Endoderm-derived Fgf3 is necessary and sufficient for inducing neurogenesis in the epibranchial placodes in zebrafish

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Accepted 22 April 2005

Development 132, 3717-3730
Published by The Company of Biologists 2005
doi:10.1242/dev.01876

Summary

In vertebrates, epibranchial placodes are transient ectodermal thickenings that contribute sensory neurons to the epibranchial ganglia. These ganglia innervate internal organs and transmit information on heart rate, blood pressure and visceral distension from the periphery to the central nervous system. Despite their importance, the molecular mechanisms that govern the induction and neurogenesis of the epibranchial placodes are only now being elucidated. In this study, we demonstrate that endoderm is required for neurogenesis of the zebrafish epibranchial placodes. Mosaic analyses confirm that endoderm is the source of the neurogenic signal. Using a morpholino knockdown approach, we find that *fgf3* is required for the majority of placode cells to undergo neurogenesis. Tissue transplants demonstrate that *fgf3* activity is specifically required in the endodermal pouches. Furthermore, ectopic *fgf3* expression is sufficient for inducing *phox2a*-positive neurons in wild-type and endoderm-deficient embryos. Surprisingly, ectodermal *foxi1* expression, a marker for the epibranchial placode precursors, is present in both endoderm-deficient embryos and *fgf3* morphants, indicating that neither endoderm nor Fgf3 is required for initial placode induction. Based on these findings, we propose a model for epibranchial placode development in which Fgf3 is a major endodermal determinant required for epibranchial placode neurogenesis.

Key words: Fgf3, Foxi1, Epibranchial placodes, Cranial ganglia, Endoderm, Pharyngeal pouches, Placode induction, Neurogenesis

Introduction

Neurogenic cranial placodes are transient ectodermal thickenings that form at the border of the neural plate and epidermis and give rise to delaminating cells that form the cranial sensory ganglia. The epibranchial (EB) placodes, positioned ventrally to the ear and dorsally to the posterior pharyngeal pouches, form sensory neurons of the epibranchial ganglia that innervate organs derived from the pharyngeal arches as well as the heart and viscera. In zebrafish, each placode is associated with a pharyngeal arch: the facial placode with the second arch, the glossopharyngeal placode with the third arch, and four vagal placodes with the four posteriormost arches.

Several studies have identified transcription factors that regulate different steps of EB placode development. In mouse, specification and delamination of neuroblasts from EB placodes is controlled by expression of the atonal-related basic helix-loop-helix (bHLH) transcription factor neurogenin 2 (Fode et al., 1998), while neurogenesis from other cranial placodes is controlled by neurogenin 1 (Neurog1) (Ma et al., 1998; Ma et al., 2000). These roles may be reversed in amphibian and avian species (Schlosser and Northcutt, 2000; Begbie et al., 2002). By contrast, a single neurogenin, Ngn1 (Neurog1 – Zebrafish Information Network) is required for the development of all ganglia in zebrafish (Andermann et al., 2002). The homeobox transcription factors *phox2a* and *phox2b* are necessary for the subsequent differentiation of EB neurons (Guo et al., 1999; Morin et al., 1997; Pattyn et al., 1997; Pattyn et al., 1999). Expression of *ngn1* is regulated in EB placodes by two other sets of transcription factors, *Eya1* and *Six1* in mouse (Zou, 2004), and the forkhead-related winged helix transcription factor *foxi1* in zebrafish (Lee et al., 2003). In *foxi1* mutants, placodal progenitors fail to express both *ngn1* and *phox2a*. However, *foxi1* is also expressed in the pharyngeal pouches during the time of EB placode neurogenesis, and mutant embryos show defective pouch morphology and marker expression (Nissen et al., 2003; Solomon et al., 2003). Thus, it remains to be determined whether the phenotype observed in *foxi1* mutants is caused by defective Foxi1 in ectoderm or by abnormal signaling from the pharyngeal pouches.

Much less is known about the inductive signals that regulate EB placode development. Previous studies have suggested that pharyngeal endoderm acts as a source of signals for EB placodes. Landacre (Landacre, 1931) first noted a close spatial and temporal association between EB ganglia and pharyngeal endoderm in axolotl. In chick, tissue recombination experiments demonstrated that pharyngeal endoderm promotes neuron production from cranial ectoderm (Begbie et al., 1999). BMP7, which is expressed in chick pharyngeal pouch endoderm, also induces neurogenesis in cranial ectoderm, and the BMP-inhibitor follistatin blocks the neurogenic effect of pharyngeal endoderm (Begbie et al., 1999). However, BMP7 is not sufficient for inducing neurons from trunk ectoderm, while trunk ectoderm is competent to form EB neurons when grafted to the head region (Vogel and Davies, 1993). This

suggests that other signal(s), in addition to BMP7, are required for EB placode induction and/or neurogenesis.

We postulated that possible candidates for signals that regulate EB neurogenesis include members of the Fgf signaling family. Fgfs play important roles in embryogenesis (e.g. development of limb bud, heart, tooth, lung, otic vesicle) as well as in the adult organism (e.g. tissue repair, angiogenesis). To date 23 members have been isolated from human and mouse (Javerzat et al., 2002). Our recent literature and database searches have revealed at least 25 candidate Fgf members in zebrafish (http://www.sanger.ac.uk/Projects/D_rerio). The effects of vertebrate Fgfs are mediated by four transmembrane receptors (Fgfr1 through Fgfr4), which are members of the receptor tyrosine kinase (RTK) superfamily (Ornitz, 2000; Scholpp et al., 2004; Sleptsova-Friedrich et al., 2001; Thisse et al., 1995; Tonou-Fujimori et al., 2002). Upon ligand binding, Fgfrs trigger, by way of Ras, a sequential activation of the mitogen-activated kinase (MAPK)/extracellular activated kinase (ERK) signaling cascade (Cobb and Goldsmith, 1995), which in turn leads to the activation of nuclear transcription. In zebrafish, the ETS transcription factor genes *erm* and *pea3* are transcriptional targets of this pathway (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001).

Although not implicated in EB placode development, Fgfs are well established as regulators of development of the otic placode (Riley and Phillips, 2003; Wright and Mansour, 2003). In particular, Fgfs have been implicated in otic placode neurogenesis in chick (Alsina et al., 2004). Inhibition of Fgf signaling blocks *ngn1* expression and formation of the acoustic ganglion, while ectopic Fgf10 promotes expression of the proneural genes *neuroD* and *neuroM*. A recent study indicated that zebrafish Foxi1 modulates cellular responses to Fgf signaling (Nissen et al., 2003), suggesting that a similar situation may occur in EB placode development.

Here, we use a genetic approach to test whether endoderm is required for EB ganglia development. We show that endoderm-deficient zebrafish embryos lack the majority of the EB ganglia sensory neurons. Mosaic analyses demonstrated that endoderm restoration could rescue EB ganglia and specifically identified endodermal pouches as a source of the inductive signal. We further implicate Fgf3 signaling as a major endodermal determinant required for the neurogenesis of the EB placodes. Tissue transplantation experiments confirmed that Fgf3 activity is specifically required in the endodermal pouches. Finally, ectopic expression experiments demonstrated that Fgf3 is sufficient for inducing sensory neurons in both wild-type and endoderm-deficient morphants. Altogether, our results demonstrate a requirement for the pharyngeal pouch endoderm and endoderm-derived Fgf3 in the neurogenesis and survival of the majority of EB sensory neurons.

Materials and methods

Fish care and SU5402 treatment

Wild-type embryos were obtained from natural spawning of *AB adults and staged according to age [hours post-fertilization (hpf) at 28.5°C] and morphological criteria (Kimmel et al., 1995). Heterozygous *ace*^{ti282a} or *foxi1*$^{+/-}$ adults were crossed to generate homozygous *ace* or *foxi1* mutant embryos, respectively (Reifers et al., 1998; Solomon et al., 2003). The Fgfr inhibitor, SU5402 (Calbiochem), was dissolved in DMSO at 100 mmol/l and then further diluted to a working concentration in embryo medium (EM) (Westerfield, 2000). Control embryos were treated with the same amount of DMSO in EM. To determine the most effective concentration of inhibitor, we (1) treated embryos for 24 hours, beginning at blastula stage, and examined them for reduction of the trunk mesoderm and otic vesicle; and (2) analyzed *erm* and *pea3* expression in the embryos treated with the inhibitor between 24 and 28 hpf. Embryos treated with a 60 μmol/l solution lacked trunk mesoderm, otic vesicle and *erm* and *pea3* expression, without exhibiting significant cell death. Thus, we used 60 μmol/l concentrations in all our subsequent treatments.

Embryo injections

Antisense morpholino oligonucleotides (MO) were obtained from GeneTools (Corvalis, OR), diluted to a working concentration in Danieau buffer (58 mmol/l NaCl, 0.7 mmol/l KCl, 0.4 mmol/l $MgSO_4$, 0.6 mmol/l $Ca(NO_3)_2$, and 5 mmol/l HEPES, pH 7.6), and 2-3 nl were pressure-injected into one-cell stage embryos. *cas*-MO, 5′-GCATCCGGTCGAGATACATGCTGTT, was injected at 2 ng/nl (Sakaguchi et al., 2001); *fgf3*-MO1, 5′-CATTGTGGCATGGAGGGATGTCGGC, was injected at 0.75 ng/nl (Maroon et al., 2002); *fgf3*-MO2, 5′- CAGTAACAACAAGAGCAGAATTATA, was injected at 5 ng/nl (Raible and Brand, 2001); *fgf4*-MOE1I1, 5′-AACTTACTGTAGCGGTTTTCGTTGT, was injected at 3 ng/nl (Jackman et al., 2004); *fgf8*-MOE2I2+*fgf8*-MOE3I3, 5′-TAGGATGCTCTTACCATGAACGTCG+5′-CACATACCTTGCCAATCAGTTTCCC, were injected at 0.5 ng/nl each (Draper et al., 2001); *fgf24*-MOE3I3, 5′-AGGAGACTCCCGTACCGTACTTGCC, was injected at 2.5 ng/nl (Draper et al., 2003); *foxd3*-MO, 5′-TGCTGCTGGAGCAACCCAAGGTAAG, was injected at 2 ng/nl (Lister et al., unpublished); *ngn1*-MO, 5′-ACGATCTCCATTGTTGATAACCTGG, was injected at 5 ng/nl (Andermann et al., 2002).

In-situ hybridization, immunolabeling, TUNEL assay and Richardson's stain

In-situ hybridization and immunolabeling experiments were performed according to the published protocols (Andermann et al., 2002). We used the following riboprobes and antibodies: *erm* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), *fgf3* (Kiefer et al., 1996), *fgfr1* (Scholpp et al., 2004), *foxi1* (Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003), *ngn1* (Korzh et al., 1998), *nrd* (*neurod* – Zebrafish Information Network) (Korzh et al., 1998), *pea3* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), *phox2a* (Guo et al., 1999), *phox2b* (Shepherd et al., 2004), anti-Hu (1:750, Molecular Probes), anti-Zn-5 (1:200, The Zebrafish International Resource Center, Eugene, OR). For TUNEL, embryos were fixed for 1 hour at room temperature in 4% paraformaldehyde in PBS (pH = 7.3). Embryos were then washed 3× with TBST (PBS with 2% Triton X-100 and 5% Tween-20, Sigma), dehydrated and rehydrated through a methanol:TBST series and transferred back into TBST. Embryos were treated with 10 μg/ml Proteinase K for 5 minutes, postfixed for 20 minutes, and then washed several times in TBST. Following equilibration in TdT dilution buffer, embryos were first incubated in the fluorescein-TUNEL labeling mix (Roche) for 1 hour on ice and then for 1 hour at 37°C. Embryos were then washed with TBST and processed to detect fluorescein using NBT/BCIP chromogenic substrate (Roche). For brightfield photography, embryos were deyolked when appropriate, flat mounted in 50% glycerol/50% PBS and photographed on a Nikon SMZ 1500 stereoscope or Zeiss Axioplan microscope using Spot CCD camera (Diagnostic Instruments). Fluorescent images were obtained using an LSM-5 Pascal confocal microscope (Zeiss). Images were processed in Adobe Photoshop. Some embryos were dehydrated, embedded in Araldite resin (Polysciences) and sectioned at 4-8 μm. For Richardson's stain, 26-hour-old embryos were fixed in 4% PFA in PBS for 2 hours at room temperature, dehydrated and embedded. Dry 4 μm sections were heated to 73°C on a hot plate and dipped into an aqueous solution of

0.0005% Azure II, 0.001% Methylene Blue, and 0.001% Sodium Borate for 80 seconds, then washed, air dried and mounted in Araldite resin.

Grafting experiments

For transplants, embryos were raised in filter-sterilized EM supplemented with penicillin (5000 U/l)/streptomycin (100 mg/l; Sigma). mRNAs were synthesized using mMessage Machine Kit (Ambion) following the manufacturer's protocol. Donor embryos were injected at the one-cell stage with 2% lysine-fixable fluorescein dextran (10,000 Mr; Molecular Probes) or 80 pg of *gfp* mRNA and 1.2 pg of *tar** mRNA (Peyreiras et al., 1998) in 0.2 mol/l KCl. Dechorionated donor and host embryos were mounted in 3% methylcellulose in Ringer's solution (Westerfield, 2000) on a glass depression slide. Ten to 20 donor cells were inserted into a host embryo. For in-situ hybridization, donor-derived fluorescein-labeled cells were detected essentially as described (Prince et al., 1998), except INT Red chromogenic substrate (Iodo Nitrotetrazolium Violet, Sigma) in PVA (Polyvinyl Alcohol) was used instead of Fast Red.

Fgf3 misexpression

HS-*fgf3* (Maves et al., 2002) and HS-*gfp* (Halloran et al., 2000) plasmids were co-injected into one-cell-stage embryos at 2.5 ng/µl. At this concentration, plasmid uptake by early blastomeres is very mosaic, leading to small clones of *fgf*-expressing cells upon heat shock. Embryos were heat shocked at 37°C for 1 hour at 22 hpf, fixed at 36 hpf and assayed for *phox2a* and *gfp* expression. Control embryos injected with HS-*gfp* alone showed normal *phox2a* expression. To detect GFP expression following RNA in-situ hybridization, embryos were processed with anti-GFP antibody (1:200, Molecular Probes) and Alexa 568 anti-mouse secondary antibodies (1:500, Molecular Probes) as described (Andermann et al., 2002).

Results

Endoderm is required for EB placode neurogenesis and survival

The initiation of EB placode neurogenesis was defined by expression of *ngn1*, which began at 24 hpf (not shown) (Andermann et al., 2002); by 36 hpf, *ngn1* was distinctly expressed in all EB placodes and delaminating neuronal precursors (Fig. 1A). Initially, the last three vagal placodes were fused together. Between 48 and 72 hpf, this large vagal placode separated into three distinct vagal placodes associated with each arch. For simplicity, we will refer hereafter to the fused vagal placode (ganglion) as the large vagal placode (ganglion). Expression of *ngn1* was followed by expression of *phox2a* and *phox2b*, which specifically mark EB neuronal precursors (Fig. 1A). Ngn1 is required for the development of all *phox2a*- and *phox2b*-positive EB sensory neurons, as this expression was eliminated after *ngn1* MO injection (Fig. 1B). By 80 hpf, EB neurons coalesced into distinct ganglia identified with the general neuronal marker, Hu (Fig. 1A).

To determine whether endoderm is necessary for the development of EB neurons, we injected zebrafish embryos with a specific MO directed against *casanova* (*cas*) mRNA (Sakaguchi et al., 2001). Cas is a Sox-related factor that is required for endoderm formation (Alexander et al., 1999; Kikuchi et al., 2001). One hundred percent of injected embryos exhibited cardiabifida and loss of the pancreas (monitored by *nrd* expression; data not shown), confirming loss of endoderm. In addition, the pharyngeal pouches were completely absent in all *cas*-MO embryos examined (Fig. 1A), as detected by expression of Zn-5, a DM-GRASP cell-surface antigen (Fashena and Westerfield, 1999). When compared to uninjected controls, *cas* morphants completely lacked *ngn1*, *nrd*, *phox2a* and *phox2b* expression in the glossopharyngeal and small vagal placodes and ganglia, while expression in the facial and large vagal placodes and ganglia were significantly reduced (Fig. 1A, Table 1). To confirm that the above phenotype did not result from developmental delay, we stained 80-hour-old wild-type and *cas* morphants with a pan-neuronal anti-Hu antibody. While the facial ganglion is difficult to ascertain at this stage because it is often fused with trigeminal and anterior lateral line ganglia, glossopharyngeal and small vagal ganglia were clearly absent and the large vagal ganglion was significantly reduced in *cas*-MO embryos (Fig. 1A). Analyses of Hu expression at 36 hpf revealed that trigeminal and lateral line ganglia were not affected (data not shown), indicating a specific defect in the EB ganglia in *cas* morphants. These experiments demonstrate that endoderm is required for neurogenesis in the EB placodes.

In chick and mouse, the sensory neurons of EB ganglia derive from two sources: neural crest contributes to the proximal ganglia, while placodes contribute to the distal ganglia (reviewed by Baker and Bronner-Fraser, 2001). Thus, the remaining neurons in the *cas* morphants might derive from the neural crest. Alternatively, a subpopulation of cells in the facial and vagal placodes might develop independently of the endoderm. To distinguish between these two possibilities, we investigated whether *ngn1* expression remaining after *cas*-MO injection was found in the ectoderm or underlying mesenchymal cells. Fig. 1C shows that in *cas* morphants *ngn1* was expressed in the ectoderm, suggesting that the remaining cells are derived from placode and not neural crest.

To determine if the EB placode cells undergo cell death in *cas*-MO embryos, we processed these embryos for TdT-mediated dUTP nick-end labeling (TUNEL) assay (Fig. 1D). While little cell death was observed in controls, an increase in cell death was observed in *cas* morphants at 26 hpf. Previous study indicated that endoderm is required for survival of the arch cartilage precursors (David et al., 2002). Indeed, we found a number of TUNEL-positive neural crest cells in the *cas* morphants. However, we also detected TUNEL-positive cells within ectoderm in the location of the EB placodes (Fig. 1D, inset). In addition to TUNEL, transverse sections through the head region of *cas* morphants revealed the presence of fragmented nuclei in the EB placodes (see Fig. 5I), indicating cell death. Thus, we concluded that endoderm is also required for survival of at least some EB placode cells.

Expression of Fgf signaling members in the EB placodes

The above experiments suggested that a diffused signal from the endoderm promotes EB placode neurogenesis. We set out to investigate whether the Fgf signaling pathway is activated in EB placodes. We incubated zebrafish embryos with the pharmacological Fgfr inhibitor SU5402 (Mohammadi et al., 1997) beginning at 19 hpf, well before any onset of *ngn1* expression. Inhibitor-treated embryos did not express *ngn1*, *phox2a* and *phox2b* in the glossopharyngeal and small vagal placode and ganglia, while facial and large vagal placode and ganglia were significantly reduced (Fig. 2A). Zn-5 expression revealed that SU5402-treated embryos had abnormal pouch

morphology (Fig. 2A) and segmentation. However, pharyngeal pouch segmentation is not absolutely necessary for the development of EB ganglia (Piotrowski and Nusslein-Volhard, 2000). We used the inhibitor to determine the timing requirement of the Fgf signal. We treated embryos every 2 hours from 18 hpf until 30 hpf and then assayed them for *nrd*

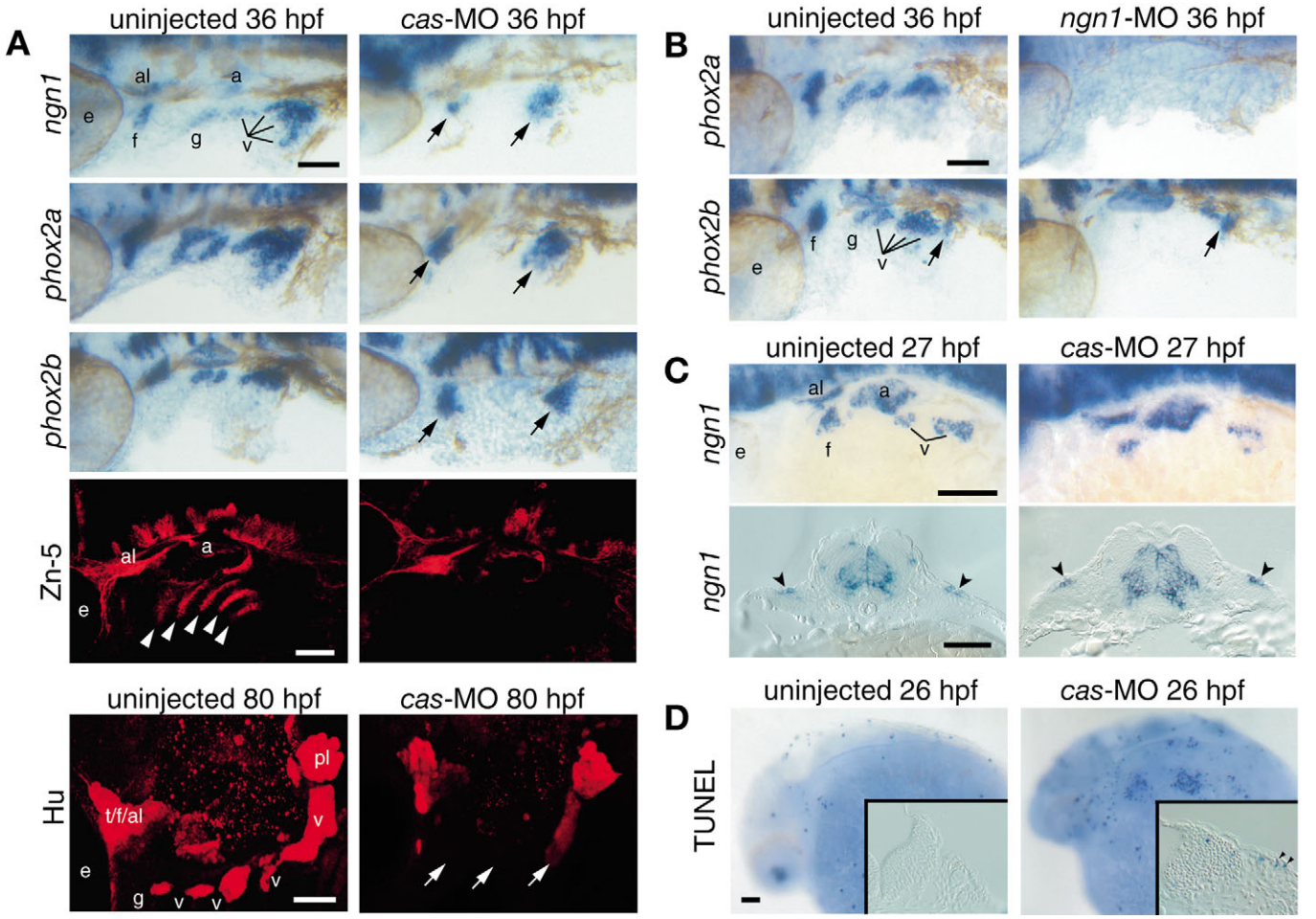

Fig. 1. Endoderm is required for the neurogenesis and survival of EB placodes. Zebrafish embryos injected with a morpholino against *cas* (A,C,D, right) or *ngn1* (B, right) and uninjected controls (A-D, left) were collected at various time points and processed for in-situ hybridization with *ngn1*, *phox2a* and *phox2b* riboprobes, immunolabeling with Zn-5 or Hu antibody, or TUNEL assay. All whole-mount panels show lateral views. Anterior is left. (A) Glossopharyngeal and small vagal placodes and ganglia are absent, while facial and large vagal placodes and ganglia are significantly reduced (arrows) after *cas* MO injection. Endodermal pouches are completely absent in *cas* morphants (Zn-5, white arrowheads). (B) *phox2a* and *phox2b* expression is absent in *ngn1* morphants. *phox2b*-positive enteric neuronal precursors (arrow) were not affected by injection of *ngn1*-MO. (C) Transverse sections through the vagal placode revealed ectodermal expression of *ngn1* (arrowheads), indicating that the remaining EB neurons in *cas* morphants derived from the placodes. (D) Endoderm is important for survival of the EB placode cells. Transverse section through the facial placode revealed TUNEL-positive cells in the *cas* morphants (arrowheads). Scale bars: 50 μm. a, acoustic ganglion; al, anterior lateral line ganglion; e, eye; f, facial placode or ganglion; g, glossopharyngeal placode or ganglion; pl, posterior lateral line placode or ganglion; t, trigeminal ganglion; v, vagal placode or ganglion.

Table 1. Neurogenesis in epibranchial placodes requires endoderm and Fgf3*

	$n^{\dagger}$	Reduced f	Absent g	Absent small v	Reduced large v
cas-MO	36	36‡ (100%)	36 (100%)	36 (100%)	36‡ (100%)
SU5402 treatment	60	60§ (100%)	60 (100%)	60 (100%)	60§ (100%)
fgf3-MO1	45	45¶ (100%)	45 (100%)	45 (45%)	60¶ (100%)
fgf3-MO2	48	25 (56%)	12 (25%)	14 (29%)	33 (69%)

*All phenotypes assessed by *phox2a/phox2b* expression at 36 hpf.
†Number of embryos in each treatment group.
‡Significantly reduced compared with to wild type, see Fig. 1A.
§Significantly reduced compared with wild type, see Fig. 2A.
¶Significantly reduced compared with wild type, see Fig. 3A.
wt, wild type; f, facial ganglion; g, glossopharyngeal ganglion; v, vagal ganglion.

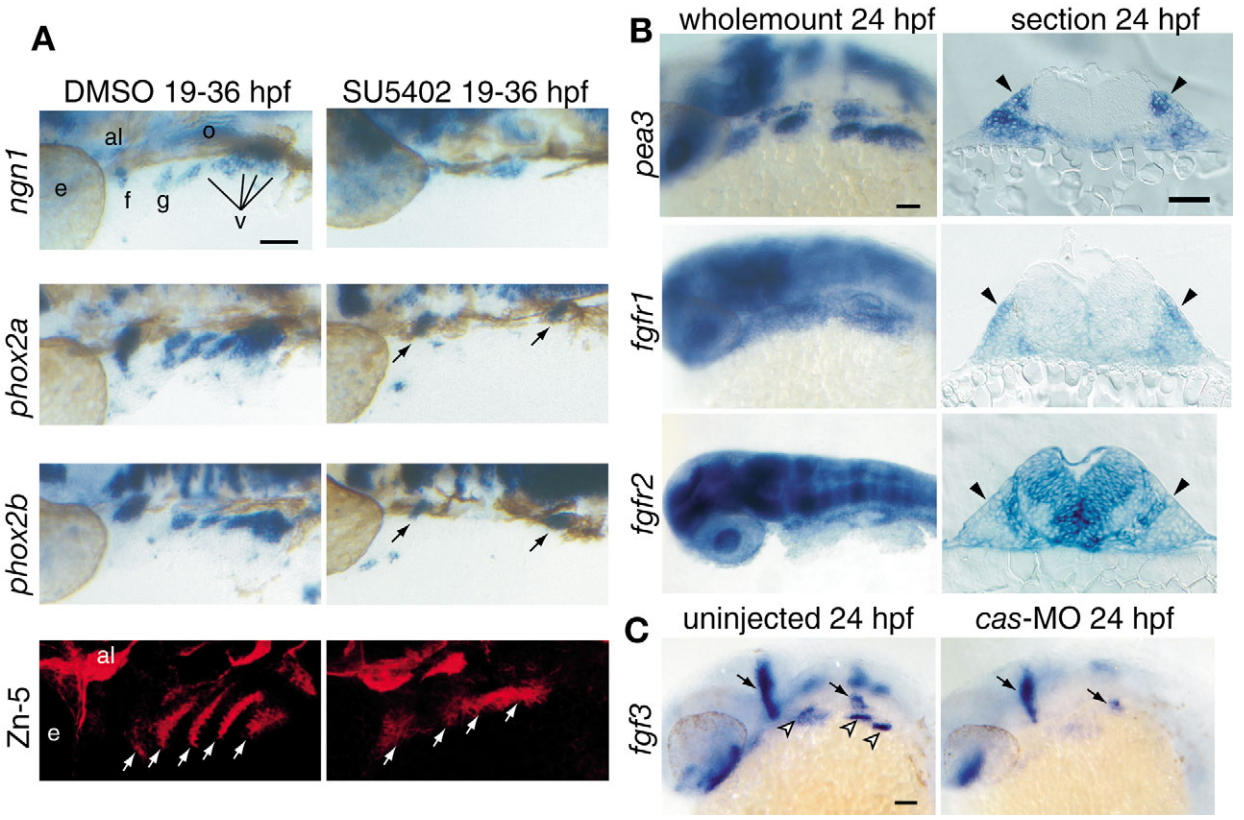

Fig. 2. Fgf signaling is active in EB placodes. (A) Zebrafish embryos treated with the Fgfr inhibitor SU5402 (right) or DMSO (left) beginning at 19 hpf were collected at 36 hpf and processed for in-situ hybridization with *ngn1*, *phox2a* and *phox2b*. All panels show lateral views. Anterior is at right. In the SU5402-treated embryos, *ngn1*, *phox2a* and *phox2b* expression is either reduced (arrows) or absent. Fgfr inhibitor treatment also affected pharyngeal pouch morphology and size, as assessed by Zn-5 immunolabeling (white arrows). (B) Expression analyses of *pea3*, *fgfr1* and *fgfr2* at 24 hpf. Whole-mount panels are lateral views of the embryos; transverse sections are at the level of the facial placode. Note characteristic ectodermal thickenings, indicating cranial placodes (black arrowheads). *pea3* and *fgfr1* were expressed in the EB placodes, while *fgfr2* was not. (C) *fgf3* was expressed in the pharyngeal endoderm (left; empty arrowheads) at 24 hpf. As expected, the endodermal *fgf3* expression was lost in *cas* morphants (right). Scale bars: 50 μm. Abbreviations are as in Fig. 1.

expression at 36 hpf (data not shown). The EB ganglia were not affected in the embryos treated after 26 hpf. By contrast, these ganglia were significantly reduced or absent in the embryos treated before 24 hpf (data not shown). This indicated that EB placode neurogenesis is regulated by an Fgf signal before or around 24 hpf.

We next examined whether components of the Fgf signaling pathway were present in EB placodes. We examined the expression of *erm* and *pea3*, transcriptional targets of Fgf signaling. While we did not detect any *erm* expression in the head ectoderm (data not shown), *pea3* was expressed in distinct domains at 24 hpf (Fig. 2B). Transverse sections through the head region confirmed robust *pea3* expression in the EB placodes and apparently in delaminating neuroblasts (Fig. 2B). We investigated expression of Fgf receptors at 18, 20, 22 and 24 hpf. *fgfr2*, *-3* or *-4* expression was not detected in the head ectoderm (Fig. 2B and data not shown). *fgfr1* displayed a broad expression pattern in the head at 24 hpf. Transverse section revealed *fgfr1* transcript in the EB placodes at 24 hpf (Fig. 2B). Finally, we assayed various Fgf members for expression in the endodermal pouches (Fig. 2C and data not shown). *fgf3* was expressed in the pharyngeal pouches during EB placode neurogenesis, around 24 hpf (Fig. 2C) (David et al., 2002). Endodermal *fgf3* expression persisted until at least 48 hpf (David et al., 2002) (data not shown). As expected, endodermal *fgf3* expression was lost in *cas* morphants (Fig. 2C). We conclude that the expression pattern of these Fgf signaling components is consistent with active Fgf signaling in the EB placodes.

Fgf3 is required for neurogenesis in the EB placodes

To determine if Fgf3 is required for EB placode neurogenesis, we injected morpholinos directed against *fgf3* mRNA. As controls, we assayed uninjected embryos as well as embryos injected with a combination of morpholinos directed against *fgf8* (Fig. 3 and data not shown). We used two non-overlapping *fgf3* morpholinos, termed here *fgf3*-MO1 and *fgf3*-MO2, which have been extensively characterized by others (David et al., 2002; Leger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001; Raible and Brand, 2001; Walshe and Mason, 2003a; Walshe and Mason, 2003b). In our hands, *fgf3*-MO1 displayed a more penetrant phenotype than *fgf3*-MO2, including significant reduction in *pax2.1* expression in the isthmus and otic placode at 18 somites as well as significant reduction of the otic vesicle, as previously described (data not shown).

Therefore we used *fgf3*-MO1 in the majority of our assays. Strikingly, *fgf3* morphants lacked *ngn1*, *phox2a* and *phox2b* expression in glossopharyngeal and small vagal placodes and ganglia, and had reduced expression in the facial and large vagal placodes and ganglia (Fig. 3A, Table 1), similar to the phenotype observed in *cas* morphants. When assayed at 80 hpf with Hu antibody, sensory neurons of the glossopharyngeal and small vagal ganglia were absent, while the large vagal ganglion was reduced (Fig. 3A). Importantly, *fgf3*-MO1 did not interfere with the pharyngeal pouch development, as indicated by normal Zn-5 expression (Fig. 3B). This is consistent with recent studies demonstrating that *fgf3* morpholino injections did not interfere with the pharyngeal pouch development (Crump, 2004; David et al., 2002). However, embryos that carry a null allele in the *fgf3* gene have defects in the pharyngeal pouch segmentation (Herzog et al., 2004). This indicates that *fgf3* morphants used in this study are hypomorphic with respect to the pharyngeal endoderm segmentation.

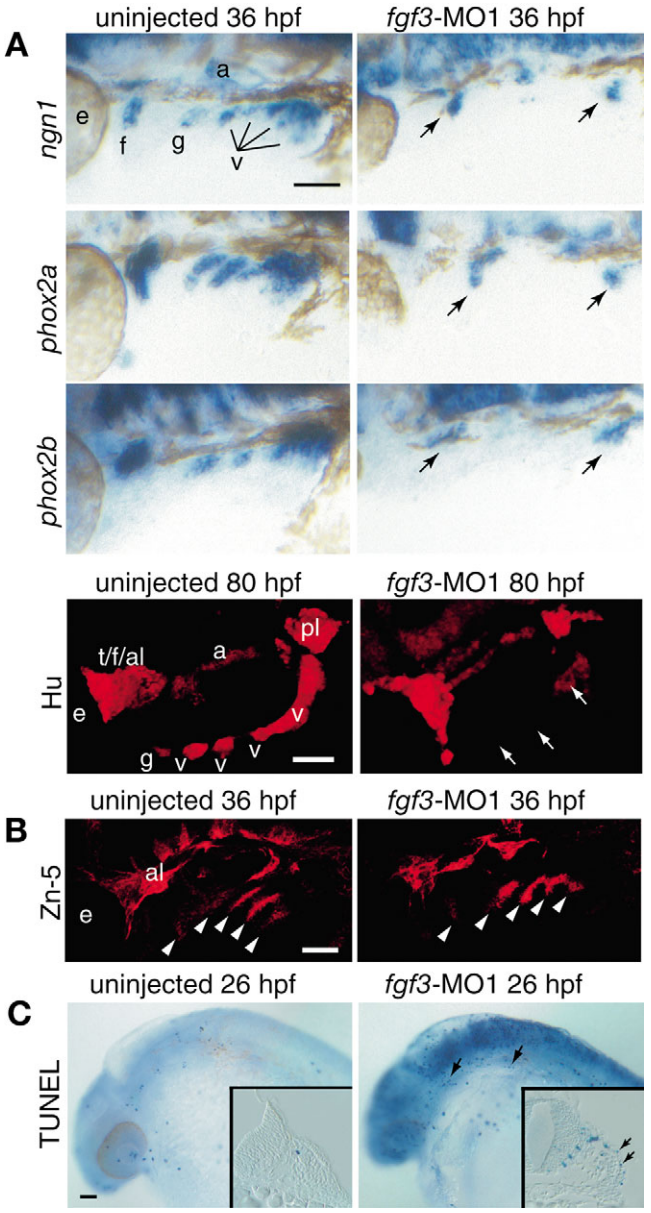

Fig. 3. Fgf3 is necessary for inducing neurogenesis in EB placodes. Embryos injected with morpholino directed against *fgf3* (A-C, right) and uninjected controls (A-C, left) were processed for in-situ hybridization with *ngn1*, *phox2a* and *phox2b* at 36 hpf, immunolabeling with Hu antibody at 80 hpf and Zn-5 antibody at 36 hpf, and TUNEL assay at 26 hpf. All whole-mount sections show lateral views. Anterior is left. (A) *ngn1*, *phox2a*, *phox2b* and Hu expression were absent in the glossopharyngeal and small vagal placodes and ganglia and significantly reduced in the facial and large vagal placodes and ganglia (arrows). (B) Zn-5 staining demonstrated that pharyngeal pouches were not affected by *fgf3*-MO1 injection (arrowheads). (C) TUNEL assay revealed dying cells in the ectoderm of the *fgf3*-morphants (arrows). Insets show transverse sections through the facial placode. Scale bars: 50 μm. Abbreviations are as in Fig. 1.

To determine whether Fgf3 is required for survival of EB placode cells, we analyzed *fgf3* morphants by TUNEL. We found an increase in TUNEL-positive cells in the head ectoderm (Fig. 3C), specifically in the ectodermal thickenings. Fig. 3C shows TUNEL-positive cells in the facial placode at 26 hpf. We also detected nuclear fragmentation on the histological sections taken through the EB placodes (Fig. 5K). Altogether, our results show that Fgf3 activity in the endoderm is required for the survival and neurogenesis of EB placode cells.

We investigated whether other Fgfs are involved in EB placode neurogenesis. *fgf8* is expressed in the endodermal pouches of chick, mouse and zebrafish (Crossley and Martin, 1995; Hidalgo-Sanchez et al., 2000; Mahmood et al., 1995a; Mahmood et al., 1995b; Reim and Brand, 2002; Veitch et al., 1999; Wall and Hogan, 1995). To determine whether Fgf8 is required for zebrafish EB placode development, we analyzed the mutant *acerebellar* (*ace*), which harbors a mutation in the *fgf8* gene (Reifers et al., 1998). Because the existing ace^{ti282a} allele is hypomorphic, we also analyzed *fgf8*-MO-induced phenotypes. Expression analyses of *ngn1*, *phox2a* and *phox2b* demonstrated that all EB placodes and ganglia were present in *ace* mutants and *fgf8* morphants (see Fig. S1 in the supplementary material; data not shown). Moreover, all EB ganglia appeared grossly normal when assayed at 80 hpf by Hu antibody (see Fig. S1 in the supplementary material). Occasionally, we noticed a shift or a fusion in the *phox2a* or *phox2b* expression domains marking glossopharyngeal and small vagal ganglia, which develop in close proximity to the otic vesicle (see Fig. S1 in the supplementary material). As Fgf8 is required for induction of the otic vesicle as well as segmentation of the branchial arches (Crump, 2004; Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001), we suspect these differences in EB neuron distribution may be indirect. Similar analyses revealed that neither *fgf4* nor *fgf24*, which are also expressed in the pharyngeal endoderm at 24 hpf (David et al., 2002; Draper et al., 2003), were required for EB placode neurogenesis (data not shown).

Chondrogenic neural crest is not required for early neurogenesis in the EB placodes

A previous study demonstrated a requirement for Fgf3 in the development of the posterior arch cartilages (David et al., 2002). Consequently, abnormal EB placode neurogenesis found after blocking Fgf3 function could be indirect. Thus, we

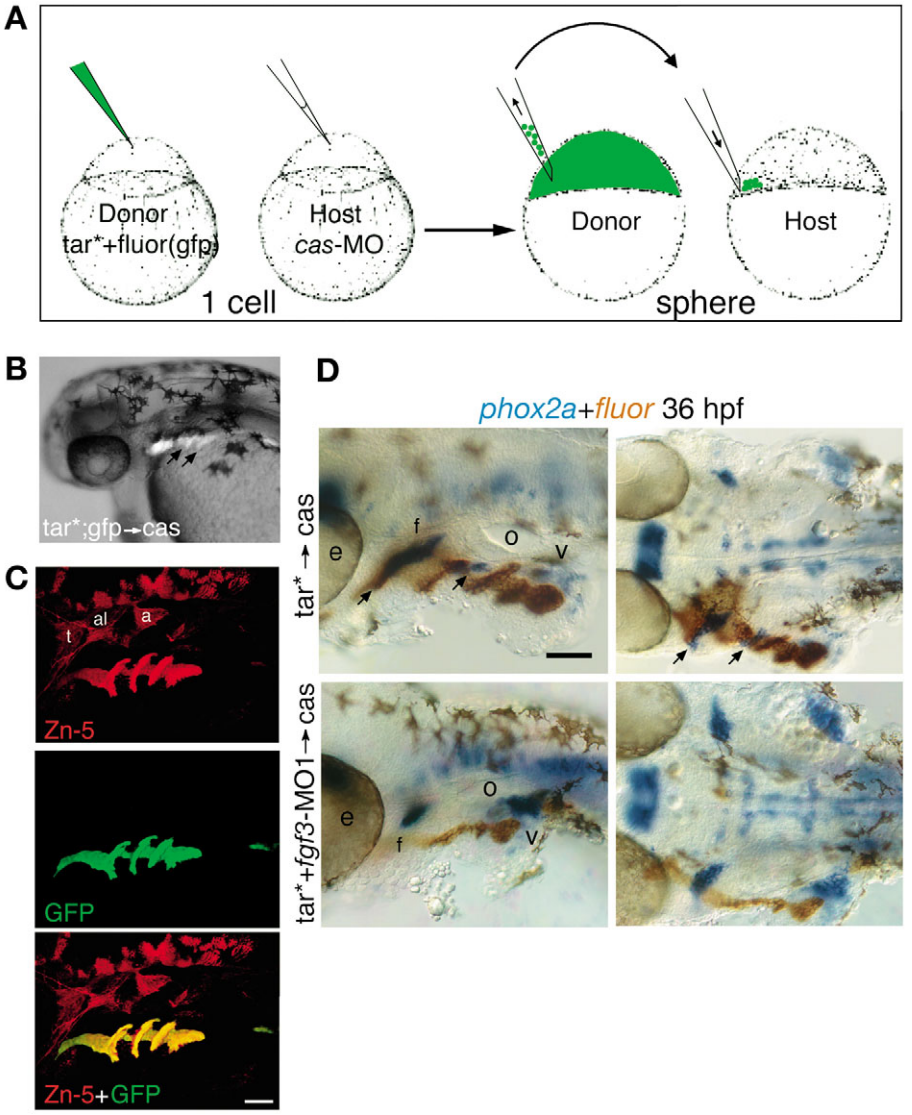

Fig. 4. Pharyngeal pouch restoration is sufficient for rescuing *phox2a*-positive EB neurons. (A) Donor embryos were injected with a *tar** mRNA and a lineage tracer (fluorescein or *gfp*), while host embryos were injected with *cas* morpholino. At sphere stage, 10-20 *tar**-positive cells were transplanted to the margin of *cas* hosts and mosaic embryos were collected at 36 hpf. (B) Side view of the live *cas*-MO embryos that received *tar** transplant. Arrows indicate endodermal pouches. (C) Lateral views of a *cas* host embryo that received *tar**+*gfp* transplant. Zn-5 (red, top) and GFP (green, middle) were detected by immunolabeling. Note that *tar**-positive cells efficiently rescue endodermal pouches (yellow overlay, bottom). (D) Side (left) and ventral (right) views of *cas* hosts that received *tar**+fluorescein transplant with (bottom) or without (top) *fgf3*-MO1. Resulting embryos were processed to detect *phox2a* expression (blue) and fluorescein (brown) at 36 hpf. Note rescue of the glossopharyngeal and facial ganglia (arrows) in the embryos that did not receive *fgf3*-MO1 (top). By contrast, *fgf3*-MO1 *tar**-transplanted cells failed to rescue EB ganglia (bottom). Scale bars: 50 μm. Abbreviations are as in Fig. 1.

sought to disrupt the migration of the posterior chondrogenic precursors by means other than reduction in Fgf3 activity. The winged helix transcription factor Foxd3 is required for the development of many NC derivatives, including posterior arch cartilages (J. A. Lister and D.W.R., unpublished). Zebrafish embryos injected with a specific morpholino directed against *foxd3* displayed defects in the posteriormost *dlx2*-positive NC stream (see Fig. S2 in the supplementary material), a phenotype nearly identical to that observed in *fgf3* morphants (David et al., 2002; Walshe and Mason, 2003a). By contrast, analyses of *ngn1* expression did not reveal any defects in early EB placode neurogenesis in *foxd3* morphants (see Fig. S2 in the supplementary material). However, at 80 hpf, Hu antibody staining revealed that various cranial ganglia, including the trigeminal, lateral line and EB ganglia, were reduced in size, and many Hu-positive neurons failed to coalesce into distinct ganglia (see Fig. S2 in the supplementary material). This observation is consistent with the previously proposed idea that *foxd3*-positive glia are required for normal organization of the cranial ganglia (Begbie and Graham, 2001). We concluded that the chondrogenic neural crest is not required for early neurogenesis in EB placodes.

Fgf3 activity in the pharyngeal pouches is required for EB placode neurogenesis

To determine whether Fgf3 activity is specifically required in the endoderm, we performed mosaic analysis in zebrafish. Activation of the Nodal/activin pathway by Tar*, an activated version of the type I TGF-related receptor Taram-A (Tar), autonomously specifies wild-type cells as endoderm (David and Rosa, 2001; Peyrieras et al., 1998). When transplanted into wild-type or *cas* morphants, *tar**-positive cells differentiate into various endodermal derivatives including pharyngeal pouches. We injected zebrafish embryos at the one-cell stage with *tar** mRNA and a lineage tracer (*gfp* or fluorescein) and transplanted 10-20 *tar**-positive cells into the margin of the *cas*-MO hosts at blastula stage (Fig. 4A). Grafting wild-type cells into *cas* morphants did not rescue pharyngeal pouches and did not restore EB ganglia (0 out of 15; Table 2). By contrast, *tar**-positive grafts rescued one or more pharyngeal pouches (19 out of 23; Fig. 4B,C and data not shown). When assayed for *phox2a* expression, *cas* morphants that received *tar** transplants showed restoration of the facial, glossopharyngeal and small vagal ganglia (Table 2; Fig. 4D). Grafted cells were always adjacent to the rescued *phox2a*-positive neurons, indicating that introduction of endoderm into *cas* morphants rescued the EB ganglia in a non-autonomous fashion, most probably by a signal derived from the pharyngeal pouches. To determine whether Fgf3 expression is specifically required in endoderm for EB placode neurogenesis, we transplanted *tar**-positive cells that also contained *fgf3*-MO1 into *cas* morphants.

Table 2. *tar-mediated restoration of EB ganglia requires endoderm-derived Fgf3***

	$n^{\dagger}$	f	g	Small v	Large v
Transplant of GFP-positive wt cells into *cas*-MO	15	0‡	0	0	n/d
Transplant of *tar** cells into *cas*-MO	45	21‡ (47%)	7 (16%)	10 (22%)	n/d
Transplant of *tar**+*fgf3*-MO1 cells into *cas*-MO§	30	2‡ (7%)	2 (7%)	2 (7%)	n/d

*All phenotypes assessed by *phox2a* expression at 36 hpf.
†Number of embryos in each treatment group.
‡Significantly larger than contralateral control, see Fig. 4D.
§$P \ll 0.01$ (χ^2 test) when compared to *tar** transplants without *fgf3*-MO1.
wt, wild type; f, facial ganglion; g, glossopharyngeal ganglion; v, vagal ganglion.

The resulting embryos were grown until 36 hpf and analyzed for *phox2a* expression in EB ganglia. Very few embryos displayed rescue of EB ganglia, often limited to only one or two *phox2a*-positive cells (Fig. 4D, Table 2). Overall, these results demonstrate that Fgf3 activity in the pharyngeal pouches is required for EB placode neurogenesis.

Neither endoderm nor Fgf3 are required for EB placode formation

Next we asked whether endoderm and Fgf3 are required for the formation of the EB placode precursors and/or placode induction. To visualize the EB placode precursors, we processed *cas* and *fgf3* morphants as well as SU5402-treated embryos for in-situ hybridization with *foxi1* (Fig. 5). Among other structures, *foxi1* is expressed in EB placode precursors and its activity is required for *ngn1* expression (Lee et al., 2003). Because there are no molecular markers available in zebrafish that mark the induced EB placodes before neurogenesis, we used modified Richardson's stain to visualize placodes in *cas* and *fgf3* morphants, and SU5402-treated embryos. Ectodermal *foxi1* expression, although somewhat altered, was present in both *cas* and *fgf3* morphants (Fig. 5B,C,F,G,J,K). *cas* morphants consistently displayed a gap in the *foxi1* expression domain that spanned the third and fourth arches (Fig. 5B). Transverse sections confirmed the presence of EB placodes in all arches (Fig. 5F,G and data not shown). The pattern of *foxi1* expression in the *fgf3* morphants was indistinguishable from controls. However, the levels of *foxi1*

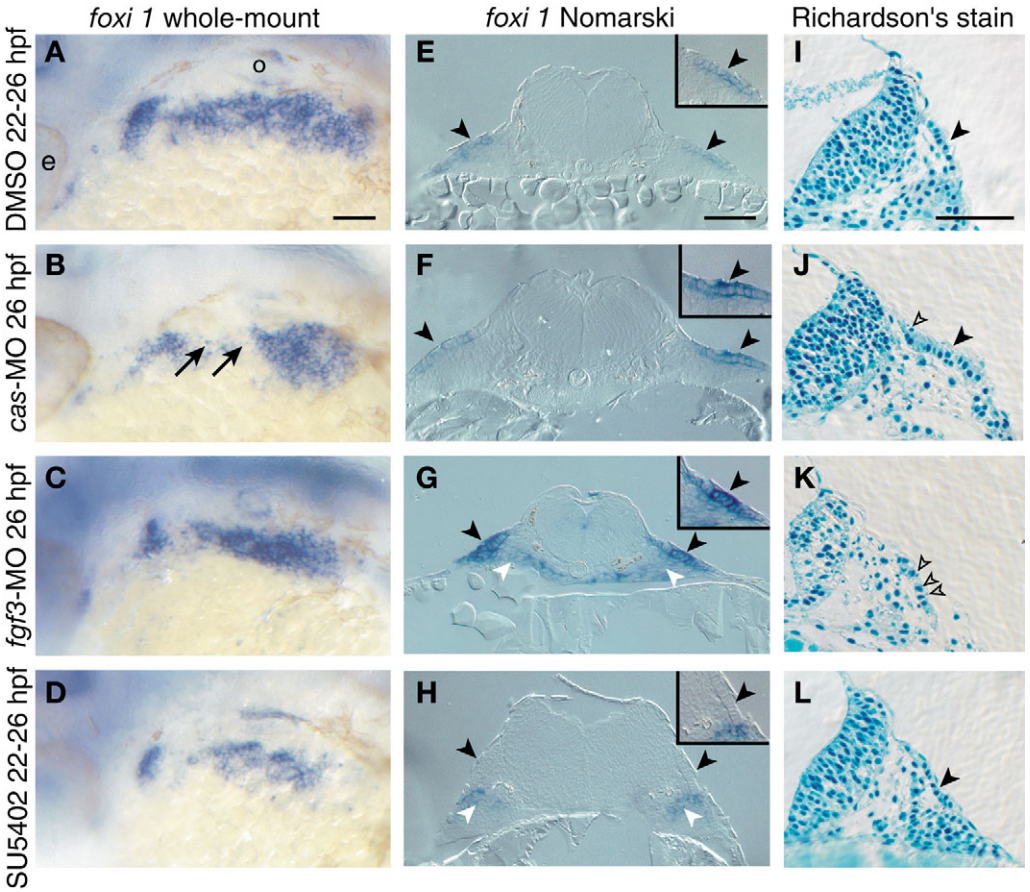

Fig. 5. Neither endoderm nor Fgf3 is required for EB placode induction. Lateral views (A-D) and transverse sections (E-L) of embryos injected with *cas* (B,F,J) or *fgf3* morpholino (C,G,K) or treated with SU5402 (D,H,L) or DMSO (A,E,I) beginning at 22 hpf are shown. Embryos were collected at 26 hpf and processed for in-situ hybridization with *foxi1* riboprobe (A-H) or modified Richardson's stain (I-L). Panels E-G show sections through the vagal placode, while panels H-L show sections through the facial placode. EB placodes are marked by black arrowheads, while pharyngeal endoderm is marked by white arrowheads. Insets in E-H show magnified views of the placodes. Note ectodermal *foxi1* expression and presence of the ectodermal thickenings in *cas* and *fgf3* morphants, indicating presence of the EB placodes. *foxi1* was not expressed in the ectoderm of the third and fourth arches (black arrows) in *cas* morphants. We consistently observed increased levels of the *foxi1* transcript in *fgf3* morphants (C,G). In addition, we observed nuclear fragmentation in EB placodes in *cas* and *fgf3* morphants (empty arrowheads in J,K), indicating dying cells. While endodermal *foxi1* expression was not affected, ectodermal expression was lost in the SU5402-treated embryos (D,H). Scale bars: 50 μm. e, eye; o, otic vesicle.

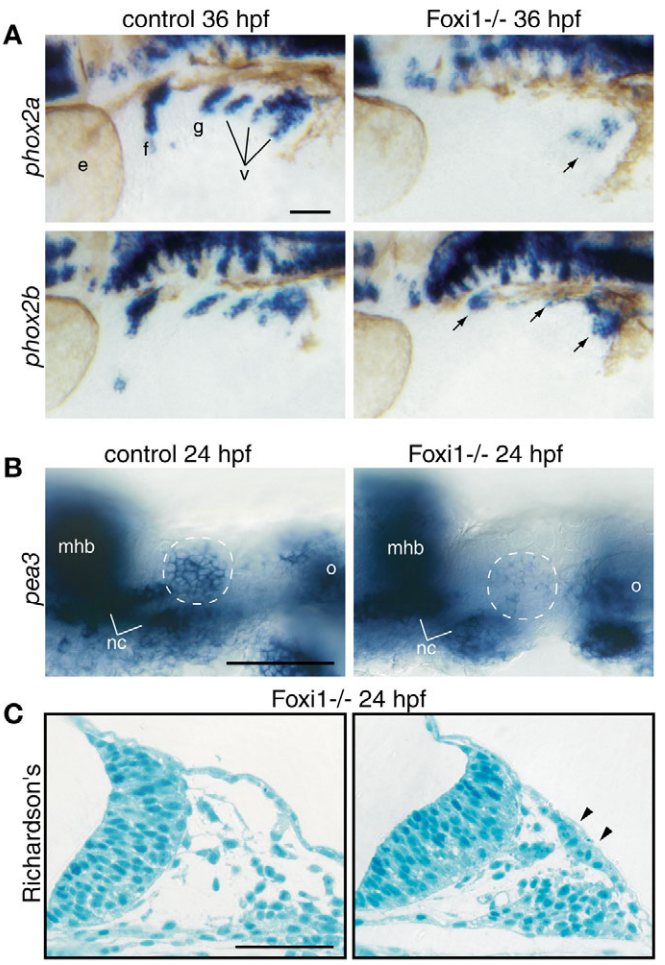

Fig. 6. Foxi1 mediates Fgf response in the EB placodes. *foxi1* mutant embryos and their wild-type sibs were processed for in-situ hybridization with *phox2a* and *phox2b* (A), *pea3* (B) and modified Richardson's stain (C). All whole-mount panels show lateral views. Anterior is left. (A) When compared with their wild-type sibs (left), *phox2a* expression is absent in the facial, glossopharyngeal and small vagal ganglia, while it is significantly reduced in the large vagal ganglion (arrow) in the *foxi1* mutants (right). By contrast, *phox2b* is still expressed in all EB ganglia, albeit at lower levels (arrows). (B) Optical sections through the facial placode (presumptive facial placode is outlined by a dashed circle). Note significant reduction of *pea3* expression in the facial placode of the *foxi1* mutant embryo (right), while other *pea3* expression domains are unaffected. (C) Histological analysis of the transverse sections through the head region revealed the presence of columnar epithelium (marked by arrowheads; compare with the ectoderm immediately proximal to the facial placode, left panel), indicating presence of placodes in the *foxi1* mutants. Scale bars: 50 μm. mhb, midhindbrain boundary; nc, chondrogenic neural crest of the first and second arches; o, otic vesicle. Other abbreviations are as in Fig. 1.

transcript were consistently higher compared with controls (Fig. 5C,G). Transverse sections confirmed that *foxi1* was expressed in the ectodermal thickenings, indicating the presence of EB placodes (Fig. 5J,K). Interestingly, SU5402-treated embryos lost ectodermal expression, while maintaining *foxi1* expression in endodermal pouches (Fig. 5D,H). This observation suggests that Fgf signaling from source(s) other

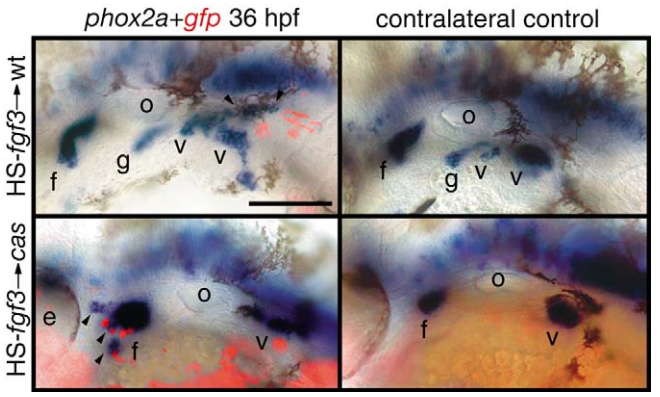

Fig. 7. Fgf3 is sufficient to induce *phox2a*-positive epibranchial neurons. Zebrafish embryos were co-injected with HS-*fgf3* and HS-*gfp* plasmids alone (top) or *cas*-MO (bottom) at the one-cell stage, heat shocked for 1 hour at 37°C beginning at 22 hpf and then assayed for *phox2a* expression (blue) and *gfp* expression (red) at 36 hpf. The majority of ectopic, *phox2a*-positive neurons (arrowheads) were immediately adjacent to the *gfp*-positive cells. Note significantly increased facial ganglion in *cas* morphant (bottom left) when compared with the contralateral control side. Anterior is at left. Scale bar: 50 μm. Abbreviations are as in Fig. 1.

than endoderm may be required to maintain ectodermal *foxi1* expression. Despite the absence of ectodermal *foxi1* expression in inhibitor-treated embryos, transverse sections revealed the presence of ectodermal thickenings, indicating the presence of placodes (Fig. 5L). Overall, these results support the argument that endoderm and Fgf3 are not required for the initial formation of the EB placodes.

Foxi1 is required for activation of Fgf signaling in the EB placodes

To determine the relationship between Foxi1 and Fgf signaling in the EB placodes, we assayed *phox2a*, *phox2b* and *pea3* expression in the embryos with a mutation in the *foxi1* gene (Solomon et al., 2003). Consistent with a previous study, *phox2a* expression was absent in all but the large vagal ganglion, a phenotype more severe than the one observed in *fgf3* morphants (Fig. 6A) (Lee et al., 2003). However, *phox2b* expression, while consistently reduced, was variable and occasionally present in all EB ganglia (Fig. 6A). Importantly, *pea3* expression, a downstream target of Fgf signaling, was reduced in the EB placodes at 24 hpf (Fig. 6B). Finally, histological sections revealed the presence of EB placodes in the *foxi1* mutant embryos, indicating that Foxi1 is not required for the initial placode induction (Fig. 6C). Overall, these results suggest that Foxi1 functions upstream of Fgf3 and is necessary for the EB placodal cells to respond to the Fgf signals.

Fgf3 is sufficient for inducing *phox2a*-positive neurons in wild-type embryos and *cas* morphants

To determine if Fgf3 is sufficient for inducing EB sensory neurons, we used a heat-shock (HS) promoter to drive *fgf3* expression in wild-type embryos and *cas* morphants (Halloran et al., 2000; Maves et al., 2002). Embryos were co-injected with HS-*fgf3* and HS-*gfp* plasmids at the one-cell stage, heat shocked for 1 hour beginning at 22 hpf, and then assayed for *phox2a* and *gfp* expression at 36 hpf (Fig. 7, Table 3). As

Table 3. Fgf3 is sufficient to induce *phox2a*-positive, epibranchial neurons in wild-type and *cas* embryos

	*n**	Embryos with ectopic *phox2a*	Ectopic *phox2a* foci†	Colocalized with GFP	II‡	III and IV	Posterior arches
HS-*gfp* → wt	61	1	1	0	1	0	0
HS-*gfp* → *cas*	74	1	1	0	1	0	0
HS-*fgf3* → wt§	72	15	28	20 (71%)	16	3	9
HS-*fgf3* → *cas*§	78	21	28	20 (71%)	21	0	7

*Number of animals assessed in each treatment group.
†A number of embryos had more than one ectopic *phox2a*-positive foci.
‡II-IV – bronchial arches.
§$P \ll 0.01$ (χ^2 test) when compared with HS-gfp injections.

controls, wild-type embryos and *cas* morphants were injected with the same amount of HS-*gfp* plasmid alone and subjected to the same treatment. HS-*fgf3* plasmid was injected at 2.5 ng/μl, because at higher concentrations (beyond 5 ng/μl), a majority of embryos were dorsalized, probably due to a leaky expression from the HS promoter. Any dorsalized embryos were excluded from the analyses. Strikingly, a significant number of wild-type embryos (21%) displayed ectopic *phox2a*-positive foci (Fig. 7, Table 3). By contrast, we found only one (2%) *phox2a*-positive site that we classified as ectopic in HS-*gfp*-injected controls. Ectopic neurons were induced in the vicinity of all EB ganglia, including facial, glossopharyngeal and vagal ganglia (Table 3). Similarly, 27% of the *cas* morphants displayed either ectopic *phox2a*-positive foci or rescue of the facial or large vagal ganglia (compared with only 2% in controls; Fig. 7, Table 3). However, by contrast to the wild-type embryos, we did not observe any *phox2a*-positive neurons in the third or fourth arch, which would indicate rescue of the glossopharyngeal and small vagal ganglia (Table 3). In both wild-type and *cas*-MO injected embryos, the majority of ectopic *phox2a*-positive neurons (71%) were adjacent to the *gfp*-positive cells. These experiments indicate that *fgf3* expression is sufficient to induce *phox2a*-positive EB neurons.

Discussion

A model of EB placode induction and neurogenesis in zebrafish

Observations described here and by others (Andermann et al., 2002; Guo et al., 1999; Lee et al., 2003) suggest a sequential step model for zebrafish EB placode development (Fig. 8). Placodes are first induced in ectoderm lateral to the neural plate/epidermis border from *foxi1*-positive precursors, as detected by morphological thickening (Fig. 8A). Signals must come from a tissue(s) other than endoderm, because we observed ectodermal thickening and expression of *foxi1* in *cas* morpholino-injected embryos. It is not clear whether an Fgf signal is required for initial placode formation, but it may play a role in its maintenance, as ectodermal *foxi1* expression was lost but placodal structure remained intact after treatment with the Fgf inhibitor. Our preliminary analysis suggests that *foxi1* expression in EB precursors might be regulated differently from the otic placode, as ectodermal *foxi1* expression is abolished at mid-somitogenesis stages following SU5402 treatment (A.N., unpublished), whereas *foxi1* expression in otic placode precursors is not dependent on Fgf signaling (Solomon et al., 2004). Overall, it will be important to confirm our observations with additional markers of early EB placode induction when they become available. Once the EB placodes are established, we suggest that neurogenesis is stimulated by endoderm-derived Fgf3 (Fig. 8B). However, a subpopulation of neurogenic cells remains in the facial and large vagal EB placodes after elimination of endoderm or Fgf3. These cells go on to delaminate and differentiate into neurons in the absence of these signals, suggesting that a second inductive pathway regulates their development (Fig. 8C). A recent study determined that Fgfr1 morphant zebrafish mimicked many aspects of *fgf8* mutants (Scholpp et al., 2004), while studies in species other than zebrafish indicate that Fgf3 could bind to the IIIb isoform of both Fgfr1 and Fgfr2 with high affinity (Mathieu et al., 1995a; Mathieu et al., 1995b; Ornitz et al., 1996). In addition, our search of the zebrafish genome sequencing database (http://www.sanger.ac.uk/Projects/D_rerio/) revealed at least three other Fgf receptor orthologs. Thus, further studies are necessary to determine the bone fide Fgf3 receptor in zebrafish.

The onset of zebrafish EB neurogenesis is highlighted by expression of *ngn1*, which is expressed in all EB placodes and transiently in delaminating neuroblasts (Fig. 8B,C). *ngn1* expression is required for the subsequent expression of three other transcription factors, *nrd*, *phox2a* and *phox2b*. The timing and position of *nrd* expression suggests that it is upstream of *phox2a* and *phox2b* (Andermann et al., 2002) (data not shown), although this idea has not been tested directly. In zebrafish, both Fgf3-dependent and Fgf3-independent neurogenic precursors give rise to *phox2a*- and *phox2b*-expressing cells, although it is unclear whether all EB neurons express these markers. It will be important to investigate whether the Fgf3-dependent and Fgf3-independent subpopulations represent particular functional subtypes within the facial and large vagal ganglia. Development of the molecular markers that recognize different subtypes of the EB sensory neurons in zebrafish will help to address this question.

Interestingly, while *fgf3* expression in wild-type embryos was sufficient to induce *phox2a*-positive neurons in all arches, in *cas* morphants it was not sufficient to rescue any glossopharyngeal or small vagal neurons, located in the third and fourth arches, respectively. This result may indicate that another endoderm-derived factor, in addition to Fgf3, is required for neurogenesis of the glossopharyngeal and small vagal placodes. Another explanation is that *foxi1* expression, which is required to respond to Fgf signals, was lost in the third and fourth arches in *cas* morphants (Fig. 5B).

In addition to promoting neurogenesis, endoderm and endoderm-derived Fgf3 may play a role in promoting survival

of the EB placodal cells. Interestingly, placodal cells in *foxi1* mutants undergo cell death between 28 and 36 hpf, right after they fail to undergo neurogenesis. We observed a similar effect in *cas* and *fgf3* morphants. On one hand, these observations may indicate that the above factors are responsible for suppressing the apoptotic pathway and/or promoting survival of the EB neurons. On the other hand, programmed cell death may be induced in the EB placodes when they fail to undergo their normal developmental program. We favor the latter explanation, because *fgf3* expression is sufficient for inducing *phox2a* expression, supporting the argument that Fgf3 is directly involved in EB placode neurogenesis.

Previous studies in avian embryos have implicated pharyngeal endoderm in the induction of EB placodes (Begbie et al., 1999). However, this study used markers that were expressed in differentiating neurons, rather than placodal precursors. Thus, it was not clear whether endoderm was required for EB placode induction or subsequent neurogenesis. Interestingly, in these studies cranial ectodermal explants generated some *phox2a*-positive neurons even in the absence of pharyngeal endoderm, while trunk ectoderm never produced neurons with or without inducing tissue. By contrast, trunk ectoderm is capable of EB neuron production after transplant in vivo (Vogel and Davies, 1993). These results are consistent with our model for two inductive signals: an endoderm-independent signal involved in placode formation, and an endoderm-dependent signal for neurogenesis.

It has been suggested that endodermally derived BMP7 plays a role in the generation of EB neurons in avian embryos (Begbie et al., 1999; Begbie and Graham, 2001). In zebrafish, *bmp7* is expressed in endoderm at 15 somites (16.5 hpf) and thus may play a role in the EB placode development even before Fgf3-dependent neurogenesis (Dick et al., 2000). Alternatively, Bmp7 signaling may represent a parallel pathway required during EB placode neurogenesis. Interestingly, expression of *phox2a* in the locus coeruleus requires both Bmp and Fgf signals (Guo et al., 1999). It will be important to determine the exact timing and pattern of *bmp7* expression and its role during EB placode induction in zebrafish.

Role of Foxi1 in EB placode development

A number of previous studies demonstrated a Foxi1 requirement during otic placode, jaw and EB placode development (Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003). During ear development, *foxi1* is expressed in the otic placode precursors and is required for these cells to respond to Fgf-inducing signals, suggesting that Foxi1 might play the role of competence factor (Solomon et al., 2004). Our experiments demonstrated that in the absence of Foxi1 activity EB placodes were still induced, while neurogenesis was impaired. Moreover, a downstream effector of Fgf signaling, *pea3*, was not activated in the EB placodes, strengthening the notion that upregulation of Foxi1 is required in EB precursors to respond to Fgf signals, a role similar to the one postulated for otic placode induction. Importantly, Lee et al. demonstrated that *foxi1* mutants undergo massive cell death in the cranial placodes between 28 and 36 hpf, shortly after neurogenesis is induced (Lee et al., 2003). This is consistent with the idea that Foxi1 may render ectodermal cells competent to undergo neurogenesis. Finally, *phox2a* and *phox2b* expression analysis in *foxi1* mutants revealed a phenotype distinct from the one observed in *cas* and *fgf3* morphants. While *foxi1* mutants displayed a more severe loss of *phox2a* expression, *phox2b* expression was only mildly affected, often persisting even in smaller glossopharyngeal and vagal ganglia. These observations indicate that *phox2a* and *phox2b* might be expressed in overlapping, but not identical, populations of the EB neurons, and that these neuronal populations might be differentially regulated by Fgf signals.

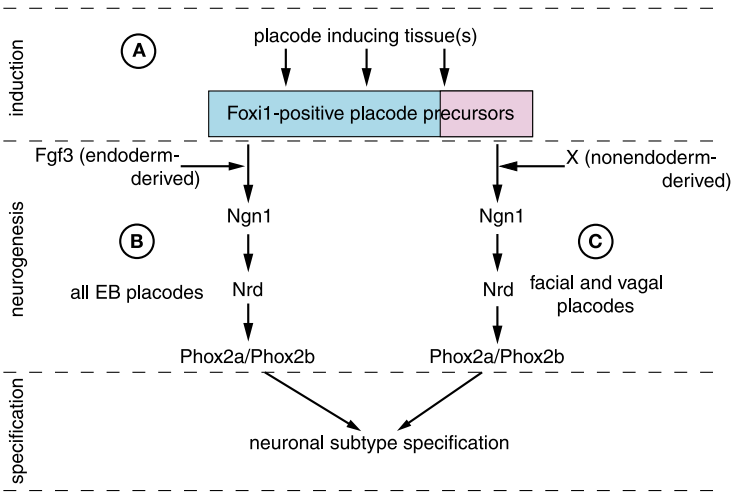

Fig. 8. A model of EB placode induction and neurogenesis in zebrafish. See text for details.

Roles of Fgf signaling during otic and EB placode development

While the roles of Fgfs have not been previously studied in EB placode differentiation, studies in zebrafish, chick and mouse have revealed that the Fgf signaling pathway plays multiple roles during early inductive events in the otic placode. Loss-of-function experiments in zebrafish demonstrated that both Fgf3 and Fgf8 are absolutely required for otic placode induction (Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002). The role of Fgf3 appears to be conserved among fish, chick and mouse. Whereas the hindbrain is the source of the Fgf3 signal in otic placode formation in zebrafish, chick and mouse, it is less clear whether the origin of the Fgf8 signal is conserved. A recent study demonstrated that an endoderm-derived Fgf8 acts upstream of other Fgf signals in mesoderm to trigger otic placode development in chick (Ladher et al., 2005). However, the source of Fgf8 signal in zebrafish and mouse is unknown. A recent study in mouse supports a model in which Fgf3 and mesendoderm-derived Fgf10 have partially redundant functions during otic placode induction (Alvarez et al., 2003; Wright and Mansour, 2003). In chick, Fgf19 is expressed in mesendoderm and acts in concert with Fgf3 to induce otic tissue (Ladher et al., 2000; Ladher et al., 2005). Recently, Fgf19 expression has been described in epibranchial ganglia in avian embryos, but its function has not been tested (Kurose et al., 2004).

In addition to roles in early otic placode induction, Fgfs are also important for otic placode neurogenesis in chick (Alsina

et al., 2004). Inhibition of Fgf signaling blocks formation of the acoustic ganglion, while ectopic Fgf10 promotes ear expression of the proneural genes such as Nrd and NeuroM. Similarly, we showed that Fgf signaling is essential to induce neurogenesis in the EB placode and yet an unidentified Fgf ligand may be required for maintaining ectodermal *foxi1* expression.

Common signaling pathways may specify neurogenic placodes

It is becoming increasingly apparent that placode development in vertebrates is a multi-step process that involves a multitude of signaling pathways, many of which are similar in different placodes (Bhattacharyya and Bronner-Fraser, 2004; Streit, 2004). For example, in zebrafish and mouse, *foxi* genes are expressed in otic and epibranchial placodes (Hans et al., 2004; Hulander et al., 1998; Lee et al., 2003; Nissen et al., 2003; Ohyama and Groves, 2004; Solomon et al., 2003). Loss of zebrafish *foxi1* results in severe disruption of otic and EB placode development (Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003). Fate maps in zebrafish and chick demonstrate that otic and EB placode precursors might be intermingled during the early stages of development (Bhattacharyya et al., 2004; Kozlowski et al., 1997; Streit, 2002), and careful analysis of gene expression in *Xenopus* supports this idea (Schlosser and Ahrens, 2004). It is tempting to speculate that despite clear differences in timing of induction, otic and EB placodes might share a common field of precursors, and in both cases *foxi* activity may confer ectoderm competence to respond to Fgf signals. It will be interesting to investigate whether overexpression of *foxi1* together with *fgf3* will induce ectopic sensory neurons in atypical locations in the embryo, including the trunk. Among other early markers, various members of Pax/Eya/Six/Dach network are required for the development of both EB and otic placodes and probably act downstream of Foxi1 in both systems (Riley and Phillips, 2003; Streit, 2004; Zou, 2004). Additional experiments in *Xenopus*, chick and mouse embryos support the idea that *pax*, *eya*, *six* and *dach* genes act early during epibranchial placode specification (Abu-Elmagd et al., 2001; Baker and Bronner-Fraser, 2000; Ishii et al., 2001; Schlosser and Ahrens, 2004; Zou, 2004), but their role in zebrafish EB placode development remains to be determined.

Overall, an emerging picture supports the idea that multiple signals from different tissues are required to specify various sensory placodes. The availability of markers that define various steps of otic placode induction, including earliest steps in conjunction with loss-of-function experiments has been particularly useful in defining functions of various inducers. Thus, identification of additional early markers involved in EB placode development in zebrafish should help to pinpoint signals that induce EB placodes.

We thank B. Draper, A. Carmany-Rampey and S. Yu for sharing reagents, K. Cooper for TUNEL protocol, and L. Maves for HS-*fgf3* construct. We are indebted to A. Fritz and R. Esterberg for providing *foxi1* mutant embryos. We also thank J. Lister for sharing unpublished results. We are grateful to B. Draper and A. Fritz for helpful discussions and critical comments on the manuscript. We thank D. White for excellent fish care. A.N. is supported by an NRSA postdoctoral fellowship from the NICHD. Research was supported by grants to D.W.R. from NIH.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/16/3717/DC1

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