From placode to polarization: new tunes in inner ear development

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Summary

The highly orchestrated processes that generate the vertebrate inner ear from the otic placode provide an excellent and circumscribed testing ground for fundamental cellular and molecular mechanisms of development. The recent pace of discovery in developmental auditory biology has been unusually rapid, with hundreds of papers published in the past 4 years. This review summarizes studies addressing several key issues that shape our current thinking about inner ear development, with particular emphasis on early patterning events, sensory hair cell specification and planar cell polarity.

Introduction

The formation of the inner ear is one of the most remarkable events in vertebrate organogenesis. However, after more than 100 years of studying this elaborate structure, our understanding of its basic morphological and cellular development is still extremely limited. Nevertheless, the pace of discovery has increased considerably in the past decade. The emergence of mice and zebrafish as in vivo model systems amenable to genetic manipulation has contributed significantly to this acceleration. In this review, we highlight some of the most important advances that have taken place within the past 4 years. Two particularly exciting areas in the field are: the identification of genes that pattern the early stages of inner ear development, including formation of the otic placode and patterning of the otocyst; and genes that pattern the sensory epithelium, particularly the specification and polarization of hair cells. For areas that are not covered in depth in this article, we urge readers to consult several excellent recent reviews (Riley, 2003; Wright and Mansour, 2003a; Riley and Phillips, 2003; Whitfield et al., 2002; Noramly and Grainger, 2002). We begin with an overview of the morphological development of 'one of the most remarkable displays of precision microengineering in the vertebrate body' (Swanson et al., 1990).

An overview of inner ear development

The first visible sign of inner ear development is the appearance of the otic placode, an ectodermal thickening located adjacent to rhombomeres 5 and 6 of the hindbrain (Noramly and Graiger, 2002; Solomon and Fritz, 2002) (Fig. 1). The otic placode then invaginates and pinches off from the surface ectoderm in birds and mice, or cavitates in fish and reptiles, to form a simple epithelial sac referred to as the otocyst or otic vesicle. Soon after the otocyst is formed, individual neuroblasts delaminate from its anteroventral region and coalesce to form the developing statoacoustic ganglion (SAG). The SAG provides the sensory innervation for all aspects of the inner ear. As development continues, morphological changes occur within the otocyst to reshape this simple sac into an elaborate fluid-filled labyrinth that includes both auditory and vestibular sensory structures. Within the labyrinth, some regions of epithelial cells become specified to develop as the specialized sensory epithelia that will act as the primary transducers of sound, motion and gravity, while other regions retain a simple cuboidal epithelial morphology. Concomitant with these changes within the labyrinth, the developing inner ear also recruits surrounding mesenchymal cells to coalesce around it to form an encasing bony labyrinth (Fig. 1).

In its adult form, the mammalian inner ear includes six sensory patches: the organ of Corti, which extends along the coil of the cochlear duct, acts as the primary auditory organ; the sacculus and utriculus, which are located in the morphological center of the inner ear, perceive linear and angular acceleration; and the three cristae associated with the three semi-circular canals are responsible for the perception and maintenance of balance. In addition to the sensory structures, the inner ear includes the endolymphatic duct (ED), which extends dorsally to communicate with the central nervous system (CNS). This communication allows the passage of cerebrospinal fluid from the CNS to inner ear, where it is modified to form endolymph, an essential component for sensory transduction (Fig. 2).

Each of the sensory patches consists of mechanosensory hair cells and non-sensory supporting cells that are arranged into a cellular mosaic. In most sensory epithelia, the mosaic is fairly simple, but in the organ of Corti the hair cells and supporting cells are organized into rows (Fig. 2). Hair cells perceive sound and/or motion through the deflections of a bundle of modified microvilli, referred to as a stereociliary bundle, which projects from the lumenal surface of the cell. Stereociliary bundles are directionally sensitive and are usually polarized such that all

the bundles within a region of the sensory epithelium are aligned in the same plane.

Although the overall structure of the inner ear is comparable in all vertebrates, with the exception of the organ of Corti in mammals, the rate at which the ear develops is highly variable. In order to make comparisons between different species, we use the following definitions throughout this review: 'early' inner ear development encompasses stages from pre-placode to stages at which all the anatomical aspects of the ear have appeared, but prior to sensory epithelial and sensory neuronal cellular maturation; and 'late' development encompasses the stages from the end of early development through to the adult form. Accordingly, the dividing line between early and late development in chick is embryonic day 5 (E5) [Hamburger-Hamilton (HH) stage 27/28], while in the mouse the line is drawn at E13.5. In the zebrafish, the divide falls at the Prim 5 stage at 24 hours of development, although the basic mature ear form does not appear until 5 days post fertilization (dpf) and the ventral part of the ear (the pars inferior) does not assume adult-like form until 20 dpf. In contrast to most other species, in which the ED develops early, the formation of the ED is late in zebrafish development and does not occur until 20 dpf (Bever and Fekete, 2002).

Formation of the otic placode

Role of transcription and growth factors (Fgfs)

Although the otic placode constitutes the first visible sign of inner ear development, recent studies in zebrafish have demonstrated that crucial changes in gene expression begin well before a placode becomes visible (Liu et al., 2003). For example, expression of Dlx family members is observed in the ectodermal cells that will form the otic placode (Ekker et al., 1992; Solomon and Fritz, 2002; Liu et al., 2003). These and other pre-placode gene markers define an 'otic space' (or pre-otic anlage) in the ectoderm that will form the placode and in the underlying mesenchyme that eventually gives rise to the periotic mesenchyme (POM). Otic induction involves *fgf3* and *fgf8* (Maroon et al., 2002; Leger and Brand, 2002) together with *dlx3b* and *dlx4b* (Solomon and Fritz, 2002). The importance of these genes was confirmed in an elegant series of experiments by Liu and colleagues (Liu et al., 2003), who used genetic and molecular techniques to show that combined loss of signaling by *fgf3* and *fgf8*, and three transcription factors that are expressed in the pre-otic anlage – *dlx3b*, *dlx4b* and *sox9a* – completely eliminated all evidence of ectodermal otic cells in the zebrafish (Liu et al., 2003). More recently, the importance of both Dlx genes and *sox9* in otic induction has been confirmed in other species, including *Xenopus* (Saint-Germain et al., 2004) and mouse (Robledo et al., 2002).

Fox genes

The forkhead (Fox) gene, *foxi1*, a winged-helix transcription factor, is expressed in the zebrafish pre-placodal ectoderm in advance of all previously identified pre-placodal markers, including *dlx3b*, *dlx4b*, *sox9a* and *pax8* (Solomon et al., 2003; Nissen et al., 2003). *foxi1* mutations in zebrafish result in the complete loss of *pax8* expression in the placode and in the loss or reduction of *pax2a* expression (an early placodal marker). By contrast, the expression of *dlx3b* and *dlx4b* is reduced but not eliminated. Although placodes are significantly affected in *foxi1* mutants, they are not always absent, indicating that neither *foxi1* nor *pax8* is absolutely required for placode formation. Therefore, whereas *foxi1* clearly plays a key role in placodal formation in zebrafish, there are parallel pathways involved in otic induction and redundancies among these pathways. Fox genes may also act redundantly in mammals, as both *Foxi1* and *Foxf2* are expressed in the early mouse and rat otic epithelium (Aitola et al., 2000), although their roles at these early times have not yet been elucidated.

The argument for evolutionarily redundant pathways in vertebrate placode formation is supported by the demonstration that deletion of *Foxi1* in mice has no obvious effect on the development of either the placode or otocyst (Hulander et al., 1998), although the early stages of ear development have not been examined in detail in these animals. Nevertheless, the absence of Foxi1 in mice does affect later stages of development, resulting in an enlarged membranous labyrinth and auditory and vestibular deficits, apparently because of an increase in the size of the ED. More recent evidence indicates that increased ED size is probably the result of complete loss of expression of pendrin (*PDS*), which encodes a molecule believed to act as an ion transporter, although the specific ions transported are still under discussion (Hulander et al., 2003). *PDS* is mutated in individuals with Pendred syndrome, which

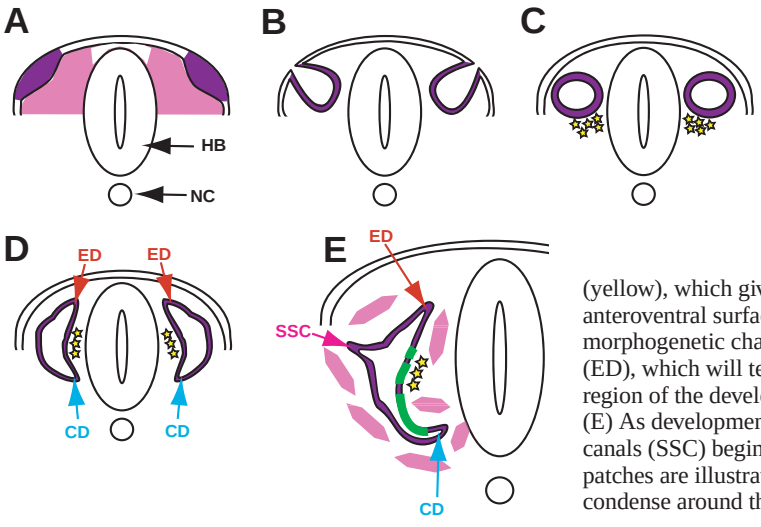

Fig. 1. Development of the otocyst. (A) Cross-section through a developing embryo at the level of the hindbrain (dorsal towards the top). The otic placode forms as a thickening of the surface ectoderm (blue) adjacent to the hindbrain (HB) and notochord (NC). The space between the hindbrain and the surface ectoderm is populated by periotic mesenchymal cells and some neural crest cells (pink). (B) As development continues, the placodes pinch off to form otic vesicles (purple). (C) Soon after closure of the otocyst (purple), neuroblasts (yellow), which give rise to the statoacoustic ganglion, delaminate from the anteroventral surface of the otocyst. (D) Next, the otocyst undergoes elaborate morphogenetic changes, including the dorsal extension of the endolymphatic ducts (ED), which will terminate in the endolymphatic sacs (not shown), in the dorsal region of the developing otocyst and the cochlear duct (CD) in the ventral region. (E) As development continues, the cochlear duct begins to coil and the semicircular canals (SSC) begin to form in the dorsal region of the ear. Developing sensory patches are illustrated in green. At the same time, periotic mesenchymal cells (pink) condense around the developing ear to form the bony labyrinth.

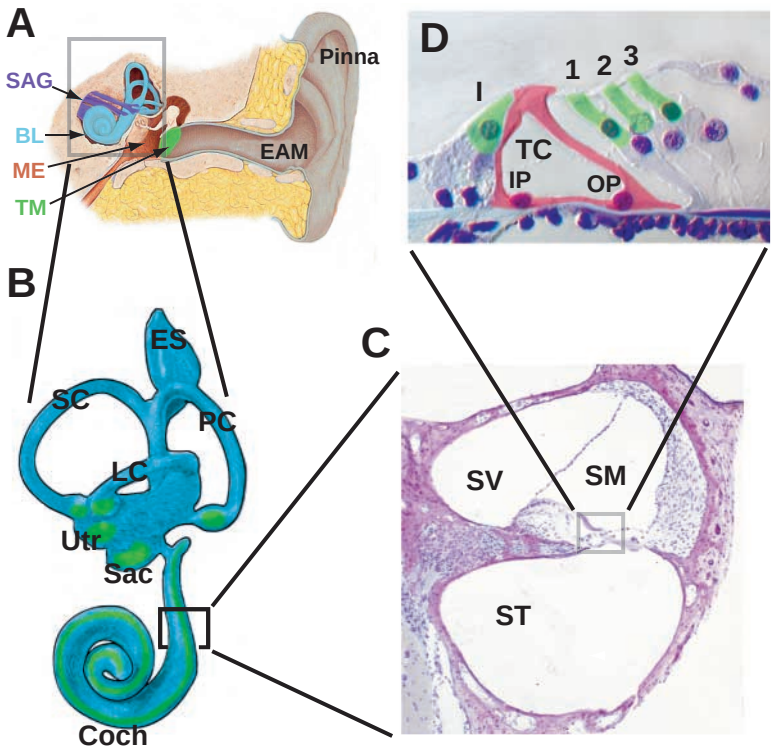

Fig. 2. Anatomy of the adult mammalian cochlea. (A) Cross-section through the human head, illustrating the position of the inner ear. The ear has three parts: the external ear, which consists of the pinna and external auditory meatus (EAM) and ends at the tympanic membrane (TM, green); the middle ear (ME, orange), which contains the three middle ear bones; and the inner ear, which includes the bony labyrinth (BL, light blue) and the statoacoustic ganglion (SAG, purple). (B) Lateral view of the mouse inner ear (boxed region in A) with sensory epithelia in green. LC, lateral semicircular canal; SC, superior semicircular canal; PC, posterior semicircular canal; Sac, saccule; Utr, utricle; Coch, cochlea; ES, endolymphatic sac. The endolymphatic duct is hidden from view but connects the ES to the semicircular canals. (C) Cross-section through the boxed region in B, illustrating the anatomy of the cochlear duct. The duct is separated into scala vestibule (SV), scala media (SM) and scala tympani (ST). The sensory epithelium of the cochlea, the organ of Corti (boxed), is located on the floor of the scala media. (D) Cross-section of the organ of Corti, containing a single inner hair cell (I, green), three outer hair cells (1-3, green) and non-sensory supporting cells (blue), which include the pillar cells (purple). Inner and outer sensory hair cells are separated by the tunnel of Corti (TC), a fluid filled structure that is bounded by inner and outer pillar cells (IP and OP, purple).

accounts for as much as 10% of hereditary deafness. The size of the ED is also affected by other proteins, notably Dan (differential screening-selected genes aberrative in neuroblastoma) (Gerlach-Bank et al., 2004) and Six1, which are both discussed later.

Therefore, although *Foxi1* still plays an important role in inner ear development in mice, the scope of its effects appears to have become markedly reduced from the role it plays in zebrafish. The basis for this change during evolution is unclear, but it seems possible that the role of *foxi1* in zebrafish may fall to another Fox family member in mice, although none of the existing mouse Fox mutants has an ear phenotype. Alternatively, multiple Fox genes may play functionally redundant roles in placode formation in mice [e.g. the *Foxf2* gene is expressed with *Foxi1* in the developing inner ears of both the mouse and the rat (Aitola et al., 2000)]. These will not be revealed until compound knockouts have been produced.

Signals from the hindbrain, neural crest and POM

The unique environment that surrounds the otocyst significantly influences its development. Signals from the hindbrain, the POM, the notochord and the neural crest all provide inductive and patterning information necessary for ear development (Fig. 3) (reviewed by Fritzsch et al., 1997; Fritzsch and Beisel, 2001; Fekete and Wu, 2002; Liu et al., 2003). In particular, members of the Fgf and Wnt signaling families originate in the hindbrain or POM, and act as either direct or indirect inducing agents for otic tissue (Ladher et al., 2000; Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Phillips et al., 2004). Fgf3 is thought to act as an otic-inducing agent in most vertebrates (Liu et al., 2003), but although disruption of Fgf3 signaling alone does lead to defects in ear development, otic placodes clearly form in all the mutant animal models tested to date (Represa et al., 1991; Mansour et al., 1993; Phillips et al., 2001). This has lead to the suggestion that other Fgfs may act in a redundant fashion.

The suggestion that different Fgfs have redundant roles was recently tested in studies by Wright and Mansour (Wright and Mansour, 2003b) and Alvarez et al. (Alvarez et al., 2003) in the mouse. In mice, *Fgf10* is expressed specifically in the POM located beneath the ectoderm that develops into the otic placode and is present here until E8.75. Mice with targeted mutations in *Fgf10* develop inner ear defects (Pauley et al., 2003). To examine potential redundancy between Fgf3 and Fgf10, embryos were generated that were deficient for both genes. Analyses at E9.5 and E10 indicated a complete lack of otocysts, although some animals had either unilateral or bilateral microvesicles in positions that were anatomically consistent with the location of an otocyst. Furthermore, expression of four different early placodal markers (*Pax8*, *Pax2*, *Dlx5* and *Gbx2*) was altered or absent in the dorsal ectoderm. Most importantly, analysis of embryos with different combinations of *Fgf3* and *Fgf10* deletions indicated a gradient of defects, consistent with dose-dependent functional redundancy.

The results discussed above demonstrate that multiple signals have roles in the induction of the placode (Fig. 3). Although the role of Fgf3 appears to be conserved across vertebrate species, alternate additional molecules seem to have been adopted in different species. Fgf10 is a second placode-inducing factor in mammals, while Fgf8 plays a similar role in zebrafish (Liu et al., 2003). In chicken, Fgf19 and Wnt8c induce the expression of placode-specific genes (Ladher et al., 2000), but in zebrafish, Wnt8c does not appear to play a role in otic induction (Phillips et al., 2004). Moreover, the murine homolog of chick Fgf19, Fgf15, induces otic markers in chick explants, but deletion of *Fgf15* in mice does not lead to obvious auditory defects (Wright et al., 2004). It is intriguing that the

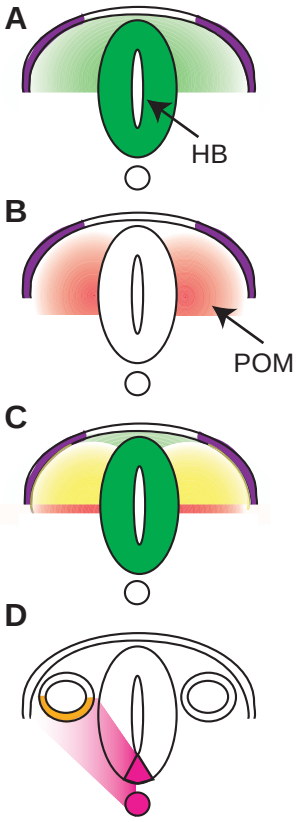

Fig. 3. Inductive interactions that regulate otocyst induction and ventral patterning. (A) Cross-section of a developing vertebrate embryo at the level of rhombomeres 4 and 5. Prior to placodal induction, a group of transcription factors, including Dlx3b, Dlx4b, Sox9a and Foxi1 are expressed in the preplacodal ectoderm (blue). Simultaneously, the hindbrain (HB) secretes Fgf3 (green), which acts as an inducer for the otic placode. (B) At the same time, periotic mesenchyme (POM) located between the hindbrain and placodal ectoderm produces either Fgf10- or Fgf19/15- (red) inducing agents, depending on the species. (C) The combined expression of Fgf3 (green) and either Fgf10 or Fgf19/15 (red) generates a combinatorial code (yellow) that induces placodal development in a specific region of the ectoderm (purple). (D) After closure of the otocyst, ventral phenotypes are induced through a combination of the expression of the transcription factor Six1 (orange) in the ventral region of the otocyst and long-range signaling by Shh (pink). Shh originates in the notochord and floorplate to influence the formation of both otocysts (although the presumed signaling interactions are only illustrated for the left side).

number of examples of genetic deletions leading to a complete loss of all aspects of otocyst development is still small and appears to be species dependent. Even some of the *Fgf3/Fgf10* double mouse mutants generate a microvesicle that must be considered to be an otic rudiment. Therefore, it seems possible that other inducing molecules remain to be discovered.

Patterning the otocyst

Early patterning events

Following induction, the otic placodes of most vertebrates invaginate to form the otocysts. In all animals examined, the development of the otocyst into the membranous labyrinth involves elaborate processes that include cellular growth, apoptosis, migration and differentiation. The molecular and cellular factors that direct this morphogenesis are still poorly understood. However, strong evidence indicates that both intrinsic and extrinsic signals begin to pattern the otocyst almost immediately after its formation at E9 in mouse (Morsli et al., 1998), the prim5 stage (24 hours post fertilization) in zebrafish (Liu et al., 2003) and HH stage 12 in chick (Fekete and Wu, 2002).

Pax genes

The transcription factor *Pax2* is among the earliest of the 'regionalized' genes in the chick inner ear. It is first expressed at around HH stage 17 in the region of the otocyst that will develop as the ED and endolymphatic sac (ES), the blind pouch at the end of the ED that is thought to remove debris from the endolymph (Hutson et al., 1999). A little later (HH stage 22), *Pax2* expression is found in the medial aspect of the otocyst, including the region that will develop as the cochlear duct. In the zebrafish, *pax2a* is first expressed at about 10.5-11 hours of development (at the initiation of somite formation) and is seen in two ovoid aspects at the medial edges of the placode. Neither *pax2a* nor *pax2b* is expressed in the ED, which forms late in the zebrafish (on day 8) (Bever and Fekete, 2002; Mendonsa and Riley, 1999; Phillips et al., 2001). Although ventromedial *pax2* expression in the chick is plastic and can be altered by otocyst axis disruption [rotation of the otocyst about the dorsoventral (DV) and mediolateral axes simultaneously], *Pax2* expression in the ED and ES is cell-autonomous by stage 13-14 in the chick and remains fixed even when the otocyst is inverted at this stage (Hutson et al., 1999). Therefore, the dorsomedial ED/ES region is among the first structures to be patterned in the chick otocyst. Similar rotation experiments have not yet been carried out in other animals.

Bmp4 and Bmp4 antagonists

Among the early regionally expressed genes are bone morphogenetic protein (Bmp4) and some of its known molecular antagonists. Bone morphogenetic proteins (Bmps) are members of the TGFβ superfamily of secreted signaling molecules (reviewed by Hogan, 1996) and interactions between Bmps and their antagonists, which include Noggin, Chordin, Follistatin and Dan family members, establish the anteroposterior (AP) and DV axes of the early embryo (reviewed by Niehrs, 1999; Nieto, 1999; Sasai, 2001) and the inner ear (Chang et al., 1999; Gerlach et al., 2000). Pathways downstream of Bmp4 and Bmp4 antagonists are also thought to be involved in sensory and neuronal cell specification (Chang et al., 1999; Gerlach et al., 2000), and it has been suggested that Bmp4 specifies the formation of the sensory patches in the chick inner ear (Oh et al., 1996; Wu and Oh, 1996; Cole et al., 2000). However, it is now thought that other genes, such as *Six1* (Oliver et al., 1995; Zheng et al., 2003; Ozaki et al., 2003), may play this role, as sensory patches develop in ears that have been exposed to either Noggin or Dan, indicating that Bmp signaling is not absolutely required for the formation of these structures.

Interactions between Bmp4 and retinoic acid

Studies by Thompson et al. (Thompson et al., 2003) have demonstrated that in the ear, all-trans retinoic acid (RA) directly regulates, and in fact inhibits, the expression of Bmp4.

Expression of Bmp4 in inner ear cell lines (Barald et al., 1997) and in vivo in the inner ears of chicks and mice is negatively regulated by RA through a novel promoter, i2, which originates from intron 2 of the Bmp4 gene (Thompson et al., 2003). Thompson et al. have demonstrated that the effects of treatment with RA on the development of the chick inner ear, which includes loss of the semicircular canals (SCCs), can be rescued by the implantation of cells producing Bmp4. Furthermore, the treatment of mice in utero with RA specifically downregulates Bmp4 expression through the i2 promoter. Choo et al. (Choo et al., 1998) have described experiments in which RA-soaked beads eliminate the development of semicircular canals at low-moderate doses, whereas sensory epithelia are apparently perturbed at higher doses. The elimination of SCC by RA in the study by Choo et al. (Choo et al., 1998) is therefore probably due to the effects of RA on Bmp4, as addition of Bmp4 blocks canal loss (Thompson et al., 2003).

Additional roles of Bmp4

In addition to its role in inner ear morphogenesis, Bmp4 is involved in patterning the surrounding mesenchymal cells that will develop into the bony labyrinth. Chang et al. (Chang et al., 2002) have compared the phenotypes of the otic capsule in ears that were infected with an avian retrovirus encoding either constitutively active or dominant-negative forms of the Bmp type IB receptor (Bmpr1b). Ectopic expression of constitutively active Bmpr1b induces cartilage overgrowth, whereas a dominant-negative form results in cartilage loss in the otic capsule. Inhibition of cartilage formation also occurs in response to treatment with Noggin, a secreted inhibitor of Bmp (Liu et al., 2002). In both cases, it is not clear whether the inhibition of cartilage development results from failure of mesenchyme to form cartilage precursor cells or from the resorption of initiated cartilage.

Dan, another Bmp antagonist, helps pattern the ED/ES

Dan is another Bmp antagonist involved in patterning during ear development. Dan is synthesized in the chick otic placode and at later stages in the medial wall of the otocyst, close to the hindbrain rhombomeres (Gerlach-Bank et al., 2002). Exogenous Dan has effects similar to Noggin, but also has profound effects on the ED/ES, including gross structural abnormalities in or elimination of these structures (Gerlach-Bank et al., 2004). Adding exogenous Bmp4 together with Dan rescues semicircular canal loss and prevents the effects on ED/ES, demonstrating that the effects of Dan are mediated through Bmps. Inhibition of Dan protein translation using antisense oligo morpholinos electroporated into the otic epithelium of chick embryos causes overgrowth of the ED/ES at the expense of canal structures. These experiments indicate that Dan may help to partition the otic epithelium of chick embryos into the SCC and the ED/ES fields. However, Dan is only expressed in extra-otic tissue in the mouse, showing that its role differs between mammals and birds.

Six1 and the ventral otic region

A group of recent studies has identified *Six1*, a homolog of the *Drosophila sine oculis* homeobox gene, as an important player in patterning of the ventral region of the otocyst, including, in the mouse, all of the sensory patches (Oliver et al., 1995; Zheng et al., 2003; Ozaki et al., 2004). *Six1* is a component of the Pax-Six-Eya-Dach genetic network that plays a key role in organogenesis in both flies and vertebrates (reviewed by Kawakami et al., 2000). *Six1* expression actually begins in the otic pit at E8.75 and becomes restricted to the ventral region of the otocyst by E9.5 (Fig. 3) (Oliver et al., 1995; Zheng et al., 2003; Ozaki et al., 2004). There is some debate regarding the expression of *Six1* during the latter stages of gestation, with one reporter mouse strain indicating expression in all developing sensory patches (Zheng et al., 2003), while a second indicates that expression of *Six1* is limited to the cochlea (Ozaki et al., 2004). The basis for this discrepancy is not clear and may be strain dependent.

Deletion of *Six1* in the mouse results in a complete loss of sensory epithelia within the ear, as well as agenesis of the cochlear duct (Zheng et al., 2003; Li et al., 2003b; Ozaki et al., 2004). It also leads to an increase in the size of the ED/ES and an increased representation of genes expressed exclusively in the dorsal region of the otocyst, such as *Dlx5*, *Hmx3* and *Nkx5.1* (Hadrys et al., 1998; Wang et al., 1998; Acampora et al., 1999; Depew et al., 1999). Also lost are ventral markers such as *Fgf10*, *Fgf3*, *Otx1* and *Otx2*, as well as lunatic fringe (*Lfng*), a marker of developing sensory patches. There is also a decrease in cellular proliferation and an increase in apoptosis in the ventral region of the otocyst in *Six1* mutants. These results show that *Six1* clearly plays an important role in multiple aspects of otocyst development. It is important to note that defects in the ears of *Six1* mutants are not observed until the latter half of gestation, indicating that *Six1* does not play a significant role in early development of the otocyst, despite its expression in the mouse otic pit at E8.75.

Six1, Eya1 and cellular proliferation

It has recently been demonstrated that eyeless 1 (Eya1) is a phosphatase that modulates the activity of Six1 (Li et al., 2003b; Rayapureddi et al., 2003; Tootle et al., 2003). *Eya1* is expressed in the developing otocyst, and its deletion in mice leads to the arrest of ear development at the otocyst stage (Xu et al., 1999), indicating that *Eya1* and *Six1* interact to mediate otocyst patterning. In mice that are double heterozygous for *Six1* and *Eya1* deletions, the overall formation of the ear is not disrupted but they do display some of the phenotypes produced by either of the homozygous deletions, including a shortened cochlea (Zheng et al., 2003). However, the phenotypic effects of the double heterozygote are quite variable and no changes were observed in the ED/ES or saccular regions of the inner ear, indicating that other factors may also be involved in this interaction. As Six1 is thought to play a general role in cellular proliferation and survival (Li et al., 2003b), these results indicate that Six1 and Eya1 act together to regulate cellular proliferation and survival in the ventral otocyst. It is not clear whether these factors also directly regulate ventral gene expression, or if the defects in gene expression are a secondary result of the defects in proliferation and survival.

Tbx1 regulates neural fate in the otocyst

Individuals suffering from DiGeorge syndrome, velocranial facial syndrome and conotruncal anomaly face syndrome experience a number of abnormalities, including auditory deficits, as a result of a chromosomal deletion on 22q11.2 (reviewed by Baldini, 2003). Several studies have demonstrated that the basis for these defects is the deletion of

the T-box gene, *TBX1* (Yagi et al., 2003). Deletion of *Tbx1* in mice or of *tbx1* in zebrafish results in small dysmorphic otocysts that have minimal or no sensory epithelia (Jerome and Papaioannou, 2001; Vitelli et al., 2003; Piotrowski et al., 2003). The gene is expressed widely throughout both the otic epithelium and POM, indicating that it could have roles in multiple patterning events within the ear. A recent analysis of the effects of either deletion of *Tbx1* or overexpression of *Tbx1* in the mouse otocyst between E9 and E13.5 indicates a role in the regulation of neurogenesis (Raft et al., 2004). Specifically, overexpression of Tbx1 in the otocyst decreases the number of neurogenin 1 (*Ngn1*)-positive neuronal precursors and reduces the size of the statoacoustic ganglion. Conversely, in *Tbx1* mutants, *Ngn1*-postive precursor numbers and ganglion size increase. Considering that neuronal precursors arise from the anterior half of the otocyst, and that *Tbx1* is apparently not expressed in those precursors, the authors suggest that Tbx1 helps regulate the development of the AP axis of the otocyst.

Sonic hedgehog and ventral patterning

Mice lacking the secreted morphogen sonic hedgehog (Shh) (Riccomagno et al., 2002) have a similar phenotype to that described in *Six1* knockouts. In the absence of *Shh*, otic development is normal until midgestation, at which point ventral otic morphogenesis fails to occur. Neither the cochlear duct nor the statoacoustic ganglion develops in the absence of *Shh*. The expression of many ventral and dorsal markers is disrupted, resulting in rearranged expression patterns similar to those reported for *Six1* mutants. Moreover, the expression of *Ngn1* and a second neuronal precursor marker, *Neurod1* (previously *NeuroD*) is significantly reduced in the absence of *Shh*. Shh may also play a role in establishing the chondrogenic lineage that produces the cartilage anlagen of the bony labyrinth (Liu et al., 2002).

Despite the presence of significant defects in the ears of *Shh* mutants, *Shh* itself is not expressed in the ear or in the surrounding POM (Riccomagno et al., 2002). The closest source of Shh is the developing notochord and floor plate (Fig. 3), as seen by in situ hybridization at E10.5. *Gli1* and *Ptch*, two genes that are dependent on Shh for expression, are expressed in both the developing otocyst and POM between E9.5 and E10.5, leading to the suggestion that Shh produced in the notochord and floorplate is directly involved in the formation of the ventral auditory system (Riccomagno et al., 2002).

The same study examined the role of Shh in formation of the ventral auditory system using a transgenic mouse model that specifically directed ectopic expression of *Shh* to the otocyst. Otic expression of Shh leads to the ectopic expression of markers of the ventral otocyst and to expanded ventral (auditory) regions of the inner ear at the expense of dorsal (vestibular) structures. Riccomagno and colleagues suggest that this phenotype could be a result of a premature truncation of the development of dorsal structures, but it is also possible that these structures form correctly and are then resorbed. This study demonstrates a role for Shh in dorsoventral patterning of the otocyst, as well as in the regulation of a number of regionally expressed genes within the ear. However, it cannot yet be concluded how many of these genes are directly regulated through Shh signaling and how many might be affected secondarily as a result of defects in dorsoventral patterning.

A model for axis formation in the inner ear

In summary, the results of a number of recent studies have resulted in a significant step forward in our understanding of the factors that pattern the dorsoventral axis of the ear. Beginning at the otic pit stage, members of the Pax-Six-Eya-Dach gene pathway, including *Pax2*, *Six1*, *Eya1* and *Dach1*, are all expressed in the ventral, ventromedial or medial region of the otocyst and appear to work together to specify these regions of the ear. The complete relationships between these genes remain to be elucidated, but existing data indicate that *Eya1* is required for *Six1* expression (Zheng et al., 2003). Expression of Pax-Six-Eya-Dach, along with *Otx1*, *Otx2*, *Fgf3*, *Lfng*, *Ngn1* and *Neurod1* coordinate the development of different ventral and/or medial aspects of the ear (Morsli et al., 1998; Morsli et al., 1999; Zhang et al., 2000; Cole et al., 2000; Ma et al., 2000; Kim et al., 2001). At the same time, dorsal determinants, including *Dlx5* and *Hmx3* (Wang et al., 1998; Acampora et al., 1999), regulate the formation of the dorsal region. Further discoveries concerning the specific relationships between these signaling pathways will lead to some fundamental and perhaps widely applicable tenets of axis formation.

Development of inner ear sensory epithelia

Cell fate specification

Among the later steps in the development of the inner ear are the specification of individual cell fates and the development of cellular pattern. Studies have demonstrated that the basic helix-loop-helix protein Math1 (Atoh1) is important for the development of hair cells in rodents (Bermingham et al., 1999; Zheng and Gao, 2000). A crucial second result of the Zheng and Gao study was that cells located within the greater epithelial ridge (GER), an embryonic structure located adjacent to the sensory epithelium of the cochlea, develop into hair cells after transfection with Math1 (Figs 4 and 5). Hair cells rarely, if ever, develop in the GER in vivo, highlighting the importance of the spatial regulation of *Math1* expression. Our present understanding of the regulation of *Math1* is limited, although analysis of its promoter region (reviewed by Ross et al., 2003) indicates that Math1 can promote its own expression (Helms et al., 2000).

Zic genes may regulate Math1 expression

A recent study identified a Zic-binding domain in the 3′-enhancer region of *Math1* that is also present in the 3′-enhancers of the Math1 homologs, *HATH1* (*ATOH1*; human) and *Cath1* (chicken) (Ebert et al., 2003). Zic genes are neural inhibitors in the developing chick spinal cord that are regulated by the Bmp signaling pathway (Nakata et al., 1997; Nakata et al., 1998; Brewster et al., 1998; Mizuseki et al., 1998; Aruga et al., 2002a; Aruga et al., 2002b). The binding of Zic1 to the Math1 3′-enhancer region (based on electron mobility shift assay studies) is sufficient to inhibit *Math1* expression in the chicken neural tube. The vertebrate Zic (zinc finger in the cerebellum) genes are homologs of the *Drosophila* pair-rule gene *odd-paired* (*opa*), and code for transcription factors that contain five tandem repeats of a C_2H_2 zinc-finger domain (Aruga et al., 1994; Aruga et al., 1996). Deletion of *Zic1* in mice results in patterning defects in the cerebellum and in particular in cerebellar granule neurons (Aruga et al., 2002a; Aruga et al., 2002b). *Zic1* and *Zic2* are expressed in the

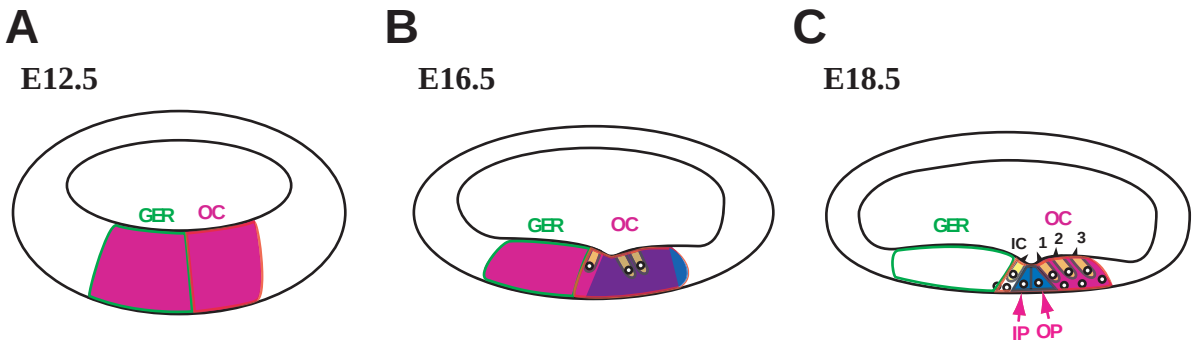

Fig. 4. Development of the cochlear duct and organ of Corti. Each drawing illustrates a cross-section through the developing mammalian cochlear duct. (A) At E12.5, the duct consists of undifferentiated epithelial cells. A subset of cells located within the duct expresses *Fgfr1* (pink). Expression of *Fgfr1* appears to correlate with the region of the duct will develop into the greater epithelial ridge (GER, green outline) and organ of Corti (OC, red outline). (B) By E16.5, individual hair cells (yellow) begin to differentiate within the OC. *Fgfr1* expression continues in the GER and OC (red outline). In addition, a subset of cells within the OC begins to express *Fgfr3* (blue) and *Fgfr1* (overlap of *Fgfr1* and *Fgfr3* expression appears as purple). (C) By E18.5, the basic cellular pattern of the OC is complete. A single inner hair cell (IC, yellow) and three outer hair cells (1-3, yellow) are present at this stage. In addition, inner and outer hair cells are separated by the developing pillar cells (labeled as OP and IP) that will give rise to the tunnel of Corti. By this time, expression of *Fgfr1* (pink) has become restricted to the outer hair cells and Deiter's cells (another type of non-sensory supporting hair cell), while *Fgfr3* expression (blue) is restricted to the pillar cells.

developing chick inner ear from the early otocyst stage onwards (Warner et al., 2003), raising the possibility that they may play a key role in regulating *Math1* expression and cellular patterning in the inner ear.

In addition to their roles in regulating Math1, Zic genes may contribute to the regulation of neurogenesis in the developing otocyst. In the chicken, both *Zic1* and *Zic2* are expressed in the same anteroventral region that gives rise to delaminating SAG precursors (Molea et al., 1999; Stone et al., 2003; Warner et al., 2003), and the onset of their expression roughly correlates with the cessation of neurogenesis in this region. It seems possible that expression of Zic genes, possibly in response to Bmp signaling, could play a role in regulating the number of neuronal precursors that delaminate to form the SAG.

Fgfrs in sensory epithelium patterning

A recent study in mice has demonstrated a very intriguing role for Fgfr1 in the patterning of the sensory epithelium of the mammalian cochlea. Using a Cre-lox strategy, Pirvola et al. (Pirvola et al., 2002) deleted Fgfr1 in a restricted set of tissues, including the otocyst, beginning at around E11.5. The gross morphology of the cochleae in these mice was normal, but there was significant disruption in the patterning of the sensory epithelium (Fig. 5). The normal pattern of hair cells and supporting cells was replaced by small islands of sensory cells that were separated by large regions of unpatterned epithelial cells. There was also decreased proliferation of cells located within the developing cochlea, leading the authors to suggest that the defect in cellular patterning might be a result of a decrease in the progenitor pool required to build the sensory epithelium. More-detailed studies will determine whether the role of Fgfr1 in the cochlea is limited solely to cellular proliferation.

Mueller et al. (Mueller et al., 2002) have demonstrated that Fgfr3 plays a key role in the differentiation of pillar cells (Fig. 5), a unique cell type found only in the cochleae of mammals. Previous results had demonstrated that deletion of *Fgfr3* leads to a loss of pillar cells (Colvin et al., 1996). Mueller et al. (Mueller et al., 2002) used an in vitro approach to examine the effects of inhibiting Fgfr3 activation at different times during development of the organ of Corti. They demonstrated that the differentiation of pillar cells is dependent on continuous activation of Fgfr3 from ~E15 at least until birth. The same study demonstrated that ectopic activation of Fgfr3 leads to an overproduction of pillar cells, indicating that a limiting amount of Fgfr3 ligand within the organ of Corti may play a key role in regulating pillar cell number and differentiation.

Planar polarity of the hair cells

Another key event that occurs during the formation of all hair cell sensory epithelia is the generation of uniform orientation of stereociliary bundles. Stereociliary bundles are directionally sensitive such that only deflections in a single plane will elicit a physiological response. Even minor deviations in the generation of the orientation of the stereociliary bundle can result in defects in auditory or vestibular function (Yoshida and Liberman, 1999).

Recent work from a number of laboratories has successfully identified many of the molecules that regulate planar cell polarization (PCP) in the eye and wing of *Drosophila* (reviewed by Mlodzik, 2002; Strutt, 2003). Using this knowledge as a starting point, several recent studies have examined the roles of vertebrate homologs of *Drosophila* PCP genes in the regulation of stereociliary bundle orientation. One of these homologs, *Vangl2*, codes for unique four-pass transmembrane proteins with a PDZ-binding motif located at the C terminus (Wolff and Rubin, 1998; Kibar et al., 2001; Murdoch et al., 2001) (Table 1). Montcouquiol et al. (Montcouquiol et al., 2003) have demonstrated significant defects in stereociliary bundle orientation in the ears of mice that carry a mutation in *Vangl2*. The overall morphology of the stereociliary bundles in these animals appears normal, but polarization decreases in a graded fashion that correlates with hair cell position. In the most severely affected regions, the distribution of stereociliary bundles is random, indicating that the polarizing signals have been lost entirely. The specific role

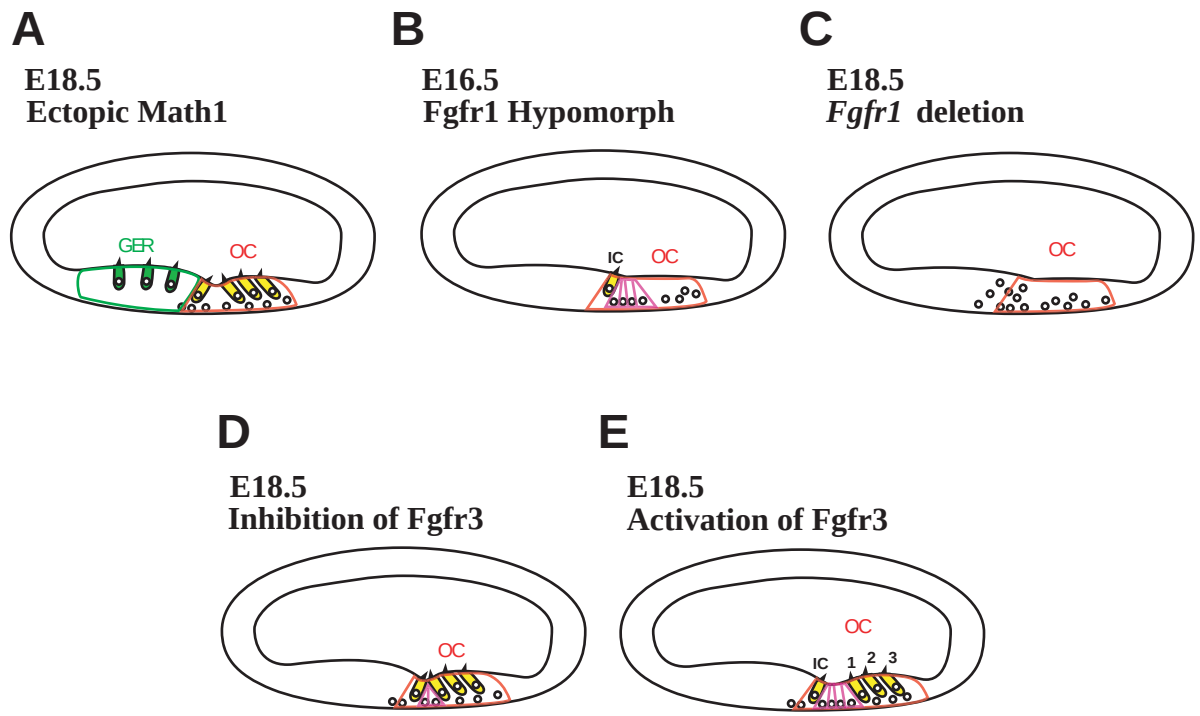

Fig. 5. Effects of modulating gene expression on development of the organ of Corti (OC). (A) Ectopic expression of Math1 (Atoh1) in individual cells within the greater epithelial ridge (GER) (green outline) results in the formation of hair cells (filled in green) in the GER. (B) Hypomorphic Fgfr1 produces small sensory islands that consist of inner hair cells (IC, yellow), an increased number of pillar cells (pink) and no outer hair cells. (C) Deletion of *Fgfr1* leads to a nearly complete disruption in the formation of the OC (red outline) with no identifiable cell types. (D) Deletion of *Fgfr3* or inhibition of FGFR3 activity results in a disruption in the differentiation of pillar cells (outlined in pink). Compare the shape of the pillar cells in this drawing with the normal shape of the pillar cells illustrated in Fig. 4. (E) Increased activation of Fgfr3 leads to an overproduction of pillar cells but no significant affects on formation of either inner hair cells (IC) or outer hair cells (1-3, yellow).

of *Vangl2* in directing the orientation of the stereociliary bundle remains unclear, but a mutation in *Vangl2* results in a defect in the initial step of stereociliary bundle orientation. As illustrated in Fig. 6, the first sign of bundle orientation is the centrifugal movement of the developing kinocilium from the center of the lumenal surface of the cell towards the peripheral edge. In wild-type individuals, this movement has a significant bias in the direction of final bundle orientation. In *Vangl2* mutants, the centrifugal movement still occurs, but the direction of this movement is disrupted. Nevertheless, as the mechanism(s) that physically determine the orientation of the stereociliary bundle have not been determined, it is not yet possible to make a definite link between the PCP signaling cascade and stereociliary bundle orientation.

In a second study examining the possible role of PCP in the ear, Dabdoub et al. (Dabdoub et al., 2003) have demonstrated that Wnt signaling is involved in the development of stereociliary bundle orientation (Fig. 6D). This is consistent with the hypothesis that Wnts play a role in the generation of an instructive morphogen gradient that could be used by developing hair cells to accurately refine the orientations of stereociliary bundles. The same study identified Wnt7a as a possible candidate for this morphogen, although no polarization defects were observed in *Wnt7a* mutant mice, indicating that, again, functional redundancy may exist. Finally, mutations in *Celsr1*, a murine homolog for the *Drosophila* PCP gene *flamingo*, lead to polarization defects in a mouse line generated through the Harwell ENU project (Curtin et al., 2003). *Celsr1* and *flamingo* code for atypical cadherins that include a seven-pass transmembrane domain that has a similarity to the serpentine G-protein receptors and, in *Drosophila*, interact with Dsh (Table 1).

Table 1. Genes that regulate planar cell polarization in vertebrates

Gene	Protein structure	Inner ear phenotype	Reference
Vangl2	Four-pass transmembrane with PDZ-binding domain	Near-random distribution of bundle orientations	Montcouquiol et al. (2003)
Scrb1	Cytoplasmic with four PDZ domains	Mild PCP defect, but strong genetic interaction with *Vangl2*	Montcouquiol et al. (2003)
Wnt	Secreted glycoprotein	Inhibition of refinement in orientation	Dabdoub et al. (2003)
Celsr1	Atypical cadherin with seven-pass transmembrane domain	Orientation defects appear comparable with Vangl2	Curtin et al. (2003)

The four genes or factors known to influence planar cell polarization (PCP) in the auditory system are listed. For each, the structure of the protein and the phenotype in the inner ear are also listed.

These experiments demonstrate that the molecular pathway(s) that regulate PCP have been conserved between flies and vertebrates. In the future, it should be possible to use the known players to identify other interacting proteins. A genetic complementation assay has already demonstrated that *Scrb1* (*Scrib*) the homolog of a tumor suppressor gene involved in apicobasal polarity in *Drosophila* called *scribble* (Brumby and Richardson, 2003), plays a role in bundle polarization in the mouse ear (Montcouquiol et al., 2003).

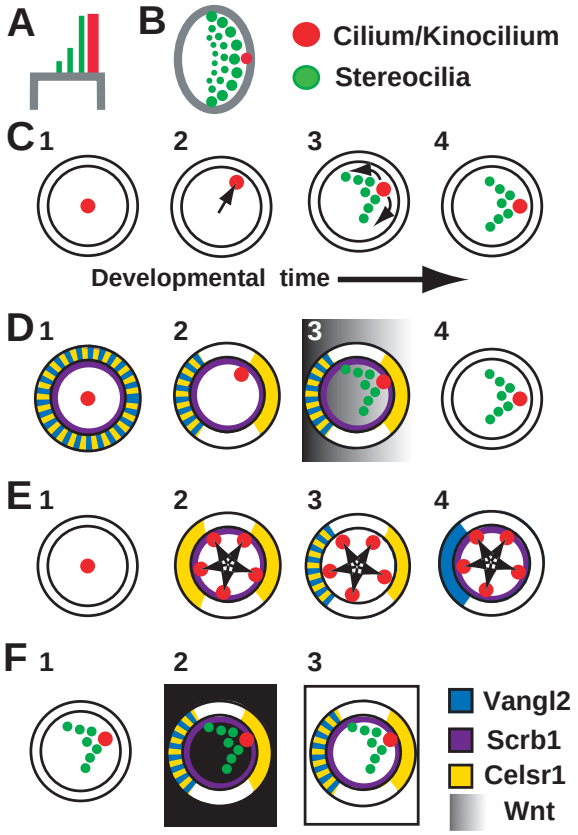

Fig. 6. Generation of stereociliary bundle orientation. (A,B) Side and lumenal views of a hair cell illustrating the morphology of the mechanosensitive stereociliary bundle. The stereocilia (green) are arranged in a staircase pattern with a single microtubule-based kinocilium (red) located at one edge. (C) Each circle illustrates the lumenal surface of a developing hair cell at different time points. (C1) Prior to bundle formation, all developing hair cells have a single cilium (red) that will develop as the kinocilium. (C2) As development proceeds, the cilium moves from the center of the lumenal surface towards the periphery (arrow). Most cilia move to a position that approximates their final orientation. (C3) Next, the developing stereociliary bundle (kinocilium in red and stereocilia in green) moves along the peripheral edge of the cell (arrows) to attain its final orientation. (C4) Progressive refinements ultimately result in the development of appropriate orientation. (D) Hypothetical expression patterns for molecules that regulate bundle orientation. (D1) Vangl2 (blue), Celsr1 (yellow) and Scrb1 (purple) are thought to be expressed initially throughout hair cells. (D2) Prior to the centrifugal movement of the cilium, Vangl2 is localized to the side of the developing cell opposite the site of stereociliary bundle formation (the proximal side) and Celsr1 is localized to both the proximal and distal sides of the cell. (D3,D4) Wnt (dark shading) is thought to be distributed in a gradient around the developing hair cell. Following centrifugal movement, outer hair cells reorient their bundles towards the point of lowest Wnt concentration. (E) Effects of different mutations on bundle orientation. Mutations in *Vangl2* (E2) result in a defect in the direction of centrifugal movement. Similar effects are assumed for *Scrb1* (E3) or *Celsr1* (E4) mutants. (F) Disruption of the Wnt gradient, either an excess of Wnt (F2) or a deficiency (F3), results in inhibition of the bundle reorientation.

Future directions and insights into human deafness

In the preceding sections, we have highlighted some of the most recent discoveries in inner ear development. Although these results clearly represent a significant advance in our understanding of how the ear forms, it is also important to recognize that these discoveries may contribute to our understanding of, and potential treatments for, human deafness. The most obvious approach would be to consider all the genes discussed here as potential candidates for human deafness genes, and to determine whether these genes are mutated in individuals or families with genetic-based hearing loss or deafness. It is particularly important to consider that hypomorphic alleles could result in proteins with levels of function that are sufficient for overall survival but insufficient for the maintenance of hearing. For example, mice with a targeted deletion in the transcription factor *Brn3c* (*Pou4f3*) begin to lose hair cells by E17 and are deaf from birth (Erkman et al., 1996), while humans with a mutation in *POU4F3* (the ortholog of *Brn3c*) have progressive hearing loss that does not begin before 18 years of age (Vahava et al., 1998). Similarly, some mutations in myosin VIIa (*MYO7A*) lead to non-syndromic deafness (Liu et al., 1997; Weil et al., 1997), while other mutations in the same gene result in Usher's syndrome, which includes both deafness and loss of sight (reviewed by Ahmed et al., 2003).

At present, there are limited options for the treatment of genetically based hearing loss, either congenital or progressive, except for cochlear implantation. However, several recent findings have indicated new avenues for treatments for deafness. Transfer of adenoviral vectors expressing *Math1* into the ears of guinea pigs resulted in the formation of 'hair cell-like' cells (Kawamoto et al., 2003). Similarly, transplantation of stem cells into the otocysts of embryonic chicks has resulted in the formation of stem cell-derived hair cells (Li et al., 2003a). Biasing stem cells towards particular inner ear cell fates, possibly by transfecting genes that are expressed during inner ear development, could potentially replace particular cell types within the ears of individuals with hearing loss or deafness. The translation of our knowledge of the genes regulating inner ear development into therapeutic strategies to correct or delay hearing loss seems closer to reality than at any time in the past.

Armed with the existing entry points into the genetic pathways that regulate different aspects of ear development, our next task must be to elucidate the genetic cascades fully, and to determine the roles of these cascades in the cellular events that must occur to create the different components of the ear. Although we are clearly a long way from even a limited blueprint for the complete development of the ear, the molecular biological tools required to complete this project are either now available or soon will be. We look forward to an increased and synergistic rate of discovery that will open even more avenues of study into this fascinating system.

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