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Mapping of Wnt, Frizzled and Wnt inhibitor gene expression domains in the avian otic primordium

Ulrike J. Sienknecht and Donna M. Fekete

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

Abstract

Wnt signaling activates at least three different pathways involved in development and disease. Interactions of secreted ligands and inhibitors with cell-surface receptors result in the activation or regulation of particular downstream intracellular cascades. During the developmental stages of otic vesicle closure and beginning morphogenesis, the forming inner ear transcribes a plethora of Wnt-related genes. We report expression of 23 genes out of 25 tested *in situ* hybridization probes on tissue serial sections. Sensory primordia and Frizzled gene expression share domains, with *Fzd1* being a continuous marker. Prospective nonsensory domains express Wnts, whose transcripts mainly flank prosensory regions. Finally, Wnt inhibitor domains are superimposed over both prosensory and nonsensory otic regions. Three Wnt antagonists, *Dkk1*, *SFRP2*, and *Frzb* are prominent. Their gene expression patterns partly overlap and change over time, which adds to the diversity of molecular micro-environments. Strikingly, prosensory domains express Wnts transiently. This includes (1) the prosensory otic region of high proliferation, neuroblast delamination, and programmed cell death at stage 20/21 (*Wnt3*, *-5b*, *-7b*, *-8b*, *-9a*, *-11*), and (2) sensory primordia at stage 25 (*Wnt7a*, *Wnt9a*). In summary, robust Wnt-related gene expression shows both spatial and temporal tuning during inner ear development as the otic vesicle initiates morphogenesis and prosensory cell fate determination.

Keywords

inner ear; otic vesicle; hearing; vestibular; sensory primordia; Wnts; Fzd; Wnt-antagonists

Introduction

Wnt proteins are a large family of secreted factors that have been linked with various developmental processes such as boundary formation, convergence-extension during gastrulation, neural tube closure, cell proliferation, axial polarity of the whole embryo as well as specific organs, cell fate specification, planar cell polarity and axon guidance. Wnts can transmit a signal through at least three intracellular signaling pathways. The canonical pathway exercises control over the stabilization versus degradation of β -catenin, which can act as a transcriptional regulator when present in the nucleus (Dale, 1998). The second pathway influences the release of intracellular calcium (Slusarski et al., 1997; Kohn and Moon, 2005), while the third involves activation of RhoA and has been linked to planar cell polarity (PCP) (Mlodzik, 2002).

The choice of intracellular pathway depends, in part, on the developmental context in which the Wnts act, including receptor availability, relative levels of Wnt inhibitors, and

availability of intracellular signaling components (Moon et al., 1997; Cadigan and Nusse, 1997; Miller et al., 1999; Jones and Jomary, 2002; Hendrickx and Leyns, 2008). For example, it was shown that Wnt5a can signal either through the canonical pathway via Fzd4 and its LRP5 co-receptor or through a calcium-independent non-canonical pathway via Ror2 receptor tyrosine kinase; furthermore these two signaling pathways act antagonistically on the readout of a downstream β -catenin-responsive reporter gene (Mikels and Nusse, 2006).

Wnts and Frizzleds are known to regulate several aspects of inner ear development at both early and late stages. At the onset of ear formation, Wnts are implicated in otic induction in chicken (Ladher et al., 2000) and *Xenopus* (Park and Saint-Jeannet, 2008). However, in zebrafish, interference with canonical Wnt signaling does not prevent otic formation (Phillips et al., 2004). Canonical Wnt signal acts instructively to determine the size of the otic placode (Ohshima et al., 2006; Jayasena et al., 2008) and this may explain the size reduction of zebrafish otic vesicles observed after overexpression of the Wnt antagonist, Dkk1 (Phillips et al., 2004). Based on Dkk1-mediated Wnt inhibition in chickens, Freter et al. (2008) interpret Wnt signaling as permissive and important during otic commitment rather than for initial otic induction. Misexpression of Wnt4, which is normally restricted to the ventral otocyst in frogs, leads to brain defects and lateral displacement of the otocyst in zebrafish and *Xenopus* (Ungar et al., 1995). Interference with β -catenin mRNA levels by antisense oligos reduces cell proliferation and the number of otic ganglion neurons in the chicken inner ear (Matsuda and Keino, 2000). Data from our lab show that Wnt/ β -catenin signaling can influence the decision between auditory and vestibular sensory fate (Stevens et al., 2003), although it is still unknown whether there is an endogenous Wnt ligand with a comparable function. Finally, Frizzleds are implicated in the establishment of cochlear hair cell polarity (Wang et al., 2006). Knockout of Fzd4 causes degeneration of the organ of Corti and hearing loss in mice (Wang et al., 2001; Xu et al., 2004).

Gene expression studies further implicate Wnt signaling at several time points of otic development, although a full spatial and temporal survey has yet to be reported for the stages under consideration here (from otic vesicle closure through early morphogenesis). Anecdotal findings include asymmetric expression of *Wnt2b*, *Wnt3a* and *Wnt6* in the otic vesicle of different vertebrate embryos (Parr et al., 1993; Zakin et al., 1998; Grove et al., 1998; Hollyday et al., 1995; Lillevall et al., 2006). Chicken *Frizzled* genes are expressed non-uniformly in the otic cup (Stark et al., 2000) and show partial overlap with a sensory marker, Serrate-1 (Lewis and Davies, 2002; Stevens et al., 2003). A *Wnt* and *Frizzled* gene expression survey in mice using Optical Projection Tomography shows several genes in the otic vesicle (Summerhurst et al., 2008). Finally, we reported expression of 22 genes in the Wnt pathway in the developing ventral ear and cochlear duct of the chicken (Sienknecht and Fekete, 2008), with a time course that begins approximately when the period discussed in this paper leaves off.

Several key morphogenetic events take place between otic cup closure and overt cochlear duct differentiation; some of these are accompanied by programmed cell death. Outgrowth of the endolymphatic duct from the dorsal otocyst begins almost immediately after the ventrolateral edge of the otic cup moves upward to meet the dorsal rim and fuses with it to separate the otic tissue from the surface ectoderm (Brigande et al., 2000). Eventually the endolymphatic duct enlarges at its most dorsal tip to form the endolymphatic sac. Formation of the endolymphatic duct is extraordinarily robust; in some cases it is virtually the only structure remaining in severely dysmorphic phenotypes resulting from gene knockouts such as *Six1* (Ozaki et al., 2004). The specification of the mouse dorsal otocyst, including the endolymphatic duct, has been shown to be controlled by Wnt/ β -catenin signaling via Wnt1 and Wnt3a expressed and presumably released from the dorsal hindbrain (Riccomagno et al., 2005).

Ventrally, the pars inferior will extend to create the cochlear duct. Outgrowth and/or specification of the cochlear duct can be regulated by Sonic hedgehog signaling emanating from the ventral midline (Bok et al., 2005; Riccomagno et al., 2002). Furthermore, the width and length of the cochlear duct are altered in mice carrying mutations in genes traditionally associated with PCP (Montcouquiol et al., 2003; Wang et al., 2005a), leading to the suggestion that cochlear duct elongation may occur through a convergence-extension process similar to that associated with axis elongation in gastrulating vertebrate embryos. Wnt4, Wnt5 and Wnt11, that preferentially signal through non-canonical pathways, have been linked to axial convergence-extension in zebrafish and *Xenopus* (Heisenberg et al., 2000; Kilian et al., 2003; Du et al., 1995; Wallingford et al., 2001). Intriguingly, earlier we reported distinct expressions of a number of Wnts, with *Wnt4*, *-5a*, and *-11* in particular, at the ventral tip of the elongating cochlear duct (Sienknecht and Fekete, 2008).

These morphogenetic changes take place concurrently with cell fate specification. As early as the otic cup stage, neuroblasts delaminate from the floor of the cup and migrate medially to generate the statoacoustic ganglion. Within the otic epithelium, patches of cells assume a sensory fate and will differentiate into either auditory or vestibular sensory organs. The process of specifying and segregating the sensory anlage is not fully understood, although it depends on the transcription factor, Sox2 (Kiernan et al., 2005). A number of marker genes allow for prosensory identification well before overt differentiation, including molecules associated with Notch, BMP, Neurotrophin, FGF and, as shown here, Wnt signaling as well as a few transcription factors (Wu and Oh, 1996; Morrison et al., 1999; Cole et al., 2000; Qiu et al., 2004; Schimmang, 2007; for reviews see Fekete and Wu, 2002; Fekete and Sienknecht, 2007).

Here we present an extensive survey of gene expression for a total of 25 probes for Wnts, Frizzleds or Wnt inhibitors over the course of the chicken's early ear morphogenesis using tissue section *in situ* hybridization methods. Shown are exemplary data of all 23 genes that have otic expression. We begin at the tear-drop shaped otic vesicle stage (Hamburger-Hamilton stage 16) and proceed through stages that include endolymphatic duct formation, the establishment of the statoacoustic ganglion, the appearance of distinct sensory primordia and the beginning of cochlear duct outgrowth (s25/26).

Material and Methods

Tissue preparation

White Leghorn chicken embryos ranging from stage (s)16 to s26 were harvested and staged according to established criteria (Hamburger and Hamilton, 1951). RNase-free treated embryos were processed as described previously (Sienknecht and Fekete, 2008). Briefly, immersion fixation overnight at 4°C in 4% paraformaldehyde in phosphate buffered saline was followed by cryo-protection in 15% sucrose in phosphate buffered saline and embedding in TFM (Tissue Freezing Medium, TBS, Triangle Biomedical Sciences). Frozen serial sections of 15µm were collected onto a set of 5–10 slides in a consecutive alternating manner. Depending on the developmental stage every second to 5th section was probed for the same gene, which allowed for a comparison of two to maximal five genes per specimen, including prosensory markers. Experiments were replicated and the order of slide series per tested probe was changed routinely to ensure the representation of different section levels for three dimensional expression pattern information. Transverse and coronal sections were obtained by cutting angles parallel or perpendicular, respectively, to the dorso-ventral axis of the otic anlage.

***in situ* hybridization on tissue sections**

Riboprobes for chicken Wnts and Wnt-related genes (Chapman et al., 2004) were made from plasmids provided by the laboratories of G. Schoenwolf and C. Tabin. The 25 genes studied are listed as follows with gene accession number, sequence and the number of experiments for stages 16–26 in parentheses:

Wnt1 (AY753286, 1 – 373, 4, no otic expression), *Wnt2b* (NM_204336, 687 – 1067, 7), *Wnt3* (NM_001081696, 621 – 1034, 10), *Wnt3a* (NM_204675, 697 – 1088, 7), *Wnt4* (NM_204783, 798 – 1191, 10), *Wnt5a* (NM_204887, 994 – 1364, 16), *Wnt5b* (NM_001037269, 656 – 1017, 9), *Wnt6* (NM_001007594, 557 – 930, 14), *Wnt7a* (NM_204292, 555 – 1244 + 3'UTR, 5), *Wnt7b* (NM_001037274, 679 – 1067, 10), *Wnt8b* (AY753292, 440 – 834, 7), *Wnt9a* (NM_204981, 72 – 1132, 10), *Wnt11* (NM_204784, 1 – 1827, 10), *Fzd1* (NM_001030337, 1148 – (1839), 12), *Fzd2* (NM_204222, 288 – 1430, 11), *Fzd4* (NM_204099, 818 – 3757, 9), *Fzd7* (NM_204221, 1034 – 3090, 17), *Fzd8* (XM_418566, 728 – 1452, 13), *Fzd9* (XM_425392, 219 – 1746, 18), *Fzd10* (NM_204098, 1 – 2244, 8), *Dkk1* (XM_421563, 478 – 1064, 9), *Cer* (NM_204823, 44 – 877, 4, no otic expression), *SFRP1* (NM_204553, 250 – 468, 9), *SFRP2* (NM_204773, 1 – 1594, 13), *Frzb* (NM_204772, 655 – 1822, 20).

In each experiment the hybridization was replicated on 2 to 5 slides, with few exceptions. In addition, different molecular markers such as *Ser1*, *Lfng* (Adam et al., 1998), and *BMP4* (Wu and Oh, 1996) were probed on selected adjacent sections to identify prosensory domains. *In situ* hybridizations were performed as described previously (Sienknecht and Fekete, 2008; Sanchez-Calderon et al., 2005) with riboprobe concentrations of ~1 µg/ml. Hybridization was detected with Anti-Digoxigenin-AP Fab fragments (Roche, 1:3500) followed by alkaline phosphatase (AP) precipitation with BM Purple AP Substrate (Roche Applied Science). Throughout the remainder of the manuscript, chicken genes and transcripts are capitalized and presented in italics to distinguish them from proteins.

Photomicrographic documentation

Photomicrographs of tissue sections of all *in situ* hybridization results were captured and stored in a large image database (Aperture 2.1, Apple Inc., Cupertino, CA) to allow filename- and keyword-based searches and data comparisons of section series. The figure panels in this study provide exemplary data to support the reported results. Cropping of photomicrographs and a minimal amount of contrast and brightness adjustment was carried out with Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA).

Axon immunohistochemistry

Identification of prosensory patches in the developing otic vesicle was aided by double labeling of *in situ* hybridized tissue sections with 3A10 antibody (1:50, Developmental Studies Hybridoma Bank, DSHB, University of Iowa, USA). 3A10 is a monoclonal against chicken ventral spinal cord that recognizes an uncharacterized antigen that is found in the auditory nerve, and is used to label the axons as previously described (Sienknecht and Fekete, 2008; Sanchez-Calderon et al., 2005).

Results

Otic vesicle expression patterns at s16-s19

The timeframe s16 to s19 includes the phase when neuroblasts delaminate from the neurogenic otic anlage (s13–21, Adam et al., 1998) and the anterior-posterior inner ear axis is specified (s17, Wu et al., 1998). The combined expression of both *Lfng* and *Ser1*, which are partially overlapping along the A-P axis, is used to indicate the sensory-competent

region of the otic vesicle (Cole et al., 2000). Neuroblasts originate from a subdomain of this neurogenic region (Alsina et al. 2004). By s19 finally, the first two prosensory patches associated with presumptive anterior (superior) and posterior cristae are evident (Wu and Oh, 1996).

As the otic cup is closing (s16/17), this epithelium expresses several Frizzled receptor genes (*Fzd1*, -2, -7, -9) and the Wnt antagonists *Dkk1*, *SFRP2*, and *Frzb* (Fig. 1). Frizzled mRNA is mainly confined to the neurosensory-competent ventral half of the otic vesicle up to and including the equatorial zone as confirmed by marker gene expression (Fig. 1A, H, K, N). *Fzd1* is strong in the anterior-medial half of the otocyst and includes neuroblasts in the ganglion (Fig. 1B). Concurrently, mRNA expression of the Wnt antagonist *SFRP2* is more restricted and concentrated rostrally in the corresponding otic region, excluding the ganglion (Fig. 1C). *Fzd9* extends from the anterior otocyst and statoacoustic ganglion into the lateral wall of the otic vesicle (Fig. 1D). *Dkk1* resembles this pattern, including some delaminating ganglion neuroblasts (Fig. 1E). Likewise, *Fzd7* is present in the anterior and lateral otocyst but only weakly in the adjacent ganglion (Fig. 1F). Partially overlapping with the *Fzd7* domain, in the lateral wall is *Frzb* (compare Fig. 1F and G, and 1I and L). By stage 18, the *Fzd7* and *Frzb* domains are more similar (Fig. 1J and M). We compared these two transcripts to the prosensory markers, *Ser1* and *Lfng*, and conclude that both *Fzd7* and *Frzb* encroach into prosensory territory. *Fzd7* partially overlaps with both *Lfng* (Fig. 1A and F) and *Ser1* (Fig. 1I and K). In contrast *Frzb* overlaps with *Ser1* (Fig. 1K and L) but not with *Lfng* (compare Fig. 1A with G, and 1H with L, and 1N with O). *Fzd2* transcripts are present in the medial-ventral otocyst (Fig. 1P), co-expressed with *Fzd7* (Fig. 1Q). Continuing this domain, the medial otocyst transcribes *Fzd1* (Fig. 1R).

None of the ligands could be detected in the inner ear as early as s16. By s18, transcripts for Wnts associated with canonical Wnt signaling, such as *Wnt2b* and -3a, are confined to the dorsal-medial otic vesicle in the anlage of the endolymphatic duct and sac (Fig. 1S, *Wnt2b*). While *Wnt3a* expression later also expands in the dorsal ear anlage to include the base of the vertical canal pouch (summarized in Fig. 5), *Wnt2b* transcription remains exclusively dorsomedial in the endolymphatic system (latest test s41, embryonic day 15). Although weakly, *Wnt5a* is also present in the prospective endolymphatic sac at s18 (Fig. 1T). In addition, the otic epithelium of the middle and posterior vesicle expresses *Wnt5a* with the signal concentrated towards the otocyst lumen (Fig. 1T).

Otocyst expression patterns at s20-s23

Axis specification in the dorsal-ventral dimension of the forming inner ear takes place at s21 to 22 (Wu et al., 1998). The first presumptive sensory macula is evident in the saccule at s20 as an extended patch of *BMP4*-expressing prosensory cells in the medial otocyst (Wu and Oh, 1996). Of the three cristae ampullares, the last to arise is the lateral crista primordium at s22/23. By s23, the prosensory field of the presumptive auditory epithelium (basilar papilla) and the vestibular lagena macula can be detected by molecular markers (Wu and Oh, 1996).

Continuing earlier stages, *Fzd1* and *Fzd7* domains complement each other in the prosensory domain with partial overlap (compare Fig. 2A, and B). *Fzd1* is transcribed in the medial-anterior otic vesicle and in the statoacoustic ganglion (Fig. 2A). *Fzd7* is more prominent in the lateral-anterior vesicle, but extends medially, and in addition it is strong in the medial-posterior epithelium (Fig. 2B and E). Overlapping with *Fzd7* (Fig. 2E) in its lateral-anterior domain is the expression of *SFRP2* and *Frzb* (Fig. 2C, D). Both inhibitor genes are transcribed to a different extent in the lateral otic vesicle (Fig. 2C, D, and R, S). New otic Frizzled expression is seen for *Fzd8* that is barely detectable until s21 and rises at s22 (Fig. 2G), and *Fzd4* of which transcription begins at s21 (Fig. 2H).

The anterior-medial otocyst includes a thickened epithelial domain located near the origin of the endolymphatic duct that corresponds to the prosensory patch of the saccular macula primordium. Extending into the ventral-medial part of the otocyst, this region is described as the area of highest cell proliferation between s16 and s21 (Alvarez et al., 1989; Hemond and Morest, 1991). In addition, Lang et al. (2000) find that it contains foci of increased programmed cell death beginning at s19. This domain expresses all Frizzled genes with the exception of *Fzd10*. Fig. 2F-H show examples: anteriorly *Fzd2* (F), and *Fzd8* (G) and more posteriorly *Fzd4* (H). Wnt inhibitor genes such as *Dkk1* (Fig. 2I), less strongly *SFRP1* (Fig. 2J) and *SFRP2* (see Fig. 3C for s24) are present as well. Transiently, limited to s20/21, the anterior-medial and medial-ventral otocyst express numerous ligand genes (*Wnt3*, *-5b*, *-7b*, *-8b*, *-9a*, and *-11*). Fig. 2K-O show examples of a weak (*Wnt7b*, K) and several more prominent Wnt-expressors in this prosensory domain: *Wnt9a* (L), *-11* (M), *-3* (N), and *-8b* (O). Wnts that are transcribed transiently by this prosensory region are otherwise characteristic of nonsensory areas at later stages, with the exception of *Wnt7b* that is transcribed extensively in the lagena macula and basilar papilla later by s34 (see Sienknecht and Fekete, 2008, Fig. 4B). Following this transient anterior-medial otic expression of *Wnt11* at s20 (Fig. 2M), this transcript disappears from the otocyst until s25 (Fig. 3I). However, we find *Wnt11* expression adjacent to the dorsal otocyst in cells that appear to be neural crest derived (Fig. 2P) between s21 and s23. As a positive control, migrating neural crest cells in the trunk region are labeled by *Wnt11* at this time point (Fig. 2P inset).

Three major nonsensory domains of the developing inner ear are: (1) the outgrowing endolymphatic sac and duct located medial-dorsal; (2) the presumptive utricular recess joint with the forming lateral ampulla located lateral-dorsal; and (3) the lateral wall of the pars inferior, the presumptive cochlear duct located ventral-lateral. Each of these 3 domains expresses transcripts for Wnt ligands, receptors and inhibitors. Specifically, the presumptive endolymphatic domain expresses *Wnt2b* (Fig. 2Q), *-3* (Fig. 2N), *-3a* (see later Fig. 3J), *-9a* (Fig. 2L), and several Frizzleds (*Fzd1*, *-2*, and *-9*, see Fig. 3D for *Fzd9* at s25), along with *Dkk1* (see Fig. 3E for s25) and *SFRP2* (not shown). Cells in the presumptive utricular recess express *SFRP2* (Fig. 2R), *Frzb* (Fig. 2S), and the forming semicircular canal ampulla expresses *Wnt5a* (see Fig. 3H for s25), as well as weakly *Wnt3a* and *-6* (both not shown). The prospective nonsensory cochlear duct transcribes *Wnt4* (Fig. 2T). Congruent with this, *Frzb* is present at s20 and extends its expression by s22 to the entire ventral-lateral wall (Fig. 2S), excluding the medial prosensory domain. Weak expression of *Wnt3*, *Dkk1* and *SFRP1* is also detectable in the ventral-lateral otocyst (cf. Fig. 2N, I, and J). In addition *Fzd2*, and *-9*, which are broadly expressed (see Fig. 3D, *Fzd9*), are present in the lateral wall of the pars inferior and *Fzd7* that extends from its prosensory domains into adjacent nonsensory areas (not shown).

Inner ear primordium expression patterns at s24-s26

According to (Wu and Oh, 1996) the last large prosensory domain, the utricular macula, is recognizable by s24. At this stage, Frizzleds demarcate all the prosensory patches of the developing inner ear (Fig. 3A, *Fzd1*). Not restricted to prosensory domains, but including the endolymphatic sac and the nonsensory cochlear duct, are the expressions of *Fzd2* and *Fzd9* (Fig. 3D, *Fzd9*) as well as *Dkk1* (Fig. 3E). The last Frizzled receptor gene, *Fzd10*, is uniquely localized in the basilar papilla by s26 (Fig. 3F), as described in more detail previously (Sienknecht and Fekete, 2008).

Expressed differentially in both prosensory and nonsensory domains are the Wnt inhibitor genes, *Frzb* and *SFRP2*, apart from the aforementioned *Dkk1*. In detail, *Frzb* is transcribed most strongly in the prospective auditory organ (the basilar papilla), less so in vestibular prosensory patches at s24 (Fig. 3B). Furthermore, the nonsensory cochlear duct (Fig. 3B) and the forming lateral ampulla region of the dorsal ear transcribe *Frzb* (Fig. 3B, arrow

head). In contrast, *SFRP2*'s highest expression levels are in vestibular prosensory patches (Fig. 3C, SacM, star; UM, bar) and in the nonsensory cochlear duct, while less strong in the presumptive basilar papilla at this time (Fig. 3C).

Wnts, in contrast, are mainly confined to nonsensory domains (Fig. 3G-J), with some transient exceptions (see below). The nonsensory cochlear duct expresses *Wnt6* (Fig. 3G), plus *SFRP2* as described before, and weakly both *SFRP1* and *Wnt5a* (Fig. 3H, *Wnt5a*). In the posterior cochlear duct, however, the latter is more apparent at the distal tip of the ventral ear (not shown, see Sienknecht and Fekete, 2008, Fig. 1L), sharing this domain with *Wnt4* and *Wnt11* (Fig. 3I, *Wnt11*). Another prominent nonsensory expression domain is the forming ampulla of the lateral crista in the dorsal ear. These cells express both *Wnt5a* (Fig. 3H) and *Frzb* in particular (the latter boldly by s25; see Fig. 3B arrow head for weak labeling at s24). Also faintly detectable in the lateral ampulla at this stage is *Wnt6* (Fig. 3G, arrow head). Medial-dorsal, the endolymphatic apparatus is distinctly labeled by *Wnt3a* (Fig. 3J), *-3* (Fig. 3P) and *-2b* (see Fig. 2Q, for a younger stage). Although not as restricted to this domain, the expression of *Wnt9a* is most intense in the forming endolymphatic anlage (see Fig. 2L). *SFRP2*, *Frzb*, and the aforementioned *Dkk1* (Fig. 3E), are present in this domain, too.

In addition to the prosensory domains, Frizzled receptor genes are prominent in the statoacoustic ganglion (namely these are *Fzd1*, *-2*, *-8*, and *-9*) (Fig. 3D, *Fzd9*). The only ligand gene expressed in the ganglion is *Wnt9a* (see Sienknecht and Fekete, 2008, Fig. 5F). All three inhibitors (e.g. Fig. 3E, *Dkk1*) are present with *SFRP2* being the weakest. *Wnt11* is expressed in glia or Schwann cells as cranial nerves enter the hindbrain and is first apparent at s23 (see s36 in Sienknecht and Fekete, 2008, Fig. 5G). Cranial nerve glia or Schwann cells also express *Wnt5a* and *Fzd7* at s24–26 and beyond (not shown).

In Figure 4, we provide a block diagram that compares regions of qualitatively scored Wnt-related gene expression in the morphogenetic inner ear from s24 to s26. This reveals several centers of high transcriptional activity and their composition of Wnt pathway genes. Wnt expression peaks at the distal tip of the pars inferior (dist.), the outgrowing cochlear duct. Wnt transcription in the endolymphatic sac (medial-dorsal) is relatively high, followed by the lateral-dorsal inner ear in the region of the forming lateral ampulla and the superior semicircular canal base (fusion plate). Peak areas for Frizzleds and Wnt inhibitors are the regions that give rise to the prosensory patches in the medial-ventral (m-v) and the lateral-dorsal (l-d) inner ear anlage.

At s25, when the prosensory patches become distinct, several Wnts are expressed transiently in these domains. For example, the dorsal ear expresses *Wnt5b* in the domain of the anterior crista (Fig. 3K). *Wnt7a* shows a weak expression primarily in the ventral ear (basilar papilla and lagena macula; Fig. 3L and inset). Yet, prosensory domains are negative again for *Wnt7a* soon thereafter. Fig. 3M reports this down-regulation for the time point two days later (s30). The window of s25/26 is also when *Wnt9a* transcripts appear transiently in the auditory primordium as shown in cross section through the cochlear duct (Fig. 3N). Shortly thereafter, at s27, *Wnt9a* has mostly vanished from the prosensory domain and remains as a flanking border along the non-innervated neural side (Fig. 3O). Another example for a transient expression is *Wnt3* that appears in the ventromedial prosensory patch at s20 (see Fig. 2N) and then disappears from the entire ventral ear by s23, maintaining only its endolymphatic sac and duct expression (Fig. 3P).

Excluding those transient expression events, Figure 5 summarizes domain maps of (1) Wnts (ligand genes, in blue), (2) Frizzleds (receptor genes, in green) and (3) Wnt antagonists (inhibitor genes, in red) during morphogenesis from s25/s26 and later. Wnts establish

mainly nonsensory and border domains, while Frizzleds demarcate areas of sensory primordia, and all these domains are superimposed by differential Wnt inhibitor gene expressions. The endolymphatic apparatus is the only otic region where all three Wnt signaling entities regularly overlap.

Discussion

Correlation of Frizzleds with neurosensory development

Our results refine and extend a previous study by (Stark et al., 2000) regarding Frizzled gene expression in the avian otic anlage as well as other cranial placodes. They observed *Fzd1*, -2, and -7 from s11/12 (otic cup) to s15/16 (closing otic vesicle), with expression diminished by s16–18. In contrast, we find the expression of these genes persists to later stages. Furthermore, we show here distinct otic expression of three Frizzled genes (*Fzd4*, -8, and -9) that they described as not prominent in cranial placodes at similar stages. These discrepancies may be due to less reliable mRNA detection when performed as whole mount *in situ* hybridization, particularly after closure of the superficial otic cup.

Frizzled family members vary in the onset of their inner ear expression. The closing otic vesicle expresses *Fzd1*, -2, -7, and -9 at s16, while *Fzd4* (s21), -8 (s22), and -10 (s25/26) show distinct otic transcription at progressively later stages of development. The early expression overlaps with the neurogenic region that gives rise to delaminating neuroblasts and expression of *Fzd1* persists in the newly-released neuroblasts at s16 (Fig. 1B). Concurrently, there is focal expression of *SFRP2* within the otic vesicle at the site of delamination (Fig. 1C) that continues in the lateral vestibule at s20 (Fig. 2R) and persists within and adjacent to the presumptive utricular macula at s25/26 (summarized in Fig. 5C). The macular region remains neurogenic through s27 (Stone et al., 2003) and shows persistent expression of one or more Frizzled genes during the interval from s16-s27 (summarized for s25/26 in Fig. 5B). This raises the interesting question of whether the binding of *SFRP2* to Frizzled receptors might be involved either in the specification or repulsion of neuroblasts.

The Frizzled genes are also associated with prosensory domains, including both ventromedial and lateral (summarized in Fig. 5B). This suggests a potential function for Wnt signaling in the development of sensory identity, which is also under the control of the proneural transcription factor, Sox2 (Uchikawa et al., 1999; Neves et al., 2007; Kiernan et al., 2005). Such a role for Frizzleds in the formation and maintenance of neural-competent precursors has been reported by Van Raay et al. (2005) for the developing *Xenopus* retina. There, blocking of either *Fzd5* or canonical Wnt signaling inhibits Sox2 expression and promotes non-neural fate. A Frizzled requirement was also reported during neurogenesis in *Drosophila* (Bhat, 1998).

Finally, expression of Frizzleds in the statoacoustic ganglion neurons raises the question of whether they might regulate axon guidance as has been shown elsewhere (Drescher, 2005; Bovolenta et al., 2006). It seems there are at least three possibilities for Frizzled-mediated responsiveness: (1) attraction via Wnt inhibitors that are abundant in the prosensory domains; (2) attraction via Wnt ligands that appear transiently in the prosensory domains (see below); or (3) repulsion via Wnt ligands that flank the sensory domains at most stages.

In some systems, such as *Drosophila*, Frizzleds appear to function redundantly (Bhanot et al., 1999), and indeed we show several examples where there are multiple overlapping Frizzled genes. Yet our results also show spatial and temporal differences in Frizzled gene expression that suggest distinct non-redundant functions during inner ear development.

Correlation of Wnts with non-sensory domains

Typically, nonsensory borders expressing Wnts flank sensory primordia presenting Frizzled receptors. This arrangement is consistent with the possibility of paracrine signaling from nonsensory to sensory territories as we noted in a previous study (Sienknecht and Fekete, 2008) and that we can now extend to earlier time points. Interactions between sensory and nonsensory domains seems crucial for inner ear development, as shown by the requirement of FGF activity in the sensory cristae to promote adjacent semicircular canal formation in chickens (Chang et al., 2004) and conversely the nonsensory expression of *Foxg1* for proper patterning of the mouse organ of Corti (Pauley et al., 2006). Transcription of several Wnt inhibitors in sensory and nonsensory domains further suggests that paracrine Wnt signaling may be tightly regulated.

Spatial expression of Wnts that commonly signal through a non-canonical pathway, such as *Wnt4*, *-5a* and *-11*, are found in association with the lateral otocyst and ventral tip of the outgrowing cochlear duct. A role for these proteins in convergent extension of the cochlear duct is conceivable based on the expression data reported here and the finding that *Wnt5a* and *Wnt11* are involved in convergent extension movements during gastrulation of birds and zebrafish (Hardy et al., 2008; Kilian et al., 2003; Zhu et al., 2006). Furthermore, *Wnt5a* mutant mice have a shortened cochlear duct, a fact that has been connected to convergent extension failure (Qian et al., 2007). In addition, *in vitro* data from *Wnt5a* mutant mice indicate involvement of this ligand in mesenchymal condensation during otic capsule chondrogenesis (Liu et al., 2008).

Finally, the ventral expression of preferentially non-canonical Wnts, such as *Wnt5a* and *Wnt11*, could suggest an antagonistic relationship with dorsal Wnts, such as the canonical *Wnt3a*. Given that both *Wnt11* and *Wnt5a* can effectively antagonize *Wnt3a*-activated β -catenin signaling (Maye et al., 2004), an otic-autonomous Wnt-Wnt interplay either establishing or refining non-overlapping canonical vs. non-canonical Wnt territories in the otocyst becomes an attractive idea.

The large number of Wnt-related genes expressed in the endolymphatic duct raises the possibility that it may be an important signaling center acting on nearby cells in or around the dorsal otocyst, including the hindbrain and migrating neural crest cells. Yet, it should also be remembered that Wnt ligands, as well as the Wnt antagonists, are secreted molecules which means the location of their transcription does not necessarily correlate directly with the proteins' functions. One possibility is that a region of high transcriptional turnover, such as the endolymphatic sac appears to be, is particularly in need of control by Wnt inhibitors to prevent Wnt signaling in loco.

Taken together, regionalized expression of several Wnts, Wnt receptors and Wnt inhibitor genes in the otic anlage indicates that the system is carefully regulating Wnt responsiveness.

Transient Wnt signaling in prosensory domains

Unexpected was the observation of Wnt transcription within prosensory domains, although temporally restricted and often short term (cf. s20/21 and s25). Because this overlaps both spatially and temporally with receptor gene expression, it suggests a probable role of autocrine Wnt signaling as well.

In the mouse otocyst, canonical Wnt activity is restricted dorsally by counteractive *Shh* signaling originating ventrally (Riccomagno et al., 2002; 2005). For the chicken otocyst, we confirm the dorsal-medial predominance of Wnt genes that activate β -catenin dependent signaling such as *Wnt2b* and *-3a*. In mice, the dorsal restriction of *Wnt1* and *Wnt3a* expression, coupled with ventral, cochlear defects in *Wnt1* and *Wnt3a* knockouts, was

initially puzzling. However, Cre-lox-mediated lineage tracing of Wnt-responding cells provided a plausible explanation: dorsally-derived cells received a canonical Wnt signal before migrating to the medial-ventral otocyst (Riccomagno et al., 2005). While we do not dispute this idea, in chickens there may be a more direct opportunity for Wnt signaling to influence cells of the medial-ventral otocyst. This region shows transient expression of numerous Wnt genes (*Wnt3*, *-5b*, *-7b*, *-8b*, *-9a* and *-11* at s20/21). Timing and location of this expression corresponds to the specification of a Sox2-expressing prosensory domain (Neves et al., 2007) that is mitotically active (Lang et al., 2000). Interestingly, canonical Wnt signaling is thought to maintain an undifferentiated, proliferating progenitor population, whereas non-canonical Wnts facilitate differentiation, as evident during osteogenesis *in vitro* in human mesenchymal stem cells (Boland et al., 2004). However, the set of genes we find co-expressed at s20/21 is per se not further instructive in this respect, although, considering recent reports, it does not contradict canonical signaling (cf. Kim et al., 2008; Lewis et al., 2008, *Wnt3*; Kuorelahti et al., 2007, *Wnt5b*, *Wnt7b*; Wang et al., 2005b, *Wnt7b*; Cadigan and Liu, 2006, *Wnt8b*; Guo et al., 2004; Person et al., 2005, *Wnt9a*; Tao et al., 2005, *Wnt11*).

Forced expression of activated β -catenin in nonsensory territories of the chicken inner ear induces the formation of ectopic sensory patches of vestibular nature during a sensitive period that ends by s23 (Stevens et al., 2003). This time frame precedes the second time point, s25, when a few Wnts (e.g. *Wnt7a* and *Wnt9a*) occur transiently in prosensory domains. Shortly thereafter Wnts disappear again from these domains. Both the observed temporal restriction of Wnt expression in prosensory domains and the functional data lead to the conclusion that a tight temporal control system is necessary for prosensory specification in the developing inner ear.

Furthermore, *Wnt7b*, one of the expressors in the prosensory domain at s20/21, disappears from prosensory territory for nearly 5 days, reappearing at embryonic day 8 (s34) in the auditory basilar papilla and the vestibular lagena macula. By that time, both sensory organ primordia show a robust and non-overlapping expression of different Wnt inhibitor genes, i.e., *SFRP2* in the basilar papilla and *Frzb* in the lagena macula (Sienknecht and Fekete, 2008). Thus, (a) autocrine canonical Wnt signaling influences are likely to be prevented in the sensory primordia for an extended time and (b) the gene expression data are consistent with a role of Wnts in conjunction with Wnt inhibitor activity in the proper formation of distinct auditory and vestibular sensory organs.

Shortly after the medial prosensory domain is established, a focus of apoptosis appears in the medial wall of the otocyst (Fekete et al., 1997; Lang et al., 2000). This leads us to consider whether Wnt signaling might influence programmed cell death during inner ear development. He et al. (2004) provided evidence that blocking *Wnt1* leads to apoptosis in human cancer cell lines, although others found the opposite in the chicken hindbrain *in vivo*, where *Wnt1* overexpression causes ectopic programmed cell death (Ellies et al., 2000). Comparison of our Wnt-related gene expression patterns to maps of cell proliferation and cell death during chicken inner ear development by Lang et al. (2000) revealed some regions of interest. *Wnt9a*, for example, shows resemblance to the distribution of proliferating cells that incorporated BrdU over development. In the region called the ventral-medial 'hot spot' of cell death (s19–23), where proliferating and apoptotic cells are intermingled, *Wnt9a* overlaps with *Dkk1* at s20 (Fig. 2L, *Wnt9a*; 2I, *Dkk1*). Interestingly, *Wnt9a* promotes cell proliferation during chicken heart development, an effect that is antagonized by *Frzb* (Person et al., 2005). Furthermore, the authors show that inhibition of *Wnt9a* by overexpression of dominant-negative *Wnt9a* results in an increase in apoptotic (TUNEL-positive) cells in cardiac atrioventricular canal cultures. A role of *Dkk1* in cell proliferation and cell death was suggested by Mukhopadhyay et al. (2001). Grotewold and Rther (2002)

provided evidence that *Dkk1* enhances apoptosis during limb development. Finally, another Wnt antagonist, *SFRP2*, modulates programmed cell death in chicken hindbrain rhombomeres via a Wnt-BMP4 feedback-loop as demonstrated by Ellies et al. (2000).

Inner ear domains under control of Wnt antagonists

Three Wnt inhibitors, *Dkk1*, *Frzb*, and *SFRP2* are notable for their robust transcription. Present from early otic stages throughout inner ear development, Wnt inhibitor genes show partial overlap in their expression patterns. Figure 6 maps six differently composed domains established by Wnt signaling modulators at two exemplary time points.

Nonetheless, dynamic changes over time are drastic. For example, *SFRP2* shifts from vestibular sensory domains to the auditory primordium. During the divergence of vestibular and auditory sensory cell fates, *SFRP2* is expressed mainly in prospective vestibular prosensory patches and the nonsensory pars inferior, thus flanking the prospective auditory epithelium (Fig. 6, s24/25). Later, (s27-s36), *SFRP2* is prominently expressed in the auditory epithelium (Sienknecht and Fekete, 2008) but not in any vestibular sensory organ primordium (at least until s39, embryonic day 13, by s40 this changes, unpublished data). *Frzb*, in contrast, is mainly present in prospective nonsensory territories at s20–22, but then becomes transcribed in the auditory primordium by s24/25 (Fig. 6). Later, during differentiation of the basilar papilla, when *SFRP2* is strongly expressed in this tissue, the transcription of *Frzb* restricts to the neural-basal basilar papilla by s30 before diminishing from the auditory organ as reported previously; *Frzb* remains in the vestibular lagena macula (Sienknecht and Fekete, 2008).

Based on the particular set of genes expressed in the medial-ventral (neurosensory) otic vesicle at s20/21, we suggested that the neurosensory domain may be responsive to β -catenin pathway signaling factors for a small time window, with canonical Wnt activity serving to promote a prosensory fate. However, such a function seems, at first glance, to be inconsistent with the high level of *Dkk1* expression we observed in this same domain. *Dkk1* is a potent inhibitor of canonical Wnt signaling (reviewed in Niehrs, 2006). One plausible explanation is that the antagonistic effect of *Dkk1* may be overruled by the accumulated presence of numerous presumed canonical-pathway Wnt ligands (e.g. Wnt7b, -8b, -9a). Moreover, *Dkk1* might actually not interfere with canonical signaling after all. In fact, there is evidence for *Dkk1* as a direct inhibitor of the non-canonical PCP pathway acting independently of β -catenin (Caneparo et al., 2007). Furthermore, Cha et al. (2008) show that during dorsal-ventral axis formation in *Xenopus*, *Dkk1* limits the site and amount of activation of Wnt target genes by modulating JNK activation, a key component of non-canonical signaling. In the light of this knowledge, *Dkk1* could very well be permissive for canonical Wnt signaling in the otic neurosensory domain.

Modulation of Wnt signaling by co-expression and therefore cumulative gene activity seems likely and could provide vast plasticity. For example, activity of both *Dkk1* and *SFRP2* in the anterior otic vesicle at s16 could be crucial to pass the threshold for inhibition of a given signaling event. Conversely, ligands with regionally overlapping expression and the tendency to form functional complexes could together reach an expression threshold to reduce the efficacy of inhibitors. A situation like this has been described by Cha et al. (2008) for *Xenopus* embryos. There, *Dkk1* antagonizes signaling of the broadly expressed Wnt5a, except in the dorsal blastula where Wnt11 mRNA is enriched. Co-expression of both ligands, which tend to form a functional complex of two homodimers, passes a threshold for activity and the *Dkk1* antagonism becomes ineffective, allowing transduction of the Wnt signal and leading to expression of Wnt target genes.

The striking prominence of secreted Wnt signaling antagonists during otic development, their differential expression and dynamic changes over time that we report here, suggests that Wnt signaling is likely to be under tight control spatially and temporally.

Summary

Expression of a plethora of Wnt-related genes is concomitant with inner ear development as the otic vesicle initiates morphogenesis and prosensory cell fate determination. Co-expression and spatiotemporal tuning create specific developmental contexts for responding cells. In addition, topological patterns and temporal dynamics of Wnt inhibitor gene expression lead to a catalog of molecular micro-environments that likely specify and regulate Wnt activities. Detailed knowledge of overlapping expression of several Wnts, Frizzleds, and/or Wnt inhibitors, respectively, may assist in the design of experiments for functional analyses by suggesting a need to target functional units rather than single genes. This may also help to overcome the common problem of analyzing functional redundancies.

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Fig. 1. mRNA expression in the otic vesicle (s16–18)

A-G and N,O: Horizontal sections through otic vesicles (horizontal level in schema). Anterior is to the left and hindbrain rhombomeres are at the top of the image. **H-M and P-T:** Transversal sections (vertical level in schema). **A:** *Lfng*, prosensory marker expression in the anterior and medial otic vesicle at the equatorial level of the vesicle, and in the adjacent SAG. **B-G:** Comparison of overlapping mRNA expression of three Frizzleds (**B,D,F**) with three Wnt inhibitors (**C,E,G**), respectively, on adjacent section levels. **H-O:** Comparison of Frizzled receptor transcripts, *Fzd7*, with the Wnt inhibitor gene, *Frzb*, and prosensory markers (*Lfng* and *Ser1*). Consecutive sections of the same embryo are: H,I and L; J and M; and N,O, respectively. Horizontal sections in N,O are through the ventral otic vesicle. **P-R:** Pro-neurosensory domain expressing Frizzleds (consecutive sections of the same embryo). **S,T:** Otic Wnt gene expression at s18. Abbreviations: ED, endolymphatic duct; D, dorsal; HB, hindbrain; L, lateral; M, medial; P, posterior; s, embryonic stage. SAG, statoacoustic ganglion (dashed line). *In situ* hybridization signals (purple) are counterstained by axon labeling with 3A10 antibody (brown) in B, Q, and R. Scale bar = 100µm.

Fig. 2. mRNA expression in the otocyst (s20–22)

A-E: Horizontal sections through the broad middle otocyst; anterior is to the left, statoacoustic ganglion (SAG) dashed line. **A,B:** Comparison of *Fzd1* and *Fzd7* on adjacent sections. **C,D:** Expression of two Wnt inhibitors, *SFRP2* and *Frzb*, compared with **E:** *Fzd7* on adjacent sections of the same ear. **F-T:** Transverse sections. **F-O:** Transcripts in the anterior and medial otocyst. **F-H:** *Fzds*. **I,J:** Wnt antagonists. **K-O:** *Wnts*. **F,G and K-M:** Anterior-middle section level through the otocyst. **H-J and N,O:** Middle-posterior otocyst section level. **P:** Neural crest derived mesenchyme cells expressing *Wnt11* adjacent to the endolymphatic sac primordium (arrow). **P inset:** *Wnt11* expression in neural crest cells and the dorsal somite at trunk levels (same embryo as in P). **Q:** Dorsal-medial expression of *Wnt2b*. **R-T:** Lateral otocyst expressors. Abbreviations: CD, cochlear duct; D, dorsal; ED, endolymphatic duct/sac; HB, hindbrain; L, lateral; M, medial; P, posterior; s, embryonic stage; SC, spinal cord; SP, sensory primordium; UR, utricular recess. *In situ* hybridization signals (purple) are counterstained by axon labeling with 3A10 antibody (brown) in A-E. Scale bar = 100µm.

Fig. 3. mRNA expression during otic morphogenesis (s23–30)

A: Prosensory domains are labeled by *Fzd1* expression including the prosensory sacculle (asterisk)/basilar papilla patch on the medial side and utricular macula (UM) laterally. **B,C:** Complementary expression patterns of two Wnt inhibitors, *Frzb* and *SFRP2* (asterisk, prosensory sacculle; bar, prosensory utricle; BP, prosensory basilar papilla). **D,E:** Broad otic expression of *Fzd9* and *Dkk1*. **F:** Exclusive expression of *Fzd10* in the distal-abneural BP primordium. **G:** Nonsensory lateral wall of the cochlear duct (nsCD, demarcated by black line) transcribing *Wnt6*. **H:** Lateral ampulla expresses *Wnt5a*. (Arrow head in **B** and **G** indicate weak transcription of *Frzb* and *Wnt6*, respectively in the same region). **I:** Ventral tip of the cochlear duct labeled by locally restricted expression of *Wnt11*. **J:** Endolymphatic sac and duct express *Wnt3a*. **K:** *Wnt5b* expression in the anterior crista (AC) region at s25. **L,L inset:** Weak *Wnt7a* transcription in the primordia of the auditory BP and vestibular lagena macula (LM) at s25. **M:** Nonsensory expression of *Wnt7a* at s30. Transcripts border the saccular macula (SacM) and flank the BP but are not present in the prosensory domains. **N:** At s25 the BP primordium is expressing *Wnt9a*, shown in cross section (neural is to the left). The neural side of the forming cochlear duct also shows some *Wnt9a* transcription. **O:** At s27 the neural side of the cochlear duct, the prospective homogeneous cell domain, expresses *Wnt9a*, flanking the BP domain. **P:** *Wnt3* transcripts label the endolymphatic sac and duct but are not present in the ventral medial domain (bar) at s23. Abbreviations: AC, anterior crista; Amp, semicircular canal ampulla; BP, basilar papilla; D, dorsal; Esac, endolymphatic sac; HB, hindbrain; L, lateral; LM, lagena macula; M, medial; nsCD, nonsensory cochlear duct; P, posterior; s, embryonic stage; SAG, statoacoustic ganglion; SacM, saccular macula,

UM, utricular macula. *In situ* hybridization signals (purple) are counterstained by axon labeling with 3A10 antibody (brown) in F. Scale bar =100µm.

Fig. 4. Relative comparison of gene expression in otic regions (s24-s26)

Qualitatively scored expression of *Wnts* (blue), *Frizzleds* (green), and Wnt antagonists (red) allow a comparison of different otic regions and the diversity of genes contributing to each expression domain. The box height refers to a qualitative score from 1–3 that indicates either a) relative expression strength/distinctiveness (signal vs. background and compared to positive control tissue of the same embryo); b) expression duration; or c) local restriction within the domain. That means, a gene with the score 1 (small box) is either expressed weakly in that domain compared to its expression elsewhere in the embryo, or a score 1 refers to a limited duration within the time window discussed, or finally a score 1 indicates a localized expression e.g. only in parts of the lateral dorsal otic anlage. The number above each diagram block is the sum of expression units (scores), representing relative gene expression per otic region. Schematic provides orientation of otocyst quadrants (m-d, m-v, l-d, l-v), illustrates locations of prosensory domains (gray), and indicates proximal and distal border domains (black). Abbreviations: Amp, semicircular canal ampulla; BP, basilar papilla; CD, cochlear duct; CD/U border, border between CD and U; D, dorsal; Esac, endolymphatic sac; L, lateral; LC, lateral crista; LM, lagena macula; LM/BP border, inter-sensory border between LM and BP (not indicated in schema); M, medial, s, embryonic stage; SacM, saccular macula, U, utricle, V, ventral.

gene expression maps (s25/26)

Fig. 5. Summary of gene expression domains of the morphogenetic inner ear (s25/26)

Schematic illustration summarizes maps of characteristic expression domains established in the regionalized otic epithelium. **A:** *Wnts* (blue). **B:** *Fzds* (green). **C:** Wnt inhibitors (red). **D:** prosensory domains (gray). Color intensities refer to co-expression of multiple genes and expression strength. Faint background color indicates broad and indistinct gene expression. Text boxes group and list expressors within the region. Comparison of **A:** *Wnt* ligands and **B:** *Fzd* receptors show mostly complementary expression domains. Prosensory areas express *Fzds*, whereas nonsensory and border domains express *Wnts*. The exception is the endolymphatic apparatus that co-expresses both *Wnts* and *Fzds*. **C:** Wnt inhibitor maps combine to differentially cover both the *Wnt* and *Fzd* domains. Abbreviations: Amp, semicircular canal ampulla; BP, basilar papilla; D, dorsal; Esac/duct, endolymphatic sac/duct; FP, semicircular canal fusion plate; L, lateral; LC, lateral crista; LM, lagenar macula; s, embryonic stage; SacM, saccular macula; SSC, semicircular canal; UM, utricular macula.

Wnt inhibitor domains

Fig. 6. Wnt inhibitor domains

Comparison of two time points, **A**: otocyst stage 20/21 and **B**: inner ear primordium stage 24/25, reveals six differently composed Wnt inhibitor domains with distinct or overlapping expression of three Wnt inhibitor genes: (1) *Dkk1* at s20 in the medial otocyst and SAG (circle); (2) *Frzb* at s20 in the lateral otocyst, and at s24/25 lateral-dorsal (in the vestibule); (3) co-expression of *Frzb* and *SFRP2* at s20 in the lateral-dorsal otocyst; (4) *SFRP2* at s24/25 in prosensory vestibular primordia; (5) co-expression with strong *SFRP2* and mild *Frzb* at s24/25 lateral-ventral (in the nonsensory pars inferior); (6) co-expression with strong *Frzb* and mild *SFRP2* at s24/25 medial-ventral (in the prosensory BP primordium). Not shown are the sporadic and weak expression of *SFRP1* (see Fig. 2J) and an underlying broad expression of *Dkk1* by s24/25 (Fig. 3E). Bold and plain font face in the legend refers to relative expression strength. Abbreviations: BP, basilar papilla; D, dorsal; L, lateral; s, embryonic stage; (SAG), statoacoustic ganglion.