Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh

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The inner ear is partitioned along its dorsal/ventral axis into vestibular and auditory organs, respectively. Gene expression studies suggest that this subdivision occurs within the otic vesicle, the tissue from which all inner ear structures are derived. While the specification of ventral otic fates is dependent on Shh secreted from the notochord, the nature of the signal responsible for dorsal otic development has not been described. In this study, we demonstrate that Wnt signaling is active in dorsal regions of the otic vesicle, where it functions to regulate the expression of genes (*Dlx5/6* and *Gbx2*) necessary for vestibular morphogenesis. We further show that the source of Wnt impacting on dorsal otic development emanates from the dorsal hindbrain, and identify Wnt1 and Wnt3a as the specific ligands required for this function. The restriction of Wnt target genes to the dorsal otocyst is also influenced by Shh. Thus, a balance between Wnt and Shh signaling activities is key in distinguishing between vestibular and auditory cell types.

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The vestibular apparatus is the component of the inner ear responsible for sensing balance, motion, and body position. It consists of three semicircular canals and two membranous sacks, the saccule and utricle. Sensory organs housed within each of these structures serve to detect angular and linear acceleration of the head and relay this information along vestibular neurons to vestibular nuclei in the brainstem (Goldberg and Hudspeth 2000). A complex interplay between the vestibular nuclei and axons connecting with the cerebellum, eyes, and spinal cord enables us to maintain balance, stabilize images while in motion, and uphold vertical posture, respectively. Disorders of the inner ear that disrupt the reception or processing of these sensory stimuli result in vertigo, nystagmus, and loss of balance (Sando et al. 2001).

The structural complexity and functional relevance of the vestibular apparatus make it a fascinating model for developmental studies. All mature inner ear structures derive from the otic vesicle, a cyst-like epithelial tissue that forms on either side of the hindbrain (Barald and Kelley 2004). In amniotes, the vestibulum develops primarily from the dorsal otocyst, while the cochlea originates mostly from ventral regions of the otic vesicle. The morphogenesis of the vestibular labyrinth from the dor-

sal otocyst is a highly intricate process. The semicircular canals emerge initially as two epithelial protrusions or canal plates. The anterior and posterior semicircular canals are generated from the vertical canal plate, whereas the lateral semicircular canal develops from the horizontal canal plate (Martin and Swanson 1993; Chang et al. 2004a). With the fusion of the opposing walls of the epithelial plates and resorption of the central portion, the three fluid-filled canals are formed.

Although simple in its morphology, the otic vesicle is highly patterned at the level of gene expression. Programming the complex array of genes transcribed in the otic epithelium is heavily influenced by extracellular cues emanating from tissues surrounding the inner ear. The initial phase of ear development in the mouse, the induction of the otic placode, is dependent on sources of Fgf derived from the hindbrain (Fgf3) and periotic mesoderm (Fgf8 and Fgf10) (Alvarez et al. 2003; Wright and Mansour 2003; Ladher et al. 2005). Immediately prior to and concomitant with otic placode formation, the expression of a number of transcription factors (*Pax2*, *Pax8*, *Dlx5*, *Gbx2*, *Hmx3*) is initiated in a broad pattern throughout the otic epithelium, presumably in response to Fgf signaling (Alvarez et al. 2003; Wright and Mansour 2003; Ladher et al. 2005). As the development of the ear progresses to the otocyst stage, the expression domains of these and other genes become confined to particular regions of the epithelium destined to give rise to specific

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inner ear structures (Fekete and Wu 2002). This refinement of gene expression within the otic vesicle is also dependent on extrinsic signals derived from neighboring tissues as evidenced by the growing number of mouse mutants exhibiting inner ear phenotypes that result from aberrant gene function in the neural tube and surrounding tissues (Chang et al. 2004a). Previous work by our group and others described a role for Shh in promoting ventral otic fates (Liu et al. 2002; Riccomagno et al. 2002). The importance of notochord-derived Shh signaling in inner ear development was realized by the lack of cochlear duct outgrowth in *Shh*^{-/-} embryos resulting from a failure to properly regulate the expression of a number of genes in the ventral otocyst (Liu et al. 2002; Riccomagno et al. 2002).

In this corollary study, our experiments focused on determining the molecular pathways required to regulate the expression of genes in the dorsal otocyst (*Dlx5/6*, *Hmx2/3*, *Gbx2*) whose function is critical for vestibular development. Insight to this problem came from the analysis of embryos carrying the Wnt-responsive Topgal reporter gene, which showed that Wnt signaling was active in dorsal regions of the otic vesicle in a pattern indistinguishable to that of *Dlx5*. Our studies indicate that *Dlx5/6* and *Gbx2* are selective targets of Wnt signaling in the dorsal otocyst. Interestingly, the restriction of these Wnt-responsive genes to the dorsal otic epithelium is also influenced by Shh. We further demonstrate that the source of Wnt impacting on dorsal otic development emanates from the dorsal hindbrain, and identify Wnt1 and Wnt3a as the specific ligands required for this function. *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos are completely devoid of vestibular structures and to our surprise also show a severe truncation in the cochlear duct. Using an inducible recombination system to permanently mark Wnt-responsive cells in the ear, we determined that the cochlear duct is populated by cells exposed to Wnt signaling at an early stage of otic development. Taken together, our data indicate essential roles for Wnt1 and Wnt3a in promoting the development of both dorsal (vestibular) and ventral (cochlear) components of the inner ear.

Results

Wnt/β-catenin signaling is active in the dorsal otocyst

Members of the Wnt family of secreted glycoproteins have been identified in several tissues that impact on the inner ear (Parr et al. 1993; Barald and Kelley 2004). To determine where and when Wnt signaling is active during early stages of inner ear development, we evaluated embryos carrying the Wnt-responsive Topgal transgene for reporter activity (Fig. 1; DasGupta and Fuchs 1999). At the time of otic placode induction (eight to 10 somites), Topgal staining was detected at the junction between the surface ectoderm and the neural tube consistent with the known site of neural crest formation (Fig. 1a). With the completion of the otic induction phase (12 somites), revealed by the thickening of the surface

Figure 1. Wnt/β-catenin activity within the developing inner ear. (a–f) Transverse sections through the otic epithelium of Topgal embryos stained with X-gal. Topgal expression is restricted to the dorsomedial region of the placode at 10 somites (10 s) (a), 12 somites (12 s) (b), and 17 somites (17 s) (c). Expression of Topgal extends throughout the dorsal otic vesicle at 21 somites (21 s) (d), 25 somites (25 s) (e), and 10.5 dpc (f). Dotted lines in d mark the tips of the lateral and medial walls of the epithelia as they fuse. The black arrowhead points to the expression of Topgal soon after it expands to the lateral side of the otocyst. (g–i) Transverse sections through the otic vesicle of a 25-somite/9.5-dpc wild-type embryo stained with β-catenin antibody (red) and Dapi (green). Boxes mark the dorsal and ventral otic regions shown at higher magnification in h and i, respectively. White arrows point to representative cells showing nuclear β-catenin staining. White asterisk marks Topgal staining in the branchial arch. (ed) Ectoderm; (hb) hindbrain; (ncc) neural crest cells; (oc) otic cup; (op) otic placode; (ov) otic vesicle.

ectoderm, Topgal staining was evident in the dorsomedial portion of the otic epithelium in close proximity to the dorsal neural tube (Fig. 1b). Topgal reporter activity retained its dorsomedial localization as the otic epithelium invaginated to form the otic cup (Fig. 1c). Interestingly, when the tips of the lateral and medial walls of the otic epithelium fused at the 21-somite stage, X-gal staining expanded to cover the entire dorsal half of the otic epithelium and remained this way throughout the period of otic vesicle maturation (Fig. 1d–f). Thus, from placode to vesicle stages, Wnt reporter activity was detected in dorsal portions of the developing inner ear.

To confirm that Topgal staining was truly reflective of activated Wnt/β-catenin signaling in the dorsal otic epithelium, the cellular distribution of β-catenin was examined. In the absence of Wnt signaling the cytoplasmic

pool of β -catenin is rapidly degraded, whereas upon Wnt pathway activation β -catenin is stabilized, is translocated to the nucleus and, in conjunction with Lef/Tcf transcription factors, is responsible for regulating the expression of Wnt target genes (Bienz and Clevers 2003; Cong et al. 2003; Tolwinski and Wieschaus 2004). At 9.5 d post-coitum (dpc), nuclear β -catenin was primarily detected in cells of the dorsal otocyst, with little evidence of staining in ventral otic regions (Fig. 1g–i). Given the similarity in expression between nuclear β -catenin and Topgal, we conclude that the dorsal otocyst is an active site of canonical Wnt signaling. Other required components of the Wnt signaling pathway, including members of the TCF transcription factor and Frizzled receptor families, were also expressed in the otic epithelium between 8.5 and 10.5 dpc, in further support of our finding that Wnt signaling is active in the dorsal otocyst (data not shown).

Dlx5 and Topgal expression overlap in the dorsal otocyst and are antagonized by Shh signaling

The homeobox gene *Dlx5* is one of several orthologs of *Drosophila distal-less (dll)* that is expressed in the vertebrate ear (Panganiban and Rubenstein 2002). Initially, *Dlx5* covers a broad patch of surface ectoderm encompassing the otic placode (Fig. 2a; Acampora et al. 1999). Over a 24-h period, the expression of *Dlx5* becomes localized to the dorsal otocyst (Fig. 2b).

While the patterns of Topgal and *Dlx5* clearly differed at the otic placode stage, they showed very similar expression in dorsal regions of the otic epithelium after 9.25 dpc (Fig. 2a–d). To determine the extent of overlap between *Dlx5* and Topgal, double-labeling experiments using α -Dlx and α - β gal antibodies were performed on otic sections from embryos between 9.5 and 11.5 dpc. The α -Dlx antibody cross-reacts with several Dlx family members (Panganiban et al. 1995); thus in the otic vesicle, it is expected to recognize both *Dlx5* and *Dlx6* since the two genes are coregulated in this and other tissues (Robledo et al. 2002). Notably, the expression of *Dlx5/6* and Topgal exhibited significant colocalization in the dorsal otocyst of wild-type embryos (Fig. 2e–g, l–n).

We previously reported that *Dlx5* expression in the otic vesicle is negatively regulated by Shh (Riccomagno et al. 2002). Since *Dlx5/6* and Topgal show a similar distribution in the dorsal otocyst, we postulated that Shh could also be antagonizing Wnt signaling activity in ventral regions of the otic vesicle. To test this hypothesis, *Dlx5/6* and Topgal expression were evaluated in Shh loss- and gain-of-function mutants. At 10.5 dpc, both *Dlx5/6* and Topgal expression showed a significant ventral expansion in *Shh*^{-/-} embryos compared with wild-type littermates, resulting in a 33% increase ($p < 0.01$) in the number of *Dlx5/6*⁺;Topgal⁺ cells (Fig. 2e–j). At this stage, proliferation rates in *Shh*^{-/-} otic vesicles are equivalent to wild type, suggesting that the observed ventral expansion is not due to preferential proliferation of dorsal otic cells (Riccomagno et al. 2002). In keeping with this result, the dorsal otic staining of *Dlx5/6* and

Figure 2. Topgal and *Dlx5* colocalize in the dorsal otocyst and are antagonized by Shh. Whole-mount staining for *Dlx5* mRNA (a,b) and Topgal expression (c,d) at 8.5 dpc (a,c) and 9.5 dpc (b,d). Black arrowheads point to the otic epithelium. (e–r) Transverse sections through Topgal (e–g,k–n), *Shh*^{-/-}; Topgal (h–j), and *ShhP1*; Topgal (o–r) embryos at 10.5 dpc (e–j) and 11.5 dpc (k–r). Antibody staining for Dlx is in green (e,h,l,p), β -galactosidase in red (f,i,m,q), and merged channels in yellow (g,j,n,r). Note that Shh expression is normally absent in the wild-type otocyst (k) and ectopically expressed in the dorsal region of the ear in *ShhP1* embryos (o). The graph shows a 33% increase in *Dlx*⁺;Topgal⁺ cells in *ShhP1* embryos compared with wild type ($p < 0.01$, cell counts were normalized to wild type). (D) Dorsal; (L) lateral.

Topgal was extinguished in *ShhP1* embryos, a transgenic line that ectopically expresses *Shh* in the dorsal otocyst (Fig. 2k–r; Riccomagno et al. 2002). From these data, we conclude that Shh functions to restrict *Dlx5/6* and Wnt signaling activity to the dorsal otocyst.

Lithium induces Wnt/ β -catenin responsive genes in the otic vesicle

The coexpression of Topgal and *Dlx5* in the dorsal otocyst, in addition to their concordant responses to modulations in Shh signaling, suggested that *Dlx5* is a transcriptional target of the Wnt pathway in the inner ear. As an initial test of this hypothesis, we dissected otic vesicles and their surrounding tissues from embryos at

9.25 dpc and cultured them in the presence or absence of lithium chloride (LiCl) for 24 h. LiCl is known to act as a Wnt/ β -catenin pathway agonist by inhibiting Gsk3 β function, a negative regulator of canonical Wnt signaling (Hedgepeth et al. 1997).

Otic explants carrying the Topgal reporter were used to evaluate the extent of Wnt pathway activation in response to increasing concentrations of LiCl. At the lowest dose tested (1 mM), LiCl had no effect on the intensity or spatial distribution of X-gal staining compared with untreated control explants (Fig. 3A, panel a; data not shown). In contrast, otic explants cultured in higher concentrations of LiCl showed a dramatic ventral expansion of Topgal reporter activity in a dose-dependent manner (Fig. 3A, panels a–f). At the highest doses of LiCl (40–50 mM), the otic vesicles became dysmorphic, showing a highly thickened epithelium and smaller overall size (Fig. 3A, panels e,f). Therefore, all subsequent experiments were performed using a 30 mM dose of LiCl, the concentration that resulted in maximal Wnt pathway activation without adversely affecting otic vesicle morphology (Fig. 3A, panel d).

Otic explants cultured in the presence of 30 mM LiCl showed a complete ventral expansion of *Dlx5* expression, similar to the pattern of Topgal staining (Fig. 3B, panels a,g). As with Topgal, the degree of ectopic *Dlx5* expression correlated with the dose of LiCl in the culture (data not shown). To address whether other dorsal otic markers were equally responsive to Wnt signaling, we evaluated the expression of *Gbx2*, *Wnt2b*, and *Hmx3*. Expression of *Gbx2*, a transcription factor normally found in cells overlapping *Dlx5* along the dorsomedial wall of the otocyst, also showed a ventral expansion in response to LiCl treatment, though limited to the medial side of the vesicle (Fig. 3B, panels b,h). In contrast, the expression of *Wnt2b* and *Hmx3* was unchanged in LiCl-treated otic explants (Fig. 3B, panels c,d,i,j). The selective response of dorsal otic markers to LiCl implies that the ventral expansion observed for some did not come about

from the complete dorsalization of the otocyst. Additional support for this conclusion stems from the observation that LiCl failed to repress the ventral expression of *Pax2* and *Ngn1* (Fig. 3B, panels e,f,k,l). In summary, forced activation of the Wnt/ β -catenin pathway is sufficient to induce the ectopic expression of some (*Dlx5*, *Gbx2*) but not all (*Wnt2b*, *Hmx3*) dorsal otic genes.

Wnt target gene expression in the dorsal otocyst is dependent on signals from the dorsal neural tube

The finding that transcriptional targets of the Wnt/ β -catenin pathway were activated in the dorsal region of the otic vesicle raised the question of which tissue was serving as the source of Wnt. Previous studies implicated the hindbrain as a source of secreted factors impacting on various aspects of inner ear development (Mansour et al. 1993; Giraldez 1998; Ladher et al. 2000; Niederreither et al. 2000; Pasqualetti et al. 2001). We thus reasoned that the dorsal hindbrain might also be expressing the Wnt ligand regulating gene transcription in the dorsal otocyst. To test the dependency of the otic vesicle on the dorsal neural tube, otic explants were isolated at 9.25 dpc, cultured in the presence or absence of the dorsal hindbrain for 24 h, and evaluated for the expression of dorsal otic markers (Fig. 4a–c). If a Wnt ligand secreted from the dorsal neural tube is required for Wnt/ β -catenin pathway activation in the dorsal otocyst, then dorsal hindbrain removal should result in a down-regulation of Topgal reporter activity as well as other Wnt-responsive genes. Moreover, if a Wnt signal is the only factor in the dorsal neural tube necessary for target gene expression in the dorsal otocyst, then LiCl should be able to substitute for the dorsal hindbrain in maintaining the dorsal otic expression of Wnt-responsive genes. Alternatively, if dorsal neural tube removal has no effect on the expression of Wnt target genes in the dorsal otocyst, then other tissue sources must be implicated. Since the ectoderm overlying the dorsal surface of the otic vesicle is also a

Figure 3. Forced activation of the Wnt/ β -catenin pathway by LiCl causes a ventral expansion of Wnt-responsive genes in the otic vesicle. (A) Transverse sections through otic explants stained for Topgal after being cultured for 24 h in the presence (panels b–f) or absence (panel a) of increasing concentrations of LiCl. Red arrowheads point to the expanded domain of Topgal in response to LiCl. (B) Gene expression analysis of otic explants cultured alone (panels a–f) or in the presence of 30 mM LiCl (panels g–l). The red brackets in panels g and h highlight the ectopic expression of *Dlx5* and *Gbx2*, respectively. (D) Dorsal; (L) lateral.

Figure 4. The dorsal neural tube is required for the expression of Wnt-responsive genes and the repression of Shh target genes in the dorsal otocyst. (a–c) Schematic representation of the manipulations performed on otic explants: untreated control explants (a), dorsal neural tube ablated explants (Δ dNT) (b), and dorsal neural tube ablated explants (c) cultured in the presence of 30 mM LiCl. (d–aa) Gene expression studies on otic explants cultured according to the conditions outlined in a–c. The numbers in each panel reflect the reproducibility of the expression pattern indicated. Arrowheads in y–aa point to the absence (black) or presence (red) of ectopic *Gli1* expression along the lateral wall of the otocyst. (D) Dorsal; (L) lateral.

source of Wnts, careful effort was made to leave this tissue intact.

Explants in which the dorsal hindbrain was removed showed a significant reduction in Topgal reporter activity in the dorsal otocyst compared with control explants (Fig. 4d,e). A similar down-regulation was observed for *Dlx5* and *Gbx2* (Fig. 4g,h,i,k). Remarkably, the expression of Wnt-responsive genes was fully restored in the otic vesicles of dorsal-hindbrain ablated explants when cultured in the presence of 30 mM LiCl (Fig. 4f,i,l). It is important to note that the absence of Topgal, *Dlx5*, and *Gbx2* expression did not result from a loss in the number of cells in the dorsal otic epithelium since mitotic and apoptotic rates were comparable in the otic vesicles of dorsal-hindbrain ablated and control explants (data not shown). Furthermore, the dorsal otocyst was not completely dependent on signals from the dorsal neural tube as evidenced by the unaltered expression of *Wnt2b* and *Hmx3* upon dorsal hindbrain removal in the presence or absence of LiCl treatment (Fig. 4m–r). Taken together, these results strongly implicate the dorsal hindbrain as a likely source of a secreted Wnt ligand required for the maintenance of *Dlx5* and *Gbx2* expression.

Factors in the dorsal neural tube limit the expression of Shh-dependent target genes in the otic vesicle

Prompted by our earlier observations that Shh signaling is necessary and sufficient to restrict the expression of certain dorsal otic genes (Fig. 2), we set out to determine whether the reciprocal is also true; that is, are targets of Shh signaling in the otic vesicle antagonized by signals from the dorsal neural tube. *Pax2* and *Ngn1* are Shh-dependent genes required for cochlear and cochlear-vestibular ganglia formation, respectively (Riccomagno et al. 2002). The expression of both *Pax2* and *Ngn1* was expanded ectopically along the lateral wall of the otocyst in explants lacking the dorsal hindbrain (Fig. 4s,t,v,w).

To confirm that the misexpression of *Pax2* and *Ngn1* resulted from an expansion of the Shh-responsive field within the otic epithelium, we analyzed the expression of *Gli1*, a molecular readout of Shh signaling. As with *Pax2* and *Ngn1*, the lateral portion of the otic epithelium, an area that does not normally respond to Shh signaling, displayed heightened activation of *Gli1* transcription (Fig. 4y,z). Interestingly, treatment of dorsal hindbrain ablated explants with 30 mM LiCl did not fully restore the expression of Shh target genes to their normal level and position (Fig. 4u,x,aa). The observation that Shh signaling is dorsally expanded in the otic vesicles of hindbrain ablated explants may explain why the ectopic expression of Topgal and *Dlx5* in response to LiCl treatment is not as broad under these conditions compared with when the hindbrain is left intact (cf. Figs. 4f,i and 3A [panel d], B [panel g]). These results suggest that cues provided by the dorsal hindbrain restrict Shh signaling to ventral and medial regions of the otic epithelium. It is unlikely, however, that Wnt is the only dorsal hindbrain signal involved in this process since lithium treatment was unable to reduce the ectopic expression of *Gli1* and could only partially restore the expression of *Pax2* and *Ngn1*.

Wnt1 and Wnt3a cooperate to maintain *Dlx5/6* and *Gbx2* expression

Results from the otic explant experiments suggested that the dorsal otocyst is a target of a Wnt ligand derived from the dorsal neural tube. A number of *Wnt* genes are expressed in the dorsal hindbrain (Parr et al. 1993). Our attention was drawn to *Wnt1* and *Wnt3a* because their expression was present in the roof of the hindbrain and absent from the inner ear at otic vesicle stages (Parr et al. 1993; data not shown). Moreover, the combined activities of *Wnt1* and *Wnt3a* were previously shown to be required for the proliferation and specification of distinct

cell types in axial and paraxial tissues (Ikeya et al. 1997; Ikeya and Takada 1998; Muroyama et al. 2002). This raised the question of whether Wnt1 and/or Wnt3a could be acting in a similar capacity in the otocyst to regulate *Dlx5* transcription. Whereas *Dlx5* was detected in the otic vesicles of *Wnt1*^{-/-} and *Wnt3a*^{-/-} single mutants in a manner indistinguishable from wild-type littermates, its expression was markedly reduced in *Wnt1*^{-/-}; *Wnt3a*^{-/-} double mutants at 10.5 dpc (Fig. 5a–d). This result was confirmed by the failure to detect α-Dlx antibody staining in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, indicating that both *Dlx5* and *Dlx6* were absent in these mutants (Fig. 5q–s). Expression of *Gbx2*, the other gene that was responsive to Wnt activity in otic explant culture, also failed to be maintained in *Wnt1*^{-/-}; *Wnt3a*^{-/-} otic vesicles (Fig. 5v).

Interestingly, the expression of *Hmx3* and *Bmp4*, which show considerable overlap with *Dlx5* in dorsolateral regions of the wild-type otocyst at 10.5 dpc, persisted in the ears of *Wnt1*^{-/-}; *Wnt3a*^{-/-} double mutant embryos, albeit in a partially compromised manner (Fig. 5e–l). This suggests that in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos there is a preferential loss of *Dlx5/6* and *Gbx2* expression rather than a uniform loss of dorsal otic cells, consistent with the results from otic explant experiments in which the dorsal hindbrain was removed (Fig. 4). The significant reduction in the distance between the anterior and posterior domains of *Bmp4* expression in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos is likely due to the reduction in otic vesicle size exhibited by these mutants (Fig. 5m–p). Since a progressive reduction in the size of the otocyst was also observed in *Dlx5*^{-/-}; *Dlx6*^{-/-} embryos (Robledo et al. 2002), the small ear phenotype displayed by *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos may be attributed to the failure to maintain *Dlx5/6* transcription.

Given that dorsal neural tube removal resulted in an expanded domain of Shh target gene activation in otic explants, we also wanted to determine whether the same was true in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos. *Pax2* and *Otx2*,

two Shh-dependent genes expressed in medial and ventral portions of the otic epithelium, respectively, retained their normal distribution in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos (Fig. 5w–bb). These findings are in agreement with the data from otic explants and argue that the restriction of Shh signaling to primarily ventral and medial regions of the otocyst is controlled by hindbrain signals other than Wnt1/3a.

Wnt1 and Wnt3a are required for inner ear morphogenesis

We next examined the morphology of the inner ears of *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos by the paint-fill technique (Martin and Swanson 1993). At the gross anatomical level, inner ear morphogenesis appeared normal in each of the *Wnt1*^{-/-} and *Wnt3a*^{-/-} single mutants at 14.5 dpc (Fig. 6a–c). In marked contrast, the inner ears from *Wnt1*^{-/-}; *Wnt3a*^{-/-} double mutant embryos were so severely disrupted that no vestibular components could be identified (Fig. 6d). All that remained was a truncated cochlear-like structure. The absence of vestibular development in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos is likely due to the down-regulation of *Dlx5/6* and *Gbx2* within the otic vesicle since overlapping phenotypes are apparent in embryos carrying mutations in these genes (Robledo et al. 2002; Lin et al. 2005).

Otic progenitors contributing to both vestibular and auditory structures are exposed to Wnt signals at early stages of inner ear development

The finding that dorsal otic derivatives were disrupted in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos is in keeping with our data showing that Wnt signaling activity is restricted to the dorsal otocyst, from where the vestibulum develops. However, the discovery that ventral otic structures were also affected in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos was more of

Figure 5. *Wnt1* and *Wnt3a* are required for the expression of *Dlx5/6* and *Gbx2* in the otic vesicle at 10.5 dpc. Transverse sections (a–l, q–bb) or lateral whole-mount views (m–p) of otic vesicles from wild-type (WT) (a, e, i, m, q, t, w, z), *Wnt1*^{-/-} (b, f, j, n, r, u, x, aa), *Wnt3a*^{-/-} (c, g, k, o), and *Wnt1*^{-/-}; *Wnt3a*^{-/-} (d, h, l, p, s, v, y, bb) embryos analyzed by RNA in situ hybridization (a–p, t–v) or antibody staining (q–s, w–bb) for the indicated markers. Red arrow in d highlights the loss of *Dlx5* expression. The expression domain of *Gbx2*, marked by brackets (t, u), is lost in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos (v). Images in s, v, y, and bb are magnified for better display of the expression patterns. The size ratio relative to wild type was unaltered for all other images. Bar, 50 μm. (D) Dorsal; (P) posterior; (L) lateral.

Figure 6. *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos show aberrant inner ear morphology. Analysis of inner ears injected with latex paint from wild type (a), *Wnt1*^{-/-} (b), *Wnt3a*^{-/-} (c), and *Wnt1*^{-/-}; *Wnt3a*^{-/-} (d) embryos at 14.5 dpc. (aa) Anterior ampulla; (asc) anterior semicircular canal; (cc) common crus; (cd) cochlear duct; (cls) cochlear-like structure; (ed) endolymphatic duct; (la) lateral ampulla; (lsc) lateral semicircular canal; (pa) posterior ampulla; (psc) posterior semicircular canal; (s) sacculus; (u) utricle.

a surprise, especially since ventral otic determinants were appropriately expressed in these mutants (Fig. 5w–bb). A number of possible explanations can account for the cochlear phenotype in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, including cell-autonomous and nonautonomous functions of Wnt target genes. Unfortunately, definitive roles for *Dlx5/6* and *Gbx2* in cochlear development have not been reported (see Discussion). Our efforts to address this problem focused on determining the fate of Wnt-responsive cells at early stages of otic development. As an approach, we made use of the tamoxifen-inducible recombination system by generating a transgenic mouse line carrying the *Cre-ERT2* fusion gene downstream of the Wnt-responsive Top promoter (*TopCreERT2*) (Das-Gupta and Fuchs 1999; Indra et al. 1999). The overall pattern of *TopCreERT2* mRNA expression in embryos was very similar to that of *Topgal*, suggesting that this transgene is responsive to Wnt signaling (data not shown). With respect to the inner ear, *TopCreERT2* mRNA expression was confined to dorsomedial cells of the otic cup at 8.5 dpc and dorsal cells of the otic vesicle between 9.5 and 10.5 dpc, in a manner highly reminiscent of *Topgal* (Fig. 7a,b).

Crossing *TopCreERT2* males with females homozygous for the *R26R* reporter allele and administering tamoxifen at specific time points during gestation achieved permanent labeling of Wnt-responsive cells. For these experiments we attempted to initiate reporter activity at or around the time of otic cup closure by administering tamoxifen between 8.5 and 8.75 dpc. The fate of these labeled cells was surveyed between 9.5 and 14.5 dpc. Remarkably, when examined at 9.5 dpc, X-gal staining was not simply detected in the dorsal otocyst, as was predicted by the pattern of *TopCreERT2* mRNA and *Topgal* expression, but included cells along most of the medial wall of the vesicle (Fig. 7c). Control mice of the same genotype fed mineral oil showed no evidence of reporter activity in the inner ear (data not shown). As inner ear morphogenesis progressed between 10.5 and 12.5 dpc, *lacZ*⁺ cells continued to occupy the medial wall of the otic epithelium, including the outgrowing endolymphatic and cochlear ducts (Fig. 7d; data not shown). When examined between 13.5 and 14.5 dpc, *lacZ*⁺ cells were observed throughout the utricle, common crus, endolymphatic duct, and the anterior and posterior semicircular canals, including their corresponding ampullae (Fig. 7e,f; data not shown). Very little X-gal staining was detected in the lateral canal likely due to the limited number of *lacZ*⁺ cells on the dorsolateral side of the oto-

cyst (Fig. 7d–f). Interestingly, X-gal staining detected along the length of the cochlea and sacculus was restricted to the sensory (medial) side of the epithelium (Fig. 7g). These results indicate that Wnt-responsive cells in the dorsomedial portion of the otic cup contribute not only to a variety of dorsally derived inner ear structures but also, unexpectedly, to ventral otic derivatives including the cochlea and sacculus. Prohibiting the exposure of cells in the dorsomedial region of the otic cup to *Wnt1* and *Wnt3a* may be the cause of the cochlear phenotype observed in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos.

Figure 7. Fate mapping of Wnt-responsive cells in the inner ear. (a,b) *TopCreERT2* expression in the inner ear was revealed by RNA in situ hybridization. Red arrow points to the ventral hinge point of the otic cup. (c–g) X-gal staining of inner ears from *TopCreERT2*; *R26R*/+ embryos administered with tamoxifen at 8.75 dpc. Cells that have responded to Wnt signaling are located along most of the medial and dorsal regions of the vesicle at 9.5 dpc (c) and 11.0 dpc (d). (e) Whole-mount view of the inner ear at 14.5 dpc. (f,g) Transverse sections through the ear shown in e revealing the contribution of Wnt-responsive cells to the vestibule (f) and medial wall of the cochlea (g). Red bracket marks the unstained lateral wall of the cochlea. (asc) Anterior semicircular canal; (cc) common crus; (cd) cochlear duct; (ed) endolymphatic duct; (lsc) lateral semicircular canal; (psc) posterior semicircular canal.

Discussion

Wnt signaling from the dorsal neural tube is required for dorsal otic patterning

Extracellular signaling molecules provide the inner ear with positional information at multiple stages of its development to produce the required patterns of growth and differentiation necessary for the formation of the vestibulum and the cochlea, the two inner ear organs responsible for sensing balance and sound, respectively (Barald and Kelley 2004). Experiments performed in the present study set out to address the nature and source of the signaling molecules required to impart dorsal (vestibular) identity within the otic vesicle.

Our data show that the inner ear is a direct target of Wnt signaling as early as the otic placode stage. Cells in the dorsomedial region of the placode were positive for the Wnt-responsive Topgal reporter. At these early stages a number of Wnt ligands, including Wnt1 and Wnt3a, are expressed in the dorsal neural tube immediately adjacent to the portion of the placode responsive to Wnt signaling (Parr et al. 1993; data not shown). By 9.5 dpc, the dorsal cells of the otic vesicle that remain closest to the source of Wnts in the dorsal neural tube continue to express the Topgal reporter gene. The distance between the dorsal otocyst and the source of Wnt1 and Wnt3a in the dorsal midline of the neural tube at 9.5 dpc is well within the range of signaling (100 μ m) proposed for these ligands in the spinal cord (Megason and McMahon 2002). The best evidence in support of a direct action of dorsal neural tube Wnt signals on the dorsal otocyst was obtained in explant experiments, which revealed that Topgal expression in the dorsal otocyst was fully dependent on the presence of the dorsal neural tube and that LiCl, a Wnt agonist, could substitute for the dorsal neural tube in maintaining Topgal activity in the ear. Interestingly, the dorsal otic expression of Topgal persists after 10.5 dpc, an age when the distance between the dorsal neural tube and ear far exceeds the limits of Wnt signaling. Local sources of Wnt signals within the ear have been described that function at these later stages to specify vestibular hair cells (Stevens et al. 2003).

Loss of Dlx5/6 and Gbx2 account for the vestibular defects in Wnt1^{-/-}; Wnt3a^{-/-} embryos

Several lines of evidence indicate that the expression of *Dlx5/6* and *Gbx2* in the dorsal otocyst is dependent on Wnt signaling. The expression of *Dlx5* and *Gbx2* was expanded in otic explants treated with LiCl and lost in otic explants in which the dorsal hindbrain was removed. Importantly, LiCl was sufficient to restore *Dlx5* and *Gbx2* expression in dorsal hindbrain ablated explants, arguing that the only dorsal neural tube signal required to maintain the expression of these genes belongs to the Wnt family (Fig. 8). In support of this claim, the expression of *Dlx5/6* and *Gbx2* was lost from the dorsal otocyst of *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos at 10.5 dpc. The early expression of these genes throughout the otic

Figure 8. Model depicting how inner ear polarity is generated in response to extracellular signals. Wnt signals secreted by the dorsal neural tube (dark green) are necessary and sufficient for the regulation of *Dlx5/6* in the dorsal otocyst (light green). This contrasts with the function of Shh emanating from the notochord (blue), which specifies ventral otic cell fates by regulating the transcription of genes, including *Pax2* and *Ngn1* (light blue). The combination of Wnt and Shh signaling activities are required for the maintenance of *Gbx2* along the dorsomedial side of the otocyst (green-blue shading). Shh also restricts Wnt pathway activation, and consequently *Dlx5/6* expression, to dorsal regions of the otic vesicle. An additional signal secreted from the dorsal neural tube, possibly a Bmp family member (red), restricts Shh target genes to the ventromedial domain of the otocyst. A balance between these dorsal and ventral signals is key to promoting regional identity within the otic epithelium with the ultimate goal of coordinating the morphogenesis of vestibular and auditory organs.

placode is unlikely to be dependent on Wnt signaling since Topgal and *Dlx5/Gbx2* only partially overlap at this stage.

The Wnt-dependent regulation of *Dlx5* expression in the mouse ear is remarkably similar to the molecular mechanism underpinning specification of the *Drosophila* antenna, which, like the vertebrate ear, houses mechanosensory structures responsible for transmitting auditory information to the brain. One of the many functions of wg (a Wnt1 ortholog) is to direct the expression of *dll* (a *Dlx5* homolog) to the antenna and other tissues (Diaz-Benjumea et al. 1994; Panganiban 2000). Regulating the expression of *Dlx5/dll* by Wnt/wg family members may represent part of a conserved pathway used in the specification of sense organs (Panganiban 2000). Interestingly, downstream targets of this regulatory module in the fly antenna are also expressed in the mouse inner ear (for review, see Panganiban and Rubenstein 2002).

The inner ear phenotype exhibited by *Dlx5*^{-/-} embryos represents a subset of those displayed by *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos. While the lack of semicircular canals is a feature common to both mutants, other inner ear structures, including the endolymphatic duct, utricle, saccule, and cochlea, all of which are absent or indistinct in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, still form to varying degrees in *Dlx5*^{-/-} mutants (Acampora et al. 1999; Depew et al. 1999; Merlo et al. 2002). As a result, the otic phe-

notype depicted by *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos cannot fully be explained by the loss of *Dlx5*. Interestingly, embryos carrying mutations in both *Dlx5* and *Dlx6* show a more drastic phenotype in several tissues, including the inner ear, compared with *Dlx5*^{-/-} embryos alone (Robledo et al. 2002). *Dlx5*^{-/-}; *Dlx6*^{-/-} mutants display a striking reduction in the overall size of the otocyst at 9.5 dpc and fail to form any dorsal otic derivatives. Thus, the down-regulated expression of both *Dlx5* and *Dlx6* that we observed in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos better explains the reduction in otic vesicle size and vestibular defects displayed by these mutants.

It is likely that the down-regulated expression of *Gbx2* also contributes in some part to the *Wnt1*^{-/-}; *Wnt3a*^{-/-} otic phenotype. *Gbx2*^{-/-} embryos show variable vestibular malformations that are generally milder than those seen in *Wnt1/3a* mutants (Lin et al. 2005). This raises the possibility that the inner ear defects in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos result from the additive effects of losing *Gbx2* and *Dlx5/6* expression.

Convergence of multiple pathways during vestibular morphogenesis

While our data indicate that Wnt signaling is necessary and sufficient to maintain the expression of some dorsal otic genes, others including *Hmx3* and *Wnt2b*, were unchanged by dorsal neural tube removal, LiCl treatment, or, in the case of *Hmx3*, targeted disruption of *Wnt1* and *Wnt3a*. *Hmx3* and *Hmx2* overlap in the dorsolateral region of the otic vesicle and together are required for the development of all vestibular structures (Wang et al. 2004). Similarly, embryos lacking *Dlx5* fail to generate semicircular canals (Acampora et al. 1999; Depew et al. 1999; Merlo et al. 2002). The apparent discordance in the mechanisms regulating *Dlx5* and *Hmx3*, coupled with the finding that the expression of each gene is maintained in embryos deficient in the other, supports the notion that two pathways are functioning at early stages of inner ear development to promote the vestibulum (Merlo et al. 2002; Wang et al. 2004). A third pathway operating at a slightly later stage was also shown to promote outgrowth of the semicircular canals (Chang et al. 2004b). In this study, Fgf signaling from the presumptive cristae, the neurosensory tissue located at the base of each semicircular canal, was implicated in canal outgrowth due to its ability to up-regulate *Bmp2* in the canal pouch. *Bmp* family members have previously been implicated in vestibular morphogenesis (Chang et al. 1999; Gerlach et al. 2000). The perturbation of *Bmp4* function in the presumptive cristae of chick embryos using the *Bmp2/4* antagonist *Noggin* resulted in a loss of semicircular canal formation. Interestingly, the absence or mislocalized expression of *Bmp4* is a feature common to *Wnt1/Wnt3a*, *Dlx5*, and *Hmx2/Hmx3* mutants (Fig. 5; Merlo et al. 2002; Wang et al. 2004). For this reason, the three pathways involved in vestibular development described above may be integrating at different levels of a *Bmp* signaling cascade. In this model, *Wnt1/3a-Dlx5* and *Hmx2/3* are required to position *Bmp4* expression

within the cristae-forming region of the otic epithelium. This source of *Bmp4* would then be involved, either directly or indirectly, in the activation of Fgf (possibly Fgf10) (see Chang et al. 2004b) within the presumptive cristae, which in turn leads to the expression of *Bmp2* in the canal genesis zone and subsequent outgrowth of the semicircular canals. Future experiments will be needed to confirm the proposed roles of *Bmp2* and *Bmp4* in vestibular development by the conditional inactivation of these genes in the otic epithelium.

Otic cup cells exposed to Wnt signaling also contribute to the cochlear duct

The spectrum of otic phenotypes observed in *Wnt1*^{-/-}; *Wnt3a*^{-/-} mutants exceeds the domains of the otic vesicle that are directly responding to Wnt signaling between 9.5 and 10.5 dpc. The failure to maintain *Dlx5/6* and *Gbx2* expression in the dorsal otocyst may adequately explain the vestibular defects in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos; however, it remains unclear as to whether the down-regulation of these genes accounts for the hypoplastic cochlea observed in these mutants. A role for *Dlx5/6* during cochlear development has yet to be reported. Moreover, only a subset (26%) of *Gbx2*^{-/-} embryos exhibit cochlear duct malformations, and it is uncertain whether this phenotype results from the loss of a non-cell-autonomous function of *Gbx2* in the ear or is the indirect consequence of a hindbrain patterning defect (Lin et al. 2005). Regardless of the potential roles that targets of Wnt signaling may have in cochlear development, our fate mapping studies provide novel insight into the contribution of Wnt-responsive cells to the formation of the cochlear duct. Using an inducible recombination system, we were able to permanently mark Wnt-responsive cells in the otic epithelium prior to closure of the otic cup. Remarkably, many mature inner ear structures stained positively for lacZ activity, including the entire medial side of the cochlear duct and much of the vestibulum. The extent to which the Wnt-responsive cells in the dorsomedial portion of the otic cup contributed to the mature inner ear was not anticipated. Fate mapping studies performed at a similar stage of otic development in the chicken showed that these cells remain exclusively in the dorsal otocyst (Brigande et al. 2000). The expansion of otic epithelial cells that we observed in the mouse is reminiscent of the scattered behavior of otic progenitor cells described in frogs (Kil and Collazo 2001). However, in contrast to this study, we do not know if the broad distribution of Wnt-responsive cells along the medial wall of the mouse otocyst results from cell migration, a rapid expansion of dorsomedial otic progenitors, or both. Regardless of the mechanism, ventral otic progenitors destined to give rise to the cochlear duct are exposed to Wnt signaling at an early stage of inner ear development. The failure to expand this population of cells in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos may explain the limited outgrowth of the cochlear duct observed in these mutants.

Opposing activities of Shh and Wnt signaling pathways: parallels in somite and inner ear development

Shh specifies ventral fates within the otic epithelium by initiating and/or maintaining the expression of several transcription factors whose function is essential for proper cochlear and cochlear vestibular ganglia formation (Liu et al. 2002; Riccomagno et al. 2002). Our observation that Wnt target genes were expanded ventrally in *Shh* mutant otocysts revealed an additional function of Shh in ensuring that Wnt signaling remains restricted to the dorsal otocyst (Fig. 8). *Six1*^{-/-} embryos also show a loss of ventral otic targets and a significant ventral expansion of *Dlx5* and other dorsally expressed genes, despite possessing normal Shh signaling activity (Zheng et al. 2003; Ozaki et al. 2004). This and additional data suggest that *Shh* and *Six1* are operating in separate pathways to pattern the ventral otocyst and consequently establish a molecular boundary along the dorsoventral axis of the otic vesicle, which delineates the vestibular (dorsal) from auditory (ventral) regions of the inner ear. The molecular boundary that is created by the opposing activities of the Wnt and Shh signaling pathways is not likely to be sharp given that both pathways are required to maintain *Gbx2* expression on the dorsomedial side of the otocyst (Fig. 8; Riccomagno et al. 2002). This can be justified if *Gbx2* expression is dependent on a low concentration of Shh and a high concentration of Wnt. Thus, the balance of Shh and Wnt signaling activities would be key in specifying distinct inner ear fates.

Our data in the ear bear remarkable similarity to the molecular mechanisms underlying the development of another paraxial tissue, the somite. Hh and Wnt signals exhibit opposing and cooperative functions in partitioning the presomitic mesoderm along its dorsoventral axis into dermomyotome (dorsal) and sclerotome (ventral) cell fates (for review, see Brent and Tabin 2002). Wnt signaling promotes the expression of the dermomyotomal marker *Pax3* at the expense of the sclerotomal marker *Pax1*, while Hh signaling mediates the converse function of inducing *Pax1* over *Pax3* expressing fates (Brent and Tabin 2002). In the mouse, Wnt1 and Wnt3a secreted from the dorsal neural tube are required for the expression of genes in dorsal and dorsomedial regions of the somite (Ikeya and Takada 1998). One target of Wnt1/3a regulation, the myogenic determinant *Myf5*, is also dependent on Shh signaling, suggesting that *Myf5* expression is coordinated by the combination of Shh and Wnt signaling activities (Ikeya and Takada 1998; Borycki et al. 1999; Gustafsson et al. 2002). As with the opposing and cooperative effects of Shh and Wnt in the inner ear, different levels of pathway activation may also dictate the specification of distinct cell fates within the somite.

While Shh is required to restrict Wnt signaling activity to the dorsal otocyst, several lines of evidence suggest that factors other than Wnts are responsible for the reciprocal role of limiting Shh responsiveness to the ventral and medial regions of the inner ear. First, Shh target genes were not expanded dorsally in *Wnt1*^{-/-}; *Wnt3a*^{-/-}

embryos. Second, Shh signaling was not reduced in LiCl-treated otic explants despite the ectopic expression of Wnt targets in the ventral otocyst. Third and perhaps of most significance, the dorsal expansion of Shh-responsive genes that resulted from dorsal hindbrain removal in otic explants was not fully restored by LiCl treatment. We cannot rule out that higher concentrations of LiCl may be needed to rescue the dorsal otic expansion of Shh target genes however, the 30-mM dose received is likely at or close to saturating levels. Drawing from further comparisons between inner ear and somite development, we propose that Bmp family members expressed in the dorsal hindbrain may be the factors that participate in restricting Shh signaling in the otocyst (Fig. 8). Support for this claim stems from the finding that Noggin, a Bmp2/4 antagonist, is required to prevent Bmp-mediated repression of Shh activity in the ventral somite (McMahon et al. 1998). Formal testing of this hypothesis must await conditional inactivation of Noggin and Bmp family members.

Materials and methods

Production and genotyping of transgenic mice

The TopCreERT2 construct was generated by replacing the β -galactosidase cDNA in the Topgal vector (DasGupta and Fuchs 1999) with the CreERT2 fusion gene (Indra et al. 1999). The TopCreERT2 construct was linearized with EcoRI and purified for pronuclear injection by standard methods (Hogan et al. 1994). Genotyping of founder animals using primers for *Cre* (F, 5'-GCGGCATGGTGCAAGTTGAAT-3'; R, 5'-CGTTCACCGGCATCAACGTTT-3') identified several independent mouse lines carrying the transgene. Evaluation of transgenic lines expressing TopCreERT2 was performed in F1 progeny at various embryonic stages by whole-mount in situ hybridization using a *Cre* riboprobe. One line showed TopCreERT2 expression in a Wnt-responsive manner similar to the pattern exhibited by Topgal (Fig. 7).

The *Wnt1*^{-/-}; *Wnt3a*^{-/-} double mutants were identified among embryos derived from matings between compound heterozygotes that were maintained on a C57/Bl6 background (Ikeya et al. 1997). The *Shh*^{+/-} animals were kindly provided by H. Westphal (National Institutes of Health, Bethesda, MD) (Chiang et al. 1996) and maintained on a CD-1 background (Charles River). The generation of *Shh* P1 mice has been described previously (Riccomagno et al. 2002). Topgal mice were kindly provided by E. Fuchs (Rockefeller University, New York, NY) (DasGupta and Fuchs 1999). R26R mice were procured from Jackson Laboratories.

Culturing of otic explants

Otic explants were cultured according to the protocol described in Jeong and Epstein (2003) with slight modifications. Embryos generated from matings between Topgal males and CD-1 females were harvested in ice-cold L15 medium (GIBCO-BRL) at 9.25 dpc. Otic vesicles and surrounding tissues, including the periotic mesenchyme, hindbrain, and branchial arches, were explanted from the embryo and cultured on Transwell filters (0.4 μ m, Costar) floating on 47.5% Dulbecco's minimum essential medium (Specialty Media), 47.5% F-12 Ham's nutrient mixture (GIBCO-BRL), 2 mM glutamine (GIBCO-BRL), 100 U/mL penicillin-streptomycin (GIBCO-BRL), and 5% rat serum (Harlan) in

the presence or absence of LiCl (10–50 mM). In some experiments the dorsal neural tube was surgically removed prior to culturing the explant. After 24 h of culture in a CO₂ incubator at 37°C, explants were fixed and stained with X-gal or processed for whole-mount RNA in situ hybridization. After staining, embryos were post-fixed in 4% paraformaldehyde, rinsed in PBS, embedded in 4% agarose, and sectioned on a vibratome at 50–75 µm.

Fate mapping and β -galactosidase staining

To follow the fate of Wnt-responsive cells, TopCreERT2 males were crossed to R26R/R26R females (Soriano 1999). Cre activity was induced by administering 7–8 mg of tamoxifen/40 g of body mass to pregnant females by oral gavage at 8.75 dpc.

Whole embryos (9.5–11.5 dpc) or inner ears (14.5 dpc) were dissected in ice cold PBS and fixed in 0.2% glutaraldehyde/1% formaldehyde wash buffer for 30 min to 1.5 h (depending on the stage of development) at 4°C. Tissues were stained with X-gal solution for 2 h to overnight (Epstein et al. 2000).

In situ hybridization and paint fill studies

Whole-mount RNA in situ hybridization was performed essentially as described (Matise et al. 1998) using digoxigenin-UTP-labeled riboprobes. Between two to five embryos of each genotype were analyzed for every probe. Representative embryos were sectioned on a vibratome as described above. Paint fill analysis was conducted according to the method of Martin and Swanson (1993).

Immunohistochemistry

Antibody staining was performed as previously described (Riccomagno et al. 2002). For detection of β -catenin, fresh tissue was embedded in OCT and flash-frozen in liquid nitrogen without prior fixation or equilibration. Primary antibodies and dilutions used were as follows: Pax2 (Zymed) 1:250; Dlx (courtesy of J. Kohtz, Northwestern University, Chicago, IL) 1:70; Otx2 (Baas et al. 2000; courtesy of F. Vaccarino, Yale University, New Haven, CT) 1:2000; β -gal (Promega) 1:200; β -catenin (Clone 15B8, Sigma) 1:200; Shh (5E1, Developmental Studies Hybridoma Bank) 1:100.

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