Development 130, 2375-2384
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doi:10.1242/dev.00448

Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea

Alain Dabdoub[1,*], Maura J. Donohue[1], Angela Brennan[2], Vladimir Wolf[3], Mireille Montcouquiol[1], David A. Sassoon[4], Jen-Chih Hseih[5], Jeffrey S. Rubin[3], Patricia C. Salinas[2] and Matthew W. Kelley[1]

[1]Section on Developmental Neuroscience, NIDCD, National Institutes of Health, Rockville, MD 20850, USA
[2]Department of Biological Sciences, Imperial College of Science, Technology and Medicine, Exhibition Road, London SW7 2AZ, UK
[3]Laboratory of Cellular and Molecular Biology, Division of Basic Sciences, NCI, National Institutes of Health, Bethesda, MD 20892, USA
[4]Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029, USA
[5]Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794, USA
*Author for correspondence (e-mail: dabdouba@nidcd.nih.gov)

Accepted 11 February 2003

SUMMARY

In the mammalian cochlea, stereociliary bundles located on mechanosensory hair cells within the sensory epithelium are unidirectionally oriented. Development of this planar polarity is necessary for normal hearing as stereociliary bundles are only sensitive to vibrations in a single plane; however, the mechanisms governing their orientation are unknown. We report that Wnt signaling regulates the development of unidirectional stereociliary bundle orientation. In vitro application of Wnt7a protein or inhibitors of Wnt signaling, secreted Frizzled-related protein 1 or Wnt inhibitory factor 1, disrupts bundle orientation. Moreover, *Wnt7a* is expressed in a pattern consistent with a role in the polarization of the developing stereociliary bundles. We propose that Wnt signaling across the region of developing outer hair cells gives rise to planar polarity in the mammalian cochlea.

Key words: Planar polarity, Organ of Corti, Sensory epithelium, Mechanosensory hair cells, Mouse

INTRODUCTION

A fundamental aspect of embryonic development in both vertebrates and invertebrates is the generation of planar polarity. Planar polarity refers to the organization of cellular structures within the plane of an epithelium such that the structures are all oriented in the same direction. The development of planar polarity has been extensively studied in flies; the details of the causal mechanisms responsible for this polarization in vertebrates remain unknown. Planar polarity is evident in the mechanosensory hair cells within the sensory epithelium of the mammalian cochlea, the organ of Corti. In particular, the stereociliary bundles present on hair cells located throughout the cochlea are all oriented towards the outer border of the cochlear duct (Fig. 1). The importance of appropriate bundle orientation has been demonstrated in several studies in which the presence of even a limited number of misoriented stereociliary bundles lead to a marked decrease in hearing sensitivity (Fujita and Orita, 1988; Comis et al., 1989; Fujita, 1990; Furness et al., 1990; Yoshida and Liberman, 1999).

A possible source of instructive cues for the determination of polarization could be a secreted molecule that is produced by cells located on either side of the organ of Corti (Cotanche and Corwin, 1991). The results of studies examining the generation of similarly polarized structures in the wing and eye of *Drosophila* have suggested that the Wingless/Wnt signaling pathway could play a key role in the generation of uniform orientation (reviewed by Shulman et al., 1998; Mlodzik, 2000; Adler and Lee, 2001).

The Wnt family encodes secreted glycoproteins that are ligands for the frizzled (Fzd) family of seven-transmembrane receptors and the low density lipoprotein receptor-related protein family of co-receptors (Wodarz and Nusse, 1998; Tamai et al., 2000; Pinson et al., 2000). At present, the Wnt family includes approximately 15 to 20 genes per species (reviewed by Miller et al., 1999). Wnt proteins can elicit various responses in the same, and different, tissues via binding to different Fzd receptors and activation of distinct intracellular signaling pathways (reviewed by Wodarz and Nusse, 1998; Bejsovec, 2000).

Wnt proteins can signal through canonical β-catenin-dependent pathways and through β-catenin-independent (non-canonical) pathways including the Wnt/Ca^{2+} pathway and the Jun-N-terminal kinase (JNK) pathway (reviewed by Huelsken and Birchmeier, 2001). Results have demonstrated a role for the JNK pathway in planar cell polarity (PCP) in some invertebrate systems and convergent extension in vertebrate ones (reviewed by Mlodzik, 2002).

Recent studies have demonstrated that *Drosophila* homologs of two deafness genes, protocadherin 23 (Fat in *Drosophila*) and myosin VIIa (Myosin VIIa/*crinkled* in *Drosophila*), are components of the *Drosophila* planar polarity pathway (Weil et al., 1995; Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001; Wilson et al., 2001; Winter et al., 2001; Wada et al., 2001; Yang et al., 2002). Moreover, stereociliary bundle formation is defective in mice with mutations in either protocadherin 23 (DiPalma et al., 2001) or myosin VIIa (Self et al., 1998), suggesting that the planar polarity pathway may play a similar role in the cochlea. Finally, mice containing a mutation in *Fzd4* are deaf, although there are no obvious defects in the cochlea, and *Fzd10* has been identified as a candidate gene for non-syndromic deafness DFNA41 (Wang et al., 2001; Blanton et al., 2002). Based on these results, and others, Lewis and Davis (Lewis and Davies, 2002) recently suggested that the ear might provide evidence for parallels between flies and vertebrates in terms of planar polarity. We have investigated the potential role of Wnt signaling in the development of stereociliary bundles and present evidence that disruption of Wnt signaling leads to defects in stereociliary bundle orientation.

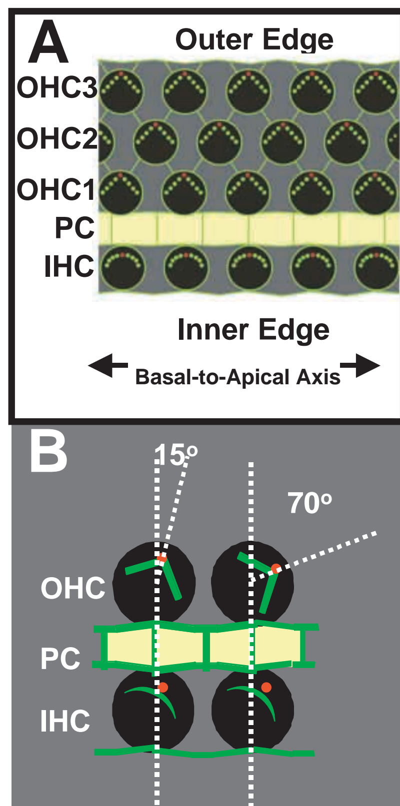


Fig. 1. Cellular structure of the organ of Corti and determination of orientation of stereociliary bundles. (A) The apical surface of the organ of Corti at P0. The organ of Corti extends in a spiral along the basal-to-apical axis (arrow) of the cochlear duct. A single row of IHC (black) is located closest to the modiolus (inner edge). Adjacent to the IHC is a row of pillar cells (PC, yellow). On the opposite side of the pillar cells, nearer to the stria vascularis (outer edge) are three or four rows of OHC1-OHC3 (black). In both the inner and outer hair cell regions, each hair cell is separated from neighboring hair cells by non-sensory supporting cells (gray). On the apical surface of each hair cell is a stereociliary bundle. At P0 this bundle is comprised of a single tubulin-based kinocilium (red) and a group of actin-based stereocilia (green dots). Stereocilia bundles are uniformly oriented towards the outer edge, and are arranged into a curved (IHC) or V-shaped (OHC) pattern with the kinocilium located at the vertex of the bundle. (B) The orientation of stereociliary bundles (illustrated as in A) was determined relative to a line (broken white line) extending perpendicular to the row of pillar cells (PC, yellow). If the vertex of the bundle aligned exactly with the perpendicular line, then the bundle was assigned an orientation of 0°. Deviations towards the apex of the cochlea were assigned a positive value and deviations towards the base were assigned a negative value. The OHC on the left has a relatively small deviation of 15° from the perpendicular line, while the OHC on the right has a larger deviation of 70°.

MATERIALS AND METHODS

Analysis of stereociliary bundle reorientation in the organ of Corti

Cochleae were dissected from E17, P0, and P10 mice and fixed for 2 hours to overnight in cold 4% paraformaldehyde. For E17, pregnant female ICR mice were sacrificed according to NIH guidelines, embryos were removed and cochleae were isolated. For P0 and P10, pups were sacrificed and cochleae were isolated. It has been our experience that delayed implantation can result in variability in the developmental stage of individual mice, even within a single female. Therefore, for the embryonic time point, individual animals were staged according to the developmental series in Kauffman (Kauffman, 1992) rather than by date of plug formation.

After fixation, sensory epithelia were exposed and developing kinocilia were labeled using a monoclonal anti-acetylated-tubulin antibody (Sigma) and an Alexa 568-conjugated anti-mouse secondary antibody (Molecular Probes). The 'Mouse-On-Mouse Kit' (Vector Laboratories) was used to reduce nonspecific labeling. Stereocilia were labeled with either Alexa 488-conjugated phalloidin (Molecular Probes) or with a biotin-conjugated *Griffonia simplicifolia* lectin (Vector Laboratories) (Lanford et al., 1999). Binding of *G. simplicifolia* was detected using the ABC Elite Staining Kit (Vector Labs).

Outer hair cell stereociliary bundles are comprised of a V-shaped group of actin-based stereocilia with a single tubulin-based kinocilium located at the vertex of the crescent (Figs 1 and 2). The orientation of each bundle was determined by extending a straight line from the single kinocilium to create an arrow with the stereociliary bundle as the head (Fig. 1). The orientation of this line was then determined relative to a line perpendicular to the row of pillar cells and passing through the center of the same cell (Fig. 1B). Bundles that were exactly aligned with the perpendicular line and oriented towards the outer edge of the epithelium were assigned an orientation of 0°. Orientations were plotted either as absolute degrees of deviation from an orientation of 0° regardless of direction or as a distribution plot between –180° and +180°, with 0° corresponding exact alignment with the perpendicular line and rotation towards the apex considered as positive.

Orientation data was collected from a minimum of three samples for each time point or experiment. For each position sampled, orientations were determined for a minimum of 12 stereociliary bundles. Significant changes were determined using an unpaired, two-tailed *t*-test.

Analysis of *Wnt7a* mutants

Cochleae from P0 and adult mice homozygous for a deletion of *Wnt7a* with obvious limb patterning defects (Parr and McMahon, 1995) were selected for analysis, isolated and fixed. Homozygous deletion of the *Wnt7a* gene was confirmed by PCR (Hall et al., 2000).

PCR for Wnt genes

Cochleae were dissected from E15, E17 and E18 mice. For E15, Trizol reagent (Invitrogen) was used to extract total RNA from tissue that included both the developing cochlear epithelial cells as well as associated mesenchymal cells and developing neuronal and glial cells from the spiral ganglion. For E17 and E18, the developing sensory epithelia and surrounding epithelial cells were separated from mesenchymal and neural cell types using thermolysin (250 μg/ml, Sigma) (Montcouquiol and Corwin, 2001) prior to RNA extraction. Degenerate primers and PCR reactions were as described by Gavin et al. (Gavin et al., 1990) and were used to amplify Wnt genes from E15 or E17 cochlear RT-DNA. PCR products were separated on agarose gels, cloned using the TA-cloning kit (Invitrogen) and then sequenced. In order to examine more fully the spectrum of Wnt genes that might be expressed in the developing cochlea, specific primer sets for individual Wnt genes were used to amplify genes from E18 cochlear RT-DNA.

Cochlear explant cultures

Cochlear explant cultures from E13 embryos were established as described previously (Kelley et al., 1993) and treated after 18 hours in vitro. Explants were maintained until hair cell stereociliary bundles had developed along the length of the sensory epithelium – a total of 6 days in vitro (DIV, equivalent to P0). At the conclusion of each experiment, explants were fixed in 4% paraformaldehyde for 45 minutes. Stereociliary bundles were labeled as described for in vivo samples.

Modulation of Wnt signaling

Cultures were either exposed to medium conditioned with Wnt7a protein or to medium conditioned by the parent cell line. Conditioned medium was generated as described by Hall et al. (Hall et al., 2000). For co-culture, Wnt7a expressing, or parent, RatB1a fibroblast cells were grown to confluence in MatTek dishes (Shimizu et al., 1997). Twenty-four hours prior to the start of each experiment, the growth media was replaced with serum-free media containing 1.0 mM sodium butyrate to stimulate expression of Wnt7a protein (also added to control media). After 24 hours in serum-free media, RatB1a cells were scraped from a small region in the center of each dish and a cochlear explant was placed in that position. Explants were maintained in serum-free media with sodium butyrate for the duration of the experiment (6 DIV).

Secreted frizzled-related protein 1 (Sfrp1; 50 μg/ml) was dissolved in the culture medium along with 1μg/ml heparin (Uren et al., 2000). The culture medium was replaced with fresh Sfrp1-containing medium after 48 hours. Control medium contained 1 μg/ml heparin.

Wnt inhibitory factor 1 (Wif1) was obtained in conditioned medium. Explant cultures were exposed to either Wif1-conditioned medium or control medium (Hsieh et al., 1998) as described.

Synthesis of heparan sulfate proteoglycans was inhibited by addition of 30 mM sodium chlorate in the medium (Kispert et al., 1996).

In situ hybridization

Wnt7a whole-mount in situ hybridization was performed on cochleae from mice at E14, E16, P0 and P3 as described previously (Lanford et al., 1999). Sections were obtained by embedding wholemounts and sectioning at 12 μm using a cryostat.

RESULTS

Cochlear stereociliary bundles reorient during development

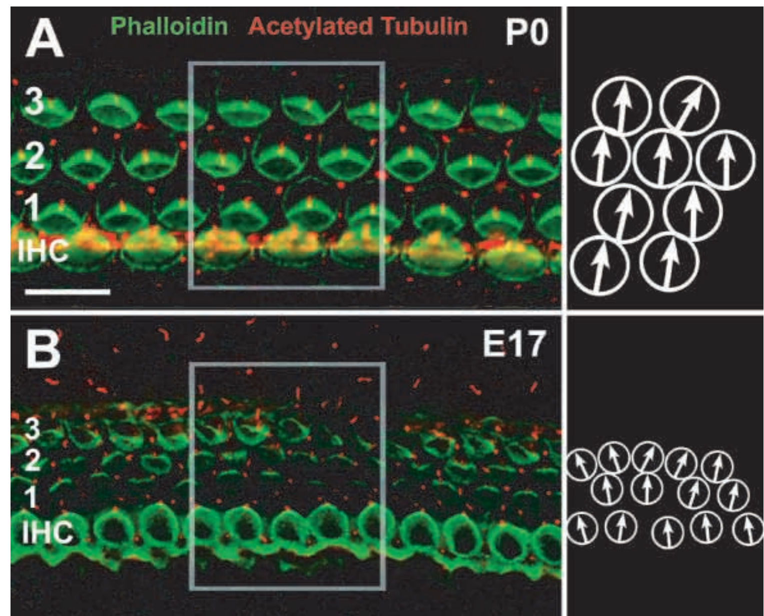
Based on previous descriptions of stereociliary development in the cochlea (Lim and Anniko, 1985), the orientations of developing stereociliary bundles were determined in cochleae from E17, P0 and P10 mice (Fig. 2A,B). Moreover, as the organ of Corti develops in a gradient that extends from base to apex (reviewed by Kelley and Bianchi, 2001), bundle orientations at each time point were determined for cells located in both the basal (more mature) and the apical (less mature) turns of the cochlea. Stereociliary bundles located on inner hair cells (IHC) and first row outer hair cells (OHC) in the apex of the cochlea at E17 were oriented with an average deviation (from a line perpendicular to the pillar cell row) of approximately 15° (Fig. 2C). Over time, the orientations of both IHC and first row OHC continued to improve such that by P0 the average deviations for IHC and first row OHC in the base of the cochlea were approximately 10° and 5°, respectively. By P10, the average deviation for bundles located on IHC in both the base and apex was approximately 2°. For first row OHC at P10, average deviations were ~3° in the base and 4° in the apex. Stereociliary bundles on OHC located in second or third rows could not be identified in the apex of the cochlea at E17 but were present in the base at the same time point (Fig. 2B). The initial average deviation from zero of stereociliary bundles on OHC located in both the second and third row was greater than for either IHC or first row OHC (Fig. 2C). In particular, the average initial deviation for stereociliary bundles located in the third row was ~30°, nearly double the value for first row OHC. However, in the more mature basal region of the cochlea at P0 the average deviation of stereocilia bundles for the second row was ~10° and for the third row it was ~13°. Finally, at P10, average deviations for second row OHC were ~6° in the apex and 2° in the base. For third row OHC, deviations at P10 were ~6° in the apex and 5° in the base.

Multiple Wnt genes are expressed in the embryonic cochlea

To determine if Wnt genes are expressed in the developing cochlea, we initially performed a degenerate PCR screen for Wnt genes in cochleae from E15 and E17 mice. Approximately 50 individual colonies were sequenced. Greater than 50% of those colonies represented *Wnt7a*, and an additional approximately 25% were *Wnt5a*. Other Wnts identified were *Wnt2* and *Wnt10b*. Specific primer sets were used to confirm expression of *Wnt7a*, *Wnt5a*, *Wnt2* and *Wnt10b*. In addition, transcripts were also detected for *Wnt4*, *Wnt7b*, *Wnt8* and *Wnt11*.

Exposure to Wnt7a-conditioned medium inhibits stereociliary bundle reorientation

As the results of our PCR screen indicated that *Wnt7a* was strongly expressed within the developing cochlear duct, and, because of its reported role in polarity (Parr and McMahon, 1995; Kengaku et al., 1998), we decided to investigate the effects of exogenous Wnt7a protein on the development of polarity in cochlear explant cultures. As reported previously (Kelley et al., 1993), explant cultures of embryonic cochleae from E13 animals developed normally in vitro, including the formation of normal stereociliary bundle orientation (all bundles oriented towards the outer border of the organ of Corti; Fig. 3A). The overall development of stereociliary bundle morphology on both IHC and OHC also appeared normal, with the formation of characteristic curved or V shapes. In cultures maintained in Wnt7a-conditioned medium, the overall



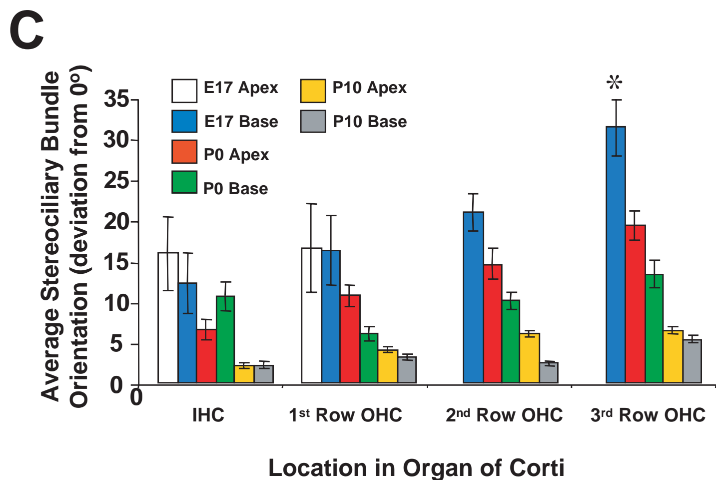


Fig. 2. Stereociliary bundles reorient during development. (A,B) Stereocilia (green) and kinocilia (red) were labeled in cochleae from P0, E17 and P10 (not shown). (A) In the basal region of the cochlea at P0, a typical chevron-shaped stereociliary bundle with a kinocilia located at the vertex of the bundle is present on each hair cell. Orientations for hair cells located in the boxed region are illustrated on the right. The locations of the single row of IHC and three rows of OHC (numbered) are indicated on the left. (B) In the mid-basal region of the cochlea at E17, chevron-shaped or flattened stereociliary bundles with asymmetric kinocilia can be identified for IHC and for OHC located in the first two rows (1,2). Bundles are present on third row OHC (3) but specific orientations could not be determined. As in A, orientations for cells located in the boxed region are illustrated on the right. Scale bar: in A 10 μm for A,B. (C) Average orientations of stereociliary bundles were determined for IHC and OHC in basal and apical regions of the cochlea at E17, P0 and P10. Distinct orientations could not be determined for stereociliary bundles located on second and third row OHC in the apical region of the cochlea at E17. Average deviation in stereociliary bundle orientation decreased with development for hair cells in all rows of the organ of Corti, indicating that orientation improves towards 0° over time. The average initial orientation for stereociliary bundles located in the third row of outer hair cells (asterisk) was significantly greater (P=0.02) than the initial orientation for bundles located in the row of IHC or in the first row of OHC.

development of the sensory epithelium, including the development of IHC and OHC, appeared unaffected. However, in the outer hair cell region, and in particular in the second and third rows, many cells were observed with deviated bundle orientations with some of the deviations equal to or exceeding 90° (Fig. 3B). Similar results were obtained for explants that were co-cultured with Wnt7a-expressing RatB1a cells (data not shown). Wnt7a exposure did not seem to impede hair cell maturation, as evidenced by the presence of stereociliary bundles, markers of maturation (Sobin and Anniko, 1984) and by the development of mature bundle morphologies including a chevron shape and asymmetrically located kinocilium (Fig. 3B). Furthermore, both control and treated cultures expressed similar levels of prestin, a protein expressed exclusively in the plasma membrane of postnatal OHC (data not shown) (Belyantseva et al., 2000).

The development of stereociliary bundle orientation in control explant cultures was comparable with development in vivo as indicated by the analysis of the average deviation from 0° at different positions along the basal half of the sensory epithelium. The overall average deviation for all OHC in the basal-most regions of E13 control explants after 6 days in vitro (DIV) was 12.4° when compared with 9.6° for the average deviation for all OHC in the basal region of P0 cochleae in vivo. Moreover, analysis of average bundle deviations at different positions along the basal half of control cultures suggests that stereociliary bundles reoriented in culture at a rate that was similar, although somewhat delayed, to that observed in vivo (Fig. 3C). As a result, in control cultures the average bundle deviation for all OHC at the mid-point of the sensory epithelium was ~40°. At more basal positions along the epithelium the average deviation gradually decreased to a minimum of ~11° at a position located 12.5% along the length of the epithelium.

In cultures exposed to Wnt7a-conditioned medium, the orientations of stereociliary bundles located at the mid-point of the sensory epithelium did not significantly differ from control (Fig. 3C). However, although the average deviation from 0° gradually decreased at more basal positions in control cultures, a similar decrease was not observed in cultures exposed to Wnt7a-conditioned medium. As a result, stereociliary bundles located in the basal regions of Wnt7a-treated cultures were significantly misoriented in comparison with controls (Fig. 3C). Moreover, in cultures exposed to Wnt7a-conditioned medium, the average deviation from 0° was approximately the same at each position along the basal half of the sensory epithelium.

The observation that there was a change in the average deviation from 0° in the presence of Wnt7a protein suggested that Wnt7a could play a role in either the reorientation of stereociliary bundles or in the overall orientation of the plane of cellular polarity within the outer hair cell region. If Wnt7a

influences the overall orientation of cellular polarity, then treatment with Wnt7a protein would lead to a uniform deviation in bundle orientation. To examine this hypothesis, the spectrum of possible stereociliary bundle orientations (–180° to +180°) was partitioned into 15° intervals. Individual bundle orientations were assigned to specific partitions to generate a histogram for the distribution of bundle orientations (Fig. 4). Analysis of the distribution of stereociliary bundle orientations along the basal half of the sensory epithelium indicates that treatment with Wnt7a protein does not lead to a uniform deviation in stereociliary bundle orientation (Fig. 4A,B). Rather, the relatively broad initial distribution of bundle orientations is maintained at positions along the length of the sensory epithelium, suggesting that the effect of treatment with Wnt7a protein is to inhibit stereociliary bundle reorientation.

Stereociliary bundle orientation is unaffected in *Wnt7a* mutant mice

Development of the cochlea and organ of Corti of P0 and adults appeared unaffected in *Wnt7a* homozygous mutants and no obvious defects in stereociliary bundle orientation were observed at either time point (data not shown).

Blocking endogenous Wnt Signaling with Sfrp1 or Wif1 inhibits stereociliary bundle reorientation

Recent studies have identified a secreted family of molecules that are related to the Frizzled family of Wnt receptors (Rattner et al., 1997; Leyns et al., 1997; Finch et al., 1997). Secreted-frizzled-related-proteins (Sfrps) have been shown to bind to multiple Wnt proteins and to prevent bound Wnt from binding to Frizzled receptors (Wang et al., 1997; Xu et al., 1998; Bafico et al., 1999; Uren et al., 2000). As a result, Sfrps can be used to inhibit a broad range of Wnt signals, including Wnt7a (Hall et al., 2000; Yoshino et al., 2001; Bergwitz et al., 2001). To investigate the effects of inhibition of endogenous Wnt signaling on the development of bundle orientation, cochlear cultures were established and exposed to medium containing Sfrp1. Sfrp1 induces a disruption in OHC stereociliary bundle reorientation that appears comparable to the effects of Wnt7a (Fig. 5A), supporting a role for endogenous Wnt signaling in stereociliary bundle reorientation.

A second Wnt antagonist is Wnt inhibitory factor 1 (Wif1), a secreted protein that binds to Wnt proteins and inhibits their activity (Hsieh et al., 1999). To further examine and confirm the role of endogenous Wnt signaling on the development of bundle orientation, cochlear explants were established and exposed to Wif1-conditioned or control medium. As significant changes in bundle orientation in response to Wnt7a or Sfrp1 were only observed at the 5% and 12.5% positions, analysis of the effects of Wif1 were restricted to these points. OHC bundle orientations in Wif1-exposed explants were significantly misoriented when compared with controls (Fig. 5B).

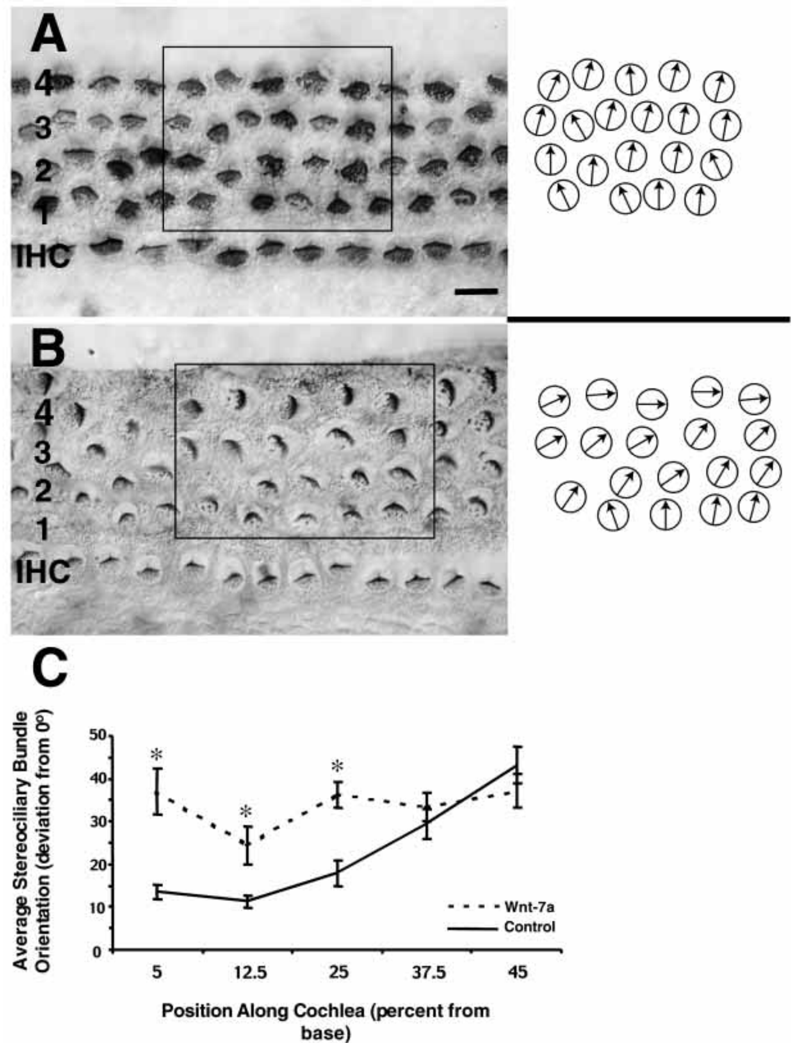


Fig. 3. Wnt7a protein influences stereociliary bundle orientation. (A) Surface view of labeled stereociliary bundles in a cochlear culture established on E13 after 6 days in vitro (DIV). Stereociliary bundles on both IHC and all rows of OHC (1-4) have developed with appropriate orientation. Orientations for the outer hair cells located in the boxed region are illustrated on the right. (B) Surface view of a cochlear culture established on E13 and maintained for 6 DIV in medium conditioned with Wnt7a protein. There are marked bundle misorientations in all rows of outer hair cells (1-4). Orientations of IHC appear unaffected. Orientations for the boxed region are illustrated on the right. (C) Average stereociliary bundle orientations were determined for outer hair cells at specific positions (determined as a percent of the total length) along the basal-to-apical axis of the cochlea in control and Wnt7a-treated cultures. In control cultures, average bundle orientation decreased progressively towards the basal end of the cochlea. This pattern is consistent with in vivo data (see Fig. 2) and suggests that bundles reorient in vitro. Exposure to Wnt7a protein did not alter the orientation of stereociliary bundles located at the 45% or 37.5% positions along the cochlea; however, there were significant changes in average stereociliary bundle orientation at the 25%, 12.5% and 5% positions (asterisks; $P<0.003$). Results are from three pairs of control and Wnt7a-treated cochlear explants. Error bars are s.e.m. (at least 12 stereociliary bundles were measured per point per culture). Scale bar in A (same in B), 10 μm.

Disruption of endogenous Wnt diffusion inhibits reorientation of stereociliary bundles

Previous studies have demonstrated that heparan sulfate proteoglycans (HSPGs) play a key role in regulating Wnt/Wg signaling, and in particular, in the extracellular distribution of Wnt/Wg (Tsuda et al., 1999; Lin and Perrimon, 1999; Baeg et al., 2001; Dhoot et al., 2001; Giráldez et al., 2002). To determine whether HSPGs play a role in bundle reorientation, cochlear explant cultures were exposed to 30 mM $NaClO_3$, a concentration that has been shown to be sufficient to inhibit the sulfation of polysaccharides and therefore to prevent the production of HSPGs (Kispert et al., 1996). Treatment with $NaClO_3$ lead to significant defects in stereociliary bundle orientations at both the 5% and 12.5% positions when compared with controls (Fig. 5B).

Expression pattern of *Wnt7a* in the embryonic cochlea

Based on the results of exposure to Wnt7a protein, the cellular distribution of *Wnt7a* was determined in whole-mount in situ on cochleae from E14, E16, P0 and P3. At E14, *Wnt7a* is expressed throughout a wide region of the developing cochlear epithelium (Fig. 6A). However, the relative intensity of expression appears to vary along both the basal-to-apical and inner-to-outer axes of the epithelium. In the relatively undeveloped apex of the cochlea at E14, *Wnt7a* is expressed

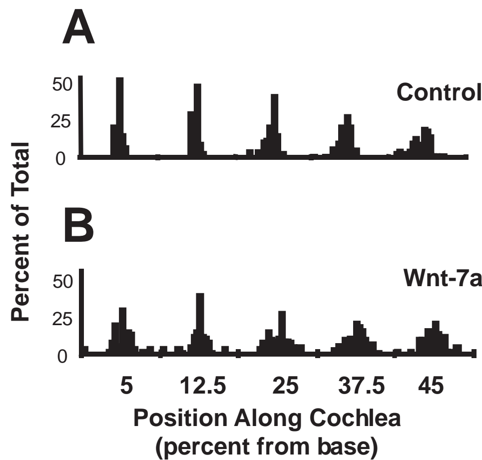


Fig. 4. Wnt7a does not bias the direction of stereociliary bundle orientation. (A) Frequency histograms for stereociliary bundle orientations at different positions along the basal-to-apical axis in control cochleae. Orientations were determined for bundles on outer hair cells and the resulting values were used to generate a frequency histogram with a class interval of 15°. In more immature regions of the cochlea (45% and 37.5% positions) there is a broad distribution of bundle orientations. By contrast, in more mature regions of the cochlea (25%, 12.5% and 5% positions), the distribution of bundle orientations becomes tightly clustered. (B) Frequency histograms, generated as in A, for stereociliary bundle orientations in cochleae exposed to Wnt7a. The distribution of bundle orientations at the 45% position appears similar to control. However, bundle orientations remain fairly broad at more basal (mature) positions, suggesting that Wnt7a inhibits bundle reorientation rather than affecting the overall polarity of the epithelium. The data in A and B represent orientations for stereociliary bundles from cochlear explant cultures established on E13 after 6 days in vitro in the presence of either control or Wnt7a-conditiond medium.

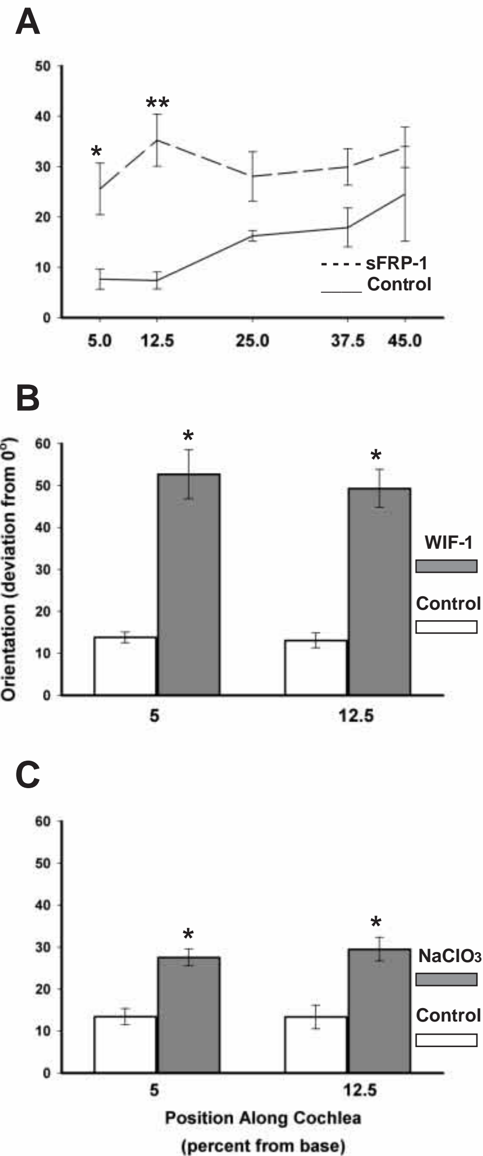


Fig. 5. Inhibition of the Wnt signaling prevents stereociliary bundle reorientation. (A) Secreted frizzled-related protein 1 (Sfrp1) inhibits stereociliary bundle reorientation. Average stereociliary bundle orientations were determined for outer hair cells at specific positions along the basal-to-apical axis of the cochlea in control and Sfrp1-treated cultures. Exposure to Sfrp1 inhibited bundle reorientation with significant differences in average stereociliary bundle orientation at both the 5% and 12.5% positions (*$P<0.02$, **$P<0.003$). (B) Wnt inhibitory factor 1 (Wif1) inhibits stereociliary bundle reorientation. Average stereociliary bundle orientations were determined for outer hair cells at the 5% and 12.5% positions along the basal-to-apical axis of the cochlea in control and Wif1-conditoned media cultures. Wif1-conditioned medium significantly inhibited stereociliary bundle reorientation at both positions (*$P<0.001$). (C) Sodium chlorate inhibits stereociliary bundle reorientation. Diffusion of Wnt protein within cochlear explants was reduced by inhibition of synthesis of HSPG with 30 mM sodium chlorate ($NaClO_3$). $NaClO_3$-treated cultures exhibited significant differences, as compared with control, in average bundle orientations at the 5%, and 12.5% positions (*$P<0.004$). Results for all three graphs are from a minimum of three control and three experimental cochleae and orientations were determined for a minimum of 12 stereociliary bundles at each position in each sample. Error bars indicate s.e.m.

broadly in the inner half of the epithelium (Fig. 6B). However, its expression ends abruptly in a region of the epithelium that correlates with the position of the spiral vessel in the outer half of the epithelium (Fig. 6B). As the spiral vessel is located beneath the region of the epithelium that will develop as the pillar cells, the edge of *Wnt7a* expression appears to correlate with the mid-point of the developing organ of Corti.

In the basal region of the cochlea at E14, expression of *Wnt7a* persists in a band of cells that is located near the mid-point of the duct (Fig. 6A). Analysis of cross-sections indicates that this band of expression correlates with cells located on the inner side of the spiral vessel (Fig. 6C). The position of the band of *Wnt7a* expression relative to the location of the spiral vessel, suggests that *Wnt7a* is expressed in cells that will develop as IHC, inner phalangeal cells and pillar cells.

At E16, *Wnt7a* is strongly expressed in a narrow band of cells that extends along the length of the cochlea in a position that is consistent with the developing sensory epithelium (Fig. 6D). Weak expression also persists in the apical region in some cells located in the inner half of the cochlear duct (Fig. 6D). Cross-sections of the base of the cochlea at E16 indicate that the strong band of *Wnt7a* expression correspond to developing pillar cells (Fig. 6E). By P0, expression is restricted to the pillar cells (Fig. 6F). Expression of *Wnt7a* in pillar cells extends at least through P3, the oldest stage analyzed (data not shown).

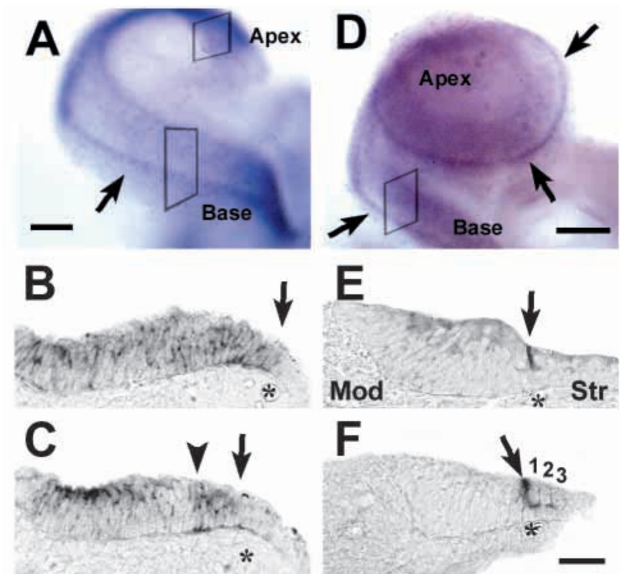


Fig. 6. *Wnt7a* is expressed in the developing organ of Corti. (A) Whole-mount in situ hybridization for *Wnt7a* in the cochlear duct at E14. A band of *Wnt7a* expression is present in the basal region (base) of the cochlea (arrow). In the apical turn (apex) of the cochlea, *Wnt7a* expression spans most of the width of the floor of the cochlear duct. Box near base indicates the region of the epithelium illustrated in B. Box near apex indicates region illustrated in C. (B) Cross-section through the apical turn of the cochlea at E14. For all cross-sections, inner is towards the left (Mod in E), outer is towards the right (Str in E). There is broad expression of *Wnt7a* throughout the epithelium. However, there is a distinct boundary of *Wnt7a* expression (arrow) that correlates with the spiral vessel (asterisk), an embryonic marker for the location of the developing organ of Corti. (C) Cross-section through the basal region of the cochlear duct at E14. *Wnt7a* expression persists in a band of cells that extends from the boundary at the spiral vessel (arrow) towards the inner-side of the duct (arrowhead). In addition, *Wnt7a* is still expressed in some cells located at the inner edge of the duct. (D) Whole-mount in situ hybridization for *Wnt7a* at E16. By this stage, expression of *Wnt7a* is restricted to a band of cells that extends along the length of the cochlea (arrows) from base to apex. Box indicates region illustrated in E. (E) Cross-section through the basal region of the cochlear duct at E16. Expression of *Wnt7a* is restricted to developing pillar cells (arrow) located directly above the spiral vessel (asterisk). (F) Cross-section through the cochlear duct at P0. *Wnt7a* expression persists in the inner and outer pillar cells (arrow), with the possibility of a small amount of expression in the Deiter's cell located underneath the first row OHC (1). Second (2) and third (3) row OHC are numbered for reference. Scale bars: 100 μm in A,D; in F, 20 μm for B,C,E,F.

DISCUSSION

The development of specific orientation of epithelial structures is critical for normal cellular function. In the ear, orientation of hair cell stereociliary bundles is particularly important for normal hearing (Fujita and Orita, 1988; Comis et al., 1989; Fujita, 1990; Furness et al., 1990; Yoshida and Liberman, 1999). Our findings demonstrate that stereociliary bundles develop initially with a non-random orientation that is biased towards the final orientation and subsequently continue to reorient towards their final position. We show that exposure to Wnt protein or Wnt antagonists results in inhibition of bundle reorientation indicating that a Wnt signaling pathway plays a role in stereociliary bundle reorientation. Moreover, *Wnt7a* is expressed in the cochlea during the developmental period when stereociliary bundles reorient and the pattern of expression of *Wnt7a* mRNA is consistent with a role for Wnt7a protein in the polarization of the developing outer hair cell stereociliary bundles.

Cochlear stereociliary bundles reorient during development

The factors that play a role in the determination of specific stereociliary bundle orientations in hair cells are largely unknown. The results of two previous studies in chick cochlea and mouse vestibular system have demonstrated that orientation apparently arises as a two-step process (Cotanche and Corwin, 1991; Denman-Johnson and Forge, 1999). During the first step, stereociliary bundles develop with an initial, non-random orientation that is biased towards the final orientation of each cell. Following initial development, individual stereociliary bundles gradually reorient to obtain their final orientation. Our observations suggest that hair cells within the mammalian organ of Corti develop in a similar manner.

In the proposed two step process of bundle orientation, a general specification of polarity must occur prior to bundle formation, and a second phase of orientation is required to achieve a final uniform polarity. A similar series of specifications appears to occur during the development of wing hair polarity in *Drosophila* (reviewed by Adler and Lee, 2001). In both vertebrate and invertebrate systems, it appears that uniform planar polarity develops through a series of interactions in which an initial overall polarity is determined

possibly through the perception of a gradient and subsequent refinements are made based on the gradient and signals produced through cell-cell signaling.

It is important to consider that there were marked differences in the uniformity of initial stereociliary bundle orientations between hair cells located in different regions of the organ of Corti in vivo. Initial orientations for IHC and first row OHC were fairly uniform (average deviation of 15°) and comparable. However, there was a progressive increase in the average deviation of the initial orientations of second (20°) and third row (30°) OHC. One explanation for this result would be that a diffusible cue plays a role in the determination of initial bundle orientation and that the source of this cue is located closer to the IHC and first row OHC. Therefore, greater distance from the source could lead to a greater degree of inaccuracy as a result of an asymptotic diffusion gradient.

A role for wnt signaling in bundle reorientation

Our results strongly support a role for Wnt signaling during stereociliary bundle reorientation. At least eight different Wnt genes are present in the developing cochlear epithelium and treatment with Wnt7a leads to a disruption in bundle reorientation. Similarly, inhibition of Wnt signaling using factors that either bind directly to Wnts (Sfrp1, Wif1) or that prevent the diffusion of Wnts ($NaClO_3$) also inhibit bundle reorientation. However, the specific mechanism(s) of Wnt signaling in bundle orientation have not been determined. Wnt signaling is mediated through binding to Fzd receptors, leading to activation of at least three downstream signaling pathways. Although the specific downstream pathways that are activated by Wnts in the cochlea have not been identified, the effects on bundle orientation would seem most consistent with the JNK/PCP pathway. If this is the case, then differential activation of Fzd receptors across developing outer hair cells could serve as an instructive cue for the re-orientation of the stereociliary bundle (Tomlinson et al., 1997). Preliminary results indicate that mRNAs for multiple frizzled genes are expressed in the developing cochlear epithelium (A.D. and M.W.K., unpublished), and mRNAs for at least five frizzled genes are expressed in the adult rat cochlea (Daudet et al., 2002). However the cellular localization of these genes has not been determined in either the embryonic or adult cochlea.

The dynamics of Wnt-dependent Fzd activation across outer hair cells has not been determined. One possibility, which will be discussed below in more detail, is that Wnts act as a soluble molecule that diffuses across the developing outer hair cells, leading to differential activation of Fzds based on distance from the Wnt source. Alternatively, in *Drosophila* although *wg* is necessary for the development of ommatidial polarity, it apparently serves a permissive, rather than instructive, role (Adler, 2002). Based on these results, it has been suggested that polarizing signals may arise through the differential localization of Fzd receptors to one side of the cell (Adler and Lee, 2001). If a similar mechanism exists in the organ of Corti, then Fzd receptors should be differentially localized to one edge of each developing OHC. However, at present there are no data regarding the cellular distribution of Fzd proteins in developing hair cells. Finally, it is important to consider that the effects of exogenous Wnt7a protein on stereociliary bundle orientation are not obviously consistent with a purely permissive role for Wnts.

Interestingly, the orientation of stereociliary bundles located on IHC did not seem affected by treatment with Wnt7a-conditioned medium or Sfrp1. One possible explanation would be that orientation of IHC stereociliary bundles could occur at an earlier developmental stage than OHC. This is supported by the finding that IHC are already differentiated at E14, at least 2 days prior to OHC as determined by the expression of the hair cell specific marker myosin-VI (M.M. and M.W.K., unpublished).

A gradient could play a role in stereociliary bundle reorientation

The effects of treatment with Wnt7a, Sfrp1, Wif1 and sodium chlorate, as well as the pattern of expression for *Wnt7a*, are consistent with a role for Wnt-signaling in the re-orientation of OHC bundles. *Wnt7a* is expressed in developing pillar cells, creating a potential line source that could result in the formation of a gradient leading to an asymmetric distribution of the soluble protein across a plane of epithelial cells. Individual cells within the plane would be able to detect this gradient and to generate a uniform cellular polarity that would correspond with the direction of the gradient. The Wg/Wnt families of signaling proteins have been shown to act as morphogens during development of various structures in *Drosophila* (Zecca et al., 1996; Neumann and Cohen, 1997; Strigini and Cohen, 2000) and as has been discussed, a gradient in the activation of Fzd receptors determines planar orientation in both the *Drosophila* eye and wing (reviewed by Mlodzik, 1999).

If detection of a gradient is an important factor in stereociliary bundle reorientation, then the diffusion dynamics of that gradient should impact on the absolute change in concentration that exists at different distances from its source (Monteiro et al., 2001). Assuming that the developing pillar cells act as a line source for Wnts then the relative drop in concentration across the first row of OHC should be greater than the drop in concentration across the third row of OHC assuming an asymptotic gradient. Under these circumstances, it seems reasonable to expect that stereociliary bundle reorientation in third row OHC might be delayed or that there might be an overall lower level of uniform orientation as a result of the relatively smaller overall change in concentration across those cells. One way to examine the level of uniformity of bundle orientation is to compare the change in average bundle deviation between first row and third row OHC during reorientation. However, as third row OHC develop with a greater initial average bundle deviation, it is difficult to make comparisons regarding changes that occur during the period of reorientation. An alternative approach would be to compare the changes in the standard deviation of the distribution of bundle orientations between first and third row OHC. Between E17 and P0 the standard deviation for bundle orientations in first row OHC decreases by 80% while the standard deviation for orientation in the third row decreases by only 52%. Although a decrease in the relative change of a concentration gradient across each row of OHC is certainly not the only explanation for these differences, the relative delays in the formation of uniform orientation in second and third row OHC is consistent with this hypothesis.

It is important to note that we do not present direct or conclusive evidence that a Wnt gradient is necessary to

generate polarization of the stereociliary bundles in the organ of Corti. Wnt could be responsible for the organization of secondary gradients of other PCP molecules that in turn influence planar polarity in the cochlea (Yang et al., 2002; Tree et al., 2002; Ma et al., 2003). Moreover, further experiments are needed to investigate whether Wnt plays a direct role in PCP signaling in the stereociliary bundles, permissively or instructively.

Alternative pathways for stereociliary bundle orientation

The observation that stereociliary bundle orientation is normal in *Wnt7a* mutant animals suggests that Wnt7a is not necessary for bundle reorientation. This result suggests that either alternative mechanism(s) for the determination of appropriate bundle orientation exist within the cochlea or that deletion of *Wnt7a* leads to the activation of compensatory mechanisms. As treatment with Sfrp1 or Wif1, generic inhibitors of Wnt signaling, leads to inhibition of reorientation, it is possible that one or more of the seven other Wnts expressed within the cochlea could be acting in either a functionally redundant or compensatory fashion in *Wnt7a* mutants. For example, Wnt7b like Wnt7a induces axonal remodeling and synapsin I clustering in mossy fibers (A. Hall, A.B. and P.C.S., unpublished).

It is also possible that other signaling pathways could act to compensate for the loss of *Wnt7a* signaling. Previous studies have suggested a role for the Notch signaling pathway in the determination of stereociliary bundle polarity in mechanosensory hair cell epithelia in both zebrafish and mice (Haddon et al., 1999; Lanford et al., 1999). Analysis of stereociliary bundle orientation in the zebrafish mutant *mindbomb*, a mutation in an as yet undetermined gene that has been shown to disrupt the Notch pathway, indicates consistent defects in the development of a normal pattern of stereociliary bundle orientation (Haddon et al., 1999). Similar effects were observed in the orientations of OHC stereociliary bundles in the organ of Corti from mice in which *Jag2* (a Notch ligand) had been deleted (Lanford et al., 1999). Therefore, it seems likely that Notch signaling also plays a role in the determination of stereociliary bundle orientation. These results, taken together with our findings, suggest the intriguing possibility that Notch and Wnt signaling may collaborate to regulate hair cell orientation as observed in other developing systems (Tomlinson and Struhl, 1999). In addition, Notch signaling could compensate for the loss of Wnt7a function or it is possible that Wnt signaling may be complementary to the short-range Delta/Notch signaling pathway.

In summary, we have presented the first study on the development of planar polarity in a mammalian system. The results presented here strongly implicate the Wnt signaling pathway in the development of the mammalian cochlea and suggest that the molecular basis of planar polarization has been conserved between vertebrates and invertebrates.

We thank Drs T. Friedman and D. Wu for comments on the manuscript, Dr A. McMahon for providing cDNA for Wnt7a, Dr J. Kitajewski for stably transfected Wnt-expressing cells, and Dr B. Kachar for providing the prestin antibody. This work was supported by the intramural program at the National Institute on Deafness and Other Communication Disorders.

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