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Wnt5a functions in planar cell polarity regulation in mice

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Abstract

Planar cell polarity (PCP) refers to the polarization of cells within the plane of a cell sheet. A distinctive epithelial PCP in vertebrates is the uniform orientation of stereociliary bundles of the sensory hair cells in the mammalian cochlea. In addition to establishing epithelial PCP, planar polarization is also required for convergent extension (CE), a polarized cellular movement that occurs during neural tube closure and cochlear extension. Studies in Drosophila and vertebrates have revealed a conserved PCP pathway, including Frizzled (Fz) receptors. Here we use the cochlea as a model system to explore the involvement of known ligands of Fz, Wnt morphogens, in PCP regulation. We show that Wnt5a forms a reciprocal expression pattern with a Wnt antagonist, the secreted frizzled-related protein 3 (Sfrp3 or Frzb), along the axis of planar polarization in the cochlear epithelium. We further demonstrate that Wnt5a antagonizes Frzb in regulating cochlear extension and stereociliary bundle orientation in vitro, and that *Wnt5a*$^{-/-}$ animals have a shortened and widened cochlea. Finally, we show that Wnt5a is required for proper subcellular distribution of a PCP protein, Ltap/Vangl2, and that Wnt5a interacts genetically with *Ltap/Vangl2* for uniform orientation of stereocilia, cochlear extension, and neural tube closure. Together, these findings demonstrate that Wnt5a functions in PCP regulation in mice.

INTRODUCTION

The mammalian auditory sensory organ, the organ of Corti, consists of four rows of sensory hair cells interdigitated with supporting cells along the length of the spiral cochlear duct. The innermost row of cells toward the center (medial aspect) are the inner hair cells (IHCs); the three rows toward the periphery (lateral aspect) of the cochlea are the outer hair cells (OHCs) (Fig. 1A, B). On the apical surface of each hair cell, bundles of finger-like extensions (stereocilia) are arranged in a “V”-shaped staircase. Invariably, the vertices of the “V”-shaped stereocilia of all the hair cells point to the periphery of the cochlea, displaying a distinctive planar cell polarity (PCP) (Gubb and Garcia-Bellido, 1982) along the mediolateral axis of the cochlea (Fig. 1B). The PCP of the cochlea is essential for auditory transduction. Deflection of the stereocilia bundle towards the tallest row of stereocilia during sound stimulation mechanically opens transduction channels near the tips of stereocilia, allowing influx of cations and depolarization of the hair cell. Thus, the coordinated orientation of stereocilia bundles is

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critical for effective function of the cochlea as it permits synchronous activation of groups of hair cells and their associated afferent neurons. During mouse development, the organ of Corti differentiates from a shorter and thicker primordium through cellular rearrangements characteristic of convergent extension (CE) (Keller, 2002) concurrently with the establishment of PCP from embryonic day 14.5 (E14.5) to E18.5 (Chen et al., 2002; McKenzie et al., 2004; Wang et al., 2005).

Genetic studies in Drosophila have identified a set of so-called core PCP genes that are required for diverse forms of planar polarity (Klein and Mlodzik, 2005; Strutt and Strutt, 2005; Tree et al., 2002). In vertebrates, a similar cassette of genes, including Frizzled (Fz), Dishevelled, Ltap/Vangl2, and Celsr1, regulate CE and the establishment of PCP (Keller, 2002; Mlodzik, 2002; Wallingford et al., 2000; Wang et al., 2006a). Mutations in mouse PCP genes cause defective planar polarization of the cochlear epithelium and consequently stereociliary bundle misorientation (Curtin et al., 2003; Lu et al., 2004; Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b). These mutations are also associated with a shortened and widened cochlear duct and a completely open neural tube, presumably resulting from defective CE during cochlear extension and neurulation, respectively (Montcouquiol et al., 2003; Wang et al., 2006a; Wang et al., 2005). During planar polarization, PCP components such as Fz3 and Fz6 (Montcouquiol et al., 2006; Wang et al., 2006b), dishevelled2 (Wang et al., 2005) , and Ltap/Vangl2 (Montcouquiol et al., 2006) are sorted asymmetrically along the mediolateral axis and display polarized subcellular localizations across the organ of Corti. Polarized membrane localization of Celsr1 was observed in the chicken auditory sensory organ (Davies et al., 2005). During CE in vertebrates, PCP proteins and other polarization complexes also display polarized subcellular distribution in polarized lamellipodial protrusions which are presumed to exert traction, pulling the cells toward one another mediolaterally. This causes the tissue to narrow along the mediolateral axis and, concomitantly, extend along a perpendicular axis (Ciruna et al., 2006; Hyodo-Miura et al., 2006; Jiang et al., 2005; Mlodzik, 2006) .

Wnts are known ligands for Fz receptors (Bhanot et al., 1996; Yang-Snyder et al., 1996), which are essential components of PCP signaling (Vinson et al., 1989; Wang et al., 2006b). However, whether Wnts are involved in PCP regulation remains controversial. Studies in Drosophila wing have pointed to a Wnt-independent mechanism (Amonlirdviman et al., 2005; Klein and Mlodzik, 2005; Strutt and Strutt, 2005; Tree et al., 2002) . Two recent studies show that Wnt is involved in orientating the denticles (actin-based cell projections) on segmentally repeated subsets of ventral epidermal cells in the Drosophila embryos (Colosimo and Tolwinski, 2006; Price et al., 2006), and that Wnt may function together with hedgehog (Hh) as instructive polarizing cues that help establishing directionality within the epidermis (Colosimo and Tolwinski, 2006). In Xenopus and zebrafish, Wnt5 and Wnt11 are required for CE, although their role may be permissive instead of instructive (Smith et al., 2000; Tada and Smith, 2000; Topczewski et al., 2001; Ulrich et al., 2003).

So far, Wnts have not been demonstrated for an in vivo role in PCP signaling in mammals. In the mouse cochlea, Wnt7a is expressed in a type of supporting cells, the pillar cells, within the organ of Corti (Dabdoub et al., 2003). Addition of Wnt7a-conditioned medium (CM) or the Wnt antagonists Sfrp1 and Wif1 leads to misorientation of stereocilia in culture (Dabdoub et al., 2003). However, no PCP defect is observed in Wnt7a knockout mice (Dabdoub et al., 2003). In this study, we used the cochlea as a model system and explored the involvement of Wnt morphogens in PCP regulation. We provide in vitro and in vivo evidence supporting a role for Wnt5a in PCP regulation in mice.

MATERIALS AND METHODS

Mouse strains and animal care

The animals used are *Wnt5a*$^{+/-}$ (Yamaguchi et al., 1999), Looptail *Ltap*$^{Lp/+}$ (Kibar et al., 2001), Cre/Esr1 (Nat) (Badea et al., 2003), Gt(Rosa)26Sor animal (Jackson Laboratory), Math1/GFP (Chen et al., 2002; Lumpkin et al., 2003) and BAT-gal (Maretto et al., 2003) animals. The *Ltap*$^{Lp/+}$ colony was in a mixed C57BL/6 × 129 background. *Wnt5a*$^{+/-}$ animals (129S7/SvEvBrd-Hprt) were bred with C57/B6Crl animals (Charles River Laboratory). To generate transgenic mice expressing Ltap-GFP fusion protein, we inserted the coding sequence for GFP before the stop codon of the Ltap gene in a bacterial artificial chromosome (BAC) (BACPAC, RP23-244J9) using a reported method (Yang et al., 1997). Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of Emory University and the University of California, Irvine. The morning after the mating of the animals is designated as E0.5. BrdU cell proliferation analyses were performed as described (Chen et al., 2002). The number of animals used for each genotype at each stage is least three and indicated where data are presented.

In-situ hybridization and histology analyses of inner ear tissues

Inner ear dissection, sectioning, immunostaining, and in-situ hybridization were performed as described (Radde-Gallwitz et al., 2004). Primary antibodies and dyes used were: acetylated α-tubulin (mouse monoclonal, 1:50-100, Sigma); FITC- and rhodamineconjugated phalloidin (5 pM, from Molecular Probes); p27/Kip1 (mouse monoclonal, 1:100, BD Transduction Laboratories); BrdU (mouse monoclonal, 1:100, Roche). Secondary antibodies were purchased from Jackson ImmunoResearch. For p27/Kip1 and BrdU staining, the sections were subjected to citric acid steam treatment prior to immunostaining.

For analysis of PCP, we stained the whole mount preparations of the organs of Corti with an antibody against acetylated α-tubulin and rhodamine- or FITC-conjugated phalloidin that label the kinocilium and the stereocilia, respectively (Wang et al., 2005). The samples were analyzed and images were acquired with Zeiss LSM510 for less than 1 μm optical sections.

cDNAs for Wnt5a and Frzb were cloned from theE14.5 inner ear epithelium by RT-PCR with oligonucleotides listed in supplemental Table I.

Scanning electronic microscope (SEM)

SEM imaging was performed as described (Wang et al., 2005). Briefly, inner ears were fixed in 2.5% glutaraldehyde in 0.7M Cacodylate buffer (pH 7.3) for 4 hours at room temperature, and transferred to PBS, pH7.4. The specimens were further treated with 1% tannic acid, followed by osmium tetroxide treatment. The specimens were dehydrated through series ethanol, and critically dried. The dried samples were mounted on metal stubs, and coated with gold particles, and ready for examination.

Organ Culture

We dissected cochleae from E14.5 embryos and cultured either the intact cochlear duct or bisected cochlear ducts on polyD-lysine- (Sigma) and fibronectin-coated glass-bottomed dishes in control, Frzb-containing, or Wnt5a- and Frzb-treated culture media for 4-6 days in vitro (DIV) as described (Wang et al., 2005). For suppression of Frzb by Wnt5a, we pre-incubated molar ratios of Frzb and Wnt5a for 20 minutes at 37°C before addition to the culture media. For cochlear grafting cultures, two cochleae were placed close or distant to each other. The mediolateral axes of the two cochleae cultured were parallel or anti-parallel to determine whether there is an intrinsic difference between the cochlear tissues in the medial and the lateral regions for the orientation of stereociliary bundles.

Preparation of Wnt5a-conditioned media and Western blot analysis

Both the control L cells (ATCC, Cat# CRL-2648) and Wnt5a producing L cells (ATCC, Cat# CRL-2814) were cultured according to ATCC instructions. The media collected from both control cells and the Wnt5a-producing cells were concentrated with Centriplus YM-10 (Millipore, Cat# 4411). The concentration of Wnt5a in the conditioned media was determined by Western blot analysis using the recombinant Wnt5a expressed in CHO cells (R&D, Cat# 645WN) as the standard and an antibody against Wnt5a (Neuromics, Cat# GT15034, 1:2500). Western blot analyses were carried out using standard protocol. Cochlear and brain protein extracts were isolated from E15.5-E16.5 embryos and subjected to Western blot analysis.

Quantification of stereociliary bundle orientation and cochlear extension

We measured the length of cochlear ducts at the beginning and end of culture using NIH ImageJ software. The lengthening in the culture was scored as the ratio of the length of the culture at the beginning to the length of the culture at the end. To determine stereociliary bundle orientation, we drew a line from the position of the kinocilium through the middle of the "V"-shaped stereocilia (bisecting line). The angle formed between this line and the line parallel to the mediolateral axis was used for quantifications. In wild type animals, this angle is closed to 0. For histogram plots of distribution of the angles, the angles forms at the right side or the left side of the line representing mediolateral axis are designated to be positive (+) or negative (−), respectively. For quantification of the average deviation from the normal orientation (angle=0), only the angles were scored without positive (+) or negative (−) designations. At least 50 hair cells in each row at the basal, middle, and apical regions were quantified for each sample. The same quantification method was used for quantification of stereociliary bundle orientation for whole mount preparations of the organs of Corti and the organs of Corti in vitro. SPSS (11.0) was used for statistic analyses.

Canonical Wnt activity assay

Embryos were collected from timed-pregnant BAT-gal (Maretto et al., 2003) transgenic females at E10.5, E12.5, E14.5, E16.5 and E18.5. Embryonic heads were partially dissected in ice-cold PBS (pH 7.4) and briefly fixed in 2% paraformaldehyde (PFA) in PBS on ice for thirty minutes. The ears were completely dissected and washed three times in fresh rinse buffer (100mM sodium phosphate pH7.3, 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40) and incubated for LacZ activity in staining solution (rinse buffer plus 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 1mg/ml X-Gal) in the dark overnight (16-20 hours) at room temperature. The samples were washed and post-fixed in 4% PFA , and further treated for whole mount or cryosection according to our standard protocol. To visualize the tissue structure, tissue sections were sometimes counterstained with eosin and mounted.

RESULTS

Wnt5a is expressed in a reciprocal pattern with a Wnt antagonist in the cochlea along the planar polarization axis

During mouse development, precursor cells giving rise to the organ of Corti withdraw from the cell cycle around E13.5-E14.5 (Chen and Segil, 1999; Ruben, 1967). Subsequently, hair cells start to differentiate in a gradient starting near the base of the cochlear duct at the IHC location and extending along the mediolateral and longitudinal axes (Fig. 1A). The development of stereocilia follows the differentiation gradient of hair cells. By E18.5, the organ of Corti is patterned along the entire length of the cochlea into four rows of hair cells. The polarization of stereocilia along the mediolateral axis is readily recognizable in the base to the middle region of the cochlear duct by E18.5.

Since Wnts are implicated in the orientation of stereocilia in vitro but Wnt7a does not appear to be essential for PCP regulation in vivo (Dabdoub et al., 2003), we sought to determine whether other Wnts are involved in planar polarization of the cochlea. We found transcripts for several Wnt molecules in the cochlear epithelium from E14.5 to E18.5 (data not shown). In particular, we detected the expression of Wnt5a at E14.5 near the base in the region medial to the p27/Kip1-expressing domain that demarcates the developing organ of Corti in the cochlear epithelium (Fig. 1C,D) (Chen and Segil, 1999). By E16.5, Wnt5a is expressed in the region medial to the organ of Corti from the base to the apex of the cochlea (Fig.1E,F).

A family of secreted Fz-related proteins, or Sfrps, shares the extracellular Wnt-binding domain of Fz. Sfrps can bind Wnts and sequester Wnts from Fz receptors, thus acting as Wnt antagonists (Rattner et al., 1997; Wang et al., 1997). In the cochlear epithelium, Sfrp3, also known as Frzb, is expressed in the region lateral to the developing organ of Corti when Wnt5a is expressed in the region medial to the organ of Corti (Fig. 1G-I). Therefore, a Wnt antagonist is expressed in a reciprocal pattern with Wnt5a along the mediolateral axis. In addition, the onset of Wnt5a expression also displays a basal-to-apical polarity along the longitudinal axis of the cochlear duct (Fig. 1).

Wnt5a and Frzb interact in vitro for stereociliary bundle orientation and cochlear extension

Wnt molecules upon binding to Fz can activate the so-called canonical signaling pathway, which culminates in the formation of nuclear β-catenin/LEF/TCF transcriptional complexes that activate expression of target genes (Logan and Nusse, 2004). We therefore examined the status of canonical Wnt signaling in the cochlea at the time of Wnt5a expression using BAT-gal reporter mice, where LacZ expression is under the control of LEF/TCF binding sites (Maretto et al., 2003). No canonical Wnt activity was detected in the cochlea at the same stage that Wnt5a is expressed in wild-type cochleae (supplement Fig.1), suggesting that Wnt5a expressed in the cochlea might function in a fashion that is independent of β-catenin-mediated transcriptional activation.

We next used organ culture to test whether Wnt5a and Frzb have any effect on the orientation of stereocilia or cochlear extension (Figs. 2,3). We isolated cochlear ducts at E14.5 and cultured the intact cochlear ducts or bisected cochlear ducts on polyD-lysine-and fibronectin-coated cover glass for 4-6 days in vitro (DIV). Under this condition, the organ of Corti will differentiate and achieve nearly normal PCP (Fig. 2A)(Wang et al., 2005). The addition of Frzb affected the polarization of stereocilia in a dose-dependent manner (Fig. 2A-D). This effect was suppressed by pre-incubation of Frzb with molar ratios of Wnt5a in Wnt5a-conditioned medium for 20 minutes before addition to the cultures (CM) (Fig. 2C,D).

Parallel to the association of cochlear extension and terminal differentiation of the organ of Corti in vivo, bisected wild-type cochleae isolated at E14.5 undergo extensive lengthening in the culture (Fig. 3A,B) (Wang et al., 2005). The addition of Frzb affected cochlear extension (Fig. 3C,D), and pre-incubation with Wnt5a partially reversed this effect (Fig. 3E-G).

Together, these results suggest that excess Frzb affects stereociliary bundle orientation and cochlear extension. Since Wnt5a can suppress the effect of excess Frzb on stereociliary bundle orientation and cochlear extension, the endogenous pathway modulated by Frzb likely involves its Wnt-binding domain.

Interestingly, however, the effect of Wnt5a-CM alone appeared less specific. No effect was observed at similar concentrations to Frzb, while gross defects in the development of stereocilia were often seen at higher concentrations (>1.5 mg/ml) (data not shown), possibly due to non-optimized culture media containing Wnt5a-CM. This may suggest that Wnt5a, if involved, plays mainly a permissive function in stereociliary bundle orientation in vitro. Alternative, the

Wnt5a protein present in the conditioned medium may be in a different biological form compared with the endogenous Wnt5a protein from cochlea (Fig. 2E) and brain (data not shown). The latter notion is supported by our observation of different mobility on SDS gel electrophoresis of the recombinant Wnt5a and the endogenous Wnt5a proteins from mouse brain and cochlea (Fig. 2E). The recombinant Wnt5a protein migrates as around 50 kD (Fig. 2E, lanes 1 and 3), as reported previously (Mikels and Nusse, 2006). However, the endogenous Wnt5a protein from mouse brain and cochlea migrates around 37 kD (Fig. 2E, lanes 5-8). Therefore, it is possible that the Wnt5a in the conditioned medium is capable of binding to Frzb while incapable of activating downstream pathways involved in regulating cochlear extension and stereociliary bundle orientation. An additional implication of this possibility is that the binding of Wnt5a in Wnt5a-CM to endogenous Frzb, or the loss-of-function of Frzb in vitro, does not affect the orientation of stereocilia.

Ablation of *Wnt5a* leads to characteristic CE defects in the cochlea

We next analyzed the function of Wnt5a in PCP regulation in vivo. PCP mutants exhibit a fully opened neural tube (craniorachischisis), misoriented stereocilia, and cochlear phenotypes characteristic of CE defects, including a shortened and widened cochlear duct (Curtin et al., 2003; Lu et al., 2004; Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b). In particular, the apical region of the organ of Corti includes additional rows of sensory hair cells in these PCP mutants (Montcouquiol et al., 2003; Wang et al., 2005).

We examined cochleae isolated from *Wnt5a*$^{-/-}$, *Wnt5a*$^{+/-}$, and wild-type littermates (Yamaguchi et al., 1999). In gross appearance, the inner ears of *Wnt5a*$^{-/-}$ animals differed slightly from those of control littermates. The normal curvature at the base of the cochlea was not apparent in *Wnt5a*$^{-/-}$ animals and the hook region constituting the curvature at the base of the cochlear duct was shortened (Fig. 4A,B). The cochlear ducts from about 35% of *Wnt5a*$^{-/-}$ animals (n = 34) were shortened and the apical tip of the cochlear duct was bent (Fig. 4A,B). The organs of Corti in these 35% of *Wnt5a*$^{-/-}$ animals that had shortened cochlear ducts (n=34) had additional rows of hair cells along the entire length of the cochlear duct (Fig. 4C,D) in comparison to control samples (Fig. 4E,F). The widening of the organ of Corti was especially prominent in the apical half of the cochlear duct (Fig. 4C,D). One *Wnt5a*$^{-/-}$ animal also had craniorachischisis observed in all known mammalian PCP mutants (Curtin et al., 2003; Lu et al., 2004; Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b), while three had the exencephaly phenotype (not shown). In the remaining 65% of *Wnt5a*$^{-/-}$ animals, the cochlear duct was slightly shortened and a discontinuous additional row of outer hair cells was observed (data not shown).

Wnt5a$^{-/-}$ animals show a general reduction in outgrowth of diverse embryonic structures whose development requires extension from the primary body axis (Yamaguchi et al., 1999). The embryos were truncated caudally including a loss of tail and significant shortening of the embryonic anterior-to-posterior axis, the snout, and manidible and tongue were truncated, reduced outgrowth of the external ear was apparent, both fore- and hindlimbs were shortened along the proximal-to-distal axis, and genital tubercle was absent (Yamaguchi et al., 1999). The outgrowth defects in *Wnt5a*$^{-/-}$ animals have been attributed partly to proliferation defects in these tissues (Yamaguchi et al., 1999). The expression of Wnt5a in the cochlea medial to the developing organ of Corti is detected at E14.5, when cells in the organ of Corti have withdrawn from the cell cycle, but the cells medial to the organ of Corti are still dividing at this stage and will undergo a few further rounds of cell proliferation (Fig. 4G, H) (Chen and Segil, 1999; Ruben, 1967). To determine whether loss of Wnt5a affects cell proliferation in regions that undergo active cell division after the expression of Wnt5a is detected at E14.5, we administered BrdU to pregnant females three times at E14.5, and then compared cell proliferation in *Wnt5a*$^{-/-}$ and wild-type embryos (Fig. 4G,H). We observed comparable

numbers of BrdUpositive cells in each cochlear section from *Wnt5a*$^{-/-}$ animals and their control littermates. Collectively, the widening of the organ of Corti and the cochlear duct, the concomitant shortening of the cochlear duct itself, and the apparently normal cell proliferation in affected regions indicate a CE defect in *Wnt5a*$^{-/-}$ animals.

Wnt5a genetically interacts with *Ltap/Vangl2* to regulate stereociliary bundle orientation, cochlear length, and neural tube closure

In contrast to 35% penetrance of shortened and widened cochleae in *Wnt5a*$^{-/-}$ animals, hair cell stereociliary bundles in *Wnt5a*$^{-/-}$ mice only showed occasional imperfect alignments (Fig. 4I-K). To explore a potential function of Wnt5a in stereociliary bundle orientation and to define its role in CE, we generated mice carrying mutant alleles for both *Wnt5a* and *Ltap/Vangl2* (Kibar et al., 2001; Montcouquiol et al., 2003) (Figs. 5,6).

Mice that are homozygous for a loss-of-function point mutation in *Ltap*, the looptail (*Ltap*^{Lp}) mutation, showed misoriented stereocilia and a shortened, widened cochlear duct, as well as craniorachischisis (Montcouquiol et al., 2003). Heterozygous *Ltap*$^{Lp/+}$ mice had normal stereociliary bundle orientation (Fig. 5A-C,I,J). In double-heterozygous *Wnt5a*$^{+/-}$; *Ltap*$^{Lp/+}$ mice, the third row of OHCs displayed high frequency of stereociliary bundle misorientation in the basal region, and almost all bundles were misorientation in the middle to apical region (Fig. 5G-I, K,L). The IHCs in *Wnt5a*$^{+/-}$; *Ltap*$^{Lp/+}$ double-heterozygous animals also showed some degree of stereociliary bundle misorientation (Fig. 5I, K).

In addition to the inner ear stereociliary bundle orientation phenotype, *Wnt5a*$^{+/-}$; *Ltap*$^{Lp/+}$ heterozygous mice displayed drastically decreased weight gain after birth; most of them died within one week. One *Wnt5a*$^{+/-}$; *Ltap*$^{Lp/+}$ heterozygous male survived and produced 16 litters, or 131 live embryos with *Wnt5a*$^{+/-}$ females. Of these embryos, four were *Wnt5a*$^{-/-}$;*Ltap*$^{Lp/+}$. All four exhibited craniorachischisis (Fig. 6), presenting a drastic increase in penetrance as compared to the craniorachischisis phenotype displayed by *Wnt5a*$^{-/-}$ (1 in 34) or *Ltap*$^{Lp/+}$ animals (0 in more than 100). The inner ears of these *Wnt5a*$^{-/-}$;*Ltap*$^{Lp/+}$ embryos were similar to those of *Ltap*$^{Lp/Lp}$ PCP mutants and had shortened, widened cochlear ducts (Fig. 6).

These observations indicate that *Wnt5a* genetically interacts with the PCP gene *Ltap/Vangl2*. The PCP-specific phenotypes in the cochlea of *Wnt5a*$^{+/-}$; *Ltap*$^{Lp/+}$ double-heterozygous animals and in the neural tube of *Wnt5a*$^{-/-}$;*Ltap*$^{Lp/+}$ animals further indicate that Wnt5a plays a role in PCP signaling in mice.

Ltap distribution in the cochlea is abnormal in *Wnt5a*$^{-/-}$ mice

The hallmark of planar polarization for CE and establishment of epithelial PCP is polarized localization of core PCP proteins. We generated BAC$^{\text{Ltap-GFP}}$ mice through BAC-mediated transgenesis to examine the subcellular localization of Ltap/Vangl2 (Yang et al., 1997). BAC$^{\text{Ltap-GFP}}$ mice carry a transgene in which the coding sequence for GFP was inserted before the stop codon of the Ltap gene.

The subcellular localization of Ltap-GFP in wild-type mice was consistent with immunostaining using an antibody against Ltap/Vangl2 (Montcouquiol et al., 2006). In the wild-type cochlea at E18.5, Ltap-GFP was localized to the junctions between hair cells and supporting cells, and apparently appears on the medial side of hair cells (Fig. 7A,B). In *Wnt5a*$^{-/-}$ littermates, cellular geometries and contacts were altered in the apical region of the cochlea (Fig. 7C,D) where strong CE defects were observed (Fig. 4). In the apical region, Ltap-GFP was localized to the junctions between supporting cells and the polarized subcellular localization to the medial sides of hair cells was absent (Fig. 7C,D). In the basal region of the

cochlear duct, Ltap-GFP showed a more normal distribution, being largely localized to the medial side of hair cells in *Wnt5a*$^{-/-}$ mice, although some abnormal localization of Ltap-GFP to the lateral side of hair cells was also observed (Fig. 7E,F). Therefore, the subcellular distribution of Ltap in the cochlea was affected by the absence of Wnt5a, particularly in the apical region. The strong spatial correlation between the extent of abnormalities in Ltap localization and cellular geometry and the extent of CE defects suggests possible cellular mechanisms that underlie the CE defects in the cochlea of *Wnt5a*$^{-/-}$ mice (Blankenship et al., 2006; Classen et al., 2005; Hyodo-Miura et al., 2006).

Mediolateral instructive cues for PCP in the cochlea

The expression and functional studies of Wnt5a (Figs. 1-7) strongly supports a role for Wnt5a in PCP regulation in mice. However, it is not clear whether there are instructive cues for PCP in the cochlea and, if so, whether Wnt5a contributes to such instructive cues. To address these issues, we grafted tissues in the organ culture to test whether instructive cues are present along the mediolateral axis of the cochlea for the orientation of stereociliary bundles (Fig. 8).

As we showed previously (Fig. 2) (Wang et al., 2005), the cochleae cultured singly showed normal PCP (Fig. 8A-C). When two cochleae were cultured together in a parallel manner so that the medial side of one cochlea (outside) was grafted to the lateral side of the second cochlea (inside), the orientation of stereociliary bundles in the outer rows of OHCs in the inside cochlea that were the nearest to the medial region of the outside cochlea was significantly altered (Fig. 8D,F,G). The orientation of stereociliary bundles of the outer rows of OHCs in the outside cochlea showed a small alteration (Fig. 8E-G).

To further test whether the effect on the orientation of stereociliary bundles of the outer rows of OHCs is due to the intrinsic properties of the cochlear tissue grafted, we increased the distance between the two cochleae in parallel cultures (Fig. 8H-J), as well as reversed the relative orientation of the two cochleae so that the mediolateral axes of the two cochleae grafted together were anti-parallel (Fig. 8K-M). When the distance between the two cochleae in parallel cultures was increased, the orientation of stereociliary bundles in both the inside and outside cochleae was normal (Fig. 8H-J, and data not shown). In contrast to the parallel cultures (Fig. 8D-G), the orientation of stereociliary bundles was not altered even in regions where the distance between the two cochleae was close in the anti-parallel cultures where the mediolateral axes of the two cochleae were reversed and the lateral sides of the two cochleae were placed next to each other (Fig. 8K-M, and data not shown). These data together indicates that there is potentially an intrinsic difference between the medial and lateral regions of the cochlear epithelium for directing the orientation of stereociliary bundles and supports the presence of instructive cues along the mediolateral axis of the cochlear epithelium for establishing PCP in the outer rows of OHCs.

However, we could not determine the contribution of Wnt5a to the difference between the medial and lateral tissues of the cochlea for PCP in vitro. We grafted the medial region of cochleae from *Wnt5a*$^{-/-}$ animals to the lateral side of wild-type cochleae (Fig. 8N-P). No statistically significant difference was observed between cultures of *Wnt5a*$^{-/-}$ and wild-type grafts (Fig. 8N-P) and wild-type and wild-type grafts in the same experiments. This observation is consistent with the low penetrance of stereociliary misorientation in *Wnt5a*$^{-/-}$ mice and suggests the presence of redundant pathways for Wnt5a function in PCP signaling.

DISCUSSION

In this study, we demonstrate that Wnt5a plays a role in mice in two cellular processes regulated by the PCP pathway, establishment of epithelial PCP and CE.

We found a reciprocal expression pattern of Wnt5a and Frzb along the axis for planar polarization in the cochlea at the time that PCP is being established (Fig. 1). We showed that Wnt5a suppresses the effect of Frzb on stereociliary bundle orientation in vitro (Fig.2). We further observed imperfect stereociliary bundle alignments in *Wnt5a*$^{-/-}$ animals (Fig. 4) and a genetic interaction between *Wnt5a* and *Ltap/Vangl2* in regulating stereociliary bundle orientation (Fig. 5). Together, these observations indicate that Wnt5a contribute to the establishment of uniform bundle orientation.

Although Frzb addition has a strong effect on PCP in vitro, only minor imperfection in the alignment of stereocilia in *Wnt5a*$^{-/-}$ mice was observed. This observation suggests that additional pathways parallel or redundant to Wnt5a are involved in PCP regulation in the cochlea. The presence of redundant pathways made it difficult to determine the molecular mechanism underlying the role of Wnt5a in PCP signaling in mice. It is not clear whether the reciprocal expression of Wnt5a and Frzb along the axis for planar polarization is involved in generating a graded Wnt signal to direct the establishment of PCP in the cochlea. The lack of demonstrated effectiveness of Wnt5a-CM on stereociliary orientation further prevented us from reversing the source of Wnt5a in vitro to test a possible instructive role for Wnt5a. However, the data from our grafted organ cultures (Fig. 8) indicates an intrinsic difference between the medial and lateral regions of the cochlear epithelium for PCP and supports the presence of instructive cues along the mediolateral axis of the cochlear epithelium.

The composition of potential mediolateral instructive cues, however, remains unknown. The putative compensatory pathways for Wnt5a may include additional Wnts (Colosimo and Tolwinski, 2006; Dabdoub et al., 2003; Price et al., 2006) and Hh (Colosimo and Tolwinski, 2006). BMPs might also contribute to PCP regulation in the cochlea, as BMP signaling can be modulated by a secreted frizzled molecule (Lee et al., 2006; Muraoka et al., 2006) and BMPs are expressed asymmetrically along the mediolateral axis of the cochlear epithelium (Morsli et al., 1998; Takemura et al., 1996). Future studies towards the understanding of additional pathways and the generation of genetic tools to interrupt or reverse the presence of Wnts and Wnt antagonists in vivo will be critical to determine the molecular role of Wnts in PCP regulation.

CE consists of cellular intercalations along the mediolateral axis and extension along the perpendicular longitudinal axis. In Xenopus and zebrafish, Wnt5 and Wnt11 are required for CE. However, their expression pattern is not consistent with an instructive but rather a permissive role (Heisenberg et al., 2000; Kilian et al., 2003; Smith et al., 2000; Tada and Smith, 2000; Ulrich et al., 2003). In the cochlea, the expression of Wnt5a is asymmetric along the mediolateral and the longitudinal axes (Fig. 1) and is appropriate to serve as a cue for CE along both axes. The effect of Frzb on cochlear extension in vitro and the suppression of this effect by Wnt5a provided the first hint that Wnts may be involved in CE in mammals (Fig. 3). Our observation of craniorachischisis and shortened and widened cochleae in some *Wnt5a*$^{-/-}$ animals (Fig. 4, and data not shown), and the genetic interaction between Wnt5a and Ltap/Vangl2 in enhancing the penetrance of cochlear CE and neural tube closure (Figs. 5,6) further supported a role for Wnt5a in the PCP pathway for CE regulation.

An intriguing question is how the mammalian PCP pathway concurrently regulates the establishment of PCP and CE in the cochlea during terminal differentiation. In *Wnt5a* mutants, phenotypes from the two processes show different penetrance. The cochlear CE defect has a higher penetrance than the stereociliary defect in *Wnt5a*$^{-/-}$ animals (Fig. 4), while *Wnt5a*$^{+/-}$*; Ltap*$^{Lp/+}$ double heterozygous animals have a 100% penetrant stereociliary orientation defect in the third row of OHCs but no apparent cochlear CE defect (Fig. 7). The dissociation of cochlear CE and stereocilia orientation defects in some *Wnt5a* mutants suggests

that the molecular mechanisms underlying CE and stereociliary bundle orientation are not identical.

Wnts can trigger both canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) downstream pathways. No canonical Wnt activity was detected in the cochlea during terminal differentiation (supplemental Fig. 1), making it unlikely that Wnt5a acts via a β-catenin-dependent pathway. This said, it remains possible that canonical activity occurs at a level below the detection threshold with the BAT-gal reporter. However, our data is consistent with the current view on the modulation of the specificity of downstream Wnt pathways by the context of intracellular factors and receptor. Candidate vertebrate cytoplasmic PCP proteins have been shown to promote the PCP pathway while inhibiting the canonical Wnt pathway (Moeller et al., 2006; Schwarz-Romond et al., 2002; Simons et al., 2005). For example, a candidate PCP gene, Ankrd6/Diversin, inhibits canonical Wnt activity while promoting PCP signaling (Moeller et al., 2006; Schwarz-Romond et al., 2002). The presence of specific Fz receptors in the cochlea may further direct Wnts in the cochlea to act via a noncanonical pathway. Fz3 and Fz6 are expressed in the developing organ of Corti and are redundantly required for uniform stereociliary orientation (Wang et al., 2006b). However, Fz3 and Fz6 fail to mediate the activation of the canonical Wnt pathway in vitro when Wnt5a is juxtaposed with a domain enabling its binding to LRP5/6, the coreceptor required for the canonical Wnt pathway (Liu et al., 2005). Our study now provides the first complementary genetic evidence to demonstrate functional involvement of Wnt5a in the PCP signaling, a noncanonical Wnt pathway, in mammals. While additional studies are clearly needed to further dissect the exact molecular role and the signaling cascade downstream of Wnt5a in PCP regulation, it is tempting to speculate that an intricate modulation or suppression of canonical Wnt signaling by multiple components of the PCP pathway may have evolved in the vertebrates to divert Wnts to PCP signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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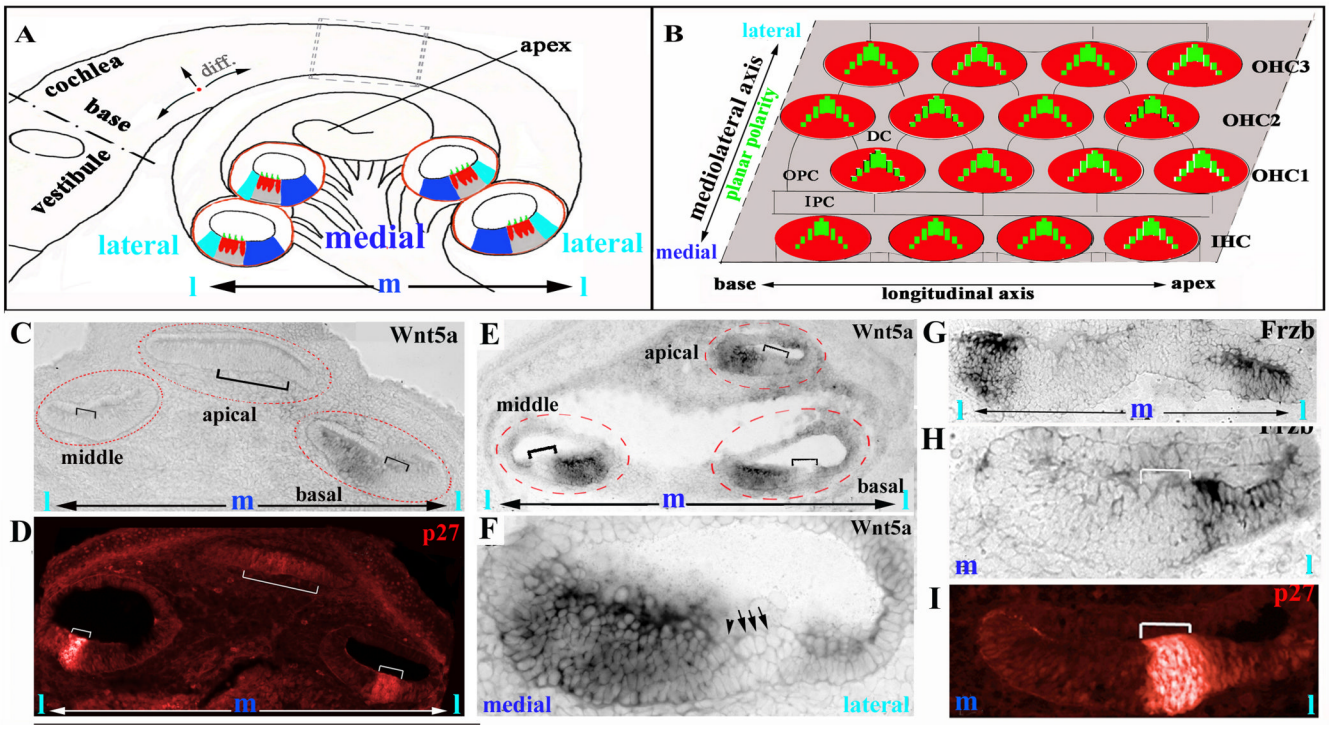

Fig. 1. Reciprocal expression of Wnt5a and Frzb along the mediolateral axis of the cochlea

(A): Diagram of a cross section of the cochlear duct at different regions along the longitudinal axis (outlined by red circles). The IHCs are localized toward the center, or medial; the three rows of OHCs are at the periphery, or lateral region. The differentiation gradient of the organ of Corti is designated by arrows near base of the cochlea (A). The dark and light blue regions correspond, respectively, to the Wnt5a- and Frzb-expressing domains.

(B): Stereocilia are uniformly oriented along the mediolateral axis illustrated in this diagram of a whole mount of the organ of Corti.

(C,D): At E14.5, Wnt5a mRNA was detected in the region medial (C) to the developing organ of Corti (indicated by brackets) at the basal region of the cochlea. The expression of p27/Kip1 (D, red) marked distinctively the developing organ of Corti at this stage. In the middle and apical regions of the cochlea, Wnt5a expression was not detected.

(E,F): At E16.5, Wnt5a is expressed in the region medial to the organ of Corti (indicated with brackets) along the length of the cochlear duct. (F) is a higher magnification view of the middle cochlear cross section of (E). The IHC and OHC are indicated by an arrowhead and arrows, respectively.

(G-I): At E14.5, Frzb is expressed in the regions lateral (H, I) to the developing organ of Corti (indicated by brackets) marked by p27/Kip1 expression (J). (I): a higher magnification view of (H). m: medial. l: lateral.

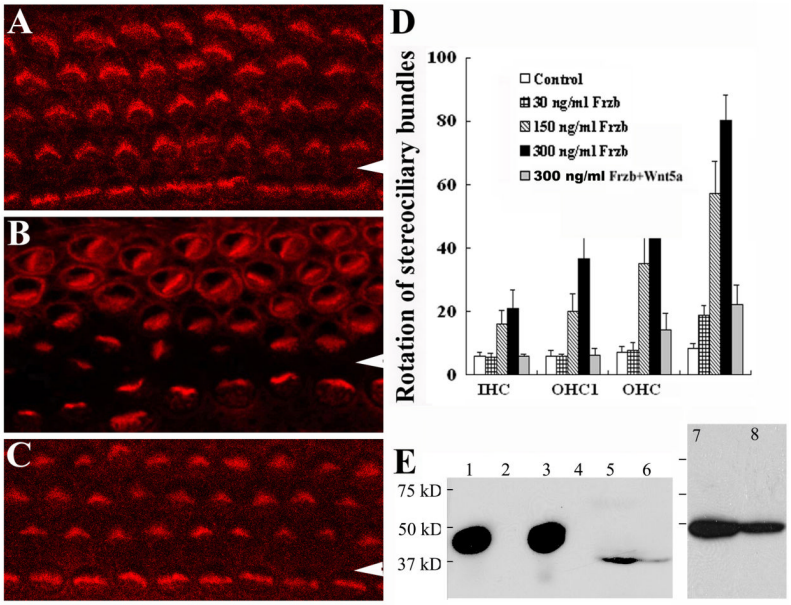

Fig. 2. Wnt5a and Frzb interact in vitro for stereociliary bundle orientation

(A-D): The cochleae from wild-type E14.5 embryos undergo differentiation in culture. Stereocilia were normally oriented in the control medium as shown by phalloidin staining (A). The addition of Frzb to the medium affected stereociliary bundle orientation (B), but this effect was suppressed by pre-incubation with Wnt5a (C). Arrowheads indicate the pillar cell region that separates the IHCs from OHCs. Stereociliary bundle misorientation in the culture was quantified (D). The OHC3 and additional rows of OHCs in culture are quantified as a single group, OHC3+. The effect of Frzb on stereociliary bundle orientation ([Frzb]> 150 ng/ml, $p<0.001$) and the rescue by Wnt5a pre-incubation ($p<0.000$) were statistically significant. (E): Western blots with an antibody against Wnt5a. Lane 1: purified recombinant Wnt5a (R&D); lanes 2 and 4: media from the control cell line; lane 3: media from Wnt5a-expressing cells (Wnt5a-CM); lanes 5, 6, 8: cochlear extracts; lane 7: brain extracts.

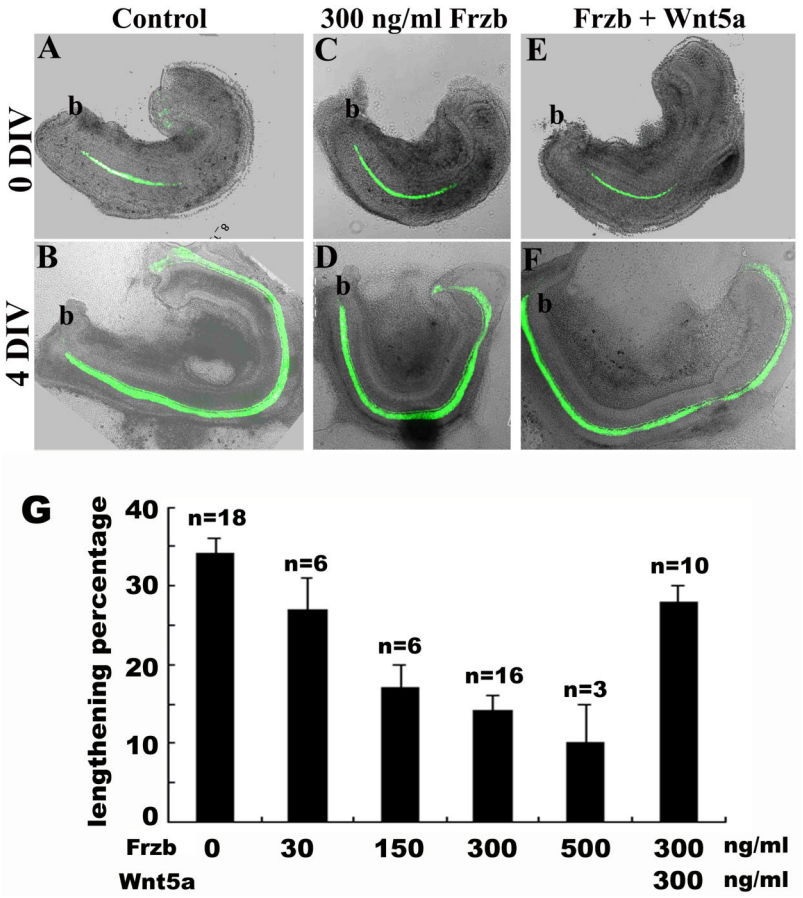

Fig. 3. Wnt5a suppressed the effect of Frzb on cochlear extension in vitro

(A-F): The extension of the wild-type cochlea in culture was reduced in the presence of Frzb (A-D). This reduced extension was partially suppressed by pre-incubation with Wnt5a (E,F). Green signals are GFP expressed under the control of Math1 enhancers marking the hair cells. At the beginning of culture, only a single row of IHCs were differentiated near the base of the cochlea (A, C, E). b: base of the cochlea.

(G): Quantification of cochlear extension in vitro. The effect of Frzb on cochlear extension ([Frzb]>150 ng/ml, $p<0.001$) and the rescue by Wnt5a pre-incubation ($p=0.001$) were statistically significant.

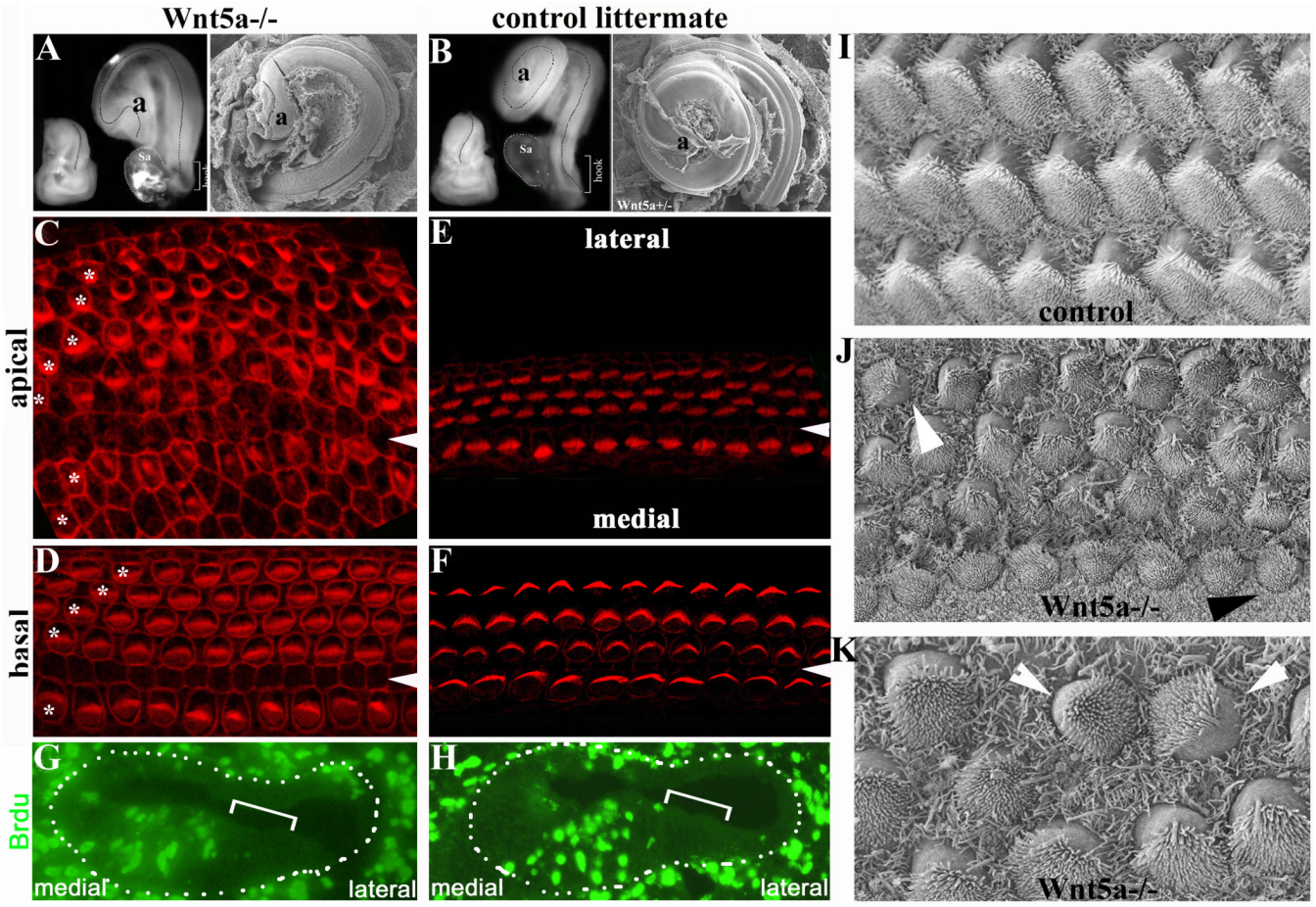

Fig. 4. Wnt5a null animals show characteristic CE defects in the cochlea

(A-B): Cochleae from E18.5 *Wnt5a*$^{-/-}$ embryos (A) were shorter than those from control littermates (B) as shown in both dissection (left) and SEM (right) images. Dashes outline the curvatures of the cochlear ducts. Sa: vestibular saccule attached to the base of the cochlea. (C-F) The organ of Corti from an E18.5 *Wnt5a*$^{-/-}$ embryo (C-D) stained with phalloidin showed additional rows of OHCs in both basal and apical regions compared with a control littermate (E,F). Asterisks mark each row of OHCs in the *Wnt5a*$^{-/-}$ sample. Arrowheads indicate the pillar cell region. The confocal plane for the *Wnt5a*$^{-/-}$ sample was close to the apical cortex of the cells in the organ of Corti, and therefore the phalloidin staining for cell-cell junctions in the *Wnt5a*$^{-/-}$ sample (C,D) appeared to be more intense than the staining in the control sample (E,F).

(G-H): BrdU-injected E18.5 cochlear sections from *Wnt5a*$^{-/-}$ (G) and control (H, *Wnt5a*$^{+/-}$) littermates stained with BrdU antibody (green) to reveal dividing cells. Brackets indicate the organ of Corti. Dashes dots outline the cochlear ducts. No statistic difference in the number of BrdU+ cells was detected in comparable cochlear sections from *Wnt5a*$^{-/-}$ and control embryos.

(I-K): In contrast to normal polarity of the stereocilia across the organ of Corti in the control littermate (I), the organ of Corti from *Wnt5a*$^{-/-}$ animals showed limited misorientation of stereocilia with varying degrees (J, K). The white arrowheads indicate a rotated outer hair cell (J) and a pair of outer hair cells facing each other (K). The black arrowhead indicates a rotated inner hair cell (J).

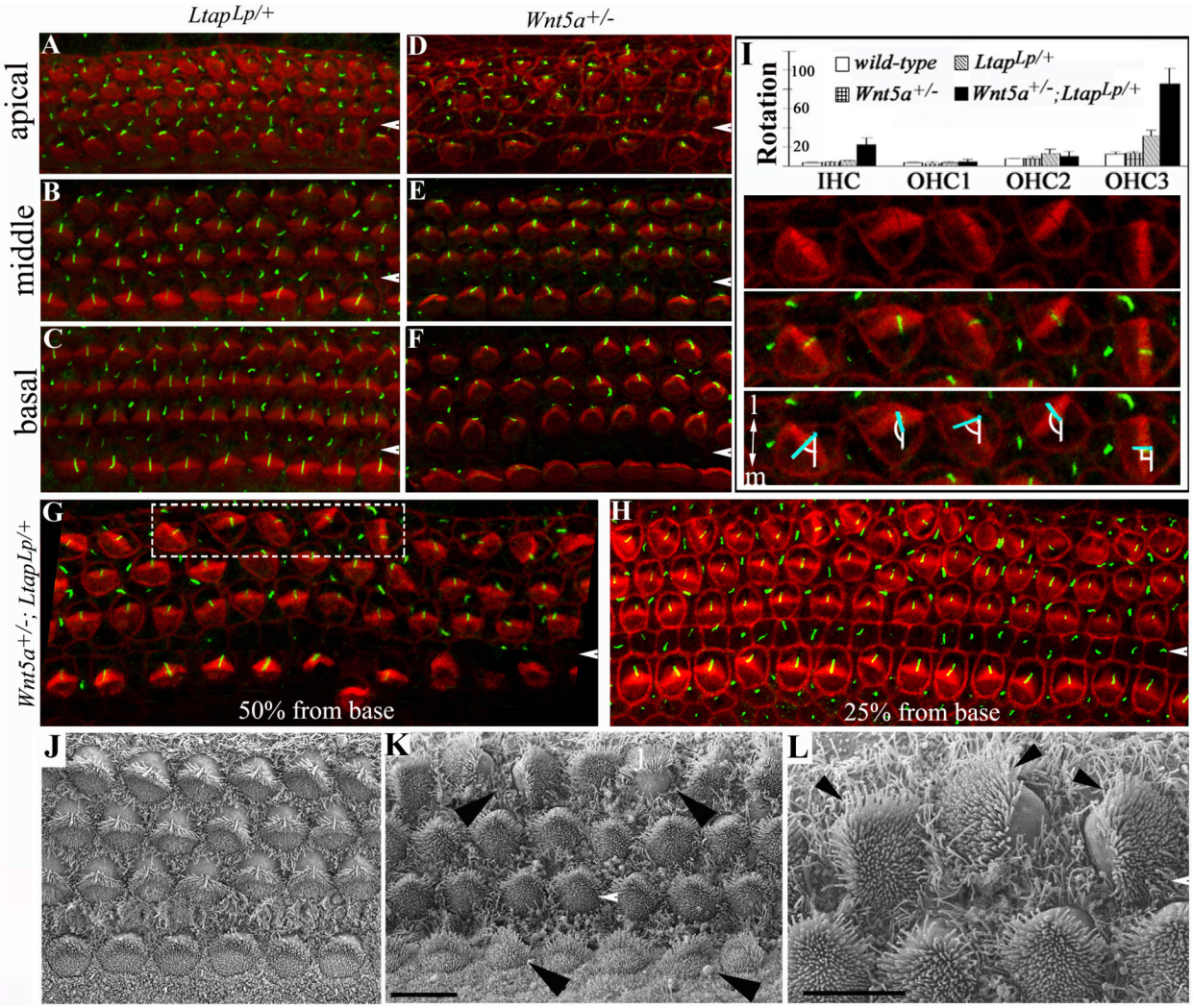

Fig. 5. Genetic interaction of *Wnt5a* and *Ltap/Vangl2* in establishing uniform orientation of stereocilia

(A-H): The cochlear ducts from E18.5 $Ltap^{Lp/+}$ (A-C), $Wnt5a^{+/-}$ (D-F), and $Wnt5a^{+/-}$; $Ltap^{Lp/+}$ (G-H) littermates stained with phalloidin (red) and acetylated α-tubulin (green). The acetylated α-tubulin antibody labels the kinocilia located at the vertices of the stereocilia and phalloidin labels actin in stereocilia. In contrast to the heterozygous $Ltap^{Lp/+}$ (A-C) and $Wnt5a^{+/-}$ (D-F) embryos, the $Wnt5a^{+/-}$; $Ltap^{Lp/+}$ double-heterozygous littermates (G-H) had misoriented stereocilia in the third row of OHCs. The white arrowheads indicate the pillar cell region separating the IHCs from OHCs.

(I): The orientation of stereocilia in $Wnt5a^{+/-}$; $Ltap^{Lp/+}$ (n= 16) mice was quantified and compared to that of $Ltap^{Lp/+}$ (n = 4), $Wnt5a^{+/-}$ (n = 4) and *wild-type* (n = 4) animals. The quantification of stereociliary orientation (I) is illustrated by hair cells from the dashed box in (G), which is shown in the panels below the histogram in (I) with the top panel showing phalloidin labeling alone, the middle panel showing phalloidin plus acetylated atubulin, and bottom panel showing the measurement of the rotation. The angle formed between the mediolateral axis (I, bottom panel, white line) and the line bisecting the "V"-shaped stereocilia (I, bottom panel, blue line) was measured and plotted in the histogram.

(J-L): SEM images of the organ of Corti from E17.5 $Ltap^{Lp/+}$ (J) and $Wnt5a^{+/-}$; $Ltap^{Lp/+}$ (K-L) embryos. Arrowheads in (K) mark two OHCs with an abnormal 90° or 180° rotation and

two IHCs with minor degrees of rotation. The misalignment of stereocilia (L, arrowheads) is more clearly seen at high magnification (L, higher magnification of the top left corner of K). Scale bars: 10 μm (J-K) and 6 μm (L).

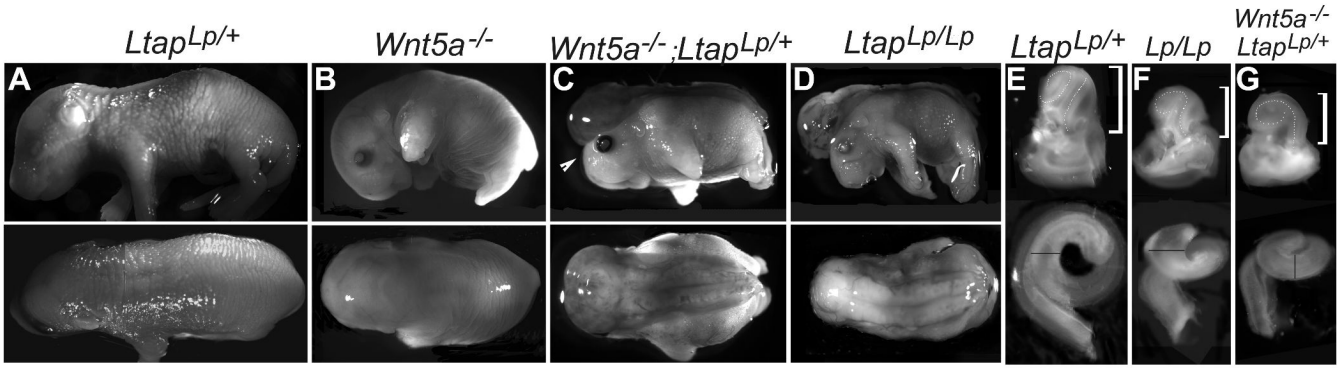

Fig. 6. Genetic interaction of *Wnt5a* and *Ltap* for neurulation and cochlear lengthening

(A-D): $Ltap^{Lp/+}$ (n>100) (A) and $Wnt5a^{-/-}$ (B, 95%, n = 26) mice had normal neural tube closure. $Wnt5a^{-/-};Ltap^{Lp/+}$ (C, 100%, n = 4) and $Ltap^{Lp/Lp}$ embryos (D, 100%) show craniorachischisis observed in all the known PCP mutants. The arrowhead in (C) indicates the open- eyelid phenotype observed in several PCP mutants. (E-G): Inner ears and dissected cochlear ducts (below) from $Ltap^{Lp/+}$ (E), $Ltap^{Lp/Lp}$ (F) and $Wnt5a^{-/-};Ltap^{Lp/+}$ embryos (G). The brackets indicate the cochlear portion. The lines were drawn from the medial to the lateral sides of the cochlear ducts in the apical region to mark the width of the cochlear ducts.

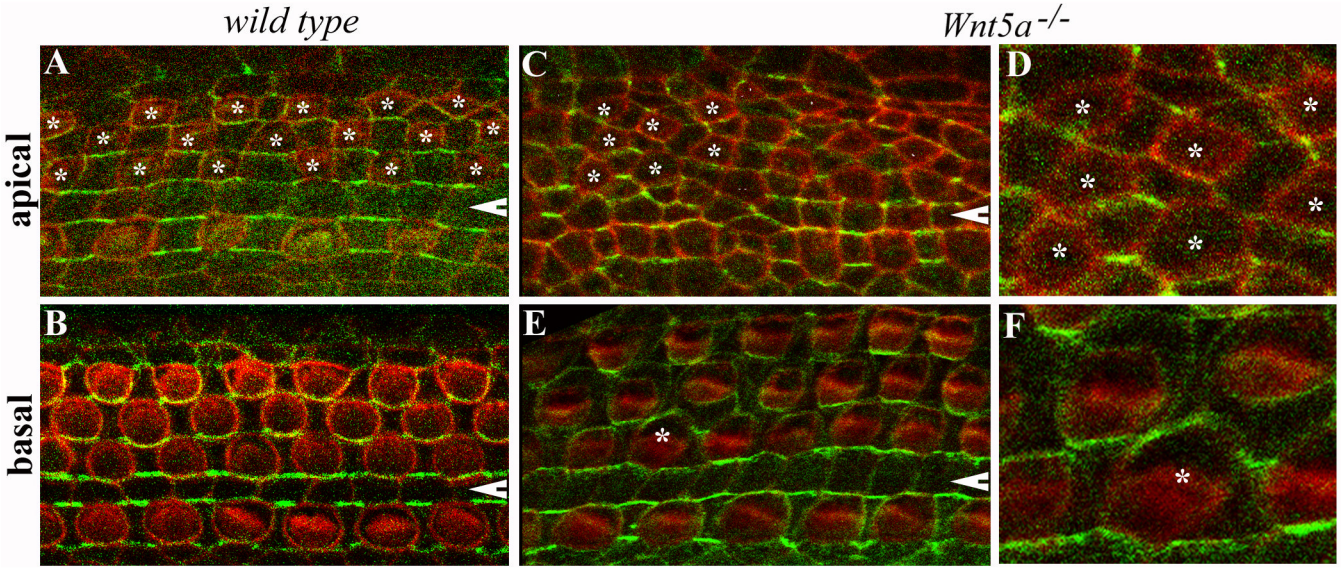

Fig. 7. Aberrant cellular geometries and distribution of Ltap in *Wnt5a*$^{-/-}$ mice

(A-F): Whole mount of the organ of Corti from E18.5 wild-type (A-B) and *Wnt5a*$^{-/-}$ (C-F) mice carrying BAC$^{Ltap\text{-}GFP}$. Hair cells were visualized by phalloidin staining (red). Ltap-GFP (green) was seen at the medial boundaries between hair cells and supporting cells in wild-type samples (A, B). In *Wnt5a*$^{-/-}$ mice, the localization of Ltap-GFP in the base region appeared normal on the medial side of hair cells (E), except around one hair cell (E, marked by an asterisk). In the apical region, cellular geometry was altered in *Wnt5a*$^{-/-}$ mice, and so was the localization of Ltap-GFP (C). OHCs in the apical region are marked with asterisks (A, C). White arrowheads indicate the pillar cell region. (D) and (F) are higher magnification images for the regions where hair cells are marked by asterisks in (C) and (E), respectively.

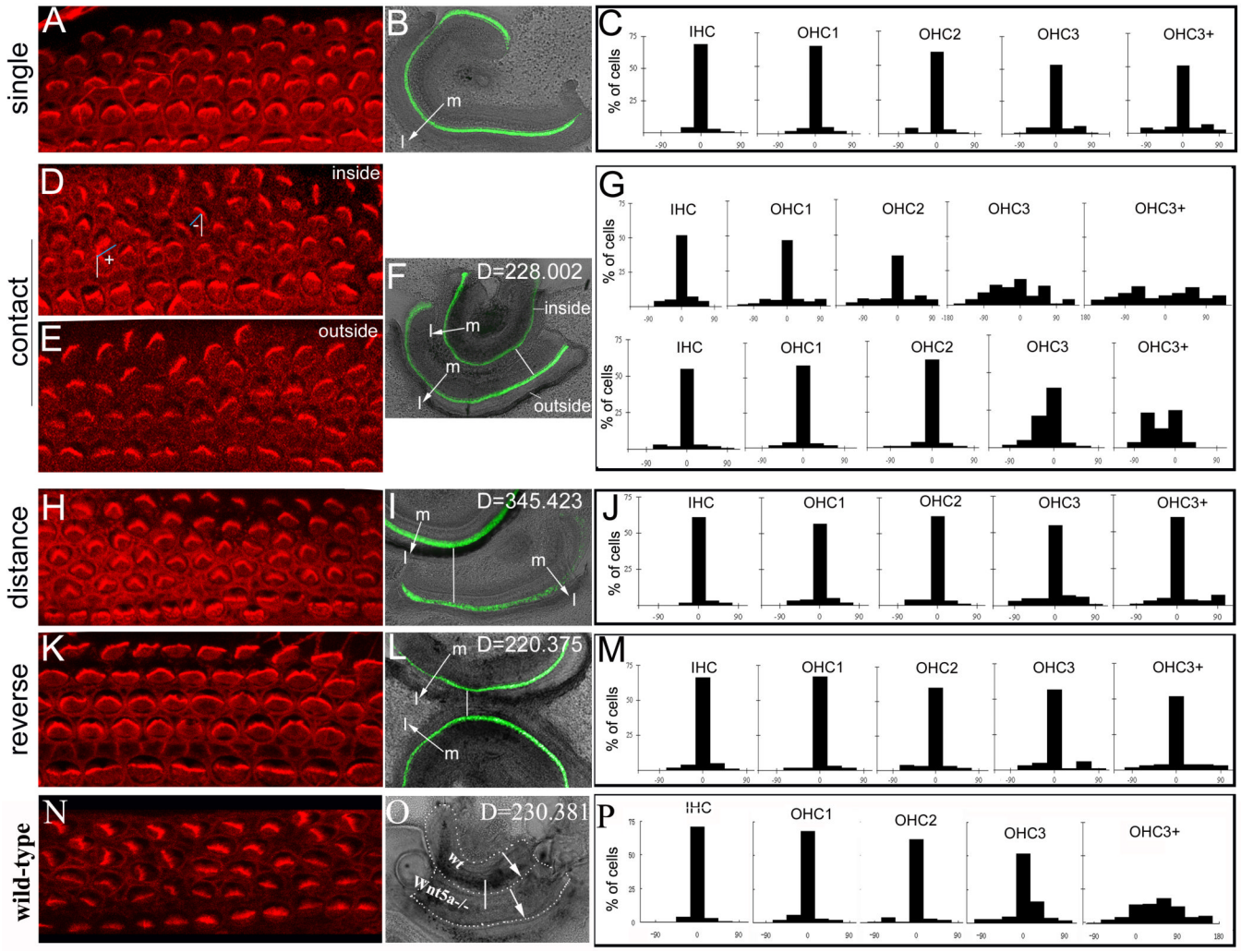

Fig. 8. Potential instructive cues along the mediolateral axis of the cochlea

(A-P): The orientation of stereociliary bundles was visualized with phalloidin staining (A,D,E,H,K,N) in wild-type cochleae cultured singly (A,B), in parallel closely (D-F), in parallel distantly (H,I), in anti-parallel (K,L), and between closely parallel *Wnt5a*$^{-/-}$ and wild-type cochleae (N,P) The distribution of stereociliary bundle orientation in each row of hair cells cochleae cultured singly (C, n=11), in closely parallel (G, n=4), in distantly parallel (J, n=4), in anti-parallel (M, n=4), and between *Wnt5a*$^{-/-}$ and wild-type cochleae (P, N=3) was quantified and plotted on the histograms. The angles formed between the mediolateral axis (D, white lines) and the lines bisecting the "V"-shaped stereocilia (D, blue lines) were used for quantifications as described in Methods. This angle is close to 0 in normally oriented stereocilia. As diagramed in (D), the angles formed at the right or the left sides of the mediolateral axis were designated to be positive or negative, respectively. The lines (F,I,L,O) and arrows (B,F,I,L,O) indicate the distance between the two cochleae and the orientation of the mediolateral axes of the cochleae, respectively. The green signal (B, F, I, L) is from GFP expressed under Math1 enhancers and marks the hair cells. In the grafts between *Wnt5a*$^{-/-}$ and wild-type cochleae (O), GFP was not present and the cochleae were outlined by dashed lines. D: units of the distance between the two cochleae cultured together using NIH ImageJ (F, I, L,O); m: the medial side of the cochlea; l: the lateral side of the cochlea.