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Chapter 2

Induction and Morphogenesis of the Inner Ear

Bruce B. Riley*
Biology Department, Texas A&M University, College Station TX, US

Abstract

As the precursor of the inner ear, the otic placode has long been a source of fascination for experimental embryologists. More recently the otic placode has attracted the attention of a growing number of developmental geneticists. Modern analysis of the otic placode has provided a wealth of information regarding many fundamental processes including cell migration and morphogenesis, cell signaling mechanisms, pattern formation and cell fate specification, gene regulation and integration of gene networks. The superficial structural simplicity of the otic placode belies a challenging degree of regulatory complexity. For this reason, genetics approaches have proven especially valuable in elucidating core molecular mechanisms. Fortunately, underlying mechanisms have proven to be highly conserved, facilitating meaningful cross-species comparisons and the ability to draw from the complementary strengths of several model organisms: chiefly mouse, chick, *Xenopus* and zebrafish. This chapter summarizes the rich literature on otic placode development from its origins in the early gastrula through early pattern formation and morphogenesis of the otic cup.

Introduction

The otic placode poses a fascinating challenge for developmental biologists. Though seemingly simple in structure, the otic placode is remarkable for its ability to quickly generate the entire structure of the inner ear, arguably one of the most complex organs in the vertebrate body. No other cranial placode shows such rich complexity. This explains why the otic

* Corresponding author: Bruce B. Riley, Ph.D., Biology Department, Texas A&M University, College Station TX 77843. Email: briley@bio.tamu.edu.

placode has attracted the attention of experimental embryologists for nearly a century. The earliest studies of otic development focused on tissue interactions required for inducing the otic placode from uncommitted ectoderm [63, 75, 78, 181, 199, 222]. Additionally, investigators found that early manipulation of the otic placode often resulted in understandable changes in later morphology, providing a convenient readout of how the morphogenetic field had responded [62, 198, 215]. With the introduction of molecular and genetics techniques came a renewed vigor in studies of early otic development. The pace of research has been quite rapid and indeed continues to accelerate. It has become clear that the otic placode originates earlier than previously thought and shares much of its early development with all other cranial placodes, which initially develop within a common field of "preplacodal ectoderm" [24, 171, 182]. Local signaling interactions subdivide the preplacodal ectoderm into various distinct cranial placodes. Much is now known about the signals that specify formation of the otic placode, as well as the genes that are activated in response [135]. Many of the same pathways continue to operate as the otic placode is further subdivided into domains that express new sets of genes [18, 170, 174, 204]. Ultimately these molecular domains undergo extensive growth, folding and differentiation to produce different parts of the inner ear. There have been fundamental breakthroughs in understanding key mechanisms of cell fate specification and epithelial morphogenesis. Still, as more and more regulatory genes are identified the task of understanding their functions and interactions becomes increasingly challenging. Fortunately, core mechanisms of otic development have been widely conserved amongst vertebrates, allowing meaningful cross-species comparisons that effectively join the complementary strengths of different model systems. The sophisticated genetics approaches available in mouse figure prominently in current otic research.

This chapter summarizes the recent advances in understanding the molecular mechanisms controlling formation of the otic placode and its early patterning and morphogenesis. Discussion of otic development is organized by developmental stage, with subheadings for discussion of specific genes, signaling molecules, or developmental processes. To facilitate understanding of data obtained from analysis of various stages in different species, Table 1 provides an alignment of embryonic staging for the four principal model organisms. According to nomenclature conventions established in each field, gene names are italicized with the first letter capitalized (mouse, chick, *Xenopus*) or all lower case (zebrafish), whereas protein names are in block print with the first letter capitalized (all species).

Table 1. Comparison of embryonic staging in different species

Stage	**Mouse**	**Chick**	**Xenopus**	**Zebrafish**
Gastrulation	E6.5 pre-streak	ST1 pre-streak	ST9	30% epiboly
	E6.75 early streak	ST2 initial streak	ST10	50% epiboly
	E7.0 mid streak	ST3/4	ST11.5	75% epiboly
	E7.25 late streak	ST4 definitive streak	ST12	90% epiboly
Neural plate	E7.5	ST5 head process	ST13/14	100% epiboly
1 somite	E8.0	ST7	ST17/18	11 hpf
7 somites	E8.4 otic thickening	ST9	ST20	12.5 hpf
10 somites	E8.5 otic placode	ST10	ST21/22	14 hpf
14 somites	E8.8 early otic cup	ST11	ST22/23	16 hpf
18-20 somites	E9.5 otic vesicle	ST14-	ST28	18 hpf

Preplacodal Ectoderm

Preplacodal ectoderm forms after gastrulation as a contiguous band of multipotent progenitor cells surrounding the anterior neural plate (Figure 1). Although it will ultimately subdivide into a diverse array of cranial placodes with distinct identities (147), cells throughout the preplacodal ectoderm initially share a common molecular identity reflecting their shared developmental history. Most of the work on specification and development of preplacodal ectoderm has come from studies in *Xenopus*, zebrafish and chick. Studies in mouse are more limited but appear consistent with the existence of a broadly conserved mechanism of preplacodal development. There have been several recent and detailed reviews of preplacodal development (24, 171, 182). Below is a brief summary of data gleaned from non-mammalian species and an explanation of how mouse data contribute to this interesting area of research.

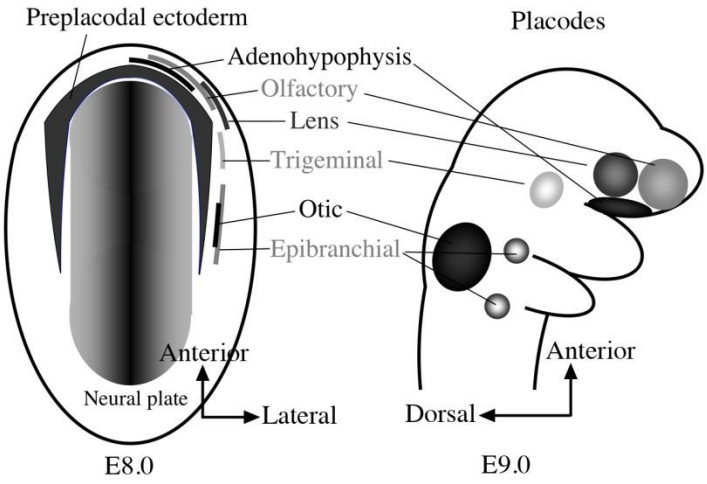

Figure 1. Formation of preplacodal ectoderm and cranial placodes in mouse. The preplacodal ectoderm is marked by expression of *Eya1/2/4*, Six1/4, *Dlx5/6*, *GATA3* and *Foxi3* genes, and is established by E8.0. Different regions of the preplacodal ectoderm contribute to different cranial placodes, as indicated.

Signaling Interactions

Explant and transplant studies in chick and *Xenopus* show that preplacodal ectoderm is specified during neurula stages [5, 107]. Inductive signals include Fgfs, Bmp-antagonists and Wnt-antagonists, which in combination are found only near the border of the anterior neural plate. Overexpression of any of these factors can broaden the endogenous domains of preplacodal markers [25, 56, 107], and co-misexpression of Fgf and Chordin in *Xenopus* and zebrafish can induce ectopic expression of preplacodal markers in ventral ectoderm [5, 90]. Furthermore, blocking Fgf signaling in chick and *Xenopus* [5, 107], or blocking both Fgf and

the partially redundant Pdgf pathway in zebrafish [90], reduces or eliminates expression of preplacodal markers. Similarly, knocking down relevant Bmp-antagonists in zebrafish [47, 89] also reduces expression of preplacodal markers.

The importance of Bmp-antagonists belies a complex role for Bmp during preplacodal development. There has been some debate as to whether Bmp-antagonists block Bmp entirely or instead titrate Bmp to some critical level in a morphogen gradient. However, work in zebrafish shows that overt specification of preplacodal fate requires complete attenuation of Bmp [90]. Despite this late requirement, Bmp signaling is absolutely required at an earlier stage to induce expression of several transcription factors that render the entire ventral/nonneural ectoderm competent to form preplacodal ectoderm [90]. Once expressed, these competence factors no longer require Bmp, hence they can be maintained after preplacodal cells begin to respond to dorsally expressed Bmp-antagonists. Preplacodal competence factors include members of the Gata, Fox and Tfap2 families of transcription factors, several of which are described in more detail below. Simultaneous knockdown of these partially redundant factors specifically and completely blocks preplacodal specification, even though requisite signaling interactions still occur [90]. Comparable functions have not yet been identified in other vertebrates.

To date, there have been no studies to establish the requirements for expression of preplacodal markers in mouse, though expression patterns of preplacodal markers and the positioning of putative inductive signals appear to be conserved [28, 34, 50, 69, 142].

Eya and *Six* Genes

Multiple members of the *Eyes absent* (*Eya*) and *Sine occulis* (*Six*) gene families specifically mark the preplacodal ectoderm in all vertebrates examined to date. Eya and Six proteins bind together to form a transactivation complex, with Six providing DNA-binding and Eya providing protein tyrosine phosphatase activity required for transcriptional activation of target genes [80, 101, 153, 187]. Multiple homologues, including *Eya1*, *Eya2*, *Eya4*, *Six1* and *Six4*, are coexpressed in the preplacodal ectoderm and appear to provide extensive functional redundancy [15, 19, 28, 37, 48, 55, 83, 85, 107, 147, 169]. Accordingly, disruption of any one gene is not sufficient to ablate formation of preplacodal ectoderm, though deficiencies in various placodal derivatives are often observed. Knockdown of *Xenopus Eya1* or *Six1* reduces expression levels of some preplacodal genes and also reduces neurogenesis in all placodes [25, 173]. Disruption or knockdown of zebrafish *eya1*, *eya4* or *six1* does not detectably alter expression of preplacodal markers, but in each case there are defects in otic development [22, 85, 200]. Loss of *eya1* also causes some cells in the zebrafish adenohypophysis [anterior pituitary] to differentiate aberrantly as nonsecretory cells [130]. Compound loss of all *Eya* or *Six* genes has not been reported in any species, but expression in chick of Six1 fused to an Engrailed repressor domain can severely impair expression of most, though not all, preplacodal genes [31]. Misexpression of *Eya1* and/or *Six1* in *Xenopus* can expand the domain of preplacodal gene expression [25, 173]. In chick, *Eya2* and *Six1* must be co-misexpressed to expand the preplacodal domain. In no case does misexpression of *Eya* or *Six* induce formation of ectopic placodal tissue. Thus, a network of *Eya* and *Six* genes appears necessary but not sufficient for establishing the preplacodal ectoderm.

Dlx Genes

Members of the *Distal-less* (*Dlx)* family of homeobox genes also mark preplacodal ectoderm but are expressed earlier than *Eya* and *Six* genes. The degree of structural conservation of *Dlx* genes is relatively high and, not surprisingly, coexpressed homologues show significant functional redundancy. In chick and mouse, linked paralogues *Dlx5* and *Dlx6* mark the preplacodal ectoderm [123, 176, 214]. Misexpression of *Dlx5* in chick causes medial expansion of the preplacodal domain of *Six4* at the expense of neural ectoderm [176]. In *Xenopus*, *Dlx3* and *Dlx5* show similar broad expression throughout the nonneural ectoderm, including the preplacodal ectoderm [110]. Misexpression of *Xenopus* Dlx3 fused to an Engrailed repressor domain can block expression of *Six1*, though this is variable [204]. In zebrafish, *dlx3b* and *dlx4b* are coexpressed in preplacodal ectoderm [6, 178]. Knockdown or deletion of both genes leads to a synthetic phenotype in which preplacodal expression of *eya1* and *six4.1* is significantly reduced and/or delayed [47, 146, 178], and placodal derivatives are severely deficient. Otic vesicles are produced but are small and severely mispatterned. Two functions have been attributed to zebrafish *dlx3b/4b*. First, these genes are required for expression of the Bmp-antagonist Crossveinless-2 (CV2) in preplacodal ectoderm and subjacent mesendoderm [47]. Indeed, forced expression of CV2 can rescue many of the placodal deficiencies seen in *dlx3b/4b*-deficient embryos. Second, Dlx proteins form inert heterodimers with closely related Muscle Segment Homeobox [Msx] proteins [217], which show overlapping expression in the preplacodal ectoderm [146]. Dlx and Msx proteins show similar DNA-binding preferences, but Msx proteins act as transcriptional repressors whereas Dlx proteins act as transcriptional activators [14]. Hence, coexpression achieves a proper balance of transcription of target genes. Loss of expression of otic placode markers seen in *dlx3b/4b* null embryos is partially rescued when relevant *msx* genes are also knocked down [146]. Thus Dlx proteins alleviate repression mediated by Msx proteins and augment expression of a Bmp-antagonist required for normal preplacodal specification.

Eya, *Six* and *Dlx* Genes in Mouse

In mouse, knockouts of putative preplacodal genes result in severe deficiencies in early otic development: In *Eya1*$^{-/-}$ and *Six1*$^{-/-}$ mutant mice, the otic placode forms and invaginates to form an otic vesicle, but the vesicle remains small and subsequent patterning is severely perturbed or arrested [138, 211, 219]. The small size of the otic vesicle reflects an increase in apoptosis and a decrease in cell proliferation. In addition to defects in otic development, cranial ganglia from other placodes show deficiencies to varying degrees, especially the olfactory and epibranchial placodes [28, 74, 220]. Disruption of *Six4* causes no morphological defects by itself, but *Six1*$^{-/-}$*;Six4*$^{-/-}$ double mutants show a much more severe deficiency in development of the olfactory placode than either of the single mutants [28, 137]. Similarly, *Eya4* is likely redundant with *Eya1*, though double knockouts have not yet been examined. Disruption of *Dlx5* causes variable defects in vestibular morphogenesis in the inner ear, as well as variable defects in development of the olfactory placode [3, 39]. A more severe and penetrant phenotype is observed in *Dlx5*$^{-/-}$*; Dlx6*$^{-/-}$ double mutant mice, which produce small mispatterned otic vesicles characterized by reduced cell division and increased cell death reminiscent of *Eya1*$^{-/-}$ and *Six1*$^{-/-}$ mutants [162]. The functions of *Eya*, *Six* and *Dlx* genes have

been largely interpreted in the light of their localized functions within the developing otic vesicle, but the early defects in morphogenesis, growth and survival in multiple placodal derivatives suggest that faulty preplacodal development may contribute significantly to these phenotypes. Conditional knockouts, and combinatorial gene inactivation, will be required to distinguish early from later requirements.

Gata3

Though not customarily viewed as a preplacodal gene, *Gata3* in mouse shows an early expression pattern that is similar to *Dlx*, *Eya* and *Six* genes [117]. A superficially similar otic arrest phenotype is also observed in *Gata3*$^{-/-}$ mutant mice, though the severity of the phenotype is variable [79]. Closure of the otic cup occurs on time in *Gata3*$^{-/-}$ mutants, but in about half of mutant embryos the otic placode bifurcates to form two independent otocysts that differentially express various patterning genes [104]. BrdU incorporation shows that mitotic indices are reduced by 10-20% between E9.0 and E10.5. In contrast to *Eya1*$^{-/-}$, *Six1*$^{-/-}$ and *Dlx5*$^{-/-}$*; Dlx6*$^{-/-}$ double mutants, rates of apoptosis are relatively normal in *Gata3*$^{-/-}$ mutants. Curiously, it has been found that maternal feeding of catecholamine precursors allows *Gata3*$^{-/-}$ mutants to survive to as late as E18.5, as opposed to E12. These longer-lived mutants often produce otic vesicles that develop further, often forming a rudimentary cochlear duct and endolymphatic duct [43]. Nevertheless, patterning and cell type differentiation remains grossly abnormal. The molecular and developmental basis for partial rescue by maternal ingestion of catecholamine precursors is unknown. A more general role for Gata3 in preplacodal development has not been demonstrated in mouse, though Gata3 serves as one of the preplacodal competence factors in zebrafish [90].

Forkhead (Fox) Genes

Several *Fox* genes have been implicated in early placodal development, and their early expression patterns suggest they may contribute to preplacodal development. Zebrafish *foxi1*, which functions as a preplacodal competence factor [90], is initially expressed throughout the nonneural ectoderm before gastrulation [179]. Expression is eventually lost from ventral ectoderm but continues in preplacodal ectoderm abutting the hindbrain. This corresponds to the portion of preplacodal ectoderm giving rise to otic and epibranchial placodes, which are severely impaired in *foxi1* mutants [98, 132, 179]. In mouse, three *Fox* genes show expression patterns suggesting a role in regulation of preplacodal ectoderm. *Foxi3* is expressed in ectoderm abutting the neural plate from E6.5, overlapping with *Dlx5* expression [134]. *Foxi2* is expressed in a salt-and-pepper pattern in cranial ectoderm by 4-5 somite stage, showing extensive overlap with the future otic and epibranchial placodes. Expression is later excluded from otic cells but continues to be expressed in epibranchial placodes and surrounding epidermal ectoderm. *Foxg1* is expressed throughout the preplacodal ectoderm by 1-2 somite stage and later marks all placode-derived cranial ganglia [66]. No functional studies have yet been reported for *Foxi2* or *Foxi3*, but *Foxg1*$^{-/-}$ mutants produce an inner ear in which the lateral semicircular canal lacks a sensory crista [73, 141], and there is also a severe deficiency in growth and differentiation of the olfactory epithelium [42]. Additional studies are needed

to establish whether the function of zebrafish *foxi1* is conserved with one or more of the mouse *Fox* genes, and whether any of these genes regulates early preplacodal development in mouse.

Status of Preplacodal Regulation in Mouse

As described above, mouse mutant phenotypes resulting from loss of *Eya*, *Six*, *Dlx*, *Gata3* or *Foxg1* do not reveal an obvious role in preplacodal development. However, given the extensive overlap in expression and likely redundancy of so many transcription factors in the preplacodal ectoderm, it is not surprising that loss of only one, or even two genes, does not globally compromise preplacodal specification and differentiation. As shown in various studies in zebrafish, it will undoubtedly prove necessary to disrupt multiple preplacodal genes in mouse to test their roles in preplacodal development.

Subdivision of Preplacodal Ectoderm

Once established, preplacodal ectoderm begins almost immediately to diverge into discrete placodal fates (Figure 1) through differential localized inductive signaling (Table 2). The otic placode is one of the first to form. The decades-long search for inducers of the otic placode led to the discovery that, in all vertebrates, otic development is initiated by Fgfs secreted by the hindbrain and nearby mesoderm and endoderm (discussed in more detail in the next section). The specificity of this inductive cue became less obvious as it was subsequently discovered that Fgf plays a vital role in specifying all other placodal fates, not to mention its earlier role in specifying preplacodal ectoderm. This raises the question of how a single signaling pathway can be reutilized to achieve such a diverse set of fates. The answer is, in part, that Fgf cooperates with combinations of other signaling molecules to help establish unique identities for the various cranial placodes. Additionally, it is likely that a prepattern of previously expressed regional specifiers also helps diversify the response to Fgf. Before the emergence of preplacodal ectoderm, there is already present in cranial ectoderm a complex pattern of regionally expressed transcription factors involved in specifying positional identity (such as *Otx*, *Gbx* and *Irx* genes). Such factors clearly affect the developmental potential of different parts of the preplacodal ectoderm [171]. Nevertheless, under some circumstances Fgf misexpression, or heterotopic transplants of putative otic-inducing tissues, can lead to production of ectopic otic placodes [109, 140, 145, 181, 196, 198, 207]. This suggests that appropriate signaling interactions can over-ride the influence of previously expressed regional specifiers. On the other hand, ectopic otic placodes rarely progress beyond the earliest steps in otic development, in part because the local signaling environment does not fully mimic that surrounding the endogenous otic placode. Studies in various vertebrate species are beginning to elucidate the unique inductive requirements for each of the cranial placodes. Table 2 summarizes the relevant signaling molecules that regulate distinct placodal fates.

Table 2. Signals required for induction of distinct cranial placodes

Placode	Inductive Signals	Species	References
Adenohypophysis (anterior pituitary)	Bmp4	Mouse	46, 189
	Fgf3, Fgf8	Mouse, Zebrafish	46, 68, 189
	Shh	Mouse, Zebrafish	44, 190, 218
	Wnt5a	Mouse	190
Olfactory	Bmp4 (transient)	Mouse	177
	Fgf8	Mouse, Chick	10, 51, 81, 93
	Shh	Mouse	93
	RA	Mouse	93
Lens	Bmp4, Bmp7	Chick	176, 202
	Fgf (transient)	Chick, Mouse	10, 49
	Notch	***Xenopus***	133
	Shh (inhibitory)	Zebrafish	44
Trigeminal	Fgf8	Chick	26, 95
	PDGF	Chick	121
	Wnt1	Chick	26, 94
Otic	Fgf3, Fgf8, Fgf10, Fgf19	Chick, Mouse, *Xenopus*, Zebrafish	8, 40, 91, 92., 99, 108, 120 140, 144, 209
	Fsta, Chd, CV2	Zebrafish	89
	Notch (maintenance)	Mouse	76
	Wnt (maintenance)	Chick, Mouse	136, 52
Epibranchial	Fgf3, Fgf8, Fgf24	Chick, Zebrafish	52, 128, 131, 139, 184
	Notch (inhibitory)	Mouse	76
	Wnt (inhibitory)	Chick, Mouse	52, 136

Signals Required for Otic Placode Induction

Fgf is the Primary Otic Inducing Signal

Based on its early pattern of expression, Fgf3 was proposed more than 20 years ago to be a good candidate for an otic inducer [205]. *Fgf3* is expressed in the caudal hindbrain from the 3 somite-stage in both mouse and chick [113, 114, 122]. *Fgf3* is also expressed in subotic mesenchyme in chick, whereas it is expressed in prospective otic ectoderm and pharyngeal endoderm in mouse, all sites that could readily augment otic induction. In seeming contradiction, however, analysis of the first *Fgf3* knockout in mouse challenged the role of Fgf3 in otic induction [118]. *Fgf3*$^{-/-}$ mutant mice were found to have variable defects in otic vesicle morphogenesis but otic placode induction appeared normal. It was concluded that Fgf3 is not required for otic induction, though the authors left open the possibility that a parallel or redundant pathway could have partially compensated for loss of *Fgf3*. Indeed, the first direct support for redundant Fgf function came from a series of studies in zebrafish showing that Fgf3 and Fgf8 together induce the otic placode [99, 108, 120, 144]. Both genes are expressed in the hindbrain and subotic mesenchyme during appropriate stages in zebrafish development (Figure 2). Knockdown of either gene alone causes modest deficiencies in otic

development, but knockdown of both genes blocks otic induction entirely. It is now clear that such redundancy amongst Fgfs typifies otic induction in all vertebrates. In mouse, in addition to Fgf3, Fgf8 and Fgf10 are also involved in otic induction (summarized in Figure 2).

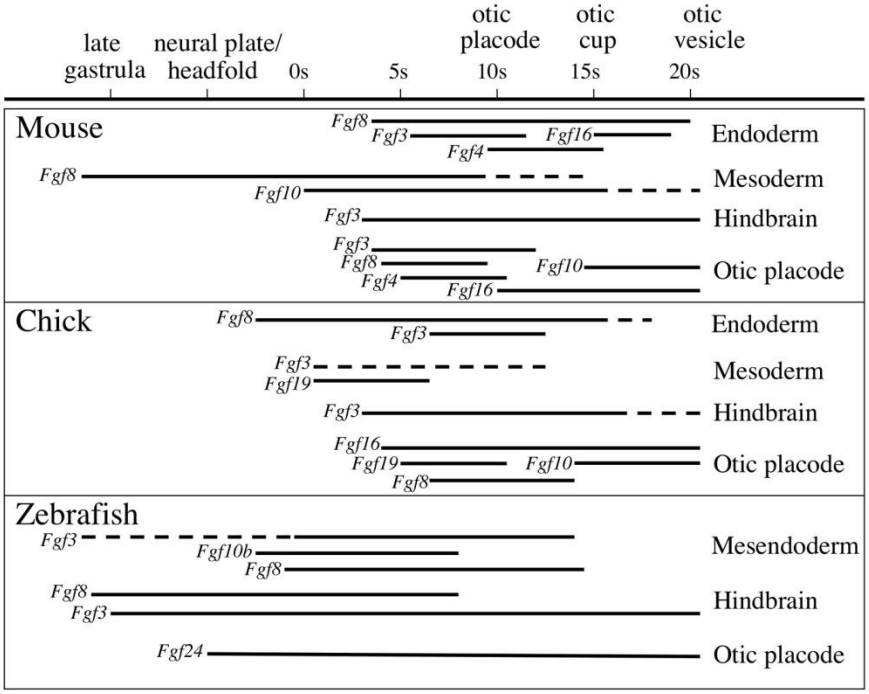

Figure 2. Expression patterns of *Fgf* genes in periotic tissues. Expression patterns are shown for mouse, chick and zebrafish. *Fgf* genes are listed along the left of corresponding expression timelines, and tissue sources are shown along the right side of the figure. Developmental stages are labeled across the top (s, somite-stage). See text for details and citations.

Fgf10 is expressed in subotic mesoderm just prior to somitogenesis stages and is later expressed weakly in the hindbrain, followed by more robust expression in the developing otic cup itself. Knockouts of *Fgf10* cause formation of a slightly reduced otic vesicle, whereas *Fgf3*$^{-/-}$*; Fgf10*$^{-/-}$ double mutants typically produce only micro-vesicles or sometimes no otic tissue at all, depending upon strains and alleles [8, 209]. *Fgf8* is expressed in cranial mesoderm from mid-gastrulation and subsequently in subotic mesoderm and pharyngeal endoderm by early somite stages. Disruption of *Fgf8* from the beginning of development leads to lethality during gastrulation, whereas a hypomorphic allele [92] or conditional knockouts that remove *Fgf8* after gastrulation [40] allows relatively normal otic development. Combining these other *Fgf8* alleles with *Fgf3* null alleles severely impairs otic induction: Otic induction is lost entirely in one background, while in another microvesicles initially form but are subsequently lost by E12. It should be noted that disruption of *Fgf3* and *Fgf8* also strongly reduces expression of *Fgf10* in subotic mesenchyme [92], explaining why *Fgf3-Fgf8* double mutants show a slightly stronger phenotype than *Fgf3-Fgf10* double mutants. In contrast to loss of Fgf function, mice mutant for two feedback inhibitors of Fgf, *Sprouty1* and *Sprouty2*, show an enlarged otic placode and vesicle [115].

Studies in chick show that Fgf3, Fgf8 and Fgf19 are all involved in otic induction (Figure 2). Moreover, these studies reveal an especially critical role for signals from subotic endoderm and mesoderm. A signaling cascade begins with *Fgf8* expression from Stage 6 in a discrete subotic domain of pharyngeal endoderm, which induces expression of *Fgf19* in subotic mesoderm [92]. Knockdown of *Fgf8* by electroporation of an siRNA vector reduces expression of mesodermal *Fgf19* and severely impairs otic induction. Localized knockdown of mesodermal *Fgf3* or *Fgf19* causes only slight impairment of otic induction whereas knockdown of both blocks production of all but a few otic cells [52]. The importance of mesodermal signals is also revealed by surgical ablation of mesoderm, which completely abolishes otic induction in chick [82].

Despite the above studies in chick, in other species it is unlikely that any single tissue bears such disproportionate responsibility for otic induction. The *Fgf8-Fgf19* signaling cascade in chick is analogous to that shown for *Fgf8* and *Fgf10* in mouse, yet the consequences of disrupting *Fgf8* are considerably milder in mouse. This is presumably because in mouse *Fgf3* and other hindbrain signals are sufficient for otic induction. In zebrafish, cranial mesendoderm expresses *fgf3, fgf8* and *fgf10b*, as well as several Bmp-antagonists that are required for normal otic development [89, 128, 131, 144; BBR, unpublished observations]. Blocking mesendoderm formation by disrupting Nodal activity delays otic induction, but small otic placodes eventually form in response to Fgf3 and Fgf8 from the hindbrain [89]. Combining loss of mesendoderm with knockdown of either *fgf3* or *fgf8* eliminates otic induction entirely, indicating that the remaining hindbrain-Fgf is no longer sufficient. Thus, the overall level of Fgf signaling may be more important than the specific source from which it is derived. Moreover, the trend amongst most vertebrates seems to be that extensive redundancy provided by multiple Fgfs emitted by multiple tissues (Figure 2) makes the process of otic induction highly robust.

Although the requirement for Fgf signaling is conserved in all vertebrates, there are conflicting data regarding the time frame during which it acts. Mesendodermal expression of *Fgf3* and *Fgf19* in chick begins to dissipate soon after the otic placode forms. Moreover, misexpression of *Fgf3* or *Fgf19* after this stage blocks expression of later markers of otic vesicle patterning. This has been interpreted as evidence that, in chick, Fgf signaling must be attenuated to permit otic development to continue [52]. However, this appears not to be the case in other vertebrates, and there are potentially conflicting data in chick as well [40]. In mouse and zebrafish, *Fgf3* continues to be expressed in the hindbrain throughout development of the otic placode and early otic vesicle [114, 122, 144]. In chick, too, *Fgf3* continues to be expressed in the hindbrain through otic vesicle stages, although expression is restricted to rhombomere boundaries during much of placodal development [113]. These data are consistent with classical tissue recombination studies showing that otic-inducing signals continue to be expressed well after the otic placode has formed [54, 57, 198, 215, 216]. Furthermore, in all vertebrates examined, various *Fgf* genes are expressed within the developing otic placode itself [Figure 2]. Mouse *Fgf4* is expressed in the pre-otic placode by 4 somite-stage and is maintained until the morphological placode forms at 9 somite-stage [210]. Chick *Fgf8* shows similar transient expression from 7-14 somite-stage [4]. In chick and mouse, *Fgf16* is expressed in the nascent otic placode by 4 somites and 10 somites, respectively, and later becomes restricted to various portions of the otic cup and vesicle [27, 210]. In mouse, chick and *Xenopus*, *Fgf10* is expressed in the otic placode by 12-14 somites and is maintained through otic vesicle stages [7, 96, 150]. In zebrafish, *fgf24* is expressed in

the pre-otic placode prior to somitogenesis and is maintained during subsequent placodal development [41]. The role of placodal *Fgf* expression has so far been tested only for zebrafish *fgf24*. Surprisingly, *fgf24* mutants and morphants display no obvious defects in otic development; instead they show a marked deficiency in development of adjacent epibranchial placodes, revealing a mechanism for serial induction of otic and epibranchial placodes through an Fgf-relay [139]. Despite the absence of otic defects following loss of *fgf24*, there is clear evidence that Fgf signaling must be maintained for normal otic development. In zebrafish, globally blocking Fgf signaling with the pharmacological inhibitor SU5402 causes loss of early otic markers when applied at 12 somite-stage, just after the placode has formed [99]. In explanted chick embryos, SU5402 blocks expression of otic markers when added at 3-6 somite-stage, whereas later addition eliminates or alters expression of various regional markers and diminishes the size of the otic cup [1]. These data, combined with Fgf expression data, suggest that in most species ongoing Fgf signaling is necessary to maintain otic fate and later to regulate the first steps in patterning and morphogenesis of the otic epithelium.

Wnt Signaling Appears to Stabilize Otic Fate

A consensus is building that canonical Wnt signaling is required for otic development after placode induction. In an early model based on experiments with chick explants [91], it was proposed that the hindbrain signal Wnt8a (formerly Wnt8c) works in combination with Fgf19 to induce the otic placode. In that study, explants of prospective otic ectoderm failed to express otic markers in response to either Fgf19 or human Wnt8a alone, but exposure to both factors induced robust expression of a host of otic markers. However, it was also noted that Wnt8a strongly induced Fgf3, which could have enhanced the effects of exogenous Fgf19. More recently, it has been shown that blocking Wnt activity in chick embryos by misexpressing *Dkk1* does not alter induction of early otic markers *Pax8* and *Pax2* [52]. However, much of the otic domain of *Pax2* is subsequently lost and later markers of otic vesicle patterning are undetectable or limited to very small domains. In contrast, expression of *Foxi2*, an epibranchial marker that is normally restricted from the otic placode, expands part way into the otic domain. In a converse approach, stimulation of the Wnt pathway by misexpressing a stabilized form of β-catenin does not affect otic induction or subsequent otic development but does strongly inhibit *Foxi2* expression as well as subsequent epibranchial development. These data support a model in which canonical Wnt signaling is not required for otic induction but instead maintains otic fate while simultaneously inhibiting epibranchial fate.

A similar model has been suggested in mouse based on the effects of using *Pax2-Cre* to conditionally knockout *β-catenin* in nascent otic tissue [136]. This manipulation causes downregulation of otic placode markers by E8.5, and the otic vesicle is reduced in size by 80% at E10.5. Additionally, expression of *Foxi2* encroaches into the otic domain. Expansion of *Foxi2* into the otic domain is also observed in *Fgf3*$^{-/-}$*; Fgf10*$^{-/-}$ double mutants, an effect attributed to the greatly reduced level of expression of *Wnt8a* in the hindbrain [191]. Conversely, using *Pax2-Cre* to misexpress stabilized *β-catenin* increases the size of the otic placode and correspondingly reduces expression of *Foxi2* and inhibits epibranchial development. Therefore, Wnt signaling is required to maintain otic fate and inhibit

epibranchial development in mouse, as it is in chick. However, only in mouse does increasing Wnt activity enlarge the otic placode. The reason for this difference is not clear.

Work in teleosts is mixed but may ultimately prove reconcilable with other model systems. In zebrafish, knocking down *wnt8* or misexpressing *dkk1* delays but does not block otic induction [145]. The delay in otic development correlates well with a delay in hindbrain expression of *fgf3* and *fgf8*. In both zebrafish and medaka, misexpression of *wnt8* or *wnt1* leads to production of enlarged and ectopic otic vesicles in the front of the head [11, 145]. In both cases, however, early Wnt activity expands the hindbrain domains of *fgf3* and *fgf8* to encompass the anterior end of the neural plate. Knocking down *fgf3* and *fgf8* blocks the ability of Wnt-misexpression to induce ectopic otic tissue in zebrafish, though this has not been tested in medaka. Together, these findings suggest that Wnt is not required for otic induction, in agreement with chick and mouse studies, though Wnt can indirectly influence the process in fish via regulation of hindbrain expression of Fgf. It has not yet been established whether manipulation of Wnt alters Fgf expression in chick or mouse.

A Role for Notch Signaling

In mouse, a number of components of the Notch pathway are expressed in the nascent otic placode, including the receptor Notch1, ligands Delta1 and Jag1, and effectors Hes1 and Hes5 [76]. Conditionally knocking out *Notch1* using *Pax2-Cre* reduces the size of the otic placode by roughly half, whereas misexpression of activated *Notch1 intracellular domain* (*NICD*) dramatically expands the domain of early otic markers. Conversely, expression of *Foxi2* expands following conditional loss of *Notch1* and contracts after misexpression of *NICD*, suggesting a fate-change like that caused by manipulating □-catenin levels. Indeed, Notch pathway genes appear to be directly activated by Wnt signaling. Furthermore, activating Notch expands the domain of Wnt signaling. These data suggest that a positive feedback loop between the Wnt and Notch pathways operates during early stages of otic placode development to maintain otic fate at the expense of epidermal/epibranchial fate. However, this function of Notch does not appear to have been conserved in zebrafish. Mutations in the gene encoding zebrafish *mind bomb*, which encodes an E3 ligase required for Delta-Notch signaling, does not detectably alter the size of the otic placode or early otic vesicle, though patterning within the otic vesicle is severely altered due to massive overproduction of sensory hair cells [58, 158]. Likewise, misexpression of *NICD* does not alter early otic development [124]. Relevant functional studies have not yet been reported in chick or *Xenopus*.

A Role for Bmp-Antagonists

In zebrafish, specification and maintenance of the otic placode requires stringent attenuation of Bmp. At least three Bmp-antagonists, Chordin (Chd), Follistatin-a (Fsta), and CV2, are secreted from cranial paraxial mesendoderm beneath the otic anlagen near the end of gastrulation [89]. As discussed above, Bmp-antagonists initially help specify the preplacodal ectoderm, but the temporal and spatial patterns of these genes suggest that the otic placode, and other posterior placodes, experience prolonged attenuation of Bmp even

after they are specified. Expression of *chd* and *fsta* begins in early gastrulation but likely does not directly influence anterior placodes because their anterior limits of expression lie close to the trigeminal placode. Expression of *chd* is lost from paraxial mesoderm soon after the onset of otic induction, but *fsta* persists in subotic mesoderm through mid-somitogenesis stages. Expression of *cv2* begins after the onset of otic induction and also persists through mid-somitogenesis. Simultaneous knockdown of *cv2*, *chd* and *fsta* results in formation of very tiny otic and trigeminal placodes. This severe deficiency does not reflect loss of preplacodal ectoderm, since preplacodal markers *dlx3b*, *eya1* and *six4.1* are still expressed, albeit at reduced levels. Moreover, anterior placodes are affected much less severely. Knocking down all three Bmp-antagonists in *fgf3*$^{-/-}$ mutants or *fgf8*$^{-/-}$ mutants blocks otic induction entirely, indicating that the remaining Fgf is no longer sufficient. Because Bmp and Fgf often act in opposition, it is possible that attenuation of Bmp is required for achieving a sufficient level of Fgf signaling. A similar role for Bmp-antagonists in otic induction has not been documented in other vertebrates. However, *Follistatin* shows a similar pattern of expression in subotic mesoderm in chick and mouse [50, 192], suggesting that it could directly influence development of the otic placode in those species.

Early Differentiation of the Otic Placode

In all vertebrates examined, the otic placode appears by 9-10 somite-stage when the otic epithelium transforms from a simple cuboidal epithelium to a thickened columnar epithelium. However, transcriptional responses to otic induction begin well before overt morphological manifestations become apparent. As described above, various preplacodal genes related to the *Dlx*, *Eya*, *Six*, *Gata*, and *Fox* gene families are initially expressed in broader domains of ectoderm and later resolve into discrete placodal primordia. In response to otic-inducing signals, expression of these genes is maintained and upregulated in the otic anlagen. In addition, new sets of otic-specific transcription factors are detected by late gastrulation through early somitogenesis stages. Chief among these are members of the *Pax2/8* and *Sox9/10* families of transcription factor genes, which have proven to play especially critical roles in early otic development. *Sox3* also appears to play an early role in diversifying otic vs. epibranchial placode identities.

Pax2/8 Genes

The earliest known marker of otic induction is *Pax8*, which in most vertebrates is expressed by late gastrulation [67, 70, 143], though the chick genome lacks *Pax8* entirely. In all species examined, the closely related *Pax2* gene is expressed in the otic anlagen slightly later, typically around the onset of somitogenesis. In zebrafish there are two *Pax2* paralogues, *pax2a* and *pax2b*, which are expressed in otic cells beginning at 3 somites and 7 somites, respectively [143]. In zebrafish, *pax8* and *pax2a/b* operate in distinct but parallel pathways: Induction of *pax8* requires both Fgf and *foxi1*, but not *dlx3b/4b*, whereas *pax2a/b* requires Fgf and *dlx3b/4b* but not *foxi1* [61, 178, 179]. It is not known whether similar parallel pathways underlie *Pax8* and *Pax2* expression in frog and mouse.

In keeping with their highly conserved structures, *Pax2* and *Pax8* provide considerable functional redundancy during otic development. In zebrafish, disruption or knockdown of *pax8* reduces the size of the otic placode by roughly half [74, 112, 139]. Knockdown of both *Pax2* paralogs, *pax2a* and *pax2b*, causes no obvious defects until otic vesicle stages, when sensory hair cells show aberrant patterning and elevated apoptosis [88, 158]. In contrast, combined knockdown of *pax8*, *pax2a* and *pax2b* leads to complete loss of otic tissue [61, 112]. Small otic placodes initially form as in *pax8* knockdown embryos, but otic tissue gradually dissipates and no otic vesicles are produced. Interestingly, otic cells do not die but persist after losing expression of placodal markers, possibly contributing to epidermis instead [112; BBR unpublished observations]. Thus, *pax2a/b* and *pax8* are together required to maintain otic fate in zebrafish. In mouse, *Pax8*$^{-/-}$ mutants show no discernable otic phenotype [119] and *Pax2*$^{-/-}$ mutants do not show defects until later stages of otic vesicle development when the cochlea fails to form properly [188]. *Pax8*$^{-/-}$*; Pax2*$^{-/-}$ double knockout mice produce small otic placodes that either fail to invaginate properly or produce vesicles much smaller than normal [20]. Chick embryos, which lack the functional redundancy normally provided by *Pax8*, appear to be more sensitive to loss of *Pax2* function than other species. Electroporation of *Pax2*-morpholino into the otic region of chick embryos causes severe morphological defects, including failure of the otic placode to invaginate [12], and expression of a subset of early otic markers is not maintained [32]. The fate of otic cells in such embryos is not known, but these findings suggest that a stringent requirement for *Pax2/8*-related functions is broadly conserved.

In chick, *Pax2* interacts genetically with another essential otic regulator, *Spalt4* [more commonly known as *Sall4*] [12]. Chick *Sall4* is initially widely expressed in the neural plate and preplacodal ectoderm. By10 somite-stage expression is lost from the hindbrain but is still strongly expressed in the nascent otic placode. *Sall4* expression is maintained in otic tissue until after the otic vesicle forms, after which it is gradually lost. Misexpression of *Sall4* can induce ectopic otic placodes, while expression of a dominant-negative form of *Sall4* causes a dramatic reduction in the size of the otic vesicle. Knockdown of *Pax2* strongly reduces expression of *Sall4*, suggesting mutual feedback between these genes is required for further placodal development. Since chick lacks *Pax8*, it is possible that *Sall4* provides a compensatory early function preceding activation of *Pax2*. It is not known whether *Sall4* function is conserved in other vertebrates, but similar patterns of *Sall4* expression are observed in zebrafish, *Xenopus* and mouse [64, 129, 201]. Furthermore, haploinsufficiency for *Sall4* causes sensorineural deafness in heterozygous mice as well as humans with Okihiro syndrome [84, 201]. It will be interesting to examine whether *Sall4* interacts with *Pax2* and *Pax8* in these other species.

Sox9/10 (SoxE) Genes

Sox9 is expressed in pre-otic cells beginning at late neurula (in *Xenopus*) or early somitogenesis stages (in mouse and zebrafish) and is maintained in all otic cells through early otic vesicle stages [13, 100, 108, 116, 166, 213]. Disruption of mouse *Sox9* causes a severe defect in which initial thickening of the otic placode occurs but all later stages of otic development are blocked [13]. Invagination fails to occur and the otic placode subsequently shrinks due to elevated apoptosis. In addition, there appears to be a defect in cellular cohesion

within the placode. High resolution electron micrographs reveal reduced cell-cell contacts, and mutants show reduced expression of Epha4 and Collagen 2a1. It is therefore possible that *Sox9* directly regulates cell adhesion and/or extracellular matrix, which are likely required for morphogenesis and cell survival. Alternatively, it is possible that cell attachments are weakened in the mutant because cells are already sick and dying. In zebrafish, there are two *sox9* paralogues, *sox9a* and *sox9b* [29]. Mutations in *sox9a* cause only mild defects in otic development, while loss of *sox9b* results in production of a small otic vesicle [108, 213]. Disruption or knockdown of both *sox9a* and *sox9b* severely impairs otic development such that the otic vesicle often fails to form, though a few residual otic cells are evident. Analysis of earlier stages has not been examined in such embryos, but it is likely that otic induction (e.g. expression of *pax8)* occurs normally because *sox9a* and *sox9b* are expressed well after *pax8*. Therefore loss of otic cells in these embryos could reflect elevated apoptosis, as seen in mouse *Sox9* mutants. In *Xenopus*, *Sox9* is coexpressed with *Pax8* during neurula stage as part of the initial response to otic inducing signals [166]. Knockdown of *Sox9* causes a correspondingly early block to development such that early otic markers including *Pax8* are eliminated. Thus, *Sox9* is required to initiate otic development in frogs but acts to maintain otic cells in mouse and zebrafish.

A putative Sox9-interacting factor, Med12, appears necessary for Sox9a/b protein function in zebrafish [152]. Related to *Drosophila Trap230/Med12*, zebrafish *med12* encodes a Mediator complex protein that activates transcription by forming a bridge between specific transcription factors and RNA polymerase II. A mutation in *med12* produces a phenotype that closely resembles *sox9a-sox9b* double mutants, including a severe deficiency of otic cells. Misexpression studies confirm that the transcriptional activities of Sox9a/b proteins require *med12* function. Disruption of *med12* impairs even the earliest stages of otic development, such that *pax8* expression is lost entirely. However, this likely reflects a Sox9-independent function as *sox9a/b* are not expressed in the ear until later stages. Similarly, *pax8* expression is also lost from the developing pronephros, which does not detectably express *sox9a* or *sox9b* at any stage. It therefore appears some other early acting transcription factor requires *med12* in order to activate *pax8* expression.

Sox10 is closely related to *Sox9*, but is expressed slightly later in the otic placode beginning at around 10 somites in mouse and 15 somites in zebrafish [21, 45]. Loss of mouse *Sox10* does not alter formation of the otic placode or vesicle but does lead to hypoplasia of the cochlear duct due to elevated apoptosis [21]. Zebrafish *sox10* mutants show a more severe phenotype, with elevated apoptosis throughout the otic epithelium and production of a small malformed otic vesicle [45]. It is likely that *Sox10* is partially redundant with *Sox9* during otic development. Indeed, knockdown of either *sox9a* or *sox9b* in zebrafish strongly enhances the *sox10* mutant phenotype [45]. Disrupting all three genes causes a more severe phenotype, with loss of all vestiges of the otic placode in some cases. Redundancy has not been examined in mouse through analysis of compound mutants. However, *Sox9*$^{-/-}$ mutants do not express *Sox10* [13] and therefore the severe otic-arrest phenotype represents the functional equivalent of a double mutant phenotype.

Sox3

Though *Sox3* is not strictly required for otic induction, it deserves mention here because its expression and function reveal a close kinship in the regulation of otic and epibranchial placodes. In mouse, chick and zebrafish, *Sox3* is expressed in nonneural ectoderm in a region that initially covers both the otic and epibranchial anlage [131, 154, 184, 208]. As development continues *Sox3* expression is gradually lost from otic cells but is maintained and upregulated in epibranchial placodes. In zebrafish, *sox3* and *pax8* are initially co-induced by the combined activities of Foxi1 and Fgf [184]. The domain of *sox3* then expands outward while it is gradually excluded from the *pax8*-positive otic domain, a transition that depends on otic expression of *fgf24* [139]. In chick, too, Fgf is required to co-induce *Sox3* and *Pax2* in overlapping otic/epibranchial domains [1]. However, chick *Sox3* continues to be expressed at low levels in the anterior otic placode and is maintained there until early otic vesicle stages. This domain appears to mark early development of the neurogenic domain of the otic epithelium. No comparable maintenance in the otic placode/vesicle has been detected in mouse [208], and there are conflicting reports in *Xenopus* [163, 172]. In any case, two non-exclusive roles can be envisioned for *Sox3*. First, it is possible that *Sox3* plays a common early role in otic and epibranchial epithelia by specifying neurogenic potential [1]. Second, shifting the balance of *Sox3* and *Pax2/8* expression could diversify placode-identity, with low *Sox3*:high *Pax2/8* specifying otic identity and high *Sox3*:low *Pax2/8* specifying epibranchial identity. Several data support this scenario. Disruption of *Sox3* in mouse and zebrafish severely impairs epibranchial development but has much less effect on otic development [38, 139, 161]. Though otic patterning has not been examined in detail in mouse *Sox3*$^{-/-}$ mutant mice, the otic vesicle appears relatively normal [161], whereas in *sox3*-depleted zebrafish embryos the otic vesicle is variably [but usually mildly] reduced in size [139, 161]. Conversely, knockdown of all *Pax2/8* function in zebrafish causes loss of all otic tissue whereas epibranchial ganglia still form, though significantly reduced in size [61, 112, 139]. Clearly more detailed studies are needed to understand how *Sox3* affects otic placode development.

Early Morphogenesis of the Otic Placode

Cytoskeletal Remodeling

In addition to inducing transcription of new sets of genes during otic induction, Fgf signaling also directly affects non-nuclear processes that are vital for placodal morphogenesis. This was recently highlighted in chick wherein Fgf signaling triggers invagination of the otic placode by regulating the redistribution of cytoskeletal F-actin [165]. Explants of otic ectoderm taken at 10 somite-stage do not invaginate in vitro unless co-cultured with Fgf-soaked beads. Moreover, the invagination response requires that Fgf beads be presented to the basal surface of the epithelium. F-actin, which is initially localized to both the apical and basal poles of otic cells, is subsequently lost basally and enriched apically. This presumably facilitates apical constriction to provide the motive force required for reshaping the otic epithelium into a cup. Interestingly, the requirement for Fgf is mediated through activation of

Phospho-lipase Cγ (PLCγ), as shown by the ability to block cytoskeletal remodeling by treatment with the PLCγ-inhibitor U73122. In contrast, blocking the MAPK pathway with a MEK1/2 inhibitor has little effect. PLCγ apparently activates Myosin Light Chain Kinase, which in turn phosphorylates and activates Myosin light chain II, which then triggers depolymerization of F-actin. Though Fgf initiates the process, invagination becomes Fgf-independent by 13 somite-stage when the initial indentation first becomes visible. This raises the question of how invagination is regulated and maintained at later stages to complete formation of a closed vesicle. Whether a similar mechanism operates in mouse remains to be tested. It will also be interesting to examine whether cytoskeletal remodeling underlying cavitation of the fish otic placode also depends on Fgf signaling.

Growth of the Otic Field

Detailed analysis of various early otic markers (e.g. *Sox9* in *Xenopus* and mouse, *dlx3b*, *pax8* and *pax2a* in zebrafish) shows that the otic expression begins in a limited group of early differentiating cells and then spreads within a few hours to encompass the rest of the otic field [6, 13, 144, 166]. While Fgf is often assumed to control proliferative expansion of the otic placode, the modest mitotic index observed in the otic placode suggests that cell division cannot fully account for rapid initial expansion of the early placode. Moreover, in zebrafish *harpy/emi1* mutants, which are blocked in mitosis soon after the onset of gastrulation, the number of Pax2+ otic cells nearly doubles between 12 hpf and 14 hpf (6-10 somites), similar to wild-type embryos [159]. This indicates that new otic cells must continue to be induced over a protracted period independent of cell division. It is possible that the time needed to respond to otic inducing factors varies directly with distance from the signaling source. Additionally, since otic cells begin to express their own Fgf genes soon after induction, they could potentially spread the range of inductive signaling, thereby recruiting outlying neighbors.

Convergence and Cell Adhesion

Despite the increasing number of cells in the otic field, the area of the field temporarily decreases as the placode becomes morphologically visible. This is probably due in part to transition from a simple cuboidal epithelium to a columnar epithelium. In zebrafish, there is also a marked convergence of the initially broad field of pre-otic cells through directed cell migration to form a more compact cluster in the nascent placode. Various cell adhesion molecules are likely involved in these aspects of morphogenesis. In zebrafish, *integrin-5* (*itga5*) is initially expressed in a *dlx3b*-like pattern in the preplacodal ectoderm and becomes locally restricted to various placodes including the otic/epibranchial region by the 12-somite stage [35]. Disruption of *itga5* reduces the size of the otic placode. Cell-motion analysis reveals that cell migration in *itga5* mutants is chaotic and undirected, resulting in delayed convergence and impaired recruitment. In other words, fewer prospective otic cells are drawn into range of localized inductive signals, limiting the number of otic cells available to build a placode. Additionally, loss of Itga5-dependent signaling leads to a dramatic increase in

apoptosis in the otic/epibranchial field, further diminishing the size of the otic placode. The cell death defect can be rescued by over-expressing Fgf, suggesting that Itga5 normally acts to augment Fgf through activation of shared pathways such as MAPK and PI3K [16]. Chick *Integrin-4* is expressed in the otic placode [111], and blocking Integrin and Laminin function with antibodies prevents otic invagination [197]. In *Xenopus*, *Paraxial protocadherin* (*Papc)* is induced in the developing otic placode and is maintained through invagination. Misexpression of dominant-negative forms of *Papc*, which likely interfere with multiple other homologs in addition to Papc, blocks expression of *Sox9* and a later marker of otic vesicle patterning, *Tbx2* [71]. Injection of morpholinos specifically targeting Papc does not block formation of the otic placode, although subsequent morphogenesis of the otic vesicle is impaired due to improper cell alignment within the otic epithelium [77]. Papc appears to mediate its effects through RhoA-dependent signaling and interactions with the Wnt5a pathway. In no other vertebrate has otic expression of *Papc* been detected, though otic expression of related genes cannot be excluded. These studies suggest diverse roles for cell adhesion in morphogenesis as well as differentiation, but the examples are too few to draw firm conclusions about detailed mechanisms or evolutionary trends. This is an area that clearly deserves greater attention.

Pattern Formation in the Otic Placode

The elaborate program of epithelial folding, regional growth and cell type diversification in the inner ear is set in motion by molecular asymmetries that can already be detected in the early otic placode. This entails shifting expression patterns in genes initially expressed throughout the placode, as well as induction of a rapid succession of new regionally expressed genes. Together these spatially restricted genes subdivide the otic epithelium into distinct territories with unique potentials and fates. Initial asymmetries in gene expression are induced by signals from surrounding tissues. In addition, cross-regulation amongst regionally expressed genes reinforces and refines otic patterning. Figure 3 illustrates the spatial distributions of the earliest genes showing asymmetric expression within the developing otic placode and otic cup. Below is a summary of data concerning these early gene functions and the signals that activate them.

The Sensory/Neural Domain

In mouse and chick, the first region to be specified in the ear is the domain from which neuroblasts of the stato-acoustic ganglion (SAG) are derived. Corresponding genes are first detected in the anterior half of the nascent otic placode in chick [1, 7, 33, 36], or in the ventral epithelium of the incipient otic cup in mouse [76, 127, 151, 194] [Figure 3]. Included are proneural genes *Ngn1* and *NeuroD* and genes in the Notch pathway such as *Delta1*, *Lfng*, and *Hes1/5/6*. These are followed shortly by co-expression of *Fgf10* in the neurogenic domain [7, 209], and *Jag1*/*Ser1*, *Bmp4* and *Tbx1* in the complementary nonneurogenic epithelium [1, 33, 76, 127, 151]. In chick, *Sox3* and *Fgf8* are also transiently expressed in the anterior domain and appear to lie upstream of the neurogenic genes [1]. Misexpression of *Sox3* causes

expansion of *Delta1* expression into the posterior otic placode, though very few *NeuroD*-positive SAG precursors are detected outside the normal anterior domain. Such embryos also accumulate a large number of apoptotic cells in the posterior placode, possibly indicating that SAG precursors are specified but are not properly maintained. Misexpression of *Fgf8* causes expansion of both *Sox3* and *NeuroD* into the posterior placode, indicating that Fgf8 can activate a broader developmental program that includes *Sox3* and stably specifies ectopic SAG precursors.

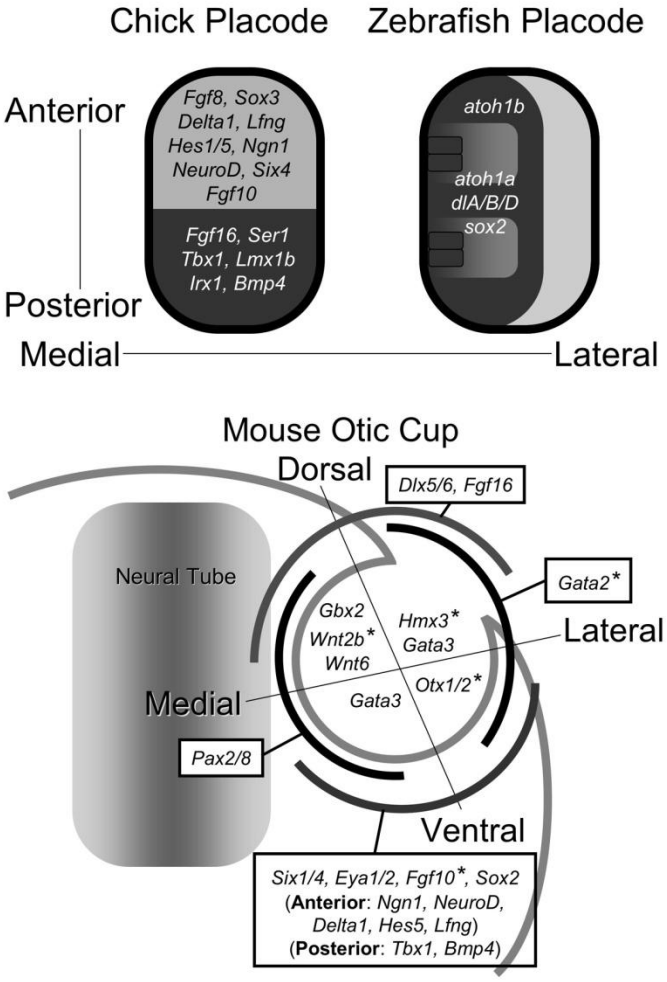

Figure 3. Localized gene expression in the otic placode/otic cup. The earliest molecular asymmetries involve establishment of the neurogenic domains in chick and mouse, and the prosensory domains in zebrafish. Other axial markers also show asymmetric expression in the mouse otic cup. See text for details and citations. Asterisks indicate genes that are uniformly expressed during placodal stages but become localized soon after formation of the otic vesicle; they are included here to facilitate discussion of mechanisms of pattern formation.

In zebrafish, the earliest clusters of hair cells form well before the SAG neurogenic domain. The pro-sensory bHLH gene *atoh1b* is first detected in the medial half of the pre-otic placode before the onset of somitogenesis [124] (Figure 3). Indeed, *atoh1b* is seen within 45

minutes of induction of *pax8*, constituting the earliest known asymmetry in otic development. The initially broad domain of *aoth1b* eventually resolves into two patches of prospective hair cells. As the morphological placode appears, *atoh1b*-positive cells begin to express a closely related homolog, *atoh1a*. This results in stable specification of tether cells, which differentiate into the first hair cells in the utricular and saccular maculae [157]. Tether cells also begin to express *deltaA, deltaB* and *deltaD* at the early placodal stage. Medial expression of *sox2* is also detected at this time and marks prospective hair cells and support cells [125]. Induction and maintenance of *atoh1a/b* requires ongoing Fgf signaling whereas expression of *deltaA/B/D* and *sox2* requires Atoh1a/b activity. Induction of the SAG neurogenic domain begins as the nascent otic vesicle forms and is also dependent on Fgf signaling [9; BBR, unpublished observations].

Cross-Regulation of Axial Patterning Genes

Despite differences between species regarding which genes show the first asymmetries in the ear, once the otic vesicle forms there is striking conservation across species in expression of many of the axial markers identified in mouse (Figure 3). For example, *Dlx3/5*, *Pax2*, *Gbx2*, *Eya1*, *Six1*, *Gata3*, *Otx1/2*, and *Tbx1* all show similar patterns in mouse, chick and zebrafish [6, 7, 17, 22, 23, 72, 85, 86, 102, 103,105, 126, 149, 151, 167, 175, 183, 210, 214, 221]. This suggests that many underlying developmental mechanisms have been conserved during evolution of the inner ear. Regionally expressed patterning genes often help reinforce positional identities by positively regulating other coexpressed genes, and in some cases by repressing genes normally found in other domains. Expression patterns and mutant phenotypes of a few key examples from mouse are summarized below and in Figure 3.

Tbx1 is expressed very early in the posterior half of the otic placode in a domain that is complementary to the neurogenic genes described above [151]. In *Tbx1* mutants, anterior neurogenic markers expand posteriorly whereas posterior expression of *Otx1* and *Bmp4* is lost.

Eya1, ***Six1***, and ***Gata3***, which initially mark all otic cells, shift to more restricted expression patterns during the otic cup stage. *Eya1* and *Six1* become restricted to cells that will make up the ventral compartment of the otic vesicle [138, 219, 221]. Disruption of either of these genes leads to reduction or loss of expression of other ventral markers and ventral expansion of dorsal markers. *Gata3* becomes restricted initially to ventromedial cells and later also marks dorsolateral cells [104]. In *Gata3*$^{-/-}$ mutants, ventral expression of *Fgf10* is blocked or severely reduced and expression of *Wnt2b*, which normally marks the dorsal otic vesicle, is not induced [43, 104]. This does not reflect a global failure of patterning as various other dorsal and ventral markers are unaffected.

Gbx2 is an example of a regionally expressed gene that acts primarily within its domain of expression. *Gbx2* is expressed in dorsomedial cells by the otic cup stage [210]. This domain forms the endolymphatic duct, one of the earliest fates to be specified in the ear. *Gbx2*$^{-/-}$ mutants produce no endolymphatic duct and dorsomedial expression of *Dlx5* is lost by the early otocyst stage [106]. Dorsolateral expression of *Dlx5* and *Hmx3* is not altered, nor is ventromedial expression of *Lfng*. Thus *Gbx2* is vital for proper development of the dorsomedial domain but does not function by repressing markers of abutting domains. On the other hand, the ventrolateral domain of *Otx2* expands medially in some specimens, though

this is highly variable. All of these examples provide a glimpse of the intricacy of the genetic networks controlling regional fates and the rapidity with which they are established once the otic placode has formed.

Signals Controlling Positional Identities

Tissue recombination studies confirm that interactions with the hindbrain and mesoderm are required for initiating the first molecular asymmetries in the ear. All of the specific signals identified to date are associated with the hindbrain, as summarized in Table 3.

Table 3. Signals required for regulating axes of the otic vesicle

	Signal	**Mouse/chick genes**	**Zebrafish genes**	**References**
DV axis	Fgf3/10	(+) *Fgf10* (-) *NeuroD, Lfng*	None identified	65, 195
	Shh	(+) *NeuroD, Ngn1, Otx1/2* (-) *Dlx5/6*	(+) *eya1* (-) *dlx3b*	59, 60, 155
	Wnt1/3a	(+) *Dlx5/6, Gbx2* (-) *Eya1, Six1, Tbx1*	(+) *dlx3b* (-) *eya1*	53, 60, 97, 156
ML axis	Fgf3/8	(+) *Gbx2, Wnt2b* (-) *Hmx3*	(+) *atoh1a/b,*	65, 124, 185
	Shh	(+) Pax2	(+) *pax2a* (-) *tbx1*	59, 155
	Wnt1/3a	(-) *Pax2, Ngn1*	None identified	97, 156
AP axis	Fgf3	*Bmp4* (post. crista) *Fgf10* (post. crista)	(+) *hmx3, pax5* (-) *pou23*	58, 65, 87, 97
	Shh	None identified	(+) *fgf8, otx2* (-) *hmx3*	58-60, 155
	Other	NeuroD	Not comparable	195

DV Axis

Regulation of the DV axis of the ear is the best characterized even though the DV axis is specified after the AP and ML axes. Wnt1 and Wnt3a from the roof plate of the hindbrain are necessary and sufficient to activate expression of dorsal otic markers *Dlx5/6* and *Gbx2* [156]. Wnt signaling also restricts expression of *Tbx1*, *Eya1* and *Six1* from the dorsal compartment [53]. Shh from the floor plate and notochord is required for proper ventral expression of *Otx1/2*, *Ngn1*, *NeuroD* and *Lfng*, as well as repression of *Dlx5* [155]. However, other signals must also be involved in DV patterning because dorsal markers *Hmx3* and *Wnt2b* are not responsive to changes in Wnt or Shh signaling. It has been suggested that Bmp from the roof

plate might regulate these and other markers, though there is currently no experimental support for this hypothesis. Finally, Fgf3 and Fgf10 from the hindbrain are required to restrict neurogenic markers *NeuroD*, *Delta1*, *Lfng*, and *Fgf10* from the dorsal epithelium [65, 195].

ML Axis

This axis in mouse also appears to rely on Fgf, Shh, and Wnt. Fgf is required for maintenance of dorsomedial markers *Gbx2* and *Wnt2b*, as well as development of the endolymphatic duct [30, 65]. Fgf3 from the hindbrain is also required to block medial expression of the dorsolateral marker *Hmx3*. Shh and Wnt appear to work together to regulate ML patterning. Shh is required for medial expression of *Pax2*, and ectopic Shh signaling is sufficient to activate *Pax2* expression in the lateral wall [155]. Lateral expansion of *Pax2* expression is also seen when the Wnt-expressing roof plate is excised, consistent with a model in which Wnt functions in part by restricting the range of Shh signaling [156]. Roof plate excision also results in lateral expansion of *Ngn1* [another Shh-responsive gene]. In contrast, treating roof plate-excised embryos with LiCl to globally activate the canonical Wnt pathway partially reverses ectopic expression of *Pax2* and *Ngn1* without eliminating their normal domains of expression. Whether Wnt can also affect ML patterning in a Shh-independent manner remains unknown.

AP Axis

In contrast to the other axes, there is still no clear notion of how the AP axis of the otic placode is regulated in mouse and chick. An attractive hypothesis has been that various signals expressed in different rhombomeres of the hindbrain are good candidates for regulating the AP axis of the ear, but supportive data remain scanty. Despite the asymmetric expression of *Fgf3* along the AP axis of the hindbrain, *Fgf3*$^{-/-}$ mutants show only minor AP defects in the otic vesicle limited to loss of the posterior crista and its markers *Fgf10* and *Bmp4* [65; Table 2]. Disruption of the hindbrain segmentation gene *Kreisler* (*MafB)* severely perturbs DV and ML patterning in the otic vesicle, whereas effects on the AP axis of the ear are much subtler. For example, *Kreisler* mutants show strong dorsal expansion of neurogenic genes, along with loss of some dorsal markers [30, 195]. There is also some ectopic expression of neurogenic genes in posterior otic tissue, but this is quite sparse. Moreover, *Lfng* does not expand posteriorly and expression of the posterior marker *Tbx1* is not altered, indicating that most aspects of AP patterning remain normal in *Kreisler* mutants. Loss of *Kreisler* severely reduces expression of *Fgf3* and *Fgf10* in the hindbrain, which likely accounts for some of the patterning defects in the ear [195]. Indeed, introducing transgenes into *Kreisler* mutants to misexpress either *Fgf3* or *Fgf10* in the hindbrain partially reverses the dorsal expansion of neurogenic genes. However, these transgenes do not eliminate ectopic posterior expression of *NeuroD*, suggesting that *Kreisler* affects some other, as yet unknown, hindbrain signal that influences neurogenesis in the ear. However, this signal apparently does not regulate most other AP patterning genes. Finally, rotating the chick hindbrain to reverse its AP polarity does not alter AP patterning in the ear [17]. By the process of elimination these findings implicate mesodermal or endodermal signals as the principal regulators of the

AP axis, though direct experimental support is lacking and candidate signals remain to be identified.

Comparative Studies

In zebrafish, signaling interactions that regulate the axes of the otic placode show some similarities to chick and mouse, though there are also a number of notable differences (Table 3). Wnt signaling appears to play a conserved role in DV patterning. Disruption of the hindbrain patterning gene *vhnf1* strongly reduces expression of *wnt1* and *wnt3a* in the hindbrain and leads to loss of the dorsal otic marker *dlx3b* and expansion of the ventral otic marker *eya1* [97]. Shh plays a conserved role in regulating the DV and ML axis in the zebrafish inner ear, with positive regulation of medial *pax2a* and ventral *eya1* and repression of dorsal *dlx3b* and lateral *tbx1* [60]. Fgf's role in ML patterning is also conserved, as hindbrain expression of *fgf3/8* is required for expression of *atoh1a/b* in the medial otic placode [124]. Moreover, Fgf signaling appears to regulate other genes that influence the zone of sensory competence. Misexpression of *atoh1a* leads to expansion of sensory epithelia to cover the entire medial wall of the otic vesicle but is not sufficient to stimulate hair cell production in the lateral wall. However, co-misexpression of *atoh1a* with *fgf8* stimulates much more widespread hair cell production, including the lateral wall [185]. Another function of hindbrain Fgf in zebrafish is to regulate the AP axis of the ear, in marked contrast to mouse and chick. Zebrafish *fgf3* is normally limited to rhombomere 4 of the hindbrain, which lies adjacent to the anterior end of the otic placode and vesicle. Disruption of hindbrain patterning genes *vhnf1* or *valentino/kreisler* leads to posterior expansion of *fgf3* in the hindbrain. There is a corresponding posterior expansion of anterior otic markers *pax5* and *hmx3* and loss of posterior markers *pou23* and *follistatin-a* [58, 87, 97]. Another difference between zebrafish and amniotes is that Shh strongly affects AP patterning of the inner ear. Loss of Shh function causes mirror image duplications of anterior markers such as *hmx3* and loss of posterior markers *otx2* and *follistatin-a* [58, 59]. Blocking Hh signaling also causes anterior duplications of the ear in Xenopus [199], suggesting that this function may reflect a fundamental divergence between amniotes and anamniotes.

Apparent differences between zebrafish and mouse are currently difficult to interpret because the list of axial regulators is clearly incomplete and functional interactions are highly complex. For example, the role of Shh in regulating the AP axis in fish and frog was initially difficult to explain because this ligand is not differentially expressed along the AP axis of the midline. However, a recent study in zebrafish [58] showed that Shh interacts with localized expression of Fgf3 to establish the AP axis. It will be important to examine whether a similar mechanism operates in mouse, especially since the primary determinant(s) of AP patterning in the mouse ear remain to be identified. Ultimately, such signaling mechanisms may prove highly conserved. This would offer a simple explanation for the broad conservation of so many regional markers within the otic vesicle. Alternatively, if signaling mechanisms have diverged significantly, it will likely prove challenging to deduce how promoter/enhancer regions of multiple target genes have coevolved to maintain ancestral expression patterns in the otic vesicle. In either case, it is clear that there are fundamental gaps in our understanding of axial patterning in the ear, and comparative studies are likely to remain a fruitful approach.

Conclusion

Mechanisms of otic placode development are now understood in considerable molecular detail. Fgf, acting in concert with Bmp-antagonists and Wnt antagonists, first establishes a broad domain of preplacodal ectoderm expressing *Eya1/2*, *Six1/2/4* and *Dlx3/4/5/6* genes. *Gata3*, *Foxi1/2/3* and *Foxg1* may also regulate preplacodal development. Subsequent upregulation of Fgf expression in the hindbrain and subotic mesendoderm induces early otic genes *Pax2/8* and *Sox9/10* near the posterior limits of the preplacodal ectoderm. These genes initiate otic differentiation and actively maintain otic fate. Fgf also triggers non-transcriptional responses, including reorganization of cytoskeletal actin, controlling early morphogenesis of the otic placode. Wnt signaling also appears necessary to maintain otic development. The first molecular asymmetries in the nascent otic placode involve specification of the neurogenic domain (in mouse and chick) or the prosensory domain (in zebrafish). These early domains express *Ngn1*, *NeuroD* and *Sox3* or *atoh1a/b* and *sox2*, respectively, which orchestrate subsequent differentiation of neuroblasts and sensory epithelia. Regional expression of other genes becomes evident in the late placode/early vesicle, setting the stage for differential growth and development of the various parts of the inner ear. Wnt1/3, Shh and Fgf3/10 from the hindbrain help establish the main axes of the otic vesicle. The extrinsic signals controlling the AP axis of the ear have not yet been identified in mouse or chick, but they likely come from mesendodermal tissues since interactions with the hindbrain have little effect on this axis. At each stage of otic development, there are complex interactions between the key regulatory factors, and many additional interacting factors have been identified in recent years.

Beyond the immediate goal of understanding the origins of the inner ear, continuing studies of otic placode induction and development are likely to provide two broader benefits. First, many of the early acting otic regulatory genes are associated with known genetic defects leading to human deafness. Included are preplacodal genes *Eya1*, *Eya4*, *Six1* and *Gata3*, otic induction genes *Fgf3*, *Pax2*, *Sox10*, *Sall4* and *Med12*, and placodal patterning genes *Sox2* and *Tbx1* [59, 84, 148, 160, 164, 168, 180, 186, 193, 203, 212]. In most cases these genes act during multiple stages of otic development so it is not clear how they impact the etiology of the associated human diseases. Genetic studies in mouse offer a powerful means of resolving many such disease mechanisms. Second, otic placode studies are now showing a level of sophistication approaching that of classical "model tissues" such as the vertebrate limb bud and the *Drosophila* eye. As such the otic placode can be seen as a general model for investigating how molecular and cellular processes interact to govern differential growth, morphogenesis, pattern formation, differentiation, survival and regeneration. In this regard comparative studies will be tremendously important, and mouse studies will continue to play a vital role.

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