Restriction of Wnt signaling in the dorsal otocyst determines semicircular canal formation in the mouse embryo

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Restriction of Wnt signaling in the dorsal otocyst determines semicircular canal formation in the mouse embryo

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Highlights

• Canonical Wnt signaling becomes restricted in the dorsal otocyst of mouse embryos.
• Forced persistence of canonical Wnt signaling delays fusion plate formation.
• Down-regulation of Wnt signaling is required for breakdown of the basal lamina.
• Canonical Wnt signaling in the otocyst regulates cell proliferation and apoptosis.
Abstract
The mouse inner ear develops from a simple epithelial pouch, the otocyst, with the dorsal and ventral portions giving rise to the vestibule and cochlea, respectively. The otocyst undergoes a morphological change to generate flattened saclike structures, known as outpocketings, in the dorsal and lateral regions. The semicircular canals of the vestibule form from the periphery of the outpocketings, with the central region (the fusion plate) undergoing de-epithelialization and disappearing. However, little is known of the mechanism that orchestrates formation of the semicircular canals. We now show that the area of canonical Wnt signaling changes dynamically in the dorsal otocyst during its morphogenesis. The genes for several Wnt ligands were found to be expressed in the dorsal otocyst according to specific patterns, whereas those for secreted inhibitors of Wnt ligands were expressed exclusively in the ventral otocyst. With the use of whole-embryo culture in combination with potent modulators of canonical Wnt signaling, we found that forced persistence of such signaling resulted in impaired formation both of the lateral outpocketing and of the fusion plates of the dorsal outpocketing. Canonical Wnt signaling was found to suppress Netrin1 expression and to preserve the integrity of the outpocketing epithelium. In addition, inhibition of canonical Wnt signaling reduced the size of the otocyst, likely through suppression of cell proliferation and promotion of apoptosis. Our stage-specific functional analysis suggests that strict regulation of canonical Wnt signaling in the dorsal otocyst orchestrates the process of semicircular canal formation.

Keywords: otocyst, semicircular canal, morphogenesis, canonical Wnt signaling, whole-embryo culture
**Introduction**

Wnt ligands are secreted proteins that regulate various biological processes including embryonic development (Cadigan and Nusse, 1997; Logan and Nusse, 2004), self-renewal of stem cells (Goessling et al., 2009; Reya et al., 2003), and carcinogenesis (Harada et al., 1999). Wnt proteins diffuse to form concentration gradients and bind to their receptor Frizzled, a protein containing seven transmembrane domains. Wnt signaling is mediated by at least three pathways: the canonical pathway, the planer cell polarity pathway, and the Ca\(^{2+}\) pathway. Which pathway operates in a given cell is thought to depend on the specific Wnt ligand and the association of Frizzled with different coreceptors. In the canonical pathway, Wnt ligands bind to Frizzled and the coreceptors LRP5 or LRP6 (Kelly et al., 2004; Pinson et al., 2000), resulting in the activation of Dishevelled by phosphorylation. Dishevelled then suppresses the activity of a degradation complex (Axin-APC-GSK3) that targets β-catenin, resulting in the accumulation of β-catenin in the cytosol and its interaction with the transcriptional regulators Tcf or Lef. The resulting complex translocates to the nucleus and activates the transcription of various target genes.

The vertebrate inner ear is a sensory organ embedded in the temporal bone and consists of the cochlea and vestibule (comprising the endolymphatic duct, semicircular canals, saccule, and utricle), which are responsible for hearing and balance, respectively (Martin and Swanson, 1993; Sher, 1971; Streeter, 1906). The inner ear (membranous labyrinth) originates from a simple epithelial sheet (the otic placode) that is induced adjacent to rhombomeres 5 and 6 (Ladher et al., 2000; Torres and Giráldez, 1998). In mice, formation of the otic placode is regulated by the balanced action of fibroblast growth factors (FGFs) and canonical Wnt ligands, both of which diffuse from the neural tube until embryonic day (E) 8.5 (Ladher et al., 2005; Ohyama et al., 2006; Vendrell et al., 2000). The first of several topological changes that occur during inner ear development is otocyst formation, in which the otic placode invaginates to form a cyst structure surrounded by mesenchymal cells. A dorsoventral axis is then conferred on the otocyst by the action of Wnt ligands and Sonic hedgehog (Shh), which emanate from the dorsal and ventral portions, respectively, of the neural tube until E10.5 (Riccomagno et al., 2005). In \(Wnt1^{-/-};Wnt3a^{-/-}\) embryos, the inner ear lacks the entire dorsal portion and a part of the ventral portion (Riccomagno et al., 2005). A second wave of topological changes involves formation of the cochlea and vestibule, including the
semicircular canals (SCs). The dorsal part of the otocyst forms a flattened sac or outpocketing at E11.5, and the opposite epithelial walls of the outpocketing approach each other and fuse in two regions at E12.5. The fused regions of the epithelium, referred to as fusion plates (FPs), subsequently disappear, resulting in the formation of hollow ducts that become the superior and posterior SCs. Concurrently, another outpocketing that forms at the lateral side of the otocyst (E12.0) gives rise to the lateral SC by a similar process (E12.5). In the chick, apoptosis in the cells of the prospective FP is involved in the fusion and subsequent disappearance of the FP (Fekete et al., 1997). A process of de-epithelialization also contributes to FP formation. The basal lamina beneath the epithelial wall of the prospective FP is broken down before the fusion event, first in the lateral and then the medial wall in the mouse (Martin and Swanson, 1993; Salminen et al., 2000) or simultaneously in both walls in the chick (Fekete et al., 1997). In the process of FP disappearance, the de-epithelialized cells of the FP are retracted into the canals in mice (Martin and Swanson, 1993).

Various molecules have been implicated in dorsal otic morphogenesis. Dlx5 and -6, Hmx2 and -3, and Gbx2, all of which are transcription factors expressed in the dorsal otocyst, are required for development of the vestibular organs (Robledo and Lufkin, 2006; Wang et al., 2004; Lin et al., 2005). Bone morphogenetic protein (BMP) signaling in the dorsolateral portion of the otocyst promotes expansion of the dorsal outpocketing by inducing a change in the shape of cells in the otic epithelium (Chang et al., 2008; Ohta et al., 2010). FGF9 and Netrin1 expressed in the prospective FP play an important role in FP formation by promoting the proliferation of surrounding mesenchymal cells (FGF9 and Netrin1) and by inducing the breakdown of the basal lamina beneath the prospective FP (Netrin1) (Pirvola et al., 2004; Salminen et al., 2000). During formation of the lateral SC, Lrig3 engages in a cross-repressive interaction with Netrin1 to define the region of the epithelium destined to form the canal rather than the FP (Abraira et al., 2008). Finally, mice deficient in Otx1 also lack the lateral SC (Acampora et al., 1996; Morsli et al., 1999). Given that development of the inner ear is dependent on the action of these various molecules at appropriate times and specific places, it might be expected that expression of the corresponding genes is coordinated by an overarching mechanism.

During our studies of inner ear development in the mouse, we noticed that canonical Wnt signaling gradually becomes restricted in the dorsal otocyst before SC formation,
suggesting that such restriction might contribute to vestibular morphogenesis. The roles of the canonical Wnt signaling pathway in inner ear formation have been examined mostly by analysis of mutant mice. However, the early defects of some such mutants have hampered characterization of the roles of this pathway in otocyst development. To overcome this hurdle, we developed a whole-embryo culture system (Cockroft, 1990) in which the endogenous canonical Wnt pathway can be readily up- or down-regulated during specific time windows. With this system, we were able to analyze the effect of canonical Wnt signaling on SC formation. Our results shed light on the mechanism responsible for restriction of Wnt activity and orchestration of the molecules that regulate SC formation.

**Materials and Methods**

**Mice**
Female ICR mice in estrus were mated with male ICR mice or male BAT-gal transgenic mice (kindly provided by H. Hamada, Osaka University, Japan). Noon on the day of vaginal plug detection was designated as E0.5, and the pregnant mice were subsequently killed for isolation of embryos at appropriate stages from the uterus. This study was approved by the Animal Care and Use Committee of Kyushu University.

**Whole-embryo culture**
Embryos isolated at E11.5 without damage to the placenta or yolk sac were transferred to Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The yolk sac was cut with microscissors to create a hole through which the embryo was placed out of the yolk sac. The amnion was torn with the use of forceps. Embryos with the placenta and membranes were then transferred to 50-ml centrifuge tubes containing DMEM supplemented with 25% rat serum, and the air in the tubes was replaced with a mixture of 5% CO₂ and 95% O₂. Embryos were cultured with rotation of the tubes in a humidified incubator at 37°C. The following compounds were added to the culture medium: CHIR99021, an inhibitor of GSK3β (Axon Medchem BV); XAV939, a Tankyrase inhibitor (Sigma-Aldrich); z-VAD-fmk, a pan-caspase inhibitor (Enzo Life Sciences); or dimethyl sulfoxide (DMSO) as a vehicle control. For labeling with bromodeoxyuridine (BrdU), BrdU (50 µM) was added to the culture medium 10 min before termination of whole-embryo culture. Culture was terminated by washing the embryos with phosphate-buffered saline (PBS) and fixation.
with 4% paraformaldehyde.

**In situ hybridization**

Embryos fixed with 4% paraformaldehyde were washed with PBS containing 0.1% Tween 20 (PBS-T), exposed to 20% (w/v) sucrose, and embedded in OCT compound (Sakura). Cryosections cut at a thickness of 10 µm were placed on MAS-GP–coated slide glasses (Matsunami) and air-dried. They were subsequently incubated with PBS-T for 5 min and then with PBS-T containing proteinase K (5 mg/ml) for 15 min, fixed again with 4% paraformaldehyde and 0.1% glutaraldehyde, incubated with 0.1 M tetrathanolamine-HCl and 0.25 M acetic anhydride (Sigma) for 10 min, and permeabilized with 0.3% Triton X-100 in PBS for 25 min. The sections were exposed to hybridization solution [50% formamide, 5× saline sodium citrate, 1% SDS, 2× Denhardt solution, yeast RNA (50 µg/ml), heparin (50 µg/ml)] alone before incubation overnight with the same solution containing a digoxigenin-labeled riboprobe. They were then washed several times with saline sodium citrate solutions of increasing stringency, incubated with alkaline phosphatase–conjugated antibodies to digoxigenin (Roche), and washed extensively before detection of immune complexes with nitroblue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (Roche). The sections were observed with a fluorescence microscope (Leica DM5000) and photographed with a digital camera (Leica DFC300FX).

**Immunohistofluorescence analysis**

Embryos fixed with 4% paraformaldehyde were washed with PBS containing 0.1% Triton X-100, dehydrated with a series of methanol solutions of increasing concentration, treated with isopropanol, and embedded in Paraplast Plus (Leica) for sectioning at a thickness of 8 µm. The sections were placed on MAS-coated slide glasses (Matsunami) and subjected to immunofluorescence analysis as described previously (Oki et al., 2007). For the detection of E-cadherin and cleaved caspase-3, the sections were first treated with 10 mM sodium citrate buffer (pH 6.0) at 105°C for 1 min or at 121°C for 5 min, respectively. Primary antibodies included those to laminin (Abcam), to β-galactosidase (Abcam), to E-cadherin (Santa Cruz Biotechnology), to cleaved caspase-3 (Cell Signaling Technology), and to BrdU (MBL). After incubation with primary antibodies, the sections were washed with PBS containing 0.1% Triton X-100 before incubation with Alexa Fluor 488– or Alexa Fluor 568–conjugated secondary antibodies (Molecular Probes). Nuclei and F-actin were stained with
4′,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488-phalloidin (Molecular Probes), respectively. Sections were observed with a fluorescence microscope (Leica DM5000) and photographed with a digital camera (Leica DFC300FX).

**Three-dimensional reconstruction**
Serial coronal sections encompassing the entire region of the otocyst were prepared from paraffin-embedded tissue as described above. All sections were photographed, and the digital images were processed for three-dimensional reconstruction with Amira software (Visage Imaging). Three-dimensional images of the otocyst were generated by volume-rendering of the otocyst lumen, and dimensions along the dorsoventral and anteroposterior axes were measured.

**Statistical analysis**
Data are presented as means ± SD and were compared with the Mann-Whitney test or Student's *t* test. A *p* value of <0.05 was considered statistically significant.

**Results**

**Gradual restriction of canonical Wnt signaling during otic morphogenesis**
Whereas canonical Wnt signaling plays an important role in induction of the otic placode and its axial determination, its contribution to otic morphogenesis at later stages has remained unclear. We therefore first examined the activity of the canonical signaling pathway in the developing otocyst with the use of the BAT-gal transgenic mouse, a Wnt reporter strain that harbors a *lacZ* (β-galactosidase) transgene driven by seven Tcf/Lef binding sites (Maretto et al., 2003). At E10.5, staining with the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was observed throughout the dorsal otocyst (*n* = 5), which is destined to become the endolymphatic duct (ED) and SCs (Fig. 1A, D). Staining apparent at the periotic mesenchyme was likely due to leakage of the pigment from the epithelium, give that β-galactosidase was not detected in the mesenchyme by immunostaining (data not shown). The staining pattern we observed is consistent with that described in a previous study with the TOP-gal transgenic mouse, another Wnt reporter strain (Riccomagno et al., 2005). We found that staining was most intense in the medial wall of the otocyst (Fig. 1D). From E11.5, dynamic morphologic changes occur in the dorsal otocyst. The dorsolateral and dorsomedial epithelial walls thus fuse with each other to form the FPs, and the lateral wall begins to form the outpocketing that gives rise to the lateral SC. At
this stage, the intensity of X-gal staining had decreased in the dorsolateral wall of the otocyst with the exception of that in a small portion of the wall \((n = 5)\) (Fig. 1B, E). At E12.2, X-gal staining was detected in the ED, the canal portion and FPs of the dorsal outpocketing, and the canal portion of the lateral outpocketing \((n = 5)\) (Fig. 1F). Given that the activity of \(\beta\)-galactosidase persists longer than \(\text{lacZ}\) expression (Schwanhäusser et al., 2011), we also performed in situ hybridization for \(\text{lacZ}\) transcripts. Expression of \(\text{lacZ}\) was found to be down-regulated in the dorsolateral otocyst from E10.5 \((n = 5)\) to E11.5 \((n = 5)\), showing a pattern similar to that of X-gal staining at E11.5 (Fig. 1G, H), and the expression was further restricted to the ED at E12.2 \((n = 8 \text{ of } 12)\) (Fig. 1I). The X-gal staining apparent in the FPs and most of the prospective SC regions at E12.2 was thus likely due to remnant \(\beta\)-galactosidase. These results indicated that the canonical Wnt pathway is activated during morphogenesis of the vestibular organs but gradually becomes restricted to the ED and the canal portions of the outpocketings.

**Differential distributions of Wnt signaling components in the developing otocyst**

To investigate how the activity of the canonical Wnt pathway becomes restricted during development of the mouse otocyst, we examined the expression patterns of genes for Wnt ligands, Wnt inhibitors, and transcription factors that contribute to this pathway (Fig. 2). At E12.2, \(Wnt6\) was expressed in the dorsal otocyst except the prospective SC region \((n = 4)\) (Fig. 2G). More restricted expression patterns were observed for \(Wnt2b\) \((n = 4)\) and \(Wnt3\) \((n = 8)\), which were expressed in the ED and lateral wall of the FPs, respectively (Fig. 2B, C). \(Wnt9b\) was expressed at a low level in the FPs \((n = 4)\) (Fig. 2H; data not shown). \(Wnt5a\), which encodes a noncanonical Wnt ligand, was expressed in the mesenchymal cells surrounding the dorsal otocyst, which are destined to become the bony labyrinth, as well as in the canal portion of the dorsal outpocketing \((n = 4)\) (Fig. 2F). \(Wnt4\), which encodes another noncanonical Wnt ligand, was expressed in the ventral otocyst \((n = 6)\), which is destined to become the cochlear duct (CD) (Fig. 2E). Expression of \(Wnt1\), \(Wnt3a\), or \(Wnt8a\) was not detected in the otocyst \((n = 4)\) (Fig. 2A, D; data not shown).

Expression of genes for soluble Frizzled-related proteins (Sfrps) and Dickkopf (Dkk), which interfere with canonical Wnt ligands in the extracellular space (Finch et al., 1997; Glinka et al., 1998; Mukhopadhyay et al., 2001; Satoh et al., 2008; Shirozu et al., 1996), was found to be largely restricted to the ventral part of the otocyst. \(Sfrp1\) was thus expressed in the epithelia of the CD region and the ventral part of the lateral
outpocketing \((n = 6)\) (Fig. 2J), whereas \(Dkk1\) expression was restricted to the lateral wall of the ventral otocyst \((n = 4)\) (Fig. 2I), which gives rise to the utricle and CD. On the other hand, \(Sfrp2\) was expressed in the ventral otocyst and the lateral wall of the dorsal FPs \((n = 4)\) (Fig. 2K). \(Frzb\) (also known as \(Sfrp3\)) was expressed in the dorsolateral mesenchymal cells surrounding the otocyst and in the medial wall of the prospective CD \((n = 4)\) (Fig. 2L). The expression of \(Sfrp5\) was not detected (data not shown, \(n = 3\)).

Finally, we examined the expression of Tcf and Lef transcription factors, which mediate canonical Wnt signaling together with \(\beta\)-catenin (Logan and Nusse, 2004). \(Tcf1\) was expressed in the dorsal otocyst \((n = 6)\) (Fig. 2M), consistent with the pattern of X-gal staining in BAT-gal transgenic embryos (Fig. 1F). \(Lef1\) was expressed in the dorsolateral otic epithelium and the mesenchyme around the ventral otocyst \((n = 4)\) (Fig. 2N). \(Tcf3\) \((n = 4)\) and \(Tcf4\) \((n = 4)\) were expressed throughout the entire otocyst (Fig. 2O, P).

Together, these results showed that the expression of canonical Wnt ligands occurs mostly in the dorsal otocyst, whereas Wnt-inhibitory factors are expressed in the ventral otocyst and mesenchyme. Such a complementary pattern might be expected to ensure that the canonical Wnt pathway is activated locally in the dorsal otocyst.

**Increased canonical Wnt signaling perturbs SC formation**

The dynamic changes in canonical Wnt signaling apparent during otic development suggest that the appropriate level of signaling may be important for formation of the vestibular organs. To explore this possibility, we performed whole-embryo culture in the presence of potent inhibitors of components of the canonical pathway. Without the input of Wnt ligands, the GSK3\(\beta\)-Axin complex destabilizes cytosolic \(\beta\)-catenin. We therefore examined the effects of CHIR99021, which inhibits GSK3\(\beta\) (Cline et al., 2002; Ring et al., 2003), as well as those of XAV939, which increases Axin stability through inhibition of Tankyrase (Huang et al., 2009); these compounds thus respectively promote and suppress canonical Wnt signaling. To optimize the concentrations of these compounds, we cultured BAT-gal transgenic embryos \((n = 3)\) at E7.5 for 18 h in the presence of various concentrations and then subjected the embryos to in situ hybridization analysis of \(lacZ\) mRNA (Fig. 3A–C). For CHIR99021, we selected a concentration of 20 \(\mu\)M, given that \(lacZ\) expression was markedly enhanced and no morphological abnormalities were apparent at this level (Fig. 3A, B). For
XAV939, we found that 100 µM was the optimum concentration, given that lacZ
expression was substantially inhibited and again no morphological anomalies were
observed (Fig. 3A, C).

We next cultured BAT-gal transgenic embryos at E11.5 with these compounds for
18 h, during which period the dorsal FPs and lateral outpocketing are formed in control
embryos. In control cultured embryos (n = 11), lacZ expression was observed in the ED
(Fig. 3D, G), similar to the pattern apparent in embryos collected at E12.2 (Fig. 1F).
Exposure to CHIR99021 resulted in a marked increase in lacZ expression throughout
the dorsal otocyst including the ED and dorsal and lateral outpocketings (n = 11) (Fig.
3E, H). Histological sections showed that formation of the lateral outpocketing was
severely affected by CHIR99021, with the width being enlarged along the dorsoventral
axis (n = 8 of 11). We further examined the abnormal morphogenesis in
three-dimensional reconstructions of the inner ear cavity generated from serial sections
of the otocyst (Fig. 4A, B, D, E). In addition to the lateral outpocketing defect (n = 4 of
5), we found that dorsal FP formation was delayed in CHIR99021-treated embryos (n =
2 of 5). Consistent with such a delay, the lateral epithelial layer of the dorsal
outpocketing was thickened compared with that in control embryos (21.5 versus 16.9
µm, n = 5; p = 0.0707, Student's t test), whereas no significant changes in epithelial
thickness were detected elsewhere (Fig. 4K, L). Given that the dorsal outpocketing
fuses and collapses after the epithelial layer thins, a low level of Wnt signaling may be
required for FP disappearance.

Expression of lacZ was not detected in the otocyst of XAV939-treated embryos (n =
11) (Fig. 3F, I), indicating that canonical Wnt signaling was eliminated, at least at the
end of the culture period. Whereas these embryos showed no apparent specific
morphological defects, three-dimensional reconstruction indicated that, although the
shape of the otocyst was normal, its size was reduced along the vertical and horizontal
axes (n = 4; p = 0.0832 and p = 0.0433, respectively) (Fig. 4A, C, D, F, J). In addition,
the epithelial layer of the lateral outpocketing in XAV939-treated embryos was thinner
than that in control embryos (Fig. 4L). These effects of both CHIR99021 and XAV939
on epithelial thickness may be due to a change in cell size, given that the epithelial layer
of the lateral outpocketing maintained its monolayered columnar morphology in the
presence of each agent (Fig. 4G–I).

Collectively, these results suggested that canonical Wnt signaling in the dorsal
otocyst from E11.5 is required for otocyst growth, and that down-regulation of such signaling in the lateral wall of the otocyst is necessary for formation of the dorsal FPs and lateral outpocketing.

**Wnt signaling increases cell number in the vestibular region**

The reduced size of the otocyst formed in the presence of XAV939 suggested that canonical Wnt signaling regulates cell number in this structure. To examine this possibility, we performed BrdU labeling as well as immunostaining of cleaved caspase-3 (Urase et al., 1998) for the detection of cell proliferation and apoptosis, respectively. In control embryos, BrdU incorporation was observed mostly in the medial wall and the lateral outpocketing at E12.2 ($n = 4$), whereas the cleaved form of caspase-3 was detected throughout the otocyst including the dorsal FPs and lateral outpocketing ($n = 7$) (Fig. 5A), consistent with the results of previous studies in which apoptotic cells were detected with the TUNEL assay (Cecconi et al., 2004; Lang et al., 2000). In CHIR99021-treated embryos, BrdU incorporation was increased in the impaired lateral outpocketing ($n = 4$) (Fig. 5B, D, F), where canonical Wnt signaling was up-regulated (Fig. 3H). Furthermore, the number of cells positive for cleaved caspase-3 was greatly reduced in the dorsal and lateral outpocketings of the otocyst ($n = 7$) (Fig. 5B, E). In contrast, in XAV939-treated embryos, the number of apoptotic cells was significantly increased in the dorsal and lateral outpocketings ($n = 7$) (Fig. 5C, E), suggesting that canonical Wnt signaling suppresses apoptosis in this region. No significant differences in BrdU incorporation were apparent between XAV939-treated and control embryos ($n = 4$) (Fig. 5A, C, F). These results thus suggested that canonical Wnt signaling not only promotes cell proliferation but also suppresses apoptosis in the otocyst. The extension of the lateral canal by CHIR99021 treatment might therefore be explained by the enhanced cell proliferation in this area.

**Canonical Wnt signaling regulates the expression of genes related to SC formation**

To understand further the defective morphogenesis of the otocyst induced by aberrant canonical Wnt signaling, we investigated the expression patterns of marker genes in the otocyst of cultured embryos. *Dlx5*, which encodes a homeobox transcription factor and is a Wnt target gene (Robledo and Lufkin, 2006), was expressed in the ED and the ventral portion of the prospective lateral SC ($n = 7$) (Fig. 6A). The level of *Dlx5* expression was slightly increased and decreased by treatment with CHIR99021 ($n = 7$) or XAV939 ($n = 7$), respectively (Fig. 6E, I), and the expression domain in the lateral
outpocketing was expanded to the dorsal side in CHIR99021-treated embryos. Gbx2, another Wnt target gene in the otocyst (Riccomagno et al., 2005), was expressed mostly in the ED, but the expression pattern was not affected by either CHIR99201 or XAV939 \((n = 4, \text{ data not shown})\). We next examined the expression of Lrig3, Otx1, and Tbx1 as markers of the ventrolateral otocyst (Abraira et al., 2008; Morsli et al., 1999; Vitelli et al., 2003). The expression level of Lrig3 was increased and decreased in CHIR99021- or XAV939-treated embryos \((n = 6)\), respectively (Fig. 6C, G, K). By contrast, Tbx1 expression was not affected by treatment with either compound \((n = 8, \text{ data not shown})\). Otx1 expression was not affected by CHIR99021 treatment \((n = 6 \text{ of } 8)\) (Fig. 6D, H, L), consistent with the previous observation that Otx1 expression is lost in the early-stage otocyst of a conditional mutant with stabilized \(\beta\)-catenin (Freyer and Morrow, 2010). Given that Otx1 mutant mice lack the lateral SC, the defect in formation of the lateral outpocketing induced by CHIR99021 is likely explained by the reduction in Otx1 expression. We finally examined the expression of Netrin1 and Fgf9, both of which contribute to FP formation (Pirvola et al., 2004; Salminen et al., 2000). Netrin1\(^{-/-}\) embryos manifest a delay in fusion of the prospective FP (Salminen et al., 2000) similar to that observed in CHIR99021-treated embryos (Fig. 4). Netrin1 was expressed in the FPs and floor plate of control embryos \((n = 10)\) (Fig. 6B). Netrin1 expression was suppressed in the prospective FP but was unaffected in the floor plate of CHIR99021-treated embryos \((n = 8)\) (Fig. 6F). On the other hand, no obvious change of Netrin1 expression was observed in XAV939-treated embryos \((n = 5 \text{ of } 8)\) (Fig. 6J). Fgf9 expression was not affected by either CHIR99021 or XAV939 \((n = 7, \text{ data not shown})\). Together, these results suggested that the persistent canonical Wnt signaling in CHIR99021-treated embryos suppresses Netrin1 expression and thereby delays FP formation.

**Integrity of the basal lamina is maintained by Wnt signaling**

The SCs are formed as a result of the disappearance of the FPs. The breakdown of the basal lamina underlying the FPs is thought to be required for their disappearance, and this process is thought to be regulated by Netrin1 (Salminen et al., 2000). To examine whether breakdown of the basal lamina is affected in CHIR99021-treated embryos, we performed immunostaining for laminin and E-cadherin, components of the basal lamina and adherens junctions, respectively. In control embryos \((n = 8)\), laminin immunoreactivity was continuously localized beneath the medial wall of the prospective
FP but became discontinuous beneath the lateral wall, indicative of breakdown of the basal lamina (Fig. 7A). Consistent with this pattern, E-cadherin immunoreactivity was also diminished only in the dorsolateral region of control embryos \((n = 6)\) (Fig. 7D). These findings suggested that the dorsolateral cells are destined to become de-epithelialized before E11.5. In CHIR99021-treated embryos \((n = 6\) of 8), laminin was detected continuously along the prospective FP (Fig. 7B), indicating that the basal lamina was not degraded. In contrast, no obvious change in the pattern of laminin immunostaining was observed in XAV939-treated embryos \((n = 8)\) (Fig. 7C). Localization of E-cadherin in the prospective FP remained uniform in about half of CHIR99021-treated embryos \((n = 4\) of 7) (Fig. 7E), whereas it was unaffected by XAV939 treatment \((n = 7)\) (Fig. 7F). The persistent activation of the canonical Wnt signaling pathway in embryos treated with CHIR99021 was thus found to prevent breakdown of the basal lamina, consistent with the observed down-regulation of Netrin1.

In summary, canonical Wnt signaling in the dorsal otocyst is down-regulated in both the lateral wall of the dorsal outpocketing and the lateral outpocketing between E11.5 and E12.2. However, forced persistence of Wnt signaling suppresses Otx1 and Netrin1 expression and enhances Lrig3 expression, possibly accounting for the associated aberrant lateral outpocketing and delay in FP formation. Proper formation of the SCs therefore requires down-regulation of canonical Wnt signaling in the center of the vestibular region, which likely results in part from the distribution of Wnt-inhibitory factors.

**Discussion**

**Dynamic regulation of Wnt signaling in the vestibular region of the otocyst**

We have found that canonical Wnt signaling changes dynamically during otocyst development. Although such signaling was initially apparent throughout the epithelial layer of the dorsal otocyst, it subsequently became restricted before the shape change. The signaling disappeared first in the lateral wall of the otocyst, with the exception of the area where protrusion of the lateral outpocketing occurs. The signaling then disappeared in the canal region and FPs of the dorsal outpocketing, becoming restricted to the ED, medial wall of the utriclo-saccular space, and dorsal wall of the lateral outpocketing by E12.2. The area of signaling at E12.2 is likely determined by the
combined expression patterns of canonical Wnt ligands and their secreted inhibitors. For example, Wnt2b and Wnt6 were found to be expressed in the ED, whereas Wnt6 was expressed in the dorsal outpocketing, with the exception of the prospective SC region, and Wnt3 was expressed in the lateral wall of the dorsal outpocketing. On the other hand, Sfrp2 was expressed in the lateral wall of the dorsal outpocketing, suggesting that Sfrp2 suppresses the activity of Wnt3 and Wnt6 in this region of the prospective FP. Furthermore, given that Netrin1 is thought to suppress canonical Wnt signaling (Lopez-Rios et al., 2008), Netrin1 expressed in the prospective FP may also suppress Wnt3 and Wnt6 activity in this area. Given that we found that the loss of canonical Wnt signaling is required for formation of the FPs and the lateral outpocketing, the balance between Wnt ligands and their antagonists would be expected to be important for SC formation. The importance of such balance may be revealed by examination of mice lacking both Wnt and Sfrp genes whose expression overlaps in the otocyst.

**Persistence of Wnt signaling affects SC formation**

The roles of canonical Wnt signaling in the otocyst have remained unclear because knockout mice for individual Wnt genes are not informative with regard to morphogenesis of the otocyst either because they die at an early stage of development (Liu et al., 1999; Logan and Nusse, 2004) or because of likely redundancy in Wnt gene expression in the otocyst (Riccomagno et al., 2005; Sienknecht and Fekete, 2009). A conditional mutant lacking β-catenin in the otocyst was recently found to show early (E10.5) defects in the otocyst (Freyer and Morrow, 2010). We have now manipulated the level of canonical Wnt signaling in cultured embryos with the use of potent modulators of such signaling. This experimental system appears reliable, given that CHIR99021 enhanced and maintained lacZ expression in the dorsal otocyst of BAT-gal embryos, whereas lacZ expression was not detected in the ventral portion of the otocyst that contributes to the saccule and CD or in the mesenchyme surrounding the otocyst. We were therefore able to analyze the effect of persistence of canonical Wnt signaling specifically in the dorsal otocyst.

Most of the lateral wall of the otocyst loses canonical Wnt signaling during its development. The persistence of canonical Wnt signaling induced by treatment with CHIR99021 was found to affect structures derived from the lateral wall, namely the lateral outpocketing and the lateral side of FPs of the dorsal outpocketing. In the chick, forced expression of β-catenin by retroviral infection at an early stage of development
was found to result in malformation of the entire otocyst (Stevens et al., 2003), with such malformation possibly including defects similar to those induced by CHIR99021 in the present study. While formation of the lateral outpocketing was impaired in most embryos when CHIR99021-treated culture was initiated before E11.5 ($n = 6$ of $7$), it was normal when the culture was initiated after E11.5 ($n = 3$ of $4$). These results suggest that the developmental program for formation of the lateral outpocketing is decided by E11.5. Importantly, we found that canonical Wnt signaling regulates the expression of *Lrig3* and *Netrin1*, both of which are involved in FP formation. CHIR99021 treatment induced down-regulation of *Netrin1* expression in the dorsal prospective FP and the dorsal wall of the lateral outpocketing. *Netrin1* expression in the prospective FP from E11.5 induces the fusion and collapse of the FP by promoting the proliferation of mesenchymal cells and degradation of the basal lamina (Salminen et al., 2000). In CHIR99021-treated embryos, we found that both E-cadherin expression in the FP as well as the associated basal lamina were preserved, suggesting that the canonical Wnt signaling pathway prevents FP de-epithelialization. On the other hand, *Lrig3* expression in the lateral outpocketing was increased by CHIR99021 treatment. In the lateral outpocketing, the FP is formed as a result of a cross-repressive interaction between *Lrig3* and *Netrin1* (Abraira et al., 2008). Given that formation of the lateral outpocketing itself was impaired by CHIR99021 in our system, we were not able to address whether canonical Wnt signaling plays a role in subsequent formation of the lateral FP.

**Regulation of cell proliferation and apoptosis by Wnt signaling**

In contrast to the severe defects observed in embryos treated with CHIR99021, XAV939 did not induce specific morphological defects in the otocyst but rather reduced the size of the entire structure. This relatively mild abnormality induced by XAV939 suggests that morphogenetic events dependent on the canonical Wnt pathway are completed by E11.5, when embryo culture was initiated. Alternatively, a more pronounced effect of XAV939 might become apparent at later stages of otic development. Our experiments with XAV939 and CHIR99021 revealed that canonical Wnt signaling increases cell number in the otocyst both by promoting cell proliferation and by inhibiting apoptosis. The canonical Wnt pathway activates the expression of various genes related to cell proliferation including those for c-Myc and cyclin D (Harada et al., 1999; Kioussi et al., 2002). In mouse embryos, the appropriate level of Wnt signaling is important for
control of cell number during both neurogenesis (Hirsch et al., 2007; Lie et al., 2005) and heart development (Kioussi et al., 2002). On the other hand, Wnt signaling also inhibits apoptosis through the action of Tcf4 (Chen et al., 2001). Given that we found that Tcf4 is expressed throughout the otocyst, the canonical Wnt pathway may suppress apoptosis through Tcf4. Canonical Wnt signaling might therefore function to ensure a sufficient number of cells before SC formation by both promoting cell proliferation and inhibiting apoptosis. Aberrant apoptosis is responsible for various congenital anomalies of the inner ear, although the stages at which apoptosis contributes to otic morphogenesis remain unclear. In mice deficient in Apaf1 or caspase-9, both cell proliferation and apoptosis are attenuated in the dorsal otocyst and the size of the anterior SC is reduced (Cecconi et al., 2004). In contrast, the posterior SC fails to form as a result of increased apoptosis in Bcl2 knockout mice (Cecconi et al., 2004). In these mutant mice, cell proliferation is regulated so as to compensate for aberrant apoptosis, probably making the phenotype complicated. We found that whole-embryo culture in the presence of z-VAD-fmk, a potent inhibitor of apoptosis, did not induce any apparent malformation of the otocyst (data not shown), although it is possible that an effect of such inhibition of apoptosis may become evident at a later stage of otocyst development. The mutant phenotype and our results suggest that the coordination between cell proliferation and apoptosis is important for the morphogenesis of dorsal otocyst. It would be interesting to see whether canonical Wnt is involved in such coordination at the later stage of otocyst development.

Finally, on the basis of our results, we propose a model for SC formation that is dependent on strict control of canonical Wnt signaling. First, the cell number increases and the integrity of the epithelium is maintained in the dorsal otocyst as a result of continuous expression of canonical Wnt ligands from an early stage of development. Second, the FPs formed in the center of the dorsal outpocketing disappear and thereby give rise to the superior and posterior SCs as a result of localized suppression of canonical Wnt signaling. The down-regulation of Wnt signaling induces the expression of Netrin1, which in turn results in breakdown of the basal lamina. Consistent with this notion, breakdown of the basal lamina occurs first in the lateral wall of the prospective FPs, in which the disappearance of Wnt signaling occurs earlier than in the medial wall. The signaling is then lost in the medial wall, which is followed by the disappearance of the FPs and formation of the SCs. Third, canonical Wnt signaling is lost by E11.5 in the
prospective lateral outpocketing region, resulting in localized Otx1 expression and formation of the lateral outpocketing.

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Figure Legends

**Fig. 1** Canonical Wnt signaling becomes restricted during otocyst morphogenesis. (A–F) X-gal staining of BAT-gal mouse embryos at E10.5 (A, D), E11.5 (B, E), and E12.2 (C, F). Full lateral views (A–C) and coronal sections through the otocyst (D–F) indicated by the broken lines in (A) through (C) are shown. The arrowhead in (E) indicates the site where the lateral outpocketing protrudes. The signal in the periotic mesenchyme (asterisks) is likely due to pigment leakage. (G–I) In situ hybridization of lacZ transcripts in BAT-gal embryos at E10.5 (G), E11.5 (H), and E12.2 (I). Ot, otocyst; ED, endolymphatic duct; DOP, dorsal outpocketing; NT, neural tube; PHV, primary head vein; CD, cochlear duct; SSC, superior semicircular canal; FP, fusion plate; LSC, prospective lateral semicircular canal. Scale bar, 100 µm.

**Fig. 2** Expression of genes related to Wnt signaling at E12.2. Coronal sections through the otocyst of mouse embryos were subjected to in situ hybridization with probes for the indicated genes. Areas including the otocyst are shown. Dorsal is to the top and lateral is to the right. Scale bar, 100 µm.

**Fig. 3** Effects of CHIR99021 and XAV939 on canonical Wnt signaling in the otocyst. BAT-gal embryos collected at E7.5 (A–C) or E11.5 (D–I) were cultured for 18 h in the presence of DMSO as a control (A, D, G), 20 µM CHIR99021 (B, E, H), or 100 µM XAV939 (C, F, I). The embryos were then subjected to in situ hybridization analysis of lacZ transcripts (A–C, G–I). Left-side (A–C) and right-side (D–F) views. Coronal sections through the otocyst at the indicated lines in (D–F) are shown (G–I). Bracket represents the impaired lateral outpocketing (H). Scale bar, 100 µm.

**Fig. 4** Effects of CHIR99021 and XAV939 on morphology of the otocyst. (A–F) Three-dimensional reconstruction of the otocyst of embryos collected at E11.5 and cultured for 18 h with DMSO as a control (A, D), 20 µM CHIR99021 (B, E), or 100 µM XAV939 (C, F). Frontal (A–C) and lateral (D–F) views are shown. Arrows and asterisks indicate the lateral outpocketing (LOP) and collapsed dorsal FP, respectively. D, dorsal; V, ventral; L, lateral; M, medial; A, anterior; P, posterior. (G–I) Staining of F-actin with Alexa Fluor 488-phalloidin (red) and of nuclei with DAPI (blue) in coronal sections at the level of the lateral outpocketing obtained from embryos treated with
DMSO (G), CHIR99021 (H), or XAV939 (I). Although the lateral outpocketing maintains its monolayered columnar epithelium, the thickness of the epithelium was decreased in XAV939-treated embryos. Scale bar, 20 μm. (J) Size of the otocyst in embryos treated with DMSO, CHIR99021, or XAV939. The length along the dorsoventral and anteroposterior axes was measured in three-dimensional images similar to those shown in (A) through (F). (K) Schematic representation of the otocyst showing sites at which epithelial thickness was measured. (L) Epithelial thickness in embryos treated with DMSO, CHIR99021, or XAV939. Data in (J) and (L) are means ± SD (n = 6 and 4 embryos, respectively). *p = 0.0433 (Mann-Whitney test), **p = 0.0109 (Student's t test). Scale bar, 20 μm.

Fig. 5 Canonical Wnt signaling promotes cell proliferation and suppresses apoptosis in the otocyst. (A–C) Immunostaining of BrdU (green) and cleaved caspase-3 (Casp3, red) in coronal sections at the level of the otocyst in embryos collected at E11.5 and cultured for 18 h with DMSO as a control (A), 20 μM CHIR99021 (B), or 100 μM XAV939 (C). Nuclei are counterstained with DAPI (blue). White bracket in (A) indicates the lateral outpocketing; that in (B) indicates an area where BrdU incorporation is increased and staining for cleaved caspase-3 is decreased by CHIR99021. (D) Schematic representation of the otocyst indicating the three areas where the numbers of cells positive for BrdU or cleaved caspase-3 were counted. In CHIR99021-treated embryos, a boundary of the impaired lateral outpocketing is recognized as the bend. The numbers of cells in this area were counted. (E) Number of apoptotic cells per unit length in the three areas of interest. Cells positive for cleaved caspase-3 were counted by observing all serial sections including the otocyst. Data are means ± SD (n = 7 embryos). (F) The ratio of BrdU-positive cells to all cells in the three areas of interest. The numbers of BrdU-positive cells and all cells in every fourth serial section including the otocyst were counted. Data are means ± SD (n = 4 embryos). *p = 0.0168, **p = 0.0293, ***p = 0.0012, ****p = 0.0114, *****p = 0.0106 (Student's t test). Scale bar, 100 μm.

Fig. 6 Canonical Wnt signaling regulates the expression of genes required for inner ear morphogenesis. Embryos collected at E11.5 were cultured for 18 h with DMSO as a control (A–D), 20 μM CHIR99021 (E–H), or 100 μM XAV939 (I–L), after which coronal sections at the level of the otocyst were prepared and subjected to in situ
hybridization with probes for Dlx5 (A, E, I), Netrin1 (B, F, J), Lrig3 (C, G, K), or Otx1 (D, H, L). Insets show Netrin1 expression in the floor plate. The expression domains of Lrig3 are indicated by dots. Scale bar, 100 µm.

Fig. 7 Canonical Wnt signaling regulates the localization of laminin and E-cadherin in the otocyst. Embryos collected at E11.5 were cultured for 18 h with DMSO as a control (A, D), 20 µM CHIR99021 (B, E), or 100 µM XAV939 (C, F), after which coronal sections at the level of the otocyst were prepared and subjected to immunostaining of laminin (red) (A–C) or E-cadherin (red) (D–F). Nuclei were counterstained with DAPI (blue). Insets show higher-magnification views of the lateral wall of the dorsal outpocketing. Scale bar, 100 µm.