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Ringing in the new ear: resolution of cell interactions in otic development

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Abstract

The vertebrate inner ear is a marvel of structural and functional complexity, which is all the more remarkable because it develops from such a simple structure, the otic placode. Analysis of inner ear development has long been a fascination of experimental embryologists, who sought to understand cellular mechanisms of otic placode induction. More recently, however, molecular and genetic approaches have made the inner ear a useful model system for studying a much broader range of basic developmental mechanisms, including cell fate specification and differentiation, axial patterning, epithelial morphogenesis, cytoskeletal dynamics, stem cell biology, neurobiology, physiology, etc. Of course, there has also been tremendous progress in understanding the functions and processes peculiar to the inner ear. The goal of this review is to recount how historical approaches have shaped our understanding of the signaling interactions controlling early otic development; to discuss how new findings have led to fundamental new insights; and to point out new problems that need to be resolved in future research.

Keywords: Otic vesicle; Hair cell; Cochlea; Macula; Crista; Vestibular; Preplacodal domain; Hindbrain

Introduction

General description of inner ear structure and function

Morphological development of the inner ear begins with formation of the otic placode. In tetrapod vertebrates, the otic placode invaginates to give rise to hollow ovoid structure, the otic vesicle. In zebrafish, the otic vesicle forms by cavitation rather than invagination (Haddon and Lewis, 1996). The walls of the otic vesicle undergo complex folding and growth to produce a series of interconnected chambers (Anniko, 1983; Lewis et al., 1985). The utricle and semicircular canals constitute the vestibular apparatus, the structure and function of which have been highly conserved in all vertebrates. In contrast, the auditory chambers have undergone extensive evolutionary modification (Fig. 1): The saccule and lagena are prominent auditory endorgans in fish but the saccule serves a vestibular role in mammals and birds, and the lagena is absent in mammals and is of uncertain function in birds. The primary auditory endorgan in birds and mammals is the cochlea, which has no known counterpart in fishes and amphibians. In amphibians, the saccule is auditory, as are the basilar papilla and amphibian papilla. Each chamber is associated with a sensory epithelium containing hair cells and support cells. In tetrapod vertebrates, sensory epithelia differentiate only after the various chambers of the ear begin to form. In fish, sensory epithelia appear much earlier, soon after the otic vesicle forms (Haddon and Lewis, 1996; Riley et al., 1997; Whitfield et al., 2002). The first hair cells form in the utricle and saccule, followed by those in the semicircular canals. The lagenar sensory epithelium forms much later during larval development in zebrafish (Riley and Moorman, 2000; Bever and Fekete, 2002). Hair cell function is stimulated by lateral deflection of ciliary bundles projecting into the lumen of the ear, providing the basis for both hearing and balance (Anniko, 1983; Lewis et al., 1985; Müller and Littlewood-Evans, 2001). The function of support cells is less clear. They are thought to perform a stem cell-like function im-

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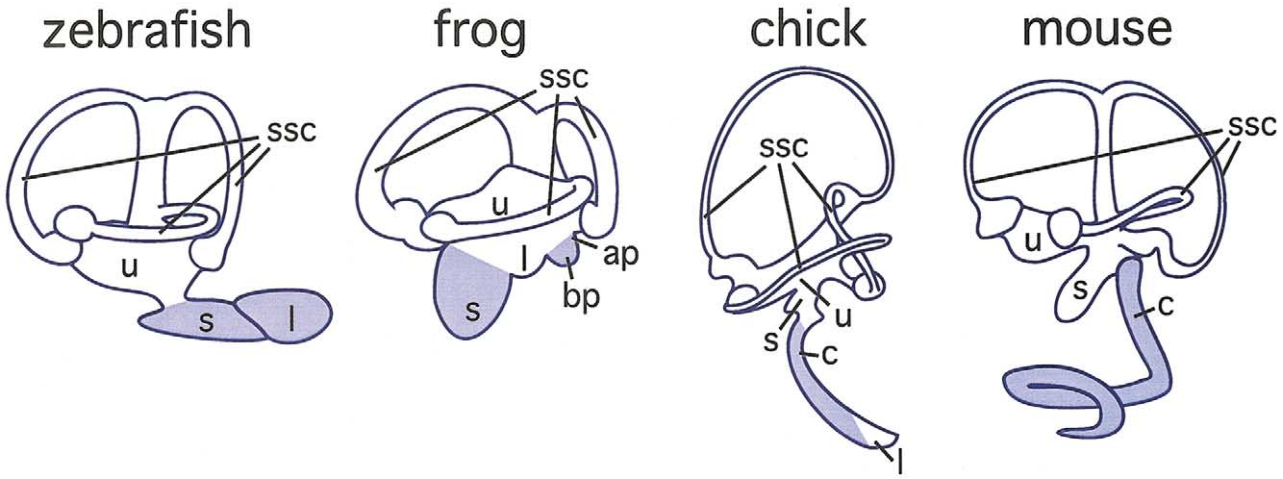

Fig. 1. General structure of the inner ear. Representations of adult inner ear structures in zebrafish, *Xenopus*, chick, and mouse. Lateral views (anterior to the left). Auditory regions are shaded blue. Abbreviations: ap, amphibian papilla; bp, basilar papilla; c, cochlea, 1, lagena; s, saccule; u, utricle.

portant for regeneration of hair cells (Presson et al., 1996; Lanford et al., 1996; Fekete et al., 1998; Baird et al., 2000; Stone and Rubel, 2000), and may also be essential for maintenance of hair cells (Eddison et al., 2000; Haddon et al., 1999). Maculae, the sensory epithelia in the utricle, saccule and lagena, are associated with otoliths (or otoconia). Otoliths are dense crystals attached to the ciliary bundles of hair cells. They facilitate vestibular and auditory function by transmitting accelerational forces and sound vibrations, respectively, to hair cell cilia. Cristae, the sensory epithelia in the semicircular canals, lack otoliths. Instead, hair cells in the cristae bear extremely long ciliary bundles that are sensitive to fluid motion caused by angular acceleration. Electrical signals from hair cells are transduced by neurons of the statoacoustic ganglion (SAG). SAG neuroblasts delaminate primarily from the anteroventral surface of the otic vesicle and later differentiate to innervate the various sensory patches in the developing inner ear. The fluid inside the inner ear, termed endolymph, has a characteristic ionic balance required for hair cell function. Its volume is maintained at proper levels by flow through the endolymphatic duct, an outgrowth from the medial wall of the inner ear. The endolymphatic duct is one of the first ear structures to form in amniotes but forms relatively late during larval development in zebrafish (Bever and Fekete, 2002). Although regulatory mechanisms have been partially characterized for each of the above structures and processes, many details are lacking and our understanding of morphogenesis is still rudimentary.

In the beginning: classical studies on otic induction

Inspired by the groundbreaking studies by Spemann and colleagues on embryonic induction, many experimental embryology studies have been conducted over the past 80 years attempting to uncover mechanisms of otic placode induction. From the beginning, it has been assumed that the process is similar in all vertebrates, and this has proven to be essentially correct. Most early studies focused on amphibian and avian species because of the ease of conducting tissue recombination experiments. The most fundamental goal of this research was to characterize the developmental timeframe of otic induction. A common approach then, and more recently, has been to transplant prospective otic ectoderm to foreign sites to establish when it becomes committed to form an otic vesicle. The answer clearly depends on the site of transplantation, as well as the criteria used to assess otic development. The ability to form a rudimentary vesicle is first detectable by the early neurula stage, just prior to the formation of a morphologically visible placode (Zwilling, 1941; Gallagher, 1996; Groves and Bronner-Fraser, 2000). As transplantation is conducted at progressively later stages, the fraction of grafts that succeed in forming a vesicle increases, and the morphology of the vesicle gradually improves (Yntema, 1933; Waddington, 1937; Swanson et al., 1990; Groves and Bronner-Fraser, 2000). In another common approach, ectoderm from foreign sites is transplanted into the periotic region at various stages to test the ability of the periotic environment to respecify uncommitted foreign ectoderm. From such experiments, it is clear that otic-inducing activity persists well beyond the stage when the otic placode normally forms. Inducing activity is finally lost rather abruptly during mid-to-late somitogenesis (Waddington, 1937; Yntema 1933, 1950; Gallagher, 1996). Assessing when otic induction begins has been more difficult, but the most telling data have come from studies seeking to identify the source(s) of otic-inducing activity. The hindbrain has long been considered an inductive source: Otic-inducing activity is localized to the lateral edges of the hindbrain (Groves and Bronner-Fraser, 2000), and transplanting hindbrain tissue to other regions

can induce ectopic otic vesicles (Stone, 1931; Waddington, 1937; Woo and Fraser, 1998). In addition, excision of the hindbrain primordium perturbs otic development to varying degrees. However, hindbrain ablation does not usually block otic development (Waddington, 1937; Harrison, 1945), suggesting that subjacent mesendoderm can also induce otic placode. Moreover, the early stage at which such results are obtained suggests that the alternate inductive tissues are already active by late gastrulation. Studies by Jacobsen (1963) indicate that signals from the hindbrain and mesoderm are partially redundant, and signals from both tissues are needed for optimal induction and normal development of the inner ear. Signals from pharyngeal endoderm also help regulate patterning of the otic vesicle but do not appear to play a role in induction. Together, these studies support the consensus that otic induction is not a singular event but reflects a gradual process involving interactions with a variety of tissues over a relatively long span of time.

Another fundamental goal has been to analyze the spatial and temporal parameters governing competence of ectoderm to respond to otic induction. By early somitogenesis stages, different regions of the ectoderm show dramatic differences in the ability to respond to otic induction, with head ectoderm near the endogenous otic placode being the most receptive and caudal and ventral ectoderm being highly refractory. Weak regional biases become discernable by late gastrulation, but in the early gastrula much if not all ectoderm seems able to respond to otic induction (Kaan, 1926; Yntema, 1933, 1950; Gallagher, 1996; Groves and Bronner-Fraser, 2000). Thus, otic competence is initially widespread but becomes increasingly localized during the course of development. The gradual restriction in otic competence is thought to reflect progressive differentiation of non-otic ectoderm to form other fates, as well as a gradual increase in competence by prospective otic ectoderm.

Identification of otic inducers

The best candidates for otic-inducing factors are members of the Fibroblast Growth Factor family of peptide ligands. Fgf3 was first proposed as an otic inducer based on its expression pattern (Wilkinson et al., 1989), but early functional studies were inconclusive. Represa et al. (1991) used antisense oligos and blocking antibodies to knockdown Fgf3 in chick explant cultures. Although this blocked formation of the otic vesicle, it did not address the process of placode induction because explants were taken at a stage when nascent placodes had already formed. It has also been pointed out that the antisense oligos used in the study were designed by using a human sequence with a poor match to chick, and attempts to replicate these results have failed (Mahmood et al., 1995). On the other hand, the antiserum was generated against a perfectly conserved peptide sequence, so the conclusion that it disrupts placode-to-vesicle development in chick explant cultures is probably still valid. Targeted disruption of mouse *Fgf3* severely perturbs patterning of the otic vesicle but does not block placode induction (Mansour et al., 1993). Together, these results were widely interpreted to mean that Fgf3 regulates postplacodal stages only. However, more recent studies suggest that Fgf3 does indeed mediate otic induction but that functional redundancy compensates for its loss. Misexpression of *Fgf3* induces ectopic otic vesicles in *Xenopus* and chick embryos (Lombardo et al., 1998; Vendrell et al., 2000), suggesting that this could reflect a normal function of *Fgf3* in these species. In zebrafish, no mutations have yet been reported in *fgf3*, but injecting zygotes with antisense morpholino oligomers (Nasevicius and Ekker, 2000) designed to specifically knockdown *fgf3* function (fgf3-MO) leads to development of embryos with small malformed otic vesicles reminiscent of *Fgf3* null mice (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002). Another candidate for an otic inducer in zebrafish is Fgf8. This gene is disrupted in *acerebellar (ace)* mutants, which also produce small malformed otic vesicles (Whitfield et al., 1996; Reifers et al., 1998). Impairing both functions, either by injection of *fgf3*-MO into *ace* mutants or coinjection of *fgf3*-MO and *fgf8*-MO, totally and specifically blocks development of the inner ear. Analysis of early markers of otic development (see below) shows that the absence of ears is caused by disruption of otic induction and not simply failure to maintain otic development. While these data support the notion that Fgf3 and Fgf8 play partially redundant roles in otic induction in zebrafish, it should be noted that loss of both gene functions significantly alters segmental identities in the hindbrain (Maves et al., 2002; Walshe et al., 2002), raising the possibility of an indirect effect. However, a recent study in mouse also supports a model in which partially redundant Fgf functions directly mediate otic induction: Disruption of *Fgf3* and *Fgf10* specifically ablates otic development but does not appreciably alter hindbrain patterning (T. Wright and S. Mansour, personal communication). In chick, Fgf19 has also been implicated in otic induction and could act in concert with Fgf3 to induce otic tissue (Ladher et al., 2000).

The temporal requirements for Fgf signaling have been examined in zebrafish by exposing embryos at various stages of development to the chemical inhibitor SU5402, an agent that specifically blocks activation of Fgf receptors (Leger and Brand, 2002; Maroon et al., 2002). Beginning treatment at any time from 30% epiboly through the two-somite stage causes severe reduction or ablation of otic tissue. If treatment is initiated after the six-somite stage, expression of early otic markers is either unaffected or reduced (but not ablated), depending on whether exposure to the inhibitor is terminated after several hours or maintained. These data suggest that otic fate becomes fixed just prior to appearance of the placode, after which development may continue in the absence of Fgf3 and Fgf8 signaling, albeit in a diminished capacity. Treatment at the otic vesicle stage ablates expression of many regionally expressed otic

markers, supporting a role for Fgf signaling in pattering of the otic vesicle.

As suggested by the above studies, the role of Fgf3 as an otic inducer appears to be conserved amongst fish, amphibian, avian, and mammalian vertebrates. In all species examined, *Fgf3* is expressed in the hindbrain primordium by the end of gastrulation and is maintained in the hindbrain well past the 9- to 10-somite stage when otic placode forms (Wilkinson, 1989; Mahmood et al., 1995, 1996; Lombardo et al., 1998; Phillips et al., 2001). This is consistent with classical studies showing that the hindbrain is a source of otic-inducing factors and that the inducing activity persists until mid–late somitogenesis. *Fgf3* is also expressed in various mesendodermal tissues in most species (but not in mouse). Although classical studies had previously supported a role for mesodermal signals, relevant mesodermal tissues were not identified. Studies of zebrafish mutants with specific mesendodermal deficiencies suggest that the prechordal plate and paraxial cephalic mesendoderm, but not chordamesoderm, play important roles in otic induction (Mendonsa and Riley, 1999). Accordingly, zebrafish *fgf3* is expressed in prechordal mesendoderm, which passes beneath the periotic region by mid gastrulation, and *fgf3* is later weakly expressed in paraxial cephalic mesoderm during the latter half of gastrulation. *Fgf3* is also expressed in paraxial cephalic mesendoderm in chick, and grafting experiments show that this tissue possesses strong otic-inducing activity (Ladher et al., 2000).

In contrast to *Fgf3*, involvement of other Fgf homologs varies considerably between species. In zebrafish, *fgf8* is coexpressed with *fgf3* in the germring and hindbrain during gastrulation, but in *Xenopus*, chick, and mouse, *Fgf8* is not expressed in periotic tissues prior to formation of the otic placode (Christian and Slack, 1997; Hidalgo-Sanchez et al., 2000; Adamska, 2001; Pickles, 2001). In addition, Fgf8-beads induce expression of some otic markers in chick embryos but cannot induce formation of ectopic vesicles (Adamska et al., 2001), further suggesting species differences in the role of this ligand. In mouse, *Fgf10* is expressed in paraxial cephalic mesendoderm beneath the prospective otic ectoderm (S. Mansour, personal communication), but in zebrafish, periotic expression of *Fgf10* is limited to the otic vesicle after it has already formed (Ng et al., 2002). Expression of *Fgf10* has not been reported in chick, but *Fgf19* is expressed in paraxial cephalic mesendoderm at the appropriate stage and could contribute to the otic-inducing activity of this tissue (Ladher et al., 2000). Homologs of *Fgf19* have not been identified in mouse, fish, or frog. The evolutionary significance of such variation is unclear. However, there are numerous examples in which relative roles of different homologs have undergone "functional shuffling" (McClintock et al., 2001) during evolution of different vertebrate lineages (see below).

The importance of alternate sources of otic inducers is further suggested by the analysis of the *pbx2* and *pbx4* genes in zebrafish (Waskiewicz et al., 2002). Pbx proteins, related to Extradenticle of *Drosophila*, are essential binding partners of Hox proteins. The functions of *pbx2* and *pbx4* are partially redundant in zebrafish. When both functions are disrupted, segmental patterning in the hindbrain is severely perturbed and all hindbrain cells adopt an r1 identity. Despite the absence of r4-specific expression of *fgf3* and *fgf8*, small otic vesicles still form in roughly the correct location. This could, in part, reflect the low level of r1-specific *fgf8* expression seen throughout the hindbrain. However, it is doubtful whether such a broad distribution of Fgf8 could properly position the otic tissue. Signals from the mesendoderm probably augment the strength of otic induction and could also specify the site of placode formation.

Another candidate for an otic-inducing factor is Wnt8. In chick, the hindbrain factor *Wnt8c* (the chick ortholog of *Wnt8*) is expressed in the hindbrain. Human Wnt8 induces a variety of otic markers in chick explant cultures, and this activity is strongly potentiated by Fgf19 (Ladher et al., 2000). This has led to the interesting hypothesis that Fgf19 secreted by paraxial cephalic mesendoderm induces expression of *Wnt8c* in the hindbrain, and that the two factors subsequently work together to induce the otic placode. One complication, however, is that Wnt8 also induces expression of *Fgf3*, leaving open the question of which factor(s) are directly responsible for otic induction. In zebrafish, disruption of *wnt8* causes a delay in periotic expression of *fgf3* and *fgf8* (Lekven et al., 2001; and unpublished observations). Otic development is also delayed, but otic markers begin to be expressed soon after the onset of expression of *fgf3* and *fgf8*. Thus, Wnt8 may regulate otic development indirectly by virtue of regulating *fgf* gene expression. The possibility remains, however, that combinatorial signaling through the Fgf and Wnt8 pathways might be important for normal otic induction.

An interesting problem raised by the above studies is why the hindbrain, being a rich source of otic-inducing factors, is not itself induced to form otic tissue. One possibility is that the factors in question act as morphogens rather than as simple inductive cues. Overexpression of *fgf3* or *fgf8* strongly dorsalizes the embryo, leading to expansion of the neural plate at the expense of placodal and non-neural ectoderm (Fürthauer et al., 1997; Koshida et al., 2002; and our unpublished observations). It is possible that otic tissue is induced only by intermediate doses of these factors, as are likely to be encountered by cells lying adjacent to the hindbrain. In addition, Fgf signaling may be strongly modified by other dorsalizing signals, such as Nodal, Chordin, Noggin, etc. In zebrafish, Nodal signaling is totally blocked by disrupting the essential cofactor One-eyed pinhead (Oep) (Gritsman et al., 1999). This prevents formation of mesendoderm and causes a delay in otic induction (Mendonsa and Riley, 1997; Phillips et al, 2001). Since *fgf3* and *fgf8* are expressed on time in the hindbrain, the delay in otic induction could result from loss of otic-inducing signals from mesendoderm. Otic vesicles are eventually produced but are small and poorly formed. From the perspective of ablating

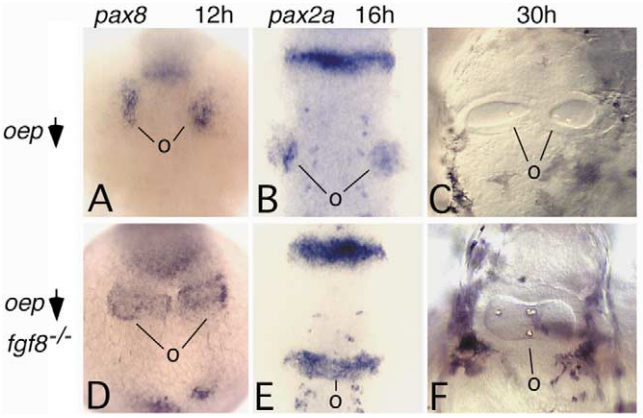

Fig. 2. Ear development in embryos deficient in *oep* and *fgf8*. (A–C) Wild-type embryos injected with *oep*-MO as previously described (Phillips et al., 2001). Small bilateral patches of *pax8* at 12 h (A) or *pax2a* at 16 h (B) mark the developing otic placodes (o). By 30 h, otic vesicles form bilaterally but often elongate medially to touch at the midline. However, they are never observed to fuse (over 300 embryos examined). (D–F) *ace*/+ intercross progeny injected with *oep*-MO. About 25% of injected progeny showed dramatic changes in gene expression and otic vesicle morphology. These are inferred to be *ace/ace(fgf8*$^{-/-}$*)* mutants. At 12 h, the otic domain of *pax8* forms bilateral transverse bands that nearly touch at the midline (D) in 27% (12/44) of embryos. By 16 h, *pax2a* is expressed in a contiguous transverse stripe through the hindbrain (E) in 19% (9/48) of embryos. At 30 h, a single large otic vesicle forms at the midline and fully spans the width of the hindbrain (F) in 27% (15/55) of embryos. All images are dorsal views with anterior to the top. Abbreviation: o, otic tissue.

additional sources of otic-inducing signals, simultaneous loss of *oep* and *fgf8* might have been expected to further reduce or ablate otic tissue, but this is not the case. Instead, a single large otic vesicle forms, spanning the width of the hindbrain (Fig. 2). Analysis of early otic markers shows that the otic placode expands medially into the *Fgf3*-expressing region of the hindbrain (Fig. 2A–D; and Leger and Brand, 2002). This phenotype is only observed when both Nodal and Fgf8 are disrupted, indicating that both signaling pathways act to restrict otic development from the neural plate. Further studies will be needed to determine whether this role of Fgf8 reflects a dosage effect or a complex interaction with other dorsalizing signals.

Molecular markers of otic induction

A number of genes expressed at early stages of otic development have proven useful as molecular markers to follow the course of otic development, and these have also provided valuable resources for analyzing the functions necessary to respond to otic induction. Groves and Bronner-Fraser (2000) followed expression of a number of such markers to readdress classical issues of otic competence and commitment in chick. From their experiments, it appears that early otic development is not regulated by a single unifying pathway or regulatory cascade. Instead, otic development proceeds through a series of discrete stages and involves parallel pathways that can be experimentally uncoupled (see below).

Pax2/5/8 genes

Pax8, which encodes a paired box transcription factor, is the earliest known marker of otic fate. It is expressed in preotic cells during the latter half of gastrulation in all vertebrates examined (Pfeffer et al., 1998; Heller and Brandi, 1999). In zebrafish, the preotic domain of *pax8* closely abuts the hindbrain domain of *fgf3* and *fgf8*, and loss of both *fgf* functions specifically blocks *pax8* expression in the ear primordium (Phillips et al., 2001; Leger and Brand, 2002). It is not known whether *Pax8* is a direct transcriptional target of Fgf signaling. In addition, there are multiple splice-variants of *Pax8* that encode proteins with distinct biological properties (Kozmik et al., 1993, 1997). How alternative splicing is regulated and what effect it has on otic development remains to be established. Loss of *Pax8* in the mouse has not been reported to have an ear phenotype (Mansouri et al., 1998), but this may warrant closer scrutiny. Knockdown of *pax8* in zebrafish reduces the size of the otic placode and impairs subsequent development of hair cells in the otic vesicle (unpublished observations).

Another important regulator of otic development is *Pax2*, a closely related homolog of *Pax8*. In all vertebrates, *Pax2* is expressed in preotic cells by early somitogenesis stages, well after *Pax8* expression is initiated (Pfeffer et al., 1998; Heller and Brandli, 1999; Groves and Bronner-Fraser, 2000). In mouse and zebrafish, loss of *Pax8* function does not prevent expression of *Pax2* (Mansouri et al., 1998; and our unpublished observations), indicating that these genes represent distinct developmental pathways. *Pax8* expression persists only through early vesicle formation and is subsequently lost, whereas expression of *Pax2* persists in the otic vesicle where it is restricted to the ventromedial wall, and is finally retained only in sensory hair cells (Nornes et al., 1990; Herbrand et al., 1998; Pfeffer et al., 1998; Heller and Brandli, 1999; Hutson et al., 1999; Riley et al., 1999; Lawako-Kerali et al., 2002). Disruption of mouse *Pax2* does not alter otic induction but prevents formation of the cochlea (Torres et al., 1996). There are two *Pax2* homologs in zebrafish, *pax2a* and *pax2b* (formerly *pax2.1* and *pax2.2*). These are coexpressed in the ear primordium, although *pax2b* is expressed later as the otic placode begins to form at 9-10 somites (Pfeffer et al., 1998). Loss of *pax2a* or *pax2b*, or both, alters various aspects of hair cell development (see below) but does not hinder development of the otic placode (Riley et al., 1999; Whitfield et al., 2002). The sequences of *Pax2* and *Pax8* are very similar, so functional redundancy probably ameliorates their respective loss-of-function phenotypes.

In zebrafish, another close family member, *pax5*, is expressed in the anterior quarter of the otic placode just before formation of the vesicle and is later restricted to the utricular

macula. Expression of *pax5* in this domain requires prior activity of *pax2a* (Pfeffer et al., 1998). The role of *pax5* is not yet known, but it marks one of the earliest known asymmetries along the AP axis of the ear. As such, it may play a role in specifying anterior identity or in conferring unique functional properties to the utricular macula. *Pax5* is not detectably expressed in the mouse ear, but a knockin of *Pax5* into the *Pax2* locus fully rescues otic development in the absence of *Pax2* function (Bouchard et al., 2000).

Pou2

Zebrafish *pou2* encodes a POU class homeodomain transcription factor related to mammalian Oct3/4. Expression of *pou2* is seen in the hindbrain in a pattern that overlaps with *fgf3* and *fgf8* (Hauptmann and Gerster, 1995; Burgess et al., 2002). This includes a longitudinal stripe, shared with *fgf8*, that directly abuts the preotic domain of *pax8* (Phillips et al., 2001). *pou2* is disrupted in *spiel ohne grenzen (spg)* mutants, which develop with ear defects strongly resembling those of *ace (fgf8)* mutants (Burgess et al., 2002). *Pou2* is required for high-level expression of *fgf3* and *fgf8* and thereby indirectly regulates otic induction (Reim and Brand, 2002; and unpublished observations). It has also been observed that *pou2* acts in the midbrain–hindbrain border to make cells Fgf-competent (Reim and Brand, 2002), but this reflects a cell-autonomous function unlikely to affect preotic cells.

Genes expressed in the preplacodal domain

Coincident with or soon after induction of *pax8* in preotic cells, a number of other genes are induced along the lateral edges of the neural plate in a "preplacodal domain" thought to give rise to the various placodes (reviewed by Baker and Bronner-Fraser, 2001; Whitfield et al., 2002). Many of the genes initially expressed in the preplacodal domain are later restricted to individual placodes and help regulate their subsequent development. Members of the *distal-less (Dlx)* and *eyes-absent (Eya)* family of transcription factor genes play especially critical roles in preotic development. In mouse, *Xenopus*, and zebrafish, *Eya1* is expressed in the preplacodal domain prior to expression of *Pax2*, but after *Pax8* (Sahly et al., 1999; David et al., 2001; Heanue et al., 2002). Chick *Eya2* shows a similar pattern of expression (Streit, 2002). Targeted disruption of mouse *Eya1* causes a particularly severe phenotype in which the otic vesicle forms but fails to undergo further differentiation (Xu et al., 1999). *Pax8* and *Pax2* are induced normally in preotic cells, but later markers of the developing ear are not expressed and the otic vesicle shows a significant increase in apoptosis. The zebrafish *eya1* gene is disrupted in *dog-eared (dog)* mutants (D. Kozlowski, personal communication). The *dog-eya1* mutant ear is not as severely disrupted as in *Eya1* null mice, probably because of redundancy provided by *eya2* and *eya4*, which are also expressed in the developing ear (D. Raible, personal communication).

There has been considerable interest in examining functional relationships between *Pax* and *Eya* genes because homologs of these genes participate, along with several other genes, in a highly conserved genetic network seen in a number of diverse systems. In the *Drosophila* eye, *eyeless (Pax6)* is required to activate expression of *Eya*, which then activates expression of *Sine Oculis (Six)* and *dachshund (Dach)* (reviewed by Desplan, 1997). All of these genes subsequently maintain each other and regulate downstream target genes required for eye development. A similar genetic circuit has been observed in the vertebrate eye and somitic muscle, which are regulated by Pax6 and Pax3, respectively (Heanue et al., 1999; reviewed by Kawakami et al., 2000; Wawersik and Maas, 2000). It is possible that a *Pax-Six-Eya-Dach* feedback loop operates in the ear as well. *Six4* is expressed in the preplacodal domain, and *Six1* and various *Dach* genes are expressed in the otic vesicle in domains that overlap with *Pax2* and *Eya1* (Oliver et al., 1995; Esteve and Bovdenta, 1999; Kobayashie et al., 2000; Ghanbari et al., 2001; Loosli et al., 2002; Hammond et al., 2002; Heanue et al., 2002). *Eya1* comes on too early to be induced by *Pax2*, but *Pax8* could serve this role. Similarly, *Pax2* might be sufficient to activate *Eya1* expression in the absence of *Pax8*. Analysis of *Eya1* expression in a *Pax2-Pax8* double mutant will settle the issue. *Six1* is not expressed in *Eya1* mutants, which is consistent with the epistatic relationship in eye development (Heanue et al., 2002). However, *Dach1* is still expressed in *Pax2* and *Eya1* mutants, indicating that not all aspects of the network are conserved. Similarly, disruption of *Six4* or *Dach1* has no discernable effect on ear development (Davis et al., 2001; Ozaki et al., 2001; Backman et al., 2003), although this could reflect redundancy. Thus, while aspects of the *Pax-Six-Eya-Dach* network in the ear might have significant differences from that seen in the eye, taking a comparative approach will undoubtedly provide useful information about inner ear development, as well as insights about how this ancient genetic network has been evolutionarily coopted and modified for different tasks.

In chick and mouse, *Dlx5* is expressed in the preplacodal domain before either *Eya1* or *Pax2* (Qiu et al., 1997; Pera et al., 1999; Luo et al., 2001). Targeted disruption of mouse *Dlx5* does not detectably alter placodal development (Acampora et al., 1999; Depew et al., 1999). *Dlx5* expression is later confined to the dorsal otic vesicle, which gives rise to the anterior and posterior semicircular canals. These structures fail to form in *Dlx5* mutants. The absence of an earlier phenotype probably results from redundancy: Vertebrate *Dlx* genes have evolved as pairs of homologs arranged in a tail-to-tail configuration, reflecting duplication and inversion of an ancestral sequence (reviewed by Kraus and Lufkin, 1999). *Dlx5* is linked to *Dlx6*, which is also expressed in early preotic cells (Qiu et al., 1997). Compared with *Dlx5* mutants, *Dlx5-Dlx6* double mutants show a much

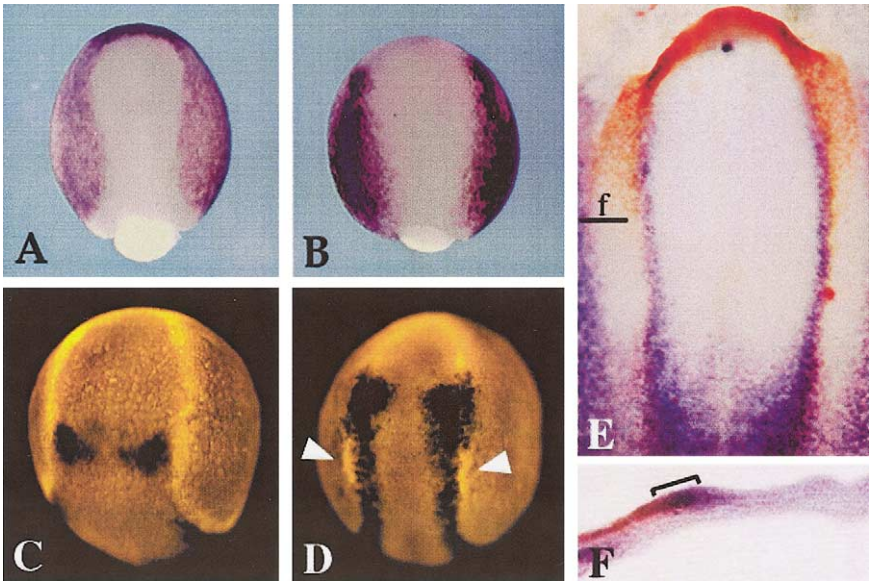

Fig. 3. Expression of *Dlx* and *Msx* genes in *Xenopus*, zebrafish, and chick. (A, B) *Xenopus* embryos at late gastrula stage showing expression of *Dlx3* (A) and *Msx1* (B). (C, D) Dorsal views of zebrafish embryos at late gastrula or early somitogenesis stages. (C) At bud stage, expression of *dlx3* (red) is limited to the preplacodal domain and does not overlap with *wnt8* in the hindbrain (black). By the 3-somite stage (D), expression of *msxB* (black) fully overlaps the *dlx3* domain (red) in the preplacodal domain, except in the anterior head and in the lateral portion of the preotic domain (arrowheads). At this stage, *msxB* expression has started to shift medially into the neural plate. By the 10-somite stage, *dlx3* and *msxB* totally separate into placodal and neural domains, respectively (not shown). (E, F) Chick embryo at stage 6 showing expression of *Dlx5* (red) and *Msx1* (blue). As seen in a wholemount specimen (E), *Msx1* overlaps with *Dlx5* except in the anterior head region. The plane of section for (F) is indicated. (F) A section confirms that *Dlx5* and *Msx1* overlap in the medial preplacodal domain (bracket). Images show dorsal views with anterior to the top (A–E) or a cross section with lateral to the left and dorsal to the top (F). With permission, (A) and (B) are reprinted from Feledy et al. (2001) and (E) and (F) are reprinted from Streit (2002).

more severe phenotype in which the otic vesicle remains small and poorly differentiated (Robledo et al., 2002). Chick and *Xenopus* embryos express both *Dlx3* and *Dlx5* in the preplacodal domain (Feledy et al., 1999; Pera and Kessel, 1999; Luo et al., 2001). In zebrafish, *dlx3b* and *dlx4b* (formerly *dlx3* and *dlx7*) are the first homologs to be expressed in the preplacodal domain and are later expressed in dorsal cells within the otic vesicle (Ekker et al., 1992; Akimenko et al., 1994; Ellies et al., 1997). These genes appear to play the same roles as *Dlx5* and *Dlx6* in the mouse and chick. Knockdown of *dlx4b* alone has little effect on the ear, whereas knockdown of *dlx3b* significantly reduces the size of the otic vesicle (Solomon and Fritz, 2002). Coinjection of *dlx3b*-MO and *dlx4b*-MO causes a strong synergistic deficiency of otic tissue, and a deletion that removes both genes blocks otic development entirely. Importantly, expression of *pax2a* is blocked, whereas early expression of *pax8* occurs normally. Thus, otic induction is initiated but is then aborted after the stage when *dlx3b* and *dlx4b* would normally begin to function.

Dlx genes are likely to function in concert with another closely allied gene family, the *muscle segment homeobox (Msx)* genes. *Dlx* and *Msx* genes are often expressed in closely apposed or overlapping domains where they mediate tissue–tissue interactions (see reviews by Kraus and Lufkin, 1999; Beanan and Sargent, 2000; Bendall and Abate-Shen, 2000). In general, Dlx proteins act as transcriptional activators that regulate positional identity or differentiation, whereas Msx proteins are transcriptional repressors that inhibit differentiation by preventing withdrawal from the cell cycle (Hu et al., 2001). Msx and Dlx proteins can also antagonize each other by forming transcriptionally inert heterodimers (Zhang et al., 1997). Paradoxically, loss of *Msx1* and/or *Msx2* in mouse indirectly perturbs differentiation in many tissues by reducing the pool of tissue progenitors, whereas overexpression causes expansion of the progenitor population followed by premature terminal differentiation (Satokota and Maas, 1994; Liu et al., 1995, 1999; Satokota et al., 2000). Thus, coexpression of *Dlx* and *Msx* genes probably serves to achieve a proper balance between proliferation and differentiation. In *Xenopus*, misexpression of either *Dlx3* or *Msx1* inhibits expression of neurectodermal markers (Feledy et al., 1999; Beanan et al., 2000). Both genes are normally expressed throughout the ventral ectoderm, including the preplacodal domain (Fig. 3A and B). Hence their anti-neural activity probably helps set the lateral boundary of the neural plate (Woda et al., 2003), and it could also be a prerequisite for promoting or permitting preplacodal development. In zebrafish, *msxB* and *msxC* are expressed in the preplacodal domain from the level of the posterior midbrain on back (Ekker et al., 1997). Within this domain, *msx* gene expression initially overlaps almost completely with *dlx3b* and *dlx4b*. (Fig. 3C and D). During early somitogenesis stages, the domains of *msx* and *dlx* expression begin to separate along the ML axis, with *msx* genes eventually occupying the dorsal neural tube and

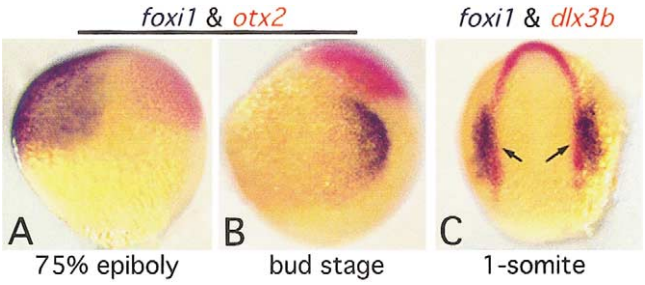

Fig. 4. Expression of *foxi1* in zebrafish. (A, B) Lateral views (anterior to the top, dorsal to the right) showing expression of *foxi1* (black) and the forebrain marker *otx2* (red). At 75% epiboly (A), *foxi1* is expressed uniformly in the anteroventral quadrant. By bud stage (B), *foxi1* has strongly upregulated in preotic placode and downregulated ventrally. (C) Dorsal view (anterior to the top) showing *foxi1* (black) and *dlx3* (red) at the one somite stage. Expression of *foxi1* fully overlaps the preotic domain of *dlx3*. Reprinted from Solomon et al. (2003) with permission.

dlx genes predominantly marking placodal tissue. A deletion that removes *msxB* causes only mild reduction in the size of the otic vesicle, and there are no obvious changes in preotic gene expression (unpublished observations). The phenotype could be ameliorated by redundancy conferred by *msxC*. In chick, *Msx1* and *Dlx5* initially overlap in the preplacodal domain, and their expression patterns shift relative to one another as in zebrafish (Streit, 2002; and Fig. 3E and F). Misexpression of *Dlx5* represses *Msx1* expression in non-neural ectoderm but causes upregulation of *Msx1* in the neural plate (McLarren et al., 2003). The context-dependent action of *Dlx5* could account for the medial shift in *Msx1* expression. Withdrawal of *Msx1* from the preplacodal domain may in turn enhance *Dlx5* function, thereby permitting the progression of otic specification or differentiation.

Foxi1 and early patterning of the ectoderm

While the early expression of *pax8* is the first clear indication of otic induction, an upstream regulator has recently been discovered in zebrafish that is expressed even earlier. *foxi1* encodes a forkhead class winged helix transcription factor that is initially expressed in the anteroventral quadrant of the early gastrula (Solomon et al., 2003; Fig. 4A). During the latter half of gastrulation, expression begins to upregulate at the lateral edges of the *foxi1* band and downregulate in more ventral cells (Fig. 4B and C). The domain of upregulation anticipates and encompasses the preotic domain of *pax8* expression. *foxi1* is disrupted in *hearsay (hsy)* mutants, which develop with variable defects in the inner ear and jaw. Preotic expression of *pax8* is not detected and, in severely affected embryos, no otic tissue forms. This is the only known example in which a single gene mutation can totally block otic induction. Misexpression of *foxi1* in zebrafish can induce ectopic *pax8* expression. It is not clear whether this is sufficient to induce ectopic otic vesicles because misexpression of *foxi1* induces apoptosis at later stages such that morphological development cannot be assessed (A. Fritz, personal communication). In mouse, disruption of *Foxi1* causes a much milder phenotype (Hulander et al., 1998, 2003). Early patterning and morphogenesis of the otic vesicle appears normal, but morphology becomes increasingly distorted after various genes fail to be expressed in the endolymphatic duct, leading to hydrops. Whether *Foxi1* functions during preplacodal development in mouse has not yet been determined, but if is does, there must be redundancy to compensate for its loss.

The expression patterns of *foxi1* and the various preplacodal genes reflect how global patterning of the ectoderm during early gastrulation sets the stage for otic induction (Fig. 5). In zebrafish, the early *foxi1* pattern resembles that of *bmp2b* (and BMP-response genes) and, indeed, *foxi1* is not expressed in Bmp pathway mutants (A. Fritz, personal communication). Our preliminary data also suggest that Wnt8 represses *foxi1* in posterior ectoderm, and organizer signals (Nodal, Chordin, etc.) repress *foxi1* in dorsal cells. Fgf signaling from the germring and hindbrain could also influence *foxi1* expression. It is noteworthy that *foxi1* upregulates at the intersection of these signaling domains, suggesting that a balance of multiple signals might be required for optimal expression. Zebrafish *msx* and *dlx* genes are also strongly influenced by Bmp, Wnt8, and axial signals (Nguyen et al., 1998, 2000; our unpublished observations). In chick and *Xenopus*, too, both Bmp and organizer signals regulate *Dlx* and *Msx* gene expression (Suzuki et al., 1997; Pera et al., 1999; Streit and Stern, 1999; Beanan et al., 2000). It therefore appears that an optimal balance of signaling interactions is achieved only at the neural non-neural interface. A critical future goal will be to assess whether this balance involves threshold responses to gradients of various signals or a mixture of multiple inducers (combinatorial signaling), or both. For now, functional studies support the notion that these extracellular signals and various intracellular mediators form a network of distinct pathways that converge to induce the otic placode (Fig. 5).

Cell lineages and gene expression domains

Domains of preplacodal gene expression reflect signaling interactions but should not be viewed as stable, lineage-restricted populations. Fate mapping studies conducted in zebrafish, chick, and *Xenopus* show that cells contributing to the otic placode converge from relatively broad areas in the early gastrula, and these areas include multiple other prospective fates (Kozlowski et al. 1997; Kil and Collazo, 2001; Streit, 2002; reviewed by Kil and Collazo, 2002). In zebrafish, the prospective otic anlagen is distributed in two large patches on the ventral side of the embryo, well within the ventral portion of the *foxil* domain—that is, far from the lateral edges of the domain where the placodes eventually form. These cells must traverse regions in which the signaling milieu is not appropriate for otic induction. Convergence eventually brings prospective otic cells into range of otic-inducing tissues. Presumably, cell populations must

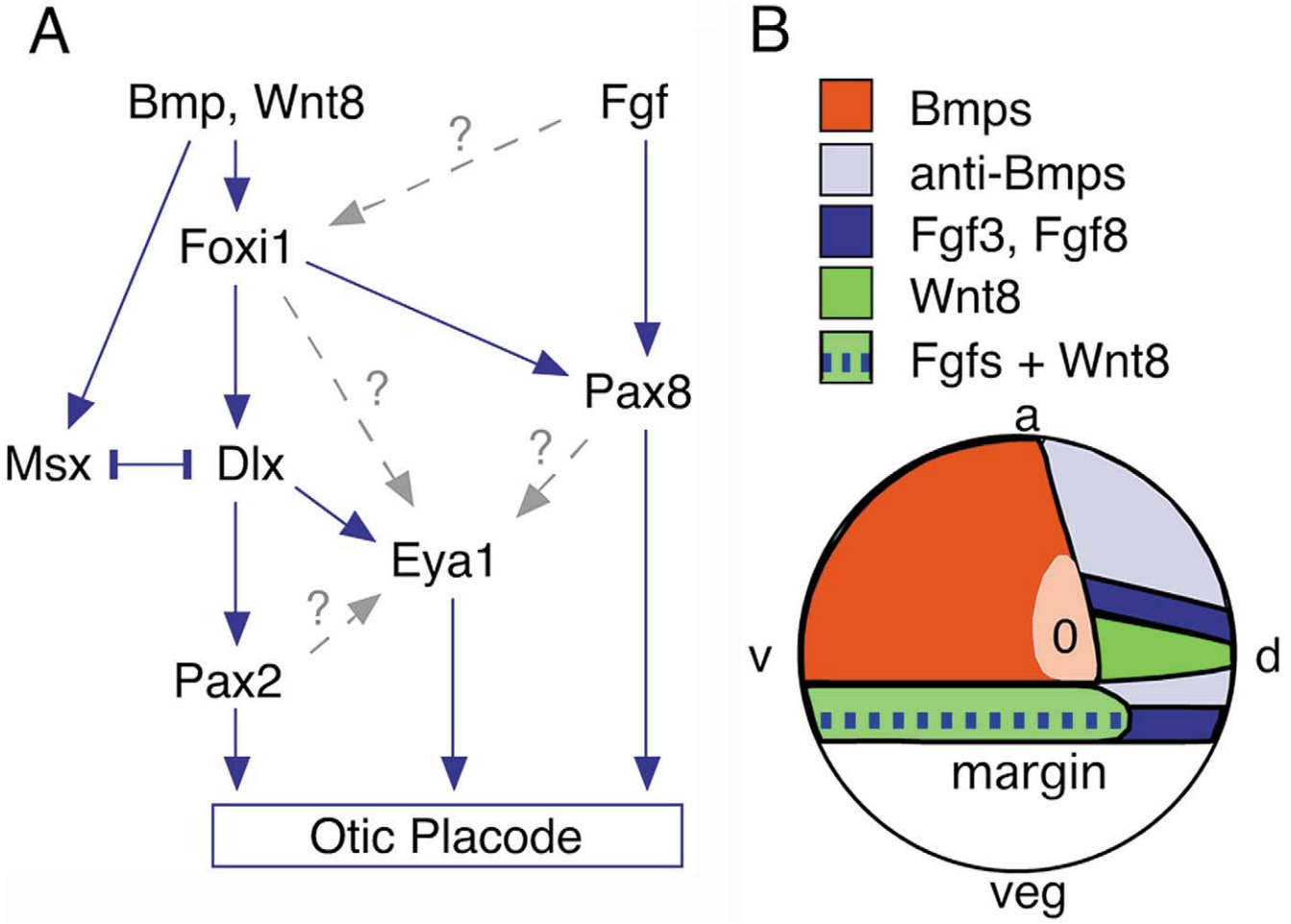

Fig. 5. Summary of genetic pathways and signaling domains involved in placode induction. (A) A putative regulatory network for placode induction. Blue arrows indicate positive regulatory relationships directly supported by genetics studies. Bars indicate negative regulation. Gray arrows indicate possible relationships that have not yet been tested. Bmp, Wnt8, and Fgf are expressed in various tissues around the otic anlagen. All other genes encode transcription factors expressed throughout the preplacodal domain or specifically within the preotic domain. See the text for discussions of specific genes. (B) Representation of a zebrafish embryo at 75–80% epiboly showing signaling domains likely to regulate expression of *foxi1* and various preplacodal genes. The margin, presumptive otic region (o), animal pole (a), vegetal pole (veg), and dorsal (d) and ventral (v) regions of the embryo are indicated. Bmp from the ventral ectoderm and Wnt8 from the ventrolateral margin induce non-neural ectoderm. Bmp antagonists, including Nodal, Chordin, and Noggin establish the neural ectoderm. Preplacodal genes are induced only at the interface between the neural and non-neural ectoderm where cells experience intermediate levels of these signals. Fgf from the hindbrain helps localized expression of preotic genes. Wnt8 in the hindbrain may act indirectly by regulating expression of Fgf genes in the hindbrain. Signals from subjacent mesendoderm (not shown) also participate in otic induction.

stabilize within the appropriate signaling domain to induce and maintain otic fate. However, a recent lineage study in chick showed that populations within the preplacodal domain are far more dynamic that previously thought (Streit, 2002). There is extensive intermixing of cells from different regions, and many cells move through the preplacodal domain relatively quickly while others persist there longer. Surprisingly, some cells that converge all the way to the neural plate and contribute to the neural fold (future dorsal neural tube) later migrate back into the prospective otic placode. This region of the neural ectoderm expresses *Msxl*, which could keep cells in an uncommitted state that facilitates their late recruitment into the ear. Populations become more stable soon after the onset of *Pax2* expression, but mixing still occurs between different regions of the developing ear even after formation of the otic vesicle. Significant intermixing has also been reported to occur in *Xenopus* after formation of the otic vesicle (Kil and Collazo, 2001). Such widespread intermixing between cells of differing lineages and gene expression domains is difficult to reconcile with known mechanisms of cell fate specification. However, both of these studies used DiI injection to label numerous cells. Tracing the movements of all labeled descendants is useful for documenting the full range of cell behaviors in a population, but this may give a distorted impression of average cell behavior. Indeed, many cells within a labeled cohort appear to remain in relatively close proximity to one another. In another set of studies in chick, smaller cohorts were labeled at the otic cup or early otic vesicle stage by DiI injection (Brigande et al., 2000a) or

retroviral infection (Fekete et al., 1998; Lang and Fekete, 2001). In these studies, resulting clones showed relatively little dispersal: Different regions of the ear appeared to arise from lineage-restricted compartments that correlate with known gene expression domains. In this case, the smaller clone sizes would have made it harder to detect compartmental intermixing, so the different studies are likely to be compatible. Although many questions remain, a number of important issues have been raised by fate mapping studies. First, many cells that pass through the preplacodal domain do not contribute to the ear. Hence, transient expression of preplacodal genes is not sufficient to specify cell fate, although it could enhance or prolong otic competence. Second, cells from other domains may contribute to the otic anlagen at relatively late stages. Third, it is vital to consider how gene expression domains are able to organize cell fates despite varying degrees of intermixing between domains. Assuming that cellular patterning reflects regional inductive signaling and known gene expression patterns, it seems unlikely that otic development involves sorting-out of mixtures of prespecified populations. Instead, cell intermixing could reflect a random process of spurious cell migration that is tolerated due to the activity of robust regulative mechanisms. Alternatively, intermixing between adjacent compartments could involve a nonrandom regulatory process that serves to adjust and refine gene expression domains and cell fate allocations. Finally, intermixing between developmental compartments must eventually cease in order to maintain spatial organization of different cell types within the ear, and domains of gene expression are certainly crucial for this process. This is a fascinating problem that clearly deserves further attention. For additional recent reviews of otic induction, see Torres and Giraldez (1998), Baker and Bronner-Fraser (2001), Streit (2001), and Noramly and Grainger (2002).

Patterning of the nascent placode

Specification of different cell fates within the otic anlagen probably begins as soon as the placode forms, and possibly earlier. Signaling interactions with surrounding tissues play a critical role in regional cell fate specification and, not surprisingly, the hindbrain is especially important in this regard (Waddington, 1937; Harrison, 1945). While *Pax2* normally becomes restricted to medial cells in the otic vesicle, excision of the hindbrain causes *Pax2* to continue to be expressed uniformly (Hutson et al., 1999). In chick, when the nascent otic vesicle is rotated 180° to invert the AP and ML axes, *Pax2* is expressed only in cells abutting the hindbrain, suggesting that cells that were originally in a lateral position are respecified as medial (Hutson et al., 1999). When ear tissue is transplanted to a more anterior location, Pax2 expression is often lost or randomized (Herbrand et al., 1998). Together, these findings suggest that interactions with the posterior hindbrain serve to specify medial fates in the otic vesicle. However, some genes do not reorient their expression following rotation of the ear, and some morphological features develop in an inverted orientation (Wu et al., 1998). These findings suggest that some aspects of axial fate are already specified by the time the otic vesicle forms. Conducting axial rotations at different stages of development shows that AP axial fates are specified first, around the time of placode formation, whereas DV and ML fates remain plastic for much longer (Harrison, 1936; Wu et al., 1998). Interestingly, specification of sensory patches occurs much earlier than nonsensory epithelia. Thus, as with placode induction, later stages of inner ear development are regulated by multiple pathways that can be experimentally uncoupled. Candidates for hindbrain factors that coordinate early pattering of the placode and vesicle include Fgf3 and Shh.

Later roles of Fgf3

In chick and mouse, *Fgf3* is initially expressed at high levels in rhombomere 4 (r4) during otic placode induction, but later upregulates in r5 and r6 where it persists through early stages of otic vesicle morphogenesis (Mahmood et al., 1995, 1996). In the hindbrain segmentation mutant *kreisler (kr)*, early expression of *fgf3* is normal, but subsequent upregulation in r5 and r6 fails to occur (Mckay et al., 1996). *kr* encodes a Maf-bZIP transcription factor that is normally expressed only in r5 and r6 (Cordes and Barsh, 1994) and is apparently required for normal upregulation of *fgf3* in that domain. The otic placode is induced normally in *kr* mutants, but morphogenesis of the otic vesicle is severely impaired (Deol, 1964). Morphological defects are highly variable and can affect virtually all chambers in the inner ear. A similar ear phenotype is also seen in *Hoxal* mutants, which also fail to express normal levels of *Fgf3* in the hindbrain (Lufkin et al., 1992; Chisaka et al., 1992; Pasqualeti et al., 2001; Fig. 6B). Remarkably, treatment of *Hoxal* mutants with retinoic acid (RA) restores normal levels of *Fgf3* expression in the hindbrain and fully rescues the inner ear phenotype (Pasqualeti et al., 2001; and Fig. 6C). *Fgf3* null mice also show variable but generally more severe inner ear defects. While these data demonstrate a role for *Fgf3* in regulating development of the otic vesicle, its mechanism of action remains unclear. Some of the morphogenetic defects seen in *kr*, *Hoxal*, and *Fgf3* null mutants probably arise secondarily from failure of the medial otic epithelium to form the endolymphatic duct and consequent buildup of excess fluid pressure (hydrops) within the otic vesicle (Deol, 1964; Brigande et al., 2000b; Fekete and Wu, 2002). On the other hand, the sensitive dependence of the cochlea on Fgf signaling (Mansour et al., 1993; Pirvola et al., 2000; see below) suggests that this structure may be directly affected by deficiency of Fgf3. Notably, none of the above hindbrain mutants have been examined with appropriate early otic markers to assess whether initial patterning of the otic cup and early vesicle occurs normally.

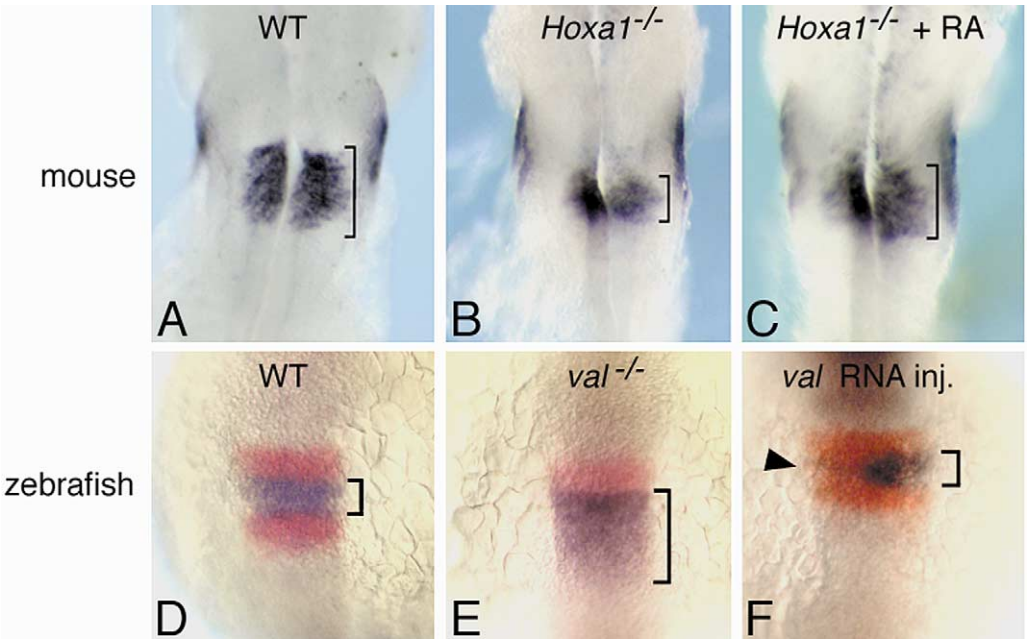

Fig. 6. Expression of *Fgf3* at comparable stages in mouse and zebrafish. Brackets are shown in all panels to help gauge the size of the *Fgf3* domain in the hindbrain. (A–C) Mouse embryos at embryonic day 8.5. (A) A wild-type embryo showing expression in r5 and r6. (B) A *Hoxa1*$^{-/-}$ mutant shows a greatly reduced hindbrain domain. (C) Brief treatment of *Hoxa1*$^{-/-}$ mutants with RA restores *Fgf3* expression to normal (Pasqualetti et al., 2001). (D–F) Zebrafish embryos at the six-somite stage showing expression of *fgf3* (blue) and *krox20* (red). (D) Wild-type embryos express *fgf3* in r4 and *krox20* in r3 and r5. (E) *val*$^{-/-}$ mutants show loss of *krox20* in r5 and expansion of *fgf3* into the r5/6 region. (F) A wild-type embryo injected with *val* mRNA at the 2 cell stage. Expression is primarily restricted to the left side of the embryo where hindbrain expression of *fgf3* is nearly extinguished (arrowhead). Expression on the right is essentially normal. Expression of *fgf8* in r4 is not altered by either loss of *val* or misexpression of *val* (Kwak et al., 2002). With permission, (A–C) are reprinted from Pasqualetti et al. (2001), and (D–F) are reprinted from Kwak et al. (2002).

In zebrafish, *fgf3* continues to be expressed in r4 after formation of the otic placode (Phillips et al., 2001; Maves et al., 2002). Unlike mouse and chick, zebrafish *fgf3* is not normally expressed in r5, and it is expressed only at a very low level in r6. However, in embryos homozygous for a mutation in *valentino (val)*, the zebrafish ortholog of *kreisler* (Moens et al., 1996, 1998), *fgf3* is expressed at high levels in an expanded domain from r4 through the r5/6 region (Kwak et al., 2002; Fig. 6D and E). Misexpression of wild-type *val* reduces or ablates expression of *fgf3* in r4 (although *fgf8* is not affected), supporting the hypothesis that Val protein normally represses *fgf3* transcription (Fig. 6F). [Note: This is the opposite of the role of Kr in mouse (McKay et al., 1996). Such species differences may have been important for evolutionary changes in the structure and function of the inner ear]. In *val* mutants, otic vesicles are small and show AP patterning defects (Kwak et al., 2002). Several anterior otic markers, which are normally limited to otic tissue adjacent to r4 (*pax5* and *nkx5.1*), are expressed throughout the medial wall of the otic vesicle in *val* mutants. In contrast, a posterior marker normally expressed in the medial wall adjacent to r5 and r6 (*zp23*), is totally ablated in *val* mutants. Differentiation of hair cells is also perturbed. The first hair cells normally form in the utricular and saccular maculae adjacent to r4 and r6, respectively. *val* mutants, however, produce hair cells throughout the medial wall of the otic vesicle, and the number of hair cells is nearly twice normal despite the small size of the otic vesicle. Knocking down *fgf3* function in *val* mutants suppresses the AP patterning defects and eliminates the excess and ectopic hair cells. Thus, abnormal expression of *fgf3* in the *val* mutant hindbrain appears responsible for the above patterning defects. These data suggest that the r4 domain of *Fgf3* normally specifies anterior fates in the otic placode and induces macular development in adjacent otic epithelium.

Role of Shh

Shh is expressed in the notochord and floorplate of the neural tube. Targeted disruption of *Shh* in mouse causes severe and widespread changes in patterning of the otic vesicle (Riccomagno et al., 2002). The dorsal marker *Dlx5* expands into the ventral region of the ear. Ventrolateral markers *Otx1* and *Otx2* are reduced or ablated. The ML axis is also partially perturbed, as *Pax2* is not maintained in medial cells. It is formally possible that these results could reflect an indirect affect on the ear by changes in hindbrain patterning. However, the otic epithelium expresses at least two Hh target genes, *Glil* and *Ptc*, indicating that otic cells do receive and respond to Hh signals. In addition, misexpression of a Shh transgene in the dorsal otic vesicle results in downregulation of *Dlx5* and upregulation of *Pax2* throughout the otic epithelium. Although not all otic markers show altered patterns of expression, these data suggest

that Shh is required to specify or maintain ventral and medial fates. Accordingly, the cochlea does not form in *Shh* mutants, and the endolymphatic duct (a dorsomedial structure) and lateral semicircular canal are initiated but later degenerate. In addition, emergence of neuroblasts from the ventral epithelium is strongly impaired and the periotic mesenchyme fails to undergo chondrogenesis (Liu et al., 2002; Riccomagno et al., 2002).

Shh also plays an important role in otic patterning in zebrafish, but with some surprising differences. Three *Hedgehog* genes are expressed in the zebrafish notochord and/or floorplate (Krauss et al., 1993; Ekker et al., 1995; Currie and Ingham, 1996), but there is only a single ortholog for *smoothened*, which encodes an essential component of the Hedgehog signal transduction pathway (Chen et al., 2001; Varga et al., 2001). This gene is disrupted in *smooth muscle omitted (smu)* mutants, which are blocked in signaling via all Hedgehog ligands. The Hedgehog pathway is also perturbed in *chameleon (con)* mutants (Schauerte et al., 1998), although the nature of the *con* gene is still unknown. Analysis of various ear markers in *con* and *smu* mutants shows that the DV and ML axes are patterned normally but, unexpectedly, AP patterning is altered (Hammond et al., 2003). Specifically, the otic vesicle forms with a mirror-image duplication of several anterior markers and loss of posterior markers. Overexpression of Shh causes duplication of posterior markers and loss of anterior markers. These changes in AP patterning are reminiscent of observations made in ear-rotation experiments in amphibians. Rotation of the axolotl ear anlagen just prior to fixation of the AP axis results in mirror image duplication of either anterior or posterior fates (Harrison, 1936). Conceivably, this could be related to the action of Shh in that species. However, it is not clear how Hedgehog genes, being uniformly expressed along the AP axis, are able to affect AP patterning in the ear. One possibility is that Hedgehog ligands are differentially processed or retained along the AP axis or, alternatively, Hedgehog signaling could be modified by other signals that are asymmetrically distributed. Another mystery is why loss or gain of Hedgehog signaling results in mirror-image duplications rather than unidirectional shifts in AP patterning. This implies an interaction with some other morphogenetic signal(s), possibly including Fgf3. For example, the low level of Fgf3 produced in r6 in zebrafish might be sufficient to anteriorize posterior otic tissue in the absence of Hh signaling, whereas overexpression of Shh might antagonize even the high level of Fgf3 produced in r4.

It is interesting that Shh seems to play such different roles in fish vs. mouse. This could reflect true species differences, but the process may ultimately prove to be more conserved than it now appears. Relatively few AP markers have been examined in mouse *Shh* mutants, and this is relevant because not all AP markers are altered in zebrafish *con* and *smu* mutants. Specifically, *pax5* is still limited to the anteromedial epithelium, and SAG neurons form only in the normal position beneath the anteroventral quadrant of the otic vesicle. Analysis of additional AP markers will clarify whether mouse and fish truly differ in this regard. On the other hand, the absence of DV and ML defects in zebrafish *Hh* mutants suggests that an alternative pathway is used in fish that is either not used or is not sufficient in the mouse. Nodal appears both necessary and sufficient for floorplate specification in zebrafish (Müller et al., 2000; Odenthal et al., 2000; Chen et al., 2001; Varga et al., 2001), whereas only Shh plays this role in mouse (Chiang et al., 1996). Importantly, the floorplate still forms in zebrafish *Hh* mutants. Thus, Nodal signaling from the notochord, or some other signal from the floorplate, might specify DV and ML patterning in the zebrafish ear.

Role of RA

RA is produced by paraxial mesoderm and has a strong posteriorizing activity on the developing nervous system. RA is also produced in the developing otic vesicle, as suggested by expression of RA biosynthetic enzymes Raldh2 and Raldh3 in the mouse ear (Mic et al., 2000; Romand et al., 2001). Embryos deficient in RA synthesis or reception show severe defects in morphogenesis and patterning of the otic vesicle, as do embryos treated with exogenous RA (Madden et al., 1996; Dupe et al., 1999; Niederreither et al., 2000; White et al., 2000; Phillips et al., 2001; Wendling, 2001; Romand et al., 2002). However, even small changes in RA levels can alter the development and signaling properties of the hindbrain (Pasqualeti et al., 2001; and Fig. 6A–C), so effects on the inner ear could be indirect. Choo et al. (1998) dealt with this problem by implanting RA-soaked beads in or near the otic vesicle in chick embryos. Such localized delivery is more likely to have a direct effect on ear development without altering hindbrain signaling. Depending on the dose, elevating RA strongly perturbs morphogenesis of the inner ear, but different regions of the ear vary in their sensitivity to RA. The anterior semicircular canal is most sensitive, followed by the lateral and posterior semicircular canals. The cochlea is much less sensitive, and the endolymphatic duct and all sensory epithelia are relatively impervious to even the highest doses of RA. Several ear markers are expressed normally despite severe disruption of morphogenesis, suggesting that RA does not affect cell fate specification. Instead, increasing RA reduces the rate of cell proliferation in the affected nonsensory epithelia, possibly indicating premature differentiation. In contrast, exogenous RA stimulates cell proliferation in the organ of Corti in cochlear explant cultures (Kelley et al., 1993).

Patterning of the otic vesicle

A compelling argument has been made that many of the ear markers used to analyze patterning of the otic vesicle

delimit discrete developmental compartments, and that signaling interactions between compartments are critical for organizing the ear as a whole (Fekete, 1996; Brigande et al., 2000b; Fekete and Wu, 2002). There is considerable evidence that, in each chamber of the ear, reciprocal signaling between the sensory patch and the surrounding nonsensory epithelium coordinates their respective development. It is also likely that cells in adjoining molecular expression domains rely on reciprocal interactions. However, testing the compartment-boundary model is difficult due to the complexity of the system. As described above, mixing of cells between compartments complicates analysis but does not necessarily constitute evidence against the model. A number of genetic studies have provided data that are consistent with the model, but they do not exclude alternative models. Despite such limitations, the compartment-boundary model is a useful paradigm for understanding molecular and cellular mechanisms that shape the inner ear.

Endogenous signaling within the otic vesicle

A variety of signaling molecules are expressed in different regions of the otic epithelium, and functional studies generally support the compartment-boundary model. Multiple *Bmp* genes cooperate to regulate development of the semicircular canals and sensory cristae. In all vertebrates, *Bmp4* is initially expressed in two patches in the nascent otic vesicle corresponding to the primordia of the anterior and posterior sensory cristae (Wu and Oh, 1996; Morsli et al., 1998; Kil and Collazo, 2001; Mowbray et al., 2001). Expression is later detected in the lateral crista and, in tetrapods, *Bmp4* is also transiently expressed in all other sensory epithelia. *Bmp7* in chick shows a broader pattern of expression that encompasses the *Bmp4*-positive sensory epithelia (Oh et al., 1996; Chang et al., 2002). In zebrafish, *bmp2b* and *bmp4* are coexpressed in all three cristae and *bmp7* is transiently expressed in the posterior crista (Mowbray et al., 2001). Unlike tetrapods, zebrafish do not express Bmp genes in the maculae. In addition to the cristae, various *Bmp* genes are also expressed in the dorsal part of the early otic vesicle in all vertebrates, and are later seen in the developing semicircular canals. Experiments in chick involving local release of Noggin from beads show that the semicircular canals are exquisitely sensitive to perturbation of Bmp signaling (Chang et al., 1999; Gerlach et al., 2000). Noggin reduces cell proliferation and increases apoptosis in the semicircular canal primordia, thereby blocking their further development. Formation of sensory cristae is also impaired, although this requires greater exposure to Noggin compared with the nonsensory epithelia. The endolymphatic duct also expresses *Bmp7*, but Noggin does not perturb this structure (Chang et al., 1999, 2002). Although the endolymphatic duct does not seem to require Bmp signaling, it may provide an additional source of Bmp needed by surrounding structures. Likely mediators of Bmp signaling are Msx proteins. In zebrafish, *msxC* and *msxD* are expressed in all three cristae, and *msxD* and *msxE* are expressed in the dorsal part of the otic vesicle (Ekker et al., 1992, 1997). *Msx1* is expressed in corresponding regions in the chick (Wu and Oh, 1996), and disruption of Bmp signaling causes downregulation of *Msxl* expression (Chang et al., 2002).

A number of *Fgf* genes are locally expressed in the otic vesicle. In zebrafish, *fgf3, fgf8,* and *fgf17* are coexpressed in the nascent utricular macula, and *fgf8* is also expressed in the saccular macula (Leger and Brand, 2002). Later in development, *fgf8* is also expressed strongly in the nascent cristae and weakly in the primordia of the semicircular canals. In mouse, *Fgf10* is widely expressed in the ventral half of the early otic vesicle, and *fgf3* is coexpressed in a portion of this domain (Pirvola et al., 2000). Both are later restricted to sensory epithelia in all chambers in the ear, and *Fgf10* is also abundantly expressed in delaminating neuroblasts. Loss of individual *Fgf* genes in zebrafish or mouse variably inhibits production of hair cells and neuroblasts, consistent with an autocrine role for Fgf signaling (Mansour et al., 1993; Adamska et al., 2000; Phillips et al., 2001; Kwak et al., 2002; Leger and Brand, 2002). However, nonsensory and dorsal structures are also perturbed, possibly indicating a paracrine role for Fgf signaling. A splice variant of mouse *Fgf receptor-2* (Fgfr2-IIIb) is expressed in a complementary pattern in the dorsal part of the otic vesicle and is later found in the endolymphatic duct, semicircular canals, and nonsensory regions of the cochlear duct (Pirvola et al., 2000). These structures fail to form in mice lacking *Fgfr2-IIIb*. Because Fgfr2-IIIb binds both Fgf3 and Fgf10, the restricted domains of Fgf secretion within the ear, as well as Fgf3 from the hindbrain, could directly regulate development of nonsensory structures. Development of sensory epithelia, which do not detectably express *Fgfr2-IIIb,* is also impaired in *Fgfr2-IIIb* knockout mice. Presumably, failed development of the dorsal epithelium prevents expression of factors required in trans for optimal development of the sensory patches.

Transcription factors expressed in the otic vesicle

Transcription factors locally expressed in the otic placode appear to be regulated by signals from surrounding tissues as well as signals from within the otic vesicle. Loss-of-function studies suggest that these transcription factors act autonomously to regulate positional identity, differentiation and morphogenesis within their domains of expression. In addition, they may have nonautonomous effects by regulating the signaling properties of expressing cells. Understanding the precise role(s) of any of these transcription factors is complicated by the fact that most are expressed dynamically and often in broader domains during earlier stages. This is exemplified by analysis of *Eyal* and *Dlx5*, both of which are initially expressed throughout the placode (Qiu et al, 1997; Heanue et al., 2002). *Eya1* is later restricted to a ventral domain in the otic vesicle. *Eya1*

mutant mice fail to express *Fgf3* in the otic vesicle (Xu et al., 1999), which probably contributes to the severe disruption of development seen throughout the ear. In the case of *Dlx5,* expression becomes restricted to the dorsolateral epithelium in the otic vesicle, and mutants fail to form anterior and posterior semicircular canals or their respective cristae (Acampora et al., 1999; Depew et al., 1999; Merlo et al., 2002). Variable defects are also seen in the lateral semicircular canal, cochlea, and vestibular maculae, possibly indicating nonautonomous effects. Indeed, *Dlx5* mutants fail to express *Bmp4* in the ear (Merlo et al., 2002), which could influence regions adjacent to the *Dlx5* expression domain. However, in neither of these mutants is it known whether disruption of earlier functions in the otic placode causes a permanent deficit in the developmental potential of cells throughout the otic vesicle.

Hmx3 (Nkx5.1) is expressed in the placode and becomes restricted to the dorsolateral wall of the otic vesicle in a domain that overlaps with *Dlx5*. Two *Hmx3* knockout alleles have been described and curiously have different phenotypes. In one case, morphogenesis of all three semicircular canals is disrupted and the lateral crista is ablated (Hadrys et al., 1998). All other sensory epithelia are present and appear normal. In the second allele, the lateral crista is ablated and the utricle and saccule and associated maculae are fused into a single endorgan (Wang et al., 1998). The three semicircular canal ducts develop normally. The cochlea and endolymphatic duct appear normal in both backgrounds. The reason for the different phenotypes is not certain but may be related to unanticipated effects on the activity of a related gene, *Hmx2,* which is closely linked to *Hmx3*. *Hmx2* is not expressed in the placode but is later coexpressed with *Hmx3* in the dorsolateral wall of the otic vesicle. *Hmx2* null mice produce a more severe phenotype that essentially phenocopies the combined effects of the two *Hmx3* alleles (Wang et al., 2001). Since mutagenic insertions can affect expression levels in nearby genes, it is possible that both loci are affected to varying degrees in one or more of these mutant lines. It is also likely that there is some functional redundancy such that knocking out both loci will be required to fully address their function(s).

GATA3 is zinc-finger transcription factor that is widely expressed in the early otic vesicle and periotic mesenchyme (Rivolta and Holey, 1999; Karis et al., 2001; Lawoko-Kerali et al., 2002). It shows a dynamic pattern of expression during subsequent ear development. Mutants produce variable but generally severe malformations of the inner ear (Karis et al., 2001). In the worst cases, the otic vesicle undergoes little morphogenesis or differentiation, forming only a rudimentary endolymphatic duct. The basis for this phenotype is likely to be complex as it could involve cell-autonomous defects or altered signaling interactions between different regions in the otic vesicle, or between the otic epithelium and the periotic mesenchyme.

Otx1 and *Otx2* are limited to ventrolateral cells and, in mouse, are not expressed earlier in the otic placode (Morsli et al., 1999; Mazan et al., 2000). The phenotype of *Otx2* cannot be assessed because homozygous mutants die at an early stage. *Otx1* null mice show defects that are usually limited to the lateral semicircular canal. $Otx1^{-/-}$, $Otx2^{+/-}$ compound mutants (retaining a half dose of *Otx2*) show a more penetrant phenotype that usually includes cochlear defects. There are no obvious defects in other compartments, but heterozygosity at the *Otx2* locus might be sufficient to allow nonautonomous functions to continue. Analysis of reciprocal gene substitutions between *Otx1* and *Otx2* indicates that their functions do partially overlap, although there are also specific functions not shared between these loci (Cantos et al., 2000).

Despite the widespread early expression of *Pax2* (Nornes et al., 1990; Lawoko-Kerali et al., 2002), defects in *Pax2* null mice are limited to agenesis of the cochlea (Torres et al., 1996). The cochlea normally emerges from the ventralmost portion of the *Pax2* expression domain in the otic vesicle. *Pax8* expression briefly overlaps with *Pax2* elsewhere in the vesicle and probably limits the severity of the *Pax2* null phenotype (Pfeffer et al., 1998; Riccomagno et al., 2002). Loss of both genes might be expected to ablate or severely compromise the otic placode, so analysis of their relative roles in the otic vesicle may prove challenging.

In summary, all of the above examples illustrate that regionally expressed transcription factors do indeed control important aspects of development within their expression domains. In most cases it is likely that they also facilitate interactions with other regions of the ear.

Differentiation of sensory epithelia

Cell fate specification

Hair cells and support cells appear to arise from a common pool of equipotential precursors (an equivalence group) in which alternate fates are specified by Delta-Notch interactions (Adam et al., 1998; Eddison et al., 2000; Müller and Littlewood-Evans, 2001; Anagnostopoulos, 2002; Fekete and Wu, 2002; Whitfield et al., 2002). All cells in the equivalence group initially express low levels of Delta and Notch and thereby mutually inhibit each other's differentiation. Inhibition is eventually overcome in a subset of centrally located cells that begin to differentiate as hair cells. Emerging hair cells strongly upregulate Delta expression, which elevates Notch activity in neighboring cells. This process, referred to as lateral inhibition, prevents the neighboring cells from differentiating as hair cells and forces them to become support cells instead.

Numerous genetic and cytological data support the above model. Cell lineage studies in chick show that hair cells and support cells are lineally related (Fekete et al., 1998; Lang and Fekete, 2001). In mouse, complete loss of *Notch1 (N1)* kills the embryo at an early stage, but $N1^{+/-}$ heterozygotes show a haploinsufficient phenotype in which cochlear hair

cells are overproduced due to weakening of lateral inhibition (Zhang et al., 2000). Disruption of *Jagged2 (Jag2),* which encodes a Delta-related ligand expressed preferentially in hair cells, also causes overproduction of hair cells (Lanford et al., 1999). The fold-increase in hair cell production is relatively modest in *Jag2* mutants, probably because another *Delta* homolog, *Delta-like 1 (Dll1),* is also expressed in hair cells and presumably continues to mediate lateral inhibition in the absence of *Jag2* (Lewis et al., 1998). Disruption of another gene, *Lunatic fringe (Lfng),* suppresses the *Jag2* phenotype (Zhang et al., 2000). Fringe proteins are glycosyl-transferases that attach O-fucose residues to sugar chains on the extracellular domain of Notch proteins as they are being trafficked to the cell membrane. Such modification differentially biases the affinity of Notch for different Delta ligands (reviewed by Justice and Jan, 2002). *Lfng* in mouse is expressed throughout the region of the ear the gives rise to sensory epithelia and appears to make N1 less sensitive to Dll1. Thus, in the absence of *Lfng*, signaling by Dll1 is strengthened sufficiently to compensate for loss of *Jag2*. In zebrafish, too, nascent hair cells express multiple *Delta* genes, including *dlA, dlB, dlC, dlD,* and *serrateB (serB)* (Haddon et al., 1998a, 1998b). Embryos homozygous for a dominant-negative point mutation in *dlA* produce a fivefold increase in hair cells and a corresponding decrease in support cells (Riley et al., 1999). Maculae and cristae are similarly affected. A more severe phenotype is seen in zebrafish *mind bomb (mib)* mutants, which produce enlarged maculae containing a 10-fold excess of hair cells but no detectable support cells (Haddon et al., 1998a; Riley et al., 1999). The maculae expand quickly and progressively to cover most of the ventromedial surface of the vesicle. Cristae, which normally form later at the edges of this domain, fail to form in *mib* mutants, possibly because the pool of precursors is recruited into the expanding maculae. Semicircular canals also fail to form, possibly due to loss of regulatory signals from the cristae. The *mib* gene encodes an E3 ubiquitin-ligase similar to *neuralized (neu)* in *Drosophila* and *Xenopus* (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001; Itoh et al., 2003). Mib protein normally targets Delta protein for destruction via the ubiquitin-proteosome pathway. Disruption of this process appears to globally block Dl-N signaling in *mib* mutants, possibly because rapid turnover of Delta is essential for sustained Delta-Notch signaling (Parks et al., 2001; reviewed by Krämer, 2001; Lai, 2002).

As a note of caution, it should be noted that none of the above mouse mutant studies fully supports the lateral inhibition model. Although loss of various Delta-Notch functions in mouse causes an increase in the numbers of hair cells, the predicted loss of support cells is not observed. Only in zebrafish *mib* and dlA^{dx2} mutants are support cells clearly deficient. However, the *mib* and dlA^{dx2} mutations cause much more complete disruption of Delta-Notch signaling making it much easier to detect the severe imbalance in cell fate specification. In contrast, the mouse mutations cause only modest impairment of lateral inhibition. It is possible that these mutations actually do reduce the number of cells specified as support cells, as subsequent support cell proliferation could correct mild-to-moderate deficiencies. Presumably $N1^{-/-}$ homozygous mutants would show a more severe phenotype if they were able to survive to the appropriate stage. Indeed, antisense knockdown of *N1* in mouse cochlear cultures does appear to reduce the number of support cells (Zine et al., 2000). Given these caveats and considerations, it is likely that the mouse does indeed follow the lateral inhibition model, although further studies are warranted.

The standard lateral inhibition model is further complicated by the fact that several Delta-like genes, including *Ser1* in chick and *Jag1* in mouse, are expressed in support cells (Lewis et al., 1998; Eddison et al., 2000; Kiernan et al., 2001). This has led to the suggestion that signaling between support cells helps to mutually maintain elevated Notch activity and thereby reinforce the initial cell fate choice imposed by lateral inhibition. Hair cells are unresponsive to Ser1 and Jag 1 because *Notch* expression is downregulated during initial stages of hair cell differentiation (Lanford et al., 1999), and hair cells also express Numb, an intracellular antagonist of Notch activity (Eddison et al., 2000). Homozygous loss of *Jag1* kills mouse embryos prior to the stage when hair cells differentiate, but $Jag1^{+/-}$ heterozygotes show a modest increase in production of inner hair cells in the cochlea (Kiernan et al., 2001). Similarly, antisense knockdown of *Jag1* increases hair cell production in cochlear cultures (Zine et al., 2000). Moreover, hair cells are produced in regions that do not normally produce sensory epithelia, a phenotype reminiscent of zebrafish *mib* mutants. $Jag1^{+/-}$ heterozygotes also frequently show loss of anterior and posterior cristae, as well as terminal truncations of the corresponding semicircular canals. It is not clear why only the anterior and posterior cristae are affected in this way.

Distinct families of the bHLH transcription factors mediate the signaling interactions that specify hair cells and support cells. In mouse, high Notch activity in prospective support cells induces *hairy*- and *Enhancer of split*-related genes *Hes1* and *Hes5,* whose gene products actively repress hair cell differentiation (Zheng et al., 2000; Zine et al., 2001). Accordingly, hair cells are overproduced when *Hes1* or *Hes5* is disrupted, and this phenotype is enhanced in double mutants. In contrast, hair cell specification requires expression of proneural genes related to *Drosophila achaete-scute* and *atonal*. In mouse, expression of *Murine atonal homolog-1 (Math1)* initially marks all cells within the equivalence group and later upregulates in emerging hair cells (Bermingham et al., 1999). This promotes hair cell differentiation and increases expression of *Delta* genes, thereby facilitating subsequent lateral inhibition. *Math1* null mice produce aberrant sensory epithelia totally lacking hair cells, instead forming uniform sheets of support cells. Overexpression of *Math1* causes overproduction of hair cells in

rat cochlear cultures (Zheng and Gao, 2000). The zebrafish homolog, *zath1,* shows a similar expression pattern, first marking the entire equivalence group and later upregulating in hair cells (Whitfield et al., 2002). Morpholino-mediated knockdown of *zath1* strongly impairs hair cell formation (unpublished observations). Thus, the function of this proneural gene appears to be conserved amongst vertebrates. The functions of proneural genes and *hairy/enhancer of split* genes are mutually antagonistic. Hence, once the balance tips to one side or the other, cell fate decisions tend to be self-reinforcing.

In addition to specifying cell fate, proneural genes often play an earlier role in establishing the entire equivalence group (reviewed by Lewis, 1996). However, *Math1* appears dispensable for this function in mouse since support cells are still produced in the absence of *Math1*. Furthermore, expression of *Math1* cannot be detected until after the onset of expression of cyclin-dependent kinase inhibitor, $p27^{kip1}$ (Chen et al., 2002), which regulates growth of the equivalence group and is required for normal development of sensory epithelia (Chen and Segil, 1999; Löwenheim et al., 1999). In *Math1* mutants, expression of $p27^{kip1}$ occurs normally and cells in the cochlear epithelium exit the cell cycle on time. Even if *Math1* is normally expressed earlier at a level too low to be detected, these data indicate that other gene(s) are able to specify the equivalence group in the absence of Math1. A second *atonal* homolog, *Neurogenin1 (Ngn1),* is also expressed in the mouse inner ear, and *Ngn1* null mutants develop with sensory patches that are significantly smaller than normal (Ma et al., 2000). However, hair cells are still produced at normal densities within the sensory patches. It is therefore possible that *Ngn1* plays an early role, perhaps in parallel with *Math1,* in specifying equivalence groups, whereas *Math1* is necessary and sufficient for subsequent hair cell specification. In mouse, yet another *atonal* homolog, *NeuroD,* is also expressed widely in the mouse inner ear and is later seen in a subset of hair cells scattered throughout the sensory epithelia (Liu et al., 2000; Kim et al., 2001). The function of *NeuroD* in these hair cells is not clear since development of sensory epithelia is essentially normal in *NeuroD* mutants. *NeuroD* might assist *Ngn1* in specification of the equivalence group.

The extracellular signals that induce proneural gene expression within equivalence groups are likely to include Fgfs and Bmps. Loss of these signals inhibits formation of maculae and/or cristae, and ectopic Fgf signaling in the hindbrain leads to formation of ectopic hair cells (Mansour et al., 1993; Chang et al., 1999; Gerlach et al., 2000; Pirvola et al., 2000; Phillips et al., 2001; Kwak et al., 2002; Leger et al., 2002).

In addition to proneural genes, *Pax2* could also play a role in hair cell differentiation. Like *Math1* and *zath1, Pax2* marks the hair cell competent epithelium in the otic vesicle and is later restricted to hair cells (Riley et al., 1999; Lawoko-Kerali, 2001). In zebrafish, it appears that *pax2a* and *pax2b* have evolved to control distinct aspects of early hair cell differentiation. *pax2a* is disrupted in *no-isthmus (noi)* mutants (Brand et al., 1996), which produce nearly twice as many hair cells as normal (Riley et al., 1999). The reason for this unexpected phenotype is that *Delta* gene expression is significantly reduced in nascent hair cells, which presumably weakens lateral inhibition. No mutations are available in *pax2b,* but injection of *pax2b*-MO into wild-type embryos inhibits production of hair cells (Whitfield et al., 2002). The same phenotype is observed when *pax2b*-MO is injected into *noi* mutants, indicating that *pax2b* is epistatic to *pax2a* (i.e., overproduction of hair cells normally seen in *noi* mutants is suppressed). Thus, *pax2b* appears necessary for specification of hair cells, whereas *pax2a* regulates the subordinate process of lateral inhibition. *Pax2* null mice have not yet been examined in sufficient histological detail to determine whether they, too, have defects in hair cell development. The functional relationship between *Pax2* and proneural genes remains to be established. *Drosophila pax2* appears to act as a downstream effector of proneural genes *achaete* and *scute* during development of sensory bristles (Kavaler et al., 1999), which bear interesting similarities with vertebrate hair cells. The situation is more complex in the vertebrate inner ear since *Pax2* is expressed throughout the placode well before proneural genes are expressed. Later, however, upregulation of *Pax2* in differentiating hair cells might require proneural function.

Differentiation and maintenance of hair cells

Following their specification, hair cells appear to rely on interactions with support cells for normal differentiation and survival. In zebrafish *mib* mutants, which produce few or no support cells, hair cell patches expand rapidly but then begin to detach from surrounding epithelia and eventually die (Haddon et al., 1999). It is not clear whether detachment is a cause or an effect of hair cell degeneration. How support cells maintain hair cells is unknown but paracrine or juxtaracrine factors or cell adhesion molecules could mediate trophic support. Hair cells in turn send signals back to support cells to facilitate monitoring of hair cell status. Hair cell damage and death stimulates a regenerative response by local support cells. The latter either transdifferentiate into hair cells or briefly reenter the cell cycle and divide asymmetrically to produce a new hair cell and another support cell (Baird et al., 2000; Stone and Rubel, 2000; Gale et al., 2002). The regeneration process is compromised in the mammalian cochlea, possibly due to unusually high expression of the mitotic suppressor $p27^{kip1}$ (Chen and Segil, 1999; Löwenheim et al., 1999). Hair cells and support cells also provide trophic support required for maintenance of SAG neurons that innervate the hair cells (see below).

Genetic studies in mouse have been instrumental in elucidating various stages of hair cell differentiation following specification. An early stage is regulated by the transcription

factor *Brn3C (Pou4f3)*, which is expressed in newly formed hair cells in all sensory epithelia. In *Brn3c* mutants, hair cells are unable to complete morphological differentiation (e.g., they do not produced stereocilia) and subsequently degenerate by apoptosis. Degeneration begins during late embryogenesis and is essentially complete by postnatal day 5. The majority of SAG neurons also degenerate due to loss of trophic support from hair cells. Mice survive but are totally deaf and exhibit profound behavioral defects (e.g., circling) indicative of loss of vestibular function (Erkman et al., 1996; Xiang et al., 1997, 1998).

Gfi1 encodes a Zn finger transcription factor that controls slightly later stages of hair cell differentiation (Wallis et al., 2003). *Gfi1* is related to *Drosophila senseless*, which is induced by proneural bHLH proteins and is required for development of the peripheral nervous system. In mouse, *Gfi1* is expressed early in the otic vesicle and is later restricted to differentiating hair cells. Hair cells are produced in *Gfi1* mutants but show aberrant patterning and morphology. While mutant hair cells express most hair cell markers, including *Brn3c*, they also inappropriately express some neural markers. Hair cells in the cochlea die during early postnatal development, followed by death of cochlear neurons. Vestibular hair cells survive but are highly disorganized, and mutant mice exhibit ataxia and circling due to loss of vestibular function.

Barhl1 is a homeobox gene required for late-stage differentiation or maintenance of hair cells (Li et al., 2002). It is expressed in all hair cells several days after their morphological differentiation. In *Barhl1* knockout mice, all hair cell markers are expressed normally and the ear appears largely normal at birth. However, cochlear outer hair cells become disorganized by postnatal day 6 and are severely depleted by 2 months. These changes correlate with significant hearing loss, especially in low frequency range. Inner hair cells in the cochlea and vestibular hair cells survive, suggesting that other functions maintain these populations.

Unexpectedly, *Caspase-3* has also been shown to play a role in hair cell maintenance in the cochlea (Takahashi et al., 2001). Caspases are cysteine proteases that normally mediate apoptotic cell death. *Caspase-3* knockout mice develop with sensory epithelia that initially appear normal, although maturation of the organ of Corti is slightly delayed. By 5 weeks, mutants show hyperplasia of support cells and significant loss of inner and outer hair cells. The few hair cells that survive show morphological abnormalities, including loss of ciliary bundles or fusion of bundles between adjacent hair cells. Cochlear neurons also undergo significant loss during this time. While production of supernumerary support cells could result from inhibition of apoptosis, the cause of hair cell degeneration is not clear. Loss of hair cells could be an indirect effect of altering the balance of signals from support cells. Another possibility is that *Caspase-3* plays a direct role in the differentiation or maintenance of hair cells. Indeed, *Caspase-3* is required to initiate differentiation of skeletal muscle, as well as Fgf-dependent terminal differentiation of lens fibers (Ishizaki et al., 1998; Fernando et al., 2002). A caspase-3-like activity has also been implicated in maintenance of cytoskeletal integrity in cultured fibroblasts (Watanabe and Akaike, 1999). How these alternative functions are regulated without triggering apoptosis is not known, but either could be required for hair cells.

Examples of functions specifically required by the vestibular system are GAP proteins Apr and Bcr. These proteins normally regulate the activity of small GTPases Cdc42 and Rac and thereby help regulate the actin cytoskeleton and cell adhesion. Loss of either *Apr* or *Bcr* has no effect on the ear, but loss of both functions causes reduction and partial detachment of the utricular and saccular maculae (Kaartinen et al., 2002). Development is initially normal but macular detachment becomes visible by E16.5. Formation of otoconia, which is normally evident by E18.5, is disrupted in *Abr-Bcr* double mutants. The utricle lacks otoconia entirely and the saccule either lacks otoconia or forms a small number of unusually large otoconia. As mutants mature, they display severe vestibular dysfunction. All other sensory epithelia appear to develop and function normally. Two other mouse mutants, *tilted head* and *head tilt*, show similar otoconial defects, but not macular detachment.

Similarly, *monolith (mnl)* mutants in zebrafish show loss of utricular otoliths and production of enlarged saccular otoliths (Riley and Grunwald, 1996). Analysis of genetic mosaics shows that the otolith deficiency results from disruption of support cell function, although hair cells and support cells appear morphologically normal. Vestibular function is so severely compromised that *mnl* mutants die during larval development. The utricular otolith deficiency can be rescued by immobilizing mutant embryos in a head-down orientation during a brief critical period of development, in which case vestibular function and survival are restored to normal (Riley et al., 1997; Riley and Moorman, 2000). The *mnl* locus has been mapped to a small interval on linkage group 1, but the affected gene has not been identified (unpublished data).

There have been numerous reviews written on other aspects of hair cell biology. Hair cell function depends on numerous structural proteins in the extracellular matrix, intercellular junctions, various transmembrane proteins, and a highly organized array of cytoskeletal elements in the ciliary bundles (Goodyear and Richardson, 2002; Müller and Littlewood-Evans, 2002; Whitfield et al., 2002). The polarity of ciliary bundles must also be coordinated throughout the epithelium. Regulation of planar polarity requires a complex interplay between cell signaling and cytoskeletal rearrangement and modification (reviewed by Bang et al., 2001; Müller and Littlewood-Evans, 2001; Lewis and Davies, 2002). Mutant forms of genes for many of these proteins are associated with various forms of human deafness (Holme and Steel, 1999; Whitfield, 2002).

Development of SAG neurons

There has long been speculation that SAG neurons and sensory epithelia are derived from a common lineage. However, recent lineage-tracing studies in chick suggest that this may not generally be the case. Labeling small clones in the chick otic cup usually marks either sensory epithelia or SAG neurons, but not both (Fekete et al., 1998; Lang and Fekete, 2001). In rare cases, it is possible to label both populations when the cells are labeled at a sufficiently early stage (T. Satoh and D.M. Fekete, personal communication). However, such colabeling has been observed in only 3 cases out of more than 100 attempts, and each colabeled clone was derived from a restricted region between the future utricular macula and lateral crista. Similarly, analysis of various molecular markers in mouse suggests the majority of SAG neuroblasts and sensory epithelia arise from complementary regions of the otic vesicle, with some areas of overlap (Farinas et al., 2001; reviewed by Fritzsch et al., 2002). In the utricular epithelium, neuroblasts appear to delaminate from regions later occupied by sensory epithelia. These data provide support for a common lineage in some regions of the otic epithelium, but in many cases neuroblasts and sensory epithelia appear to come from distinct lineages. How lineage is related to mechanisms of cell fate specification remains to be established.

In mouse, *atonal*-related proneural genes *Ngn1* and *NeuroD* regulate distinct stages of SAG differentiation. Both genes are expressed weakly in the otic cup and are later found in developing neuroblasts. *Ngn1* is expressed transiently in nascent neuroblasts, and SAG neurons fail to form in *Ngn1* mutants (Ma et al., 1998). The absence of *Dll1* expression, which is normally expressed in SAG precursors, suggests that differentiation is blocked at an early stage. In contrast, *NeuroD* is maintained in SAG neurons as they differentiate. *NeuroD* mutants produce delaminating neuroblasts capable of forming neurons, but nearly all die after projecting axons towards the sensory epithelia (Liu et al., 2000; Kim et al., 2001). Death appears to be caused by failure to express TrkB and TrkC, high-affinity receptors for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), respectively. BDNF and NT-3 are produced in vestibular and cochlear sensory epithelia, respectively, and are required for trophic support of the SAG neurons (Farinas et al., 1994; Enfors et al., 1995). Double mutants lacking both *NT-3* and *BDNF* also show death of nearly all SAG neurons, as do double mutants lacking both *TrkB* and *TrkC*. (Enfors et al., 1995; Schimmang et al., 1997; reviewed by Fritzsch et al., 1999). Thus, *NeuroD* appears to regulate a late stage of differentiation required to make SAG neurons competent to receive trophic support from their targets.

Although the function of NeuroD has not been examined in zebrafish, the function of Ngn1 appears similar to that in mouse (Andermann et al., 2001). Zebrafish *ngn1* is first expressed in ventromedial cells of the nascent otic vesicle. It soon becomes restricted to a small anteroventral patch corresponding to the region from which the majority of neuroblasts delamintate. Neuroblasts lose *ngn1* expression soon after delaminating, and knockdown of *ngn1* totally ablates formation of SAG neurons. No other inner ear defects have been noted.

Conclusion: new ear resolutions

Progress in understanding the mechanisms of early otic development has been remarkably rapid during the last 10 years. Cellular interactions controlling induction of the otic placode can now be understood based on molecular mechanisms, as many of the relevant signaling molecules and intracellular mediators have been identified. Nevertheless, there is undoubtedly much to learn. For example, it is not clear how the various gene functions recently identified are integrated and processed. Furthermore, as shown by the recent and unanticipated discovery of *foxi1* as an early otic regulator (Solomon et al., 2003), it is likely that additional key regulators are yet to be identified.

Patterning of the otic vesicle, and further testing of the compartment-boundary model, poses a significant challenge due to the complexity and dynamic nature of gene expression. A number of available techniques will be increasingly called upon to fully address this functional complexity. The Cre-Lox system in mouse is a powerful means of selectively disrupting gene functions in specific spatial domains (Zuo, 2002). In chick, use of electroporation (Muramatsu et al, 1997), viral infection, and implanting beads are all useful techniques for altering gene functions at a desired time and place. Implanting beads is also useful in *Xenopus*, as is the relative ease of misexpressing wild-type or dominant-negative gene constructs. Mutagenesis screens in zebrafish (Malicki et al., 1996; Whitfield et al., 1996) are likely to continue to identify new functions and could generate temperature-sensitive alleles useful for distinguishing between early vs. late functions (Dick et al., 2000; Rawls and Johnson, 2001; Poss et al., 2002). Heat shock-inducible constructs (Shoji et al., 1998) can also be used to control the timing of gene activity, and genetic mosaics are easily produced and can readily distinguish between autonomous vs. nonautonomous functions (Riley and Grunwald, 1996; Whitfield et al., 2002).

One of the most difficult goals remaining is to achieve better understanding of how morphogenesis of the intricate structure of the inner ear is regulated. Although many genes have been identified whose functions are required for normal morphogenesis, in most cases it is not obvious how these functions help orchestrate the fine balance of growth, death, sculpting and remodeling of the epithelium. This problem is certainly not limited to the inner ear, but epithelial morphogenesis is such a conspicuous aspect of ear development that it underscores the paucity of mechanistic data. In addition to the difficulties in addressing the com-

plexity of gene expression, it is likely that morphogenesis involves tight regulation of numerous general effectors and "house-keeping" functions. Hence, new techniques may be needed to modulate gene functions with finer control, and to visualize subtle changes in the level and distribution of gene products during ear development. Various molecular screens and microarray techniques are also likely to play an important role in the near future (Chen and Corey, 2002; Heller, 2002). Given the rapid pace of recent studies, there is every reason to be optimistic that these difficulties will be overcome.

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