuronal cell types in the cerebellum and ventral neural tube (Dahmane and Ruiz i Altaba, 1999; Rowitch et al., 1999; Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000; Cayuso et al., 2006). Proliferation was reduced by 47% in the neurogenic domain of Smo\textsuperscript{cko} embryos. The resulting deficit in cvg progenitors did not selectively eliminate one class of neurons, as both vestibular and spiral neurons were present in Smo\textsuperscript{cko} embryos. Interestingly, the vestibular neurons in Smo\textsuperscript{cko} embryos innervated their sensory targets in the cristae and maculae, whereas the auditory neurons failed to survive, which is likely to be due to a lack of trophic support normally provided by cochlear hair cells (Ernfors et al., 1995).

It is intriguing to speculate why a normal pattern of vestibular innervation was achieved in Smo\textsuperscript{cko} embryos despite the significant reduction in cvg neurons. As with many neuronal cell types, progenitors are usually generated in excess, which over time undergo apoptosis after failing to compete for a limited number of synaptic targets. The process of eliminating surplus neurons by cell death is highlighted in Bax\textsuperscript{−/−} mice, which lack a key apoptotic regulator and have an 83% increase in vestibular ganglia (Hellard et al., 2004). In chick, 24% of vestibular neurons undergo apoptosis during synapse formation, suggesting that this mechanism is not limited to mammals (Ard and Moster, 1984). Given the large fraction of vestibular neurons that are normally lost to cell death, the reduction in cvg neurons in Smo\textsuperscript{cko} embryos is unlikely to profoundly affect the elaboration of vestibular neural circuitry.

Tbx1 expression is indirectly regulated by Shh

An additional role for Shh in regulating cvg neurogenesis was indicated by the reduced expression of Ngn1 in the anteroverentral otic region of Shh\textsuperscript{−/−} embryos (Riccomagno et al., 2002). We now show that Shh signaling does not regulate Ngn1 transcription directly, but rather is required to exclude Tbx1, a known repressor of Ngn1, from the neurogenic domain (Raft et al., 2004). This conclusion is supported by the observation that Ngn1 expression and many of the cvg neuroblasts are restored in Shh\textsuperscript{−/−};Tbx1\textsuperscript{−/−} compound mutants (Figs 4 and 6). Nevertheless, the anterior otic expansion of Tbx1 appears to be a secondary consequence of losing Shh, as Tbx1 and Ngn1 are unaffected in Smo\textsuperscript{cko} embryos. We determined that heightened Wnt signaling is likely to be responsible for this neurogenic phenotype, given that it correlates with the anterior otic expansion of Tbx1 in both Shh\textsuperscript{−/−} mutants and embryos treated with LiCl (Fig. 5) (Riccomagno et al., 2005).

The antagonism of Wnt signaling by Shh is not mediated in a cell-autonomous manner within the otocyst. Therefore, the negative interaction between these two pathways must take place in tissues extrinsic to the ear (Fig. 9B,C). One way that this might occur is if Shh limits the effective range of Wnt ligands secreted from the neural tube. Wnt1 and Wnt3a in the dorsal hindbrain are known to regulate Dlx5 expression in the dorsal otocyst (Riccomagno et al., 2005). The range of these Wnts appeared to expand in Shh\textsuperscript{−/−} embryos, as evidenced by the ventralized expression of Dlx5 and the Wnt-responsive Topgal reporter (Riccomagno et al., 2005). A similar mechanism might also explain the anterior expansion of Tbx1 in the otic vesicles of Shh\textsuperscript{−/−} embryos (Fig. 9C).

Although the upregulation of Wnt signaling activity may explain the anteroposterior polarity defects observed in the otic vesicle of Shh\textsuperscript{−/−} embryos, it remains unclear what role Wnts normally play in the regulation of Tbx1 expression. The conditional inactivation of β-catenin, a transcriptional mediator of canonical Wnt signaling, at early stages of otic development causes a profound reduction in the size of the otic vesicle, yet Tbx1 expression remains properly localized (Freyer and Morrow, 2010). Interestingly, retinoic acid was recently shown to positively regulate Tbx1 expression in the posterior domain of the otic vesicle (Bok et al., 2011). Thus, Wnt signaling might only impact on Tbx1 expression in the absence of Shh, whereas retinoic acid normally activates Tbx1 in the otic epithelium.

The regulation of Tbx1 by Shh is context dependent. As described above, Shh negatively regulates Tbx1 in the neurogenic domain of the otic epithelium. However, within the pharyngeal and periotic mesoderm, Shh promotes Tbx1 expression (Garg et al., 2001; Riccomagno et al., 2002; Yamagishi et al., 2003). Mice lacking Tbx1 in the periotic mesoderm show reduced expression of Cyp26 family members, which regulate the catabolism of retinoic acid (Braunstein et al., 2009). Consequently, retinoic acid signaling is upregulated in the otic epithelium of these mice, resulting in cochlear outgrowth defects. Therefore, cochlear development is dependent on Shh signaling in both the otic epithelium and periotic mesenchyme.

In summary, we found that Shh signaling acting directly on the otic epithelium is necessary for the establishment of ventral otic identity and the proliferation of cvg neural progenitors. Furthermore, Shh acting in periotic tissues regulates the anteroposterior expression of Tbx1 in the otic vesicle, possibly through a canonical Wnt intermediate. Overall, these studies contribute to the understanding of Shh signaling in the developing ear and refute the idea that discrete amounts of Gli-R are required for vestibular development.

Acknowledgements

We thank J. Johnson for Ngn1\textsuperscript{−/−} embryos; D. Dolson for technical assistance; and Staci Rakoveviecki, Meera Sundaram, Nadia Dahmane and Jean-Pierre Saint-Jeannet for helpful comments on the manuscript. This work was supported by NIH grant R01DC006254 from NIDCD (D.J.E.). A.S.B. was supported by Cell and Molecular Biology (T32-GM07229) and Genetics (T32-GM008216) training grants. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066126/-/DC1

References

Ear conditional knockout of Smo


