Forced activation of Wnt signaling alters morphogenesis and sensory organ identity in the chicken inner ear

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Abstract

Components of the Wnt signaling pathway are expressed in the developing inner ear. To explore their role in ear patterning, we used retroviral gene transfer to force the expression of an activated form of β-catenin that should constitutively activate targets of the canonical Wnt signaling pathway. At embryonic day 9 (E9) and beyond, morphological defects were apparent in the otic capsule and the membranous labyrinth, including ectopic and fused sensory patches. Most notably, the basilar papilla, an auditory organ, contained infected sensory patches with a vestibular phenotype. Vestibular identity was based on: (1) stereociliary bundle morphology; (2) spacing of hair cells and supporting cells; (3) the presence of otoliths; (4) immunolabeling indicative of vestibular supporting cells; and (5) expression of Msx1, a marker of certain vestibular sensory organs. Retrovirus-mediated misexpression of Wnt3a also gave rise to ectopic vestibular patches in the cochlear duct. In situ hybridization revealed that genes for three Frizzled receptors, c-Fz1, c-Fz7, and c-Fz10, are expressed in and adjacent to sensory primordia, while Wnt4 is expressed in adjacent, nonsensory regions of the cochlear duct. We hypothesize that Wnt/β-catenin signaling specifies otic epithelium as macular and helps to define and maintain sensory/nonsensory boundaries in the cochlear duct.

Keywords: β-Catenin; Wnt3a; Wnt4; Msx1; p75ngfr; Frizzled; Serrate; Inner ear development; Cochlea; Hearing; Vestibular

Introduction

The vertebrate inner ear arises from placodal ectoderm that invaginates to form the otic vesicle, which grows and develops into the three semicircular canals, the vestibule, the cochlea, the endolymphatic duct and the eighth cranial ganglion. The avian inner ear houses eight sensory organs. The vestibular sensory organs include three cristae (one for each semicircular canal) and four maculae (those of the utricle, saccule, and lagena, and the macula neglecta) (Kido et al., 1993; Landolt et al., 1975). The auditory sensory organ is the basilar papilla, located within the cochlear duct. Each sensory organ is composed of mechanosensory hair cells interspersed among supporting cells and is capped by a specialized extracellular matrix. However, the sensory organ types differ in the composition of their overlying matrices and in the morphology, polarity, and spatial arrangement of their constituent cells. The maculae, with one exception in the chicken, are covered by a proteinaceous extracellular matrix encasing otolith crystals of calcium carbonate (Ballarino et al., 1985; Kido et al., 1993). Cristae are capped by a gelatinous matrix devoid of otoliths (Goodyear and Richardson, 2002). The basilar papilla is covered by the fibrous tectorial membrane, which has attachments to the superior wall of the cochlear duct and does not contain otoliths (Goodyear and Richardson, 2002). The spacing between hair cells also varies based on organ type. Vestibular hair cells are separated from each other by the broad apical surfaces of the supporting cells (Kruger et al., 1999). In contrast, hair cells in the basilar papilla are separated by thin apical projections of the underlying supporting cells.
(Goodyear and Richardson, 1997; Hirokawa, 1978). The genetic mechanisms that specify the identity of each sensory organ during inner ear development are unknown, although there are some differences in their gene expression profiles prior to overt differentiation of the organs (Wu and Oh, 1996).

Wnts are secreted factors involved in patterning, tissue polarity, and cell fate specification in many developing systems (Dale, 1998; Huelsken and Birchmeier, 2001), and thus are candidates for signaling roles in the embryonic ear. Wnt genes are the vertebrate homologues of wingless (wg) in Drosophila, and in vertebrates they form a large gene family (Nusse and Varmus, 1992). Wnts transmit their signal through at least three distinct intracellular pathways. One, the so-called canonical pathway, involves translocation of β-catenin to the cell nucleus. The others involve, respectively, release of intracellular Ca\(^{2+}\) and activation of RhoA, which leads to effects on the actin cytoskeleton and on planar polarity (Dale, 1998; Huelsken and Birchmeier, 2001; Kuhl et al., 2000). The choice of pathway is dependent on the characteristics of the receptors, called Frizzled proteins that are members, like the Wnt proteins, of a large family. Wnt proteins bind preferentially to certain Frizzled receptors (He et al., 1997). In addition, certain Frizzled receptors signal preferentially through either the β-catenin, RhoA, or Ca\(^{2+}\) pathways (Hartmann and Tabin, 2000; Medina et al., 2000; Slusarski et al., 1997), although the extent of this specificity may vary with developmental context. The focus of this paper is on the Wnt/β-catenin pathway, in which Wnt binding to its receptor initiates an intracellular signaling cascade that rescues β-catenin from targeted degradation (Hart et al., 1998; Ikeda et al., 1998). Cytoplasmic β-catenin levels rise and the protein translocates to the nucleus where it forms a complex with LEF/TCF transcription factors to stimulate transcription of Wnt target genes (Branon et al., 1997; Huber et al., 1996).

In the developing otocyst, Wnt2b and Wnt3a are expressed dorsally in mouse and chicken (Hollyday et al., 1995; Jasoni et al., 1999), while Wnt4 is expressed ventrally in Xenopus (McGrew et al., 1992). In the early chicken otocyst, the genes for several Frizzled receptors are expressed in complex spatial patterns that can overlap with the Wnts (Stark et al., 2000). The functional significance of Wnts and Frizzleds in the developing chicken inner ear is unknown.

Two of the Wnts present in otocysts, Wnt3a and Wnt4, have been associated with the canonical β-catenin signaling pathway in the developing chicken limb (Hartmann and Tabin, 2000; Kengaku et al., 1998). To determine the role of Wnt/β-catenin signaling in chicken ear development, we took a gain-of-function approach by using retrovirus-mediated gene transfer to overexpress either a truncated form of Xenopus β-catenin that leads to constitutive activation of the canonical Wnt pathway (Funayama et al., 1995) or a full-length copy of chicken Wnt3a. Infection with these reagents resulted in malformed inner ears that were defective in sensory organ patterning, although the axial specification of the ear remained intact. Of special interest was the presence of ectopic vestibular sensory patches within the auditory territory of the ear; many of these ectopic patches had assumed a macular identity. We also show that several Frizzled genes are expressed in sensory territories of the ventral ear, while Wnt4 is expressed immediately adjacent to the sensory primordia, in nonsensory territory. These data, together with the gain-of-function phenotypes, suggest that Wnt/β-catenin signaling is involved in establishing or maintaining distinctions between sensory and nonsensory domains, and in specifying the identity of vestibular versus auditory sense organs.

Materials and methods

Retrovirus preparation and injection

Virus stocks were prepared from plasmids encoding replication-competent retroviral vectors. RCASBP(A) was used as a control (or parent) viral construct and is hereafter referred to as RCAS. Two different constructs were used to misexpress genes: RCAS/Wnt3a, containing a gene for a constitutively active form of β-catenin tagged with the influenza hemagglutinin epitope (Funayama et al., 1995; Kengaku et al., 1998); and RCAS/Wnt3a, containing the full-length chicken Wnt3a gene (Kengaku et al., 1998). After transfecting the DF-1 chicken cell line, culture supernatant was concentrated to 10\(^{9}\) infectious units/ml as described (Fekete and Cepko, 1993). Fertilized White Leghorn chicken eggs (SPAFAS, Inc.) were incubated at 38°C, windowed on embryonic day (E) 2, and assigned stages (s) according to Hamburger and Hamilton (1951). Approximately 0.05–0.1 μl of viral stock was delivered by bathing the surface of the otic placode/otic cup on E1.5–E2 (s8–s12), or by filling the otic vesicles on E2.5–E4 (s15–s25) (Hamburger and Fekete, 1996). Some embryos received injections into the hindbrain ventricles and multiple injections into the mesenchyme both anterior and posterior to the otocyst on E2, followed by injections into the otocyst on E3.

Paint filling of ears

Specimens were collected on E8–E9 (s32–s34) and processed for paint filling of the inner ear (Bissonnette and Fekete, 1996).

Histological analysis by immunostaining

Embryos ranging from E6 to E17 were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. For whole-mount immunostaining of hair cells in the intact membranous labyrinth, we followed a published protocol (Wu et al., 1998) based on alkaline phosphatase detection. For staining of sections, embryos were processed
through graded sucrose solutions, embedded in sucrose–gelatin, frozen, and sectioned at 15–25 μm. In some cases, sections were saved as three series to stain adjacent sections with different combinations of antibodies. Primary antibodies include: mouse IgG, HA.11 (1:1000; Covance) directed against the hemaglutinin flu epitope tag on activated-β-catenin; mouse IgG, anti-HCA (Hair-Cell- Antigen, 1:1000) (Bartolami et al., 1991) that labels the apical surfaces of hair cells; mouse IgG2a, HCS-1 that labels hair cell bodies (1:200) (Gale et al., 2000); mouse polyclonal antibodies to β-tubulin I-II (1:200; Sigma-Aldrich); mouse IgM gm-2 that labels vestibular supporting cells (1:500) (Goodyear et al., 1994); mouse IgG, 3C2 that labels the viral gag protein (1:10 of hybridoma culture supernatant) (Potts et al., 1987); and rabbit polyclonal anti-chick neurofilament-70 (NF-70; 1:500) (Hollenbeck and Bray, 1987). Some samples were double-labeled with rhodamine-phalloidin to label f-actin. A variety of secondary antibodies were used at 1:200–1:500 dilution. These included biotinylated anti-mouse IgM or anti-mouse IgG (Vector Laboratories) and AlexaFluor 488- or 568-conjugated antibodies (Molecular Probes). Biotinylated secondary antibodies were detected by using AlexaFluor streptavidin conjugates (Molecular Probes) or the ABC kits (Vector Laboratories) followed by alkaline phosphatase or diaminobenzidine histochemistry as appropriate. Specimens were preblocked and all antibodies were diluted in 3% bovine serum albumin with or without Triton X-100 (0.05% for sections, 0.4% for whole mounts). Antibody incubation times were 1 h (at room temperature) to overnight (at 4°C) for sections, and 2 days (at 4°C) for whole mounts.

Images of whole-mounted, immunostained ears were captured by using a SPOT digital camera mounted on a Leica MZFLIII stereofluorescent microscope or a Nikon EFD-3 microscope with a BioRad MRC-1024 laser scanning confocal imaging system. Images of sections were digitized with a SPOT camera mounted on a Nikon E800 photomicroscope.

Three-dimensional reconstruction

E9 embryos were sectioned at 15 μm in a transverse plane and stained with hair cell-specific antibodies. Virus infection was monitored by immunostaining every 10th section with mouse monoclonal antibody HA.11. Five ears from 4 E9 embryos were serially reconstructed. Using NIH Image and Photoshop, hair cell staining and the lumen of the ear were selected and pasted onto a black background. Hair cell staining was false-colored in yellow and the ear lumen filled with 50% gray. Images were aligned in Photoshop, converted to grayscale, and loaded as a stack into VoxelView 2.5 (Vital Images) to generate a single 3-dimensional volume. Within VoxelView, the ear lumen was rendered in blue and hair cell staining in yellow. These images were rotated and selected views imported back into Photoshop.

Scanning electron microscopy

Embryos injected on E2.5 (s15–s16) were sacrificed on E16–E17 and the temporal bones housing the entire inner ear were fixed in 2% glutaraldehyde, 0.1 M sodium phosphate buffer at pH 6.8 (PB) for 3–4 h. Cochleae were removed, washed in PB, and the basilar papillae exposed. In some specimens, the tectorial membrane was removed by using fine forceps after 30–60 s in 0.05 mg/ml Type 24 protease (Sigma) in PB. Cochleae were postfixed for 30 min in 1% osmium tetroxide in PB (pH 7.2), washed in PB, dehydrated through graded ethanol, and critical point dried. After sputter coating with gold-palladium, samples were viewed and digital images captured with a JEOL JSM-840 scanning electron microscope.

In situ hybridization

Plasmids for Msx1, p75ngfr, cSerl1, cWnt3a, cWnt4, and cFrizzleds were linearized and used to make hapten-labeled anti-sense RNA probes utilizing digoxigenin–UTP. For hybridization of all but the Frizzled probes, specimens were collected on E3–E12, fixed overnight in 4% paraformaldehyde under RNase-free conditions, and prepared for frozen sectioning. Adjacent transverse 20-μm sections were treated with RNA probes or antibodies, including neurofilament and HA.11. In situ hybridization was performed as published (Xu and Wilkinson, 1998), and probes were detected by using anti-digoxigenin–alkaline phosphatase (Roche). Slides were reacted with NBT-BCIP color reagent for 4–16 h, washed, and postfixed for 1–3 h in 4% paraformaldehyde.

Frizzled riboprobes were derived from plasmids containing partial cDNAs (gift of Stefan Heller) and were 500–1500 nt in length. For hybridization with Frizzled probes, embryos were dissected to aid penetration of the fixative and fixed in 4% paraformaldehyde for 90–150 min. They were washed in PBS/0.1% Tween 20, embedded in 1.5% agarose/5% sucrose in water, and equilibrated in 30% sucrose overnight. Frozen sections cut at 15 μm were hybridized with probes and immunostained with an antibody to Serrate1 as described (Adam et al., 1998; Eddison et al., 2000). Hybridized probe was visualized as above but using Fast Red (Roche) as final fluorochrome.

Results

Activated β-catenin altered the gross morphology of the inner ear

Wnt3a expression in the dorsal ear begins before vesicle closure (Hollyday et al., 1995; and our unpublished observations). To study the possible effects of Wnt signaling on dorsal–ventral specification in the ear, we reasoned that it would be necessary to manipulate gene expression in the ear
as early as possible. Thus, injections of replication-competent RCAS/*β-catenin were initially performed at s8–s12 to ensure overexpression of activated β-catenin in the otic epithelium and mesenchyme shortly after otic vesicle closure. Embryos were collected at E9, a time when the canals, cochlea, and endolymphatic duct have formed, and all of the sensory organs are evident. Embryos injected with RCAS parent virus developed normally, including their ears (Fig. 1A). Embryos injected with RCAS/*β-catenin exhibited multiple defects of the epithelium and mesenchyme, including abnormal feather buds, a shortened upper beak, abnormal retinal morphology, and ectopic scleral papillae (not shown). Immunostaining for the hemagglutinin tag on activated β-catenin confirmed its presence throughout the head (not shown). Experimental ears showed gross morphogenetic defects in both vestibular and auditory components (Fig. 1B). In 9/28 experimental ears, the lateral canal was hypomorphic or missing (Fig. 1B, asterisk). In 2 of the 9, the superior and posterior canals were also hypomorphic, consisting of either a pouch or a shorter duct (Fig. 1B, arrow). Many ears also showed cochlear abnormalities; the cochlear duct was enlarged and exhibited projections along its medial wall (Fig. 1B, arrowhead). Although the endolymphatic space of the vesicle was sometimes dramatically enlarged, the endolymphatic duct and sac were always present and located in the correct anatomical positions (Fig. 1B).

Gross dissection of RCASBP/*β-catenin-infected temporal bones at older embryonic ages (E11–E17) was difficult because the otic capsule was softer and more pliable than usual. Examination of histological sections confirmed that cartilage development was abnormal in infected specimens. The cartilage capsule was infiltrated with an intricate network of small, noncartilaginous mesenchymal cells. Immunostaining revealed that these abnormal regions were infected with virus and expressed truncated β-catenin protein (data not shown).

**Activated β-catenin altered the spatial distribution of sensory organs in the vestibule**

Several methods were used to examine sensory organ distribution by immunostaining for hair cells, and all patches in the experimental (D arrow). In the cochlear duct, medial-projecting invaginations of the basilar papilla contain hair cells (D arrowhead). (E) Surface view of the sensory epithelium of the anterior vestibule in an E17 experimental ear. HCA labeling (red) shows a large, abnormally shaped sensory patch in the position of the lateral crista/utricular macula (lc/um). The superior crista is separate. Abbreviations (sensory patches are italicized): A, anterior; bp, basilar papilla; cd, cochlear duct; D, dorsal; ed, endolymphatic duct; es, endolymphatic sac; lsc, lateral semicircular canal; L, lateral; lc, lateral crista; lm, lagena macula, M, medial; mn, macula neglecta; pc, posterior crista; psc, posterior semicircular canal; sc, superior crista; sm, saccular macula; ssc, superior semicircular canal; um, utricular macula. Scale bars: (A, B) 250 μm; (E) 500 μm.
Fig. 2. Ectopic sensory patches in the cochlear duct following RCAS/β-catenin injections at s10. (A) Control E12 cochlear duct with the tegmentum vasculosum removed to show immunostaining of sensory organs with anti-HCA. (B) E12 specimen processed as in (A) showing disruptions in the basilar papilla and ectopic hair cells along the superior wall of the duct (arrowheads). (C) Experimental E12 cochlear duct showing large regions of ectopic hair cells (arrowheads). (D) E17 cochlear duct showing ectopic hair cells (arrowhead) and two types of abnormal hair cell patches on the basilar papilla: flat (yellow arrow) and invaginated (white arrow). Bars in (A-D), 300 μm. (E) Vestibular-like hair cells in the basilar papilla. A nonauditory patch of hair cells in the proximal basilar papilla at E18 following injection of β-catenin virus at s10. Green depicts β-tubulin immunostaining and red is rhodamine phalloidin-labeling of f-actin. The hair cell bundles in the large patch have the elongated shape of vestibular hair cells including possession of a kinocilium (white arrow region shown as inset). Normal auditory hair cells (yellow arrow) surround the abnormal patch. A patch containing only two vestibular-like hair cells is evident (arrowhead). Bar in (E), 50 μm. Abbreviations: bp, basilar papilla; lm, lagena macula; sm, saccular macula.
Fig. 3. Ectopic vestibular hair cells and otoliths within the auditory organ following infection with RCAS/β-catenin. (A) Normal utricular macula at E17; hair bundles each have a single kinocilium (arrow). Hair cells are separated by broad apical projections of supporting cells. (B) Normal basilar papilla at E17. Hair cells are separated by narrow projections of supporting cells (arrow). (C) Otolith crystals of the lagenar macula at E17. (D) Cochlea of an E16 experimental embryo injected with virus at s17. The tectorial membrane (tm) is intact over the distal half. An abnormal region midway along the basilar papilla is shown at higher power in (E) and (F). (E) Ectopic otolithic crystals embedded in an extracellular matrix typical of otolithic membranes. (F) Stereocilia bundles with vestibular characteristics. (G) A different E17 specimen injected with virus at s18. Vestibular-like hair cells are found in an invagination contiguous with regions of auditory hair cells. Some of the bundles are small and immature (arrow). All bars are 10 μm, unless indicated.
showed abnormalities in the spatial distribution of the sensory patches within the vestibule of experimental ears. Serial sections through experimental ears (n = 5) and one age-matched control (Fig. 1C) were reconstructed in their entirety to aid in identifying the sensory organs. In addition, whole mounts of the vestibules were processed by using either alkaline phosphatase (n = 2) or fluorescent (n = 3) immunodetection methods. Hair cell staining revealed that sensory organs in the anterior part of the vestibule appeared to be fused in E9–E17 experimental ears, most commonly the lateral crista with the utricular macula. These two organs could not be distinguished as separate in 7/10 experimental ears, and were closely apposed but distinct in the remaining 3 cases. In 1 specimen, the superior crista seemed to contact and perhaps merge with a large lateral sensory patch (Fig. 3). In 2 specimens, the superior crista seemed to contact and perhaps fuse with the utricular macula, and in the remaining case, the superior crista seemed to contact and perhaps merge with a large lateral sensory patch (Fig. 3). In 1 specimen, the superior crista seemed to contact and perhaps merge with the utricular macula. These two organs could not be distinguished as separate in 7/10 experimental ears, and were closely apposed but distinct in the remaining 3 cases. In 1 specimen, the superior crista seemed to contact and perhaps merge with a large lateral sensory patch (Fig. 3). In 2 specimens, the superior crista seemed to contact and perhaps fuse with the utricular macula, and in the remaining case, the superior crista seemed to contact and perhaps merge with a large lateral sensory patch (Fig. 3).

Activated β-catenin generated ectopic vestibular hair cells in the cochlear duct

Whole-mount immunostaining of cochlear ducts with anti-HCA showed unusual hair cell patches within and adjacent to the basilar papilla. Compared with normal ears (Fig. 2A), infected ears had patches of ectopic hair cells located beyond the superior edge of the basilar papilla (Fig. 2B–D, arrowheads). Ectopic hair cells were observed at this location in 17/21 basilar papillae processed as whole mounts at E12–E17. No ectopic hair cells were detected in 3/3 control ears.

Additional abnormalities in hair cell distribution were found within the basilar papilla itself. Patches of unusual hair cells could be separated into two distinct phenotypes. Some patches were flat foci encompassing dozens of hair cells (Fig. 2D, yellow arrow; Fig. 2E, white arrow), while others were deep invaginations of the basilar papilla, lined with hair cells (Fig. 2D, white arrow; see also Fig. 1D). The abnormal patches of hair cells were examined at higher power to study their morphology. Confocal microscopy revealed that hair cells in these patches bore apical bundles with long tapering stereocilia and a single long kinocilium (Fig. 2E, white arrow and inset). These features are characteristic of vestibular hair cells and differ markedly from surrounding auditory hair cells that have broader stereociliary bundles lacking kinocilia as development progresses (Fig. 2E, yellow arrow). Patches of vestibular-like hair cells were found at various locations along the proximal–distal axis of the basilar papilla, and ranged in size from 1–2 hair cells (Fig. 2E, arrowhead) to over 100.

The vestibular character of the hair cells was explored further by using scanning electron microscopy (SEM). Differences in bundle morphology are shown both for vestibular (Fig. 3A) and auditory (Fig. 3B) hair cells at late embryonic stages in normals. Fig. 3D shows a lower power micrograph of a cochlear duct that displayed a cluster of hair bundles with vestibular morphology (Fig. 3F), evidenced by long stereocilia and a single kinocilium. A further similarity with vestibular sensory organs was that hair cells were separated by relatively large apical processes of supporting cells, identified by the short microvilli on their surfaces (compare Fig. 3F with Fig. 3A). Auditory hair cells, by comparison, were separated by narrow apical processes of supporting cells richly endowed with microvilli (Fig. 3B). In another specimen, an invagination of the basilar papilla was found to contain a low density of hair bundles, some of which had an obvious, elongated kinocilium and others of which appeared to be immature bundles (Fig. 3G, arrow).

The identity of the ectopic hair cells on the superior wall of the cochlear duct was also of interest. Whole-mount hair cell stained and SEM indicated that the ectopic cochlear hair cells were predominantly of the vestibular phenotype. However, we have observed two cases in which the ectopic hair cells located above the superior edge of the basilar papilla had an auditory phenotype (not shown).
ectopic otoliths, or gm-2 immunolabeling (i.e., Fig. 4B, arrow).

Once ectopic otoliths were detected, we performed additional experiments to score for the penetrance of this phenotype. Embryos injected at s8–s12 had ectopic cochlear otoliths in 62% of the cases (n = 56). Additional embryos received viral inoculum into the otocyst at s15–s21, which confined initial infection to the inner ear and

Fig. 4. Vestibular supporting cells within the auditory organ following RCAS/*β-catenin injection. (A) An E17 experimental cochlea sectioned through an abnormal patch in the basilar papilla. The region between the arrowheads is immunopositive for gm-2 (green). Double-labeling for HCA (red) reveals the borders of the basilar papilla. (B) An adjacent section immunolabeled for the hemagglutinin epitope (green), demonstrates that the region with strong gm-2-immunoreactivity expresses the activated β-catenin transgene (between arrowheads). Not all regions expressing the transgene bear hair cells or express high levels of gm-2 (arrow). Some virus immunoreactivity was also seen in the tectorial membrane (tm), perhaps due to shedding of viral particles into this acellular structure. The tectorial membrane is labeled with gm-2 even in control ears, so staining of this structure occurs independently of gene transfer (see also Fig. 6I). Scale bar, 50 μm.

Fig. 5. Molecular identity of abnormal sensory patches in RCAS/*β-catenin-infected ears. (A–C) Sections of an E7 embryo following virus injection into the right otic vesicle at s17. (A) Left (uninjected) ear. Msx1 is expressed in the lateral crista (arrowhead) and not in the cochlear duct. (B, C) Right (injected) ear from same animal as (A). (B) Distortions of the cochlear duct express Msx1 (arrowheads). Ectopic patches of Msx1 are present on the lateral wall of the duct distally (asterisk) near the normal expression in the lagena macula (arrow). In the vestibule, the Msx1 domain is expanded medially (bracket). (C) In an adjacent section, p75ngfr is not present in the cochlea. In the vestibule, p75ngfr expression (bracket) partially overlaps the Msx1 domain seen in (B). (D–G) Sections of an E6 experimental ear injected at E2. (D) The cochlear duct has broad patches of ectopic Msx1 expression (arrowheads). (E) p75ngfr is not expressed in the cochlear duct (arrowheads). p75ngfr expression in the vestibule appears to be expanded (arrow). Msx1 expression was also seen in this same region in nearby sections (not shown). (E, F) Staining for neurofilament proteins (NF-70) and HA.11 indicate that the expanded region expressing p75ngfr in (E) is contacted by nerve fibers (F, arrow), and that much of the ear, including the endolymphatic duct and canals, is well infected with the β-catenin virus (G). Abbreviations: cc, common crus; cd, cochlear duct; ed, endolymphatic duct; es, endolymphatic sac; la, lateral ampulla; s, saccule; u, utricle.
adjacent mesenchyme, significantly reducing neural tube infection as well as the frequency and severity of head defects. Ectopic otoliths were evident in 79% of 19 ears injected at s15–s17 and 100% of 4 ears injected at s20–s21. These data suggest that the abnormal ear phenotypes were unlikely to be due to indirect effects arising from forced activation of β-catenin in the hindbrain. Injections of the otic vesicle in 21 embryos at s23–s25 did not yield ectopic otoliths in the cochlear duct. Thus, we can define a critical period after which virus injection does not generate an apparent conversion from auditory to vestibular identity.

Activated β-catenin altered sensory organ identity

Otolith crystals are a characteristic feature of the macular sensory organs. The areas of the basilar papilla capped by otoliths are therefore presumed to have acquired macular identity. Three sensory organs in the inner ear bear otoliths: the lagener, saccular, and utricular maculae. The fourth macula, the macula neglecta, is very small and does not have otoliths. We sought to confirm and narrow the macular identity of the ectopic vestibular patches by using a pair of molecular markers, Mxs1 and p75ngfr1, that distinguish among several vestibular sensory organs in the chicken embryo (Wu and Oh, 1996). The lagener macula and the macula neglecta both express the transcription factor, Mxs1, but not the low affinity receptor for nerve growth factor, p75ngfr. The other two maculae (utricular and saccular) express neither marker. Mxs1 is also expressed during the initial formation of the basilar papilla as well as in a non-sensory posterior arm of the cochlear duct, but becomes restricted to the lagener macula by E6. Finally, because not all ectopic vestibular patches could be associated with otoliths, we sought to distinguish between macula and crista fate using p75ngfr. Cristae have a central domain of Mxs1 that is flanked on either side by p75ngfr (Fig. 5A).

In situ hybridization of experimental ears injected on E2–E3 showed ectopic Mxs1 expression in the basilar papilla at E6–E7. Expression was seen in noninvaginated sections that varied from small, scattered foci (not shown, n = 4) to broad regions (Fig. 5D, n = 7). Abnormal invaginations of the basilar papilla also expressed Mxs1 (Fig. 5B). Hybridization of adjacent sections showed no p75ngfr expression correlating with this Mxs1 expression (Fig. 5C and E, arrowheads), arguing against a crista identity and suggesting the presence of lagener macula or macula neglecta fates. Staining adjacent sections for the hemagglutinin epitope of transduced β-catenin revealed widespread infection in these cochleas, showing that while ectopic Mxs1 patches were invariably infected, there were many other infected areas that did not express Mxs1 (Fig. 5G). Examination of age-matched uninfected embryos (not shown) or the contralateral ear of otic vesicle-injected embryos (Fig. 5A) showed that Mxs1 was no longer expressed in the basilar papilla by E7. This indicates that Mxs1 foci in the basilar papilla of experimental ears are likely to be genu-

Fig. 6. Auditory-to-vestibular conversion does not require infection of mesenchyme. This embryo received RCAS parent virus injection into the tissue surrounding the right ear at s12, followed by injection of RCAS/*β-catenin virus into the right otocyst at s17. Cross-sections through the cochlear ducts were analyzed on E11. (A, B) Nomarski image. (C, D) HCS-1 immunostaining. (E, F) 3C2 immunostaining. (G, H) HA.11 immunostaining. (I, J) gm-2 immunostaining. Left ear: (A, C, E) are the same section, and (G, I) are from an adjacent section. Right ear: (B, D, F) are the same section, and (H, J) are from a section 30 microns away. While both ears had virus spread throughout the mesenchyme and ectoderm, activated β-catenin was primarily restricted to the right otic ectoderm. An infected patch on the right basilar papilla was invaginated and expressed ectopic gm-2 and contained hair cells (arrows). An ectopic hair cell can be seen in the homogene cell region (arrowhead, D). Abbreviations: bp, basilar papilla; hm, homogene cell region; hy, hyaline/cuboidal cell region; tm, tectorial membrane; tv, tegmentum vasculosum.
inarily ectopic. However, we cannot definitively rule out that Msx1 expression seen in infected ears is actually a prolongation of its normal expression in the cochlear duct.

The patterns of Msx1 and p75ngfr expression in the vestibules of injected embryos were complex, and deformities of the epithelium made it difficult to identify individual organs with certainty. Examples are shown of enlarged sensory domains, positive for Msx1 and/or p75ngfr, extending from the lateral ampulla to the utricle in experimental ears (Fig. 5B–E).

*Sensory phenotypes induced by activated β-catenin do not require infection of mesenchyme*

Retrovirus injections into the otic region inevitably generated infection of both mesenchyme and otic ectoderm, even when injections were initially confined to the otocyst after E2.5 (beyond s16). This raised the question of whether the effects seen were autonomous to the ectoderm or transmitted indirectly through the infected otic mesenchyme. To address this question, we designed experiments to confine activated β-catenin primarily or exclusively to the otic epithelium on one side of the embryo. Embryos were inoculated with two different viruses on successive days. First, RCAS parent virus was directed into the periotic mesenchyme and adjacent hindbrain at E2 (s11–s13). The next day, the right otic vesicle was filled with RCAS/β-catenin inoculum at s17–s18. Our rationale was two-fold: empirically we knew that virus spread across the basal lamina from mesenchyme to ectoderm is relatively inefficient, and, once infected, cells cannot subsequently be superinfected with a second virus of the same envelope subgroup. The idea was to give the parent virus a "head start" at infecting and spreading within the mesenchyme, while at least some of the otic ectoderm would remain uninfected and thus would be susceptible to β-catenin virus when it was delivered directly into the otocyst the following day. Six embryos receiving this injection protocol were processed on E9 (n = 2) or E11 (n = 4). None displayed the head defects seen when β-catenin virus alone was delivered on E2, suggesting that broad misexpression of β-catenin was indeed reduced. Nonetheless, all six embryos showed evidence of phenotypic conversion of β-catenin-infected patches in the right basilar papilla, with the left side serving as a parent-virus-infected control (Fig. 6). Evidence of auditory-to-vestibular conversion correlated with expression of activated β-catenin and included deep invaginations in the basilar papilla (Fig. 6B) filled with hair cells (Fig. 6D), ectopic otoliths (Fig. 6B), focal absence of the tectorial membrane (not shown), and ectopic expression of gm-2 (Fig. 6J). Staining adjacent sections for the viral gag epitope confirmed that both ectoderm and mesoderm were well-infected with parent virus on both sides (Fig. 6E and F), while activated β-catenin protein was indeed confined almost exclusively to patches of cells in the right otic epithelium (compare Fig. 6G and H). Mesenchymal cells, when infected with the β-catenin virus, were sparse and confined to the periotic region on the right side. Converted supporting cells and/or ectopic hair cells were restricted to the basilar papilla itself and to the anterior (superior) wall of the duct which normally gives rise to homogene cells (Fig. 6D, arrowhead).

Within the vestibule, abnormal undulations of sensory patches were also observed and were always correlated with β-catenin infection (not shown). Canal morphology was normal in all six specimens, although β-catenin infection was sparse in these structures. As a result, we were unable to draw any conclusions about the relative requirement of β-catenin misexpression in periotic mesenchyme versus otic epithelium in the generation of canal dysmorphogenesis.

*Misexpression of Wnt3a altered inner ear morphogenesis and generated ectopic vestibular patches*

Misexpression of Wnt ligands should allow binding and activation of the Wnt signaling pathway wherever the appropriate Frizzled receptors are present and Wnt inhibitors are absent or ineffective. The effect of Wnt misexpression was tested by exposing the ear ectoderm to RCAS/Wnt3a at several stages. Injections at s10 resulted in a high level of mortality; most embryos died prior to E6 (11/64 survived). Histology confirmed massive infection of the head epithelium and mesenchyme, with severe defects in the brain and neural tube, including an enlarged telencephalon (data not shown). The neural tube defects discouraged inner ear analysis, because it would be difficult to distinguish direct effects of Wnt3a on the inner ear from indirect effects mediated through the brain.

Survival rates were improved by delivering virus directly into the otic vesicles at s17–s22, with 98/125 embryos surviving to E6. Nonetheless, only 15/125 embryos survived to be processed on E11 for SEM (n = 2) or sections (n = 6), or on E12–E15 as whole mounts (n = 7). Infection with RCAS/Wnt3a phenocopied several features of the activated β-catenin virus, with normal ears (Fig. 7A) or uninfected left ears serving as controls. Abnormalities due to Wnt3a misexpression included presence of ectopic otoliths along the cochlear duct in 7/7 (data not shown), ectopic vestibular hair bundles along the anterior (superior) wall of the duct in 7/7 (Fig. 7B and C), and gm-2-positive sensory patches midway through the cochlear duct (Fig. 7D) corresponding to infected regions (Fig. 7E). There were other features of the Wnt3a-induced phenotype that differed from that induced by activated β-catenin. Instead of finger-like invaginations of the basilar papilla, in all seven cases, the surface of the organ bulged into the cochlear duct (Fig. 7B). One basilar papilla had a more severe phenotype, with an obvious gap in the middle of the basilar papilla that was populated by scattered hair cells (data not shown). Finally, the lagenar macula appeared undulating (Fig. 7B) compared with either normals or following infection with activated β-catenin. In summary, misexpressing Wnt3a caused hair cell patterning defects throughout the basilar papilla and
Expression of Wnt genes in the otic vesicle

Because forced expression of both Wnt3a and activated β-catenin yielded defects in the sensory organs, we examined the normal expression of Wnts in the ear by in situ hybridization of sections, focusing on the early stages of sensory organ specification. We confirmed that Wnt3a expression was confined to the dorsal-most part of the otocyst beginning at our earliest time point on E2.5 (s15). Expression persisted in the endolympathic duct out to our latest time point on E6 (data not shown). In contrast, Wnt4 came on later and was expressed only in the ventral part of the otocyst. Its expression in the central nervous system served as an internal positive control (data not shown) for sections hybridized at E3 (s18–s19), E4, E5, E5.5, and E6. Wnt4 was not detected in the ear until E5, at which time it was expressed in only two foci in the entire ear: at the ventral tip of the cochlear duct and at the dorsal lip of the duct on the anterior–lateral wall where it meets the utricle. There was also a faint hint of Wnt4 expression between these two foci, along the lateral (nonsensory) wall of the cochlear duct (Fig. 8A). By E6, the lateral wall expression was stronger, although highest levels were still located at the two extremes of this domain, both dorsally and ventrally (Fig. 8C).

On E5 and E6, alternate sections were probed with Mx-1 and Ser1 to ask what the relationship might be between the Wnt4 domain and sensory primordia. In fact, the Wnt4 expression domain abuts but does not overlap with sensory patches at both extremes of its domain. At the ventral edge, Wnt4 forms a boundary with the lagena macula primordium, defined by its expression of Ser1 and Mx-1 (arrowhead, Fig. 8B–D). At its dorsal edge, Wnt4 meets the Ser1-positive primordium of the utricular macula (arrow, Fig. 8C and D). A similar result was seen on E5 (data not shown). A summary figure showing the boundaries formed between strong domains of Wnt4 expression and adjacent tissues on E5–E6 is shown in Fig. 8E.

One E6 ear was sectioned perpendicular to the long axis of the cochlear duct to map the edges of the domain through the middle part of the duct where Wnt4 expression is generally weak. The Wnt4 domain appears to end directly next to the bulge of the forming basilar papilla on its posterior edge, while leaving a broad gap on the anterior edge (data not shown). The Wnt4-positive cells immediately adjacent to the posterior (future inferior) edge of the basilar papilla will become the hyaline/cuboidal cells, while the Wnt4-negative cells adjacent to the anterior (future superior) edge of the basilar papilla will become the homogene cells. These preliminary findings suggest that the primordia of the hyaline cells and the tegmentum vasculosum both lie within the weak Wnt4 expression domain, while the primordia of the basilar papilla and the homogene cells are Wnt4-negative (shown schematically in Fig. 8E), although confirmation will require additional markers. Interestingly, it is along the superior edge of the duct, where Wnt4 is apparently absent, that misexpression of either Wnt3a or activated β-catenin generates ectopic sensory patches and otolith crystals.

Expression of Frizzled genes in the inner ear

We have shown that ectopic activation of WNT/β-catenin signaling alters the patterning of the sensory organs, but does this correspond to a physiological function of Wnt signaling in normal ear development? If so, we should expect the normal otocyst to express Wnt receptors, that is, Frizzled proteins, at sites of Wnt action. We used in situ hybridization to investigate the normal patterns of Frizzled expression in inner ear development, combining this with immunostaining for Serrate-1 as a marker of the future sensory patches (Adam et al., 1998). Out of the seven chicken Frizzleds examined (c-Fz1, c-Fz4, c-Fz5, c-Fz6, c-Fz7, c-Fz9, and c-Fz10), five (c-Fz1, c-Fz5, c-Fz6, c-Fz7, and c-Fz10) were expressed in the ear. At s23, c-Fz1 and c-Fz7 expression was detected both within and in regions adjacent to the Serrate-1-positive patches corresponding to the locations of sensory primordia of the sacculus/basilar papilla (which are not yet separate) and the utricle (Fig. 9A–D). c-Fz6 expression was seen in a dorsal nonsensory part of the otocyst (not shown). Expression of c-Fz10 was seen in the basilar papilla by E7 (Fig. 9E) but not in any of the cristae or maculae; no expression of c-Fz10 was seen at E4, E5, or E6. c-Fz5 expression was not detected until E8, when it was restricted to hair cells of the sacculus and utricular maculae (see Lewis and Davies, 2002; Fig. 1).

Discussion

Our main finding is that activation of the Wnt signaling pathway, during an early critical phase of otic development, can convert sensory epithelium from an auditory to a vestibular character. Before discussing this observation in detail, however, we must comment on some other aspects of our results.

Wnt/β-catenin signaling and inner ear morphogenesis

Wnt2b and Wnt3a are both expressed in the dorsal otic vesicle in chickens (Hollyday et al., 1995; Jasoni et al., 1999), and several Frizzled receptors for Wnts are available for receiving a Wnt signal at these early stages (Stark et al., 2000). Although this pattern suggests that Wnts may specify dorsal otic identity, the gain-of-function data of this study are not consistent with a dorsal conversion of the ventral ear. Even in severely deformed ears, the dorsal–ventral polarity of the ear appeared intact, with the cochlea forming ventrally and the canals located dorsally. Dorsal–ventral axis specification of the gross divisions of the ear (but not
the sensory patches) is already fixed by s16 (Wu et al., 1998). Therefore, an experimental design intended to perturb dorsal–ventral specification might require high levels of protein expression prior to s16. In the present study, virus delivery at s9–s11 should accomplish this. Transduced protein is detectable within 9–12 h and is widespread by 18–20 h following infection (Homburger and Fekete, 1996), which corresponds to s15–s16. However, earlier injections, or a more rapid method of gene transfer (such as electroporation) might be needed to fully assess the role of Wnt signaling in dorsal–ventral axis specification. Finally, our β-catenin experiments cannot rule out the possibility that Wnt2b and/or Wnt3a specify dorsal identity by signaling through a noncanonical Wnt pathway.

The early, dorsal expression of Wnt3a was associated with the endolymphatic duct from its inception. This suggests that Wnt3a could play a direct role in endolymphatic duct specification. However, we saw no evidence that ectopic ducts were generated by infection with the activated β-catenin or Wnt3a viruses.

Both types of virus did, however, produce striking disturbances in the ventral, sensory regions of the otocyst.

Fig. 7. Defects in the basilar papilla after injection with RCAS/Wnt3a. (A–C) Basilar papillae stained as whole mounts with anti-HCA. (A) Uninfected specimen at E12. (B) Experimental E15 specimen injected with virus at s20. The surface of the basilar papilla is undulated and the lagenar macula (lm) is distorted compared to normal. (C) Magnified view of the region indicated by a box in (B). Ectopic hair cells along the superior wall have two distinct bundle morphologies: punctate and broad crescents (see enlarged inset). The latter appear to be auditory while the former could be either immature bundles or vestibular-like bundles. (D, E) Cross-sections through the central part of an E11 cochlear duct injected with virus at s19. (D) A large part of the basilar papilla and superior wall (bracketed by arrowheads) stains for gM-2 and has hair cells. This area is directly adjacent to the auditory region, which is identifiable by absence of gM-2-immunoreactivity, presence of hair cells, and presence of overlying tectorial membrane. (E) Adjacent section stained with anti-gag (3C2) shows that the gM-2-positive region in (D) is infected with retrovirus (between white arrowheads) unlike the adjacent auditory region. Bars: (A, B) 300 μm; (C) 100 μm; (D) 50 μm. Abbreviations: bp, basilar papilla; lm, lagenar macula; tm, tectorial membrane.
These effects imply a sensitivity of the cells in these regions to activation of the canonical Wnt pathway, but since Wnt3a is not normally expressed ventrally, it seems likely that activation of the pathway in these regions is normally governed by some member of the Wnt family other than Wnt3a, with Wnt3a behaving as a surrogate for this other Wnt in our experiments. As we now discuss, Wnt4 is a strong candidate for this ventral role.

**Wnt4 signaling in the developing cochlear duct**

We detected Wnt4 expression in the otocyst beginning on E5, just prior to the onset of sensory organ differentiation. Expression was limited to the cochlear duct. Several Frizzleds are also expressed in sensory primordia of the duct, including c-Fz1 and cFz-7 by E4 and c-Fz10 beginning on E7, and at least some of these are likely to be responsive to Wnt4. For example, exogenous Wnt4 can promote chondrogenesis in the developing chick limb, an effect that may be mediated through Fz1 or Fz7 (Hartmann and Tabin, 2000). Both Fz1 and Fz7 belong to a class of Frizzled proteins that can act through the β-catenin pathway (Sheldahl et al., 1999).

The Wnt4 expression domain in the cochlear duct directly abuts three different sensory primordia: the lagenar macula, the basilar papilla, and the utricular macula. The approximate location of these primordia is already marked out by expression of Serrate-1 before the onset of detectable Wnt4 expression. Thus, Wnt4 is a candidate for a role in adjusting, maintaining or sharpening sensory/nonsensory boundaries between Serrate1-positive and Serrate1-negative territories, as well as a role in governing the character of the cells within the sensory patches by diffusing across them from the adjacent Wnt4 expression domains.

The expression patterns in conjunction with the results of the viral misexpression studies hint more specifically at
certain possibilities. Thus, Wnt signaling in the ventral cochlea, perhaps mediated by Wnt4, might directly specify lagenar macula fate in those sensory cells that confront the highest source of Wnt ligand. The idea is that Wnt4 could signal across the sensory/nonsensory boundary to the adjacent sensory progenitors. One downstream target gene thus activated could be the lagenar macula marker, Msx1. In fact, ectopic Msx1 expression was observed in basilar papilla cells transduced with activated β-catenin.

At the dorsal extreme of the Wnt4 expression domain, a different scenario must be envisioned, because unlike the lagenar macula, the utricular macula, developing next to this dorsal Wnt4 domain, does not normally express Msx1. However, ectopic activation of β-catenin within the vestibule appeared to enlarge the Msx1 domain normally associated with the lateral crista. This expanded domain may be responsible, in part, for the fusion of organs seen in the utricle following retroviral misexpression.

Along the length of the cochlear duct, the primordium of the basilar papilla abuts a lower level of Wnt4 than either the lagenar or utricular macula, raising the possibility that relative Wnt4 levels could be differentially instructive to the sensory primordia in the ventral ear, particularly given that they differ in Frizzled expression. Moreover, in addition to the early effects that we have noted, it is possible that Wnt signaling may have an influence on later processes such as the development of planar cell polarity in the auditory epithelium. Wnt4 protein could, for example, diffuse across the developing basilar papilla from the cells that flank it along one edge, controlling hair cell polarity through an action on Fz10, whose relatively late expression in the basilar papilla would be consistent with such a role.

To distinguish among the possible functions of Wnt/β-catenin signals during normal ear development, it would be helpful to be able to identify cells in which the signaling pathway is in fact activated. In other systems, it has been possible to identify Wnt activity because it leads to stabilization of cytosolic β-catenin followed by its translocation to the nucleus, where the protein can be detected by immunostaining (Noramly et al., 1999; Schneider et al., 1996). Our attempts to detect nuclear localization of β-catenin in normal ears on E2, E4, E5, and E8 have been inconclusive: heavy immunostaining at cell membranes throughout the ear was observed in immersion-fixed specimens. However, the incipient lagenar macula showed particularly intense cytoplasmic labeling of β-catenin on E5 in perfusion-fixed specimens (S.B. and D.M.F., unpublished observations). Perhaps this reflects the stabilization of a cytosolic pool of β-catenin in response to Wnt signaling. It may be that nuclear localization of β-catenin is transient or exquisitely sensitive to fixation conditions, or that the appropriate stages have not yet been examined.

**Wnts and cell fate specification in the inner ear**

Our most striking observation, supported by a battery of different markers, is that activation of the Wnt/β-catenin pathway within the basilar papilla can convert the auditory epithelium to a vestibular character. How does this effect fit into the overall scheme of cell determination in the inner ear? It has been proposed that cell fate specification in the vertebrate inner ear may require sequential and mostly binary fate choices (Fekete and Wu, 2002). For a typical vestibular sensory patch, the first choice, corresponding to determination of the otic placode, would be between otic and epidermal character. Next, in a sequence that is still uncertain, would come the nonneuronal vs. neuronal, sensory vs. nonsensory, and sensory organ type (macula, crista, or auditory) decisions. Finally, there would be the choice of sensory cell type (hair cell vs. supporting cell) and cell subtype (e.g., type I vs. type II hair cells). Which of these putative decision points might be influenced by Wnt-mediated signaling? The conversion of an auditory patch to a vestibular patch supports a rather direct role for Wnts in the specification of sensory organ type. However, it is not clear if this corresponds to a choice made directly from among all possible sensory organ subtypes, or to just one of a series of binary choices leading to the ultimate specification as basilar papilla, lagenar macula, utricular macula, saccular macula, or crista. For example, Wnt signaling might simply govern the choice between auditory and vestibular character, while other signals might dictate the differences between the various vestibular patches. Our data show that at least some of the aberrant patches of vestibular cells have acquired a fate that could be interpreted as lagenar macula (otoliths and Msx1 expression), but it is difficult to say whether all aberrant patches have this identity. Likewise, the merging of sensory patches in the vestibule has made it difficult to determine whether expansion of the Msx1 domain between the lateral crista and the utricular macula indicates the ectopic presence of another sensory organ.

In addition to conversions from auditory to vestibular character, we saw production of hair cells in infected regions of the cochlear duct that are normally nonsensory. This indicates that Wnt signaling can also play a role in the choice between sensory and nonsensory epithelial development. The data suggest, however, that not all regions of the otocyst are equally capable of generating ectopic sensory patches, since these were seen in only a few locations despite widespread infection with retrovirus in many ears. For example, ectopic hair cells were never observed in the semicircular canal ducts or the endolymphatic apparatus. Within the cochlear duct, nonsensory-to-sensory conversions were confined to cells located next to the anterior (future superior) edge of the sensory epithelium, a region normally devoid of Wnt4 gene expression that normally gives rise to homogene cells. Topologically, this edge most likely derives from the anteroventral part of the otocyst, close to or within the L-fng-positive domain thought to mark a broad sensory-competent zone (Cole et al., 2000). It may be that the homogene cell region initially has sensory competence and then loses this with time as a result of the absence of Wnt signaling. In other words, Wnt signaling...
might here play a permissive rather than an instructive role in the sensory versus nonsensory cell fate decision. Based on this hypothesis, we presume that the relatively restricted locales of ectopic or merged sensory patches correspond to regions that are initially specified as sensory-competent but then normally become blocked in the execution of this fate, the block being lifted where the Wnt pathway is artificially activated.

**Cell autonomy and phenotypic conversion**

The effects of ectopic β-catenin appear to be direct and cell-autonomous within the basilar papilla and homogene cell regions. That is, converted (gm-2-positive, Msx1-expressing) cells were only found in patches with ectopic activated β-catenin. However, not all β-catenin-expressing patches were converted. This holds true even within the basilar papilla proper, where one might expect the cells to be all equally competent to respond. Why would an apparently uniform patch of cells respond differently to activation of the Wnt pathway? A simple explanation may be that the viral infection takes time to spread, and that conversion requires exposure to β-catenin within the critical period—that is, before s23; thus, nonconverted cells may have simply become infected too late. Within the basilar papilla, we did not notice any systematic distribution of infected, non-responding cells that might offer an alternative biological explanation for resistance to Wnt/β-catenin signaling (such as differential presence of a transcriptional inhibitor or absence of a transcriptional coactivator).

With regard to the issue of cell autonomy, we caution that the punctate staining pattern of gm-2 as a supporting cell marker, and the absence of a specific marker of vestibular hair cells, impedes definitive confirmation of cell autonomy at the single cell level. But at the tissue level, we can say with some confidence that the epithelium is behaving autonomously in its response to β-catenin: the phenotypic conversion of the epithelium is not triggered by an influence from the mesenchyme, as conversion occurs even in the absence of mesenchymal infection.

**Comparisons with other systems**

Wnt/β-catenin signaling is known to control cell fate and stem-cell commitment in many other vertebrate tissues, including central nervous system, epidermis, gut endoderm, haemopoietic tissue, and adipogenic connective tissue (reviewed in Huelsken and Birchmeier, 2001). For example, in the epidermis of the chick embryo, misexpression of activated β-catenin causes formation of ectopic feather bud placodes in trunk regions that normally do not form feathers (Noramly et al., 1999). This may be analogous to the production of ectopic hair cell patches in the nonsensory territory of the cochlear duct in our experiments. Moreover, in *Drosophila*, Wnt signaling has been shown, among its many other functions, to play a part in defining the sites of formation of sensory bristles—sense organs that may be homologous to the sensory patches in the vertebrate ear. Thus, in the fly, overexpression of β-catenin (Armadillo) within the area of the wing pouch causes the formation of ectopic wing margin bristles (Greaves et al., 1999).

In summary, examples are accumulating in which manipulation of the Wnt/β-catenin pathway causes a conversion of cell fate. The present data add a new system to this list, with specification of sensory organ phenotype and of auditory versus vestibular identity in the vertebrate inner ear being subject to control by activated β-catenin. Our findings imply that Wnt and Frizzled proteins, as regulators of β-catenin activity, are not only expressed in the ear, but also have a critical function there. The Wnt/Frizzled signaling pathway, however, governs other intracellular events in addition to those dependent on β-catenin. It remains to be seen whether these other branches of the pathway may have additional functions in ear development. The present work represents a first step toward defining and disentangling the specific contributions that Wnt signaling makes to the patterning of the ear.

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