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Fgf3 and Fgf8 are required together for formation of the otic placode and vesicle

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SUMMARY

Fgf3 has long been implicated in otic placode induction and early development of the otocyst; however, the results of experiments in mouse and chick embryos to determine its function have proved to be conflicting. In this study, we determined *fgf3* expression in relation to otic development in the zebrafish and used antisense morpholino oligonucleotides to inhibit Fgf3 translation. Successful knockdown of Fgf3 protein was demonstrated and this resulted in a reduction of otocyst size together with reduction in expression of early markers of the otic placode.

***fgf3* is co-expressed with *fgf8* in the hindbrain prior to otic induction and, strikingly, when Fgf3 morpholinos were co-injected together with Fgf8 morpholinos, a significant number of embryos failed to form otocysts. These effects were made manifest at early stages of otic development by an absence of early placode markers (*pax2.1* and *dlx3*) but were not accompanied by effects on cell division or death. The temporal requirement for Fgf signalling was established as being between 60% epiboly and tailbud stages using the Fgf receptor inhibitor SU5402. However, the earliest molecular event in induction of the otic territory, *pax8* expression, did not require Fgf signalling, indicating an inductive event upstream of signalling by Fgf3 and Fgf8. We propose that Fgf3 and Fgf8 are required together for formation of the otic placode and act during the earliest stages of its induction.**

Key words: Fgf, Otocyst, Placode, Embryo, Induction, Zebrafish

INTRODUCTION

The otic placode arises as an ectodermal thickening adjacent to the posterior hindbrain in all vertebrates. It invaginates and pinches off from the ectoderm to form the otic vesicle, which subsequently gives rise to the sensory structures of the inner ear and neurones of the vestibuloacoustic ganglion. Thus, it has been central to the development of balance, coordination and, later, hearing during vertebrate evolution and there has been considerable interest in its embryonic origin.

Experimental studies of the tissue interactions involved in induction and development of the otic placode span much of the last century and include contributions from such luminaries as Yntema (Yntema, 1933), Harrison (Harrison, 1935), Waddington (Waddington, 1937) and Zwilling (Zwilling, 1941) working on amphibian and avian embryos. These and the many subsequent studies have implicated two tissues either acting alone or in combination in the induction of the otic placode: the underlying mesoderm and the adjacent hindbrain. The most generally accepted model is that the surface ectoderm receives an inductive signal from the underlying head mesoderm that renders it competent to form an otic placode. A second signal from the posterior hindbrain is then believed to induce the otic placode and determine its location (for reviews, see Fritsch et al., 1998; Torres and Giraldez, 1998). However, the experimental data upon which this hypothesis is based are inconclusive.

A search for the molecular players that regulate development of the otic placode has strongly implicated the fibroblast growth factors (Fgfs). For example, Fgf3 was first detected in rhombomeres (r) 5 and 6 of the mouse hindbrain, adjacent to the developing otic placode (Wilkinson et al., 1988). These expression studies were extended by Mahmood et al. (Mahmood et al., 1995a; Mahmood et al., 1996) who reported expression additionally in r4 of the mouse and in r4, 5 and 6 in the chick embryo. However, various attempts to determine Fgf3 function in otic development of these embryos have provided conflicting information. Targeted disruption of the *Fgf3* gene in transgenic mice results in a relatively mild otic phenotype, defective formation of the endolymphatic duct, and then only with incomplete penetrance and expressivity (Mansour et al., 1992). By contrast, application of antisense oligonucleotides to chick embryos resulted in an invagination defect (Represa et al., 1991) while ectopic expression of *Fgf3* in cranial ectoderm using a viral vector resulted in the formation of ectopic vesicles expressing some otic markers (Vendrell et al., 2000).

Several years ago, we reported the cloning of zebrafish *fgf3*

and also determined the biological properties of its protein product including its receptor specificity (Kiefer et al., 1996a; Kiefer et al., 1996b). However, we were unable to determine its developmental role(s) in zebrafish, because of the lack of a means of inhibiting its function. Recently, chemically modified oligonucleotides called morpholinos have been used to specifically inhibit gene function in amphibian and zebrafish embryos (Heasman et al., 2000; Nasevicius and Ekker, 2000) and this approach potentially allows targeted 'knockdown' of multiple genes in a single embryo. Morpholinos function by binding to and inhibiting translation of mRNA and are resistant to degradation. This development has allowed us to assess Fgf3 function in zebrafish otic development. We show that embryos with reduced levels of Fgf3 have smaller otic placodes and vesicles, a similar phenotype to that reported in the *ace* zebrafish which is mutated in the *fgf8* gene (Reifers et al., 1998). Moreover, when we co-inject morpholinos targeted against both *fgf3* and *fgf8*, a significant proportion of embryos fail to develop otic placodes.

MATERIALS AND METHODS

Fish strains, microinjection of oligonucleotides and incubation with Fgfr inhibitors

Zebrafish, *Danio rerio*, of the King's wild type (kwt) strain were used throughout these studies. They were maintained at 28°C and embryos staged according to Kimmel et al. (Kimmel et al., 1995). Morpholino oligonucleotides (GeneTools) were dissolved in distilled water at a concentration of 20 μg/μl and diluted to 1.5 μg/μl in 120 mM KCl, 20 mM Hepes pH 7.5, 0.25% (w/v) Phenol Red prior to injection. Oligonucleotides were injected in a volume of 5 nl (containing 7.5ng of each oligonucleotide) into the yolk cell of one-to eight-cell embryos just beneath the animal cell(s) as described (Holder and Xu, 1999).

Oligonucleotide sequences were as follows (bold letters indicate nucleotide substitutions in control sequences):

Fgf3 morpholino, 5′-CATTGTGGCATGGCGGGATGTCGGC-3′;

Fgf3 control morpholino, 5′-CATT**A**TG**T**CATGGCGGGA**G**GT-**G**GGC-3′;

Fgf8 morpholino, 5′-GAGTCTCATGTTTATAGCCTCAGTA-3′; and

Fgf8 control morpholino, 5′-GAGT**A**TCA**G**GTTTATAG**A**CT**A**A-GTA-3′.

After injection, embryos were collected in aquarium water containing methylene blue (Westerfield, 1995) and grown at 28°C in Petri dishes to the desired developmental stages.

For incubation with the Fgfr inhibitor, SU5402 (Calbiochem), embryos were dechorionated by incubation in 1 mg/ml pronase (Sigma) in aquarium water for 7 minutes. Chorions were removed by gently swirling the embryos in several changes of aquarium water, which also served to remove the pronse by serial dilution. Embryos were allowed to develop in petri dishes with a layer of 1% agarose on their base in aquarium water containing methylene blue. Upon reaching the desired stage, they were incubated in 60 μM SU5402 diluted in aquarium water from a 500× stock solution dissolved in DMSO. Control embryos had an equivalent volume of DMSO added to the aquarium water. Following incubation, embryos were washed gently in several changes of aquarium water and then grown to the desired developmental stages.

In situ hybridisation

Embryos were dechorionated as above. In situ hybridisation using NBT/BCIP and Fast Red substrates was performed as described (Shamim et al., 1999; Irving and Mason, 2001). For colour reactions using p-iodonitrotetrazolium violet (Sigma) as the second substrate embryos were incubated in 0.188 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.2 mg/ml p-iodonitrotetrazolium violet (Liang et al., 2000).

Zebrafish Fgf3 protein, anti-Fgf3 serum and western blots

Production of zebrafish Fgf3 protein and the monoclonal antibody, MSD-1, against Fgf3 have been described previously (Mathieu et al., 1995a; Mathieu et al., 1995b; Kiefer et al., 1996b). SDS PAGE and immunoblotting techniques were modified from those of Jackson and Dickson (Jackson and Dickson, 1999). Dechorionated embryos were extracted in sample buffer (4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% v/v glycerol, 0.125M Tris.HCl pH 6.8, Bromophenol Blue) using at least 5 μl per embryo. Extracts were immediately heated at 100°C for 5 minutes, centrifuged and stored at –80°C. Proteins were separated on 12% Laemmli SDS-PAGE gels at 150 V alongside molecular weight markers (BioRad). Gels were briefly equilibrated in 25 mM Tris, 190 mM glycine, 20% v/v methanol prior to electroblotting onto Immobilon P (Millipore) membranes at 110 V for 1.5-2 hours with cooling. Membranes were briefly dried at room temperature, rewet in TBS, 1% Tween-20 for 30 minutes, washed once in TBS, 0.1% Tween-20 and nonspecific binding sites were blocked in TBS, 0.1% Tween-20, 5% w/v non-fat dried milk (Marvel) for 2 hours at room temperature or at 4°C overnight. Filters were washed twice each for 5 minutes in TBS, 0.05% Tween-20 before incubation in MSD-1 diluted 1/1000 in TBS, 1% w/v milk for 2 hours at room temperature on a rocking platform. Filters were washed three times in TBS, 0.05% Tween-20 and incubated for 1 hour in 1/20,000 dilution of goat anti-mouse IgG coupled to horseradish peroxidase in TBS, 1% milk with rocking. Blots were then washed three times in TBS, 0.05% Tween-20 and excess wash buffer drained onto paper towels after the final wash. Filters were placed protein side uppermost on Saranwrap and 0.1 ml/cm^2 of lumigen solution (ECL kit, Amersham-Pharmacia) applied for 5 minutes. Excess lumigen solution was drained from the filters, which were wrapped in Saranwrap and exposed to x-ray film (X-Omat-AR, Kodak).

Detection of cell death and proliferation

Dividing cells were detected using an anti-phosphorylated histone H3 (Ser 128) antiserum (Calbiochem) according to the method of Saka and Smith (Saka and Smith, 2001). Embryos were dechorionated as above and fixed overnight in 4% (w/v) paraformaldehyde at 4°C. They were transferred to 100% methanol and stored at –20°C. Embryos were rehydrated through a graded series (75%, 50% and 25%) of methanol in PBS and then washed twice in PBS before being bleached for 1 hour in 1% v/v H_2O_2, 5% v/v formamide, 0.5×SSC for 30 minutes. They were rinsed with PBS and then with PBSBT [0.2% BSA (fraction V, Sigma), 0.1% v/v Triton X-100 in PBS] and incubated in 10% v/v goat serum in PBSBT for 1 hour. Embryos were then incubated in 1/1000 dilution of anti-phosphorylated histone H3 antiserum overnight at 4°C. They were washed briefly in 10% serum in PBSBT, washed four times each for 1 hour in PBSBT, and then for an hour in 10% goat serum in PBSBT before incubation in 1/250 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (Amersham-Pharmacia) overnight at 4°C. After a brief wash in 10% serum in PBSBT and four washes each for 1 hour in PBSBT, embryos were equilibrated in 0.3 mg/ml DAB in PBS for 10 minutes and then H_2O_2 was added to 0.03% v/v final. Colour reaction was allowed to develop for 15-30 minutes before being stopped by several rinses with water, then in PBS. Embryos were cleared in 90% glycerol, PBS.

Apoptotic cells were detected using the DeadEnd™ colourimetric detection kit (Promega). Embryos were dechorionated and fixed overnight in 4% w/v paraformaldehyde in PBS. They were then incubated in methanol for 30 minutes and rehydrated through a graded series of methanol (75%, 50%, 25%) in PBST (PBS, 0.1% Tween-20), washed twice in PBST and twice in PBS. They were incubated in proteinase K (5 μg/ml in PBS) for exactly 5 minutes, washed in

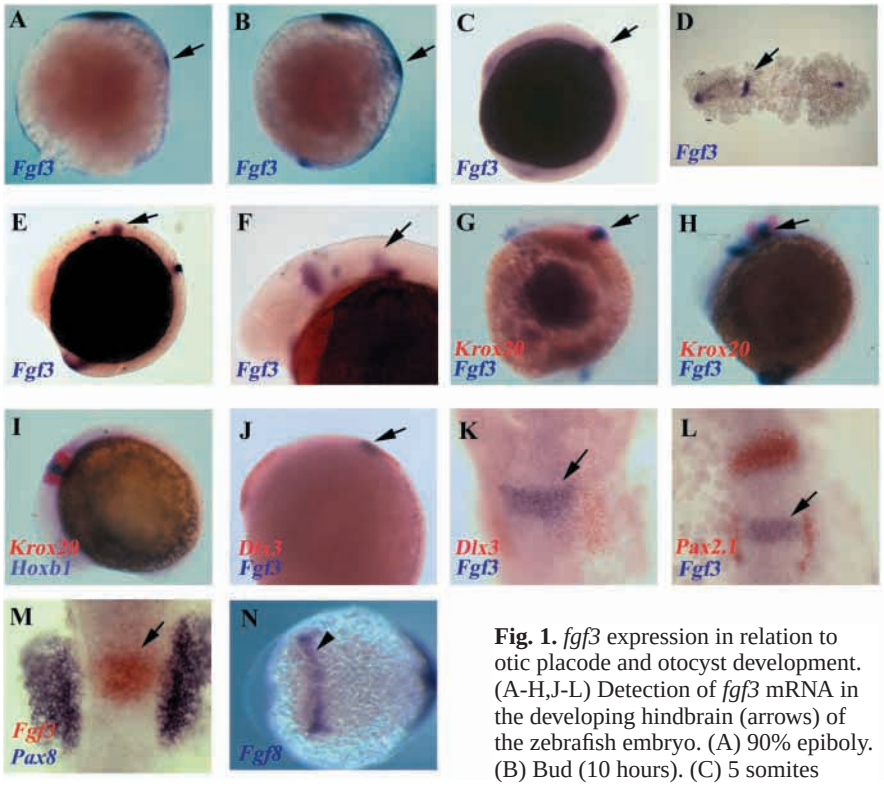

Fig. 1. *fgf3* expression in relation to otic placode and otocyst development. (A-H,J-L) Detection of *fgf3* mRNA in the developing hindbrain (arrows) of the zebrafish embryo. (A) 90% epiboly. (B) Bud (10 hours). (C) 5 somites (11.75 hours). (D) Flatmount of a 5 somite (11.75 hours) embryo; anterior towards the left. (E) 14 somite (16 hours). (F) 20 somites (19 hours). (G,H) Hybridisation to *fgf3* (blue) and *krox20* (red) in 4 somite (G) and 14 somite (H) embryos. (I) Detection of *hoxb1* (blue) and *krox20* (red) in a 12 somite embryo. (J,K) *fgf3* (blue) and *dlx3* (orange) expression in 3 somite embryos; (K) flatmount. (L) Flatmount showing *fgf3* (blue) and *pax2.1* (orange) in a 5 somite embryo. (M) Flatmounted 10 somite embryo showing *fgf3* (orange) and *pax8* (blue). (N) Dorsal view of an embryo at 90% epiboly to show onset of *fgf8* expression (arrowhead) in the presumptive r4 territory.

PBS and post-fixed in 4% w/v paraformaldhyde in PBS for 20 minutes. The embryos were then washed three times in PBS and three times in acetone, being stored at –20°C for 10 minutes in the final acetone wash. They were rinsed three times in PBS before being incubated for 10 minutes with equilibration buffer (DeadEnd kit). The equilibration buffer was removed and replaced with the terminal transferase reaction mix containing biotinylated UTP (DeadEnd kit) and the reaction was incubated at 37°C for 3 hours. Embryos were washed in 2×SSC for 30 minutes, three times in PBST and three times in PBS before being incubated in streptavidin-HRP 1/500 in PBS for 1 hour. After three washes in PBS, the colour reaction was performed using the DAB substrate and reaction mix provided with the kit. The reaction was stopped with several washes in distilled water, then in PBS and embryos were cleared in 90% glycerol, PBS.

RESULTS

fgf3 expression in relation to otic placode and otocyst development

We determined the distribution of *fgf3* transcripts during the period of otic placode induction and otocyst formation in the zebrafish embryo. *fgf3* mRNA is first detected by in situ hybridisation in the neural plate adjacent to the prospective otic territory at 90% epiboly (Fig. 1A). At this and later stages it is also expressed in other tissues, e.g. forebrain and tailbud (Fig. 1A-H) (J. W., H. M., R. M. and I. M., unpublished). The initial domain of expression in the hindbrain is quite broad (Fig. 1A,B) but rapidly refines to a single rhombomere by early somite (s) stages (Fig. 1C-E). It is downregulated at 20 somites (19 hours; Fig. 1F) and is undetectable in the hindbrain by 26 somites (22 hours; data not shown).

To determine the precise location of *fgf3* mRNA in the hindbrain, hybridisation was performed with a probe for *krox20* expressed in r3 and r5 (Oxtoby and Jowett, 1993). The domain of *fgf3* was always flanked by the *krox20*-positive territories. At early somite stages, before rhombomeric domains are segregated, there appeared to be some overlap in expression at the anterior and posterior boundaries of the *fgf3*-positive territory (Fig. 1G). However, *fgf3* transcripts eventually became confined to r4 (Fig. 1H) and were never detected in r5 or r6 as observed in mouse and chick embryos (Wilkinson et al., 1988; Mahmood et al., 1995a; Mahmood et al., 1996). Expression initially spanned the mediolateral extent of the neural plate (Fig. 1D) and after neurulation occupied the entire dorsoventral extent of r4 at early neural tube stages (Fig. 1E,G). However, by 14 somites (16 hours), expression was confined to ventral r4 (compare Fig. 1H with 1I and see Fig. 1F).

As is the case in avian embryos (Groves and Bronner-Fraser, 2000), the morphological manifestation of the zebrafish otic placode is presaged by the expression of transcripts of a number of genes. *dlx3*, which is initially expressed at the neurectoderm-ectoderm interface of the cranial neural plate, becomes upregulated in the prospective otic territory by bud stages (10 hours) and then becomes restricted to the otic placode (Ellies et al., 1997; Mendonsa and Riley, 1999). *pax8* is expressed in ectoderm in the region of the prospective otic placode from bud stages (10 hours) and expression is confined to the otic placode when the latter becomes visible morphologically. Two other members of the Pax family, *pax2.1* and *pax2.2*, are expressed in a similar manner in the prospective otic territory from two somites and three somites, respectively (Pfeffer et al., 1998). Thereafter, the placode can be distinguished using Normarski optics from 9 somites, invaginates to form a vesicle by 19 somites and otoliths are visible from 25 hours (prim 7) (Kimmel et al., 1995). Thus, our study indicates that the onset of *fgf3* expression precedes that of the first molecular markers of prospective otic ectoderm.

We have compared the spatial relationship of *fgf3* transcripts with three of these markers, *dlx3*, *pax2.1* and *pax8*, at early stages of otic placode development. *dlx3* expression is detected adjacent to the r4 *fgf3* expression domain and extends approximately two rhombomere lengths posterior to r4 (Fig. 1J,K). The *pax2.1* and *pax8* domains also lie adjacent

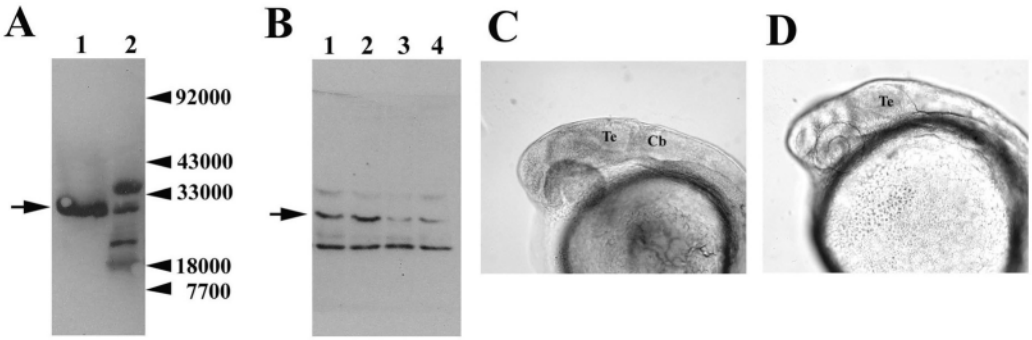

Fig. 2. Inhibition of Fgf3 and Fgf8 using morpholinos. (A) Immunoblot detection of Fgf3 in extracts of zebrafish embryos probed with an anti-Fgf3 antibody. Lane 1: zebrafish Fgf3 produced in and purified from a mammalian cell line. Lane 2: Recognition of zebrafish Fgf3 in an extract of four 5 somite embryos. The other crossreacting bands were detected variably from blot to blot and were not sensitive to knockdown by Fgf3mo. (B) Reduction in Fgf3 protein in immunoblots of individual Fgf3mo-injected embryos. Lanes 1-4: detection of Fgf3 in protein extracts of individual 5 somite embryos. Lane 1: uninjected embryo. Lane 2: embryo injected with control Fgf3 morpholino. Lanes 3 and 4: embryos injected with Fgf3mo. (C,D) Fgf8mo phenocopies homozygous *ace* zebrafish at 26 hours of development. (C) Embryo injected with control Fgf8 morpholino. (D) Embryo injected with Fgf8mo lacks cerebellar tissue. Te, telencephalon; Cb, cerebellum.

to r4 but extends both anteriorly, adjacent to r3, and posteriorly, adjacent to r5 and r6 (Fig. 1L,M), and thus may define a region of otic competence rather than the presumptive placode itself.

Transcripts of a second member of the Fgf family, *fgf8* which signals from the isthmus to pattern midbrain and anterior hindbrain (Reifers et al., 1998), was also found to be expressed in the presumptive r4 territory at 90% epiboly prior to its expression in the isthmus (midbrain-hindbrain junction; Fig. 1N) (Phillips et al., 2001) (J. W., H. M. and I. M., unpublished). Thus, at least two Fgfs are expressed in the hindbrain adjacent to the presumptive otic territory before the first molecular markers of the specification of the latter.

Inhibition of Fgf3 and Fgf8 together results in absence of the otic vesicle

To investigate Fgf3 function in the zebrafish embryo, we used morpholino oligonucleotides (Nasevicius and Ekker, 2000) injected into one- to eight-cell stage embryos to inhibit translation of Fgf3 protein. Zebrafish Fgf3 is a protein with an M_r of about 28,000 when detected on immunoblots (Fig. 2A). Fgf3 protein in blots of total protein extracts from individual embryos that had been injected with Fgf3 morpholino (Fgf3mo) was either greatly reduced or undetectable when compared with either wild-type fish or fish injected with a control morpholino which differed by only four nucleotides from the Fgf3mo (Fig. 2B) (J. W., H. M. and I. M. submitted and data not shown). The level of repression of translation varied between different Fgf3mo-injected embryos and thus it was expected that a hypomorphic series would be generated as previously reported by others (Nasevicius and Ekker, 2000). In addition, using a morpholino against *fgf8* (Fgf8mo), we were also able to phenocopy the homozygous *ace* zebrafish mutant which is mutated in the *fgf8* gene. Defects observed in these embryos included loss of cerebellar tissue (Fig. 2C,D), somite defects (data not shown) and reduction in otocyst size. All of these defects were previously reported in homozygous *ace* fish (Reifers et al., 1998).

Embryos injected with Fgf3mo were allowed to develop to 26 hours (prim8) and their otocysts were examined under Normarski optics. While otocysts were present in all cases and these generally had two otoliths, a large number of embryos exhibited a reduction in otocyst size of about 30-35% (Fig. 3A,B; Table 1). This phenotype was remarkably similar to that reported for homozygous *ace*/*fgf8* mutant fish. *fgf8* is expressed initially in the presumptive r4 territory, although at later stages its domain of expression extends from the isthmus to r4 prior to becoming restricted to the isthmus (Reifers et al., 1998) (data not shown). Consistent with the study of Reifers et al. (Reifers et al., 1998), we saw similar reductions in otocyst size after Fgf8mo injections (Fig. 3C), indeed the resultant

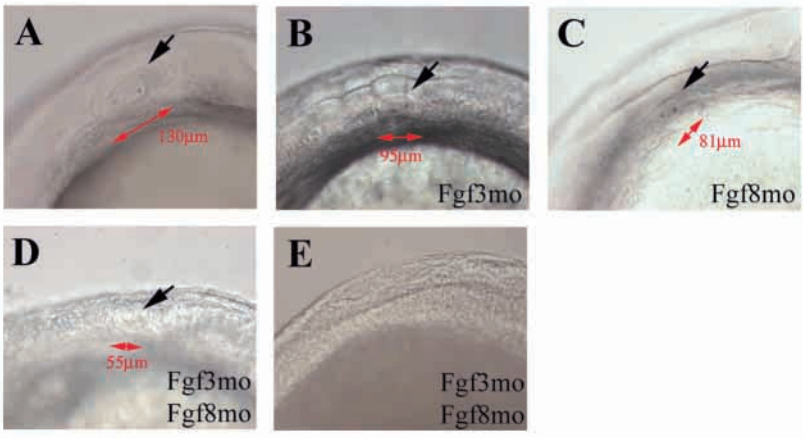

Fig. 3. Size reduction and loss of otic vesicles in fish injected with Fgf3 and Fgf8 morpholinos. (A-E) 26 hour (prim 8) embryos photographed at the same magnification using Nomarski optics. Where otic vesicles are present (arrows), their maximum diameter (anteroposterior axis) is measured and indicated in red. (A) Embryo injected with control Fgf3 morpholino. (B) Embryo injected with Fgf3mo. (C) Embryo injected with Fgf8mo. (D,E) Embryos co-injected with Fgf3mo and Fgf8mo showing further reduced size (D) or absence of an otocyst (E).

Table 1. Effects of Fgf3 and Fgf8 morpholinos (mo) on early and late otic development

	Control	Fgf3mo	Fgf8mo	Fgf3mo + Fgf8mo
Small otic vesicle (26 hours)	0/57	31/54	38/58	40/116
Maximum vesicle diameter in affected embryos	128±7 μm	93±8 μm	79±5 μm	52±12 μm
Absence of otic vesicle (26 hours)	0/57	0/54	0/58	52/116
Reduced *dlx3*	0/28	46/58	66/80	13/56
Loss of *dlx3*	0/28	0/58	0/80	35/56
Reduced *pax2.1*	0/48	32/41	12/20	12/48
Loss of *pax2.1*	0/48	0/41	0/20	27/48
Reduced *pax8*	0/40	0/37	0/44	12/23
Loss of *pax8*	0/40	0/37	0/44	1/23

otocysts were slightly smaller than those obtained following Fgf3mo injections (Table 1).

These data, together with the co-expression of both *fgf3* and *fgf8* in r4, prompted us to inject simultaneously morpholinos against both into embryos. After such injections, we obtained a range of phenotypes consistent with our predictions of generation of a hypomorphic series and with the likelihood of mosaic uptake of the morpholinos (Table 1). Embryos were mostly more severely affected by co-injection of Fgf3mo and Fgf8mo than by injection of each alone. Phenotypes ranged from otocysts that were even smaller than those observed after injection of single morpholinos and which lacked otoliths, to complete absence of otic vesicles (Fig. 3D,E; Table 1). The complete absence of an otic vesicle correlated well with absence of cerebellar tissue (predicted from loss of Fgf8 function) and defects in the production of the most posterior somites (caudal to somite 23) in the same embryos. The latter phenotype was seen in embryos injected with Fgf3mo alone, consistent with tail defects observed in mice homozygous null for *fgf3* (Mansour et al., 1992) (J. W., H. M., R. M. and I. M., unpublished). The tail defect was never seen in Fgf8mo-injected embryos. Thus, absence of an otocyst was detected in embryos in which gene knock-down appeared to have been achieved most efficiently.

Cell death and division are unaffected by inhibition of Fgf3 and Fgf8

The effects of Fgf3 and Fgf8 morpholinos on otic development might be due to influences on cell death or proliferation in the otic territory. We examined injected embryos for effects on apoptosis using the TUNEL procedure at the time of morphological appearance of the otic vesicle. Apoptotic cells were only infrequently detected in embryos injected with control morpholinos (Fig. 4A,B; *n*=28), Fgf3 morpholinos (Fig. 4C,D; *n*=33) or Fgf8 morpholinos (Fig. 4E,F; *n*=23). By contrast, many more apoptotic cells were detected in embryos

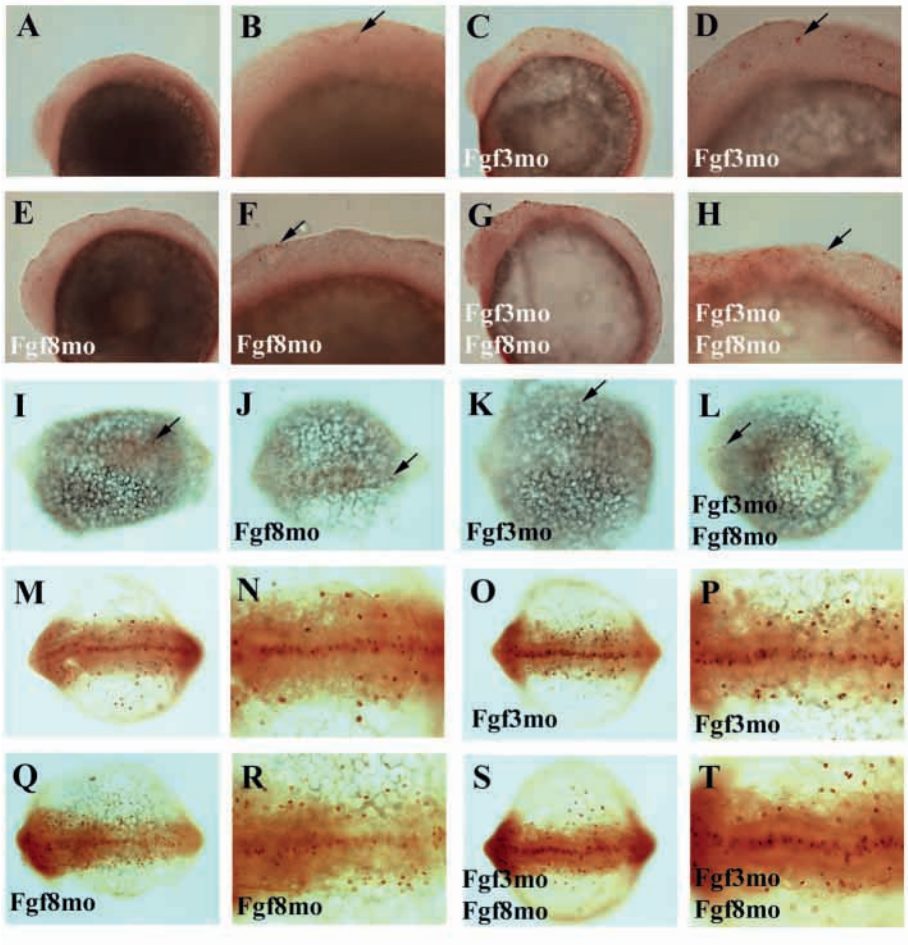

Fig. 4. Detection of apoptotic and dividing cells in embryos injected with Fgf3 and Fgf8 morpholinos. Apoptotic (A-L) and dividing cells (M-T) detected in lateral views of zebrafish embryos at 14 somites photographed at low and high magnification (A-H) and in dorsal views of the hindbrain and otic territory of embryos at 8 somites (I-T). (A,B,I) Embryos injected with control Fgf3 morpholino. (C,D,K) Embryos injected with Fgf3 morpholino. (E,F,J) Embryos injected with Fgf8 morpholino. (G,H,L) Embryos injected with both Fgf3 and Fgf8 morpholinos; note large number of apoptotic cells in the dorsal midline of the neural tube in 14 somite embryos. (M-T) Low magnification (M,O,Q,S) and high magnification (N,P,R,T) views of dividing cells in the hindbrains and adjacent otic territory of embryos injected with both control morpholinos together (M,N), Fgf3 morpholino (O,P), Fgf8 morpholino (Q,R) and Fgf3mo together with Fgf8mo (S,T).

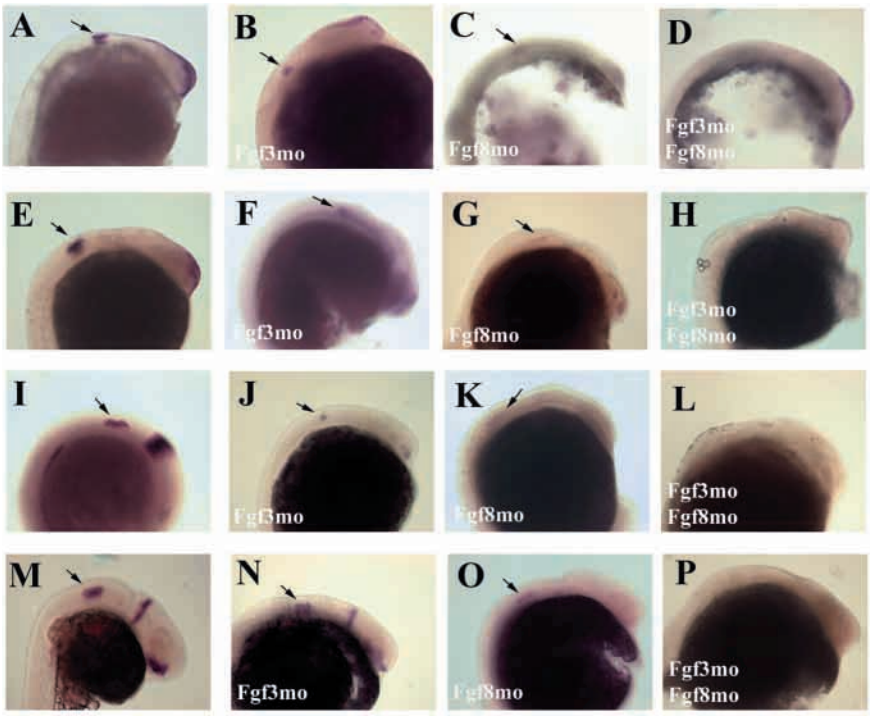

Fig. 5. Loss of early otic placode markers in embryos injected with Fgf3 and Fgf8 morpholinos. Embryos were hybridised for *dlx3* (A-H) and *pax2.1* (I-P) after incubation to 2-5 somite (A-D and I-L) or 18-21 somite (E-H and M-P) stages after injection with morpholinos. (A,E,I,M) Embryos injected with control Fgf3 morpholino are indistinguishable from uninjected embryos. (B,F,J,N) Embryos injected with Fgf3mo show reduction in level and extent of *dlx3* (B,F) and *pax2.1* (J,N) expression. (C,G,K,O) Embryos injected with Fgf8mo show reduction in level and extent of *dlx3* (C,G) and *pax2.1* (K,O) expression. (D,H,L,P) Embryos injected with Fgf8mo and Fgf3mo together show loss of *dlx3* (D,H) and *pax2.1* (L,P) expression. Arrows indicate position of *dlx3*- and *pax2.1*-expressing cells as appropriate.

injected with Fgf3 and Fgf8 morpholinos in combination (Fig. 4G,H; *n*=46). However these dying cells were localised primarily to the dorsal neural tube region throughout its length (Fig. 4G) and the prospective otic ectoderm adjacent to the hindbrain showed no increase in numbers of apoptotic cells (Fig. 4H). We also examined embryos at the 8-somite stage, prior to appearance of the otic placode but when molecular markers suggest that the otic ectoderm has been specified. We found very few apoptotic cells either in control embryos or those that had been injected with morpholinos (Fig. 4I-L)

We also used an antibody against phosphorylated (Ser28) histone H3 to detect mitotic cells between G2 and telophase (Saka and Smith, 2001). Numerous dividing cells were observed but no differences in numbers of cells in mitosis were found in ectoderm adjacent to the hindbrain in embryos injected with morpholinos either singly or in combination when compared with control embryos (Fig. 4M-T).

Early defects in otic development of Fgf3 and Fgf8 knockdown zebrafish

The early onset of *fgf3* and *fgf8* expression adjacent to the presumptive otic territory, together with the lack of obvious effects on cell death after morpholino injections, suggested that the absence or reduced size of otic vesicles might be due to defects in induction of the otic ectoderm. To determine whether or not Fgf3 and Fgf8 are required at the earliest stages of otic placode development, we examined expression of two early placode markers, *dlx3* and *pax2.1*, in embryos injected with Fgf3mo and Fgf8mo singly and in combination. We found that neither Fgf3 or Fgf8 morpholinos injected alone prevented early or late expression of otic *dlx3* and *pax2.1* (Fig. 5B,C,F,G,J,K,N,O). However, expression was both weaker and less extensive along the anteroposterior axis than observed in control embryos (compare with Fig. 5A,E,I,M; Table 1). Other sites of expression were also affected, for example isthmic *pax2.1* is lost in Fgf8mo-injected fish as expected from previous studies (Reifers et al., 1998).

In contrast to effects of the individual morpholinos, when both Fgf3mo and Fgf8mo were co-injected a significant number of embryos lacked either early or late expression of *dlx3* and *pax2.1* (Fig. 5D,H,L,P; Table 1). Others showed reduced expression similar to that observed in injections of single oligonucleotides and consistent with predictions of a hypomorphic series expressing varying levels of protein being generated (data not shown). Taken together, these data suggest that Fgf3 and Fgf8 are required at the earliest stages of otic placode induction and/or specification.

Inhibition of Fgf signalling confirms its requirement during early otic development

To confirm our findings and to investigate the temporal requirement for Fgf signalling further, we incubated embryos, denuded of their chorions, in SU5402, a widely used inhibitor of Fgf receptor (Fgfr) activation. Predictions based upon structures of co-crystals of Fgf receptor tyrosine kinase domain and SU5402 suggest that it should block kinase activity and therefore signalling by all Fgf receptors (Mohammadi et al., 1997). Embryos were incubated in inhibitor for 5 hours starting at 30% epiboly, 60% epiboly and 5-somite stages, reaching tailbud, 2- to 3-somite and 16-somite stages, respectively, at the end of the incubation period. To demonstrate the effectiveness of the treatment, a subset of the embryos were fixed immediately and processed for in situ hybridisation for transcripts of the Ets family transcription factor, *erm*. Erm is a member of a subfamily of Ets proteins that are transcriptionally activated by activated Erk1 and Erk2 (MAP kinases), which themselves are activated following Fgfr activation. Distribution of activated Erk protein is identical to *erm* mRNA distribution during early zebrafish development (J. W. and I. M., unpublished) and closely parallels distribution of *fgf3* and *fgf8* transcripts. Moreover, treatment with SU5402 abrogates *erm* expression (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001).

erm transcripts were either absent or barely detectable in the otic region and hindbrain of all embryos at the end of

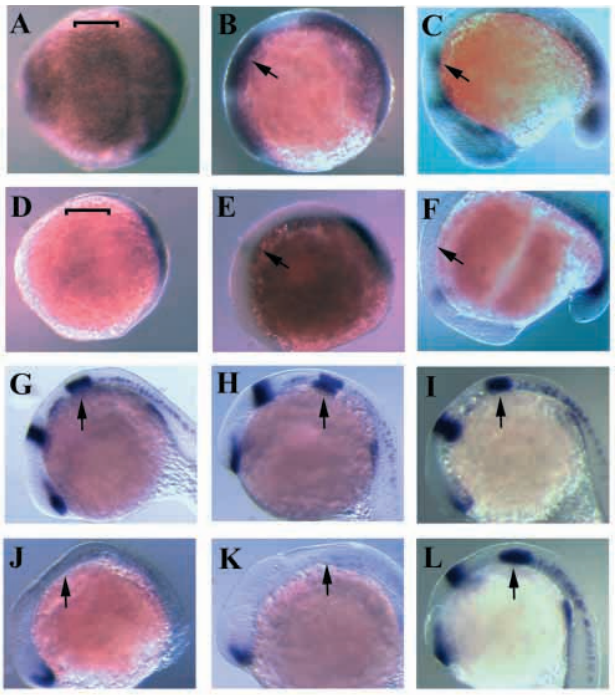

Fig. 6. Loss of *pax2.1* expression in embryos treated with an inhibitor of Fgfr signalling. Embryos were treated with either DMSO (A-C,G-I) or SU5402 (D-F,J-L) for 5 hours between 30% epiboly and tailbud (A,D,G,J), 60% epiboly and 2-3 somite (B,E,H,K), or 5-somite and 16-somite stages (C,F,I,L). Embryos were either fixed immediately and hybridised for *erm* transcripts (A-F) or allowed to develop until 16 somites and then hybridised for *pax2.1* (G-L). (A-F) *erm* transcripts are reduced or abrogated in the otic region and hindbrain (brackets in A and D, arrows in B,C,E,F). (G-L) *pax2.1* transcripts (arrows) are absent in embryos exposed to inhibitor at early stages (G,H,J,K) but not at later stages (I,L).

incubation with SU5402 (Fig. 6D-F), whereas mRNA was detected in the hindbrain and adjacent presumptive otic territory of control embryos at all stages (Fig. 6A-C). Thus, bathing embryos in the inhibitor was sufficient to inhibit Fgfr activity. Other embryos from the same experiment were allowed to develop to the 16-somite stage and then analysed for expression of *pax2.1*.

Embryos exposed to inhibitor between 30% epiboly and tailbud and between 60% epiboly and 1- to 2-somite stages showed absence of *pax2.1* transcripts (compare Fig. 6G,H with 6J,K), whereas those incubated from 5-16 somites showed no effect on expression (Fig. 6I,L; Table 2). These data indicate that expression of early markers of the presumptive otic territory is dependent upon Fgf signalling during a period between 60% epiboly and tailbud stages; the period of co-expression of *fgf3* and *fgf8* in the adjacent developing hindbrain.

An Fgf-independent event initiates the specification of the otic ectoderm

We examined the requirement of Fgf3 and Fgf8 for the induction of the earliest molecular marker of the presumptive otic territory, *pax8*. In contrast to their effects on *pax2.1* and *dlx3*, neither Fgf3 nor Fgf8 morpholino injections reduced either the apparent abundance or spatial extent of *pax8* transcripts (Fig. 7A-C). Moreover, injection of both morpholinos together reduced but did not abrogate *pax8* expression (Fig. 7D; Table 1). To determine whether signalling by another Fgf, acting either alone or in combination with Fgf3 and Fgf8, induces *pax8*, we examined expression following treatment with SU5402. Embryos exposed to inhibitor between either 30% epiboly and tailbud or 60% epiboly and 2-3 somites still expressed abundant *pax8* transcripts, although their spatial extent along the anteroposterior axis was slightly reduced (Fig. 7E-H). Thus, we conclude that an Fgf-independent event induces *pax8* expression in the presumptive otic territory.

DISCUSSION

Fgf3 is required for otic development

Investigation of *fgf3* expression in relation to otic development in the zebrafish (this study) (Phillips et al., 2001) showed that transcripts were detected within the prospective hindbrain (90% epiboly) before the time of appearance of the first otic markers (tailbud stage) (Pfeffer et al., 1998), consistent with a role in regulating early otic development. As hindbrain segmentation proceeded, *fgf3* mRNA was confined to r4, initially throughout that segment, but at later stages only ventrally. This pattern of expression is similar to that reported in *Xenopus* (Lombardo et al., 1998) but contrasts with the more complex expression observed in this region of the mouse or chick embryo head. In avian and mouse embryos, *fgf3* is dynamically expressed in posterior hindbrain segments, initially in r4 but soon thereafter in r5 and r6, while becoming downregulated in r4. Transcripts then become undetectable in the bodies of each of these three rhombomeres but are detected in the inter-rhombomeric boundary cells. By contrast, *fgf3* expression was not found in zebrafish rhombomere boundary cells. In addition, while *fgf3* mRNA was never detected in tissues adjacent to the hindbrain in zebrafish, it has been found in ectoderm including the presumptive otic territory in both

Table 2. Effects of inhibition of Fgf signalling with SU5402

Gene	Treatment	Stage at treatment	No effect	Reduced expression	Loss of expression
pax2.1	DMSO	30% epiboly	22/22	0/22	0/22
	SU5402	30% epiboly	0/25	13/25	12/25
	DMSO	60% epiboly	20/20	0/20	0/20
	SU5402	60% epiboly	0/19	0/19	19/19
	DMSO	Five somites	12/12	0/12	0/12
	SU5402	Five somites	47/47	0/47	0/47
pax8	DMSO	30% epiboly	20/20	0/20	0/20
	SU5402	30% epiboly	37/37	0/37	0/37
	DMSO	60% epiboly	32/32	0/32	0/32
	SU5402	60% epiboly	37/37	0/37	0/37

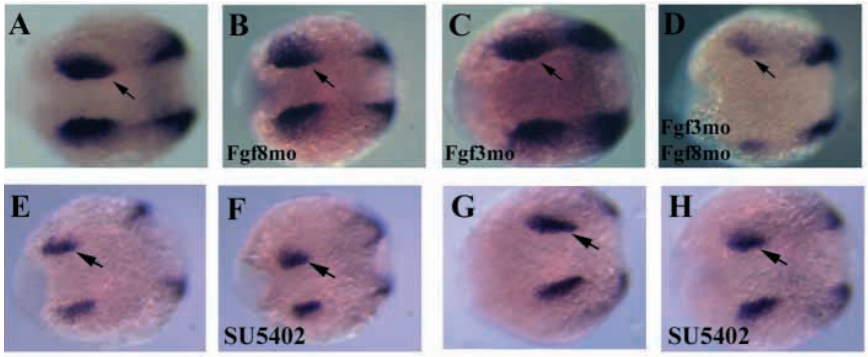

Fig. 7. Induction of *pax8* in presumptive otic ectoderm is independent of Fgf signals. (A-D) *pax8* transcripts are detected in control embryos (A) and embryos injected with Fgf8 morpholino (B), Fgf3 morpholino (C), and Fgf3 and Fgf8 morpholinos together (D), and allowed to develop to 2 somites. (E-H) Treatment with SU5402 does not abolish *pax8* expression. (E,F) Embryos treated between 30% epiboly and tailbud. (E) Control. (F) SU5402 treated. (G,H) Embryos treated between 60% epiboly and 2-3 somites. (G) Control. (H) SU5402 treated. Arrows indicate *pax8* expression in presumptive otic territory.

chick and mouse (Mahmood et al., 1995a; Mahmood et al., 1996).

Comparison of the relationship of the *fgf3* expression domain in the hindbrain with those of *pax2.1*, *pax8* or *dlx3* in adjacent ectoderm showed that Pax genes were expressed in a region adjacent to rhombomeres 3 to 6. However, *dlx3* was only detected adjacent to rhombomeres 4 to 6. This more extensive Pax-positive region has been reported by others in avian embryos (Groves and Bronner-Fraser, 2000) and may represent a region of competence to form otic placode, while the smaller *dlx3* domain may reflect a response to a signal that specifies the otic primordium proper. Data on expression of *erm*, which can be considered to be a direct indicator of cells receiving an Fgf signal, indicates that cells throughout the Pax-positive ectoderm are directly responding to Fgf (Raible and Brand, 2001).

Inhibition of Fgf3 in zebrafish embryos using morpholinos produced a hypomorphic series as evinced by the variable extent of Fgf protein loss and variability in severity of effects on otic development. The most severely affected embryos showed a reduction in otocyst size of about 30% and this correlated with reductions in level and spatial extent of early molecular markers of the otic placode. The reduction of expression of early otic markers being indicative of an early requirement for Fgf3 in otic placode development.

Embryos in which both Fgf3 and Fgf8 are inhibited lack otic vesicles

The effects of Fgf3 knockdown on otocyst size were remarkably similar to those observed in *fgf8* mutant zebrafish (Reifers et al., 1998) and the latter were reproduced by injection of *fgf8* morpholinos in this study. Taken together with observations that *fgf3* and *fgf8* were co-expressed in the presumptive r4 territory of the hindbrain adjacent to the region in which the otic placode arises, this suggested that they might function together during otic development. This was confirmed in experiments in which morpholinos against both were co-injected, resulting in complete loss of the otocyst in a significant proportion of embryos and more severe reduction of otocyst size in others compared with experiments using single morpholinos. We interpret this range of phenotypes as reflecting the generation of a hypomorphic series after morpholino injections as has been reported by others (Nasevicius and Ekker, 2000) and consistent with variation between complete and partial loss of Fgf3 protein in individual embryos. While this manuscript was in revision, similar findings were reported by others after injection of Fgf3 morpholinos into *ace* mutant zebrafish embryos (Phillips et al., 2001). However, Phillips et al. did not investigate specificity of the Fgf3 morpholinos and did not demonstrate knockdown of Fgf3 protein, the latter being important in interpreting variability between injected embryos. In support of redundancy in signalling to induce the otic vesicles, it is noteworthy that mutant fish, which lacked otic vesicles, were not isolated from mutagenesis screens (Whitfield et al., 1996).

Fgf3 and Fgf8 are required early during induction of the otic placode

Loss of the otocyst in such embryos was presaged by absence of early markers of otic placode; both *pax2.1* and *dlx3* were not expressed in embryos injected with morpholinos against both Fgfs. Injections of individual morpholinos resulted in considerable reductions in both the spatial extent and relative abundance of transcripts for both of these genes. This effect correlates well with the reduced sizes of otic vesicles observed in older embryos after such treatments and indicates that the extent of the otic primordium may be determined by the level of Fgf signal from the hindbrain.

We examined both cell death and division in the ectoderm at early stages of otic development in such embryos but found no differences when compared with control embryos or those injected with individual morpholinos. Taken together, our data suggest that Fgf3 and Fgf8 are required at the earliest stages of otic placode development. To confirm the requirement for Fgf signalling and provide further data concerning when it is required, we treated embryos at a range of developmental stages with SU5402, a potent inhibitor of Fgfr activity. Embryos treated between 30% epiboly and tailbud and between 60% epiboly and 2-3 somites showed absence of *pax2.1* expression, whereas older embryos were unaffected. This suggests that Fgf signalling is required only between 60% epiboly and tailbud stages, correlating well with the onset of *fgf3* and *fgf8* expression in the hindbrain.

Fgf3 and Fgf8 act directly on presumptive otic ectoderm

A key question concerns whether or not these Fgfs act directly upon presumptive otic ectoderm or indirectly via effects mediated within the developing hindbrain. Certainly, loss of Fgf3 and Fgf8 function has dramatic effects on the patterning of the hindbrain, for example transcripts for *valentino*/*kreisler*/*mafb* are not detected in such embryos (J. W., H. M. and I. M., unpublished). However, evidence suggests that they also act directly on adjacent ectoderm. First, absence of *dlx3*

transcripts occurs at tailbud stages, simultaneous with absence of the earliest affected marker of hindbrain patterning, *valentino* (Moens et al., 1996) (J. W., H. M. and I. M., unpublished). More importantly, expression of *erm* correlates exactly with cells in which Erk1 and Erk2 have been activated by Fgf signalling (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). *erm* transcripts occupy a broad domain within the zebrafish hindbrain at the time of *fgf3* and *fgf8* signalling (Fig. 5A,B) (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001) but importantly this also includes the *pax2.1*-positive ectoderm territory (Raible and Brand, 2001). Taken together, these data indicate a direct action of hindbrain Fgfs on presumptive otic ectoderm.

The dramatic effects of inhibition of both Fgf3 and Fgf8 together when compared with inhibition of either alone suggest that they might perform redundant functions during otic development. We have determined the receptor specificity of mouse, *Xenopus* and zebrafish Fgf3 proteins, and in all cases (Kiefer et al., 1993; Mathieu et al., 1995a; Mathieu et al., 1995b; Kiefer et al., 1996b) Fgf3 binds and activates IIIb splice variants of Fgf receptors, particularly of Fgfr2, whereas Fgf8 preferentially activates IIIc isoforms (Ornitz et al., 1996). Such considerations suggest that each may signal through a different Fgfr isoform and that redundancy may be at the level of activation of intracellular signalling pathways.

Fgf signalling and otic development in other vertebrate classes

Inhibition of Fgf3 function or its ectopic expression in chick or mouse embryos has provided conflicting data concerning its function(s) during otic development. Homozygous *Fgf3* null mice exhibit defects in endolymphatic duct formation but with incomplete penetrance and expressivity (Mansour et al., 1992). In addition, kreisler mouse mutants lack hindbrain *Fgf3* expression but again have relatively mild otic defects (McKay et al., 1996). Attempts to inhibit Fgf3 function in chick embryos using antisense oligonucleotides affected otocyst invagination but not placode induction (Represa et al., 1991), while ectopic Fgf3 expression in cranial ectoderm resulted in formation of small vesicular structures expressing some otic markers (Vendrell et al., 2000). The relatively mild phenotypes associated with Fgf3 inhibition are consistent with our finding that Fgf8 must be inhibited with Fgf3 to prevent otic placode formation.

Fgf8 expression has not been detected in the hindbrains of chick or mouse embryos despite several detailed studies having been undertaken (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995b) and otic defects have not been reported in a murine *Fgf8* hypomorphic series (Meyers et al., 1998). This raises the possibility that another Fgf acts alongside Fgf3 in these vertebrate classes, although low level expression of *Fgf8* cannot be discounted. Interestingly, we have previously reported *Fgf4* transcripts in hindbrain neurectoderm at open neural plate stages in the chick embryo (Shamim and Mason, 1999), where it is co-expressed with *Fgf3* (Mahmood et al., 1995a). At similar stages, *Fgf4* is also co-expressed with *Fgf15/Fgf19* in mesoderm underlying the prospective otic placode territory (Shamim et al., 1999; Ladher et al., 2000). However, evaluation of the role(s) of both of these in vertebrate otic development awaits appropriate inhibition studies.

The initial stage of otic induction is Fgf-independent

pax8 is currently the earliest molecular marker of the presumptive otic territory (Pfeffer et al., 1998) and, in only one embryo was its expression absent after injections of Fgf3 and Fgf8 morpholinos. Both *Fgf4* and *Fgf15/Fgf19* are expressed in mesoderm underlying the prospective otic territory in avian embryos prior to the appearance of markers of otic induction. Recent studies have suggested that Fgf15/Fgf19 might be a mesodermal inducer for the otic placode (Ladher et al., 2000), although its unique specificity for Fgfr4 (Xie et al., 1999) and the lack of otic defects in Fgfr4-null mice (Weinstein et al., 1998) indicate that it might not act alone. To further investigate the potential role of other Fgfs, including those that are mesodermally expressed, we examined embryos treated with SU5402 from 30% epiboly for expression of *pax8* transcripts and failed to find any evidence of a requirement for Fgf signalling in its induction.

Ladher et al. (Ladher et al., 2000) also implicated Wnt8c, which is expressed in the developing hindbrain, as a component of the inductive cascade in avian embryos. However, inhibition of retinoic acid signalling in the chick embryo results in absence of *Wnt8c* but otic induction is unaffected (Dupe and Lumsden, 2001), Thus, a different non-Fgf signal is most probably involved in initiation of otic induction. It is possible that this initiation signal is provided, at least in part, by the cranial mesendoderm, as *pax8* expression is delayed in *one-eyed pinhead* zebrafish mutants, which lack that tissue (Mendonsa and Riley, 1999; Phillips et al., 2001), but is subsequently rescued possibly by neurectodermal Fgf signalling.

We conclude that a non-Fgf signal(s), possibly of mesendodermal origin, provides the first inductive cue in specification of the otic ectoderm. Shortly thereafter, signalling by Fgf3 and Fgf8 from the hindbrain induce the second phase of gene expression within the latter territory further elaborating its developmental programme. Inhibition of both Fgfs results in a failure to execute that programme beyond its earliest stages.

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