THE ROLE OF FGF AND ITS DOWNSTREAM EFFECTORS IN OTIC AND EPIBRANCHIAL DEVELOPMENT IN ZEBRAFISH

A Dissertation

by

MAHESH PADANAD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Genetics

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Approved by:

Chair of Committee,	Bruce B. Riley
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	Vladislav Panin
Interdisciplinary Faculty Chair,	Craig Coates

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ABSTRACT

The Role of Fgf and Its Downstream Effectors in Otic and Epibranchial Development in Zebrafish. (August 2011)

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In vertebrates, the otic placode forms inner ear and epibranchial placodes produce sensory ganglia within branchial clefts. Fibroblast growth factor (FGF) family of protein ligands from the surrounding tissues are responsible for otic and epibranchial placode induction. Members of *pax2/5/8* family of transcription factors function as mediators during otic induction. To understand the temporal and spatial requirements of Fgf and their interaction with *pax2/8* for otic induction, we used heat shock inducible transgenic lines of zebrafish to misexpress *fgf3/8* and *pax2a/8* under the control of *hsp70* promoter. Loss of function studies were done to examine the functions of *pax2/8* genes in regulating otic and epibranchial development.

We show that global transient activation of *hs:fgf3* or *hs:fgf8* at mid-late gastrula stages (7-8 hpf) severely impairs otic induction, in part by disrupting formation of the principal signaling centers in the hindbrain. Additionally, mosaic studies show that high-level misexpression blocks otic fate cell-autonomously, whereas low to moderate levels promote otic development. At later stages high-level Fgf misexpression, both

globally and locally does not inhibit otic fate, but rather causes a dramatic expansion of endogenous otic domains. Misexpression of *hs:pax2a* or *hs:pax8* also expands endogenous otic domains but is not sufficient to bypass the requirement for Fgf signaling. Co-misexpression of Fgf with *pax2a* or *pax8* leads to production of ectopic otic tissue in a broad range of cranial ectoderm. These data show that changes in timing, distribution and level of Fgf signaling and its downstream effectors influences otic induction.

We show that otic and epibranchial placodes are induced at different times and by distinct mechanisms. Initially, Fgf from surrounding tissues induces otic expression of *pax8* and *sox3*, which cooperate synergistically to establish otic fate. Subsequently, *pax8* along with *pax2a/pax2b* downregulate *foxi1* expression in otic cells, which is necessary for further otic development. Additionally, *pax2/8* activate otic expression of *fgf24*, which induces epibranchial expression of *sox3*. Blocking functions of *fgf24* or *sox3* causes severe epibranchial deficiencies but has little effect on otic development. These results support the model whereby the otic placode forms first and induces epibranchial placodes through *pax2/8*-dependent Fgf24 signaling.

DEDICATION

I dedicate this dissertation and research work to my wife, *Roopa*, for her unconditional love, endless support and for being a constant source of strength, inspiration and motivation throughout this adventurous journey.

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Finally, I am grateful to almighty God for blessing me with precious family members, friends, great health, opportunities, strengths to overcome difficulties and providing eternal love.

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CHAPTER I

INTRODUCTION

PREPLACODAL ECTODERM AND CRANIAL PLACODES

During gastrulation, the ectoderm is subdivided into three distinct domains; the dorsal neurectoderm, the ventral epidermal ectoderm and the preplacodal ectoderm (Fig. 1). Preplacodal ectoderm forms as a contiguous band of multipotent progenitor cells in the head region along the neural-nonneural interface. Preplacodal ectoderm is marked by expression of a characteristic set of genes. Most notably, transcription factors belonging to the *Eya* (*eyes absent*), *Six* (*sine oculis*), *Dlx* (*distalless*) gene families are expressed throughout the preplacodal ectoderm by the end of gastrulation (Baker and Bronner-Fraser, 2001). The important signals responsible for preplacodal ectoderm induction include Fgfs, Bmp- antagonists and Wnt-antagonists from the dorsal tissue of the neural plate (Ahrens and Schlosser, 2005; Litsiou et al., 2005, Kwon et al., 2010). In zebrafish, the transcription factors Foxi1, Gata3, Tfap2a and Tfap2b function as partially redundant competence factors required for preplacodal development (Kwon et al., 2010).

This dissertation follows the style and format of Developmental Biology.

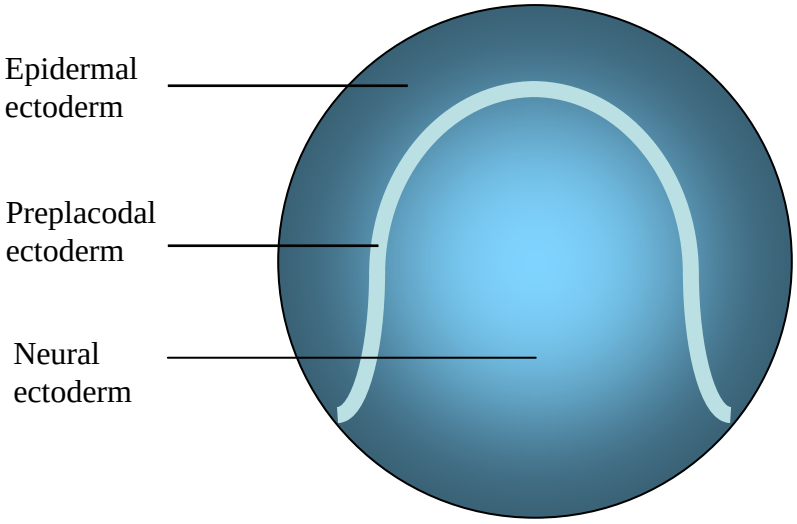


Figure 1. Cartoon showing head ectoderm of vertebrate embryo at late gastrula stage. The three distinct ectodermal domains are indicated. Image shows dorsal view with anterior to the top.

In vertebrate embryos, cranial placodes comprise a series of columnar epithelial thickenings that are formed in the head around the anterior neural plate. These placodes contribute to paired sensory structures of the head (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser, 2006). All placodes are generated from preplacodal ectoderm in response to different regional signals (Streit, 2007) (Fig. 2). Nasal placodes produce the sensory apparatus of the olfactory epithelium. The pituitary placode give rise to parts of pituitary gland. The lens placode forms the lens of the eye. The trigeminal placode produces sensory neurons of cranial ganglion V, which includes receptors for heat, cold, pain and tactile stimulation throughout the facial region. The otic placode, which gives rise to the inner ear and its associated statoacoustic ganglion (cranial ganglion VIII), has been the most characterized of all the cranial placodes.

Epibranchial placodes form a series of sensory ganglia associated with the mouth and throat, including the facial (cranial ganglion VII), glossopharyngeal (cranial ganglion IX) and vagal ganglia (cranial ganglion X). Development of the otic placode and epibranchial placodes is the primary focus of this dissertation.

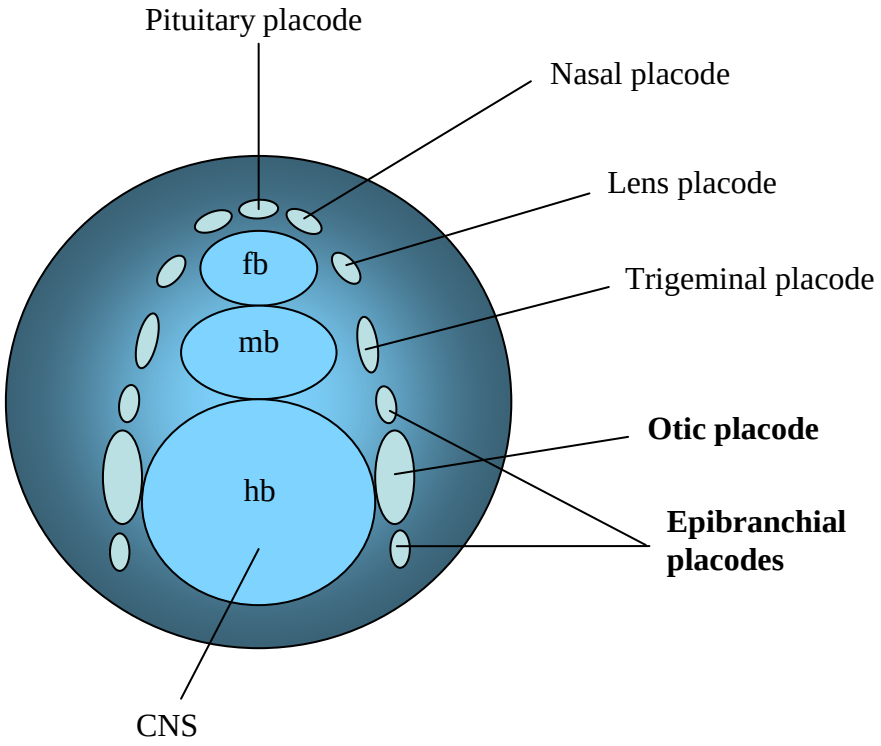


Figure 2. Schematic representation of cranial placodes. The individual cranial placodes derived from preplacodal ectoderm are indicated. fb; forebrain, mb; midbrain, hb; hindbrain, CNS; central nervous system. Cartoon shows dorsal view with anterior to the top.

INNER EAR ANATOMY

The vertebrate inner ear is a complex sensory organ with several interconnected chambers responsible for vestibular and auditory function (Haddon and Lewis, 1996; Torres and Giraldez, 1998; Riley and Phillips, 2003). The zebrafish inner ear has 6 chambers in total (Fig. 3). The utricle and three semicircular canals are responsible for vestibular function. The structure and function of these four chambers are highly conserved amongst vertebrates (Fig. 3). The posterior two chambers, the saccule and lagena provide auditory function in zebrafish but the saccule provides vestibular role in mammals and birds. The lagena is absent in mammals and its function is unknown in birds. In contrast, the cochlea, which is absent in zebrafish, is the primary endorgan for auditory function in mammals and birds (Reviewed by Whitfield et al., 2002; Riley and Philips, 2003).

Each chamber of the inner ear has a sensory epithelium comprising hair cells and support cells. The sensory epithelia of the utricle, saccule and lagena are referred to as maculae whereas sensory epithelia of semicircular canals are called cristae. The maculae of the inner ear are associated with otoliths whereas cristae lack otoliths. Otoliths are dense crystals of calcium carbonate which transmit accelerational forces (like gravity or inertia) and sound vibrations to hair cell cilia, thereby facilitating vestibular and auditory functions respectively. Hair cells act as mechanosensory transducers that are stimulated by lateral deflection of ciliary bundles responsible for hearing and balance. Support cells are thought to perform dual roles; 1) play a role in

hair cell maintenance by providing trophic factors; 2) acts as progenitor cells mediating hair cell regeneration. The neurons of the statoacoustic ganglion (SAG) (VIII cranial ganglia) transduce the signals from hair cells to the hindbrain. SAG neurons are derived from neuroblasts that originate and delaminate from the otic vesicle (Haddon and Lewis, 1996). SAG neurons are bipolar neurons that innervate all sensory patches of the inner ear and corresponding nuclei in the hindbrain.

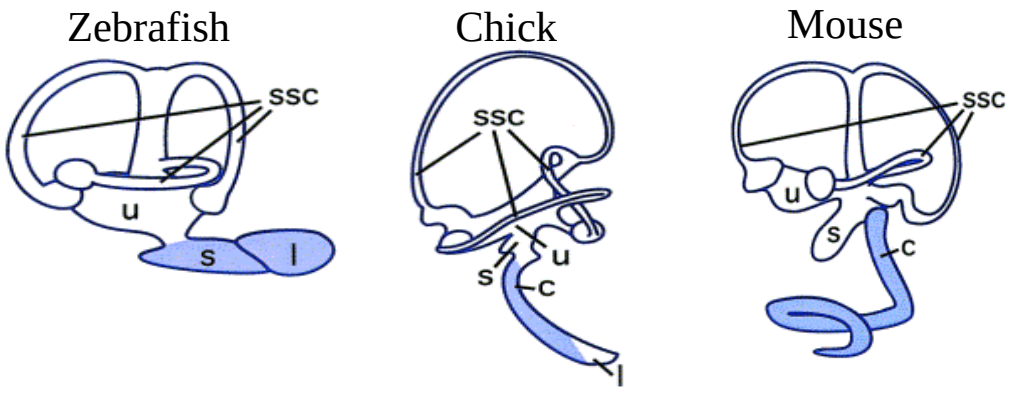


Figure 3. General structures of the vertebrate inner ears. Chambers shaded in blue color are auditory chambers; all other chambers are vestibular chambers.
ssc; semicircular canals, u; utricle, s; saccule, l; lagena, c; cochlea. (Adapted from Riley and Phillips, 2003).

INNER EAR DEVELOPMENT

In all vertebrates, the inner ear forms adjacent to the hindbrain from a transient thickening in the ectoderm known as the otic placode. The otic placode is morphologically visible by 10 somite stage (14 hpf) in zebrafish (Fig. 4b). However the preotic placode can be detected much earlier based on the expression of specific factors, for example *pax8* (Fig. 4a). Once the otic placode is formed it transforms into a fluid filled vesicle by 18hpf (Fig. 4c). In mammals and birds this vesicle is formed by invagination of the otic placode transiently forming a cup. The vesicle then pinches off and sinks into the mesenchyme under the surface ectoderm. In zebrafish the otic vesicle forms by cavitation, a process by which the cells at the center of the placode lose cell-cell contact forming a slit-like lumen (Haddon and Lewis, 1996). The otic vesicle grows rapidly with an increase in both number and size of otic cells. Extensive folding of the otic epithelium produces a complex structure with a series of interconnected chambers (Fig. 4d).

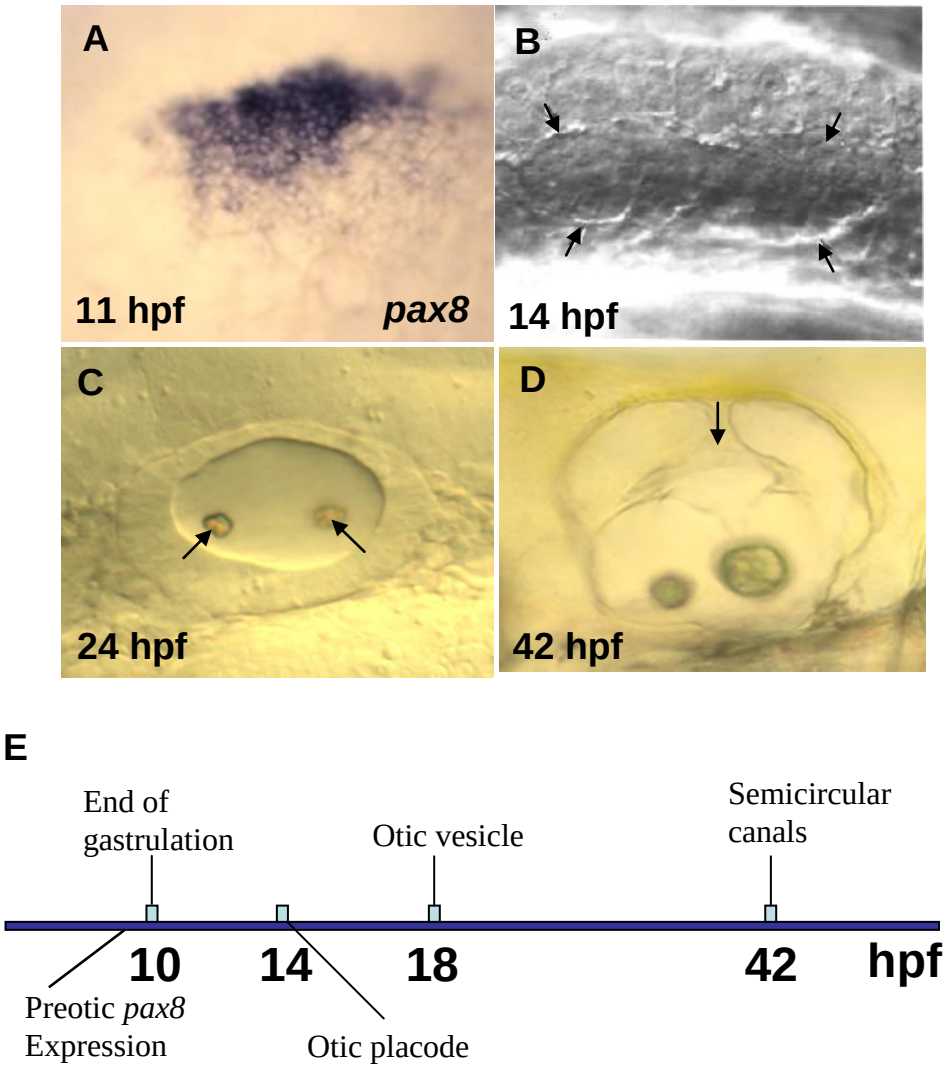


Figure 4. Otic development in zebrafish. (A) Preotic cells can be distinguished by *pax8* expression by 9.5 hpf, shown here at 11 hpf. (B) Otic placode at 14 hpf in a live embryo. Arrows indicate the surface of the otic placode (Adapted from Kimmel et al., 1995). (C) Otic vesicle at 24 hpf in a live embryo. Arrows indicate two otoliths that are formed in anterior and posterior regions of the otic vesicle. (D) Otic vesicle at 42 hpf with protrusions (arrow) of otic wall to initiate formation of semicircular canals. (E) Schematic diagram showing important stages of inner ear development. hpf; hours post fertilization. (A-D) images show lateral views with anterior to the left.

OTIC PLACODE INDUCTION

The complex inner ear develops from a simple ectodermal structure, the otic placode. Classic embryological experiments in amphibians and birds have shown that formation of the otic placode is an inductive process. Signals from adjacent tissues are necessary to instruct naïve ectoderm to initiate otic development. Understanding the characteristics of the tissue(s) responsible for otic induction and which cells are competent to form the otic placode has been the focus of many studies over the last century. Previous experiments in various species have shown that (a) initially, ectoderm competent to form otic placode is widespread and gradually becomes restricted to the otic region; (b) the prospective otic ectoderm becomes committed to otic fate by mid-somitogenesis; (c) the ability of inductive periotic tissues to re-specify uncommitted foreign ectoderm to the otic tissue persists until mid to late-somitogenesis stages; (d) adjacent hindbrain and subjacent mesendoderm are the sources of otic inducing signals (reviewed in Baker and Bronner-Fraser, 2001; Normaly and Grainger, 2002; Whitfield et al., 2002; Riley and Phillips, 2003).

OTIC PLACODE INDUCING FACTORS

The following factors such as Fgf, Wnt, Foxi1 and Dlx are shown to be important for otic placode induction.

1) **Fgf:** Fgf represent a family of secreted protein ligands that binds to FGF receptors (FGFRs), which are tyrosine receptor kinases (RTKs) and thereby activate the downstream intracellular signal transduction pathways such as RAS/MAP kinase and phospolipase C-γ pathways. FGFs and FGFRs also bind to heparin sulphate proteoglycans (HSPGs) with high affinity. HSPGs consist of a core protein and long unbranched chains of disaccharides. FGF ligands associated with HSPG binds to FGFR and results in dimerisation of FGFR and subsequent cross phosphorylation of specific tyrosine residues in the intracellular domain of the FGFR. Upon FGFR phosphorylation, FGFR substrate 2 (FRS2) binds to receptor and recruits an adaptor protein GRB2 linked to son of sevenless (SOS), a nucleotide exchange factor which activates Ras, a small GTP binding protein. Ras activation results in cascade of phosphorylation events involving Raf (a MAPK kinase kinase), MEK (a MAPK kinase) and finally ERK (MAPK, mitogen activated protein kinase). Upon phosphorylation, the active form of ERK translocates into the nucleus and phosphorylates specific members of the Ets family of transcription factors. These Ets transcription factors in turn activate transcription of Fgf target genes (Figure 5). The other transduction pathway activated by Fgf involves the phospholipase C-γ (PLC γ). PLC γ interacts with activated FGFRs and then hydrolyzees the phosphotidyl-inositol -4, 5-diphosphate (PIP2) to inositol -1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates release of intracellular calcium, whereas DAG activates protein kinase C-δ (PKCδ), which then in turn activates Raf by phosphorylation (Figure 5) (Thisse and Thisse, 2005).

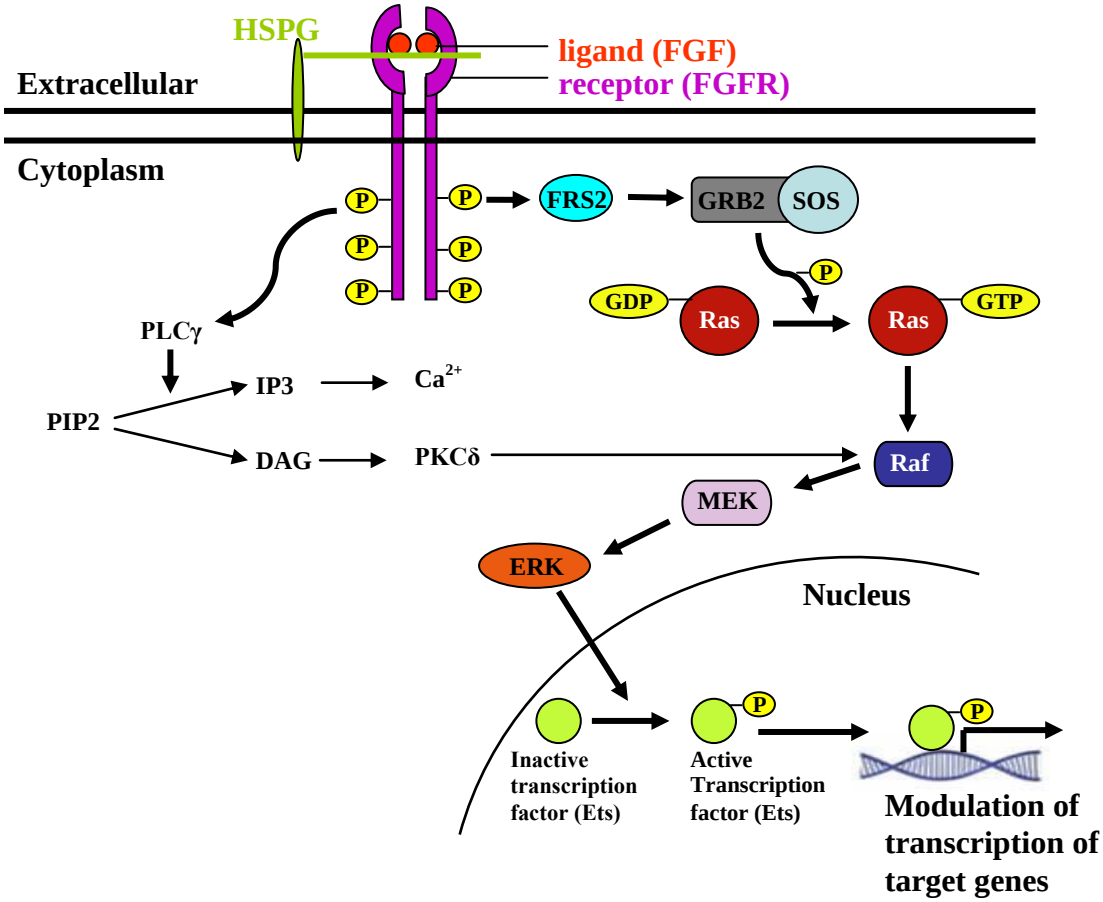


Figure 5. Schematic representation of Fgf signal transduction pathways.

Different signaling pathways from the surrounding neural tissue and mesoderm are thought to play an important role in otic placode induction. To date, several studies in vertebrates indicate that members of Fibroblast Growth Factors (FGF) are the primary otic inducing signals. Depletion of *Fgf3* in chick explants using antisense morpholinos or antibodies inhibits formation of otic vesicles (Represa et al., 1991), whereas misexpression of Fgfs using viral vectors induces ectopic ears in the head and trunk ectoderm (Vendrell et al., 2000). Disruption of *Fgf3* in mouse perturbs otic vesicle patterening but not otic placode induction (Mansour et al., 1993). These earlier studies led to the conclusion that Fgf signaling regulates postplacodal stages of otic vesicle development and not required for otic placode induction. However, the authors did not address the possibility of a parallel or redundant pathway that might have partially compensated for loss of *Fgf3*. The first evidence for redundant function among Fgfs in otic placode induction came from studies in zebrafish (Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003). In zebrafish, *fgf3* and *fgf8* are expressed in the hindbrain and subotic mesenchyme at appropriate developmental stages. Knock down of either *fgf3* or *fgf8* shows modest deficiencies in otic development, whereas knocking down both genes prevents otic placode induction completely (Phillips et al., 2001; Leger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003). Subsequently, studies in other species support a broadly conserved role for Fgf. In mouse Fgf3, Fgf8 and Fgf 10 are involved in otic induction. Disruption of both *Fgf3* and *Fgf10* ablates otic placode formation in mouse (Alvarez et al., 2003; Wright and Mansour, 2003). Disruption of *Fgf8* function in *Fgf3* null mutants strongly reduces the

expression of *Fgf10* in subotic mesenchyme and subsequently impairs otic induction (Ladher et al., 2005). The latter study suggests that Fgf8 regulates expression of *Fgf10* which inturn acts along with *Fgf3* in otic placode induction. In chick Fgf3, Fgf8 and Fgf19 are all shown to be involved in otic placode induction. Knockdown of *Fgf8* using siRNA reduces expression of *Fgf19* in the mesoderm and severely impairs otic induction. Localized knockdown of *Fgf3* and *Fgf19* in mesoderm blocks production of otic cells whereas knockdown of either one causes slight impairment of otic induction (Freter et al., 2008). Overall, these loss-of function studies in various vertebrate species support the idea that Fgfs are necessary for normal otic placode induction and development. Furthermore, these data indicates that there is functional redundancy among Fgf genes that act to induce otic placode formation.

Misexpression studies have suggested that Fgfs are not only necessary but also sufficient for otic placode induction (Lombardo and Slack, 1998; Phillips et al., 2004; Solomon et al., 2004; Vendrell et al., 2000). However, there are discrepancies in the literature regarding the sufficiency of Fgf3 or Fgf8. Misexpression of *Fgf3* can induce formation of ectopic otic placode in chick (Vendrell et al., 2000). A similar result of ectopic otic induction was observed by implanting Fgf8 or Fgf2 coated beads (Adamska et al., 2001). In contrast, misexpression of *Fgf19* does not induce expression of any otic markers in chick explants of uncommitted ectoderm. Furthermore, in zebrafish, misexpression of either *fgf3* or *fgf8* by plasmid DNA injections containing Fgf cDNA under the control of constitutive promoter induces ectopic otic vesicles around the anterior neural plate (Phillips et al., 2004). In contrast, misexpression of *fgf8* using a

stable transgenic line under the control of a heat shock inducible promoter leads to formation of larger endogenous otic vesicles, but does not induce ectopic otic vesicles (Hans et al., 2007). Similarly, application of Fgf-coated beads can expand the endogenous otic domain but does not lead to production of otic tissue in ectopic locations (Léger and Brand, 2002). The above discrepancies regarding Fgfs being sufficient for otic induction is probably due to different techniques followed for misexpression of Fgfs which produced marked differences in the onset and duration of signaling, the level of signaling, and the spatial distribution of signaling. Any or all of these parameters could influence the response to Fgf signaling leading to different outcomes. I reexamined the spatial and temporal requirements of Fgf for otic placode induction and the effects of misexpression of *fgf8* and *fgf3* on otic development are discussed in Chapter II of this dissertation.

Once otic placode induction begins, several *Fgf* genes are expressed within the developing otic placode. For example, in mouse *Fgf4* is expressed within the pre-placode by 4 somite-stage and continues to be expressed until the placode is morphologically visible (9 somite-stage). In addition, *Fgf16* is also expressed weakly in otic placode at 10 somite-stage, but later becomes more restricted to the posterior regions of otic cup (Wright et al., 2003). In chick, *Fgf8* is transiently expressed from 7-14 somite-stage (Adamska et al., 2001) and *Fgf16* is expressed in the nascent otic placode by 4 somites-satge and further restricted to different regions of the otic vesicle (Chapman et al., 2006). Furthermore, *Fgf10* is expressed in the otic placode of mouse, chick and *Xenopus* by 12-14 somites and continues to express through otic vesicle stages

(Pirvola et al., 2000; Alsina et al., 2004; Lea et al., 2009). In zebrafish, *fgf24* a member of *fgf8/17/18* subfamily of Fgf ligands is expressed in otic precursor cells by 10.5 hpf (Draper et al., 2003) and continues to express in the otic placode and otic vesicle at later stages of otic development. However, the function of these placodal *Fgf* genes such as *Fgf4, Fgf10, Fgf16 and Fgf24* has not been specifically examined in any species. It is most likely that theses placodal *Fgfs* might augment or expand inductive Fgf signaling from the surrounding tissues. The role of *fgf24* in otic placode induction is examined later in this dissertation (Chapter III).

2) **Wnt**: There is growing evidence that in addition to Fgfs, Wnt signaling is also necessary for otic development after otic placode induction. In chick, it has been postulated that Wnt8a signaling from the hindbrain works in concert with Fgf19 to induce otic placode formation (Ladher et al., 2000). Chick explants of prospective otic ectoderm, when cultured with both human Wnt8a and Fgf19 expressed a broad range of otic markers such as *Pax2*, *Nkx5.1*, *SOHo-1*, and *Dlx-5*. In contrast, culturing the explants with either Wnt8a or Fgf19 alone failed to express the above otic markers (Ladher et al., 2000). This led the authors to conclude that activities of both Wnt and Fgf cooperate in otic induction. However, it was also observed that Wnt8a induces *Fgf3* whose role in otic induction in chick has already been shown. A recent study in chick has shown that blocking Wnt activity by misexpressing an antagonistic factor *Dkk1* does not affect expression of early otic marker *Pax2*. However, the otic domain of *Pax2* is subsequently lost and later otic patterning markers are either smaller in domain or absent (Freter et al., 2008). These results indicate that canonical Wnt signaling is important for

otic fate maintenance rather than otic placode induction. A similar conclusion has been made from the studies in Mouse (Ohyama et al., 2006). β-catenin is a member of the armadillo family of proteins, which is an integral component of the Wnt signaling pathway. In the presence of Wnt protein ligand, β-catenin is not phosophorylated by the Axin-GSK3-APC complex. Thus the levels of β-catenin builds up in the cytosol and is subsequently translocated into the nucleus, which in conjunction with LEF/TCF activate specific target genes important for dvelopmental processes. Conditional knockdown of β-catenin using *Pax2-Cre* in mouse nascent otic tissue causes downregulation of otic placode markers at early stages (E8.5) and a reduction in otic vesicle size at later stages (E10.5). In the converse approach, misexpression of stabilized β-catenin using *Pax2-Cre* increased the size of the otic placode. These data support the model that Wnt signaling is necessary to maintain otic fate in mouse. However, knockdown of *wnt8* or misexpression of *dkk1* in zebrafish delays, but does not inhibit otic induction. This delay in otic induction is attributed to the delay in expression of *fgf3* and *fgf8* in the hindbrain, which regulate actual induction of otic placode in the adjacent ectoderm (Phillips et al., 2004). Misexpression of *wnt8* results in production of bigger and ectopic otic vesicles. In addition, hindbrain domains of *fgf3* and *fgf8* are expanded into the anterior neural plate. However, the ability of *wnt8* misexpression to induce ectopic otic vesicles is lost when the function of *fgf3* and *fgf8* is blocked. Similarly, misexpression of *wnt1* in medaka results in formation of bigger and ectopic otic vesicles and expanded domains of hindbrain *fgf3* and *fgf8* (Bajoghli et al., 2009). These data suggest that at least in teleosts, Wnt signaling is not directly required for otic induction but appears to function

indirectly by regulating expression of hindbrain Fgfs. However, the regulation of Fgfs by Wnt signaling has yet to be addressed in mouse and chick.

3) **Foxi1:**Forkhead class (Fox) winged helix proteins play an important role in a broad range of biological functions such as early patterning and morphogenesis (Pogoda et al., 2000), cell fate specification (Miller et al., 1993) and regulation of genes in differentiated tissues (Clevidence et al., 1994). In zebrafish *foxi1* plays a redundant role as a competence factor required for general preplacodal specification (Kwon et al., 2010) and is uniquely vital for induction of otic and epibranchial placodes because in *foxi1* mutants epibranchial placodes fail to form and otic placode is severely reduced (Hans et al., 2007; Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003). The expression pattern of *foxi1* is very dynamic. In early gastrula, *foxi1* is expressed in the ventral ectoderm including epidermal and preplacodal tissue. Later *foxi1* expression is downregulated in ventral ectoderm and is upregulated in future otic and epibranchial placodes. Furthermore, *foxi1* is no longer expressed in otic cells after 12 hpf, but continues to express in the adjacent epibranchial ganglia through at least 36 hpf (Lee et al., 2003). These results indicate that initially at gastrula stage, Foxi1 is required for establishing preplacodal ectoderm, from which all the cranial placodes are derived in response to specific regional signals. Once the preplacodal ectoderm is specified, Foxi1 mediates generation of otic and epibranchial placodes regionally within the broad preplacodal ectoderm. Furthermore, downregulation of *foxi1* specifically in otic cells may help to diversify the fates of otic and epibranchial cells, which otherwise share similar developmental histories.

In *foxi1* mutants epibranchial placodes fail to form and otic placode is severely reduced. Otic expression of *pax8* is absent in *foxi1* mutants (Nissen et al., 2003; Solomon et al., 2003). These results suggest that *foxi1* acts as an upstream activator of *pax8* in preotic cells. Additionally, disruption of *foxi1* and *pax8* together by morpholino injection causes defects in otic development that are similar to *foxi1* morphants. This is consistent with the notion that *foxi1* and *pax8* act in a linear pathway in otic induction (Mackereth et al., 2005). Moreover, *foxi1* is considered to function as a competence factor required for proper response to Fgf in otic placode induction (Hans et al., 2004; Hans et al., 2007). Although the requirement of *foxi1* is transient, Fgf signaling must be maintained throughout placodal development. Despite the importance of *foxi1*, the functional relationship between Fgf and Foxi1 remains unclear. For example, there are discrepancies as to whether Fgf inhibits or enhances *foxi1* expression, possibly reflecting differences in misexpression techniques, such as misexpression using a stable heat shock inducible transgenic line vs. bead implantation using Fgf8b coated beads (Hans et al., 2007; Nechiporuk et al., 2007). Thus, further studies are necessary to clarify this discrepancy. To understand the relationship between Fgf and *foxi1*, I examined whether Fgf enhances expression of *foxi1* in response to Fgf in the otic placode in Chapter II of this dissertation. *pax2a* is still expressed in *foxi1* morphants, albeit in a small domain. This has led to the realization that expression of *pax2a* represents a distinct second pathway that mediates the effects of Fgf (see below).

4) **Dlx**: The other important set of transcription factors expressed in preplacodal ectoderm are the members of the *Distal-less* (*dlx*) family of homeobox transcription

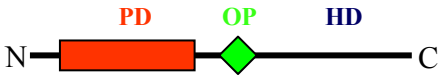
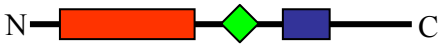
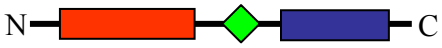
factors. In zebrafish, *dlx3b* and *dlx4b* are coexpressed in the preplacodal domain and knocking down function of both genes together inhibits otic induction (Solomon and Fritz, 2002). Moreover, *dlx3b/4b* regulates otic expression of *pax2a/2b* and not *pax8* (Hans et al., 2004). Similarly, double knockout mutants of mouse for *Dlx5* and *Dlx6* produce small and poorly differentiated otic vesicles (Robledo et al., 2002). These studies indicate that members of *Dlx* genes play a role in otic development.

TRANSCRIPTION FACTORS EXPRESSED IN THE OTIC PLACODE

The otic placode is morphologically visible by 9-10 somites stage in all vertebrates examined. Several transcription factors are expressed in response to the inducing signals well before the overt morphological changes are observed. Initially, many preplacodal genes related to the gene families of *Eya* (*eyes absent*), *Six* (*sine oculis*), *Dlx* (*distalless*), *Gata* and *Fox* are expressed in a broad domains of ectoderm, but later become restricted to individual placodal primordia. The expression of these genes is maintained and upregulated in the otic anlagen. In addition, transcription factors belonging to the family of Pax2/5/8 are activated between late gastrulation to early somitogenesis stages. *Sox3*, a member of sex determining region (SRY) related high mobility group box (HMG) transcription factors is also expressed in the otic anlagen by late gastrulation. All these factors are shown to be critical for early otic development.

1) **Pax2/8:** Pax proteins are known to play an important role in embryonic development and organogenesis including inner ear development. Members of the *pax* gene family encode highly related transcription factors that have conserved domains with specific functions (Czerny, et al., 1997). In mammals, a total of nine *Pax* genes have been identified and are classified into four subgroups based on their composition of functional domains such as paired domain, octapeptide domain, homeodomain, transactivation and inhibitory domain (Table 1) (Reviewed in Buckingham and Relaix, 2007; Chi and Epstein, 2002; Dahl et al., 1997; Mansouri et al., 1996; Robson et al., 2006). Members within a subgroup have high sequence similarity within the paired domain and similar expression patterns during embryogenesis. The highly conserved N-terminal 128 amino acid DNA binding domain, the paired domain of Pax proteins mediates recognition of their target genes. The C-termial domain consisting of transactivation/inhibitory domain controls the transcription of their target genes. The other conserved domains like octapeptide motif are involved in protein-protein interaction, for example the octapeptide domain of Pax5 interacts with Groucho protein to repress transcription of target genes (Eberhard et al., 2000). Homeodomain which is present in all *Pax* genes except the members of subgroup I, serves as an additional DNA binding domain which can recognize TATA sequence in DNA (Wilson et al., 1993). However, a partial homeodomain present in *Pax2/5/8* gene family serves as an interaction surface between retinoblastoma and TATA binding proteins (TBP) (Cvekl et al., 1999; Eberhard and Busslinger, 1999).

Table 1. The Pax gene family of transcription factors in vertebrates. Nine *Pax* genes and their primary expression domains during embryonic development are listed. *Pax* genes are classified into four groups based on their protein structure. Schematic diagram shows positions of different functional domains. Red boxes indicate the conserved paired domain (PD), green diamonds indicate octapeptide domain (OP) and blue boxes indicate homeodomain (HD). CNS: central nervous system, N: amino-terminus, C: carboxyl-terminus.

Pax family group	Structure/ domains	Gene family members	Expression domains in developing tissue/organs
I		*Pax1* *Pax9*	Skeleton, Thymus, Parathyroid Skeleton, Teeth, Thymus
II		*Pax2* *Pax5* *Pax8*	CNS, Kidney, Ear CNS, B-lymphocytes, Ear CNS, Kidney, Thyroid, Ear
III		*Pax4* *Pax6*	Pancreas, Gut CNS, Pancreas, Gut, Nose, Eye
IV		*Pax3* *Pax7*	CNS, Craniofacial tissue, Neural crest, Skeletal muscle CNS, Craniofacial tissue, Skeletal muscle

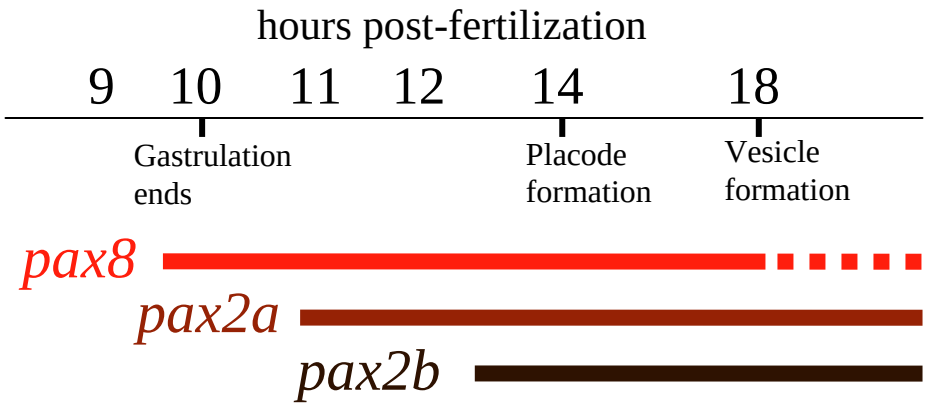


Figure 6. Temporal expression of *pax2a/2b/8* genes in otic region of zebrafish. Dashed line indicate phase of downregulation.

The members of the zebrafish *pax2/5/8* family of transcription factors are expressed dynamically in the ear at various important stages indicating their role in inner ear development (Pfeffer, et al., 1998) (Fig. 6). Otic expression of *pax8* begins during late gastrulation and is the earliest known marker of otic induction in most vertebrates, except in chick which lacks a *Pax8* ortholog in the genome. The closely related *Pax2* gene is expressed in the otic anlagen of all species examined by early somitogenesis stages. The zebrafish genome has two *pax2* paralogues, *pax2a* and *pax2b*. Expression

of *pax2a* in the preotic placode begins by 1-2 somites stage (11hpf) and *pax2b* by 9 somites stage (13.5hpf) (Pfeffer et al., 1998). In mouse, null mutants for *Pax8* show no otic phenotype and *Pax2a* null mutants show defects only at later stages with abnormal development and differentiation of the auditory apparatus (Torres et al., 1996). Disruption of *Pax2a* function in chick embryos results in severe otic morphological defects (Barembaum and Bronner-Fraser, 2010). The redundant functions of Pax2 and Pax8 in regulating early otic development have been most extensively studied in zebrafish (Hans et al., 2004; Mackereth et al., 2005). Expression of *pax8* and *pax2a/b* are regulated by distinct but parallel pathways. Preotic expression of *pax8* requires both Fgf signaling and the otic competence factor Foxi1, but not Dlx3b/Dlx4b (Fig. 7)(Hans et al., 2004; Hans et al., 2007; Phillips et al., 2001; Solomon et al., 2003; Solomon et al., 2004). In contrast, expression of *pax2a/b* in otic cells requires Fgf signaling and Dlx3b/Dlx4b but not Foxi1 (Fig. 7) (Hans et al., 2004; Léger and Brand, 2002; Solomon et al., 2003; Solomon et al., 2004). Although *pax8* and *pax2a/b* are differentially regulated, they provide partially redundant functions in otic development (Fig. 7). Knockdown of *pax8* function results in formation of a small otic placode (Hans et al., 2004; Mackereth et al., 2005). Knocking down both *pax2a* and *pax2b* has negligible effect on otic induction, however abnormal patterning and death of sensory hair cells occurs at otic vesicle stage (Riley et al., 1999; Kwak et al., 2006). Knock down of

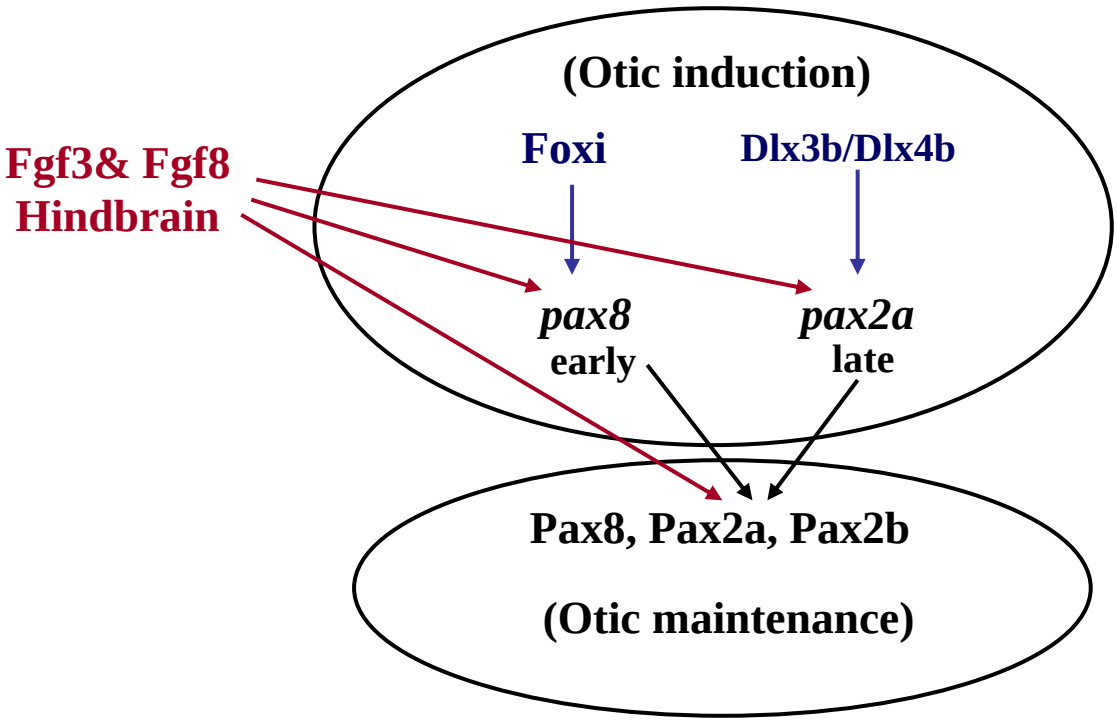


Figure 7. Genetic interactions during otic placode induction and maintenance. Pax8 and Pax2a function redundantly in otic development which are induced in preotic domain by two distinct parallel pathways. *pax8* is induced by the action of Fgfs and Foxi1 at early satges in preotic domain beginning 9 hpf whereas *pax2a* is activated by the action of Fgfs and Dlx3b/Dlx4 at later stages of 11.5 hpf. hpf; hours post fertilization.

pax8/pax2a/pax2b together leads to loss of otic fate by 24 hpf (Mackereth et al., 2005). These results indicate that *pax2/8* genes together are required for induction and maintenance of the otic placode. However, whether *pax2/8* genes are sufficient for otic induction and/or maintenance have not been investigated. We will examine the effects of misexpression of *pax2/8* genes in Chapter II of this dissertation. Functional analysis of how these *pax2/8* mediate otic development is examined later in this dissertation (Chapter III).

2) **Sox3:** *Sox3* is a member of group B1 Sox genes that encodes a sex determining region (SRY) related high mobility group box (HMG) transcription factors. In zebrafish, mouse and chick, *Sox3* is initially expressed in a broad region which includes both otic and epibranchial primordium (Rex et al., 1997; Wood and Episkopou, 1999; Nikaido et al., 2007; Sun et al., 2007). As embryonic development proceeds, at later stages of otic placode development, expression of *sox3* is downregulated in otic cells, but is upregulated and maintained in the adjacent epibranchial placodes. For example, in zebrafish, *sox3* is expressed in otic primordium until 11 hpf, but downregulated in otic cells by 12 hpf, but continues to express in adjacent epibranchial placodes (Sun et al., 2007). Moreover, *sox3* is initially co-expressed with *pax8* in the otic primordium. Similar to *pax8*, otic expression of *sox3* is regulated by the combined activities of Fgf signaling and competence factor Foxi1 (Lee et al., 2003; Nechiporuk et al., 2007; Sun et al., 2007). Similarly, co-expression of *Sox3* and *Pax2* in otic/epibranchial placodal domains in chick requires Fgf. Moreover, even at later stages of development, expression of *Sox3* is maintained in anterior regions of the otic vesicle (Abelló et al.,

2010). In mouse and zebrafish, impairment of *Sox3* function leads to mild-to-moderate reduction in the size of the otic vesicle (Rizzoti and Lovell-Badge, 2007; Dee et al., 2008; Okuda et al., 2010). However, otic patterning has not been examined in detail in these backgrounds. Additionally, genetic interaction of *pax8* and *sox3* has not been addressed, which raises the question of whether these genes cooperate to mediate otic induction. Functional analysis of how *pax8* and *sox3* interact to regulate otic induction is examined later in this dissertation (Chapter III).

EPIBRANCHIAL PLACODES AND THEIR DEVELOPMENT

Epibranchial placodes are the ectodermal thickenings that give rise to sensory neurons of facial (cranial ganglion VII), glossopharyngeal (cranial ganglion IX) and vagal ganglia (cranial ganglion X), which are responsible for transmitting the sensory information from taste buds and pharyngeal organs. In all the vertebrates examined, *Sox3* is one of the earliest markers expressed in the epibranchial placodes (Penzel et al., 1997; Abu-Elmagd et al., 2001; Ishii, et al., 2001; Nikaido et al., 2007). Knockdown of *Sox3* in mouse and zebrafish severely impairs epibranchial placode development (Rizzoti and Lovell-Badge, 2007; Dee et al., 2008). In zebrafish, the same Fgf signals and Foxi1 that regulate early otic placode development are required for epibranchial placode development (Lee et al., 2003; Nechiporuk et al., 2007; Nikaido et al., 2007; Sun et al., 2007). Moreover, in response to these upstream activators, *sox3* is co-

expressed with *pax8* in both otic and epibranchial placodes. These similarities have led to the hypothesis that Fgf initially induces a common otic/epibranchial field, which later divides into adjacent compartments with distinct fates. However, this model does not take into account the observation of a dramatic transition of *sox3* expression that occurs between 3 to 6 somites-satge (11-12hpf), wherein *sox3* is downregulated from the otic domain but upregulated in the lateral presumptive epibranchial domain (Nikaido et al., 2007; Sun et al., 2007). This pattern of *sox3* expression along with close examination of early markers suggests that otic and epibranchial placodes are induced at different times, possibly by distinct mechanisms. Initially, *pax8* and *sox3* are co-expressed within a relatively small domain adjacent to rhombomere 4 of the hindbrain. This appears to correspond to the otic domain in zebrafish because at least two otic-specific markers, *atoh1b* and *fgf24*, are soon induced within the same domain (Draper et al., 2003; Millimaki et al., 2007). How *sox3* is differentially regulated in otic and epibranchial placodes is therefore an important issue requiring further study. I will examine the factors responsible for differential regulation and functional significance of *sox3* in otic/epibranchial placodes development in Chapter III of this dissertation.

DISSERTATION OBJECTIVES

The objectives of this dissertation are to address the temporal and spatial requirements of Fgf for normal otic induction and their interaction with *pax2/8* genes

and to examine the functions of *pax2/8* genes in regulating otic and epibranchial development using zebrafish as a model system.

Although Fgfs are required for otic induction, there are controversies as to whether Fgfs are sufficient for otic induction. Chapter II readdresses the temporal and spatial requirements of Fgf for otic induction. I performed misexpression studies using stable transgenic lines under the control of heat shock promoter *hsp70*. The effects of misexpression are level and developmental stage dependent. No ectopic otic tissue was induced by misexpression of Fgf at any developmental stage. Misexpression of *pax2/8* expands otic domain and co-misexpression with Fgf induces ectopic otic vesicles.

Chapter III addresses how *pax2/8* factors coordinate otic and epibranchial placodes. In this study I show that *pax2/8* represses expression of *foxi1* in otic cells to maintain their fate during otic development. Furthermore, I show that *pax2/8* activate *fgf24* expression within otic cells which in turn repress *sox3* in otic cells, but induces *sox3* in adjacent epibranchial domain. These data indicate the Pax2/8 coordinate otic and epibranchial placodes by differentially regulating *foxi1*, *fgf24* and *sox3*.

CHAPTER II

SUFFICIENCY OF FGF AND PAX2/8 PROTEINS IN OTIC INDUCTION

INTRODUCTION

The vertebrate inner ear develops from a simple epithelial thickening called the otic placode. In all vertebrate species examined to date, the otic placode is induced from uncommitted ectoderm lateral to the developing hindbrain in response to localized Fgf signaling (Alvarez et al., 2003; Ladher et al., 2000; Ladher et al., 2005; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Riley and Phillips, 2003; Wright and Mansour, 2003). In zebrafish embryos, for example, *fgf3* and *fgf8* are expressed in the hindbrain primordium during gastrulation and serve as the principal inducers of otic development. Subotic mesoderm also expresses *fgf3* and *fgf8* and contributes to induction and maintenance of the otic placode (Léger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001). Disruption of *fgf3* and *fgf8* blocks the earliest known steps in otic development (Léger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001). Moreover, application of the Fgf-inhibitor SU5402 after the onset of otic induction shows that Fgf signaling must continue through mid-somitogenesis stages to maintain otic fate (Léger and Brand, 2002; Maroon et al., 2002).

Although there is widespread acceptance that Fgf is required for otic induction, there have been contradictory findings regarding the sufficiency of Fgf. Application of Fgf-coated beads can expand the endogenous otic domain but does not lead to production of otic tissue in ectopic locations (Léger and Brand, 2002). Similar findings have been found following global activation of a heat shock-inducible transgene expressing *fgf8* (Hans et al., 2007). In contrast, injection of plasmid expression-vectors at the 8-cell stage to achieve mosaic misexpression of *fgf3* or *fgf8* can expand endogenous otic domains and induce ectopic otic placodes in cranial ectoderm anterior to the somites (Phillips et al., 2004). The reason for the different outcomes in these experiments is not clear, but the various techniques used likely produce marked differences in the onset and duration of signaling, the level of signaling, and the spatial distribution of signaling. Any or all of these variables could influence the response to Fgf signaling.

Members of the Pax2/5/8 family of transcription factors are important mediators of Fgf signaling during otic induction (Hans et al., 2004; Mackereth et al., 2005). Expression of *pax8* marks the earliest known response to Fgf during late gastrulation (Léger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001), and is critical for setting the size of the otic placode; knockdown of *pax8* reduces the size of the otic placode by nearly half (Hans et al., 2004; Mackereth et al., 2005). *pax2a* and *pax2b* expression normally begins during early somitogenesis stages. Both genes are partially redundant with *pax8* (Mackereth et al., 2005). Knockdown of all three *pax* genes leads to loss of otic fate by 24 hpf, indicating that these genes are needed to maintain otic fate

(Mackereth et al., 2005). Whether Pax2/8 function is sufficient as a downstream response to Fgf has not been previously examined.

In addition to Fgf signaling, the transcription factor Foxi1 is required for induction of *pax8* in prospective otic tissue (Hans et al., 2004; Hans et al., 2007; Solomon et al., 2003; Solomon et al., 2004). Although otic expression of *pax2a* and *pax2b* is induced independently of *foxi1*, their expression domain is much smaller in *foxi1* mutants. Despite the importance of *foxi1*, the functional relationship between Fgf and Foxi1 remains unclear. For example, there are discrepancies as to whether Fgf inhibits or enhances *foxi1* expression, possibly reflecting differences in misexpression technique (Hans et al., 2007; Nechiporuk et al., 2007). Additionally, because *pax2a/b* expression depends on Fgf but not *foxi1* (Hans et al., 2004; Léger and Brand, 2002; Solomon et al., 2003; Solomon et al., 2004), appropriate misexpression of Fgf might be expected to expand the domain of *pax2a* and bypass the need for *foxi1*.

Here we used heat-shock inducible transgenes to examine key parameters that influence the ability of Fgf to induce otic development. The effects of transient misexpression of Fgf were dependent on the stage and level of misexpression. Global transient activation of *hs:fgf3* or *hs:fgf8* at mid-late gastrula stages (7-8 hpf) severely impaired otic induction, in part by disrupting formation of the principal signaling centers in the hindbrain. Additionally, mosaic studies showed that high-level misexpression blocks otic fate cell-autonomously, whereas low to moderate levels promote otic development. At later stages high-level Fgf misexpression, either global or local, was no longer inhibitory but instead caused a dramatic expansion of endogenous otic domains.

At all stages after mid-gastrulation, Fgf misexpression upregulated *foxi1* expression in ectoderm abutting the anterior neural plate. Moreover, the ability of Fgf to expand otic tissue required *foxi1*. Nevertheless, despite the increased domain of *foxi1*, Fgf alone did not result in production of ectopic otic tissue at sites distant from endogenous otic placodes. Misexpression of *hs:pax2a* or *hs:pax8* also expanded endogenous otic domains but was not sufficient to bypass the requirement for Fgf signaling. Co-misexpression of Fgf with *pax2a* or *pax8* led to production of ectopic otic tissue in a broad range of cranial ectoderm rostral to somites. Our data document the extent to which even small changes in the timing, distribution and level of Fgf signaling and its downstream effectors can influence otic induction. Furthermore, the data clarify functional relationships between Fgf, *foxi1* and *pax2/8* genes.

MATERIALS AND METHODS

Strains and developmental conditions

Wild type embryos were derived from AB line (Eugene, OR). Transgenic lines used in this study include *Tg(hsp70:fgf8a)*^{x17} (Millimaki et al., 2010), *Tg(hsp70:fgf3)*^{x27}, *Tg(hsp70:pax8)*^{x22}, *Tg(hsp70:pax2a)*^{x23}. For convenience, these transgenes are referred to in the remainder of the text as *hs:fgf8*, *hs:fgf3, hs:pax8 and hs:pax2a* respectively. Embryos were developed at standard conditions of 28.5^{0}C in fish water containing methylene blue and were staged based on standard protocols (Kimmel et al., 1995).

Misexpression

Misexpression studies using heat shock inducible transgenic lines were conducted in a water bath at varying temperatures between 37^0C to 39^0C for 30 minutes at specific time points mentioned in the results. After heat shock at desired temperatures, the embryos were incubated at 33^0C until fixation.

Cell transplantation

A lineage tracer (lysine-fixable biotinylated dextran, 10000 MW, in 0.2 M KCl) was injected into the donor embryos at the one-cell stage. Labeled cells from donor embryos at blastula stages were transplanted into non-labeled hosts of the same stage. Transplanted cells were identified in the hosts by streptavidin-FITC antibody staining.

In situ hybridization

In situ hybridization was carried out as described previously (Jowett and Yan, 1996; Phillips et al., 2001).

Morpholinos

For gene knockdown experiments, morpholino oligomers obtained from Gene Tools Inc. were injected into embryos at one-cell stage. Morpholino sequence for *foxi1* has been previously published (Solomon et al., 2003).

SU5402 treatment

SU5402 was dissolved in DMSO to make a stock solution of 20mM. Embryos are incubated with their chorions intact in a working concentration solution of 30μM SU5402 starting from 10.5 hpf, and then fixed at 13 hpf to examine the changes in *pax8* or *pax2a* expression.

RESULTS

Previous studies have not fully examined the effects of dose, timing and distribution of Fgfs that are required for otic induction. To readdress the temporal and spatial parameters that influence Fgfs role in otic induction, we generated heat shock inducible transgenic lines to misexpress *fgf8* or *fgf3* under the control of *hsp70* promoter in a dose and developmental stage dependent manner.

Effects of misexpression of *hs:fgf8* or *hs:fgf3*

We first examined the effects of misexpression of *fgf8* or *fgf3* at different stages of development. Consistent with previous findings (Hans et al., 2007), we observed that transient misexpression of *fgf8* or *fgf3* at mid gastrula stages(7-8 hpf) cause dorsalisation and blocked otic induction (Fig. 8A-F). In contrast, misexpression of *fgf8* or *fgf3* at later stages after gastrulation (10-11hpf) caused expansion of the early otic marker *pax2a*

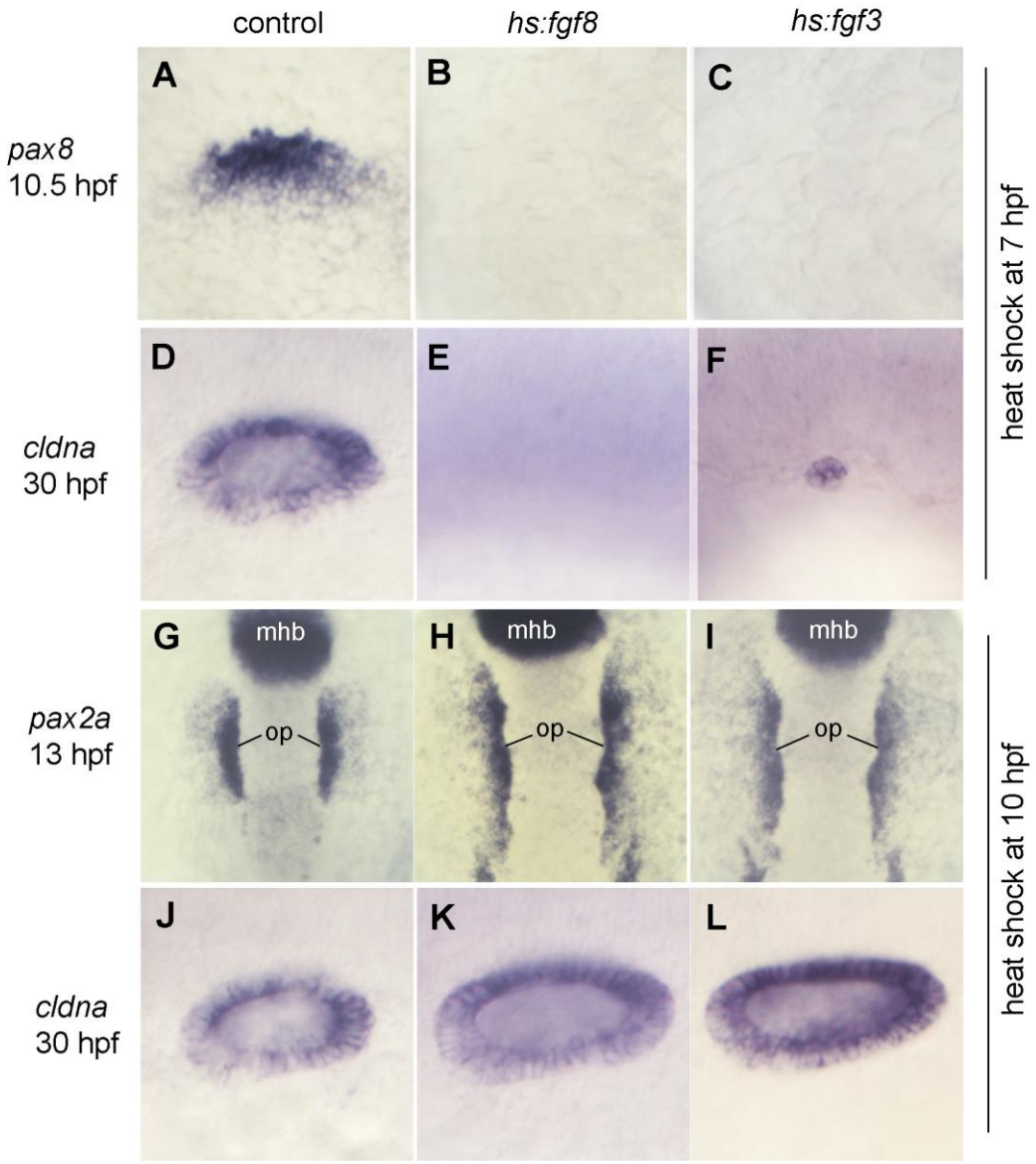


Figure 8. Effects on otic development following misexpression of *hs:fgf8* or *hs:fgf3* at different developmental stages. (A-C) *pax8* expression in the otic domain at 10.5 hpf in a control embryo (A), *hs:fgf8* transgenic embryo (B) and *hs:fgf3* transgenic embryo (C). (D-F, J-L) Expression of *cldna* in the otic vesicle at 30 hpf in a control embryos (D, J), *hs:fgf8* transgenic embryos (E, K) and *hs:fgf3* transgenic embryos (F, L). (G-I) *pax2a* expression in the otic domain at 13 hpf in control embryos (G), *hs:fgf8* transgenic embryo (H) and *hs:fgf3* transgenic embryo (I). Positions of (mhb) midbrain-hindbrain boundary and (op) otic placode are indicated. Images show lateral views with anterior to the left (A-F, J-L); dorsal views with anterior to the top (G-I).

(Fig. 8H, I) and increased the size of the otic vesicle (Fig. 8K, L). Thus misexpression of Fgfs at early stages impaired otic development, whereas misexpression at later stages promotes otic development.

Misexpression of Fgfs at early stages impairs otic induction by two mechanisms

The observation that misexpression of Fgf at mid gastrula stage blocked otic development was paradoxical because this is the stage when otic induction is thought to begin during normal development. We considered two possible explanations; first, we hypothesized that early transient activation of *hs:fgfs* disrupt endogenous signaling centers needed to induce and maintain otic development. Indeed, global activation of *hs:fgf8* at 7 hpf caused loss of expression of *fgf8* and *fgf3* in the hindbrain by 9.5 hpf (Fig. 9C, D). Similar results were observed when *hs:fgf3* was overexpressed (Fig. 9E, F). These data show that early misexpression of Fgfs disrupts later expression of Fgf in endogenous signaling centers, thereby blocking induction and maintenance of otic placodes. Second, we hypothesized that Fgf acts as a morphogen and that excess levels could block otic induction. Likewise, Fgf function might vary depending on whether it acts cell autonomously or non-autonomously. To test this we generated mosaic embryos by transplanting *hs:fgf8* transgenic cells into non-transgenic host embryos. Transgene activity levels of *hsp70* can be regulated by treatment with varying temperatures ranging from 35^0C to 39^0C (Adam et al., 2000 and our unpublished data). Full activation of

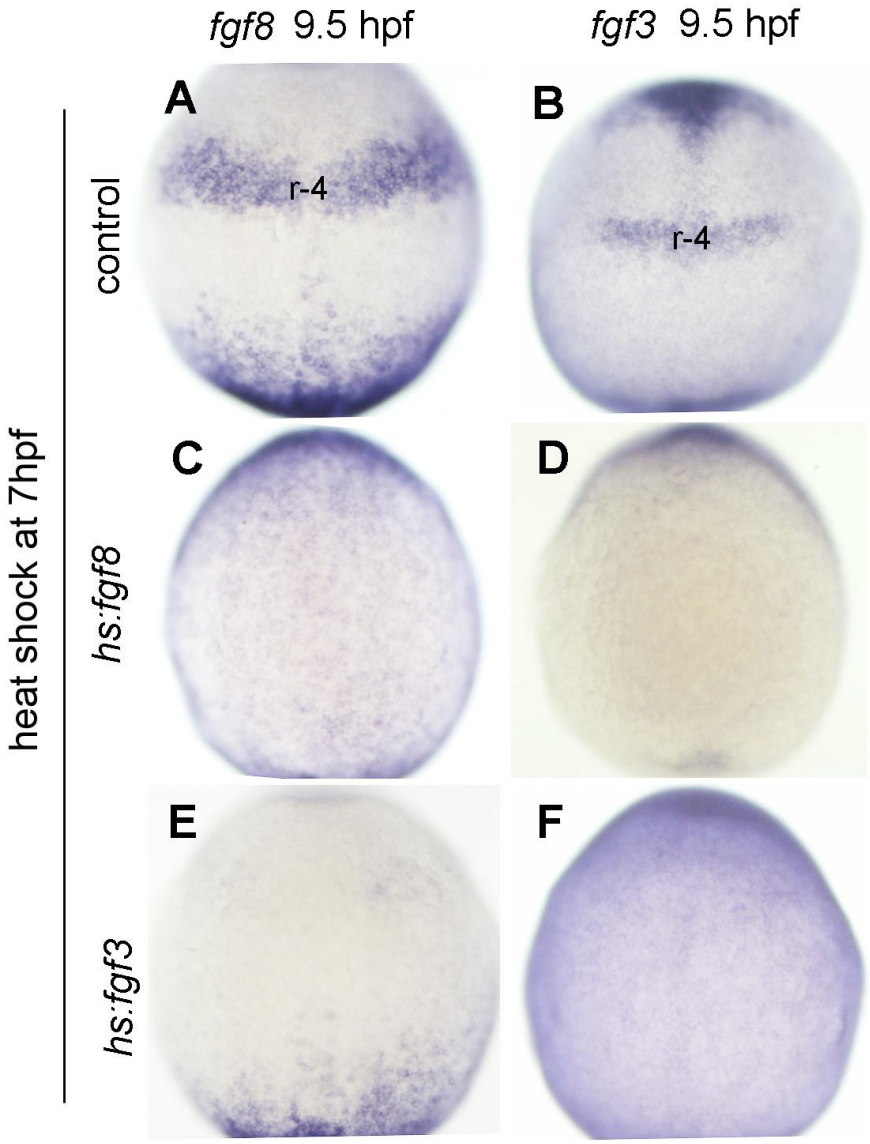


Figure 9. Misexpression of Fgfs perturbs endogenous signaling centers. Expression of *fgf8* and *fgf3* in r-4 of hindbrain at 9.5 hpf in control embryos (A, B), *hs:fgf8* transgenic embryos (C, D) and *hs:fgf3* transgenic embryos (E, F) respectively. Positions of rhombomere 4(r-4) are indicated. All images show dorsal views with anterior to the top.

hs:fgf8 at 8 hpf with a pulse of 39^0 C for 30 minutes in mosaic embryos caused cell autonomous loss of *pax8* expression in transgenic cells within the otic region (Fig. 10B, C). However, we observed an expanded domain of *pax8* expression in cells adjacent to the transgenic cells (Fig. 10B, C) indicating cell non-autonomous induction of otic markers. In contrast, a lower level of activation of *hs:fgf8* at 37^0C in mosaic embryos did not repress *pax8* expression cell autonomously (Fig. 10E, F). In this case too, *pax8* expression was induced in cells adjacent to transgenic cells (Fig. 10E, F). These results indicate that high levels of Fgf inhibit otic fate cell-autonomously, whereas low to moderate levels promote otic fate. High level activation of *hs:fgf8* both globally and in mosaic embryos at later stages of early somitogenesis (11 hpf) caused expansion of otic domain of *pax2a* (Hans etal., 2007, Fig. 10G, H, and data not shown). Also, there was no cell autonomous loss of *pax2a* within the transgenic cells in the mosaic embryos even after high level misexpression (Fig. 10G, H). Thus, once the otic development has been initiated, Fgf signaling reinforces otic fate. Taken together, these results strongly suggest that high level Fgf signaling at mid gastrula stages blocks otic fate cell-autonomously and impairs endogenous signaling centers, whereas at later stages of development, high level Fgf signaling promotes otic fate.

Misexpression of *pax2/8* expands endogenous otic field

Loss of function studies led to the conclusions that *pax2/8* genes are required for normal otic induction and maintenance (Hans et al., 2004; Mackereth et al., 2005). Here

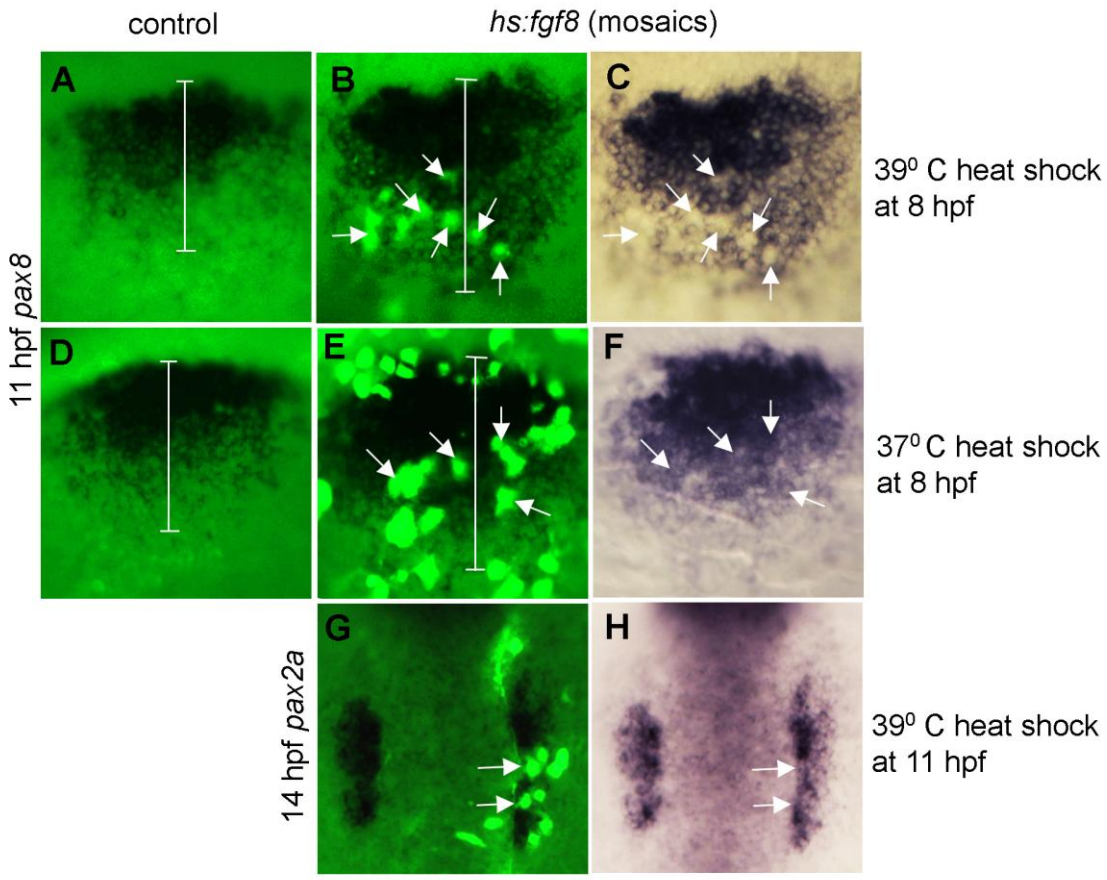


Figure 10. Cell autonomous inhibition of otic fate by high level misexpression of *hs:fgf8* at 8 hpf. (A-F) Expression of *pax8* at 11 hpf in otic domain of control embryo (A, D) and mosaic embryo (B, C, E, F) as seen under fluorescence (A, B, D, E) and bright field imaging(C, F). Note the absence and presence of *pax8* expression in transgenic cells (white arrows) in mosaic embryos after heat shock at 39^0C (B, C) and 37^0C (E, F) respectively. White bars indicate the difference in the size of the otic domain in control embryos (A, D) and mosaic embryos (B, E). (G, H) *pax2a* expression at 14 hpf in mosaic embryos after heat shock at 39^0C at 11 hpf. Note the presence of *pax2a* expression in the transgenic cells (white arrows). Images show lateral views with anterior to the left (A-F); dorsal views with anterior to the top (G, H).

we generated heat shock inducible transgenic lines to test the effects of misexpression of *pax8* or *pax2a*. Activation of *hs:pax8* at mid to late gastrula stage (8.5 hpf) caused expansion of endogenous otic domain as analyzed by early otic markers *pax2a, fgf24* and *cldna* (Fig. 11B, D and data not shown) and increased the size of the otic vesicle (Fig. 11F). Misexpression of *pax8* at tailbud stage (10 hpf) also caused expansion of the endogenous otic domain (data not shown). Similar results were observed by misexpression of *pax2a* at 8.5 hpf and 10 hpf (Fig.11H, J and data not shown). These results suggest that misexpression of *pax2/8* expands the otic field regardless of the stage of activation.

Misexpression of *pax2/8* cannot bypass the need for Fgf signaling

Although *pax2/8* act downstream of Fgf and are essential, the extent to which Pax2/8 proteins mediate the full range of effects of Fgf is not clear. We tested whether heat shock activation of *pax2a* or *pax8* can bypass the requirements of Fgf signaling for otic induction. Accordingly, we activated the transgenes at tailbud stage (10 hpf) just after the onset of otic induction and blocked Fgf signaling using SU5402, a pharmacological inhibitor of Fgf signaling. Under these conditions, expression of otic markers was blocked completely (Fig. 12D-F) indicating that *pax2/8* genes require ongoing Fgf signaling for otic induction.

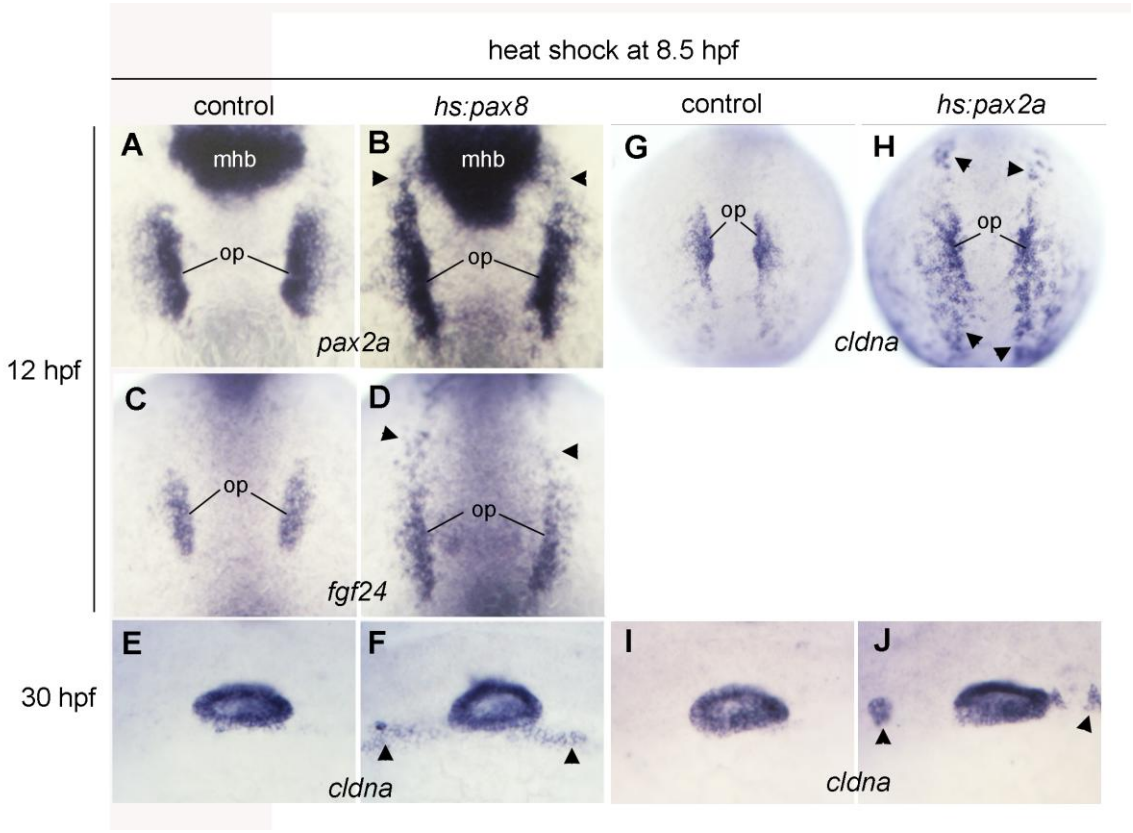


Figure 11. Expansion of otic markers following activation of *hs:pax8* or *hs:pax2a*. (A, B) Expression of *pax2a* at 12 hpf in a control embryo (A) and a *hs:pax8* transgenic embryo (B). (C, D) Expression of *fgf24* in otic domain at 12 hpf in a control embryo (C) and *hs:pax8* transgenic embryo (D). (G, H) Expression of *cldna* at 12hpf in otic domain in a control embryo (G) and *hs:pax2a* transgenic embryo (H). Black arrow heads indicate the expanded domain of otic markers. (E, F, I, J) *cldna* expression in otic vesicle at 30 hpf in control embryos (E, I), *hs:pax8* transgenic embryo (F) and *hs:pax2a* transgenic embryo (J). Black arrow heads indicate expanded domain of *cldna* (F) and expression in additional microvesicles (J). Positions of the midbrain-hindbrain boundary (mhb) and otic placode (op) are indicated. Images show dorsal view with anterior to the top (A-D, G, H); lateral view with anterior to the left (E, F, I, J).

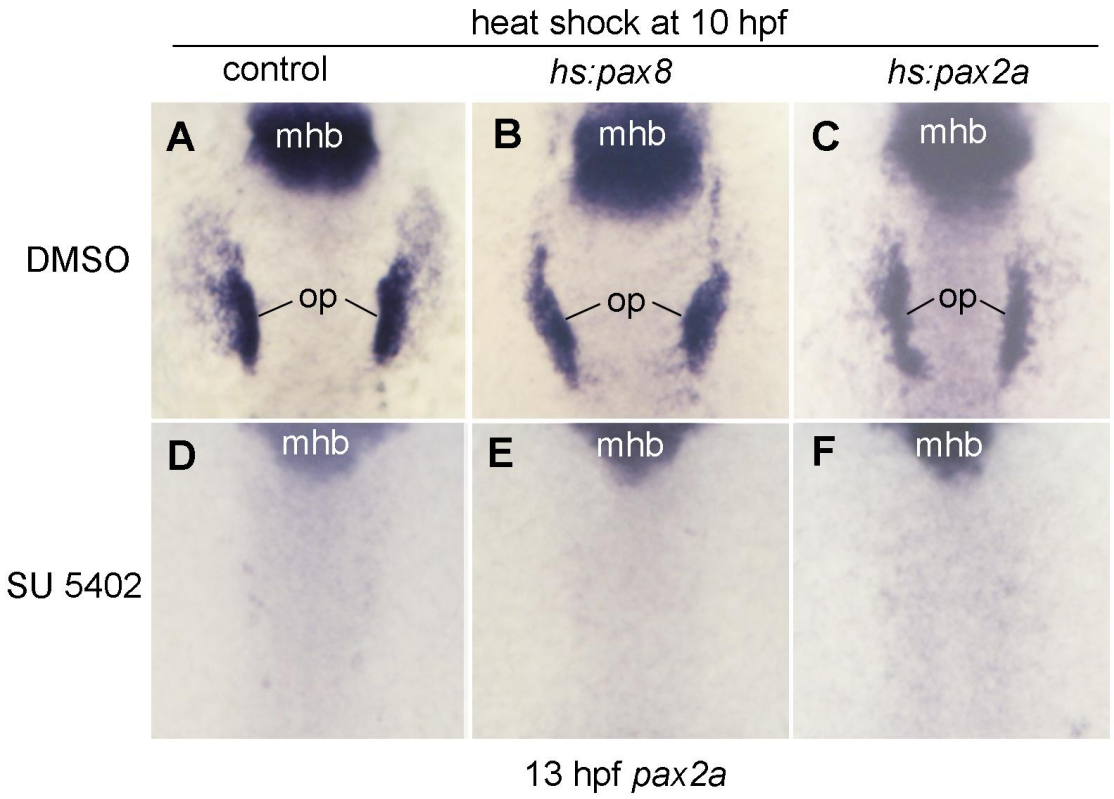


Figure 12. Elevated levels of *pax2a/8* cannot bypass the need for Fgf signaling in otic development. (A-F) *pax2a* expression at 13 hpf after heat shock at 10 hpf followed by DMSO or SU5402 treatment. (A-C) *pax2a* expression in DMSO treated control embryo (A), *hs:pax8* transgenic embryo (B), *hs:pax2a* transgenic embryo (C). (D-F) *pax2a* expression in SU5402 treated wild type (D), *hs:pax8* transgenic embryo(D) and *hs:pax2a* transgenic embryo (F). Note complete loss of *pax2a* in otic domain of SU5402 treated control and transgenic embryos. Positions of midbrain-hindbrain boundary (mhb) and otic placode (op) are indicated. All the images show dorsal views with anterior to the top.

Effects of co-misexpression of Fgf8 and Pax8/ Pax2a

We speculated that *pax2a/8* might influence the effects of Fgf on otic development. To test this idea we examined the effects of co-misexpression of *hs:fgf8* with either *hs:pax8* or *hs:pax2a*. Misexpression of *hs:fgf8* alone at 10 hpf induced formation of larger endogenous otic vesicle, but never produced microvesicles in ectopic locations (Fig. 13D and data not shown). Misexpression of either *hs:pax8* or *hs:pax2a* at 10 hpf often resulted in formation of microvesicles within the periotic tissue (between posterior edge of midbrain-hindbrain border and anterior edge of the first somite) and rarely, sometimes *hs:pax2a* embryos showed ectopic otic vesicles in the trunk region (Fig. 13B, C and Table 2 and data not shown). Co-misexpression of *hs:fgf8* with *hs:pax2a* and *hs:pax8* showed synergistic effect on induction of ectopic microvesicles (Table 2). Moreover, these vesicles expressed a full complement of otic patterning markers such as *cldna*, *dlx3b*, *otx1*, *pax2a* and *atoh1a* at 30 hpf (Fig. 13E-J and data not shown). There was three fold increase in the average number of microvesicles produced in *hs:fgf8/hs:pax2a* embryos compared to activating *hs:pax2a* alone and 90%(9/10) of these embryos had ectopic microvesicles in anterior head region and/or posterior trunk region (Fig 13F, I, J and Table 2 and data not shown). Similarly, there was two fold increase in average number of microvesicles in *hs:fgf8/hs:pax8* embryos and 60% (9/15) of the embryos had ectopic otic vesicles (Fig. 13E, G, H, and Table 2 and data not shown). These results indicate that co-misexpression of *hs:pax2a* or *hs:pax8* with *hs:fgf8* can dramatically influence the ability of Fgf to induce ectopic otic vesicles.

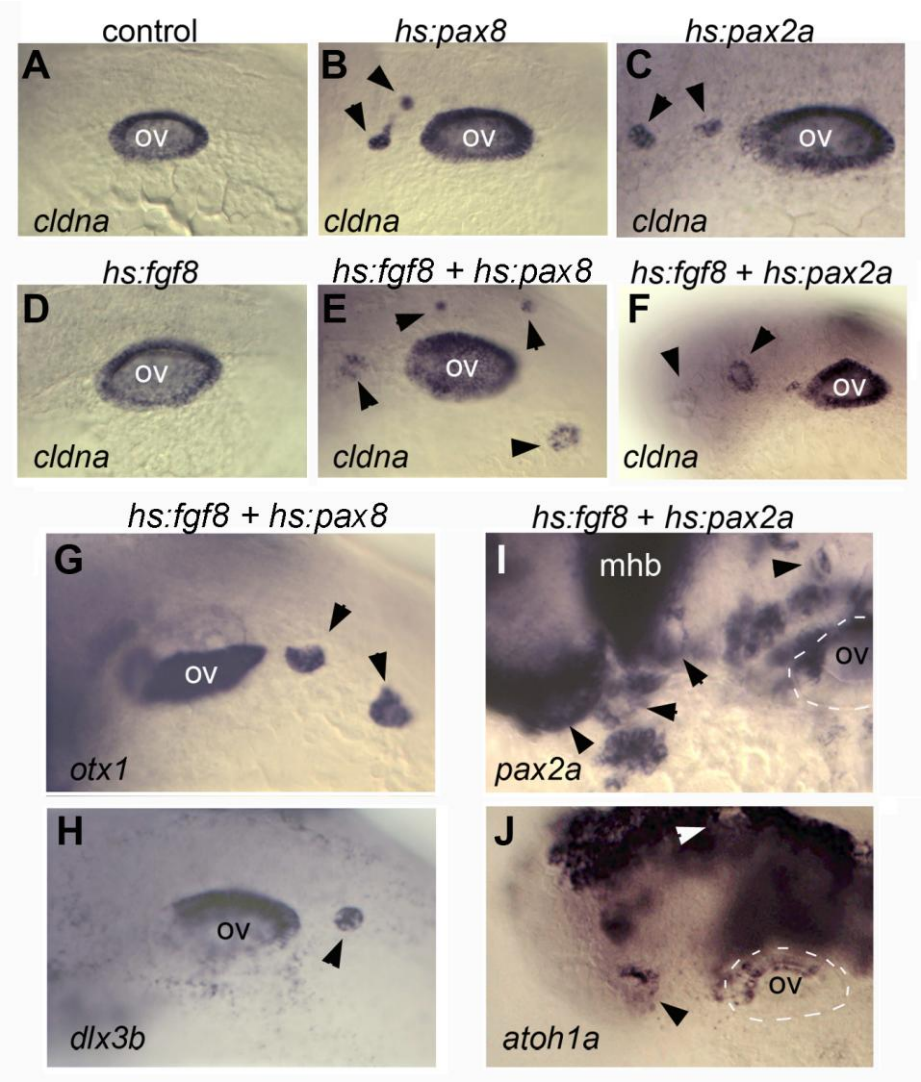


Figure 13. Co-misexpression of *fgf8* with *pax2a* or *pax8*. (A-F) Expression of *cldna* at 30 hpf after heat shock at 10 hpf in control embryo (A), *hs:pax8* transgenic embryo (B), *hs:pax2a* transgenic embryo (C), *hs:fgf8* transgenic embryo (D), *hs:fgf8;hs:pax8* transgenic embryo (E) and *hs:fgf8;hs:pax2a* transgenic embryo (F). Arrow heads in B, C indicate *cldna* expression in microvesicles formed in periotic tissue. Arrow heads in E, F indicates expression of *cldna* in ectopic microvesicles produced in brain (E) and in eye (F). Expression of *otx1* (G) and *dlx3b* (H) at 30 hpf in *hs:fgf8;hs:pax8* transgenic embryos. Expression of *pax2a* (I) and *atoh1a* (J) at 30 hpf in *hs:fgf8;hs:pax2a* transgenic embryos. White and black arrows in G-J indicate expression of respective markers in microvesicles produced in ectopic regions of trunk (G, H) and eyes and brain (I, J). Position of endogenous otic vesicle (ov) is indicated. All images show lateral views with anterior to the left.

Table 2. Production of microvesicles following global misexpression of *hs:fgf8* and/or *hs:pax2a/hs:pax8*.

	Number of embryos	Average number of microvesicles per embryo[a]	Fraction of embryos showing ectopic microvesicles
hs:pax2a	10	6.7 ± 2	2/10
hs:pax2a/hs:fgf8	10	17.4 ± 3.7	9/10
hs:pax8	12	4.4 ± 2	0/12
hs:pax8/hs:fgf8	15	9.7 ± 2.3	9/15
hs:fgf8	17	0	0/17

[a] values expressed as mean ± SD.

Misexpression of *fgf8* induces ectopic expression of *foxi1*

Hans et al. (2007) showed that misexpression of *fgf8* represses *foxi1* expression in the otic placode. This observation was paradoxical because normally *foxi1* expression is upregulated in the otic domain, a region of active Fgf signaling (Solomon et al., 2003). Other studies have shown that Fgf8 can stimulate *foxi1* expression (Nechiporuk et al., 2007; Phillips et al., 2004). So, we readdressed the relationship between Fgf and *foxi1* using the *hs:fgf8* transgenic line. Indeed, misexpression of *fgf8* at both mid gastrula (8 hpf) and tailbud (10 hpf) stages upregulated expression of *foxi1* around the anterior neural plate (Fig. 14B and data not shown). Thus Fgf signaling stimulates *foxi1* expression. Because high levels of Fgf expand otic tissue at 10 hpf and also expands *foxi1*, we tested whether Foxi1 is required for Fgf's ability to expand otic tissue.

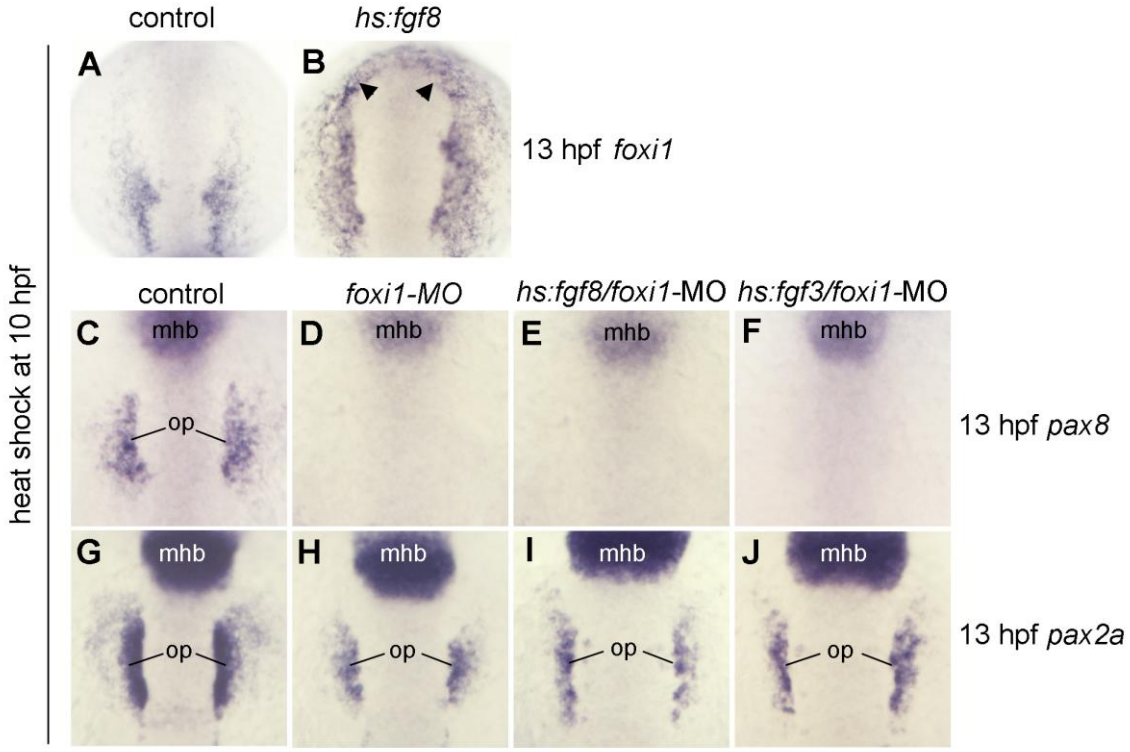


Figure 14. Interaction between Fgfs and Foxi1. (A, B) Expression of *foxi1* at 13 hpf after heat shock at 10 hpf in a control embryo (A) and *hs:fgf8* transgenic embryo (B). Black arrow heads in (B) indicates upregulation of *foxi1* expression in the anterior head region. (C-F) Expression of *pax8* at 13 hpf in control embryo (C), *foxi1* morphant (D), *hs:fgf8* transgenic embryo injected with *foxi1*-MO (E) and *hs:fgf3* transgenic embryos injected with *foxi1*-MO(F). (G-J) Expression of *pax2a* at 13 hpf in control embryo (G), *foxi1* morphant (H), *hs:fgf8* transgenic embryo injected with *foxi1*-MO (I) and *hs:fgf3* transgenic embryos injected with *foxi1*-MO (J). Positions of midbrain-hindbrain boundary (mhb) and otic placode (op) are indicated. All images show dorsal views with anterior to the top.

When Fgf was misexpressed in *foxi1* morphants, no *pax8* expression was detected in the otic domain (Fig. 14C-F). Because *pax2a* expression is dependent of Fgf but not *foxi1*, we anticipated that the domain of *pax2a* would be increased. However, activating *hs:fgf8* had little effect on the domain size of *pax2a* (Fig. 14G-J). This indicates that *pax2a* is not entirely independent of *foxi1*, which is responsible for establishing the overall size of the otic domain during induction. Thus elevating Fgf signaling can not bypass the need for *foxi1* during otic induction.

DISCUSSION

Requirements of Fgf signaling for otic induction

Our findings provide new insights into the spatial and temporal requirements for Fgf to induce otic placode. Consistent with previous findings by Hans et al. (Hans et al., 2007), our results show that global activation of either *hs:fgf8* or *hs:fgf3* at mid gastrula stages (7-8 hpf) leads to either complete loss of otic tissue or formation of very small otic vesicles (Fig. 8E, F). This observation was surprising and paradoxical because this is the time when endogenous *fgf3* and *fgf8* are expressed in the prospective hindbrain responsible for normal otic induction. Our findings show that activation of either *hs:fgf8* or *hs:fgf3* at early to mid gastrula stages (7-8 hpf) perturbs hindbrain patterning thereby disrupts the endogenous Fgf signaling center that normally induces the otic placode. In contrast, misexpression of Fgfs at tailbud stage (10 hpf) expands spatial expression of

early otic markers (Fig. 8H, I and data not shown) and leads to the formation of larger otic vesicles (Hans et al., 2007 and Fig. 8K, L) probably because of stability of endogenous signaling centers and maturation of preplacodal ectoderm to properly interpret high Fgf. Taken together our findings show that misexpression of Fgfs at early stages of embryonic development impairs otic development by perturbation of hindbrain patterning, whereas at later stages promotes otic development.

Our results show that response of cells to Fgfs greatly relies on levels of Fgf signaling and the developmental stage of the embryo. Misexpression study by plasmid injections has shown that *fgf* expressing cells induce expression of preotic markers only in the neighboring cells but never induced preotic markers within misexpressing cells, suggesting that Fgf blocks otic fate cell autonomously (Phillips et al., 2004). Consistent with previous findings, high level activation of *hs:fgf8* in mosaic embryos induces *pax8* only in the neighboring cells but never in transgenic cells themselves (Fig. 10B, C). The cell autonomous inhibition of otic fate by Fgfs is presumably because high level Fgf signaling is not compatible for otic development. Furthermore, we find that reducing the levels of transgene activity in *hs:fgf8* mosaic embryos by heat shocking at lower temperature of 37^0C for 30 minutes induces *pax8* both within *hs:fgf8* transgenic donor cells and also within the neighboring host cells (Fig. 10E, F). These results indicate that during the early phases of otic development, high levels of Fgfs blocks otic fate, whereas moderate to low levels promote otic fate cell autonomously. Most importantly, activation of *hs:fgf8* at tailbud stage (10 hpf) even at higher temperature of 39^0C for 30 minutes, which induces high levels of Fgfs, does not inhibit otic fate in donor cells

carrying *hs:fgf8* (Fig. 10G, H). We also see expansion of the otic domain when *hs:fgf8* is globally activated at 10 hpf (Fig 8H). The simplest explanation for this data is that once the otic development has been initiated, high level Fgf signaling reinforces otic fate. These findings demonstrate that level and timing of Fgf signaling are critical for otic induction.

Pax2/8 expands endogenous otic domain

Pax2/8 factors are shown to mediate otic induction downstream of Fgf signaling. Previous studies have shown that Pax2/8 play redundant functions in induction and maintenance of the otic placode (Hans et al., 2004; Mackereth et al., 2005). However, whether *pax2/8* genes are sufficient for otic development has not been addressed. Here our data show that *pax2* and *pax8* can expand the endogenous otic domain with subsequent larger otic vesicles. Misexpression of *hs:pax8* at 8.5 hpf expand the otic domain of *pax2a* and *fgf24* (Fig. 11B, D). Interestingly, misexpressing *hs:pax8* at 10 hpf led to formation of microvesicles in periotic tissue around endogenous otic vesicle, but these do not express most of the otic patterning markers except *cldna*. Our studies also show that misexpression of *hs:pax2a* at 8.5 hpf and 10 hpf expands the otic domain and induced formation of microvesicles within periotic tissue. Microvesicles formed by misexpression of *hs:pax2a* at 10 hpf express all the patterning markers analyzed (*cldna, pax2a, dlx3b, otx1, atoh1a*). The difference in outcome of *hs:pax2a* as compared to *hs:pax8* could be due to the difference in the strength of the transgene. Our findings also demonstrate that elevated levels of either *pax8* or *pax2a* after otic induction has begun is

not sufficient to bypass the need for Fgf signaling. These findings indicate that there is continuous requirement of ongoing Fgf signaling during otic development because presumably there are other unknown additional factors that are induced by Fgf signaling may co-operate with *pax2/8* genes for normal otic development.

Fgf positively regulate *foxi1* expression

Discrepancies in the literature exist regarding effects of Fgf on *foxi1*expression in the otic domain. Hans et al. (2007) showed that misexpression of *fgf8* using a transgenic line represses *foxi1* expression, whereas other studies showed that Fgf stimulates expression of *foxi1* (Nechiporuk et al., 2007; Phillips et al., 2004). We also note that *foxi1* normally shows upregulation in the preotic domain in the otic cells to the Fgf source in the hindbrain. Our data confirm that Fgf locally upregulates *foxi1* throughout preplacodal ectoderm. Foxi1 plays a general role in specification of preplacodal ectoderm, a process requiring Fgf. In this general role there is redundancy conferred by *gata3, tfap2a and tfap2c*. However, induction of *pax8* by Fgf has an absolute requirement for Foxi1 as a competence factor. Not even elevated levels of Fgf can bypass this requirement. Otic expression of *pax2a* does not require Foxi1 directly. However, Foxi1 indirectly affect setting the size of the otic domain. Hence, elevating Fgf has only a slight effect on the *pax2a* domain in *foxi1* morphants due to failure to express *pax8*.

Ectopic otic induction by co-misexpression of *hs:fgf8* and *hs:pax2a/hs:pax8*

Misexpression of either *hs:fgf8* and/or *hs:fgf3* globally or in mosaic embryos at any developmental stage failed to produce ectopic otic vesicles. However, misexpression of *hs:fgf8* or *hs:fgf3* after tailbud stage (10 hpf) resulted in production of larger endogenous otic vesicles. The possible reasons for no ectopic otic vesicles could be that the levels of the transgene may not be sufficient to induce ectopic otic vesicles in the *hs:fgf8 or hs:fgf3* heterozygous embryos. Further studies with *hs:fgfs* homozygous embryos may resolve this issue of optimum levels of Fgf necessary for ectopic otic induction. Furthermore, the transgene does not remain active long enough to either induce or to maintain the otic fate after a single pulse of heat shock. As previously shown, activation of *hsp70* heat shock promoter typically results in elevated transcripts levels of the transgene for 90 minutes followed by gradual decay over the next few hours (Hans et al., 2007 and our unpublished data). Also a study in zebrafish has shown that ongoing Fgf signaling is necessary after otic induction to maintain otic fate and to regulate patterning in the otic vesicle (Leger and Brand, 2002). Moreover, global misexpression of *hs:fgf8* or *hs:fgf3* does not imitate the normal process of otic induction from a point source(eg. Fgf signals from rhombomere-4 of hindbrain). Furthermore, the domain of otic competence may become increasingly localized as development proceeds, presumably reflecting specification or differentiation of other cell fates at other sites. Consistent with our reasoning, studies in *Xenopus* and chick have shown that, at early gastrula stages, most of the ectoderm is capable of responding to otic induction, but by late gastrula or early somitogenesis stages, regional biases become

discernable in the ability of ectoderm to respond to otic induction (Gallagher et al., 1996 ; Groves and Bronner-Fraser 2000). It is also possible that foreign sites may lack essential co-factors needed for normal Fgf activity or alternatively other regionally expressed factors may interfere with Fgf, there by inhibiting induction of ectopic otic vesicles.

Our findings show that co-misexpression of *hs:pax2a* or *hs:pax8* with *hs:fgf8* have synergistic effects on otic development resulting in induction of ectopic otic vesicles in the anterior head regions of eye and forebrain and in posterior trunk regions. In addition, the size of the endogenous otic vesicle was larger with several microvesicles within periotic tissue. Co-misexpression of Pax2/8 with Fgf8 induces formation of ectopic otic vesicles. Presumably Pax2/8 appears to act as tissue specific or regional specifiers, wherein *pax2a/8* genes might influence the ability of cells in ectopic locations to respond to Fgf signals appropriately, leading to formation of ectopic otic vesicles.

CHAPTER III

PAX2/8 PROTEINS COORDINATE SEQUENTIAL INDUCTION OF OTIC AND EPIBRANCHIAL PLACODES THROUGH DIFFERENTIAL REGULATION OF *foxi1*, *sox3* AND *fgf24* *

INTRODUCTION

In vertebrate embryos, cranial placodes form as a series of epithelial thickenings around the anterior neural plate and contribute to sensory structures of the head (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser, 2006). All placodes are derived from a contiguous zone of preplacodal ectoderm, which forms in the head along the neural-nonneural interface during gastrulation (Streit, 2007). The preplacodal ectoderm then generates the diverse array of placodal fates in response to different regional signals. The otic placode, which gives rise to the inner ear, has been the most extensively characterized of all cranial placodes.

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Otic development is initiated by Fgf ligands secreted by the hindbrain and subjacent mesendoderm (Alvarez et al., 2003; Ladher et al., 2000; Ladher et al., 2005; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Riley and Phillips, 2003; Wright and Mansour, 2003). Some of the earliest markers of otic development are members of the Pax2/8 family of transcription factors (Pfeffer et al., 1998). The functions of Pax2 and Pax8 in regulating early otic development have been most extensively studied in zebrafish (Hans et al., 2004; Mackereth et al., 2005). Otic expression of *pax8* begins during late gastrulation and requires both Fgf signaling and the otic-competence factor Foxi1 (Hans et al., 2004; Hans et al., 2007; Phillips et al., 2001; Solomon et al., 2003, 2004;). By early somitogenesis stages, expression of related genes *pax2a* and *pax2b* is also detected in the preotic placode (Pfeffer et al., 1998). Otic expression of *pax2a/b* requires Fgf, but not *foxi1* (Hans et al., 2004; Léger and Brand, 2002; Solomon et al., 2003, 2004). Despite these slight differences in regulation, *pax8* and *pax2a/b* function together and provide substantial redundancy during otic development. Impairment of both *pax2a* and *pax2b* has little effect on otic induction, whereas impairment of *pax8* leads to production of a reduced otic placode (Hans et al., 2004; Mackereth et al., 2005). In *pax2a/pax2b/pax8*-depleted embryos, a small otic placode initially forms but eventually disperses as cells lose otic identity (Mackereth et al., 2005). Thus, *pax2/8* genes are together necessary for normal induction and maintenance of the otic placode. How *pax2/8* genes mediate these functions is still unknown. Moreover, because some otic tissue initially forms in the

absence of *pax2/8* function, there must be additional genes that help mediate the effects of Fgf during otic induction.

Another gene coexpressed with *pax2/8* in the otic primordium is *sox3* (Nikaido et al., 2007; Sun et al., 2007). Like *pax8*, otic expression of *sox3* also requires Fgf and *foxi1* (Lee et al., 2003; Nechiporuk et al., 2007; Sun et al., 2007). In mouse and zebrafish, disruption of *Sox3* causes mild-to-moderate reduction in the size of the otic vesicle (Dee et al., 2008; Okuda et al., 2010; Rizzoti and Lovell-Badge, 2007). However, otic patterning has not been examined in detail in these backgrounds. Additionally, genetic interactions between *sox3* and *pax8* have not been investigated, leaving open the question of whether these genes cooperate to mediate otic induction.

Epibranchial placodes constitute a distinct set of placodes with fates quite different from the otic placode, yet there are striking parallels between early development of these placode-types (Ladher et al., 2010). Epibranchial placodes give rise to a series of sensory ganglia associated with the mouth and throat, including the facial, glossopharyngeal and vagal ganglia. Like the otic placode, epibranchial placodes require the same upstream regulators, Fgf and Foxi1, and both express *pax8* and *sox3* as early response factors. Moreover, fate-mapping studies show that otic and epibranchial precursors lie close together during early development, with epibranchial placodes emerging from an arc of ectoderm wrapping around the lateral edge of the otic territory (Streit, 2002; Sun et al, 2007). These similarities have led to the hypothesis that Fgf initially specifies a common otic/epibranchial field, which later splits into adjacent compartments with distinct fates (Freter et al., 2008; Ladher et al., 2010; Ohyama et al.,

2006; Schlosser and Ahrens, 2004; Sun et al., 2007). However, close examination of early markers suggests that otic and epibranchial placodes are induced at different times, possibly by distinct mechanisms. Initially, *pax8* and *sox3* are coexpressed within a relatively small domain adjacent to rhombomere 4 of the hindbrain. This appears to correspond to the otic domain in zebrafish because at least two otic-specific markers, *atoh1b* and *fgf24*, are soon induced within the same domain (Draper et al., 2003; Millimaki et al., 2007). A dramatic transition occurs Between 3 and 6 somites stage (11 hpf and 12 hpf) as *sox3* downregulates within the otic domain and spreads outward into the prospective epibranchial domain (Nikaido et al., 2007; Sun et al., 2007). Similarly, expression of *foxi1* is abruptly lost from otic cells but is maintained at high levels in epibranchial cells. In contrast, *pax8* and *pax2a* remain highly expressed in the otic domain but shows only weak expression in the epibranchial domain (Pfeffer et al., 1998; Phillips et al., 2001). The regulation and functional significance of these dynamic changes have not been established.

Here we have reexamined early regulation of otic and epibranchial development. Our data confirm that the otic placode forms first and that *pax8* and *sox3* interact synergistically to promote otic induction. Subsequently, *pax8* works redundantly with *pax2a* and *pax2b* to promote two distinct functions in the otic placode. First, *pax2/8* repress otic expression of *foxi1*. This is necessary to maintain otic fate, as artificially maintaining *foxi1* expression blocks further otic development. Second, *pax2/8* activate otic expression of *fgf24*. Fgf24 in turn downregulates *sox3* in the otic domain and induces *sox3* in the epibranchial domain. Knockdown of *fgf24* has little effect on otic

development but causes a severe deficiency of epibranchial ganglia, similar to knocking down *sox3* directly. These data support a new model wherein the otic placode forms first and subsequently induces formation of epibranchial placodes through *pax2/8*-dependent Fgf24 signaling. The data also support a key role for *pax8* in orchestrating the dynamic changes in early gene expression that distinguish otic from epibranchial fates.

MATERIALS AND METHODS

Strains and developmental conditions

The wild type strain was derived from AB line (Eugene, OR). The noi^{tu29a} mutation is a null allele (Lun and Brand, 1998) and was used to assess function of *pax2a*. Transgenic lines used in this study include *Tg(hsp70:fgf8a)*^{x17} (Millimaki et al., 2010), *Tg(hsp70:foxi1)*^{x19} (Kwon et al., 2010) and *Tg(brn3c:gap43-GFP)*^{s356t} (Xiao et al., 2005). For convenience, these transgenes are referred to in the remainder of the text as *hs:fgf8*, *hs:foxi1* and *brn3c:GFP*, respectively. Embryos were developed at standard conditions of 28.5^{0}C in fish water containing methylene blue and were staged based on standard protocols (Kimmel et al., 1995).

In situ hybridization

In situ hybridization was carried out at 67^0C as described previously (Jowett and Yan, 1996; Phillips et al., 2001).

Morpholinos

Translation–blocking morpholino oligomers (MOs) obtained from Gene Tools Inc. were used to block gene function. MOs were injected into embryos at one-cell. All MO sequences used in this study have been previous described and tested for efficacy and specificity. To knockdown *pax8*, wild-type embryos were injected with 2.5 ng each of variant 1 MO (5'-GTTCACAAACATGCCTCCTAGTTGA-3') and variant 2/3 MO (5'- GACCTCGCCCAGTGCTGTTGGACAT-3') as previously described (Mackereth et al., 2005). To knock down *fgf24*, embryos were coninjected with 5 ng *fgf24*-MO, 5'-GACGGCAGAACAGACATCTTGGTCA-3' (Fischer et al., 2003) and, to inhibit non-specific cell death, 7.5 ng of *p53*-MO (Robu et al., 2007). Other morpholinos used in this study include *pax2b*-MO, 5'-GGTCTGCCTTACAGTGAATATCCAT-3' (5 ng/embryo, Mackereth et al., 2005); and *sox3*-MO1 5'-TACATTCTTAAAAGTGGTGCCAAGC-3' (5 ng/embryo, Okuda et al., 2010).

Gene misexpression

To misexpress *foxi1* or *fgf8* from heat shock-inducible transgenes, heterozygous transgenic embryos were heat shocked at 39 °C for 30 min at the indicated times. After heat shock, embryos were incubated at 33°C until fixation.

Cell transplantation

Donor embryos were injected with lineage tracer (lysine-fixable biotinylated dextran, 10000 MW, in 0.2 M KCl) at the one-cell stage. Labeled cells were transplanted from blastula stage donors into non-labeled hosts of the same stage. Transplanted cells were identified in the hosts by streptavidin-FITC antibody staining.

RESULTS

Previous studies have shown the importance of Fgf signaling in otic and epibranchial induction, but there is still much to learn about the factors that mediate Fgf signaling. Fgf initially induces expression of *pax8* and *sox3* in the otic primordium by 9.5 hpf (late gastrulation). Another otic marker, *pax2a*, is coexpressed in the otic domain by 11 hpf (1-3 somites stage). By 12 hpf, expression of *sox3* begins to downregulate in the otic placode as it expands outward into prospective epibranchial ectoderm (Nikaido et al., 2007; Sun et al., 2007, Fig. 15D). Weaker expression of *pax8* and *pax2a* is also detected in the epibranchial anlagen by 12 hpf, whereas higher levels persist in otic domain (Fig. 15A, B). By comparison, expression of *fgf24* remains restricted to the otic domain throughout placodal development (Draper et al., 2003; Fig. 15C).

The roles of *pax8* and *pax2a* in otic induction have been partially characterized, but their roles in epibranchial development have not been determined, nor have the roles

of *sox3* and *fgf24* been determined. To address these questions, we injected morpholino oligomers (MOs) to knock down these genes and assessed the effects on otic and epibranchial development.

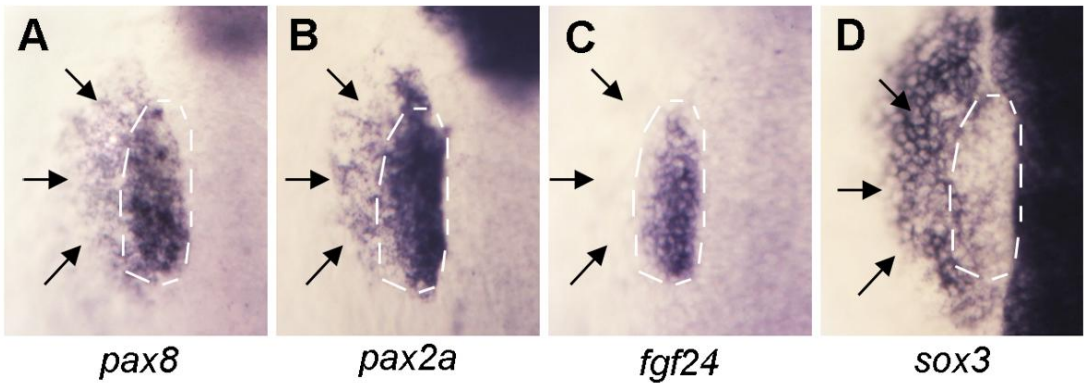

Figure 15. Spatial domains of otic and epibranchial markers at 12 hpf. Dorsal views showing expression of *pax8* (A), *pax2a* (B), *fgf24* (C) and *sox3* (D) in wild-type embryos at 12 hpf. Otic domains (white dashed lines) and epibranchial domains (black arrows) are indicated. Unlike the other genes, *fgf24* expression is limited to the otic domain.

***sox3* and *pax8* cooperate to regulate otic and epibranchial induction**

We first examined the effects of knocking down the earliest otic markers, *pax8* and/or *sox3*, on otic development. Knockdown of *sox3* alone caused a 9±2% reduction in the area of the otic/epibranchial domain of *pax2a* at 12 hpf, but subsequent formation of the otic vesicle was nearly normal (Fig. 16B, F). Consistent with previous findings (Mackereth et al., 2005), knockdown of *pax8* reduced the area of *pax2a* expression to 63±4% of normal, with a similar reduction in the size of the otic vesicle (Fig. 16C, G). The expression domains of *pax8* and *sox3* were similarly reduced at 10 hpf (not shown). Knockdown of both genes caused further reduction in otic development, such that the area of the *pax2a* domain was only 38±2% of normal and the otic vesicle was similarly reduced (Fig. 16D, H). Patterning in the otic vesicle was relatively normal in embryos knocked down for *pax8* and/or *sox3*, though expression domains of all markers were reduced in proportion to the overall size of the otic vesicle (Fig. 17A-L). Thus *sox3* and *pax8* both regulate otic placode induction. *pax8* function appears more critical than *sox3*, but the strong enhancement of otic deficiency in *pax8-sox3* double morphants shows that each gene provides unique functions required for early otic development.

Because Sox3 has been implicated in regulation of sensory-neural regions of the otic vesicle in chick (Abelló et al., 2010), we also examined formation of sensory epithelia and neurons of the stato-acoustic ganglion (SAG) in embryos knocked down for *sox3* and/or *pax8*. *sox3* morphants produced sensory epithelia with roughly normal

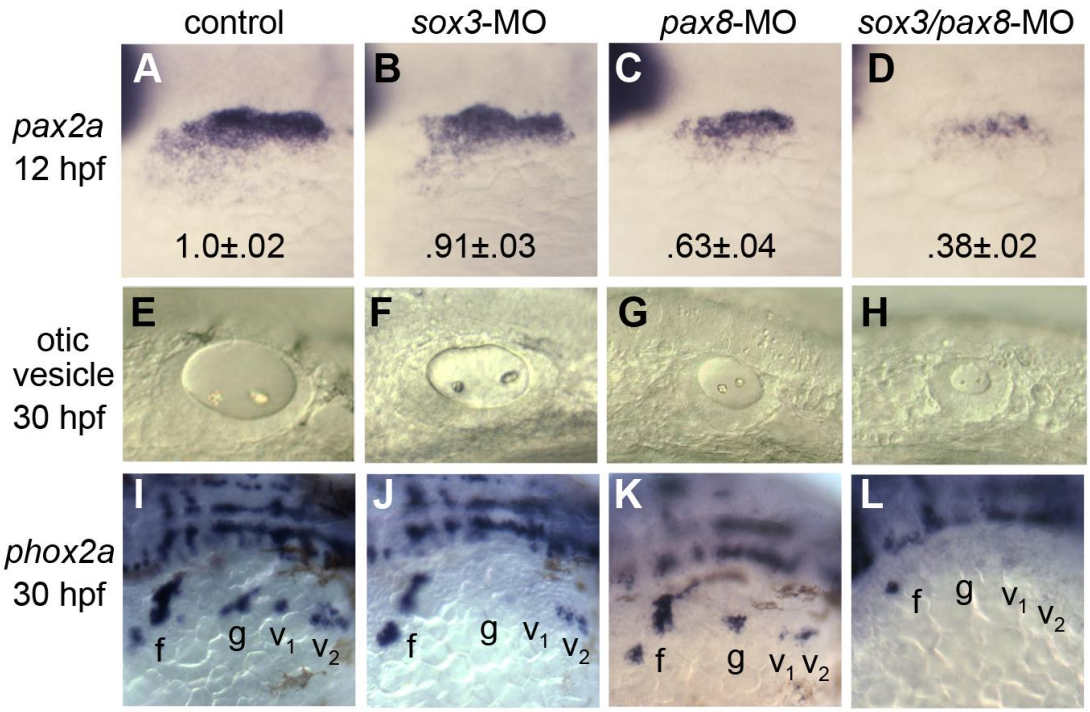

Figure 16. *pax8* and *sox3* interact in otic and epibranchial induction. (A-D) *pax2a* expression in the otic/epibranchial domain at 12 hpf in a control embryo (A), *sox3* morphant (B), *pax8* morphant (C) and *sox3-pax8* double morphant (D). Numbers indicate normalized values for the mean ± standard deviation of the area of the *pax2a* expression domain (n=10 specimens for each background). Area was calculated by outlining otic-epibranchial domains in Photoshop and measuring the number of pixels within. Differences the morphants and the control were highly significant ($p<.0005$) as determined by t-tests. (E-H) otic vesicles at 30 hpf in a live control embryo (E), *sox3* morphant (F), *pax8* morphant (G) and *sox3-pax8* double morphant (H). (I-L) expression of *phox2a* at 30 hpf in a control embryo (I), *sox3* morphant (J), *pax8* morphant (K) and *sox3-pax8* double morphant (L). Positions of the facial ganglion (f) glossopharyngeal ganglion (g), and vagal ganglia (v) are indicated. All images show lateral views with anterior to the left.

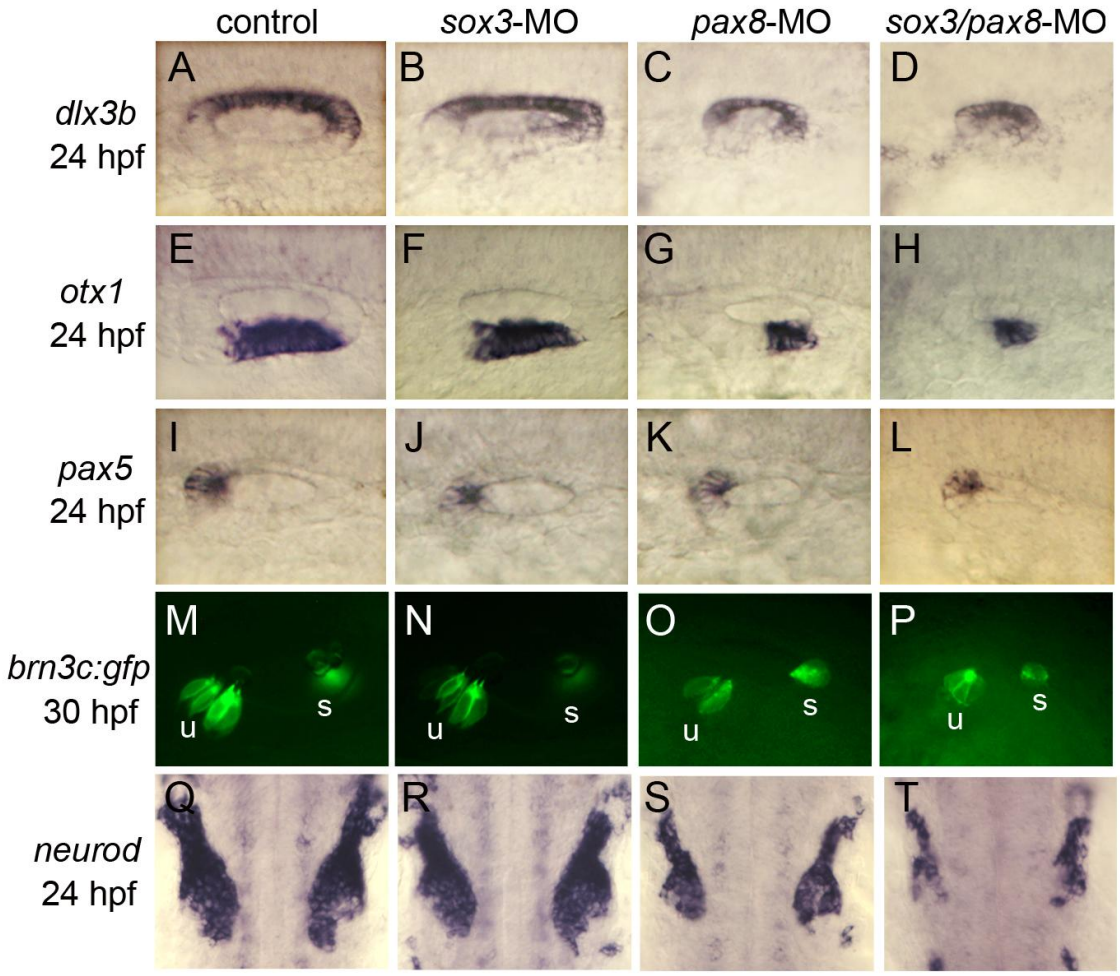

Figure 17. Patterning of the otic vesicle in *sox3–pax8* morphants. Expression of *dlx3b*, *otx1*, *pax5*, and *neurod* in the otic vesicle of control embryos (A, E, I, Q), *sox3* morphants (B, F, J, R), *pax8* morphants (C, G, K, S) and *sox3–pax8* double morphants (D, H, L, T) at 24 hpf. (M–P) *brn3c:GFP* expression at 30 hpf in a control embryo (M), *sox3* morphant (N), *pax8* morphant (O) and *sox3–pax8* double morphant (P). Positions of utricular (u) and saccular (s) maculae are indicated. Images show dorsolateral views with anterior to the left (A–P); dorsal views with anterior to the top (Q–T).

numbers of hair cells, as marked by *brn3c:GFP* expression. SAG development was also normal based on expression of proneural gene *neuroD*, as well as accumulation of mature Islet1-positive SAG neurons (Fig. 17M-T, Table 3). In *pax8* morphants and *pax8-sox3* double morphants, sensory epithelia and SAG neurons formed but were reduced in size as expected from the diminished size of the otic vesicle. These data suggest that *pax8* and *sox3* are not directly required for development of sensory epithelia or SAG neurons in zebrafish. Instead these genes interact to control the amount of otic tissue induced, which indirectly affects that amount of sensory-neural tissue produced.

To monitor epibranchial development following gene knockdown, we examined expression of *phox2a*, which marks all epibranchial ganglia by 30 hpf (Begbie et al., 1999; Lee et al., 2003; Nechiporuk et al., 2005). Previous studies have shown that *sox3* is required for normal development of epibranchial ganglia (Dee et al., 2008; Rizzoti and Lovell-Badge, 2007). We confirmed that *sox3* morphants develop with a substantial deficiency of *phox2a*-expressing epibranchial ganglia, with almost total loss of the glossopharyngeal and anterior vagal ganglia (Fig. 16J). Pax8 has not previously been shown to regulate epibranchial placode development, but we tested this possibility because *pax8* is expressed at a low level in at least part of the epibranchial primordium by 12 hpf (Hans et al., 2004; Phillips et al., 2001; Fig. 15A). Although *pax8* morphants developed with only a slight reduction in epibranchial ganglia (Fig. 16K), *pax8-sox3* double morphants showed complete loss of all epibranchial ganglia (Fig. 16L). Thus, *pax8* and *sox3* are together indispensable for development of epibranchial ganglia.

Table 3. Number of SAG neurons and hair cells in 30 hpf embryos.

	Control	*sox3*-MO	*pax8*-MO	*sox3/pax8*MO	*fgf24*-MO
No. of SAG neurons [a]	28.9 ± 2.1 n = 20	24 ± 2.2 n = 20	12.5 ± 1.8 n = 20	8.1 ± 1.4 n = 20	27.2 ± 1.9 n = 20
No. of hair cells in the utricular macula [a]	6.3 ± 0.6 n = 14	6.2 ± 0.4 n = 14	4.8 ± 0.4 n =14	3.1 ± 0.5 n = 14	6.3 ± 0.7 n=20
No. of hair cells in the saccular macula [a]	3.9 ± 0.8 n = 14	3.8 ± 0.7 n = 14	2.2 ± 0.4 n = 14	2.1 ± 0.3 n = 14	3.8 ± 0.4 n=20

[a] values expressed as mean ± SD. n, sample size. MO, morphants, hpf, hours post fertilization.

Downregulation of *foxi1* and *sox3* in the otic placode

Although *pax8* and *sox3* are initially coinduced in the otic anlagen by Fgf, *sox3* soon downregulates in otic cells as they develop. Because *pax8* expression persists in the otic domain, we speculated that *pax8* might directly or indirectly repress otic expression of *sox3*. Indeed, downregulation of *sox3* in the otic placode was delayed by at least 3 hours in *pax8* morphants (Fig. 18E, and data not shown). Surprisingly, induction of *sox3* in the epibranchial domain was also delayed by 3 hours, consistent with a non-autonomous role for *pax8* (see below).

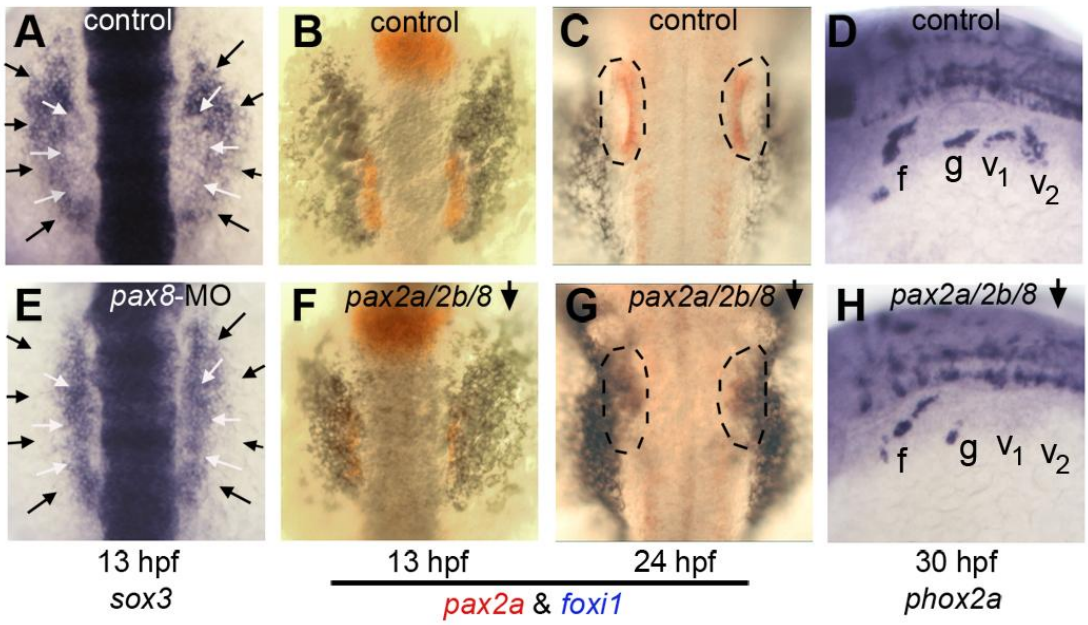

Figure 18. Requirement for *pax2/8* in otic and epibranchial development. (A, E) expression of *sox3* at 13 hpf in a control embryo (A) and *pax8* morphant (E). White arrows indicate the lateral edges of the otic domain and black arrows indicate the edges of the epibranchial domain. (B, C, F, G) two color *in situ* hybridization of embryos at 13 hpf (B, F) and 24hpf (C, G) showing expression of *pax2a* (red) and *foxi1*(blue). Outlines indicate the otic vesicle (C) or vestigial otic region (G). Expression patterns are shown in control embryos (B, C) and *pax2a/pax2b/pax8*-deficient embryos (F, G). (D, H) expression of *phox2a* at 30 hpf in a control embryo (D) and *pax2a/2b/8*-deficient embryo (H). Images show dorsal views with anterior to the top (A-C, E-G); dorsolateral views with anterior to the left and dorsal to the top (D, H). Positions of the facial ganglion (f), glossopharyngeal ganglion (g) and vagal ganglia (v_1 and v_2) are indicated.

We also tested whether *pax8* modulates *foxi1* expression during otic/epibranchial development. Foxi1 initially serves as a competence factor for establishing the entire preplacodal ectoderm (Kwon et al., 2010), and its expression later becomes restricted to the otic and epibranchial primordia where its function is especially critical (Hans et al., 2007; Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003). As development proceeds, *foxi1* expression normally begins to downregulate in the otic domain by 11 hpf whereas it is maintained in epibranchial ganglia through at least 36 hpf (Lee et al., 2003). In *pax8* morphants, however, exclusion of *foxi1* from the otic placode was delayed by about 2 hours (data not shown). Because *pax2a* and *pax2b* are later coexpressed in the otic placode and are partially redundant with *pax8*, we tested the effects of disrupting all known *pax2/8* function. In *pax2a/pax2b/pax8*-deficient embryos, strong *foxi1* expression was maintained in the otic region through at least 24 hpf (Fig. 18F, G), by which time otic identity is lost (Mackereth et al., 2005). These data show that Pax2/8 proteins directly or indirectly repress *foxi1* transcription in the otic placode.

Although *foxi1* is required to initiate otic development, we hypothesized that failure to downregulate *foxi1* at later stages impedes further otic development. To test this idea, we made use of a stable transgenic line to misexpress *foxi1* under the control of a heat shock promoter (Kwon et al., 2010). Global activation of *hs:foxi1* expression at 11 hpf caused a dramatic reduction in the size of the otic placode by 14 hpf (Fig. 19B). Additionally, otic expression of *pax2a* was irregular and spotty. Because global misexpression of *foxi1* possibly interferes with essential signals from other tissues, we

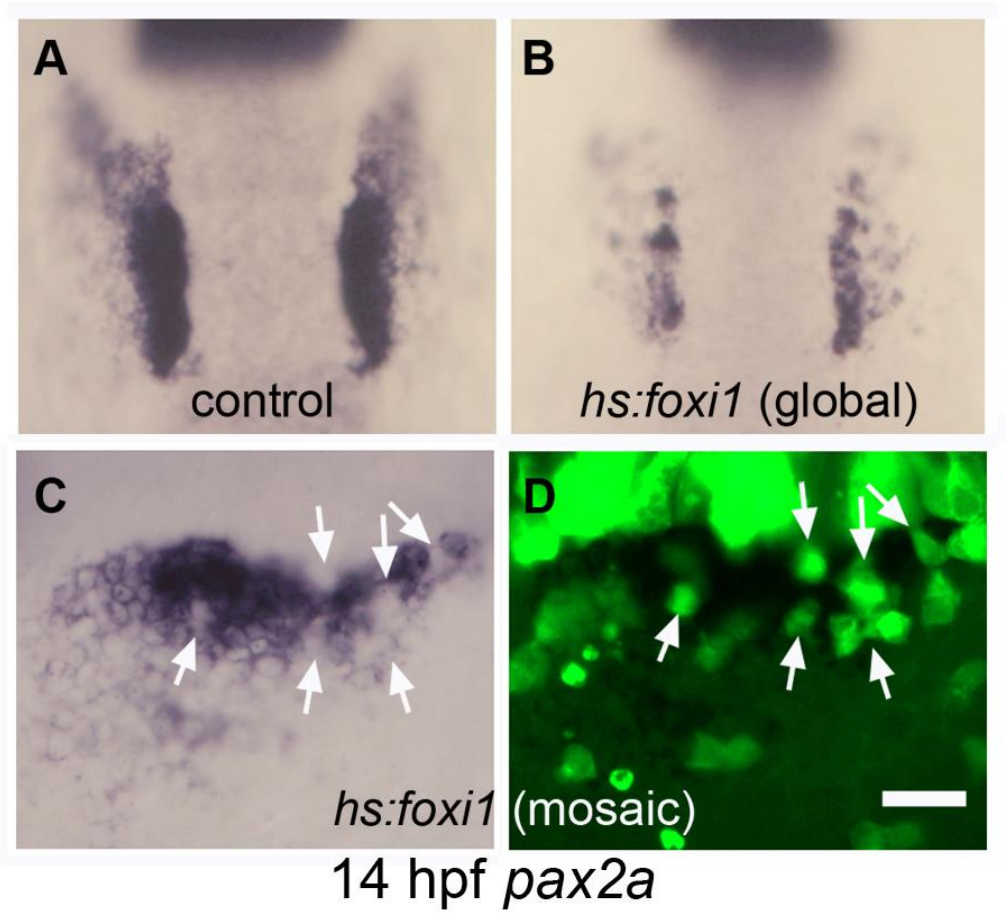

Figure 19. Misexpression of *foxi1* inhibits otic development. (A, B) expression of *pax2a* at 14 hpf in a control embryo (A) and a *hs:foxi1* transgenic embryo (B) heat shocked at 11 hpf. (C, D) expression of *pax2a* at 14 hpf in a mosaic embryo as seen under bright field (C) and fluorescence imaging (D). The mosaic was produced by transplanting lineage-labeled *hs:foxi1* transgenic cells (green fluorescence) into a non-transgenic host. The embryo was heat shocked at 11 hpf to activate the transgene. Note the absence of *pax2a* expression in transgenic cells (white arrows). Images show dorsal views with anterior to the top (A-B); lateral views with anterior to the left (C-D). Scale bar, 50 µm (A, B), 25 µm (C, D).

generated mosaic embryos by transplanting cells from *hs:foxi1* transgenic embryos into non-transgenic host embryos. Activation of *hs:foxi1* in mosaic embryos caused loss of expression of *pax2a* in transgenic cells within the otic region (Fig. 19C, D). These data indicate that maintaining *foxi1* expression after 11 hpf impairs completion of otic development in a cell-autonomous manner. This could explain why otic cells eventually lose otic identity in *pax2a/pax2b/pax8*-deficient embryos (Mackereth et al., 2005).

Loss of otic fate in *pax2a/pax2b/pax8*-deficient embryos does not involve death of the otic placode, as these cells persist in the otic-epibranchial area through at least 24 hpf (Mackereth et al., 2005). We hypothesized that some of these cells might switch fate and contribute to epibranchial tissue instead. However, development of epibranchial ganglia was severely impaired in *pax2a/pax2b/pax8*-deficient embryos (Fig, 18H). These data are consistent with loss of epibranchial expression of *sox3* (Fig. 18E, and data not shown), further indicating that *pax2/8* genes are required directly or indirectly for development of epibranchial placodes.

Pax2/8 regulate expression of *fgf24* in the otic placode

Expression of *fgf24* is limited to the otic placode and is first expressed there by 10.5 hpf, shortly after the onset of *pax8* expression (Draper et al., 2003; Fig. 15C, and our unpublished observation).

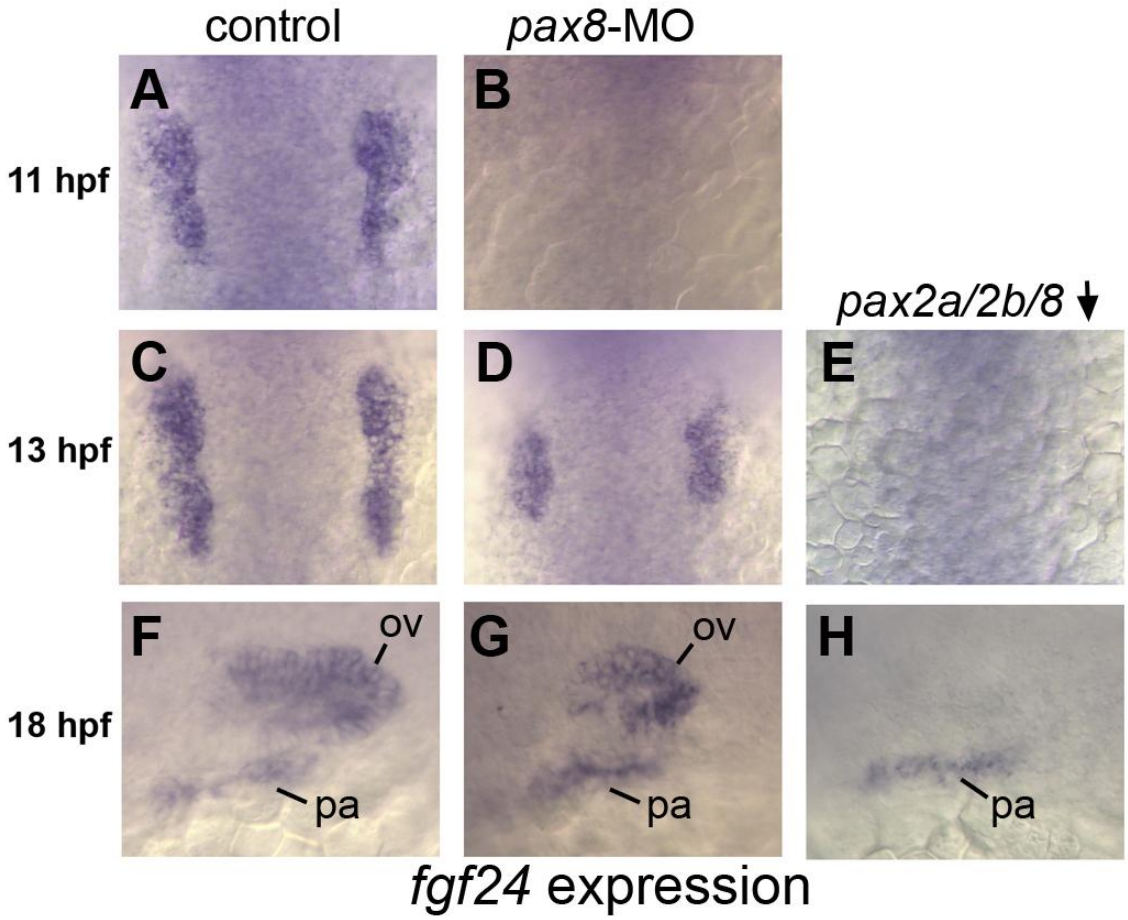

Figure 20. *pax2/8* regulates otic expression of *fgf24*. (A, C, F) *fgf24* expression in the otic placode in control embryos at 11 hpf (A), 13 hpf (C) and 18 hpf (F). (B, D, G) *fgf24* expression in *pax8* morphants at 11hpf (B), 13 hpf (D) and 18 hpf (G). Expression of *fgf24* is lost from preotic placodes in *pax8* morphants at 11 hpf (B) and is reduced in *pax8* morphants at 13 hpf (D) and 18 hpf (G). (E, H) *noi* (*pax2a*) mutants co-injected with *pax8*-MO and *pax2b*-MO showing loss of otic expression of *fgf24* at all time points. Expression in pharyngeal (pa) arches and the otic vesicle (ov) is indicated. Images show dorsal views with anterior to the top (A-E); lateral views with anterior to the left and dorsal to the top (F-H).

We therefore tested whether *pax8*, which is critical for controlling the size of the otic placode, is required to activate this domain of *fgf24* expression. Indeed, otic expression of *fgf24* is delayed until 13 hpf in *pax8* morphants (Fig. 20B, D). We hypothesized that belated expression of *fgf24* reflects the activation of *pax2a* and *pax2b*. In support, *pax2a/pax2b/pax8*-deficient embryos fail to express *fgf24* in otic tissue through at least 18 hpf, although *fgf24* expression occurs normally in pharyngeal arches (Fig. 20F-H). Thus, one of the functions of Pax2/8 during otic induction is to activate expression of *fgf24*. In contrast, knockdown of *sox3* had no effect on the onset of *fgf24* expression (not shown).

Fgf24 is not required for otic development

The function of *fgf24* in otic development has not been investigated. To test this we injected wild-type embryos with morpholino to knockdown *fgf24*. *fgf24* morphants develop with a normally sized otic placode, judging by expression of *pax8* at 11 hpf (Fig. 21E). Like *pax8* morphants, *fgf24* morphants fail to downregulate expression of *sox3* in the otic placode (Fig. 21F). In contrast, expression of *foxi1* showed a normal pattern of exclusion from otic cells in *fgf24* morphants (Fig. 21C, G). Furthermore, we could detect no changes in expression of regional markers within the otic vesicle, nor in development of sensory epithelia and SAG neurons (Fig. 22 and Table 3). Thus, *fgf24* is not required for otic placode induction or subsequent development and patterning of the otic vesicle. Additionally, the data show that failure to downregulate *sox3* in the otic placode in *fgf24* morphants does not adversely affect patterning and differentiation

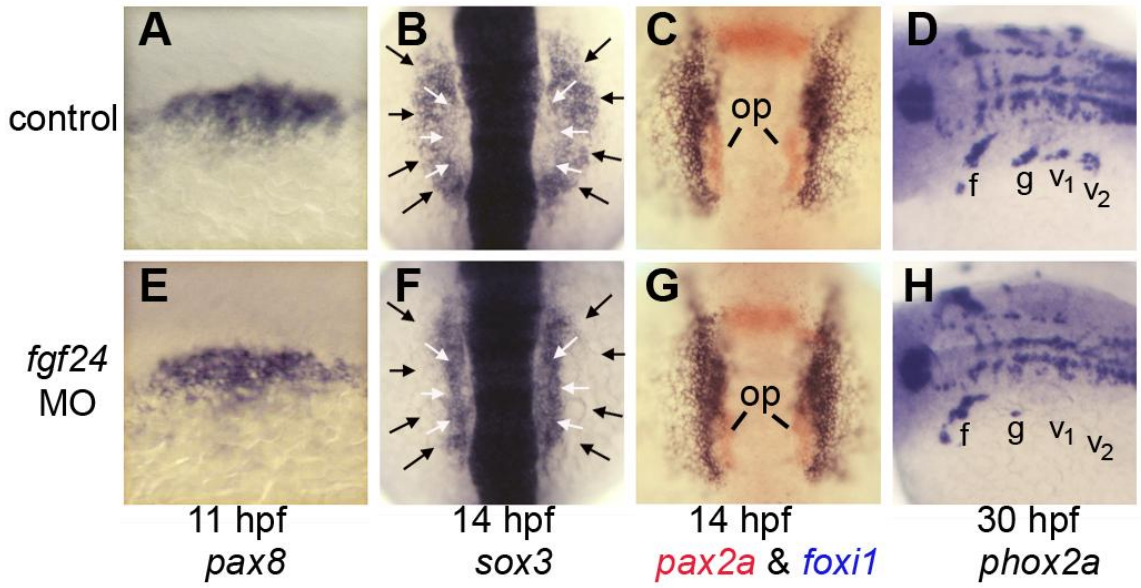

Figure 21. *fgf24* is required for epibranchial development. (A, B) expression of *pax8* at 11 hpf in a control embryo (A) and *fgf24* morphant (E). (B, F) expression of *sox3* at 14 hpf in a control embryo (B) and *fgf24* morphant (F). White arrows indicate the lateral edges of the otic domain and black arrows indicate the lateral edges of the epibranchial domain. (C, G) two color *in situ* hybridization showing *pax2a* (red) and *foxi1* (blue) in a control embryo (C) and *fgf24* morphant (G) at 14 hpf. Positions of otic placodes (op) are indicated. (D, H) expression of *phox2a* at 30 hpf in a control embryo (D) and *fgf24* morphant (H). Positions of the facial ganglion (f), glossopharyngeal ganglion (g) and vagal ganglia (v_1 and v_2) are indicated. Images show dorsolateral views with anterior to the left (A, D, E, H); dorsal views with anterior to the left and dorsal to the top (B, C, F, G).

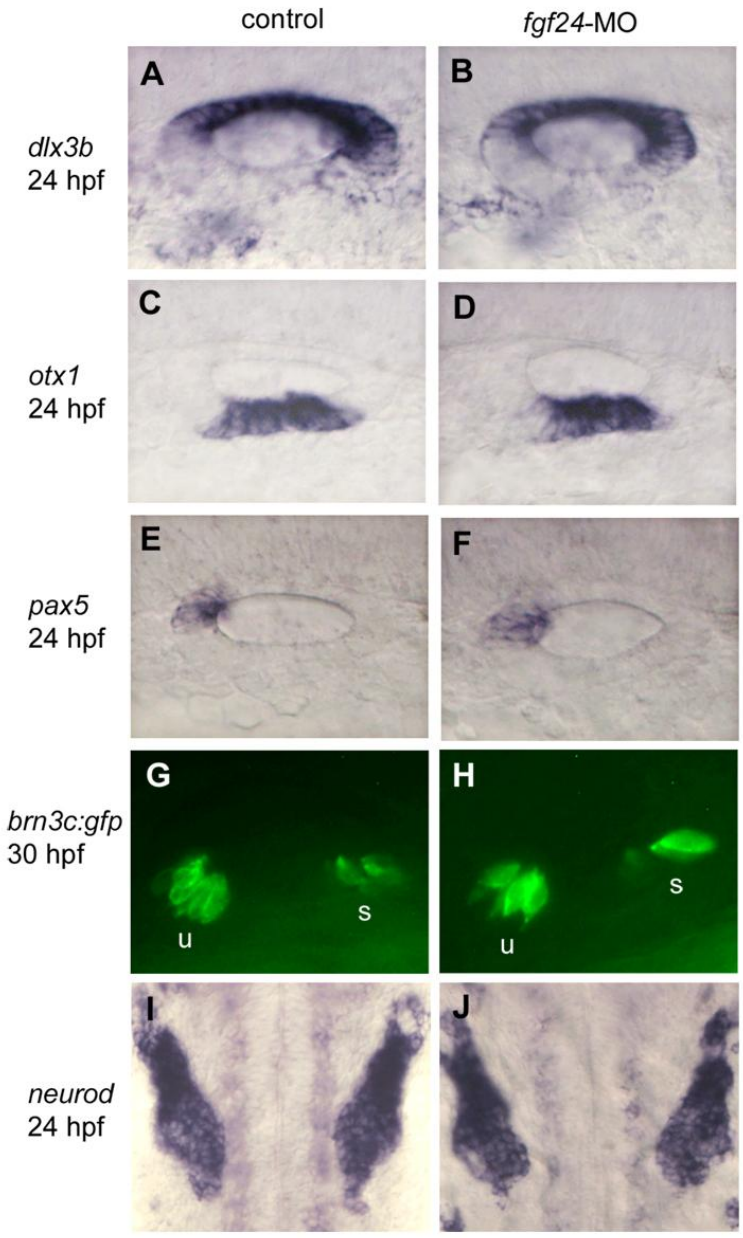

Figure 22. Patterning of the otic vesicle in *fgf24* morphants. Expression of *dlx3b*, *otx1*, *pax5*, and *neurod* in the otic vesicle of control embryos (A, C, E, I) and *fgf24* morphant (B, D, F, J) at 24 hpf. *brn3c:GFP* expression at 30 hpf in control embryo (G) and *fgf24* morphant (H). Positions of utricular (u) and saccular (s) maculae are indicated. Images show dorsolateral views with anterior to the left (A–H); dorsal views with anterior to the top (I, J).

within the otic placode and vesicle. The latter conclusion was further supported by the finding that elevating *sox3* expression by activating a heat shock-inducible transgene at 11.5 hpf does not detectably alter patterning or neurogenesis within the otic vesicle (Fig. 23).

Fgf24 regulates epibranchial development

We next examined whether the otic domain of *fgf24* acts non-autonomously to regulate epibranchial development. As in *pax8* morphants, *fgf24* morphants fail to show expansion of *sox3* into the epibranchial domain (Fig. 21F). Moreover, development of glossopharyngeal and vagal ganglia was almost completely blocked (Fig. 21H). These are the same ganglia adversely affected in *sox3* morphants (Fig. 16J), suggesting that the role of Fgf24 is to induce expression of *sox3* in these primordia. In contrast, development of the facial ganglion was relatively normal in *fgf24* and *sox3* morphants, indicating that other genes are able to compensate in these cells. The facial ganglion arises from the anterior-most region of the epibranchial domain, relatively far from the otic domain of *fgf24*. It is possible that some other source of Fgf regulates development of the facial ganglion, and that subsequent expression of *pax8* can partially compensate for loss of *sox3*.

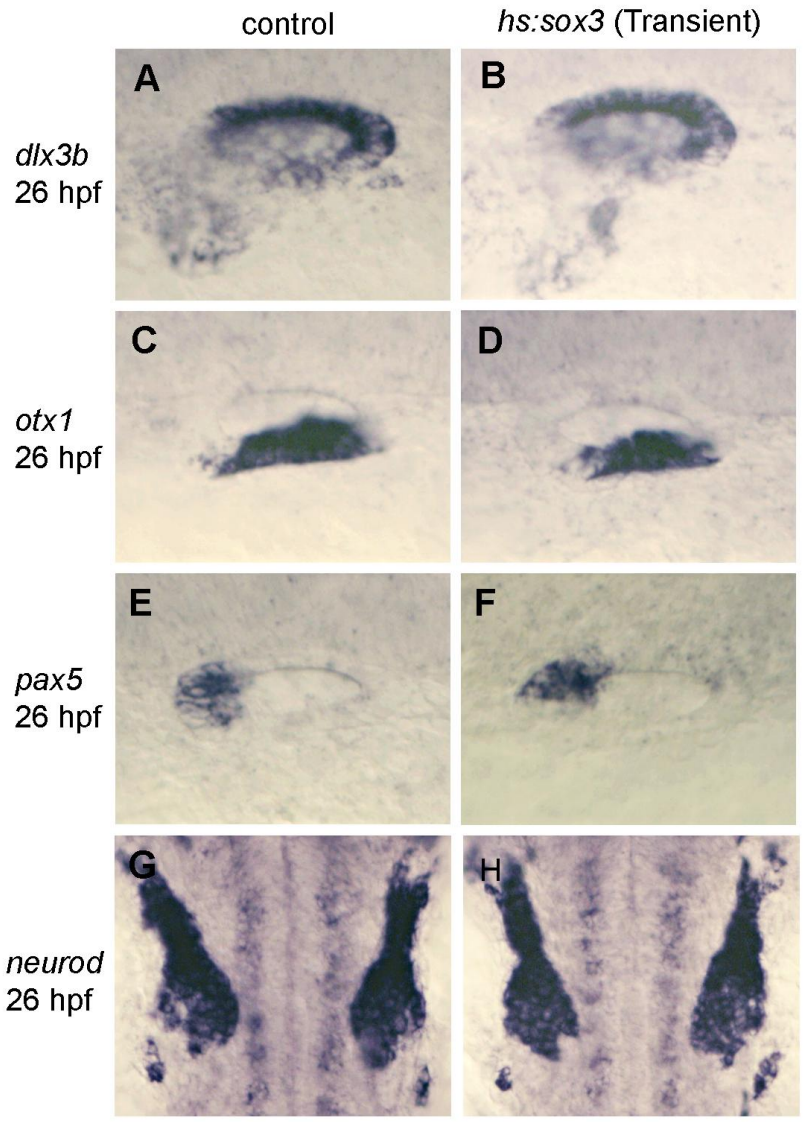

Figure 23. Patterning of the otic vesicle following misexpression of *sox3*. Expression of *dlx3b*, *otx1*, *pax5*, and *neurod* in control embryos (A, C, E, G) and in embryos transiently transfected with *hs:sox3* (B, D, F, H). Embryos were heat shocked for 30 min at 39 °C beginning at 11.5 hpf, after which embryos were maintained at 33 °C. These conditions yield maximal expression of the heat shock vector for 90 min, followed by sustained lower level misexpression until fixation at 26 hpf.

Modulation of *sox3* by a threshold response to Fgf

Although Fgf signaling is required to activate *sox3* expression, the observation that *fgf24* is required to downregulate *sox3* in the otic domain suggested that *sox3* is subject to repression by high levels of Fgf signaling. To test this, we used a heat shock line to misexpress *fgf8* beginning at 11 hpf. This caused *sox3* to be expressed throughout the otic and epibranchial domains, but at a significantly reduced level compared to the control embryo (Fig. 24). The low level of *sox3* expression in *hs:fgf8* embryos was comparable to the level normally seen in the otic domain of control embryos (compare Figs. 24A and C). In another control experiment, heat shock did not alter the effects of *fgf24* knockdown; *sox3* expression remained elevated in the otic domain and failed to expand into the epibranchial domain (Fig. 24B). These data support the hypothesis that *sox3* shows two distinct responses to Fgf signaling, explaining how otic expression of *fgf24* differentially regulates *sox3* in the otic and epibranchial domains.

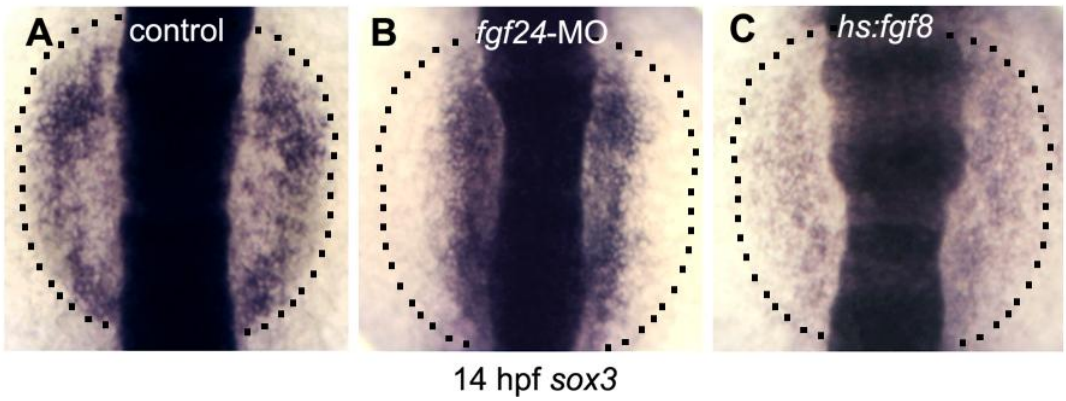

Figure 24. Response of *sox3* to elevated Fgf signaling. Expression of *sox3* at 14 hpf in a control embryo (A), *fgf24* morphant (B) and *hs:fgf8* transgenic embryo (C). All embryos were heat shocked at 11 hpf.

DISCUSSION

The data provided here clarify early steps in otic placode development and support a new model for sequential induction of otic and epibranchial placodes (Fig. 25). The otic placode forms first by a previously established mechanism involving Fgf3 and Fgf8 acting locally within a broader domain of *foxi1* expression (Hans et al., 2004; Hans et al., 2007; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Nissen et al., 2003; Phillips et al., 2001; Solomon et al., 2003). As an initial response, *pax8* and *sox3* are coinduced in the otic domain (Nikaido et al., 2007; Sun et al., 2007). Otic expression

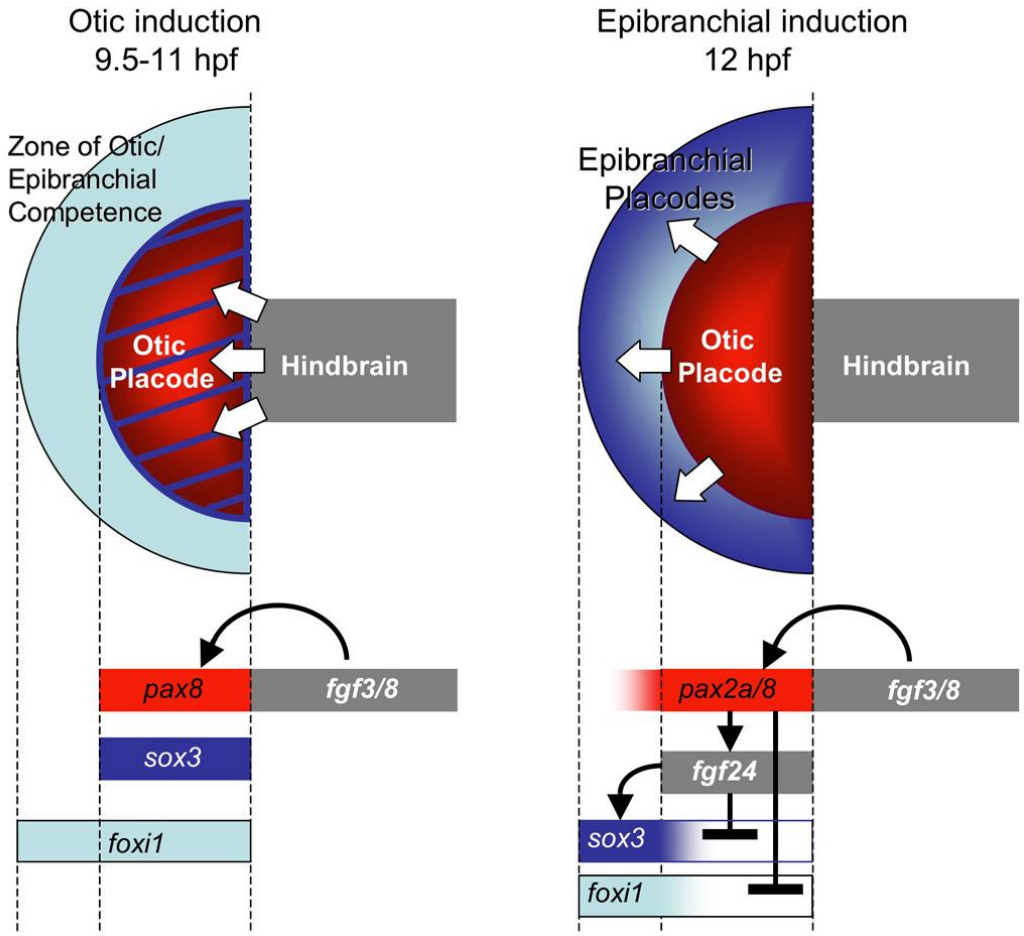

Figure 25. Summary and model of otic and epibranchial induction. During otic induction (9.5-11 hpf), Fgf3/8 from the mesendoderm (not shown) and hindbrain (gray) induce expression of *pax8* (red) and *sox3* (blue) in preotic cells. Specific responsiveness to Fgf requires the competence factor Foxi1, which becomes restricted to the otic and epibranchial regions during this period. By 12 hpf, pax8 and pax2a have induced expression of *fgf24* and repressed otic expression of *foxi1*. Fgf24 in turn downregulates otic expression of *sox3* and induces strong expression of *sox3* in adjacent epibranchial cells. Arrows represent positive regulation and cross-bars indicate negative regulation.

of *pax8* stabilizes otic fate through downregulation of *foxi1*, and non-autonomously induces the majority of epibranchial placodes through activation of *fgf24*. This model is compatible with previous studies showing that Fgf3 and Fgf8 regulate otic and epibranchial development but adds important mechanistic details, as described below. Only the facial ganglion appears to develop independently of Fgf24, and its regulation will be considered separately.

It should be noted that while we assessed epibranchial development using *sox3* as an early specification marker and *phox2a* as a terminal differentiation marker, we also examined expression of *ngn1*, a general marker of neurogenesis. Despite the deficiency of definitive epibranchial markers in *fgf24* morphants, *pax8-sox3* double morphants, and *pax2a/pax2b/pax8*-deficient embryos, small disorganized clusters of *ngn1*-expressing cells were still produced in each of these backgrounds (not shown). However, these clusters appear to be derived from neural crest, as simultaneous ablation of neural crest eliminates residual neurogenesis in the epibranchial region (our unpublished observations).

The roles of *pax8* and *sox3* in early otic development

Our data provide important new insights into the mechanisms by which Fgf-target genes control early otic development. Expression of *pax8* is especially critical for establishing the size of the otic placode, as shown by the production of roughly half-sized otic placodes in *pax8* morphants (Mackereth et al., 2005, and Fig. 16G). Because *pax8* is required to activate otic expression of *fgf24*, we initially hypothesized that this

additional source of Fgf would serve to recruit more distant cells into the otic placode. Surprisingly, however, *fgf24* appears to provide no essential function for otic development: *fgf24* morphants and mutants show no deficit in the size of the otic placode, and there appear to be no defects in subsequent patterning in the otic vesicle (Fig. 22). Instead, the primary function of otic *fgf24* is to initiate epibranchial development through induction of *sox3* in the surrounding ectoderm (described in more detail below). How then, does *pax8* control the size of the otic domain? Because *Pax2/8* genes are auto-regulatory in other developmental settings (Lun and Brand, 1998; Pfeffer et al., 1998), we speculate that Pax8 forms a feedback amplification loop in pre-otic cells, allowing cells further from the Fgf signaling source to achieve detectable expression of *pax8* and *sox3*. In *pax8* morphants, therefore, otic induction is limited to a smaller field of cells closer to the Fgf source where signal amplification is less critical. Another Fgf target gene, *sox3*, cooperates with *pax8* during otic induction. Knockdown of *sox3* alone causes only a 9% reduction in the size of the otic placode. However, knocking down both *pax8* and *sox3* causes a synergistic loss of nearly two-thirds of otic tissue. In this case, we presume that only cells immediately adjacent to the sources of Fgf are able to initiate otic development through the activation of additional as yet unknown target genes.

After helping to establish the otic placode, *pax8* later represses *foxi1* in the otic domain. This function is shared with *pax2a* and *pax2b* and appears to be essential for maintaining otic fate. In *pax2a/pax2b/pax8*-deficient embryos, *foxi1* expression persists in the otic domain (Fig. 18F, G) and expression of all otic markers is lost by 24 hpf

(Mackereth et al., 2005). Furthermore, experimentally maintaining expression of *foxi1* from an inducible transgene also causes loss of otic markers. It is not clear why *foxi1* must be repressed in the otic domain since it is absolutely required for Fgf's ability to induce otic development in the first place. However, our analysis of the early role of *foxi1* in establishing preplacodal ectoderm indicates that it functions in part by repressing other regulatory genes (Kwon et al., 2010 and our unpublished observations). Thus, *pax8*-dependent downregulation of *foxi1* may alleviate repression of other genes necessary for otic differentiation.

After the onset of otic development, the later role(s) of *sox3* are still unclear. Although the otic vesicle is slightly smaller than normal in *sox3* morphants, all regional markers are expressed normally. Based on studies in chick it has been suggested that *Sox3* regulates formation of the sensory-neural domain of the otic vesicle (Abelló et al.). However, we find that knockdown of *sox3* causes no appreciable deficiency in development of sensory epithelia or SAG neurons (Fig. 17N, R and Table 3). Otic development in *Sox3* null mice has not been studied in detail, but otic vesicles appear grossly normal and produce at least some SAG neurons (Rizzoti and Lovell-Badge, 2007). It is possible that other *SoxB1* genes compensate for loss of *Sox3* in mouse, but no other appropriately expressed genes have been identified in zebrafish. It is interesting that *fgf24* morphants fail to downregulate *sox3* in the otic placode (Fig. 21F), yet all other aspects of otic development appear normal (Fig. 22). Likewise, misexpressing *sox3* from a heat shock-inducible transgene does not detectably alter otic development. However it must be acknowledged that failure to downregulate *sox3* could cause defects

too subtle to detect using the markers at hand, even though such changes could be quite deleterious in the long-run.

The role of Fgf24

A novel finding central to our model is that Fgf24 emitted by the nascent otic placode is essential for development of all epibranchial placodes posterior to the facial placode (Fig. 21H). A prominent target of Fgf24 appears to be *sox3*. Within 1-2 hours of activation of *fgf24* in the otic placode, *sox3* begins to downregulate in the otic domain while it is induced in the abutting epibranchial domain. Differential spatial regulation of *sox3* could reflect a threshold response to changing levels of Fgf24 within a diffusion gradient. Indeed, overexpression of Fgf8 causes *sox3* to be expressed at a low level throughout the domain of *foxi1* expression (Fig. 24). In the absence of Fgf24, *sox3* remains highly expressed in the otic domain and is not detected in the epibranchial domain. Disruption of *sox3* has little effect on otic development but blocks all epibranchial development posterior to the facial ganglion. This phenotype strongly resembles that of *fgf24* morphants, again supporting the notion that *sox3* is the primary mediator of Fgf24 signaling. Otic expression of *fgf24* is in turn regulated redundantly by *pax2* and *pax8* genes. Accordingly, loss of *pax8* alone causes a 2-3 hour delay in *fgf24* expression, with negligible effects on epibranchial development. In contrast, disruption of all *pax2/8* function eliminates otic expression of *fgf24* entirely and causes a deficiency in epibranchial development comparable to *fgf24*-MO. Together these data support the

existence of a pathway in which otic expression of *pax8* activates expression of *fgf24*, which induces formation of epibranchial placodes in adjacent ectoderm through *sox3*.

In contrast to *sox3*, *pax2/8* genes are normally maintained at a high level in the otic placode but show only weak expression in the epibranchial domain. This pattern remains unchanged in *fgf24* morphants. Epibranchial expression of *pax2/8* appears after otic expression, possibly reflecting a delayed response to low levels of Fgf3 and Fgf8 from the hindbrain and subotic mesendoderm (Alvarez et al., 2003; Freter et al., 3008; Ladher et al., 2000; Ladher et al., 2005; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Nechiporuk et al. 2007; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Riley and Phillips, 2003; Wright and Mansour, 2003). It is possible that *pax2/8* provides a cell-autonomous requirement for epibranchial development, but such function(s) are evidently not sufficient to support epibranchial development in the absence of Fgf24.

Our model is distinct from an earlier model proposing that epibranchial placodes are induced by Fgf3 and Fgf8 from paraxial cephalic mesoderm (Nechiporuk et al., 2007). It is formally possible that mesodermal Fgf3 and Fgf8 work in parallel with otic Fgf24 to regulate early epibranchial development. On the other hand, we find that genetic ablation of mesoderm does not block otic induction (Kwon and Riley, 2009; Mendonsa and Riley, 1999), nor does it block early epibranchial development. Specifically, *sox3* is initially induced within the otic domain, followed by downregulation in the otic domain an upregulation in the epibranchial domain (our unpublished observations). However, we have confirmed the results of Nechiporuk and colleagues that ablation of mesoderm blocks later development of epibranchial neurons

(Nechiporuk et al., 2007; and our unpublished observations). We speculate that epibranchial fate is specified under these conditions, but is not properly maintained in the absence of mesodermal Fgf.

Regulation of the facial ganglion

Epibranchial placodes and ganglia appear to follow similar regulation in general, but our data show that the facial (geniculate) placode shows key differences from more posterior epibranchial placodes. First, development of the facial ganglion does not require *fgf24* (Fig. 19H). Similarly, there are only minor deficiencies in the facial ganglion following knockdown of *sox3* alone, *pax8* alone, or all *pax2/8* functions, whereas the other epibranchial ganglia are severely impaired or ablated under these conditions (Figs. 16J, 16K and 18H). However, combined knockdown of *sox3* and *pax8* ablates formation of facial ganglion (Fig. 16L). This indicates that *sox3* and *pax8* serve redundant functions in the facial placode, unlike more posterior epibranchial placodes. Such early differences in regulation could confer unique functional attributes to the facial ganglion that distinguish it from other epibranchial ganglia.

Other essential signals

In chick, frog and zebrafish, Fgfs and various Bmps secreted from pharyngeal endoderm are also required for development of epibranchial ganglia (Begbie et al., 1999; Holzschuh et al., 2005; Nechiporuk et al., 2005; Nikaido et al., 2007). However, these signals operate at a later stage, well after Fgf-dependent induction of *sox3*, and are

required to initiate neurogenic differentiation. It is still unknown whether these endodermal signals act sequentially or are required as parallel inputs.

In mouse and chick, Wnt8a from the hindbrain is thought to distinguish otic from epibranchial fates. Accordingly, disruption of Wnt signaling blocks completion of otic development whereas elevating Wnt signaling expands otic tissue as it blocks epibranchial development (Freter et al., 2008; Ohyama et al., 2006). At first glance this appears to be an entirely different mechanism than what we describe for zebrafish, but this is not necessarily the case. Chick and mouse embryos show prominent otic expression of multiple *Fgf* genes around the time of otic induction, the functions of which have not been examined (Adamska et al., 2001; Alsina et al., 2004; Chapman et al., 2006; Pirvola et al., 2004; Wright et al., 2003). Conceivably, Wnt signaling could help modulate expression of these otic *Fgf* genes, or work in parallel with them, to affect epibranchial development. In zebrafish, Wnt signaling influences otic development indirectly through modulation of hindbrain expression of *fgf3* and *fgf8* (Phillips et al., 2004). Additional studies are needed to assess the degree to which underlying mechanisms have been conserved between zebrafish and amniotes.

CHAPTER IV

SUMMARY AND DISCUSSION

This dissertation focuses on genetic interactions responsible for the development of the otic and epibranchial placodes. Although there is clear evidence that Fgf is required for otic induction, there have been controversies as to whether Fgf is sufficient for otic induction. Therefore, I reexamined the effects of Fgf misexpression as presented in chapter II. The effect of Fgf on otic placode induction depends on dose, level and timing of the expression. At initial stages before the end of gastrulation, a high level of Fgf is detrimental to otic development, but at later stages high levels of Fgf promote otic development. Co-misexpression of Fgfs with their targets, *pax2a/8* induces ectopic otic placodes whereas misexpression of either one alone was not sufficient. Previous studies demonstrated that, like the otic placode, the epibranchial placode requires Fgf and Foxi1 as upstream regulators. Both otic and epibranchial placodes express *pax8* and *sox3* as early response factors. How cells can attain different identities responding to the same set of factors has not been investigated. In chapter III, I showed that *pax2/8* genes induced in the presumptive otic region are responsible for distinguishing otic from epibranchial placodes by differentially regulating *fgf24, foxi1,* and *sox3*. This study, as a whole, provides important new insights into the mechanisms by which Fgfs and their target genes control early otic and epibranchial placode development.

TEMPORAL AND SPATIAL REQUIREMENTS OF Fgf FOR OTIC INDUCTION

Previous studies demonstrated that misexpression of Fgf has different outcomes for otic induction depending upon the levels and techniques used. Hans et al., (2007) used a similar approach to mine for Fgf misexpression, but did not explore variables affecting responses to Fgf, nor did they resolve the mechanistic basis for impairment of otic development following early misexpression. I demonstrated that at early developmental stages, before the end of gastrulation, high levels of Fgf impairs otic development by disrupting endogenous signaling centers in the hindbrain that are required for normal otic induction. Furthermore, analysis of mosaic embryos generated by transplanting *hs:fgf8* donor cells into non-transgenic hosts showed that high levels of Fgf8 at early stages induced expression of the preotic marker *pax8* only in the neighboring cells, however we never observed *pax8* expression within *hs:fgf8* transgenic cells. Moderate to low levels of Fgf induced expression of *pax8* both in transgenic donor cells and neighboring host cells. Likewise misexpression of *fgf8* or *fgf3* by plasmid injections induced *pax8* expression in the neighboring cells but never autoinduced within the cells that are expressing high levels of Fgf (Phillips et al., 2004). Thus high levels of Fgf inhibit otic fate whereas moderate to low levels promote otic fate cell autonomously. These results provide important information about the spatial requirement of Fgfs for otic induction. However, the fate of transgenic cells that are expressing high levels of Fgf and have lost otic markers is not clear. It is likely that high

levels of Fgfs are involved in maintaining cell plasticity, thereby inhibiting those cells experiencing high levels of Fgf from differentiation to otic fate. As in other cell types, for example overexpression of Fgf2 in adult neural stem/progenitor cells of mice inhibits their neural differentiation and maintains the undifferentiated state of isolated adult neural stem/progenitor cells (Li et al., 2008). In contrast, high levels of Fgf, after tailbud stages, results in formation of larger endogenous otic vesicle and promote otic fate cell autonomously, probably because endogenous signaling centers are stable and preplacodal ectoderm is matured allowing proper interpretation of high Fgf. Furthermore, once otic development has been initiated, high levels of Fgf signaling may reinforce otic fate. These findings demonstrate that level, timing and location of Fgf signaling are critical for otic induction.

Misexpression of either *fgf8* or *fgf3,* even at later stages does not induce formation of ectopic otic vesicles. There are a number of possible reasons for this; (a) misexpression of Fgfs using heat shock inducible transgene may not produce optimum levels of Fgf necessary for otic induction or alternatively may not last long enough to initiate otic fate after a single pulse of heat shock; (b) Global activation of transgene does not mimic localized signaling from a point source, for example formation of endogenous otic vesicle in response to Fgfs from the hindbrain; (c) the domain of otic competence may become increasingly localized as development proceeds, presumably reflecting specification or differentiation of other cell fates at other sites;(d) foreign sites may lack essential co-factors needed for normal Fgf activity or alternatively other

regionally expressed factors may interfere with Fgf, there by inhibiting induction of ectopic otic vesicles.

GENETIC INTERACTIONS BETWEEN *fgf8* AND *pax2a/pax8*

Pax2/8 factors are required for otic induction and maintenance (Hans et al., 2004; Mackereth et al., 2005). However, whether *pax2/8* are sufficient for otic development has not been investigated. My findings show that misexpression of *hs:pax8* or *hs:pax2a* expands the endogenous otic domains and induces formation of several microvesicles around the endogenous otic vesicle, but does not produce ectopic otic vesicles in more rostral regions. Presumably there are other unknown additional factors induced by Fgf signaling that co-operate with *pax2/8* genes for normal otic development. Elevated levels of *hs:pax2a/hs:pax8* does not bypass the need for Fgf signaling. Misexpression of *hs:pax8/hs:pax2a* followed by blocking Fgf signaling using pharmacological inhibitor SU5402, showed complete loss of *pax2a* at 13 hpf. These results suggest that there is continuous requirement of Fgf signaling for normal otic development even after otic induction has begun. It is possible that there are other unknown additional factors that are induced by Fgf signaling may co-operate with *pax2/8* genes for normal otic development.

Because misexpression of either *fgf3/8* or *pax2a/8* genes did not induce ectopic otic vesicles, I speculated that co-misexpression of *hs:fgf8* and *hs:pax2a/hs:pax8* might

influence each others function thereby inducing ectopic otic vesicles. Co-misexpression of *hs:fgf8* and *hs:pax2a/hs:pax8* showed a synergistic effect on otic induction in the following ways; 1) Larger endogenous otic vesicles were induced compared to misexpression of *fgf8*, *pax2a* or *pax8* alone. 2) A two to three fold increase in the average number of microvesicles was observed compared to *hs:pax8* or *hs:pax2a*. Note that misexpression of *hs:fgf8* by itself does not induce formation of microvesicles. 3) Ectopic microvesicles were formed in many regions like the eyes, forebrain, midbrain and trunk, where otic vesicles are not normally induced in these regions. These results indicate that even though *pax2/8* genes are induced by Fgfs, they appear to provide regional or tissue type specifier function and accordingly *pax2a/8* genes influence the ability of cells in ectopic locations to respond to Fgf signals appropriately, thereby leading to formation of ectopic otic vesicles. Pax genes are involved in development of specific organs in specific regions of the embryo based on their spatial expression pattern and also Pax proteins have been implicated to influence the fate of tissue specific lineages (Reviewed by Buckingham and Relaix, 2007; Lang et al., 2007). In brain, Pax2 and Pax6 factors mediate positioning of a sharp molecular boundary between future diencephalon and mesencephalon by negatively cross regulating each other (Matsunaga et al., 2000). This type of negative regulation leads to separation of different cell fates. It is possible that similar kind of negative cross regulation between the Pax genes may impart regional identity to placodes. Thus, Pax2a and Pax8 may impart otic identity and Pax6 gene expressed in the rostral preplacodal domain may specify anterior lens and olfactory placodes.

ROLE OF Foxi1 IN OTIC INDUCTION

A fork head winged helix transcription factor *foxi1* is dynamically expressed in the early stages of zebrafish development. Whether Fgf signaling regulates *foxi1* expression has been controversial. Hans et al. (2007) showed that Fgf represses *foxi1* expression whereas others have shown that *foxi1* is upregulated or induced in response to Fgf (Phillips et al., 2004; Nechiporuk, et al., 2007). Moreover, during the normal course of embryonic development, at late gastrula stages, *foxi1* expression is upregulated in the region lateral to the hindbrain, which includes preotic and epibranchial primordia, and is downregulated in other ventral regions (Hans et al., 2007; Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003). So, I reexamined whether *foxi1* expression is regulated by Fgf. Global activation of *hs:fgf8* results in upregulation of *foxi1* expression in the entire preplacodal ectoderm indicating that Fgf positively regulate *foxi1* expression (Fig. 24). This contradicts to the findings of Hans et al. (2007) but is consistent with other misexpression studies (Nechiporuk et al., 2007; Phillips et al., 2004).

Foxi1 act as an upstream activator of *pax8* expression in the otic placode (Solomon et al., 2003). Expression of *pax8* also requires Fgf signaling (Hans et al., 2004; Phillips et al., 2004; Solomon et al., 2004). *foxi1* expression begins to be downregulated in the otic domain by 11 hpf, but is maintained in the epibranchial domain through at least 36 hpf (Lee et al., 2003). My findings show that *pax2/8* downregulates *foxi1* expression within the otic placode. In support of this, *pax2a/pax2b/pax8* deficient embryos show retention of *foxi1* in the otic vesicle through

at least 24 hpf, by which time otic identity is lost. These results indicate that Pax2/8 represses *foxi1* expression in the otic placode (Fig. 24). Hence even though Fgf initially coordinately upregulates *foxi1* and induces otic expression of *pax8*, Pax8 inturn acts as a feedback inhibitor of *foxi1* within the otic domain. This modulation of *foxi1* expression in the otic placode appears to be a very important step for the cells to maintain their otic identity during later phase of otic development. Otherwise *foxi1* might promote development of epibranchial fate.

Although *foxi1* is necessary to initiate otic development, its expression is downregulated within the otic placode beginning 11 hpf. I speculated that failure to downregulate *foxi1* at later stages impairs otic dvelopment. Experimentally maintaining *foxi1* expression using a heat shock-inducible transgene shows loss of otic markers suggesting that retention of *foxi1* in otic cells at later stages impedes otic development. It is not clear why *foxi1* has to be dowregulated when it is absolutely required for otic induction initially. Retention of *foxi1* in otic cells probably represses the genes that are necessary for otic differentiation. For example, Foxi1 appears to act as a repressor for various other genes involved in preplacodal ectoderm competence, including negative regulation of *foxi1* itself. Therefore Foxi1 might act as a repressor of genes necessary for otic differentiation. Thus downregulation of *foxi1* may alleviate repression of these genes such that normal otic development occurs.

Next I asked whether elevated levels of Fgf can bypass the requirement of Foxi1 for otic induction, after the otic induction process has begun. Embryos with high levels of Fgf, but knocked down for *foxi1* show loss of *pax8* expression in the otic domain.

This result suggests that Foxi1 is essential for *pax8* induction irrespective of the levels of Fgf. In contrast, *pax2a* expression was detected in the otic placode albeit in a smaller domain. In addition, unlike the lateral expansion of *pax2a* in *hs:fgf8* embryos, knock down of *foxi1* restricts the expression domain of *pax2a* close to the hindbrain, indicating that *foxi1* does regulate *pax2a* expression to a certain extent directly or indirectly. Thus Foxi1 plays an important role in establishing the initial size of the otic domain by regulating *pax8* expression and high levels of Fgf cannot bypass the need of Foxi1 for normal otic induction.

REGULATION OF *sox3* EXPRESSION

Initially *sox3* is coexpressed with *pax8* in the otic anlagen. Between 11 and 12 hpf, a dynamic transition is seen, wherein *sox3* expression is downregulated in otic cells but expanded laterally into the adjacent presumptive epibranchial placodes (Sun et al., 2007). However, *pax8* expression persists in the otic placode. Therefore I speculated that *pax8* may regulate *sox3* expression. I showed that loss of *pax8* function results in a delay in the downregulation of *sox3* from otic cells as well as a delay in onset of *sox3* expansion into epibranchial placodes. This repression of *sox3* in otic cells is likely an indirect effect because there is a long lag between the onset of *pax8* expression and *sox3* downregulation in otic cells. One probable candidate to mediate downregulation of *sox3* is *fgf24*, which is activated by *pax8* in the otic cells. As with *pax8* morphants, loss of

fgf24 leads to the failure of *sox3* downregulation from the otic cells and its expansion into epibranchial domain. These data suggest that after activation of *fgf24* by Pax8 within the otic placode, *fgf24* downregulates *sox3* expression in otic cells, but induces *sox3* expression in adjacent epibranchial placodes (Fig. 26). Differential spatial regulation of *sox3* by *fgf24* may reflect a threshold response to changing levels of Fgf24 within a diffusion gradient. In support of this, misexpression of *hs:fgf8* induces low levels of *sox3* throughout the domain of *foxi1* expression which encompasses both otic and epibranchial placodes.

ROLES OF *pax8* AND *sox3* IN OTIC AND EPIBRACNHIAL DEVELOPMENT

pax8 and *sox3* are coexpressed in the otic primordium by 9.5 hpf. By 12 hpf, *sox3* expression begins to be downregulated in the otic placode as it expands laterally into the adjacent epibranchial placodes (Nikaido et al., 2007, Sun et al., 2007). Little is known about the function of *sox3* in otic development. In this study I examined genetic interactions between *pax8* and *sox3* in regulating otic development. My findings show that knockdown of *sox3* alone causes a slight reduction in the size of the otic placode. However knocking down both *pax8* and *sox3* causes a dramatic reduction in the size of the otic placode. These results show that *pax8* and *sox3* both regulate otic induction, but *pax8* function appears to be more critical than *sox3*.

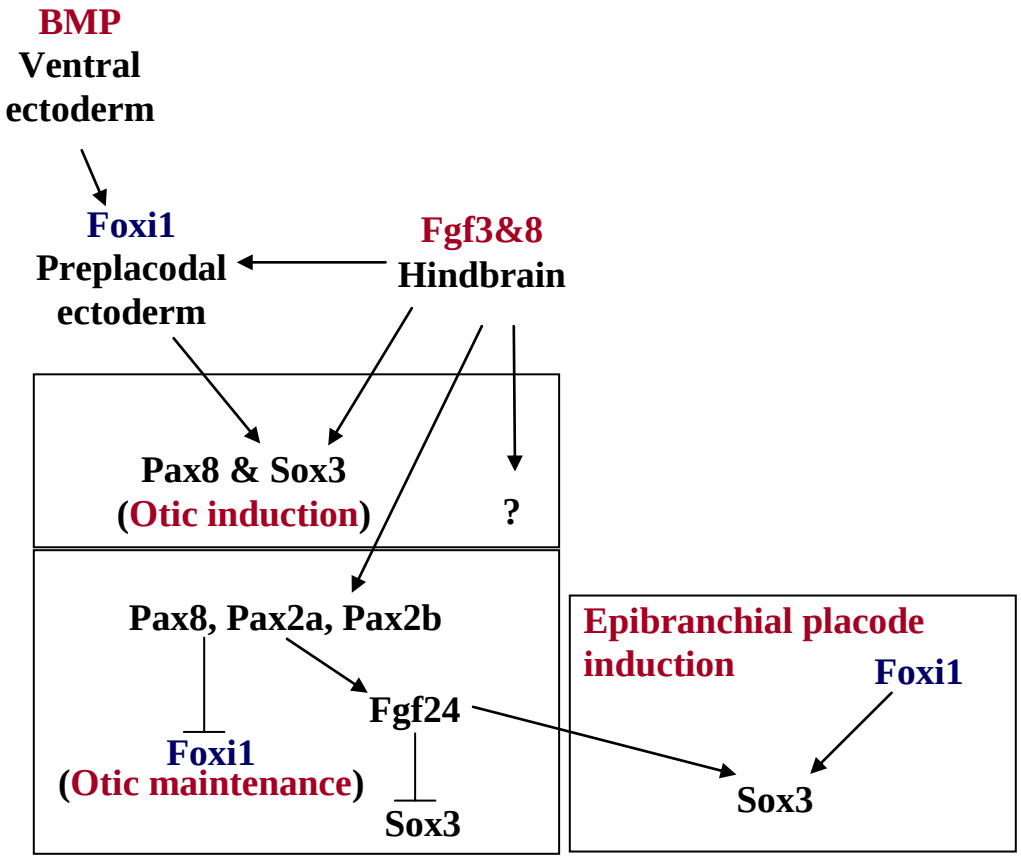

Figure 26. Summary of genetic interactions for induction of otic and epibranchial placodes. Solid arrow indicates positive regulation; inverted T indicates negative regulation of target genes; question mark indicates unknown target genes.

A later role of *sox3* in otic development is not clear. Although the size of the otic vesicle is slightly smaller in *sox3* morphants, all regional markers are expressed normally. Studies in chick suggested that *sox3* regulates formation of the sensory-neural domain of the otic vesicle (Abello et al., 2010). However, my finding shows that knock down of *sox3* does not affect the development of sensory epithelia or SAG neurons. Similarly, otic vesicles in *Sox3* null mice appear normal and produce some SAG neurons (Rizzoti and Lovell-Badge, 2007). Other *SoxB1* genes may compensate for the loss of *Sox3*, however, no such appropriately expressed genes have been identified in zebrafish. Although *sox3* expression is maintained at high levels in *fgf24* morphants, otic development appears normal in these animals. Similarly misexpression of *sox3* using a heat shock inducible transgene does not alter otic development. It is important to note that retention of *sox3* in the otic placode might cause some defects that are undetectable using available molecular markers. Further functional analysis of *sox3* in otic development is needed.

Weaker expression of *pax8* and *pax2a* is detected in the epibranchial anlagen by 12 hpf, whereas strong expression persists in otic placode. In contrast, *sox3* is strongly expressed in epibranchial placodes and weaker expression is seen in otic placode. The role of *pax2/8* genes in epibranchial development has not been investigated previously. However, previous studies have shown that *sox3* is required for normal development of epibranchial ganglia (Dee et al., 2008; Rizzoti and Lovell-Badge, 2007). My findings show that knockdown of *pax8* function results in a slight reduction of epibranchial ganglia domains marked by *phox2a* expression, whereas *pax8/sox3* double morphants

show a complete loss of all the four domains of the epibranchial ganglia. These data indicate that *pax8* and *sox3* cooperate to regulate epibranchial development. Most importantly, *pax8* along with *pax2a* and *pax2b* regulate epibranchial development non-autonomously through activation of *fgf24* in the otic placode. As with *fgf24* morphants, *pax8/pax2a/pax2b* deficient embryos show loss of glossopharyngeal and vagal ganglia. Taken together, *pax8* and *sox3* cooperate to regulate both otic and epibranchial development. This raises the question of what distinguishes otic from epibranchial fate. It is possible that the combination of different levels of upstream regulators Fgf and *foxi1* with downstream effectors *pax2/8* and *sox3* distinguishes otic from epibranchial fate; high levels of Fgf and *pax2/8* with low levels of *foxi1* and *sox3* specifies otic fate, whereas low levels of Fgf and *pax2/8* with high levels of *foxi1* and *sox3* induces epibranchial fate (Fig. 27)

Previous studies suggest that otic and epibranchial placodes are derived from the common otic-epibranchial primordium. This conclusion is based on similarities observed between otic and epibranchial placodes, such as the common upstream regulators, Fgf and Foxi1, and common expression of *pax8* and *sox3* as early response factors (Freter et al., 2008; Ladher et al., 2010; Ohyama et al., 2006; Schlosser and Ahrens, 2004; Sun et al., 2007). However, my findings show that these placodes are induced in sequence, otic placode being first followed by epibranchial placodes. Once the otic placode is established, *pax8* along with *pax2a* and *pax2b* repress *foxi1* expression in the otic placode to maintain otic fate. Furthermore, *pax8* with *pax2a* and *pax2b* activate expression of *fgf24* in the otic placode. Most importantly, *fgf24* expressed within the otic

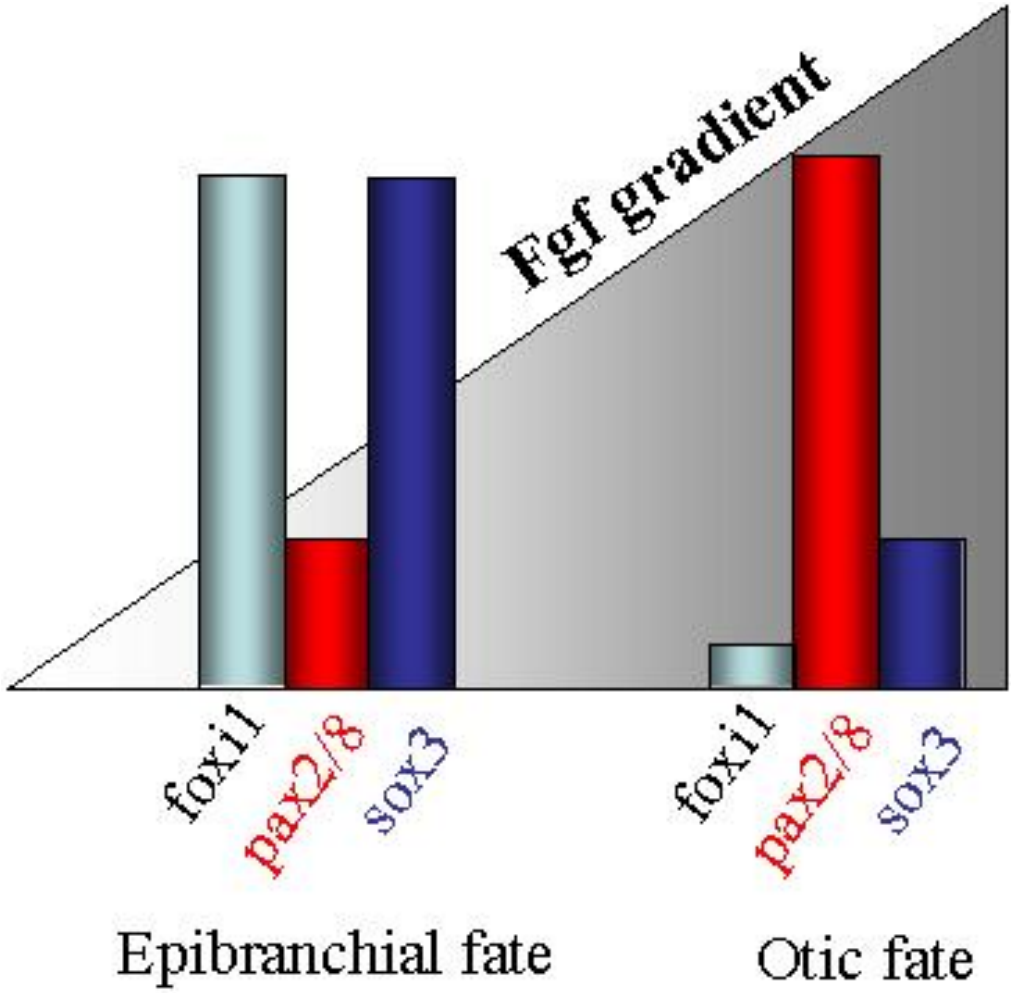

Figure 27. Combinatorial codes for otic and epibranchial fate specification.

placode is responsible for epibranchial induction. Thus Pax2/8 mediate maintenance of otic fate by repressing *foxi1* and regulate epibranchial development indirectly through activation of *fgf24* in the otic placode (Fig. 26).

Fgf24 IS REQUIRED FOR EPIBRANCHIAL PLACODE DEVELOPMENT

Expression of *fgf24* is first detected in the otic placode by 10.5 hpf, shortly after the induction of *pax8* expression in otic cells (Draper et al., 2003). I therefore reasoned that Pax8, which is required to produce a normal sized otic placode, may act upstream to activate *fgf24*. My studies show that *pax8* along with closely related genes *pax2a* and *pax2b* regulate expression of *fgf24* within the otic cells (Fig. 26). *pax8/pax2a/pax2b* deficient embryos show a loss of *fgf24* expression specifically in the otic placode at least through 18 hpf. Although *fgf24* is expressed in the preotic placode by 10.5 hpf, it is not required for otic development. Loss of *fgf24* has little effect on otic development. In contrast, *fgf24* is essential for development of all epibranchial placodes except the facial placode. Disruption of *fgf24* results in elevated expression of *sox3* in the otic cells and loss of *sox3* expansion into the adjacent presumptive epibranchial placodes. Subsequently, by 30 hpf, development of glossopharyngeal and vagal ganglia are completely blocked. These are the same ganglia affected in *sox3* morphnats. These data show that *fgf24* acts non-autonomously to regulate epibranchial development through induction of *sox3* in the epibranchial placodes (Fig. 26).

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