A direct role for Fgf but not Wnt in otic placode induction

Bryan T. Phillips, Elly M. Storch, Arne C. Lekven and Bruce B. Riley*

Biology Department, Texas A&M University, College Station, TX 77843-3258, USA
*Author for correspondence (e-mail: briley@mail.bio.tamu.edu)

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Summary

Induction of the otic placode, which gives rise to all tissues comprising the inner ear, is a fundamental aspect of vertebrate development. A number of studies indicate that fibroblast growth factor (Fgf), especially Fgf3, is necessary and sufficient for otic induction. However, an alternative model proposes that Fgf must cooperate with Wnt8 to induce otic differentiation. Using a genetic approach in zebrafish, we tested the roles of Fgf3, Fgf8 and Wnt8. We demonstrate that localized misexpression of either Fgf3 or Fgf8 is sufficient to induce ectopic otic placentas and vesicles, even in embryos lacking Wnt8. Wnt8 is expressed in the hindbrain around the time of otic induction, but loss of Wnt8 merely delays expression of preotic markers and otic vesicles form eventually. The delay in otic induction correlates closely with delayed expression of fgf3 and fgf8 in the hindbrain. Localized misexpression of Wnt8 is insufficient to induce ectopic otic tissue. By contrast, global misexpression of Wnt8 causes development of supernumerary placentas/vesicles, but this reflects posteriorization of the neural plate and consequent expansion of the hindbrain expression domains of Fgf3 and Fgf8. Embryos that misexpress Wnt8 globally but are depleted for Fgf3 and Fgf8 produce no otic tissue. Finally, cells in the preotic ectoderm express Fgf (but not Wnt) reporter genes. Thus, preotic cells respond directly to Fgf but not Wnt8. We propose that Wnt8 serves to regulate timely expression of Fgf3 and Fgf8 in the hindbrain, and that Fgf from the hindbrain then acts directly on preplacodal cells to induce otic differentiation.

Key words: Otic induction, Hindbrain patterning, pax8, foxi1, erm, dickkopf, Preplacodal domain, Zebrafish

Introduction

General mechanisms of neural development are conserved broadly amongst metazoans, but components of a number of sensory organs in vertebrates are derived from evolutionarily unique structures known as cranial placentas. The inner ear in particular is remarkable because virtually the entire organ system and the neurons that innervate it are derived from a single rudiment, the otic placode (reviewed by Baker and Bronner-Fraser, 2001; Whitfield et al., 2002; Riley and Phillips, 2003). The induction and development of the otic placode has long been a popular subject of experimental embryology studies because it is readily accessible and undergoes such a complex morphogenesis. Considerable study has shown that even the initial steps in otic induction are highly complex. Naive ectoderm is induced to form the otic placode through a series of interactions with surrounding tissues during the latter half of gastrulation. The molecular players involved in otic induction have only recently begun to come to light.

A number of studies now point to members of the Fgf family of peptide ligands as the best candidates for otic-inducing factors produced by periotic tissues. In particular, Fgf3 appears to play a highly conserved role in otic induction. In all vertebrates examined to date, Fgf3 is expressed in the hindbrain directly between the developing otic anlage during mid-late gastrulation (Wilkinson et al., 1989; Mahmood et al., 1995; Mahmood et al., 1996; McKay et al., 1996; Lombardo et al., 1998; Phillips et al., 2001), and misexpression studies in chick and Xenopus show that Fgf3 can induce formation of otic placentas in ectopic locations (Vendrell et al., 2000, Lombardo et al., 1998). Loss of Fgf3 function does not prevent otic induction in either mouse or zebrafish, although later otic development is clearly impaired (Mansour et al., 1993; Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Kwak et al., 2002). The reason for continued otic induction is that other Fgf homologs provide redundancy in the inductive pathway. In zebrafish, fgf8 is coexpressed with fgf3 in the hindbrain, and loss of both leads to complete failure of otic induction (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al., 2003). Fgf8 does not play a comparable role in tetrapods, but it is likely to regulate later stages of otic development (reviewed by Riley and Phillips, 2003). Instead, other Fgfs provide redundancy. In the mouse, Fgf10 is expressed in mesoderm just beneath the preplacode, and loss of both Fgf3 and Fgf10 ablates otic development (Wright and Mansour, 2003). The above studies do not exclude a role for other inductive signals but, taken together, they indicate that Fgf signaling is both necessary and sufficient for otic induction.

By contrast, an alternative model was proposed recently in which Fgf must cooperate with another factor, Wnt8, to induce the otic placode (Ladher et al., 2000a). In chick, Fgf19 is expressed initially in subjacent mesoderm and is found later in hindbrain between prospective otic placodes. By itself, Fgf19 does not induce expression of any otic markers in explants of uncommitted ectoderm but it does induce expression of the hindbrain factor Wnt8c, the chick ortholog of Wnt8 (Schubert et al., 2000). Exogenous Wnt8c weakly induces a subset of otic markers in explant cultures, whereas Fgf19 plus Wnt8 strongly induce the full range of otic markers. Thus, it was proposed...
that Fgf19 in the mesoderm induces expression of Wnt8c in the hindbrain, and that the two factors synergize to induce the otic placodes. This model has not been tested previously in vivo. In addition, a complication of the model is that Wnt8c and Fgf19 also strongly induce expression of Fgf3, which may have played a direct role in inducing the full range of otic markers. Because FGF19 has no known ortholog in zebrafish, we addressed the question of whether known zebrafish otic inducers, Fgf3 and Fgf8, are sufficient to induce otic tissue or must cooperate with Wnt8. Our data demonstrate that Fgf signaling is both necessary and sufficient for otic induction whereas Wnt8 is neither necessary nor sufficient. Expression of Fgf and Wnt reporter genes indicates that Fgf, but not Wnt, signals directly to the otic anlage. Instead, Wnt8 appears to be indirectly involved in otic induction by virtue of its requirement for timely hindbrain expression of Fgf genes.

Materials and methods

Strains and developmental conditions

The wild-type strain was derived from the AB line (Eugene, OR). The Dfw8 mutation was induced by γ irradiation (Lekven et al., 2000; Lekven et al., 2001). Embryos were developed in an incubator at 28.5°C in water containing 0.008% Instant Ocean salts.

In situ hybridization

Embryos were fixed in MEMFA [0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde]. In situ hybridizations (Stachel et al., 2001) were performed at 67°C using probes for pax2.1 (Krauss et al., 1991), fgfg (Reifers et al., 1998), pax8 (Pfeffer et al., 1998), TOPdGFP (Dorsky et al., 2002), ermA (Roehl and Nusslein-Volhard, 2001; Raible and Brand, 2001), wnt8 ORF2 (Lekven et al., 2001), fox1 (Solomon et al., 2003) and krox-20 (Oxtoby and Jowett, 1993) transcripts. The fgfg construct was generated by amplifying the coding sequence of fgfg (GenBank Accession Number NM 131291) and ligating it into the ClaI and EcoRI sites of pCS2+. Two-color in situ hybridization was performed essentially as described by Jowett (Jowett, 1996), with several modifications. RNase inhibitor (100 units ml⁻¹, Promega) was added during antibody incubation steps to help stabilize mRNA. Fast Red (Roche) was used in the first alkaline phosphatase reaction to give red color and fluorescence. Afterward, alkaline phosphatase from the first color reaction was inactivated by incubating embryos in a 4% formaldehyde solution for 2 hours at room temperature and then heating for 10 minutes at 37°C. NBT-BCIP (Roche) was used for the second alkaline phosphatase reaction to give blue color. For sectioning, embryos were embedded in Immunobed resin (Polysciences No. 17324) and cut into 4 μm sections.

Morpholino oligomer injections

Morpholino oligomers (Gene Tools Inc) were diluted in Danieaux solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES (pH 7.6)] to concentrations of 2.5 μg ml⁻¹ fgfg-MO, 2.5 μg ml⁻¹ fgfl8-MO, 1.25 μg ml⁻¹ wnt8 ORF1-MO, 1.25 μg ml⁻¹ wnt8 ORF2-MO. Filtered green food coloring was added to a concentration of 3% to visualize fluid during injections. Approximately 1-5 nl was injected into the yolk of one- to two-cell stage embryos. Embryos were injected and maintained in Holtfreter’s solution [60 mM NaCl, 0.6 mM KCl, 0.9 mM CaCl2, 5 mM HEPES (pH 7.4)] with 50 units ml⁻¹ penicillin and 50 μg ml⁻¹ streptomycin. Morpholino used were: fgfg-MO (Phillips et al., 2001); fgfg8-MO (Furthauer et al., 2001); wnt8 ORF1-MO; and wnt8 ORF2-MO (Lekven et al., 2001).

Misexpression

To misexpress Fgfs, we tried several approaches in which mRNA and DNA (10-100 ng ml⁻¹) were injected into embryos between one- and 16-cell stages. Two methods were used to achieve mosaic misexpression of Fgf mRNA: injection at one-cell stage followed by blastomere transplantation into uninjected hosts; and injection between four- and 16-cell stages. Both methods resulted in embryos that were too severely dorsalized to study otic development. Alternatively, pCS2+ plasmid DNA containing a constitutive cytomegalovirus promoter upstream of the coding sequence of interest was injected between one- and 16-cell stages. The method that resulted in the greatest frequency of ectopic otic tissue was eight-cell injection of fgfg plasmid at a concentration of 30 ng ml⁻¹. Mosaic misexpression of Wnt8 was achieved by eight-cell injection of 30-40 ng ml⁻¹ of ORF1 or ORF2 plasmid. Global misexpression of Wnt8 was achieved by one-cell injection of 80 ng ml⁻¹ ORF1 or ORF2 plasmid. Global misexpression of Dkk1 was accomplished by one-cell injection of either 40 ng ml⁻¹ or 80 ng ml⁻¹ plasmid. In all cases, injection volume was 1-5 nl. Filtered, green food coloring was added to a concentration of 3% to visualize fluid during injections.

Results

Wnt8 is not required for otic induction

In zebrafish, Wnt8 is the closest ortholog of chick Wnt8c (Schubert et al., 2000). The zebrafish wnt8 locus encodes a bicistronic message consisting of two complete open-reading frames, ORF1 and ORF2, which encode distinct, but highly homologous ligands. Both open-reading frames are expressed at 50% epiboly in the ventral and lateral marginal zone (Kelly et al., 1995; Lekven et al., 2001). At 75% epiboly (8 hours post fertilization, hpf) ORF2 transcripts can be detected in rhombomeres 5 and 6 (r5/6), immediately adjacent to the otic placode anlage, and persist until at least the six somite stage. To test the possibility that preotic cells require Wnt8, we examined otic development in embryos injected with morpholinos directed against ORF1 and/or ORF2. Knockdown of ORF1 alone causes mild dorsalization but no apparent otic defects (not shown). By contrast, ORF2-MO injected embryos consistently produced small otic vesicles shortened by ~50% (not shown). To ensure more complete loss of Wnt8 function, embryos were coinjected with ORF1-MO and ORF2-MO (hereafter termed wnt8 morphants). Ear development was impaired to roughly the same degree as in embryos injected with ORF2-MO alone (Fig. 1A,D). Despite the small size of these otic vesicles, they always contained anterior and posterior sensory maculae and associated otoliths, indicating that key aspects of morphogenesis and differentiation occurred normally. To ascertain whether the observed ear defects were caused by faulty otic induction, we examined the expression of the preotoc marker, pax8. Preotoc expression of pax8 begins by 90% epiboly (9 hpf) in wild-type embryos (Pfeffer et al., 1998; Phillips et al., 2001), but was still not evident at tailbud stage (10 hpf) in wnt8 morphants (Fig. 1G,H). Similarly, preotoc pax8 was not observed at tailbud stage in embryos homozygous for a chromosomal deficiency, Dfl/LG14wnt8+ (termed Dfw8), which deletes both wnt8 open-reading frames (Lekven et al., 2001) (data not shown). However, pax8 is eventually expressed in the preotoc domain in both Dfw8 mutants and wnt8 morphants by the six-somite stage (12 hpf), 2 hours later than normal (Fig. 1K and not shown). This demonstrates that Wnt8 is not necessary for otic induction per se, but is required for timely initiation of the otic field.

To address the possibility that another, as yet unknown, Wnt
protein partially compensates for the loss of Wnt8, we misexpressed the Wnt antagonist Dickkopf 1 (Dkk1). Zebrafish Dkk1 is a homologue of Xenopus Dkk1, which is a potent extracellular antagonist of Wnt activity in vivo (Glinka et al., 1998; Hashimoto et al., 2000). In total, 165/239 (69%) of dkk1 plasmid injected embryos displayed a dorsalized and anteriorized phenotype characterized by severe truncation of posterior tissues similar to wnt8 morphants (Fig. 1A,B). These dkk1-injected embryos possessed otic vesicles. The remainder (74/239, 31%) exhibited a more severe loss of posterior structures, including hindbrain, than was observed for wnt8 morphants (Fig. 1C). These severe embryos did not appear to possess otic vesicles. However, analysis of pax8 expression at six-somite stage (12 hpf) showed that otic induction had occurred in all (21/21) dkk1-injected embryos (Fig. 1J). These data demonstrate that placode induction can occur despite globally compromised Wnt function.

**Wnt8 regulates timely expression of fgf3 and fgf8 in the hindbrain**

To clarify whether the delay in otic induction observed in Wnt8 loss-of-function embryos was caused by indirect effects, we examined expression of previously identified otic inducers, Fgf3 and Fgf8, in embryos lacking Wnt8 function. Normally, fgf3 is expressed in r4 by 90% epiboly (9 hpf). However, the hindbrain domain of fgf3 was barely visible at tailbud stage (10 hpf) in over half (71/128) of wnt8 morphants and is undetectable at this stage in Dfw8 mutants (Fig. 2B,C). Strong r4 expression of fgf3 becomes evident by the six-somite stage (12 hpf) in Dfw8 homozygotes (Fig. 2D). The hindbrain domain of fgf8 becomes evident by 75% epiboly (8 hpf) in wild-type embryos but was only weakly expressed in most (61/81) wnt8 morphants even as late as 90% epiboly (9 hpf). Furthermore, 10% of wnt8 morphants still had reduced expression at tailbud stage (10 hpf, Fig. 2F). Expression of fgf8 was also delayed in Dfw8 homozygotes, in which expression cannot be detected in the hindbrain until tailbud stage (10 hpf, Fig. 2G). Dfw8 mutants and wnt8 morphants show strong fgf8 expression by the six-somite stage (12 hpf, Fig. 2H and data not shown). This indicates that Wnt8 is necessary for timely expression of both fgf3 and fgf8 in the hindbrain. The delay in Fgf expression correlates well with the delay in otic induction and indicates that the otic defects observed in these embryos may be an indirect effect resulting from a deficiency in Fgf signaling. The possibility remains, however, that Wnt signaling regulates later aspects of otic development (see Discussion).

**Fgf signaling regulates wnt8 in the hindbrain**

To more fully understand the epistatic relationship between Wnt and Fgf signaling, we examined wnt8 expression in embryos knocked down for Fgf3 and Fgf8. Expression of ORF2 in the r5/6 domain normally begins by 75% epiboly (8 hpf) but did not begin until 90% epiboly (9 hpf) in embryos depleted for Fgf3 and Fgf8 (Fig. 2I). ORF2 continued to be expressed at lower than normal levels in the r5/6 domain through tailbud stage (10 hpf, Fig. 2L). However, the wnt8 germring domain appeared unaffected. The finding that Fgf and Wnt positively regulate each other is reminiscent to the model proposed by Ladher et al. (Ladher et al., 2000a), in which chick Fgf19 is proposed to induce expression of Wnt8c and Wnt8c induces expression of Fgf3 (see Discussion).

**Misexpression of Fgf3 or Fgf8 induces ectopic otic tissue**

Although loss-of-function studies indicate that Fgf3 and Fgf8 but not Wnt8 are necessary for otic induction, we sought to test whether any of these factors are sufficient for otic induction. To misexpress either Fgf3 or Fgf8, we injected at various stages...
either synthetic RNA or plasmid DNA containing Fgf cDNA under the control of a constitutive promoter. We found that embryos are extremely sensitive to Fgf misexpression because both mRNA and early stage plasmid injection led to severe dorsalization and expansion of the neural plate at the expense of epidermal and preplacodal ectoderm (data not shown). This most likely reflects an early function of Fgf signaling in dorsal/ventral patterning (Fürthauer et al., 1997; Koshida et al., 2002). However, injection of plasmid into wild-type embryos at the eight-cell stage resulted in belated, mosaic Fgf expression. With this technique, some embryos still exhibited moderate dorsalization, but by co-staining injected embryos for neural marker and Fgf expression, we determined that the majority had only small, scattered patches of expressing cells and did not show overt signs of dorsalization. Of the non-dorsalized class, 26% (30/118) of Fgf3-misexpressing embryos and 15% (14/94) of Fgf8-misexpressing embryos showed ectopic patches of *pax8* expression and/or significant expansion of the endogenous preotic domain. Such expression did not result from expansion of the otic-inducing portion of the hindbrain because *krox-20* expression was normal (Fig. 3B). Instead, sites of ectopic *pax8* correlated with sites of Fgf3 or Fgf8 misexpression (Fig. 3C,D and data not shown). Furthermore, Fgf misexpression was able to induce ectopic domains of expression of *foxi1*, which encodes an upstream regulator of *pax8* (Solomon et al., 2003) (Fig. 3F). Fgf misexpression also led to ectopic or expanded expression of later preotic markers *pax2a* and *dlx3b* (Fig. 3G and data not shown). When allowed to develop further, 9% (17/196 non-dorsalized) of embryos injected with *fgf3* plasmid and 8% (37/464 non-dorsalized) of embryos injected with *fgf8* plasmid displayed ectopic vesicles containing differentiated sensory patches and associated otocephals (Fig. 3H-J, Table 1). Formation of ectopic vesicles was limited to the periphery of the anterior neural plate, although during earlier developmental stages isolated *pax8* expressing cells were occasionally observed elsewhere, including the neural plate (not shown). Importantly, co-injection of *fgf8* and *wnt8* plasmids did not significantly increase the number of embryos displaying ectopic vesicles, indicating that Wnt8 does not augment the ability of Fgf to induce otic tissue.

To address the possibility that Fgf acts by inducing ectopic Wnt8, we injected Fgf plasmid into *wnt8* morphants. We found that 8% (5/64) of Fgf3 misexpressing and 9% (5/58) of Fgf8 misexpressing *wnt8* morphants showed ectopic patches of *pax8* expression in the head (not shown). In another experiment, 12% (2/17) of *wnt8* morphants injected with *fgf8* plasmid produced ectopic otic vesicles (Fig. 3K). As an additional test, embryos were injected with *dkk1*-plasmid at the one-cell stage, followed by *fgf8* plasmid at the eight-cell stage. The dorsalizing effects of *dkk1* and *fgf8* strongly potentiate each other so that severely affected embryos were more numerous when compared to *fgf8* injection alone. Hence, embryonic patterning cannot be easily interpreted in most (176/196) embryos. However, of the more moderately affected embryos, 15% (3/20) formed ectopic otic vesicles (not shown). Thus, Fgf misexpression can still induce ectopic otic tissue in embryos depleted for Wnt8 or otherwise blocked in Wnt signaling activity.

**Wnt8 cannot induce ectopic otic tissue without Fgf**

To test whether mosaic misexpression of Wnt8 is sufficient to induce ectopic otic tissue, we injected *wnt8* ORF1 or ORF2 plasmid into wild-type embryos at the eight-cell stage. None of the embryos injected with ORF2-plasmid showed ectopic otic vesicles (n=50). A small fraction (5/249) of embryos injected with ORF1-plasmid produced supernumerary otic vesicles. In these few cases, embryos appeared to be severely posteriorized; they showed bilateral loss of nasal pits and eyes, and no morphological development of the epiphyses or midbrain-

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**Table 1. Effects of Fgf misexpression**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Morphology at 30 hpf</th>
<th>Expression of preotic markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>fgf3</em> injected</td>
<td><em>fgf3</em> injected</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>132</td>
</tr>
<tr>
<td>Ectopic otic nerve</td>
<td>17 (8.7%)*</td>
<td>29 (16%)*</td>
</tr>
<tr>
<td>Enlarged otic tissue</td>
<td>n.d.</td>
<td>20 (11%)*</td>
</tr>
<tr>
<td>Other head defects</td>
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<td>N/A</td>
</tr>
<tr>
<td>Dorsalized</td>
<td>190</td>
<td>151</td>
</tr>
<tr>
<td>Total</td>
<td>386</td>
<td>332</td>
</tr>
<tr>
<td>Non-dorsalized</td>
<td>196</td>
<td>181</td>
</tr>
</tbody>
</table>

*Percentages reflect ratio of indicated class to non-dorsalized embryos. N/A, not applicable.

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**Fig. 2.** Cross-regulation of Wnt8 and Fgf. (A-D) *fgf3* expression in the hindbrain of tailbud stage wild-type embryo (A), tailbud stage *wnt8* morphant (B), tailbud stage *Dfw8* homozygote (C) and six-somite stage *Dfw8* homozygote (D). (E-H) *fgf8* expression in the hindbrain of tailbud stage wild-type embryo (E), tailbud stage *wnt8* morphant (F), tailbud stage *Dfw8* homozygote (G) and six-somite stage *Dfw8* homozygote (H). (I-K) *wnt8* ORF2 expression in the hindbrains of a wild-type embryo at 90% epiboly (I), wild-type embryo injected with *fgf3* and *fgf8*-MOs at 90% epiboly (J), wild-type embryo at tailbud stage (K) and tailbud stage wild-type embryo injected with *fgf3* and *fgf8*-MOs (L). Arrowheads indicate hindbrain domain. p, prechordal plate. Dorsal views with anterior upward. Scale bar: 200 μm.
To determine whether Fgf signaling acts directly on preotic cells, we examined expression of the Fgf reporter gene \( \text{erm} \). Erm is a member of the ETS family of transcription factors that is expressed in response to Fgf signaling, and its expression is ablated by disrupting Fgf signaling (Roehl and Nusslein-Volhard, 2001; Raible and Brand, 2001). Accordingly, \( \text{erm} \) is expressed in a pattern corresponding to known Fgf expression domains, including tissues surrounding the prechordal plate, the hindbrain and the germring (Fig. 5A). When visualized with \( \text{fgf8} \), which marks the lateral edge of the hindbrain abutting the otic anlage (Phillips et al., 2001), \( \text{erm} \) expression appears to encompass all or most of the preotic field (Fig. 5B,C). Thus, preotic cells respond directly to Fgf signaling.

To ascertain whether the otic anlage actively responds to Wnt signaling, we examined the expression of the Wnt reporter gene, \( \text{TOPdGFP} \) (Dorsky et al., 2002). This is a transgene consisting of a GFP-coding sequence downstream of a minimal promoter and four \( \text{Lef} \) binding sites. Although the transgene does eventually lead to detectable levels of GFP fluorescence, wholemount in situ hybridization is a more sensitive means of detecting transgene expression during early stages of development (Dorsky et al., 2002). \( \text{TOPdGFP} \) is expressed in a pattern similar to that of \( \text{wnt8} \) (Fig. 4D). Moreover, \( \text{TOPdGFP} \) expression is dependent on Wnt8 function because both \( \text{wnt8} \) morphants and \( \text{Df} (\text{w8}) \) homozygotes lack expression (not shown). Thus, \( \text{TOPdGFP} \) expression faithfully reports Wnt8 activity during late gastrula stages. Although reported previously to be expressed only in mesendoderm during gastrulation (Dorsky et al., 2002), we find upon sectioning that \( \text{TOPdGFP} \) is also expressed in dorsal ectoderm (Fig. 4F).
staining of TOPdGFP and fgf8 reveals a small group of TOPdGFP-expressing cells lying posterior and lateral to the hindbrain domain of fgf8 (Fig. 4E-G). These cells could mark the posterior edge of the preotic domain. However, the majority of preotic cells do not express TOPdGFP. Thus, preotic cells may not respond directly to Wnt signaling, or if they do the level is too low to activate expression of the transgene.

**Discussion**

We have assessed two competing models for otic induction. In one model, Fgfs expressed in the hindbrain and subjacent mesendoderm are necessary and sufficient for otic induction. In the other, Fgf must cooperate with Wnt8 to fully induce otic development. Our data indicate that, in zebrafish, Fgf signaling is directly responsible for otic induction whereas Wnt8 acts indirectly by promoting timely expression of Fgf3 and Fgf8 in the hindbrain. As discussed below, the roles of Fgf and Wnt signaling are likely to be conserved from teleosts through tetrapods.

**A direct role for Fgf signaling in otic induction**

Comparative studies in zebrafish, *Xenopus*, chick and mouse indicate that Fgf, especially Fgf3, plays a broadly conserved role in otic induction. However, these model systems have used different experimental approaches, each of which only partially addresses the nature of Fgf function. Misexpression studies in chick and frog show that Fgf signaling can induce ectopic otic tissue (Vendrell et al., 2000; Lombardo et al., 1998), but this need not reflect the normal function of the specific ligands under study. Loss-of-function studies in zebrafish and mouse confirm an essential role for Fgf3 and, in addition, show that Fgf8 and Fgf10 have partially redundant roles in otic induction (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al., 2003; Wright and Mansour, 2003). However, these studies did not address whether any of these ligands are sufficient for otic induction. We show here that misexpression of either Fgf3 or Fgf8 can induce ectopic otic tissue in zebrafish (Fig. 3), demonstrating for the first time in a single species that Fgf is both necessary and sufficient for otic induction.

Although we cannot exclude the possibility that Fgf3 and Fgf8 induce expression of another hindbrain signal that is directly responsible for otic induction, this seems unlikely for several reasons. First, the Fgf reporter gene *erm* is expressed in ectoderm adjacent to the hindbrain during late gastrulation, indicating that preotic cells receive and respond to Fgf signals (Fig. 5). Furthermore, preplacodal expression of *erm* is ablated in embryos depleted of Fgf3 and Fgf8 (our unpublished observations). Finally, mosaic misexpression of Fgf can induce ectopic otic development without inducing hindbrain markers such as *krox20* and *wnt8* (Fig. 3B and data not shown). The simplest interpretation of these data is that Fgf3 and Fgf8 act directly on preplacodal ectoderm to induce the otic placode.

The function of Fgf signaling is clearly context-dependent. Fgf misexpression induced ectopic otic tissue only in ectoderm immediately surrounding the anterior neural plate. This probably corresponds to the preplacodal domain, a distinct domain of the ectoderm lying between neural and epidermal ectoderm. The preplacodal domain is marked by expression of
a number of transcription factors genes, including Six, Msx, Dlx and Eya-related homologs (reviewed by Baker and Bonner-Fraser, 2001; Whitfield et al., 2002; Riley and Phillips, 2003). The signaling interactions that regulate these genes are not well understood, but BMP signaling from ventral tissue is required for expression of Msx and Dlx genes, and signals from the organizer and/or neural plate are also required (Feledy et al., 1999; Pera et al., 1999; Beanan et al., 2000; McClarren et al., 2003). A balance of these competing axial signals may be crucial for establishing an uncommitted preplacodal region along the neural non-neural interface, which is then subdivided into different kinds of placodes by specific local cues. The hindbrain domain of Fgf3 and Fgf8 appears to constitute an essential part of the local trigger for otic development. It is interesting to note that Fgf3 and Fgf8 are also expressed in more anterior tissues, including the prechordal plate and midbrain-hindbrain boundary, but these sources do not normally trigger otic development in more anterior locations. This might reflect insufficiency in the level, timing and duration of Fgf signaling, and the presence of other factors could modify the response to Fgf. In any case, locally augmenting Fgf signaling can overcome the restrictions on otic development in more anterior regions. It is also noteworthy that Fgf misexpression did not induce formation of ectopic otic tissue in regions posterior to the endogenous otic placodes. This might be because retinoic acid, a posteriorizing agent that is synthesized by posterior mesoderm, strongly modifies the response to Fgf signaling (Kudoh et al., 2002).

An indirect role for Wnt8 in otic induction

Although wnt8 is expressed in the hindbrain by 75% epiboly – at the right time and place to influence otic induction – it is neither necessary nor sufficient for this process. Loss of all wnt8 activity delays but does not block expression of the preotic marker pax8 (Fig. 2). The initial delay in otic induction is probably caused by a similar delay in the expression of fgf3 and fgf8 in the hindbrain. Most embryos knocked down for wnt8 ORF1 and ORF2, and embryos that misexpress the Wnt antagonist Dkk1, produce small, well differentiated otic vesicles containing sensory maculae and associated otoliths (Fig. 1). Misexpression of wnt8 did occasionally lead to production of supernumerary otic vesicles. However, all such embryos appeared severely posteriorized, failing to develop any anterior sensory structures, midbrain-hindbrain border and epiphysis. Analysis at earlier stages confirmed that misexpression of wnt8 caused the hindbrain domains of fgf3 and fgf8 to shift almost to the anterior limit of the embryo (Fig. 4). Moreover, the lateral edges of the hindbrain domain extend forward to form a U-shaped arc of staining that is complementary to an inverse arc of preotic pax8 that wraps around the anterior limit of the neural plate. Knockdown of fgf3 and fgf8 blocked preotic pax8 expression and totally ablated formation of otic vesicles in all embryos injected with wnt8-plasmid. These data support the conclusion that Wnt8 acts indirectly in otic induction by influencing expression of fgf3 and fgf8 in the hindbrain.

Additional evidence for an indirect role for Wnt8 is that expression of TOPdGFP, a Wnt-inducible transgene, is not detected in preotic cells during gastrulation (Fig. 5). It should be pointed out that one limitation of this transgene is that it reports only transcriptional activation by Lef1, a mediator of the canonical Wnt pathway, but it does not reflect signaling via the alternate Wnt mediators, Tcf3 and Tcf3b (Dorsky et al., 2002). Analysis of Tcf3 and Tcf3b in zebrafish indicates that these proteins normally act as transcriptional repressors that are inactivated by Wnt signaling (Kim, 2000; Dorsky, 2003). As yet, no genes have been identified that specifically report Wnt-mediated derepression of Tcf3 activity. Despite this caveat, the failure to detect TOPdGFP expression shows that Wnt8 signaling is not sufficient to strongly activate the Lef1-dependent pathway in preotic cells. It is also worth noting that none of the known Frizzled receptors examined in several vertebrate species are expressed at appreciable levels in prospective otic ectoderm during late gastrulation, when otic development is initiated (Deardorf and Klein, 1999; Stark et al., 2000; Momoi et al., 2003). Expression of multiple Frizzled genes is detected later within the nascent otic placode, indicating that Wnt signaling could play a role in later stages of otic development. Indeed, TOPdGFP expression is first detected in prospective otic ectoderm between 12-13 hpf (6-8 somites), just prior to morphological formation of the otic placode (data not shown). This is also consistent with the observation that, in rat, periodic accumulation of nuclear β-catenin is first detected just after formation of the otic placode (Matsuda and Keino, 2000). In addition, secreted Frizzled proteins, which are induced by Wnt signaling, are expressed in chick otic tissue only after formation of the otic placode (Baranski et al., 2000; Ladher et al., 2000b; Esteve et al., 2000; Terry et al., 2000). Although we found no evidence to support a direct role for Wnt8 in otic induction, zebrafish embryos lacking Wnt8 function produce smaller vesicles indicating that Wnt8 signaling might stimulate proliferation in the developing otic placode. Thus, later Wnt signaling could also regulate morphogenesis or differentiation of ear tissue during post-placodal stages.

Although ORF1 and ORF2 show very close sequence homology, their functions are not identical. Knockdown of ORF1 alone has negligible effects on inner ear development whereas knockdown of ORF2 alone significantly delays otic induction and leads to production of small otic vesicles. These effects are not significantly worsened by knockdown of both ORF1 and ORF2, suggesting a more crucial role for ORF2. It is possible that this reflects the proximity of the hindbrain domain of ORF2 to r4, the site of expression of both fgf3 and fgf8. By contrast, misexpression of ORF2 had only mild effects and did not induce excess or ectopic otic tissue, whereas misexpression of ORF1 posteriorized the neural plate and led to production of supernumerary otic vesicles in 2-5% of embryos. This could reflect enhancement of an early posteriorizing function normally associated with the germring domain of Wnt8. It is not clear why global misexpression of ORF2 does not have similar effects, but sequence differences between the ligands could be critical for differential receptor binding.

Feedback between the Fgf and Wnt pathways

Although Wnt8 is required for normal expression of fgf3 and fgf8 in the hindbrain, Fgf signaling is also required for proper expression of wnt8-ORF2 in the r5/6 domain. It is not known whether this mutual regulation is direct or indirect, but it could reflect the activity of a positive-feedback loop operating within the hindbrain. The purpose of such a feedback loop could be
analogous to that of the midbrain-hindbrain boundary, wherein an anterior domain of Wnt1 abuts a posterior domain of Fgf8, and the two factors cooperate to organize surrounding brain tissue (reviewed by Wurst and Bally-Cuif, 2001). Induction of both genes is under the control of several upstream regulators. Both factors are required to maintain the midbrain-hindbrain boundary and, therefore, indirectly they require each other. The r4 region of the hindbrain appears to be a second signaling center that helps pattern the hindbrain. Expression of fgf3 and fgf8 in the r4 domain is necessary to establish the identities of r5 and r6 (Walsh et al., 2002; Maves et al., 2002; Wiellette and Sive, 2003). This could partly explain why Fgf signaling is required for proper expression of wnt8 in the r5/6 region.

The requirement for Wnt8 ORF2 on hindbrain patterning has not been examined, but this domain may help to establish and stabilize the r4 signaling center and thereby provide a sustained source of Fgf3 and Fgf8 required for otic induction. Whether a similar mechanism operates in other vertebrates remains to be fully tested. In chick and mouse, Wnt8 is expressed in a domain in the hindbrain, consistent with the role proposed in our study (Hume and Dodd, 1993; Bouillet et al., 1996). The only functional analysis of this domain in amniotes is a study by Ladher and colleagues (Ladher et al., 2000a) examining the effects of Fgf19 and Wnt8c on gene expression in chick explant cultures. From that study it was proposed that Fgf19 from periotic mesendoderm induces expression of Wnt8c in the hindbrain, and the two factors then induce otic development in adjacent ectoderm. However, a key observation was that exogenous Wnt8c induced prospective otic ectoderm to express Fgf3, which was interpreted as a marker of early otic differentiation. This presents a conundrum because Fgf3 is not expressed in the chick ear until well after formation of the otic vesicle, but Wnt8c did not induce expression of any earlier markers of otic development. By contrast, Fgf3 is expressed in the chick hindbrain by the one-somite stage (Mahmood et al., 1995), raising the possibility that induction of Fgf3 by Wnt8c mimics an early aspect of hindbrain development. In this scenario, Wnt8c could facilitate a feedback loop that augments and maintains Fgf signaling long enough to induce otic development. Thus, the ability to induce a full range of early otic markers of otic development in adjacent ectoderm. However, a key observation was that exogenous Wnt8c induced prospective otic ectoderm to express Fgf3, which was interpreted as a marker of early otic differentiation. This presents a conundrum because Fgf3 is not expressed in the chick ear until well after formation of the otic vesicle, but Wnt8c did not induce expression of any earlier markers of otic development. By contrast, Fgf3 is expressed in the chick hindbrain by the one-somite stage (Mahmood et al., 1995), raising the possibility that induction of Fgf3 by Wnt8c mimics an early aspect of hindbrain development. In this scenario, Wnt8c could facilitate a feedback loop that augments and maintains Fgf signaling long enough to induce otic development. Thus, the ability to induce a full range of early otic markers of otic development in adjacent ectoderm. However, a key observation was that exogenous Wnt8c induced prospective otic ectoderm to express Fgf3, which was interpreted as a marker of early otic differentiation. This presents a conundrum because Fgf3 is not expressed in the chick ear until well after formation of the otic vesicle, but Wnt8c did not induce expression of any earlier markers of otic development. By contrast, Fgf3 is expressed in the chick hindbrain by the one-somite stage (Mahmood et al., 1995), raising the possibility that induction of Fgf3 by Wnt8c mimics an early aspect of hindbrain development. In this scenario, Wnt8c could facilitate a feedback loop that augments and maintains Fgf signaling long enough to induce otic development. Thus, the ability to induce a full range of early otic markers of otic development in adjacent ectoderm.

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References


