Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification

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SUMMARY

The vertebrate inner ear develops from the otic placode, an ectodermal thickening that forms adjacent to the presumptive hindbrain. Previous studies have suggested that competent ectodermal cells respond to signals from adjacent tissues to form the placode. Members of the Fgf family of growth factors and the Dlx family of transcription factors have been implicated in this signal-response pathway. We show that compromising Fgf3 and Fgf8 signaling blocks ear development; only a few scattered otic cells form. Removal of dlx3b, dlx4b and sox9a genes together also blocks ear development, although a few residual cells form an otic epithelium. These cells fail to form if sox9b function is also blocked. Combined loss of Fgf signaling and the three transcription factor genes, dlx3b, dlx4b and sox9a, also completely eliminates all indications of otic cells. Expression of sox9a but not dlx3b, dlx4b or sox9b requires Fgf3 and Fgf8. Our results provide evidence for Fgf3- and Fgf8-dependent and -independent genetic pathways for otic specification and support the notion that Fgf3 and Fgf8 function to induce both the otic placode and the epithelial organization of the otic vesicle.

Key words: dlx3b, dlx4b, Inner ear, Morpholino, Olfactory placode, sox9a, sox9b, Zebrafish

INTRODUCTION

In vertebrates, the initial morphological event in inner ear development is the formation of the embryonic otic placode, a thickening of the head ectoderm adjacent to the developing hindbrain. Through interactions with adjacent tissues and incorporation of additional cells from the neural crest and mesoderm, the placode develops into the otic vesicle, also called the otocyst, an epithelial structure with sharply defined borders (Fritzsch et al., 1997; Noden and van de Water, 1986; Couly et al., 1993). Later, the otic vesicle forms the inner ear including its neurons and most of its structural elements. A variety of studies suggest that cells are specified to form the otic placode in response to inductive signals from neighboring tissues (Fritzsch et al., 1997; Torres and Giráldez, 1998; Baker and Bronner-Fraser, 2001; Whitfield et al., 2002) including the underlying mesendoderm (Jacobson, 1963; Mendonsa and Riley, 1999) and the adjacent hindbrain (Stone, 1931; Harrison, 1945; Waddington, 1937; Woo and Fraser, 1998; Hutson et al., 1999).

Although the precise molecular nature of the signals that induce cells to form the otic placode is still unknown, several studies implicate Fgf3 and Fgf8, members of the Fgf family of signaling peptides. In zebrafish, the genes that encode these peptides are expressed in the presumptive hindbrain by late gastrula stages and fgf3 is also expressed at this stage in the underlying mesendoderm (Phillips et al., 2001). Fgf3 and Fgf8 mediate inter-rhombomere signaling required for hindbrain patterning (Maves et al., 2002). Loss of Fgf3 function in chick (Repressa et al., 1991) or of both Fgf3 and Fgf8 functions together in zebrafish (Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002) is sufficient for near or total ablation of otic tissue, and ectopic expression of Fgf3 (Vendrell et al., 2000) or Fgf2 (Lombardo and Slack, 1998) results in the formation of ectopic otic vesicles in frog and chick.

To understand how Fgf signals may specify cells to form the otic placode, we studied the functions of four transcription factors expressed by otic placode precursor cells in zebrafish, dlx3b (previously called dlx3) (Ekker et al., 1994), dlx4b (previously called dlx7) (Stock et al., 1996; Ellies et al., 1997), sox9a (Chiang et al., 2001; Yan et al., 2002) and sox9b (Chiang et al., 2001; Li et al., 2002). The dlx3b and dlx4b genes are closely linked, as are their mammalian orthologues (Nakamura et al., 1996; Morasso et al., 1997), probably because of ancestral tandem duplication in the lineage giving rise to vertebrates (Stock et al., 1996). We have previously shown that early precursor cells of the otic placode express dlx3b before any overt morphological signs of differentiation (Ekker et al., 1992; Akimenko et al., 1994); dlx4b has a similar expression pattern (Ellies et al., 1997). By prim-5 stage (24 hours), only a subset of cells in the otic vesicle still expresses dlx3b (Ekker et al., 1992).
In humans, a small deletion in the DLX3 gene is thought to be responsible for Trichodentoosseous syndrome (TDO) (Price et al., 1998). Individuals with TDO exhibit various clinical problems, including ear, tooth and skull defects (Shapiro et al., 1983), consistent with the expression pattern of Dlx3 in mice (Robinson and Mahon, 1994) and zebrafish (Akimenko et al., 1994). A knockout mutation of Dlx3 in mice results in embryonic lethality due to placental insufficiency before the ear forms (Morasso et al., 1999). Analysis of dlb3b and dlb4b in zebrafish suggested that they may serve redundant roles in otic development (Solomon and Fritz, 2002).

We have previously shown that the zebrafish sox9a and sox9b genes are duplicate orthologues of the human S0X9 gene and that both genes are expressed in the otic placode (Chiang et al., 2001). In humans, S0X9 haploinsufficiency results in campomelic dysplasia, characterized by abnormal development of the long bones and associated sex reversal (Foster et al., 1994; Wagner et al., 1994; Hageman et al., 1998; Cameron et al., 1996; Huang et al., 2001; Vidal et al., 2001). Most patients die of respiratory distress during the neonatal period, but one who survived through adolescence had hearing loss (Houston et al., 1983). Studies in mouse have shown that Sox9 is expressed in the developing otic capsule (Kanzler et al., 1998). Heterozygous Sox9 mutant mice show phenotypes similar to individuals with campomelic dysplasia and die at birth (Bi et al., 2001). Our analysis of zebrafish mutants demonstrated that sox9a is required for cartilage development (Yan et al., 2002).

To analyze the potential functions of dlb3b, dlb4b and sox9a in otic development, we characterized a deficiency mutation in zebrafish (Df(LG12)dlb3b b380), called Df(b380) (Fritz et al., 1996) that lacks all three genes. We found that homozygous Df(b380) mutants completely lack otic placentes and fail to form a differentiated otic vesicle or inner ear, although a few residual cells express genes characteristic of the developing inner ear. Knock down of all three genes by injection of morpholino antisense oligonucleotides (MOs) produces these phenotypes in wild-type embryos, and injection of MOs on dlb4b and sox9a, but not dlb3b or dlb4b, expression depends on Fgf signaling, and that the residual otic cells in homozygous Df(b380) mutants fail to form if sgf8 function is also absent. Our results demonstrate that Fgf3- and Fgf8-dependent (sox9a) and -independent (dlb3b and dlb4b) transcription factors are required for specification of the otic placode. Moreover, we found that the residual otic cells form a small epithelial ball characteristic of the early otic vesicle in Df(b380) mutants but not in the absence of Fgf3 and Fgf8 signaling, thus indicating a role of Fgfs in induction of both the otic placode and the epithelial organization of the otic vesicle.

**MATERIALS AND METHODS**

**Animals**

Embryos and adults were obtained from the University of Oregon zebrafish facility. Animals were maintained and embryos produced using standard procedures (Westerfield, 2000). Embryos were staged according to standard criteria (Kimmel et al., 1995) or by hours post fertilization at 28°C (h).

The wild-type line used was AB (University of Oregon, Eugene, OR). The Df(LG12)dlb3b b380 strain name has been approved by the zebrafish nomenclature committee (http://zfin.org/zf_inf/ nomen_comm.html) and we refer to homozygous mutants as Df(b380). The acerebellar228a (ace) line, a strong hypomorphic allele of sgf8, has been described previously (Brand et al., 1996); we refer to the homozygous mutants as sgf8–. Homozygous mutants for both Df(b380) and sgf8– genotypes were obtained by crossing Df(b380) and acerebellar228a carriers. Homozygous Df(b380) sgf8– embryos were scored by their lack of somites; homozygous acerebellar228a mutant embryos were scored by their loss of the cerebellum. Homozygous Df(b380) sgf8– embryos were scored by both criteria.

**Genes, markers and mapping**

Approved gene and protein names that follow the zebrafish nomenclature conventions (http://zfin.org/zf_inf/nomen.html) are used according to http://zfin.org. To map the deletion boundaries and the genes and markers missing from the deficient region, we used primers for genes and markers on LG12 to amplify expected bands from Df(b380) genomic DNA by PCR.

**In situ hybridization, mRNA synthesis and rescue**

cDNA probes that detected the following genes were used: dlb3b (Ekker et al., 1992); dlb4b (Stock et al., 1996); sox9a (Chiang et al., 2001); egr2 (krox-20) (Ottoy and Jowett, 1993); clnda (Kollmar et al., 2001); fn1 (Zhao et al., 2001) and pax2a (Krauss et al., 1991). Probe synthesis and single or double-color in situ hybridization (whole-mount) were performed essentially as previously described (Thiese et al., 1993; Jowett and Yan, 1996; Whitlock and Westerfield, 2000), except for minor modifications. We purified the in vitro synthesized mRNA probes and used them as probes in RNAeasy mini column (Qiagen GmbH). Instead of NBT/BCIP (Boehringer), we used KM purple (Boehringer) to develop color at room temperature for more than 40 hours. We usually removed the yolks from young embryos using forceps. Embryos were mounted in phosphate-buffered saline (PBS) and photographed using a Zeiss Axiohot 2 microscope.

In vitro mRNA synthesis was performed using an RNA synthesis kit (Ambion). A partial dlb3b CDNA with a complete ORF (980 bp) was subcloned into pXT7 (Ambion). The plasmid was restricted with XbaI, and the linear DNA served as a template to generate dlb3b mRNA using T7 RNA polymerase. The complete dlb4b ORF was amplified by PCR and cloned into the pCRT7/CT-TOPO vector (Invitrogen). The resulting plasmid was linearized with PmeI and used as a template to synthesize dlb4b mRNA using T7 RNA polymerase. The recognition site of dlb4b-MO (see Morpholinos) on dlb4b mRNA was eliminated. This dlb4b mRNA could partially restore dlb4b activity if co-injected with dlb4b-MO, as judged by a restoration of dlb4b-MO-induced reduction of the median fin fold (data not shown). A full-length sox9a cDNA was cloned into pCDNA3 vector (Clontech). After linearizing with Apal, the plasmid was used as a template to synthesize sox9a mRNA with T7 RNA polymerase.

For rescue experiments, we injected wild-type RNA into Df(b380) embryos. We also injected a YAC clone that contains both the dlb3b and dlb4b genes (a gift from Angel Amores) and a shorter cosmid clone that contains the dlb3b gene with 0.8 kb of 5’ sequence. We obtained similar rescue as with dlb3b mRNA alone. The combination of the dlb3b-dlb4b YAC or cosmid DNA with a sox9a expression
vector (driven by a CMV promoter/enhancer) resulted in similar rescue as obtained with injection of all three wild-type mRNAs (dlx3b, dlx4b and sox9a). For both DNA and RNA injections, we delivered about 1 nl of solution into the cytoplasm of one-cell stage embryos. The concentrations of the injection solutions were 50-100 ng/µl (DNA) and 200-500 ng/µl (RNA). For injection of all three mRNAs, we used no more than 750 ng/µl total mRNA.

**Morpholinos**

Morpholino antisense oligonucleotides (MOs) were obtained from Gene Tools (Philomath, OR). Translation blocking MOs were: dlx3b-MO, 5′-ATGTCCGTCCTACCTTTAATAA-3′; dlx4b-MO, 5′-GCCCGATGTAGTTGTCGTCGC-3′; sox9a-MO, 5′-TCTAGGATGGTGAGAGTTCATCT-3′; sox9b-MO, 5′-GGTCCCAATCAAGAATGACCTT-3′; and fgf3-MO, 5′-TCTCGCTGGGAATAGAAAAGCTGGCC-3′. Splice blockers were: sox9bE111, 5′-GTGTTGTTCGACGAGTTGTGCGA-3′; and sox9bE212, 5′-GTGTTGTTCGACGAGTTGTGCGA-3′. Snail blockers were: sox9aL10, 5′-GCCCTGAGACTG-3′; and sox9bL5, 5′-GCCCTGAGACTG-3′.

We have previously cloned the zebrafish dlx3b gene and showed that cells of the developing otic placode express dlx3b and sox9a (Ekker et al., 1992; Akimenko et al., 1994; Yan et al., 2002). Scattered cells on the ventral side of the embryo begin expressing dlx3b by mid-gastrula stages (Akimenko et al., 1994). As gastrulation proceeds, a band of strongly expressing cells coalesces at the lateral edge of the presumptive neural plate (Fig. 1A), while expression on the ventral side disappears. When the neural plate forms and cells move toward the dorsal midline in convergence, dlx3b expression is progressively restricted to two groups of cells in this band. These two groups of dlx3b-expressing cells probably correspond to the presumptive olfactory (Whittlock and Westerfield, 2000) and otic (Akimenko et al., 1994) placodes, even though there is no sign of morphological differentiation of the placodes until a few hours later. Shortly before the otic placode is visible morphologically, dlx3b expression is restricted to cells in the position where the placode will form and from then onwards, placode cells express dlx3b (Fig. 1B,C) (Akimenko et al., 1994).

The dlx4b gene, closely linked to dlx3b (Stock et al., 1996), has a similar although non-identical expression pattern (Fig. 1D-F). Expression of dlx4b first appears slightly later than dlx3b and, unlike dlx3b, on the dorsal side of the embryo. By the end of gastrulation, dlx4b expression is concentrated in a band of cells at the lateral edge of the neural plate and overlaps dlx3b expression throughout segmentation stages.

sox9a expression starts at about the same developmental stage as dlx3b. Unlike the two Dlx genes, sox9a-expressing cells are not observed around the lateral edge of the neural plate or in the olfactory placode. Instead, initial sox9a expression in the region of the hindbrain is limited to the presumptive otic placode, where it overlaps with pax2a expression (not shown). By segmentation stages, cells in the region of rhombomere 4 also express sox9a, although at somewhat lower levels than in the placode (Fig. 1G,H).

The Df(b380) deficiency lacks the dlx3b, dlx4b and sox9a genes and blocks formation of the ear

To study the potential functions of Dlx genes in specification of the otic placode, we isolated a deficiency mutation, Df(LG12)dlx3b(b380) (Df(b380)) (Fritz et al., 1996), that removes the dlx3b locus. We identified the mutation in a screen for deficiencies based on multiplex PCR amplification of genomic DNA from haploid offspring of females carrying γ-ray induced mutations (Fritz et al., 1996). We initially identified the Df(b380) mutation by the absence of a PCR amplification product from the dlx3b gene. We subsequently mapped the dlx3b gene to LG12 of the zebrafish genetic map and showed that the Df(b380) mutation removed 21-24 cM of LG 12 that also contains excess antibody, embryos were incubated in secondary antibody goat anti-rabbit or anti-mouse Alexa Fluor 488 (Molecular Probes) at 1:200 dilution in PBDTX with 2% NGS for 5 hours at room temperature or at 4°C overnight. Embryos were then rinsed in PBS and analyzed using a Zeiss Axioptot 2 microscope.

**RESULTS**

Cells of the presumptive otic placode express three transcription factor genes, dlx3b, dlx4b and sox9a

We have previously cloned the zebrafish dlx3b gene and showed that cells of the developing otic placode express dlx3b and sox9a (Ekker et al., 1992; Akimenko et al., 1994; Yan et al., 2002). Scattered cells on the ventral side of the embryo begin expressing dlx3b by mid-gastrula stages (Akimenko et al., 1994). As gastrulation proceeds, a band of strongly expressing cells coalesces at the lateral edge of the presumptive neural plate (Fig. 1A), while expression on the ventral side disappears. When the neural plate forms and cells move toward the dorsal midline in convergence, dlx3b expression is progressively restricted to two groups of cells in this band. These two groups of dlx3b-expressing cells probably correspond to the presumptive olfactory (Whittlock and Westerfield, 2000) and otic (Akimenko et al., 1994) placodes, even though there is no sign of morphological differentiation of the placodes until a few hours later. Shortly before the otic placode is visible morphologically, dlx3b expression is restricted to cells in the position where the placode will form and from then onwards, placode cells express dlx3b (Fig. 1B,C) (Akimenko et al., 1994).
Fig. 1. Cells of the otic placode and vesicle express three transcription factor genes, *dlx3b*, *dlx4b* and *sox9a* required for inner ear development. (A,D,G) Cells of the presumptive otic placode express all three genes at the four-somite stage (4s); other cells along the lateral edge of the neural plate express *dlx3b* and *dlx4b*. (B,E,H) By the 10-somite stage (10s) cells throughout the placode express all three genes. (C,F,I) Later, by prim-5 stage (24 h), a subset of cells in the vesicle expresses *dlx3b* and *dlx4b*; other cells express *sox9a*. Throughout these stages, *dlx4b* expression is similar to but weaker than *dlx3b* expression. Other sites of expression are not shown. (J,L) Wild-type embryos exhibit an otic vesicle in live embryos (DIC, differential interference contrast optics) at prim-5 (24h) stage (J) and *pax2a* expression in the presumptive otic placode at the six-somite stage (L). (K,M) Neither the vesicle (K) nor early *pax2a* expression in the presumptive placode (M) is apparent in *Df b380* mutants (38/38 *Df b380* embryos). (N) The *dlx3b*, *dlx4b* and *sox9a* genes are closely linked on the same chromosome and are removed by the *Df b380* deficiency mutation. (Left) Schematic map of LG12 showing the region of the *Df b380* deficiency in red (not to scale). (Right) We localized *dlx3b*, *dlx4b* and *sox9a* to LG12 by mapping them relative to SSLP markers and other genes (as shown) using the LN54 radiation hybrid panel (Hukriede et al., 1999). We estimated the extent of the *Df b380* deficiency based on our ability to amplify flanking SSLP markers, z1176 and z1473, by PCR using *Df b380* homozygous mutant DNA. These two SSLP markers are separated by 21 cM on the HS and 24 cM on the MGH meiotic panels, and by 296 cR on the T51 radiation hybrid panel (http://zfin.org). Missing genes are shown in red. (A,B,D,E,G,H,L,M) Dorsal views, anterior towards the left; (C,F,I,J,K) side views, anterior towards the left, dorsal towards the top. Scale bar in N: 60 μm for A,D,G; 33 μm for B,E,H; 22 μm for C,F,I; 77 μm for J,K; 145 μm for L,M; 5 cM for N.

Several other genes (Fig. 1N), including *dlx4b* (Stock et al., 1996) and *sox9a* (Chiang et al., 2001).

Homozygous *Df b380* mutants fail to form an ear. We observed no otic placode or vesicle in live mutant embryos using Nomarski optics (Fig. 1K) and initial expression of *pax2a*, a marker of otic precursor cells in this region, is also absent (Fig. 1M). Other sites of *pax2a* expression (Püschel et al., 1992) appear normal in *Df b380* mutants, including optic stalk, mid-hindbrain junction and pronephros, although *pax2a* expression is elevated in the branchial arches (data not shown). Homozygous *Df b380* mutants also fail to form olfactory organs. Because olfactory organs form from ectodermal placodes that, like the otic placodes, express *dlx3b* and *dlx4b*, this associated phenotype may indicate a common pathway mediated by *dlx3b* and *dlx4b* for specification of these two sensory structures, as we (Akimenko et al., 1994) and others (Torres and Giraldez, 1998; Solomon and Fritz, 2002) have previously suggested. Heterozygous (*Df b380/+*) individuals develop with no obvious abnormalities.

Despite this apparent lack of otic induction, a few residual cells express characteristics of otic cells in *Df b380* mutants. By prim-5 stage (24 h), ~30% of *Df b380* mutant embryos form a patch of 10-20 *pax2a*-expressing cells (Fig. 2B) lateral to hindbrain rhombomere 5 in the region where the otic vesicle normally develops in wild-type embryos. This *pax2a*-positive patch of cells is apparent in 80% of embryos (n=43) by prim-5 (30 h) and in all embryos (n=14) by the second day of development. Consistent with their differentiation as otic cells, these cells express other otic markers, including *fn1* (Fig. 2E) and *claudin a* (*cldna*), a marker of the otic epithelium (Fig. 2H). Although cells in other regions of the embryo also express *fibronectin 1* (*fn1*) (Zhao et al., 2001), *cldna* expression marks the ear (Kollmar et al., 2001) and the posterior lateral line (not shown). In contrast to wild-type embryos, however, these residual otic cells form only a tight cluster, resembling an epithelial ball, and never produce a vesicle or other morphological features of the ear.

**Knockdown of *dlx3b*, *dlx4b* and *sox9a* functions is sufficient to block formation of the ear**

Our analysis of *Df b380* mutants suggested that the functioning of some combination of the *dlx3b*, *dlx4b* and *sox9a* genes is required for specification of the otic placode. Based solely on this analysis, however, we could not rule out the possibility that other genes are also required, because the deficiency is rather large. To distinguish between these possibilities, we used morpholino antisense oligonucleotides (MOs) that block translation or splicing of mRNA from individual target genes when injected into zebrafish embryos (Nasevicius and Ekker, 2000; Draper et al., 2001).

Knockdown of all three transcription factors phenocopies the *Df b380* deficiency mutant phenotype. When *dlx3b*-MO,
dlx3b-MO and sox9a-MO are injected in combination into wild-type embryos, formation of the otic placode fails and we observe only a few residual pax2a-expressing cells as in Df[h380] mutants (Fig. 2C). These residual cells form a small epithelial ball and also express fn1 and cldna (Fig. 2F,I), as do Df[h380] mutants (Fig. 2E,H). These results demonstrate that the combined loss of Dlx3b, Dlx4b and Sox9a functions is sufficient to account for the Df[h380] mutant otic phenotype. To study the requirements and individual roles of these three transcription factors in otic specification, we then injected MOs directed against single genes or combinations of two genes.

Dlx3b function is required for early and complete maturation of the otic placode and vesicle. Injection of dlx3b-MO into wild-type embryos delays specification of the otic placode as indicated by delayed and reduced pax2a expression (Fig. 3A-D) and morphological maturation (Fig. 3G,H,K,L). The dlx3b-MO also affects later differentiation of the otic vesicle; the vesicle fails to achieve either its normal
size or numbers of otoliths (Fig. 3H,L) and sensory hair cells (Fig. 3I,J). Formation of epithelial protrusions and subsequent semicircular canals also fails (Fig. 3KL). To ensure that the dlx3b-MO effectively knocks down dlx3b function, we raised a monoclonal antibody specific for Dlx3b protein and demonstrated absence of labeling after dlx3b-MO injection (Fig. 3E,F). These results are consistent with an absence of detectable levels of protein and provide additional support for the conclusion that Dlx3b is required for otic development. However, because the effects of dlx3b-MO on the ear are less severe than the Df b380 mutant phenotype, other genes missing in the Df b380 deficiency must also be required for otic placode specification.

Dlx4b has a less significant function than Dlx3b in otic development. Injection of dlx4b-MO has little effect on specification of the placode during early segmentation stages, as indicated by pax2a expression and by morphological observations (data not shown). By contrast, dlx4b-MO injection produced a very severe reduction in development of the medial fin fold (data not shown), another site of dlx4b expression (Ellies et al., 1997) suggesting that dlx4b-MO effectively blocks Dlx4b function. By prim-15 (30 h) stage, the otic vesicle is slightly smaller than normal after dlx4b-MO injection, but the normal number of otoliths and hair cells form. However, when the dlx4b-MO and dlx3b-MO are combined, an additive effect is seen; otoliths fail to form in the majority of embryos (Solomon and Fritz, 2002), the otic vesicle is smaller than after injection of either morpholino alone and essentially no sensory hair cells or epithelial protrusions form (data not shown). Nevertheless, the combined effect of both morpholinos is less severe than the Df b380 mutant phenotype.

Knockdown of Sox9a function by morpholino (sox9a-MO) injection has a relatively mild effect on otic specification, resulting in a slightly reduced vesicle with a normal number of otoliths (data not shown). To ensure that Sox9a function was blocked, we used morpholinos directed against splice donor and acceptor sites that would be expected to interfere with splicing of the sox9a pre-mRNA. Consistent with this interpretation, we found, using mRNA in situ hybridization, that sox9a message localizes in nuclei after sox9a-MO injection as we previously reported (Yan et al., 2002), suggesting that the morpholinos effectively block splicing. We obtained similar results with sox9a translation blocking and mRNA splice blocking MOs. The effect of sox9a-MO on otic development is more severe than the otic phenotype of jef (sox9a) mutants (data not shown), probably because of partial early function of this mutant allele (Yan et al., 2002).

**Function of Dlx3b, Dlx4b and Sox9a transcription factors is sufficient to rescue otic placode specification in Df b380 mutants**

If combined loss of the dlx3b, dlx4b and sox9a genes is responsible for the absence of otic placode specification in Df b380 mutants, then restoring wild-type function of only these genes should rescue the mutant phenotype. To test this prediction, we injected wild-type mRNAs for each gene into Df b380 mutant embryos. Although neither dlx3b nor sox9a mRNA rescues the mutant phenotype, dlx3b mRNA partially restores otic placode specification as indicated by pax2a expression and morphology (Fig. 4C,G). Injection of a combination of all three mRNAs into Df b380 mutants produces a much more robust rescue; the size of the placode, the number of pax2a-expressing cells and the level of pax2a expression are comparable with wild-type values (Fig. 4D,H). Rescue of later otic development is variable and less complete, probably because of degradation of the injected mRNAs, as we have previously shown for injected DNA (Westerfield et al., 1992).

**sox9b also participates in otic specification**

Our observation that a few residual cells express otic markers in Df b380 mutants (Fig. 2) suggests the possibility that factors in addition to dlx3b, dlx4b and sox9a participate in otic specification. We have previously shown that the sox9a duplicate, sox9b, is also expressed in the otic placode and vesicle (Chiang et al., 2001). To examine its potential role in otic specification, we compromised its function using morpholino injection. Reduction of either sox9a function, in jef (sox9a) mutants (Fig. 5C), or sox9b function by morpholino injection (Fig. 5B) has little or no effect on formation of the otic vesicle. Compromising both genes results in a more severe reduction in otic specification, although, as in Df b380 mutants, some residual scattered cells still express otic markers (Fig. 5D). Knockdown of sox9b in Df b380 mutants completely blocks all signs of otic specification (Fig. 5F). Thus, like Sox9a, Sox9b participates in otic specification; Sox9b function in Df b380 mutants presumably accounts for the few residual otic cells that form in the absence of dlx3b, dlx4b and sox9a.
**sox9a but not dlx3b, dlx4b or sox9b expression requires Fgf3 and Fgf8 signaling**

Recent studies have implicated combined, redundant functions of Fgf3 and Fgf8 in zebrafish otic placode induction (Philips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002). To learn whether Fgf3 and Fgf8 function in a common pathway with Dlx3b, Dlx4b, Sox9a and/or Sox9b, we examined expression of fgf3 and fgf8 in Df(b380) mutants and expression of the four transcription factors in fgf8– mutants (Reifers et al., 1998) and after injection of fgf3 morpholinos (fgf3-MO) into wild-type embryos. Expression of fgf3 and fgf8 is relatively normal in Df(b380) mutants; we observed no change in fgf8 expression and only a slight increase in fgf3 expression in rhombomere 4 (data not shown).

Reduction of Fgf8 function in fgf8– mutants significantly reduces sox9a otic expression (Fig. 6C), whereas knockdown of Fgf3 by fgf3-MO injection, results in a more modest reduction of sox9a otic expression (Fig. 6B). Injection of fgf3-MO into fgf8– mutants results in complete absence of sox9a expression in the region where the otic placode would normally form (Fig. 6D). Thus, Fgf3 and Fgf8 appear to act synergistically to support sox9a expression, just as they apparently act together to support otic development (Philips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002). Reduction of Fgf signaling has a similar although somewhat less significant effect on sox9b expression (Fig. 6E-H). Residual sox9b expression in the otic area, even when both fgf3 and fgf8 are compromised, is consistent with Fgf-independent regulation of sox9b.

By contrast, dlx3b expression is relatively unaffected by reduction of Fgf signaling. Knockdown of Fgf3 function by injection of fgf3-MO, has no significant effect on the dlx3 otic expression pattern (Fig. 6J). Loss of Fgf8 function in fgf8– mutants or fgf3-MO injection into fgf8– mutants results in only a slight narrowing of the dlx3b (Fig. 6K,L) and dlx4b (not shown) expression domains and significant numbers of Dlx3b-expressing cells remain, although they are scattered along the side of the hindbrain (Fig. 7D,F) rather than being well organized into an otic epithelium (Fig. 7C,E).

The reduction in size of the dlx3b expression domain when Fgf3 and Fgf8 signaling is blocked is probably due to the loss of Sox9a function. Consistent with this hypothesis, the dlx3b
and sox9a expression domains largely overlap in the otic region (Fig. 8A,B) and we found that knockdown of sox9a by itself results in a reduction in the dlx3b expression domain (Fig. 8C,D) similar to loss of Fgf3 and Fgf8 function (Fig. 6L). Reciprocally, knockdown of dlx3b produces a reduction in the sox9a expression domain (Fig. 8E,F). By contrast, knockdown of sox9b reduces sox9a expression slightly (Fig. 8G,H) but has little effect on dlx3b or dlx4b expression (not shown). Knockdown of dlx4b had only a slight effect on sox9a or sox9b expression (not shown), but dlx3b-MO combined with dlx4b-MO had a greater effect than either alone (Fig. 8I,J and data not shown). Together, these results suggest that the function of Fgf3 and Fgf8 in otic induction is independent of Dlx3b and Dlx4b, but is mediated at least partly by Sox9a and Sox9b.

Moreover, we found that the dlx3b-dlx4b and sox9a genes reciprocally regulate each other’s expression.

Residual otic cells that form in Df b380 mutants require Fgf signaling

Our observation that some aspects of Fgf3 and Fgf8 function in otic specification are independent of Dlx3b and Dlx4b raised the possibility that the residual otic cells that form in Df b380 mutants require Fgf3 and Fgf8 function. We tested this possibility by examining otic specification in Df b380 mutants with compromised Fgf signaling. Although a few otic cells form in Df b380 mutants injected with fgf3 MO (Fig. 9C), we observed no signs of otic cells morphologically or by pax2a expression in Df b380;fgf8 double mutants (Fig. 9D). These results support the interpretation that in the absence of dlx3b, dlx4b and sox9a, Fgf signaling is still capable of inducing a few cells to express characteristics of otic cells, although these cells fail to form a functional ear. These are presumably Fgf-dependent sox9b-expressing cells (Fig. 6E-H), because all signs of otic specification are also missing when we knockdown sox9b expression in Df b380 mutants (Fig. 5F). The few otic cells that develop in Df b380 mutants or when either Fgf3 or Fgf8 signaling is compromised, form a small epithelial ball that resembles the early stages of otic vesicle formation (Fig. 9B,C,F,G). Further development fails. Knockdown of both Fgf signals, when dlx3b, dlx4b and sox9a are still intact,
results in development of a similar number of cells that express otic markers such as *pax2a*, but in contrast to *Dlx3b*, *Dlx4b* and *Sox9a* depletion, these putative otic cells are scattered along the side of the hindbrain and fail to assemble into an epithelial structure (Fig. 7D,F; Fig. 9H).

**DISCUSSION**

Previous studies have implicated the signaling molecules Fgf3 and Fgf8 (Vendrell et al., 2000; Phillips et al., 2001; Léger and Brand, 2002), and the transcription factors *Dlx3b* and *Dlx4b* (Ekker et al., 1992; Akimenko et al., 1994; Ellies et al., 1997; Solomon and Fritz, 2002) in induction and specification of the vertebrate inner ear. To help define how these various factors interact and their specific roles in otic induction, we analyzed zebrafish *fgf8*-mutants, *Df* *b380* deficiency mutants that lack *Dlx3b*, *Dlx4b* and *Sox9a*, and embryos injected with morpholinos to knockdown the functions of these genes. To analyze Fgf3 and Sox9b functions, we used *fgf3*-MO and *sox9b*-MO, respectively, because mutations in the *fgf3* and *sox9b* genes have not yet been identified in zebrafish.

**Fgf3- and Fgf8-dependent and -independent pathways of otic development**

Our results provide evidence for Fgf3- and Fgf8-dependent and -independent pathways in otic development (Fig. 10). We found that loss of either Fgf3 and Fgf8 function or functions of *Dlx3b*, *Dlx4b* and *Sox9a* transcription factors missing in the *Df* *b380* deficiency mutation results in nearly complete loss of otic tissue, although a few residual cells express otic markers. Loss of both Fgf3 and Fgf8 function, and functions of the three transcription factors completely blocks all indications of otic induction. The Fgf-dependent and -independent pathways appear to act synergistically. The number of otic cells is drastically reduced in *Dlx3b* and *Dlx4b*-deficient or Fgf3- and Fgf8-deficient embryos. Providing either *Dlx3b* and *Dlx4b* or Fgf3 and Fgf8 function produces only limited otic specification, much less than when both pathways are active.

Previous studies, using three markers, *pax8*, *pax2a* and *dlx3b*, to examine the Fgf dependence of initial induction of the otic placode, produced somewhat differing results (Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002). Using morpholinos to knockdown Fgf3 and Fgf8, Léger and Brand (Léger and Brand, 2002) reported loss or strong reduction of *pax2a* and *pax8* expression in the otic region; Maroon et al. (Maroon et al., 2002) found less severe defects. By injecting *fgf3*-MO into *fgf8*-mutants, Léger and Brand and Phillips et al. found strong reduction of *pax2a* and *pax8* (Léger and Brand, 2002; Phillips et al., 2001) Using SU5402, a general inhibitor of Fgf receptors, Léger and Brand (Léger...
and Brand, 2002) reported complete loss of pax2a and pax8; Maroon et al. (Maroon et al., 2002) obtained similar results for pax2a but found that pax8 was unaffected unless the inhibitor was applied at later stages. Using SU5402, Léger and Brand (Léger and Brand, 2002) found that initial dlx3b expression is independent of Fgf signaling, but later expression in the otic region is reduced. They reported similar results with fgf3-MO + fgf8-MO. Although Maroon et al. did not examine the earliest stages of dlx3b expression, they found that dlx3b expression at later stages was lost in some experiments but only reduced in others after Fgf-MO injections. Phillips et al. did not examine dlx3b expression (Phillips et al., 2001). Although the results of these studies differ somewhat, possibly because of differences in experimental conditions, all three groups similarly conclude that Fgf3 and Fgf8 are required for initial otic induction. Our results show that although most signs of otic induction are missing when Fgf3 and Fgf8 signaling is blocked, a few residual otic cells form as indicated by expression of pax2a, dlx3b, fn1 and cldna. The previous studies may well have missed these remaining otic cells we have identified, probably because they are few in number and typically scattered along the side of the hindbrain, rather than being organized into an easily recognizable vesicle. Our results also provide an explanation for apparent discrepancies among the previous studies of the Fgf dependence of dlx3b expression. We show that sox9a expression requires fgf3 and fgf8; sox9a, in turn, is required for normal expression of dlx3b and dlx4b. Thus, induction and early patterning of dlx3b and dlx4b expression are unaffected by blocking Fgf3 and Fgf8 signaling, but their later expression in the otic region is reduced because of loss of sox9a.

Consistent with previous studies that suggested Fgf3 and Fgf8 have redundant functions in hindbrain (Maves et al., 2002; Walshe et al., 2002) and otic development (Philips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002), we found that reduction of either Fgf3 or Fgf8 signaling produces a partial loss of sox9a and sox9b expression (Fig. 6B,C,F,G) and a smaller or somewhat disorganized otic vesicle (Fig. 9F,G). Reduction of both Fgfs (Fig. 6D,H; Fig. 9H) produces a greater defect than reduction of either alone. However, recent studies suggest that Fgf3 and Fgf8 have different downstream targets (Reifers et al., 2000). Loss of Fgf8 function (in fgf8- mutants) consistently produces a more severe phenotype than knockdown of Fgf3 function alone by injection of fgf3 MO, suggesting that although Fgf3 and Fgf8 may have overlapping functions, Fgf8 apparently plays a more significant role in otic induction than Fgf3, perhaps because of its earlier and more widespread expression (Reifers et al., 1998; Maves et al., 2002).

**Genetic interactions of sox9a and sox9b with fgf3, fgf8, dlx3b and dlx4b**

Part of Fgf3 and Fgf8 function in otic development is mediated by Sox9a and Sox9b. We found that in the otic region after Fgf3 and Fgf8 reduction, sox9a expression is lost (Fig. 6D) and sox9b expression is reduced although not completely eliminated (Fig. 6H). The dlx3b and dlx4b expression domain is reduced by knockdown of sox9a expression, and there is a similar reduction in sox9a expression by knockdown of dlx3b and dlx4b (Fig. 8C-F). Depletion of Fgf3 and Fgf8 signaling similarly reduces dlx3b and dlx4b expression (Fig. 6H), presumably because of loss of sox9a function. By contrast, reduction of sox9b expression has little effect on dlx3b and dlx4b expression, although sox9b expression depends on Dlx3b and Dlx4b (Fig. 8I,J). These results indicate that sox9a interacts genetically with both the Fgf3- and Fgf8-dependent and -independent pathways. Thus, sox9a apparently plays a central role in coordinating otic development.

Previous studies have shown that pax8 expression is also reduced or lost in fgf8- mutants injected with fgf3-MO (Philip et al., 2001; Léger and Brand, 2002) (but see Maroon et al., 2002). In addition, pax8 expression persists in the absence of the dlx3b, dlx4b and sox9a genes (Solomon and Fritz, 2002) (D.L., H.C. and M.W., unpublished). Thus, Fgf3 and Fgf8 signaling appears to act through at least two genetically distinct pathways in the otic placode: one dependent upon Sox9a and the other mediated by something else, possibly Pax8 or other, as yet unidentified factors. Although pax8 expression is unaffected by reduction of sox9a, we have not yet determined whether Pax8 acts in the same or different pathways with Sox9a and Sox9b.

**Fgf3 and Fgf8 functions in otic induction and morphogenesis**

Our results may provide insight into how Fgf3 and Fgf8 growth factors function in otic development. Previous studies have indicated that FGFR3 directs morphogenesis of the avian otic vesicle (Vendrell et al., 2000). We found that the residual otic cells in Fgf3- and Fgf8-deficient embryos failed to form an epithelial structure (Fig. 7D,F; Fig. 9H), whereas the residual otic cells in Df(1X)k380 mutants, with Fgf3 and Fgf8 signaling still intact, form small epithelial balls (Fig. 2B,E,H; Fig. 9B). This might indicate that Fgf3-Fgf8 signaling that is normally localized to rhombomere 4 ‘organizes’ the placode cells into an epithelium that subsequently forms the otic vesicle. We have recently provided evidence for a similar Fgf3- and Fgf8-dependent activity of rhombomere 4 in organizing posterior hindbrain segments (Maves et al., 2002). Our previous fate map analysis of the nose demonstrated that cells from a relatively large area at the lateral edge of the neural plate converge to form the olfactory placode and subsequent epithelium (Whitlock and Westerfield, 2000). A similar mechanism may occur during otic placode development. Perhaps in the absence of Fgf3 and Fgf8 signals from rhombomere 4, cells fail to converge properly to this organizing region and, hence, cannot form the otic epithelium.

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