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

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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 placodes 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 placodes/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 placodes. 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 placodes 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 synergyze 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 $MgSO_4$, 3.7% formaldehyde]. In situ hybridizations (Stachel et al., 1993) were performed at 67°C using probes for *pax2.1* (Krauss et al., 1991), *fgf8* (Reifers et al., 1998), *pax8* (Pfeffer et al., 1998), *TOPdGFP* (Dorsky et al., 2002), *erm* (Roehl and Nusslein-Volhard, 2001; Raible and Brand, 2001), *wnt8* ORF2 (Lekven et al., 2001), *foxi1*(Solomon et al., 2003) and *krox-20* (Oxtoby and Jowett, 1993) transcripts. The *fgf3* construct was generated by amplifying the coding sequence of *fgf3* (GenBank Accession Number NM 131291) and ligating it into the *Cla*I and *Eco*RI 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 $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES (pH 7.6)] to concentrations of 2.5 μg μl^{-1} *fgf3*-MO, 2.5 μg μl^{-1} *fgf8*-MO, 1.25 μg μl^{-1} *wnt8* ORF1-MO, 1.25 μg μl^{-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 $CaCl_2$, 5 mM HEPES (pH 7.4)] with 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Morpholino used were: *fgf3*-MO (Phillips et al., 2001); *fgf8*-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 μl^{-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 *fgf* plasmid at a concentration of 30 ng μl^{-1}. Mosaic misexpression of Wnt8 was achieved by eight-cell injection of 30-40 ng μl^{-1} of ORF1 or ORF2 plasmid. Global misexpression of Wnt8 was achieved by one-cell injection of 80 ng μl^{-1} ORF1 or ORF2 plasmid. Global misexpression of Dkk1 was accomplished by one-cell injection of either 40 ng μl^{-1} or 80 ng μl^{-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 anlagen, 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 preotic marker, *pax8*. Preotic 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, preotic *pax8* was not observed at tailbud stage in embryos homozygous for a chromosomal deficiency, *Df(LG14)wnt8*^{w8} (termed *Dfw8*), which deletes both *wnt8* open-reading frames (Lekven et al., 2001) (data not shown). However, *pax8* is eventually expressed in the preotic 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

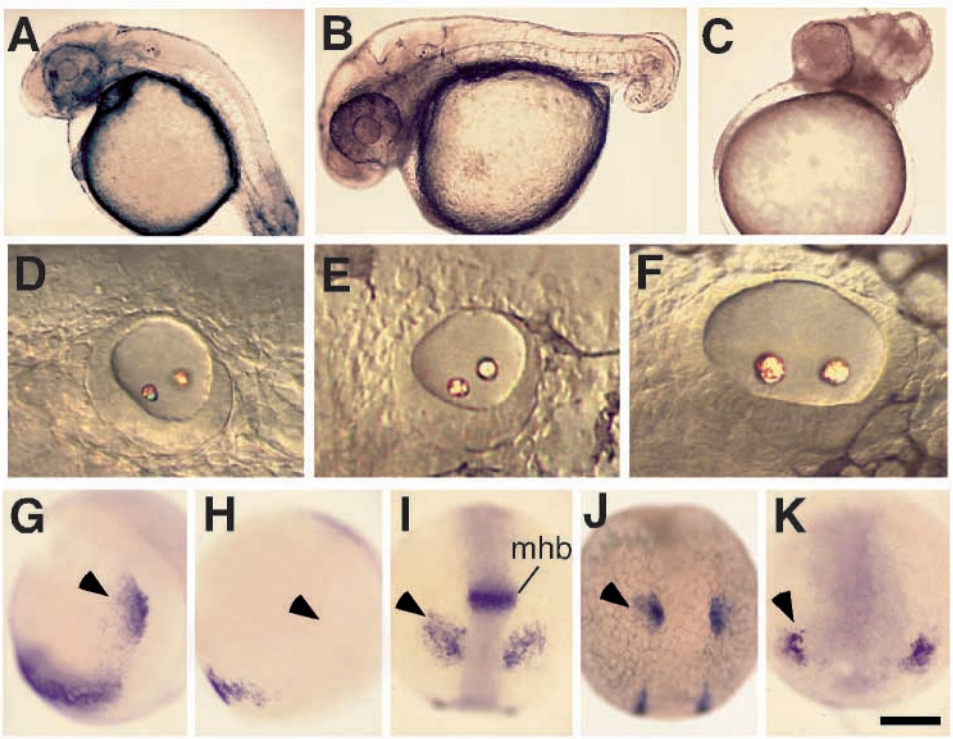

Fig. 1. Effects of disrupting Wnt8 function. (A-F) Lateral views of live embryos at 30 hpf. *wnt8* morphant (A), moderately affected *dkk1*-injected wild-type embryo (B) and severely affected *dkk1*-injected wild-type embryo lacking hindbrain and otic tissue (C). Enlarged view otic vesicles of *wnt8* morphant (D), moderately affected *dkk1*-injected wild-type embryo (E) and wild-type embryo (F). (G,H) Lateral views of *pax8* expression at tailbud stage in a wild-type embryo (G) and *wnt8* morphant lacking the otic domain (H). (I-K) Dorsal views of *pax8* expression at six-somite stage in a wild-type embryo (I), severely affected *dkk1*-injected embryo (J) and *Dfw8* homozygote (K). Arrowheads indicate the preotic region. Abbreviation: mhb, midbrain-hindbrain border. (A-F) Anterior is to the left and dorsal upward. (G,H) Lateral views with anterior upward. (I-K) Dorsal views with anterior upward. Scale bars: A-C, 150 μm; D-F, 30 μm; G-K, 200 μm.

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) *dkk-1*-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 hfp) 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. 2J). 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

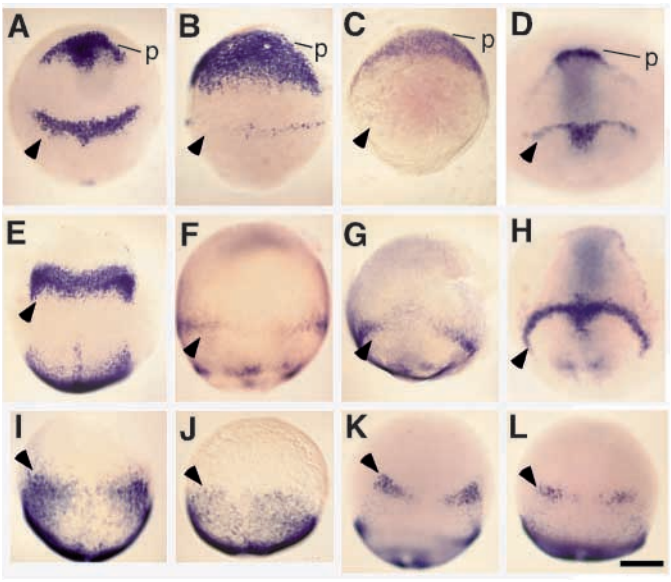

Fig. 2. Cross-regulation of Wnt8 and Fgf. (A-D) *fgf3* expression in the hindbrain of tailbud stage wild-type embryo (A), tailbud stage *wnt8* morphant (B), tailbud stage *Dfw8* homozygote (C) and six-somite stage *Dfw8* homozygote (D). (E-H) *fgf8* expression in the hindbrain of tailbud stage wild-type 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.

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 otoliths (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.

Table 1. Effects of Fgf misexpression

	Morphology at 30 hpf		Expression of preotic markers	
Phenotype	*fgf3* injected	*fgf8* injected	*fgf3* injected	*fgf8* injected
Normal	75	270	132	99
Ectopic otic nerve	17 (8.7%)*	37 (8.0%)*	29 (16%)*	16 (12.8%)*
Enlarged otic tissue	n.d.	n.d.	20 (11%)*	10 (8%)*
Other head defects	104	157	N/A	N/A
Dorsalized	190	48	151	15
Total	386	512	332	140
Non-dorsalized	196	464	181	125

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

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 epiphysis or midbrain-

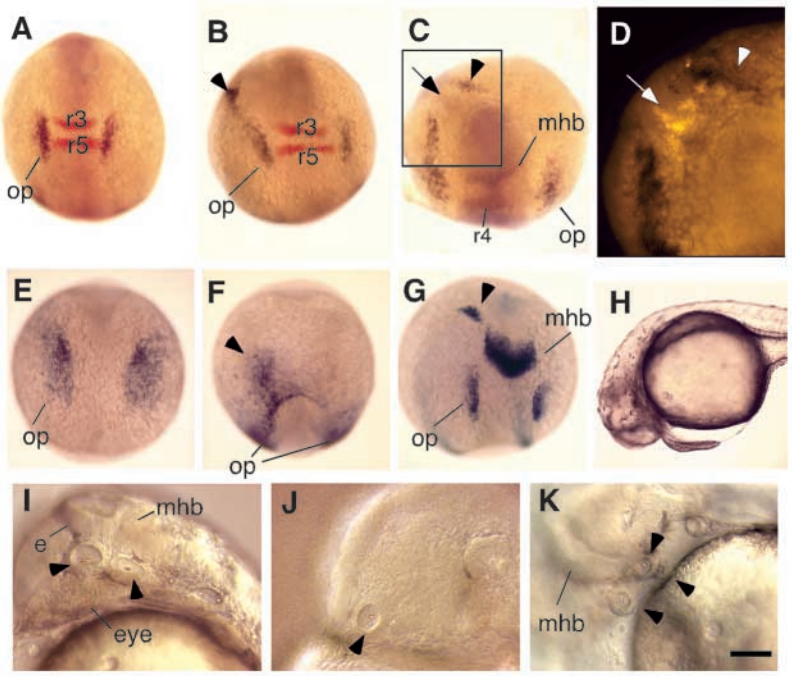

Fig. 3. Effects of Fgf misexpression. (A,B) Two-color in situ hybridization of three-somite stage. Wild-type (A) and *fgf3* (B) plasmid-injected embryos showing *pax8* expression (blue) and *krox-20* expression (red). The left preotic domain is enlarged significantly and a region with ectopic upregulation of *pax8* (arrowhead) is evident. (C,D) Two-color *in situ* hybridization of three-somite stage embryo injected with *fgf3* plasmid showing *fgf3* expression (blue) and *pax8* expression (red). (C) Brightfield and (D) fluorescent images showing relationship between ectopic *fgf3*-expressing cells (arrow) and ectopic *pax8*-expressing cells (arrowhead). The boxed area in C is enlarged in D. The endogenous preotic domain on the left is enlarged in the vicinity of misexpressed *fgf3*. The r4 domain of *fgf3* is faintly visible in C. (E,F) *foxi1* expression at three-somite stage in wild-type (E) and *fgf8* plasmid-injected embryo (F). Ectopic expression in anterior region of the injected embryo is indicated (arrowhead). (G) Ectopic *pax2.1* expression (arrowhead) adjacent to the midbrain of a four-somite stage embryo injected with *fgf3*-plasmid. (H) Low magnification view of a 30 hpf wild-type embryo injected with *fgf8*-plasmid showing that overall axial development is essentially normal, although development of anterior sensory structures is perturbed. (I) Higher magnification of the embryo shown in H. Ectopic otic vesicles are indicated (arrowheads). Development of adjacent eye tissue is perturbed, but general features of brain development, such as the epiphysis and midbrain-hindbrain boundary are produced. (J) Frontal/lateral view of an embryo injected with *fgf3*-plasmid. An ectopic otic vesicle is indicated (arrowhead). Development of adjacent nasal and eye tissue is severely perturbed. (K) Lateral view of a *wnt8* morphant injected with *fgf8*-plasmid. Ectopic otic vesicles (arrowheads) are seen next to the midbrain-hindbrain boundary. Abbreviations: e, epiphysis; mhb, midbrain-hindbrain border; op, endogenous otic placode; r3, rhombomere 3. (A-E,G) Dorsal view with anterior upward. (F) Dorsal-anterior view. (H,I,K) Lateral views with anterior to the left. (J) Frontal-lateral view with anterior to the left. Scale bars: A-C,E-G, 150 μm; D, 60 μm; H, 175 μm; I-K, 75 μm.

hindbrain boundary (not shown). We infer that these are phenotypes resulting from more widespread expression of Orf1. To test the effects of increasing Wnt8 signaling, we doubled the concentration of *wnt8* plasmid and injected embryos at the one-cell stage. Injection of ORF2-plasmid caused mild posteriorization in some embryos but had no visible effect on otic development (n=118, data not shown). By contrast, 73% (129/177) of embryos injected with ORF1-plasmid were strongly posteriorized, and these included the 5-6% (10/177) of embryos that produced supernumerary otic vesicles (Fig. 4E). Analysis at earlier stages showed that 22% (10/45) of ORF1-misexpressing embryos produced enlarged domains of *pax8* wrapping around the anterior neural plate (Fig. 4C). This correlates with expanded hindbrain domains of *fgf3, fgf8* and *erm*, a reporter of Fgf activity (Fig. 4A,B and data not shown) reminiscent of the patterns seen in embryos posteriorized with retinoic acid (Phillips et al., 2001). When *fgf3*-MO and *fgf8*-MO were coinjected with ORF1-plasmid at the one-cell stage, preotic expression of *pax8* was severely reduced or ablated (n=150; Fig. 4D,F). At later stages, most embryos appeared posteriorized but none produced any ectopic otic tissue (n=240). This finding was highly significant (P<0.0005) compared to the moderate level of ectopic ear formation in embryos injected with ORF1-plasmid alone. These data indicate that Wnt8 cannot directly induce otic tissue in the absence of Fgf.

Fgf, but not Wnt, reporter genes are expressed in preotic cells

To determine whether Fgf signaling acts directly on preotic cells, we examined expression of the Fgf reporter gene *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, *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 *fgf8*, which marks the lateral edge of the hindbrain abutting the otic anlage (Phillips et al., 2001), *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, *TOPdGFP* (Dorsky et al., 2002). This is a transgene consisting of a GFP-coding sequence downstream of a minimal promoter and four 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). *TOPdGFP* is expressed in a pattern similar to that of *wnt8* (Fig. 4D). Moreover, *TOPdGFP* expression is dependent on Wnt8 function because both *wnt8* morphants and *Dfw8* homozygotes lack expression (not shown). Thus, *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 *TOPdGFP* is also expressed in dorsal ectoderm (Fig. 4F). Co-

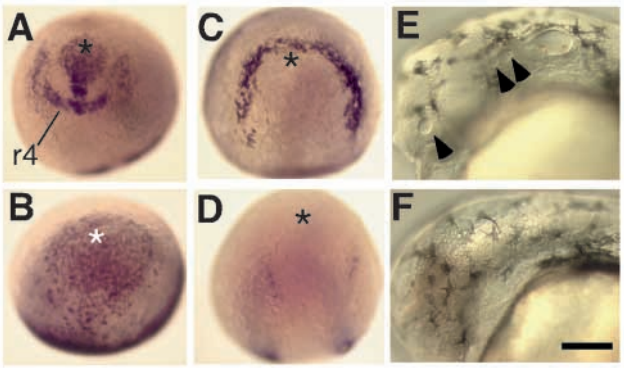

Fig. 4. Effects of Wnt8 misexpression. (A-C) Three-somite stage embryos globally expressing *wnt8* ORF1 showing expression of *fgf8* (A), *erm* (B) and *pax8* (C). The r4 domain of *fgf8* is indicated. The weaker anterior *fgf8* expression corresponds to midbrain-hindbrain boundary. Asterisks mark the anterior limit of the neural plate. (D) *pax8* expression in an embryo globally expressing ORF1 and coinjected with *fgf3*-MO and *fgf8*-MO. Preotic expression is nearly ablated. (E) Ectopic otic vesicles (arrowheads) in a live embryo at 30 hpf globally expressing ORF1. Note that anterior sensory structures and morphological landmarks in the brain such as the midbrain-hindbrain boundary and epiphysis are not produced. (F) Loss of otic tissue in a 30 hpf embryo globally expressing ORF1 and coinjected with *fgf3*-MO and *fgf8*-MO. (A-D) Dorsal views with anterior upward. (E,F) Lateral views with anterior to the left. Scale bars: A-D, 200 μm; E-F, 75 μm.

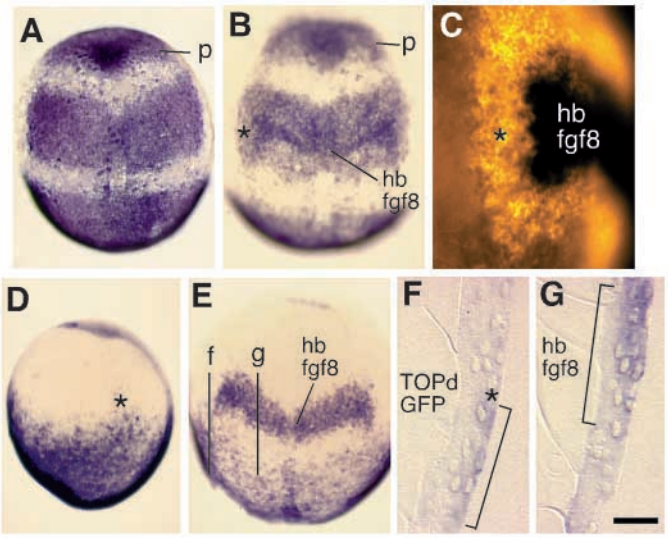

Fig. 5. Expression of Fgf- and Wnt-inducible reporter genes. All images show gene expression patterns in wild-type embryos at tailbud stage. (A) Expression of *erm*. (B) Costaining of *erm* and *fgf8* (darker staining). (C) Two-color staining showing expression of *erm* (red) and *fgf8* (blue). (D) *TOPdGFP* expression. (E) Costaining of *TOPdGFP* and *fgf8*. (F,G) Parasagittal sections at the locations indicated in E showing *TOPdGFP* expression in a domain lateral and posterior to the hindbrain (F) and *fgf8* expression in the hindbrain (G). Preotic domains are indicated by asterisks. Abbreviations: p, prechordal plate; hb, hindbrain. (A,B,E) Dorsal views with anterior upward. (C) Dorsolateral view with anterior upward. (D) Lateral view with anterior upward and dorsal to the right. (F and G) Dorsal is to the right and anterior is upward. Scale bars: A,B,E, 150 μm; C, 40 μm; D, 175 μm; F,G, 25 μm.

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, periotic 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 (Walshe 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 in cultures exposed to Fgf19 and Wnt8c might reflect the additive effects of exogenous Fgf19 plus newly synthesized Fgf3. More complete analysis of the relative roles of Fgf and Wnt signaling will require Wnt8 misexpression in vivo and loss-of-function studies using morpholinos in chick (Kos et al., 2003) and gene-knockouts in mouse.

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