Specification of the mammalian cochlea is dependent on Sonic hedgehog

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Organization of the inner ear into auditory and vestibular components is dependent on localized patterns of gene expression within the otic vesicle. Surrounding tissues are known to influence compartmentalization of the otic vesicle, yet the participating signals remain unclear. This study identifies Sonic hedgehog (Shh) secreted by the notochord and/or floor plate as a primary regulator of auditory cell fates within the mouse inner ear. Whereas otic induction proceeds normally in *Shh*$^{-/-}$ embryos, morphogenesis of the inner ear is greatly perturbed by midgestation. Ventral otic derivatives including the cochlear duct and cochleovestibular ganglia failed to develop in the absence of Shh. The origin of the inner ear defects in *Shh*$^{-/-}$ embryos could be traced back to alterations in the expression of a number of genes involved in cell fate specification including *Pax2*, *Otx1*, *Otx2*, *Tbx1*, and *Ngn1*. We further show that several of these genes are targets of Shh signaling given their ectopic activation in transgenic mice that misexpress *Shh* in the inner ear. Taken together, our data support a model whereby auditory cell fates in the otic vesicle are established by the direct action of Shh.

[*Keywords:* Shh; *Pax2*; *Ngn1*; otic vesicle; cochlea; cochleovestibular ganglia]

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The mammalian inner ear is a complex sensory organ comprised of auditory and vestibular structures that serve to coordinate the senses of hearing and balance, respectively. The inner ear develops over a protracted period originating from a thickening of surface ectoderm, the otic placode, which forms at the level of the prospective hindbrain upon inductive influences from neighboring tissues (Groves and Bronner-Fraser 2000; Ladher et al. 2000). Once induced, the otic placode invaginates to form the otic cup and shortly thereafter pinches off from the surface ectoderm to give rise to the otic vesicle. Over the next several days the otic vesicle undergoes an intense period of proliferation, differentiation, and morphogenesis culminating in the establishment of the ventrally derived auditory component of the inner ear, the cochlea, as well as the more dorsally derived vestibular apparatus, comprising the semicircular canals, utricle, and saccule (for review, see Torres and Giraldez 1998).

Grafting and lineage tracing experiments performed in the chick, in addition to mutational analyses performed in the mouse, have confirmed that the fate of inner ear progenitors is specified early in development (Baker and Bronner-Fraser 2001). By the otic vesicle stage, numerous genes showing restricted patterns of expression compartmentalize the otic epithelium along its three major axes (Fekete and Wu 2002). With respect to the auditory component of the inner ear, the expression of several genes in the ventral and ventromedial regions of the otocyst, including the overlapping expression of the homeobox transcription factors *Otx1* and *Otx2* as well as the paired-box gene *Pax2* mark the location of cochlear duct outgrowth (Fekete and Wu 2002). For vestibular development, the homeobox transcription factors *Hmx2*, *Hmx3*, and *Dlx5* in the dorsolateral region of the otocyst mark the territory contributing to semicircular canal formation (Fekete and Wu 2002). Loss-of-function studies in the mouse confirm that each of these genes participates actively in establishing regional identity within the inner ear (Acampora et al. 1996, 1999; Torres et al. 1996; Hadrys et al. 1998; Wang et al. 1998, 2001; Depew et al. 1999; Morsli et al. 1999).

In addition to the establishment of regional identity, a number of genes have also been identified that have an impact on the specification of distinct cell fates within the otocyst. The inner ear is a self-contained organ in that the majority of cell types contributing to its development including sensory, nonsensory, and neurogenic are derived from the otic epithelium (Torres and Giraldez 1998). For instance, within the anteroventral region of the otic vesicle, cells expressing the bHLH transcription factors *Neurogenin-1* (*Ngn1*) and *NeuroD* form the neuronal lineage, giving rise to the cochleovestibular

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ganglia (cvg; VIIIth cranial nerve; Ma et al. 1998). A similar domain in more posterior regions of the otic vesicle, where *Ngn1* and *NeuroD* expression is absent, marks cells fated to become sensory in character (Fekete and Wu 2002).

Despite our recent understanding of the contribution of individual loci in establishing patterns of growth and differentiation within distinct domains of the otic epithelium, little is known of how these patterning genes are themselves regulated. Surgical manipulations of the otic vesicle in chick, either by altering its location along the anteroposterior neuraxis or rotating its position in situ, have led to a common conclusion that local environment dictates cell fate within the otic epithelium (for review, see Baker and Bronner-Fraser 2001). Further indication that signals from surrounding tissues impact on otic vesicle patterning comes from studies of mouse mutants showing inner ear phenotypes that are a consequence of altered gene function in the neural tube (Mark et al. 1993; McKay et al. 1996; Gavalas et al. 1998; Dupe et al. 1999; Niederreither et al. 2000). Moreover, ablation of the ventral or dorsal neural tube results in an expansion or restriction, respectively, in the expression of *Lmx1b*, a marker of the dorsal otocyst, supporting the regional segregation of patterning activities within the neural tube (Giraldez 1998).

The notochord and floor plate are sources of the secreted protein Sonic hedgehog (Shh) that functions in both short- and long-range signaling events to promote growth and differentiation of progenitor cells in the ventral neural tube and paraxial structures including the somite (Jessell 2000; Bailey et al. 2001). The juxtaposition of the otic vesicle with respect to the neural tube is similar to that of the somite at more anterior levels of the embryonic axis. Given that the inner ear is reliant on signals from the ventral neural tube and/or notochord for its formation and that notochord-derived Shh signaling is required for patterning paraxial structures, we assessed whether Shh plays an active role in inner ear development by assessing loss- and gain-of-function Shh mutants.

Initiation of otic development proceeded normally in $Shh^{-/-}$ embryos, whereas morphogenesis of the inner ear was greatly perturbed by midgestation. In mouse embryos lacking Shh function, ventral otic derivatives including the cochlear duct and cvg failed to develop. The origin of the inner ear defects in $Shh^{-/-}$ embryos could be traced back to alterations in cell fate specification within the ventral otic epithelium and periotic mesenchyme at earlier stages of inner ear development. Genes previously attributed with required functions in the specification of cochlear (Pax2, Otx1, and Otx2), neuroblast (Ngn1, NeuroD), and chondrogenic (Brn4, Tbx1) lineages were identified in our study as being dependent on Shh. To determine whether Shh is sufficient to induce ventral otic fates, we analyzed a new transgenic mouse line that results in the misexpression of *Shh* in the otic vesicle. A phenotype reciprocal to that manifested by $Shh^{-/-}$ embryos ensued, namely a loss of dorsal (vestibular) structures at the expense of expanded ventral (auditory) cell fates. Further assessment of this gain-of-function mutant identified *Pax2*, *Ngn1*, *Brn4*, and *Tbx1* as bona fide target genes of Shh signaling for inner ear development. Our data thus support a model whereby auditory cell fates in the otic vesicle are established by the direct action of Shh signaling from the notochord and/or floor plate.

Results

Aberrant inner ear morphogenesis in $Shh^{-/-}$ *embryos*

A simple assay to assess the anatomy of the inner ear during development relies on filling the lumen of the membranous labyrinth with latex paint and observing the three-dimensional structures of the inner ear by conventional light microscopy (Martin and Swanson 1993). To determine the impact of Shh signaling on inner ear morphogenesis, this paint-fill technique was applied to wild-type and $Shh^{-/-}$ embryos at 15.5 days postconception (dpc), a stage when the majority of inner ear structures have completed their development. In embryos lacking Shh, paint-fill analysis revealed multiple irregularities to the morphology of the inner ear (Fig. 1). Instead of the three prominently displayed semicircular canals normally present in wild-type embryos, $Shh^{-/-}$ mutants possess poorly formed posterior and anterior canals, with the lateral canal being absent (Fig. 1a,b). At the gross anatomical level, no distinct development of the five vestibular sensory chambers was evident in $Shh^{-/-}$ embryos including the three ampullae, utricle, and saccule (Fig. 1a,b), even though distinct sensory patches were found within the main membranous chamber (see below). The endolymphatic duct was also noted to be missing in $Shh^{-/-}$ embryos at this stage. A particularly striking feature of the inner ear phenotype in embryos lacking Shh was the complete absence of the cochlear duct. In place of this ventral inner ear derivative was a rudimentary structure resembling the otocyst, thus suggesting an early arrest in the developmental progression of ventral inner ear structures in $Shh^{-/-}$ embryos (Fig. 1a,b).

The lack of both medial (endolymphatic duct) and lateral (lateral semicircular canal and ampulla) structures in *Shh* mutants at E15.5 suggested that the specification of the medial/lateral axis might be perturbed in these mice. This prompted us to examine inner ears at E12.5, to determine whether earlier phases of semicircular canal formation were altered in $Shh^{-/-}$ embryos. Each semicircular canal develops from an epithelial outpocket of the otocyst. Over time, the opposing epithelia in the central region of the outpocket come together, fuse, and resorb, leaving behind a tube-shaped canal (Martin and Swanson 1993). In the wild-type inner ear, this process is almost complete by 12.5 dpc. A delay in resorption was observed in the prospective anterior and posterior canals from age-matched $Shh^{-/-}$ embryos (Fig. 1c,d). Interestingly, despite being absent at later stages, the horizontal outpocket, which forms the lateral canal, was present in $Shh^{-/-}$ embryos at E12.5 (Fig. 1c–f). No evidence of en-

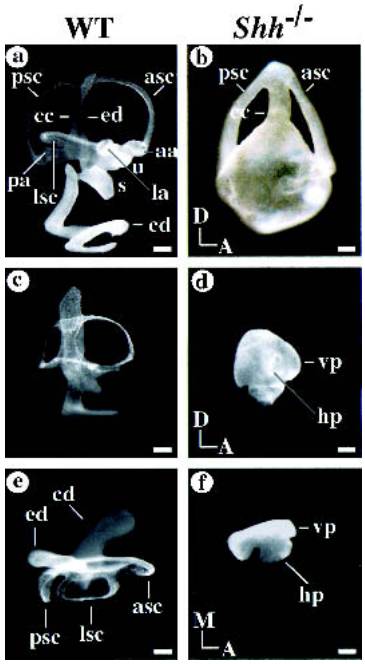


Figure 1. Morphological assessment of *Shh*$^{-/-}$ inner ears. The membranous labyrinth of wild-type (*a*,*c*,*e*) and *Shh*$^{-/-}$ (*b*,*d*,*f*) inner ears is shown after injection with latex paint. (*a*,*b*) Lateral views of inner ears from 15.5 dpc embryos. Note that the inner ear from the *Shh*$^{-/-}$ embryo appears to lack all six sensory chambers including the cochlear duct, utricule, saccule, and three ampullae. The lateral semicircular canal and endolymphatic duct are also absent in *Shh* mutants. Lateral (*c*,*d*) and dorsal (*e*,*f*) views of inner ears from 12.5 dpc embryos. The primordial structures for the semicircular canals, the vertical (vp) and horizontal (hp) outpockets are present in *Shh*$^{-/-}$ embryos, but resorption is not yet evident in these structures. aa, anterior ampulla; asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; ed, endolymphatic duct; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle. A, anterior; D, dorsal; M, medial. Bar, 100 µm.

dolymphatic duct outgrowth was detected at E12.5 in *Shh*$^{-/-}$ embryos; however, initial outgrowth of this structure was observed at E10.5 (Fig. 3k,l, see below). These results suggest that specification of the medial/lateral axis of the inner ear is normal in *Shh*$^{-/-}$ embryos, whereas morphogenesis of the endolymphatic duct and semicircular canals is dependent on Shh.

Shh is required to pattern the otic vesicle along the dorsal-ventral axis

With the complete lack of a coiling cochlea in late-gestational-stage Shh mutant embryos, we decided to address the molecular nature of these defects at otic vesicle stages of development. The ventralmost cells of the otic vesicle are fated to give rise to the cochlear duct and are marked by the overlapping expression of the homeobox genes *Otx1* and *Otx2* (Morsli et al. 1999). *Otx1* expression also extends dorsally along the lateral wall of the otic vesicle where it contributes to the formation of the lateral semicircular canal and ampulla (Morsli et al. 1999). To address whether Shh signaling is required in the ventralmost cells of the otic vesicle, we surveyed the expression of *Otx* genes in *Shh*$^{-/-}$ embryos. Interestingly, the expression of *Otx2* was not detected in the otic vesicles of *Shh*$^{-/-}$ embryos either at the time of its initiation at 10.5 dpc or any time thereafter (Fig. 2a,e; data not shown). Moreover, the domain of *Otx1* expression was reduced to a small ventral patch (Fig. 2b,c,f,g).

The altered expression of *Otx1* and *Otx2* in *Shh*$^{-/-}$ embryos could indicate that Shh signaling regulates the transcription of *Otx* genes, or that Shh is required for the specification of ventral otic vesicle cells that express *Otx* genes, or both. To begin to distinguish between these possibilities we assessed the expression of additional markers localized along the dorsal-ventral axis of the otic vesicle. If the ventralmost cells of the otic vesicle are not specified in *Shh*$^{-/-}$ embryos, then a possible outcome could be the ventral expansion of dorsal markers, reminiscent of the neural tube patterning defects observed in *Shh*$^{-/-}$ embryos (Chiang et al. 1996). The homeobox gene *Dlx5*, which marks the dorsal half of the otic vesicle, is expanded ventrally in *Shh*$^{-/-}$ embryos, suggesting that *Shh* signaling normally antagonizes *Dlx5* expression (Fig. 2d,h). This is not a general feature for all dorsal otic markers, as *Wnt2b* and *Lmx1b* did not expand ventrally in embryos lacking Shh (Fig. 3k,l; data not shown).

The ventral expansion of *Dlx5* expression in the otic vesicles of *Shh*$^{-/-}$ embryos did not include the ventralmost cells. To determine the identity of these cells, we surveyed the expression of other markers expressed in intermediate regions of the otic vesicle. In the anteroventrolateral compartment of the otic vesicle, *Fibroblast growth factor-3* (*Fgf3*) and high levels of *Lunatic Fringe* (*Lfng*) are coexpressed in a sensory competent zone from where a portion of the cvg and possibly the macula of the utricle will form (Fig. 2i,j,k). Low levels of *Lfng* can also be found in the ventralmost portion of the otic epithelium (Fig. 2j). Cells immediately dorsal to the high *Lfng* and *Fgf3* domains in the lateral otic vesicle express *Bone morphogenetic protein-4* (*Bmp4*; Fig. 2l). In the otic vesicles of *Shh*$^{-/-}$ embryos, the expression patterns of *Fgf3*, *Lfng*, and *Bmp4* were each found to shift ventrally, with *Fgf3* and high levels of *Lfng* occupying the ventralmost portion of the otocyst (Fig. 2m–p). The low level of *Lfng* that normally occupies the ventralmost region of the otic vesicle was absent in *Shh*$^{-/-}$ embryos (Fig. 2n). Despite the lack of Shh, *Bmp4*-expressing cells preserved their spatial relationship with *Fgf3* and *Lfng*, lying immediately dorsal in position. From these results we conclude that Shh signaling is required for the specification of the ventralmost cells of the otic vesicle from where the cochlear duct emerges. In the absence of Shh there is an expansion of some dorsal inner ear markers and a shift in the positioning of some markers of lateral otic cell fates.

Shh signaling acts directly on the otic epithelium and periotic mesenchyme

The effects of Hedgehog (Hh) signaling can be detected over short- or long-range distances. Long-range Hh sig-

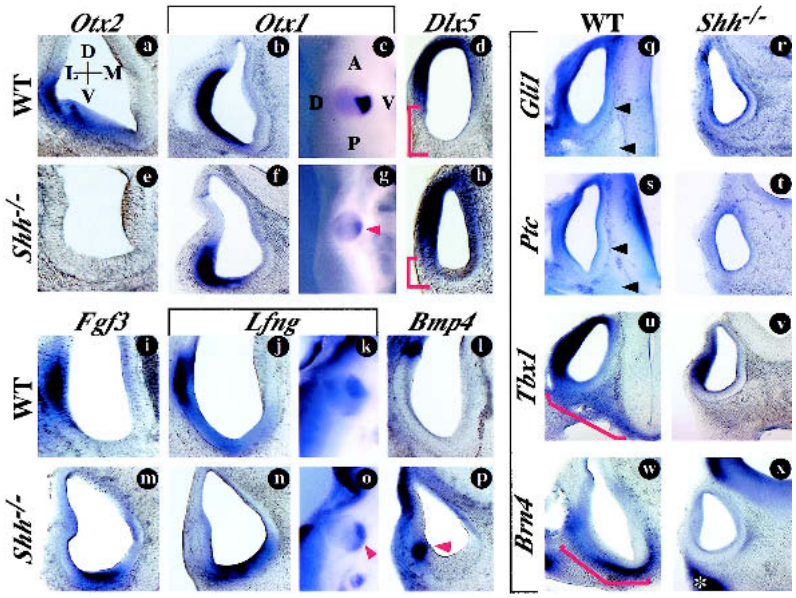


Figure 2. *Shh* regulates the expression of markers along the dorsoventral axis of the otic vesicle. Transverse section and whole-mount views of otic vesicles from wild-type (*a–d,i–l,q,s,u,w*) and *Shh*$^{-/-}$ (*e–h,m–p,r,t,v,x*) embryos analyzed for gene expression by RNA in situ hybridization. (*a,e*) *Otx2* is absent from the ventral otic epithelium of *Shh*$^{-/-}$ embryos; (*b,c,f,g*) *Otx1* expression is reduced in *Shh*$^{-/-}$ embryos. Red arrowhead in *g* points to the reduced domain of *Otx1* expression. (*d,h*) *Dlx5* is expanded ventrally in the absence of Shh. Brackets mark the distance between the ventral limit of *Dlx5* expression and the ventral extent of the otic vesicle. (*i,m*) *Fgf3*. (*j,k,n,o*) *Lfng*, the ventral shift in *Lfng* expression in *Shh*$^{-/-}$ embryos (*o*) is marked by a red arrowhead. (*l,p*) *Bmp4*; red arrowhead points to the *Bmp4* expression domain that is shifted ventrally in *Shh*$^{-/-}$ embryos. (*q,r*) *Gli1*and (*s,t*) *Ptc* are detected in the otic epithelium and periotic mesenchyme of wild-type but not *Shh*$^{-/-}$ embryos. Arrowheads in *q* and *s* point to faint expression of *Gli1* and *Ptc* in the periotic mesenchyme and medial wall of the otic vesicle. The semblance of expression of *Gli1* in *r* is the result of trapping rather than specific staining. (*u,v*) *Tbx1*; expression is detected in the lateral wall of the otic vesicle in both wild-type and *Shh*$^{-/-}$ embryos. However, the periotic mesenchyme expression of *Tbx1* (bracket) is absent in *Shh*$^{-/-}$ mutants. (*w,x*) *Brn4*; expression in the condensing mesenchyme (bracket) is absent from *Shh*$^{-/-}$ embryos at 10.5 dpc. Asterisk in *x* marks the branchial arch expression of *Brn4,* which is unaffected in *Shh*$^{-/-}$embryos. All sections are from embryos at 10.5 dpc except *d* and *h*, which are from embryos at 9.5 dpc. M, medial; L, lateral; D, dorsal; V, ventral.

naling can act directly, by an unresolved mechanism that transports Hh via its lipid linkages over several cell diameters, or indirectly, through a secondary-signal relay (Ingham and McMahon 2001). This raises the question as to whether Shh acts directly or indirectly to pattern the otic epithelium. In this regard we assessed the expression of two transcriptional targets of Shh signaling, the zinc-finger containing transcription factor *Gli1* and the Shh receptor *Patched* (*Ptc*), both of which serve as molecular readouts for pathway activation (Goodrich et al. 1996). Between 9.5 and 10.5 dpc, *Gli1* and *Ptc* transcripts are expressed broadly in the otic epithelium and ventral periotic mesenchyme (Fig. 2q,s; data not shown). The absence of *Gli1* and *Ptc* expression from these tissues in *Shh*$^{-/-}$embryos (Fig. 2r,t) excludes the possibility that other members of the hedgehog family might be signaling to the otic vesicle at these stages, lending support for a direct role of Shh in inner ear development.

With the observation that Shh signaling is active in the periotic mesenchyme, we next determined whether this tissue was also compromised in *Shh*$^{-/-}$ embryos. The transcription factors *Tbx1* and *Brn4* are markers of the periotic mesenchyme and condensing mesenchyme, respectively (Fig. 2u,w). Condensation of the mesen-

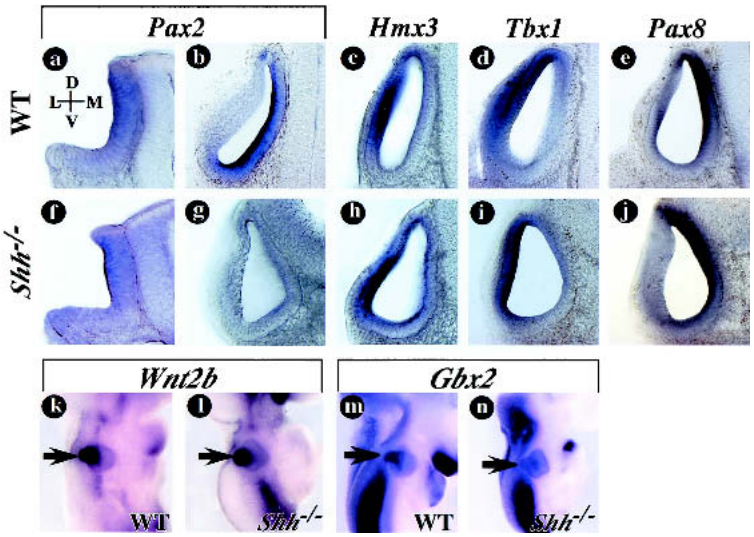


Figure 3. Shh regulates the expression of markers along the mediolateral axis of the otic vesicle. Assessment of otic markers in wild-type (*a–e,k,m*) and *Shh*$^{-/-}$ (*f–j,l,n*) embryos by in situ hybridization. (*a,b,f,g*) Transverse sections through the otic vesicles probed for *Pax2* expression at otic cup (*a,f*; 8.5 dpc) and otic vesicle (*b,g*; 9.5 dpc), stages of development. Despite being expressed at otic cup stages, *Pax2* is not maintained in the medial side of the otic vesicle at 9.5 dpc in *Shh*$^{-/-}$ embryos. (*c,h*) *Hmx3* expression along the lateral wall of the otic vesicle is unaffected in *Shh*$^{-/-}$ embryos. (*d,i*) *Tbx1*; neither the lateral epithelial expression of *Tbx1* nor the medial epithelial expression of *Pax8* (*e,j*) were altered in *Shh*$^{-/-}$ embryos at 9.5 dpc. (*k–n*) Lateral views of embryos stained by whole-mount in situ hybridization for *Wnt2b* (*k,l*) and *Gbx2* (*m,n*) at 10.5 dpc. Arrows mark the initial outgrowth of the endolymphatic duct.

chyme initiates within a subpopulation of periotic cells immediately adjacent to the ventral otic epithelium and, subsequently, the mesenchyme will encompass the inner ear to form the bony labyrinth. In the absence of Shh, neither *Tbx1* nor *Brn4* are detected in the mesenchyme at 10.5 dpc (Fig. 2v,x). In contrast, *Tbx1* expression along the lateral wall of the otic vesicle is maintained in the absence of Shh, suggesting that the two inner ear domains of *Tbx1* expression are regulated independently (Fig. 2u,v). By 15.5 dpc, *Brn4* transcripts are detected in *Shh*$^{-/-}$embryos, indicating the existence of an alternate pathway for *Brn4* activation (data not shown). Our findings suggest that Shh signaling impacts directly on both the otic epithelium and periotic mesenchyme and identify *Tbx1* and *Brn4* as two potential targets of the Shh signaling pathway in the development of the inner ear.

Maintenance of Pax2 *expression is dependent on Shh*

The paired-box gene *Pax2* is an early marker of otic fate, expressed initially throughout the otic placode and later within cells along the medial side of the otic vesicle (Nornes et al. 1990; Puschel et al. 1992; Groves and Bronner-Fraser 2000). As it was reported that *Pax2*$^{-/-}$ embryos lack the cochlear duct, we reasoned that *Pax2* expression might be altered in the inner ears of mice lacking Shh (Favor et al. 1996; Torres et al. 1996). *Pax2* was expressed at the otic cup stage in *Shh*$^{-/-}$embryos but was lost from the medial wall of the otic vesicle by 9.5 dpc (Fig. 3a,b,f,g). The failure to maintain *Pax2* expression in the absence of Shh is unlikely to be the only cause for the lack of ventral cells in the otic vesicle of *Shh*$^{-/-}$ embryos, because *Otx2*, which is also essential for cochlear development, was absent in *Shh*$^{-/-}$ mutants (Fig. 2a,e; Morsli et al. 1999). We therefore conclude that the lack of cochlear duct outgrowth in *Shh*$^{-/-}$ embryos is likely explained by a combination of defects, that is, failure to maintain *Pax2* expression in conjunction with the lack of ventral otic cell specification.

In addition to promoting cochlear duct outgrowth, Pax2 has been postulated to participate in the establishment of a compartmental boundary within the otic vesicle, at the dorsal juncture of the medial-lateral axis (Brigande et al. 2000). The homeobox- and T-box-containing transcription factors *Hmx3* and *Tbx1*, respectively, mark the complementary side of the proposed boundary within cells along the lateral wall of the otic vesicle (Fig. 3c,d). A suggested purpose for the creation of such a boundary is to establish a site from where the endolymphatic duct will emerge, at the dorsal intersection between the medial-lateral and anterior-posterior axes (Brigande et al. 2000). If Pax2 is required to maintain the medial-lateral boundary within the otic vesicle, then in its absence *Hmx3* and *Tbx1* expression should be altered and endolymphatic duct outgrowth inhibited. Interestingly, in the otic vesicles of *Shh*$^{-/-}$ embryos, the lateral expression of *Hmx3* and *Tbx1* remained fixed (Fig. 3c,d,h,i). Moreover, initial phases of endolymphatic duct outgrowth occurred normally in *Shh*$^{-/-}$ embryos as assessed by the dorsal expression of *Wnt2b* (Fig. 3k,l). The failure to maintain endolymphatic duct outgrowth in *Shh*$^{-/-}$ embryos may have more to do with the down-regulation of *Gbx2* at 10.5dpc, than with possible alterations to the compartmental boundary (Fig. 3m,n). The persistence of a medial-lateral boundary within the otic vesicle of *Shh*$^{-/-}$ embryos may be explained in part by the presence of *Pax8*, which maintains its expression along the dorsomedial wall of the otic vesicle in embryos lacking Shh (Fig. 3e,j). Pax8 does not compensate for Pax2 in all regions of the otocyst, given its inability to rescue the cochlear duct defects in either *Shh*$^{-/-}$ or *Pax2*$^{-/-}$ embryos. These findings suggest that Pax2 function is dispensable for medial-lateral boundary formation in the otocyst.

The cvg fail to form in Shh$^{-/-}$ *embryos*

The cvg derive from anteroventral regions of the otic vesicle. Once specified these neuronal precursors delaminate from the otic epithelium, migrate ventrally, and aggregate to form the cvg at the base of the otocyst. The cvg later segregate into the cochlear and vestibular nerves, which function to innervate the thousands of sensory hair cells of their respective structures. The requirement of Shh in specifying ventral otic cell fates makes the neuronal precursors of the cvg a potential target of Shh signaling.

Prior to their delamination from the otic epithelium, neuronal precursors express the bHLH transcription factors *Ngn1* and *NeuroD* (Ma et al. 1998). *Ngn1* is the earliest of the neuronal determinants to be expressed in cvg progenitors, at E9.0. followed by *NeuroD* at E9.5. Both the levels and domains of *Ngn1* and *NeuroD* transcripts were markedly reduced in the otic vesicles from *Shh*$^{-/-}$ embryos compared to age-matched wild-type littermates (Fig. 4a–d), inferring that Shh is involved in specifying the number of cvg progenitors by regulating *Ngn1* transcription.

To further investigate the dependence on Shh for neural fate specification in the inner ear, we looked to the ganglion proper at slightly later stages. By 10.5 dpc, many of the wild-type neuroblasts expressing *NeuroD* had delaminated from the otic epithelium, migrated ventrally, and aggregated to form a compact ganglion (Fig. 4e). This contrasts with the situation in *Shh*$^{-/-}$ embryos, where a smaller proportion of *NeuroD*-expressing precursors are found scattered below the otic vesicle, with the majority remaining confined to the otic epithelium (Fig. 4f). Assessment of a second cvg marker, *Fgf10*, is consistent with this result (Fig. 4g,h). Moreover, few neurons contributing to the cochlear ganglia and no neurons contributing to the vestibular ganglia were identified at 14.5 dpc (data not shown). The reduction in delaminating neuroblasts from the otic epithelium of *Shh*$^{-/-}$ embryos bears remarkable similarity to the phenotype displayed by *NeuroD*$^{-/-}$ embryos and suggests that NeuroD activity may be modulated by Shh (Liu et al. 2000; Kim et al. 2001).

Because the neurogenic domain is thought to partially overlap with the sensory competent region in the inner

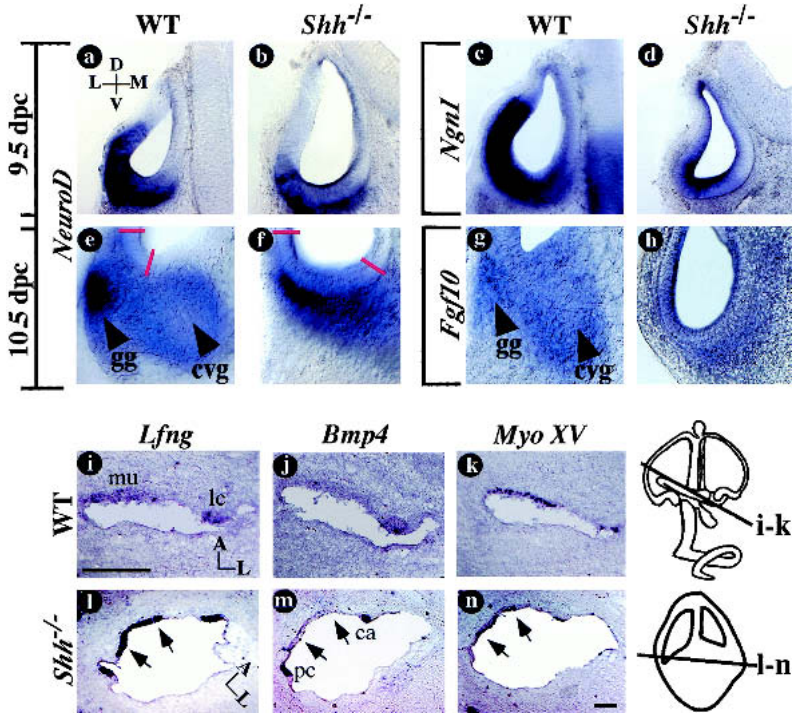


Figure 4. *Shh* is required for the specification and delamination of cvg precursors. Transverse sections through the anterior portion of the otic vesicles of wild-type (*a*,*c*,*e*,*g*) and $Shh^{-/-}$ (*b*,*d*,*f*,*h*) embryos. *NeuroD* (*a*,*b*) and *Ngn1* (*c*,*d*) expression is downregulated within cvg precursors of $Shh^{-/-}$ embryos at 9.5 dpc. *NeuroD* (*e*,*f*) and *Fgf10* (*g*,*h*) expression is reduced within the cvg and geniculate ganglia of $Shh^{-/-}$ embryos at 10.5 dpc. Red lines in *e* and *f* highlight the protracted expression of *NeuroD* in the mutant vs. wild-type otic epithelium. Adjacent sections probed with *Lfng* (*i*,*l*), *Bmp4* (*j*,*m*), and *Myo XV* (*k*,*n*) expression from wild-type and $Shh^{-/-}$ inner ears at 15.5 dpc. Arrows point to the positive *Lfng* and *Myo XV* domains that are negative for *Bmp4*. The level of the sections is shown in each representative diagram. ca, crista ampullaris; gg, geniculate ganglia; lc, lateral crista; mu, macula utriculi; pc, posterior crista. Bar, 100 μm.

ear, we investigated the development of the sensory patches in $Shh^{-/-}$ embryos at 15.5 dpc using established sensory organ markers. In the wild-type ear, all sensory patches express *Lfng* by 13 dpc and only the three cristae express *Bmp4* (Fig. 4i,j; Morsli et al. 1998). In $Shh^{-/-}$ inner ears, a broad *Lfng*-positive domain was observed on the medial side of the membranous chamber (Fig. 4l). This *Lfng* expression is thought to give rise to the maculae of the utricle and saccule and the organ of corti, but none of these sensory structures was distinguishable in *Shh* mutants. Nevertheless, Bmp4-positive crista-like structures were detected (Fig. 4m) and sensory hair cells did form in each sensory patch, as indicated by the expression of an early hair cell marker, Myosin XV (Fig. 4k,n). Thus, Shh is required to specify the majority of the neurogenic but not sensory lineages in the inner ear.

Otic targets of the Shh signaling pathway are upregulated in ShhP1 *embryos*

Two questions arise from the results described thus far; first, what is the source of Shh responsible for patterning the otic vesicle, and second, is Shh sufficient to induce ventral otic fates. To address the first question we assessed *Shh* expression in and around the developing inner ear. Between 8.5 dpc and 10.5 dpc, no discernible expression of *Shh* mRNA or protein could be detected in the otic epithelium or surrounding mesenchyme, suggesting that the likely source of Shh responsible for patterning the inner ear emanates from the notochord and/or floor plate (Figs. 5b, 6Aa).

With regard to the second question, we took advantage of a transgenic mouse line that ectopically expresses *Shh* in the otic vesicle to determine whether Shh signaling is sufficient for establishing ventral otic fates. Independent mouse lines carrying a 100-kb P1 clone (*ShhP1*) overlapping the *Shh* open reading frame exhibit behavioral abnormalities related to vestibular apparatus dysfunction including circling, hyperactivity, and head bobbing. Paint-fill analyses of inner ears from *ShhP1* transgenic mice reveal an absence of identifiable vestibular structures including semicircular canals, utricle, and saccule (Fig. 5a,e). The appearance of the cochlear duct was also irregular, with limited outgrowth and coiling (5/6 embryos).

To determine the molecular nature underlying the phenotype exhibited by embryos carrying the *ShhP1* transgene, we first assessed the expression of *Shh* at early stages of otic development. Whereas wild-type embryos show no evidence of *Shh* expression at any stage of inner ear development between 8.5 dpc and 10.5 dpc, embryos carrying the *ShhP1* transgene show ectopic expression of *Shh* in the otic epithelium at 9.5 dpc (Fig. 5b,f). The ectopic expression of *Shh* likely results from the absence of a negative regulatory element in the *ShhP1* transgene that normally functions to repress *Shh* transcription from the otic vesicle (D. Epstein, unpubl.). Indication that Shh signaling is active from this ectopic source was confirmed by the significant upregulation of *Gli1* and *Ptc* transcription (Fig. 5c,d,g,h). Although X-gal staining of embryos carrying the Ptc^{lacZ} allele recapitulates most aspects of *Ptc* expression, lacZ activity was only detected in the otic epithelium of *ShhP1* transgenic and not wild-type embryos. The failure to observe *lacZ* expression in the otic vesicles of wild-type embryos presumably results from altered transcription from the targeted allele,

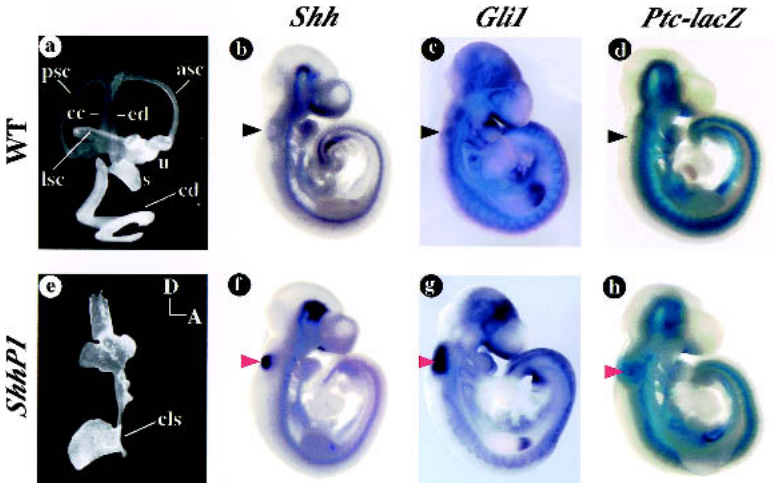


Figure 5. Embryos carrying the *ShhP1* transgene show impaired inner ear morphogenesis as a result of ectopic *Shh* expression. Analysis of paint-fill injections of inner ears from wild-type (*a*) and *ShhP1* (*e*) embryos at 15.5 dpc. Note the absence of dorsal structures in *ShhP1* compared to wild-type inner ears. Lateral views of embryos stained by whole-mount in situ hybridization for *Shh* (*b,f*) and *Gli1* (*c,g*) in wild-type (*b,c*) and *ShhP1* (*f,g*) embryos at 9.5 dpc. X-gal staining of $Ptc^{lacZ/+}$ (*d*) and $Ptc^{lacZ/+}$; *ShhP1* (*h*) embryos at 9.5 dpc. The absence of *Shh*, *Gli1*, and *Ptc* expression in the dorsal otic vesicle is marked with a black arrowhead in *b–d*, whereas ectopic expression of these markers in *ShhP1* embryos is highlighted with red arrowheads in *f–h*. cls, cochlear-like structure.

because *Ptc* mRNA is normally present in wild-type otic vesicles (Fig. 2s).

Localization of ectopic *Shh* expression in *ShhP1* embryos is confined to the dorsal portion of the otic epithelium (Fig. 6Aa,e). To address the consequence of this new source of Shh on otic vesicle patterning, we surveyed a number of genes that were shown previously to depend on Shh for their expression. Remarkably, the domain of Pax2, which normally is maintained by Shh on the medial side of the otic epithelium, was found to encompass the entire otic vesicle in *ShhP1* embryos (Fig. 6Ab,f). In contrast, the expression of *Dlx5* and *Hmx3* were reduced in the otic vesicles of *ShhP1* embryos by 9.5 dpc and almost completely absent by 10.5 dpc (Fig. 6Ac,d,g,h; data not shown). Given that both of these genes are essential for establishing the vestibular apparatus, their downregulation in *ShhP1* embryos likely explains the absence of semicircular canals, utricle, and saccule in these transgenic mice (Hadrys et al. 1998; Wang et al. 1998; Acampora et al. 1999; Depew et al. 1999). These

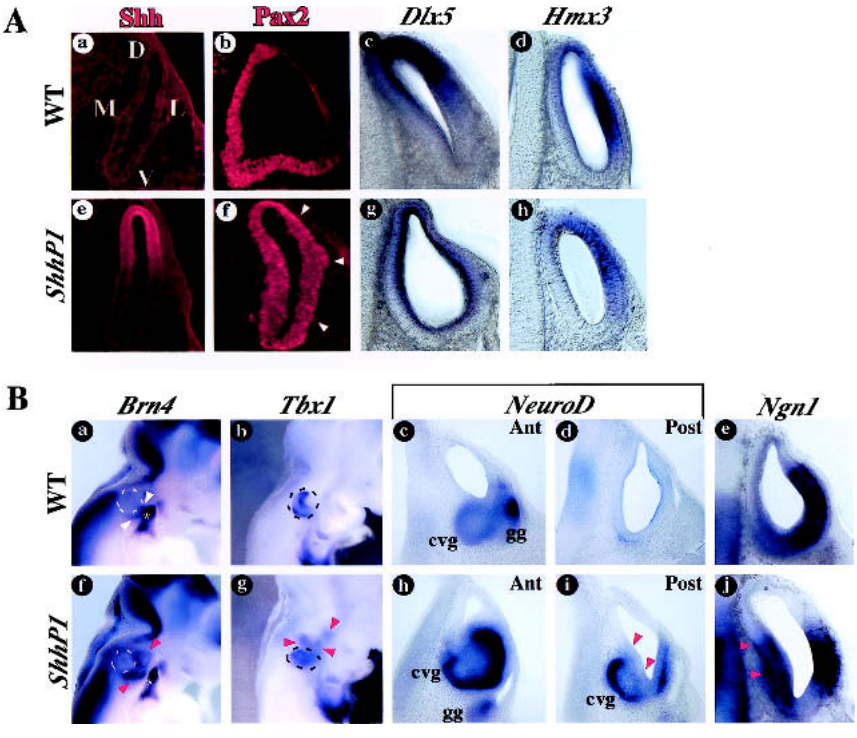


Figure 6. Ectopic expression of Shh-responsive genes in *ShhP1* embryos. (*A*) Upregulation of cochlear and downregulation of vestibular markers in *ShhP1* embryos. Antibody staining for Shh (*a,e*) and Pax2 (*b,f*) on transverse sections through the otic vesicles of wild-type (*a,b*) and *ShhP1* (*e,f*) embryos at 9.5 dpc. Shh protein is ectopically expressed in the dorsal region of the otic vesicle of *ShhP1* embryos (*e*). White arrowheads in *f* mark the ectopic expression of Pax2 protein on the lateral side of the otic vesicle. *Dlx5* (*c,g*) and *Hmx3* (*d,h*) expression is downregulated in *ShhP1* embryos compared to wild-type littermates. (*B*) Markers of the periotic mesenchyme and cvg precursors are upregulated in *ShhP1* embryos. *Brn4* (*a,f*) and *Tbx1* (*b,g*) expression in lateral views of whole-mount stained wild-type and *ShhP1* embryos at 10.5 dpc. Dashed circles mark the position of the otic vesicle. Expression of *Brn4* in the condensing mesenchyme of *ShhP1* embryos is expanded (red arrowheads in *f*) compared to wild-type embryos (white arrowheads in *a*). The asterisks in *a* and *f* mark the branchial arch expression of *Brn4*. (*g*) Red arrowheads point to the ectopic expression of *Tbx1* in *ShhP1* embryos. *NeuroD* (*c,d,h,i*) and *Ngn1* (*e,j*) expression on transverse sections through the otic vesicles of wild-type (*c–e*) and *ShhP1* (*h–j*) embryos. Note the expansion of cvg precursors in anterior and posterior locations of the otic vesicle in *ShhP1* embryos (red arrowheads in *i,j*). The ectopic ganglia depicted in *i* correlate with the ectopic *Ngn1* expression (red arrowheads in *j*). All sections are from embryos at 10.5 dpc except *e* and *j*, which are from embryos at 9.5 dpc.

results offer further support of the mutual antagonism between Dlx5 and Shh in establishing dorsal and ventral otic fates.

Since the ventralmost cells of the otic vesicle are dependent on Shh for their specification and the ventral limit of Pax2 may partially overlap these cells, we sought to address whether ventralmost cells are represented in the expanded domain of Pax2 expression in *ShhP1* embryos. *Otx2* expression remained restricted to the ventral portion of the otic vesicle in *ShhP1* embryos, confirming that dorsal Shh expression is insufficient to activate either *Otx2* transcription or ventralmost cell fates (data not shown). However, within the periotic mesenchyme, ectopic Shh is sufficient to expand the expression domain of *Brn4* ventrally and activate ectopic *Tbx1* expression dorsally (Fig. 6Ba,b,f,g). These results suggest that in some contexts Shh signaling is sufficient to activate target gene expression—*Pax2* in the otic epithelium and *Brn4* and *Tbx1* in the periotic mesenchyme—whereas in other contexts, Shh is likely acting in conjunction with cooperating signals, for instance in the specification of *Otx2*-expressing ventral cell fates.

As Shh is required for cvg formation, we next investigated whether ectopic Shh affects the neuronal lineage. Interestingly, the number of *NeuroD*-expressing neurons contributing to the cvg is expanded more than twofold in *ShhP1* embryos compared to age-matched wild-type littermates (Fig. 6Bc,h). Moreover, *NeuroD*-expressing precursors migrate from ectopic locations within the otic epithelium of *ShhP1* embryos (Fig. 6Bd,i). In comparison to wild-type littermates, a notable expansion of *Ngn1*-expressing neuronal precursors was detected in the otic epithelium of *ShhP1* embryos prior to their delamination (Fig. 6Be,j). No enhancement in cell survival or proliferation was detected in the vicinity of the expanded neuroblast lineage in *ShhP1* embryos compared to wild-type littermates (data not shown). It is possible that the expansion of neuronal progenitors comes at the expense of sensory cells, because only one or two sensory patches were evident in *ShhP1* ears at 15.5 dpc (data not shown). These findings reinforce the view that Shh is both necessary and sufficient for the specification of neuronal cell fates within the otic vesicle.

Discussion

Shh patterns the otic vesicle by specifying distinct ventral cell fates

The morphogenetic programs that shape the inner ear into auditory (ventral) and vestibular (dorsal) components are established early during otic development and are heavily influenced by extrinsic cues from surrounding tissues (Torres and Giraldez 1998; Baker and Bronner-Fraser 2001). In the present work we demonstrate that Shh secreted from the notochord and/or floor plate acts as a long-range signal to promote ventral cell identity within the adjacent otic vesicle. In mouse embryos lacking Shh function, ventral otic derivatives including the cochlear duct and cvg failed to develop. A direct action of Shh signaling on ventral otic cells is suggested by the expression of *Gli1* and *Ptc* in the otic epithelium and periotic mesenchyme, two tissues that show altered expression of these and other target genes in $Shh^{-/-}$ embryos.

As the cochlear duct and cvg emerge from different locations within the otic epithelium, we propose that ventral otic progenitor cells respond differentially to Shh signaling. Consistent with this hypothesis, in the otic vesicles of $Shh^{-/-}$ embryos we detected specific alterations in the expression of ventral otic genes localized along the anteroposterior axis (Fig. 7). For instance, in posterior regions of the otic vesicle, ventral cells contributing to the cochlear duct marked by the overlapping expression of *Otx1*, *Otx2,* and possibly *Pax2* were absent in $Shh^{-/-}$ embryos, whereas the medial expression of *Pax2* was not maintained (Fig. 7). In anterior regions of the otic vesicle where neuronal precursors are derived, the expression of *Ngn1* and *NeuroD* was significantly downregulated in $Shh^{-/-}$ embryos, resulting in a failure to generate the cvg (Fig. 7).

That the misexpression of Shh in the dorsal otocyst of *ShhP1* transgenic embryos was sufficient to induce the ectopic expression of some of these markers including *Pax2* and *Ngn1* is further indication that Shh acts in an instructive rather than permissive manner to pattern the otic vesicle (Fig. 7). How Shh differentially regulates the expression of ventral otic genes along the anteroposterior axis is unclear, but given that cooperative interactions between Shh and other signaling pathways specify distinct neuronal progenitor cells along the anteroposterior axis of the neural tube, it is conceivable that a similar strategy is employed to pattern the otic vesicle (Jessell 2000). Interestingly, Fgf family members are differentially expressed within the otic vesicle (Pickles 2001).

Although Shh is required for the specification of ventral otic cells expressing *Otx2*, ectopic *Otx2* expression was not detected in the otic vesicles of *ShhP1* embryos. Any of several interpretations may explain these results. First, *Otx2* may be a marker of ventral otic cells that is not regulated by Shh signaling. Second, Shh may require cooperating signals to activate *Otx2* expression. Third, Shh may be sufficient to activate *Otx2* expression but the level of Shh emanating from the dorsal otocyst of *ShhP1* embryos is below a critical threshold. A final possibility is that the loss of *Otx2* in $Shh^{-/-}$ embryos is an indirect consequence of the altered neural patterning in these mutants and hence ectopic *Otx2* expression would not be an expected outcome in *ShhP1* embryos. We feel this final possibility to be the least likely given that (1) known Shh target genes including *Gli* and *Ptc* are expressed ventrally in the otic vesicle, (2) Shh is sufficient to activate other otic markers in *ShhP1* embryos, and (3) Shh is known to signal to other paraxial tissues.

In addition to the loss of ventral otic cell fates, the absence of Shh impacts the positioning of dorsal and lateral markers. *Dlx5*, a gene required for dorsal (vestibular) cell fates, was expanded ventrally in $Shh^{-/-}$ embryos (Fig. 7). Further indication that Shh antagonizes the expression of *Dlx5* stems from the downregulation of *Dlx5* in

response to ectopic Shh in *ShhP1* embryos. The role of Shh in patterning the otic vesicle along the dorsal/ventral axis thus bears some similarity to the manner by which Shh establishes ventral fates in the neural tube (Ericson et al. 1996). Both processes involve the initial repression of dorsal markers in order to promote ventral cell fates. It will be interesting to determine whether other similarities exist between inner ear and neural patterning, for instance whether BMPs from the surface ectoderm and/or dorsal neural tube function as dorsalizing signals in the otic vesicle acting in opposition to the ventralizing effects of Shh.

Pax2 is a mediator of Shh signaling responsible for cochlear duct outgrowth

The failure in cochlear duct outgrowth in $Shh^{-/-}$ embryos is most likely mediated by the lack of *Pax2*, *Otx1*, and *Otx2*, genes previously ascribed with required roles in this process (Favor et al. 1996; Torres et al. 1996; Morsli et al. 1999). Furthermore, our observations that Shh is both necessary and sufficient for the expression of *Pax2* along the medial wall of the otic vesicle implicates Pax2 as a downstream effector of Shh signaling in the otocyst (Fig. 7). The regulation of *Pax2* by Shh in inner ear development resembles the relationship between *Pax2* and Shh in the formation of another placode-derived sensory organ, the eye. In generating the proximal-distal axis of the optic cup, Shh signaling from the ventral forebrain promotes *Pax2*-expressing proximal fates (optic fissure, optic stalk) at the expense of *Pax6*-expressing distal fates (prospective retina, pigmented epithelium, and lens; Ekker et al. 1995; MacDonald et al. 1995; Chiang et al. 1996; Zhang and Yang 2001). To maintain the border between proximal and distal lineages, Pax2 and Pax6 antagonize each other by mutual transcriptional repression (Schwarz et al. 2000). The commonality in response by *Pax* genes to Hh signaling can be broadened to include *Pax1* in the ventral somite and *Pax6* in the ventral neural tube (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Ericson et al. 1997; Zhang et al. 2001). In both of these cases, Pax family members with opposing functions are expressed adjacent to sites of Pax1 and Pax6 activity (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Ericson et al. 1996). This is not a general rule, as *Pax* genes are not expressed complementary to *Pax2* in the inner ear, although other transcription factors may be fulfilling an antagonistic role in this tissue (Brigande et al. 2000). Our observations thus add to the growing list of functions for Pax transcription factors in mediating cellular responses to Shh signaling (Mansouri et al. 1996).

Pax2-independent functions of the Shh signaling pathway in otic development

The downregulation of *Pax2* expression may contribute to the failure of cochlear duct outgrowth in embryos lacking Shh function. However, the absence of the lateral semicircular canal, endolymphatic duct, and cvg in $Shh^{-/-}$ embryos, structures that are present in $Pax2^{-/-}$ embryos, reveals a more complex role for Shh than simply regulating *Pax2* expression (Favor et al. 1996; Torres et al. 1996). The lack of lateral semicircular canals in $Shh^{-/-}$ embryos may be explained in part by the initial reduction and ventralward shift in expression of *Otx1*, a gene required for the formation of this structure (Acampora et al. 1996; Morsli et al. 1999). However, since the primordium of the lateral canal, the horizontal outpocket, is still present in $Shh^{-/-}$ embryos, it is unlikely that Otx1 function is completely compromised in the absence of Shh. Alternatively, the disruption in lateral canal formation may stem from the disregulated growth of ventral otic cells in $Shh^{-/-}$ embryos. The ventral overgrowth that appears after 12.5 dpc in $Shh^{-/-}$ mutants could interfere with the development of intermediate structures such as the lateral canal. Similar reasoning is unlikely to explain the failure to maintain endolymphatic duct outgrowth in $Shh^{-/-}$ embryos, however, because this dorsally derived structure arrests at 10.5 dpc, prior to the appearance of the ventral cyst. Interestingly, the arrest in endolymphatic duct development is concomitant with the downregulation of *Gbx2* expression in this tissue. Whether maintenance of *Gbx2* expression is a direct response to Shh signaling and whether Gbx2 is required for endolymphatic duct outgrowth are two questions that remain to be addressed.

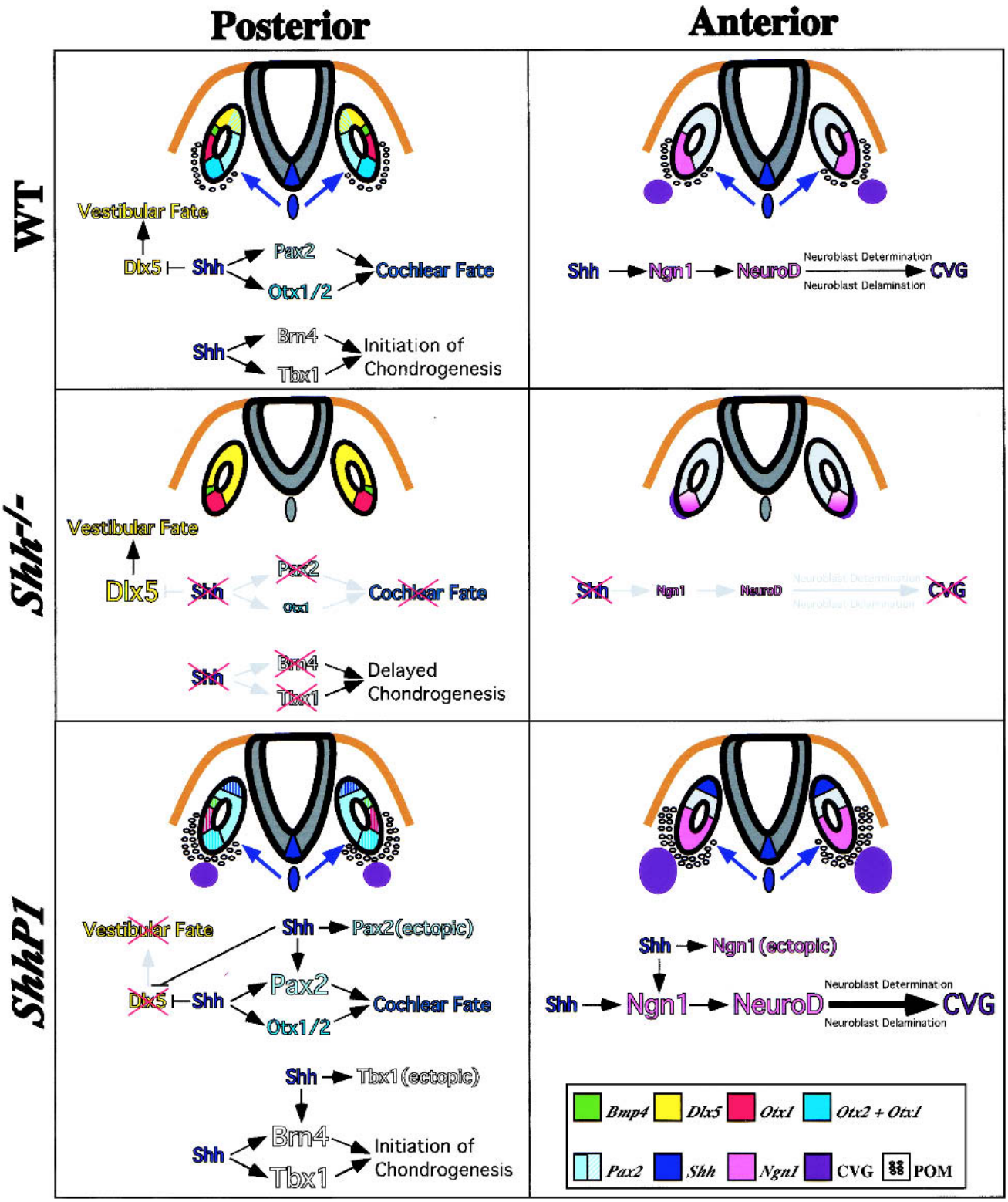
The downregulated expression of *Tbx1* and *Brn4* in the periotic mesenchyme of $Shh^{-/-}$ embryos at 10.5 dpc indicates that this tissue is also responsive to Shh signaling. Moreover, since *Tbx1* and *Brn4* are upregulated in *ShhP1* embryos, it is likely that these two genes are transcriptional targets of the Shh pathway. Interestingly, *Tbx1* has also been shown to be dependent on Shh in the pharyngeal arches (Garg et al. 2001). Although the contribution of signals from the periotic mesenchyme in patterning the otic epithelium is not well understood, Brn4 is involved in forming the mature bony labyrinth surrounding the inner ear (Phippard et al. 1999). The expression of *Brn4* in $Shh^{-/-}$ embryos at later stages in conjunction with the presence of a cartilage capsule suggests that a compensatory pathway functions to promote chondrogenesis in the absence of Shh (data not shown).

Neuroblast precursors of the cvg are dependent on Shh signaling

The mechanism by which neuronal precursors of the cranial sensory ganglia are specified is dependent on a genetic cascade mediated by basic helix-loop-helix (bHLH) transcription factors. With regard to the cvg, Ngn1 is at the top of the genetic hierarchy required to instruct a population of naïve otic epithelial cells to adopt a neuronal precursor fate (Ma et al. 1998). NeuroD, which is activated half-a-day later than Ngn1, is required for the delamination of neuroblasts from the otic epithelium as well as for their survival during the differentiation process (Ma et al. 1998; Liu et al. 2000; Kim et al. 2001). Although in many contexts activation of proneural genes in the neuronal determination pathway is regulated by

Notch-Delta signaling, a growing body of evidence indicates that the initial selection of cvg precursors is controlled by alternative mechanisms (Ma et al. 1998).

Our studies suggest that the expression of *Ngn1* in cvg precursors is established in part by the Shh signaling pathway. *Ngn1* is downregulated in cvg precursors from



(*Figure 7 legend on facing page*)

Shh$^{-/-}$ embryos and upregulated in an expanded pool of neuroblasts in *ShhP1* transgenic mice that ectopically express *Shh* (Fig. 7). Additional secreted factors acting independently of Shh are likely responsible for the specification of some cvg precursors, because a small number of *Ngn1*-expressing neuroblasts persist in *Shh*$^{-/-}$ embryos. Of the cvg precursors that are specified in *Shh*$^{-/-}$ embryos, it is unclear as to why the majority fail to emerge from the otic epithelium to form what would be perhaps a smaller ganglion. The answer may point once again to a function of Ngn1. A consequence of *Ngn1* downregulation in *Shh*$^{-/-}$ mutants is a reduction in the level of *NeuroD* expression. Given the requirement for NeuroD in promoting neuroblast delamination from the otic epithelium, it is possible that the reduction in *NeuroD* expression in *Shh*$^{-/-}$ mutants prevents the emergence of cvg precursors from the otic epithelium. We favor this explanation over a cell survival role for Shh because at the stages of neuroblast delamination examined (between 9.5 and 10.5 dpc), there was no detectable increase in the number of dying cells in a comparison of wild-type and *Shh*$^{-/-}$ embryos (data not shown). Similar conclusions were drawn in the analysis of *NeuroD*$^{-/-}$ embryos (Liu et al. 2000).

Overlapping roles for Shh and retinoids in otic vesicle patterning

Retinoic acid (RA) signaling has been shown to play an active role in patterning the otic vesicle (Dupe et al. 1999; Niederreither et al. 2000; Pasqualetti et al. 2001). Interestingly, the inner ears from *Shh*$^{-/-}$ embryos bear some resemblance to the phenotype displayed by mice lacking *Raldh2*, a gene that catalyzes RA formation (Niederreither et al. 2000). In otic vesicles from both mutants, *Pax2* expression is not maintained and *Hmx3* expression is expanded ventrally (the present study; Niederreither et al. 2000). Unfortunately, further comparisons are not possible due to the early lethality of *Raldh2*$^{-/-}$ embryos. RA can also rescue the *Hoxa1*$^{-/-}$ inner ear phenotype, which mimics features of *Shh*$^{-/-}$ embryos including a lack of cochlear duct outgrowth (Pasqualetti et al. 2001). Numerous examples exist supporting the actions of Shh and RA in the same or parallel pathways in embryonic development (Riddle et al. 1993; Ogura et al. 1996; Pierani et al. 1999; Schneider et al. 2001). Thus, the possibility exists that Shh and RA signaling may converge in the patterning of the otic vesicle.

Since the otic phenotype in *Raldh2* and *Hoxa1* mutants is attributed to a hindbrain patterning defect rather than a primary effect on the otic vesicle per se, it raises the question as to whether aspects of the *Shh*$^{-/-}$ otic phenotype can be explained by alterations in the expression of genes that pattern the hindbrain (Niederreither et al. 2000; Pasqualetti et al. 2001). The hindbrain expression of *kreisler* and *Fgf3* in rhombomeres 5 and 6 is altered in a number of mouse mutants with inner ear phenotypes, including *Raldh2*$^{-/-}$, *Hoxa1*$^{-/-}$, *kreisler*$^{-/-}$, and *Fgf3*$^{-/-}$ embryos (Mansour et al. 1993; McKay et al. 1996; Niederreither et al. 2000; Pasqualetti et al. 2001). Nevertheless, the spatial and temporal domains of *Kreisler* and *Fgf3* expression were unaffected in the hindbrain of *Shh*$^{-/-}$ embryos, suggesting that the *Shh*$^{-/-}$ otic phenotype does not result from misregulation of these hindbrain genes (data not shown). It will be interesting to determine whether the similarities in inner ear phenotypes of *Shh*$^{-/-}$, *Raldh2*$^{-/-}$, and *Hoxa1*$^{-/-}$ embryos stem from crosstalk between the Shh and RA pathways at other junctures or result from mutually independent mechanisms.

Similar usage of the Shh signaling pathway to pattern dissimilar paraxial structures

The otic vesicles and somites originate from two different tissue sources, the surface ectoderm and paraxial mesoderm, respectively, yet they share several features common to their development. For instance, both are transient structures that form adjacent to the neural tube. As a consequence, the two tissues are subjected to the same inductive signals expressed broadly along the anteroposterior axis as well as distinct signals expressed from localized sources.

Previous studies, in addition to our present work, support a general role for Shh in patterning paraxial structures (for review, see Bailey et al. 2001). What is particularly intriguing about the common use of this signaling pathway is the similarities in the types of genes used to

Figure 7. Schematic representation of the Shh loss and gain-of-function phenotypes revealed in the inner ear. In posterior regions of the otic vesicle, Shh is required for the specification of the ventralmost cells of the otic epithelium marked by the overlapping expression of *Otx1*, *Otx2*, and possibly *Pax2* (royal blue) that contribute to the outgrowth of the cochlear duct. Shh is also required for the maintenance of *Pax2* expression along the medial wall of the otic vesicle (light blue). In the absence of Shh function, there is a ventral expansion in the expression of some dorsal genes including *Dlx5* (yellow) and a shift in the expression of markers of lateral fates (red, green) to the ventralmost portion of the otic vesicle. Within the condensing mesenchyme (small white circles underlying the otic vesicle), there is a delay in *Brn4* and an absence of *Tbx1* expression. In comparison, *ShhP1* embryos, which exhibit ectopic expression of *Shh* in the dorsal otocyst (dark blue), depict a phenotype reciprocal to that of *Shh*$^{-/-}$ embryos in that dorsal markers are downregulated (loss of yellow) and *Pax2* expression (light blue) expands throughout the circumference of the otic vesicle. In addition, there is an increase in the population of condensing mesenchyme expressing *Brn4* and ectopic *Tbx1*, as well as ectopic ganglia formation (purple). Hatched portions of the otic vesicle represent coexpression with *Pax2*. In the anterior portion of the otic vesicle, Shh is required for the specification of the majority of cvg precursors through the regulation of *Ngn1* expression (pink). The domain and intensity of *Ngn1* transcription are substantially reduced in *Shh*$^{-/-}$ embryos, and consequently the size of the cvg (purple) is diminished. In *ShhP1* embryos, the ectopic expression of *Shh* in the inner ear (dark blue) causes an expansion of the *Ngn1*-positive domain (pink) and an increase in the size of the cvg. For the purpose of illustration, not all genes expressed in the anterior otic vesicle are shown.

affect disparate processes of differentiation. For instance, within the dorsal somite, Shh signaling specifies the epaxial musculature lineage through the direct activation of *Myf5* expression in the dorsomedial lip of the dermamyotome (Gustafsson et al. 2002). In the ventral somite, both Shh and Indian hedgehog cooperatively regulate sclerotome differentiation by controlling *Pax1* expression (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Zhang et al. 2001). Myf5, like Ngn1 in the otic vesicle, is a bHLH transcription factor that functions as a primary determinant of cell fate (for review, see Bailey et al. 2001). Similarly, Pax1 and Pax2 are paired box transcription factors responsible for mediating differentiation programs in a variety of contexts (Mansouri et al. 1996). Greater insight into the mechanisms by which genes utilized by Shh to pattern paraxial structures are recruited will require further unraveling of the regulatory networks underlying otic vesicle and somite gene expression.

Materials and methods

Production and genotyping of transgenic mice

A P1 bacteriophage library (Genome Systems) was screened with primers directed against exons 1 and 3 of the *Shh* locus to isolate P1 5527, a 100-kb clone overlapping the *Shh* open reading frame and extending approximately 10 kb upstream and 90 kb downstream of the Shh translational start site. The P1 clone was linearized with *Sal*I and purified for pronuclear injection by standard protocol at a concentration of 1ng/μL (Hogan et al. 1994). Genotyping of founder animals using primers against the *sacB* gene contained in the P1 vector (J50, 5′-GGTCGGCGACAACTCAATCG-3′; J51, 5′-GTGAGGGTCTCTCAGCGTATG-3′) as well as Southern blot hybridization with *Shh*-specific probes identified two independent mouse lines, each carrying 4–6 copies of an intact P1 5527 transgene (*Shh P1*). The two lines of mice exhibited similar behavioral abnormalities including hyperlocomotor activity, circling, and head bobbing. The transgenic lines were maintained on a CD-1 background (Charles River). The $Shh^{+/-}$ animals were kindly provided by H. Westphal (NIH; Chiang et al. 1996) and maintained on a CD-1 background (Charles River). $Ptc^{lacZ/+}$ mice were obtained from Jackson Laboratories.

Paint-fill studies

Mouse embryos for paint-fill analysis were harvested in PBS and placed overnight in Bodian fixative at room temperature. Specimens were subsequently dehydrated in ethanol and cleared in methyl salicylate. A 0.1% latex paint solution in methyl salicylate was injected into the lumen of the membranous labyrinth using a micromanipulator (Martin and Swanson 1993; Morsli et al. 1998).

In situ hybridization and whole-mount β-galactosidase staining

Whole-mount RNA in situ hybridization was performed essentially as described (Matise et al. 1998) using digoxigenin-UTP-labeled riboprobes. Three to five embryos of each genotype were analyzed for every probe. After whole-mount staining, representative embryos were postfixed in 4% paraformaldehyde, rinsed in PBS, embedded in 4% agarose, and sectioned on a vibratome at 50–75 μm. The assessment of β-galactosidase activity in $Ptc^{lacZ/+}$ embryos was performed by histochemical staining using X-gal (GIBCO-BRL) as substrate (Epstein et al. 2000).

Immunohistochemistry

Embryos of various genotypes were fixed for 1 h in 4% PFA, sunk in 30% sucrose overnight, embedded and frozen in OCT, and sectioned at 16–20 μm on a cryostat. Primary antibodies used and dilutions were as follows: Pax2 (Zymed) 1:250; Shh (5E1, DSHB) 1:100. Detection of primary antibodies was achieved using Cy3-conjugated goat anti-rabbit (Pax2) and goat anti-mouse (Shh) secondary antibodies (Jackson ImmunoResearch Laboratories).

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