Specification of epibranchial placodes in zebrafish

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In all vertebrates, the neurogenic placodes are transient ectodermal thickenings that give rise to sensory neurons of the cranial ganglia. Epibranchial (EB) placodes generate neurons of the distal facial, glossohyrygeal and vagal ganglia, which convey sensation from the viscera, including pharyngeal endoderm structures, to the CNS. Recent studies have implicated signals from pharyngeal endoderm in the initiation of neurogenesis from EB placodes; however, the signals underlying the formation of placodes are unknown. Here, we show that zebrafish embryos mutant for fgf3 and fgf8 do not express early EB placode markers, including foxi1 and pax2a. Mosaic analysis demonstrates that placodal cells must directly receive Fgf signals during a specific crucial period of development. Transplantation experiments and mutant analysis reveal that cephalic mesoderm is the source of Fgf signals. Finally, both Fgf3 and Fgf8 are sufficient to induce foxi1-positive placodal precursors in wild-type as well as Fgf3- plus Fgf8-depleted embryos. We propose a model in which mesoderm-derived Fgf3 and Fgf8 signals establish both the EB placodes and the development of the pharyngeal endoderm, the subsequent interaction of which promotes neurogenesis. The coordinated interplay between craniofacial tissues would thus assure proper spatial and temporal interactions in the shaping of the vertebrate head.

KEY WORDS: Fgf3, Fgf8, Foxi1, Pax2a, Epibranchial placodes, Cranial ganglia, Cephalic mesoderm, Zebrafish

INTRODUCTION

In vertebrates, placodes are transient epithelial thickenings within non-neural ectoderm that give rise to sensory neurons of the cranial ganglia as well as the lens and sensory structures of the nose and ear. The neurogenic placodes include trigeminal placodes that form neurons of the trigeminal ganglia, and epibranchial (EB) placodes that generate sensory neurons of the distal facial, glossohyrygeal, and vagal ganglia. EB neurons innervate internal organs to transmit information such as heat rate, blood pressure, and visceral distension from the periphery to the CNS. In zebrafish, the EB placodes are positioned ventrally to the ear at the dorsal aspect of the glossohyrygeal arches: the facial placode is associated with the second arch, the glossohyrygeal with the third, and four vagal placodes with the four posterior-most arches. Despite their functional importance and evolutionary conservation, the signals underlying neurogenic placode induction are not known.

EB placode induction is likely to be a multi-step process (Streit, 2004). A growing body of evidence suggests that along with the various other placodes, EB placodes are derived from a common territory, named pre-placodal or panplacodal ectoderm (PPE), positioned around the border of the anterior neural plate. PPE is defined by expression of specific transcription factors, including the homeobox factor Six1 and its binding partner Eya1 (Bessarab et al., 2004; Pandur and Moody, 2000; Sahly et al., 1999; Schlosser and Ahrens, 2004; Kou et al., 2004). Initial formation of the PPE is regulated by Bmp, Wnt and Fgf signals (Ahrens and Schlosser, 2005; Brugmann et al., 2004; Litsiou et al., 2005). Transplantation and fate-mapping studies support the idea that the PPE represents the earliest stage in placode induction (Jacobson, 1963; Kozlowski et al., 1997; Martin and Groves, 2006; Streit, 2002). Initially, cells destined to form EB placode are intermingled within the PPE with precursors of the otic placode (Streit, 2002) in what may be a PPE subdomain (Schlosser and Ahrens, 2004). Cells then segregate via an unknown mechanism to form EB placodes. Once placodes are formed, Fgf3 and Bmp signal from the endodermal pouches to induce neurogenesis (Begbie et al., 1999; Holzschuh et al., 2005; Nechiporuk et al., 2005; Trokovic et al., 2005).

Different stages of EB placode development are revealed by transcription factor expression. Foxi1 is a forkhead-related winged helix transcription factor expressed in EB placode precursors, and in foxi1 mutants, placodal progenitors fail to undergo neurogenesis and subsequently die (Lee et al., 2003). Pax2 is a paired domain transcription factor that is upregulated in the EB placodes in chick and Xenopus prior to neurogenesis (Baker and Bronner-Fraser, 2000; Schlosser and Ahrens, 2004). ngn1 (also known as neurog1 – Zebrafish Information Network) is the earliest proneural gene expressed in zebrafish EB placodes (Andermann et al., 2002). Similarly, neurogenin genes are expressed in EB placodes in other species (Fode et al., 1998; Schlosser and Ahrens, 2004). In zebrafish, Ngn1 activity is required for expression of phox2a and phox2b (Nechiporuk et al., 2005), homeobox transcription factors that are in turn necessary for subsequent differentiation of EB neurons (Dauger et al., 2003).

Although signals that regulate EB placode formation are unknown, Fgf signals are required for otic placode development in zebrafish, chick and mouse (Ladhner et al., 2005; Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Wright and Mansour, 2003). Loss-of-function experiments in zebrafish demonstrated that Fgf3 and Fgf8 are redundantly required at multiple stages of otic placode induction (Leger and Brand, 2002; Solomon et al., 2004). Foxi1 is necessary during otic placode induction and at that point foxi1 expression is independent of Fgf signals (Nissen et al., 2003; Solomon et al., 2003; Solomon et al., 2004). In contrast, we have previously shown that Fgf signaling is necessary to maintain expression of foxi1 in EB placodal precursors, suggesting that Fgf signals might also be important for early specification of EB placodes (Nechiporuk et al., 2005).

In this study, we show that global disruption of Fgf signaling blocks EB placode induction and subsequent neurogenesis. Using transplantation techniques, we demonstrate that Fgf signaling is required cell autonomously in EB placodes. Analyses of zebrafish...
mutants strongly suggest that inducing signals are mesoderm-derived, and that two mesodermally expressed fgf genes, fgf3 and fgf8, are required for EB placode formation. Transplantation of wild-type mesoderm into fgf3+/+ morphants rescues EB ganglia, and ectopic Fgf3 or Fgf8 are sufficient to induce foxi1-positive EB precursors and phox2b-positive EB neurons. Overall, our results revealed a combined role for Fgf3 and Fgf8 during EB placode induction, and suggest a model where interactions between cranial mesoderm, ectoderm and endoderm are coordinated to assure proper development of the vertebrate head.

MATERIALS AND METHODS

Fish care, fish strains, SU5402 and heat-shock treatments

Wild-type embryos were obtained from natural spawning of +AB adults and staged by age (hours post-fertilization (hpf)) at 28.5°C and morphological criteria (Kimmel et al., 1995). The following alleles were used to produce mutant embryos: fgf8, ace221a (Reifers et al., 1998); foxi1, hsp86 (Solomon et al., 2003); fgf3, hsa241a (Herzog et al., 2004); and pax2a, no252a (Lun and Brand, 1998). Maternal-zygotic one-eyed pinhead (MZoep) embryos were generated by crossing adult oep257 mutant fish, which had been rescued by injecting 20 pg of oep mRNA at the one-cell stage (Gritsman et al., 1999). The Fgfr inhibitor, SU5402 (Calbiochem), was dissolved in dimethyl sulfoxide (DMSO) at 50-100 mM and then further diluted to a working concentration in embryo medium (EM) (Westefeld, 2000). Control embryos were treated with the same amount of DMSO in EM. Embryos treated with a 60 μmol/L solution lacked trunk mesoderm, otic vesicle, and ermA expression, without exhibiting significant cell death. Thus, we used 60 μmol/L concentration in all our subsequent treatments. To obtain hsp70::dnfgf1-positive embryos we crossed [Tg(hsp70::dn-fgf1)]94 heterozygotes (Lee et al., 2005) to wild-type fish, heat shocked embryos at 38.5°C for 30 minutes and sorted embryos 2-12 hours later using epifluorescence. GFP-negative embryos were used as controls.

Generation of phox2b:egfp transgenic line

We modified a phox2b-containing bacterial artificial chromosome (BAC) clone by Escherichia coli-based homologous ET-recombination (Zhang et al., 1998). BAC clone C192B19 contains approximately 92 kb of sequence upstream and 32 kb downstream of phox2b (http://www.sanger.ac.uk/Projects/D_reis/mapping.shtml). Following recombination, the modified BAC clone contained an egfp gene positioned at an endogenous start site. The accuracy of recombination was evaluated by PCR, sequencing, and by transient expression assays. phox2b::egfp BAC faithfully recapitulated endogenous phox2b expression in cranial ganglia and other organs. To obtain a germline, we injected linearized BAC DNA into zebrafish embryos, raised injected fish to adulthood, and screened their expression in cranial ganglia and presumptive facial placodes beginning at 16-18 hpf (see Fig. S1A in the supplementary material). Expression of phox2b was analyzed its expression in zebrafish (see Fig. S1A in the supplementary material). To detect Fgf-myc or GFP expression following RNA in-situ hybridization, embryos were processed with mouse monoclonal anti-Myc (1:1000; Cell Signaling) or rabbit polyclonal anti-GFP antibody (1:1000, Molecular Probes), and Alexa 568 or Alexa-488 secondary antibody (1:1000, Molecular Probes) as described (Andermann et al., 2002).

Bead-implantation experiments were performed essentially as described (Maves et al., 2002). Briefly, 20 μm polystyrene beads (Polysciences) were rinsed in PBS, treated with 0.5 mg/mL heparin for 20 minutes at room temperature, then incubated in 250 μg/mL mouse FGFR8b (R&D Systems) with 0.5% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. Control beads were incubated in 0.5% BSA in PBS. Embryos were mounted similar to transplantation experiments. A small incision was made with a glass needle approximately half way between the first somite and the anterior limit of the neural plate. One to three beads were placed under the ectoderm either lateral to or into the neural plate tissue. To test the efficacy of FGFR8b beads, embryos with implanted beads were stained for pax2a (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). In total, 15 out of 17 embryos showed a ring of pax2a expression around the FGFR8b bead.

RESULTS

pax2a is expressed in zebrafish EB placodes prior to neurogenesis

To assess initial EB placode formation, we searched for markers that are upregulated in placodes before the onset of neurogenesis at 24 hpf. As Pax2 is expressed in the EB placodes in chick and Xenopus (Baker and Bronner-Fraser, 2000; Schlosser and Ahrens, 2004), we analyzed its expression in zebrafish (see Fig. S1A in the supplementary material). Expression of pax2a is found in presumptive facial placodes beginning at 16-18 hpf (see Fig. S1A in the supplementary material; data not shown) and extends caudally to include presumptive glosopharyngeal and vagal placodes between 20-24 hpf. Transverse sections of 24-hour-old embryos
revealed pax2a mRNA in thickened columnar placodal epithelium (see Fig. S1A in the supplementary material, arrows) but only in its ventral part (see Fig. S1A in the supplementary material, arrowheads). Between 28 and 36 hpf, pax2a expression is maintained in individual placodes and in internalized neuroblasts, but downregulated in differentiated Hu-positive EB neurons (see Fig. S1B in the supplementary material; data not shown). The zebrafish pax2a null mutant no isthmus (not<sup>264</sup>) (Lun and Brand, 1998) displayed about 50% reduction in EB neurons (see Fig. S1C in the supplementary material). Foxi1 activity is required for pax2a expression; pax2a message was absent or strongly reduced in foxi1 mutant embryos (see Fig. S1D in the supplementary material). Altogether these data indicate that pax2a is expressed in the EB placodes prior to and during neurogenesis and is necessary for their development.

**Fgf signaling is required for EB placode induction**

To analyze the role of Fgf signaling in EB placode induction, we examined foxi1, pax2a, and phox2b expression in zebrafish embryos treated with a Fgf receptor (Fgfr) inhibitor, SU5402 (Fig. 1) (Mohammadi et al., 1997). Zebrafish embryos were incubated with SU5402 or DMSO (control) beginning at 10 hpf. Alternatively, we used a zebrafish transgenic line that carries a heat-shock-inducible form of dominant-negative Fgfr1 fused to GFP (hsp70::dn-fgfr1) (Lee et al., 2005). foxi1 expression was strongly reduced at 19 hpf in both SU5402 and hsp70::dn-fgfr1 embryos (Fig. 1). pax2a expression in the EB placodes was completely absent at 24 hpf, and phox2b expression was either absent or strongly reduced at 36 hpf (Fig. 1). Thus, Fgf signaling is required for expression of early placodal markers and subsequent neurogenesis.

We used the SU5402 inhibitor and the hsp70::dn-fgfr1 transgenic line to determine the temporal requirement for Fgf signaling. In zebrafish the SU5402 block is reversible, with Fgf-mediated signaling restored within 1-2 hours after the inhibitor removal (Crump et al., 2004; Maroon et al., 2002; Phillips et al., 2001). We incubated embryos in SU5402 for periods of 1.5-4.5 hours, and analyzed them at 24 hpf using Pax2 antibody (Fig. 2A). In parallel, hsp70::dn-fgfr1 embryos were heat shocked at the same time points as for inhibitor application (Fig. 2B,C). In both sets of experiments, Pax2 expression in the facial placode was either absent or strongly reduced when Fgf signaling was inhibited between the 10- and 16.5-hour stages, with the strongest requirement found between 10 and 11.5 hpf. Pax2 expression in the glossohypoglossal and vagal placodes was most strongly affected later, after 11.5 hpf, suggesting that the EB placodes are induced in an anterior to posterior fashion. Together, these experiments demonstrate a strong Fgf requirement between 10 and 16.5 hpf for all EB placodes (Fig. 2C), well before the onset of neurogenesis at 24 hpf.

To determine whether Fgf signaling is active in EB placode precursors, we analyzed expression of fgf receptors, and the Fgf transcriptional targets erm and pea3 (see Fig. S2 in the supplementary material). Fgfr1 has been suggested to mediate Fgf8 signals (Scholpp et al., 2004), whereas erm and pea3 are activated in response to both Fgf8 and Fgf3 (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Transverse sections through 13-14-hour-old embryos revealed that fgfr1, erm and pea3 are expressed in the ectoderm in presumptive EB placode precursors (see Fig. S2 in the supplementary material). In contrast, fgfr2, fgfr3 and fgfr4 were not differentially expressed in the ectoderm at this stage (see Fig. S2 in the supplementary material; data not shown). Overall, these data demonstrate that Fgf signaling is active in EB placode precursors and is required for placode formation.

**Fgf signaling is required cell autonomously in EB placodes**

Both drug treatment and the hsp70::dn-fgfr1 transgene expression globally block Fgf signaling throughout the embryo. We therefore generated mosaic embryos with cells from wild-type and hsp70::dn-fgfr1 lines to determine which tissue(s) required receipt of the Fgf signal for proper EB placode formation. We labeled hsp70::dn-fgfr1 donor embryos with fluorescein-dextran tracer and transplanted 25-30 cells to the prospective placodal domain of a wild-type host embryo at shield stage (Fig. 3A). Resulting embryos were heat shocked between 10 and 11 hpf and analyzed for Pax2 expression at 24 hpf. Whereas wild-type cells readily contributed to EB placodes in wild-type host embryos, hsp70::dn-fgfr1 cells were largely...
excluded from them (Fig. 3A and Table 1). Moreover, we often observed that when transgenic donor cells resided within the EB placodes, they did not express Pax2 (Fig. 3A). In reciprocal experiments, wild-type cells were transplanted into hsp70::dn-fgfr1 embryos. Resulting embryos were heat shocked at 13.5-16 hpf and analyzed for Pax2 expression at 24 hpf (Fig. 3B). The majority of transplanted wild-type cells remained dorsally, at the level of the otic vesicle; however, some migrated ventrolaterally and contributed to EB placodes (Fig. 3B). When compared with the contralateral control side, the total number of Pax2-positive cells on the transplanted side was significantly increased (54 versus 42, \( P < 0.004 \), paired t-test; Fig. 3B and Table 1). If embryos were heat shocked earlier (10-13.5 hpf), wild-type cells were completely excluded from the presumptive EB placode region (data not shown). Overall, these data demonstrate a cell-autonomous requirement for Fgf signaling, consistent with our previous observations that Fgf signaling is active in the EB placodes.

Mesoderm is likely source of EB placode-inducing signal

To find a potential source of a placode-inducing signal, we analyzed foxi1 and pax2a expression in mutant embryos where cephalic mesoderm and endoderm were missing (Fig. 4A). Maternal-Zygotic one-eyed pinhead (MZoep) mutants are deficient in Nodal signaling and completely lack cephalic mesoderm and endoderm, whereas gross specification of the neural tube is normal (Gritsman et al., 1999). In contrast, embryos mutant for the sox-related transcription factor casanova (cas) are missing endoderm (Alexander et al., 1999; Kikuchi et al., 2001). foxi1 expression was severely reduced, whereas pax2a expression was absent in MZoep mutants, a phenotype identical to that of foxi1 and pax2a.
resulting from reduction in Fgf signaling (Fig. 4A). However, foxi1 expression was normal and pax2a expression only slightly altered in embryos injected with a cas morpholino (MO) (Fig. 4A). These results strongly suggest that mesoderm is the source of a placode-inducing signal.

These experiments and the time-course analysis described earlier suggest that the Fgf ligands responsible for EB placode induction would be expressed in cephalic mesoderm between 10 and 16.5 hpf. Both fgf3 and fgf8 were expressed in two bilateral stripes adjacent to the neural plate beginning at 10-11 hpf (Fig. 4B) (Reifers et al., 2000). Expression began as a narrow stripe lateral to the mid-hindbrain boundary and thombomere 4 (r4). With time, expression extended both rostrally and caudally adjacent to r6-7. These patterns of expression were largely unchanged in cas-MO-injected embryos, but completely absent in MZoop embryos (see Fig. S3 in the supplementary material), arguing that fgf3 and fgf8 are expressed in mesoderm, not endoderm (Fig. 4B).

Transverse sections through the 11- and 14-hour-old embryos confirmed that fgf3 and fgf8 were expressed in the underlying tissue and not endoderm (Fig. 4C). Interestingly, the expansion of ectodermal foxi1 expression closely correlated with changes in mesodermal fgf3 and fgf8 expression (Fig. 4B). foxi1 expression was upregulated just anterior to the otic placode between 11 and 12 hpf (Fig. 4B), and then extends posteriorly between 12 and 16 hpf (Fig. 4B). Thus, both fgf3 and fgf8 are expressed in the cephalic mesoderm at the right stage, suggesting their involvement in EB placode induction.

**FGF3 and FGF8 are required for EB placode induction**

To test whether Fgf3 and/or Fgf8 are required for EB placode induction, we crossed limabsent (lia)/fgf3 mutants (Herzog et al., 2004) with acerebellar (ace); fgf8 mutants (Reifers et al., 1998). Whereas the fgf3 (lia2419/3) allele is null (Herzog et al., 2004), the fgf8 (ace202/3) allele is a hypomorph that retains about 25% of Fgf8 activity (Draper et al., 2001). Resulting trans-heterozygous fish were intercrossed to generate all genotypic combinations (including fgf3+/8 double mutants), which were analyzed by in-situ hybridization using PPE, EB placode and EB ganglia markers, photographed and genotyped. As expected, pax2a expression is lost from the isthmus in fgf8 mutants and the otic vesicle is missing in fgf3;fgf8 double mutants (Fig. 5A) (Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Reifers et al., 1998). In addition, fgf3 mutants lacked ngn1 and phox2b expression in the glosopharyngeal and small vagal placodes and ganglia, as Fgf3 is needed for EB neurogenesis at later stages, as we previously reported (Fig. 5A) (Nechiporuk et al., 2005).

Reduction in Fgf3 and Fgf8 levels, either alone or together, did not affect formation of PPE as assessed by eya1 and six1 expression (Fig. 5A and data not shown). Ectodermal foxi1 levels were strongly reduced in fgf3;fgf8 double mutants but were not affected in either single mutant (Fig. 5A). Similarly, pax2a, ngn1 and phox2b expression in all EB placodes and ganglia was absent or strongly reduced in fgf3;fgf8 double mutants. We consistently observed some limited ngn1 and phox2a-staining, but not phox2b staining, in the large vagal placode and ganglion (Fig. 5A and data not shown). Interestingly, foxi8–/–; foxi3–/– embryos displayed an intermediate phenotype between the wild type and fgf3;fgf8 double. Because we did not observe any phenotype in foxi8–/– or foxi3+/– embryos alone, we concluded that Fgf3 and Fgf8 genetically interact.

To visualize epithelial morphology, we obtained transverse sections from wild-type and fgf3;fgf8 double mutant embryos processed for foxi1 and pax2a in situ hybridization (Fig. 5B,C). In wild type, the epithelium was well organized and displayed columnar morphology, with foxi1 expression mostly limited to the outer ectodermal layer. In contrast, epithelium was disorganized and remaining foxi1 expression extended to multiple cell layers in fgf3;fgf8 double mutants (Fig. 5B,C). Altogether, these results strongly argue that Fgf3 and Fgf8 are required for EB placode induction.

### Restoration of cephalic mesoderm is sufficient to rescue EB ganglia

Both fgf3 and fgf8 are expressed in the neural tube at the time of EB placode induction in addition to cephalic mesoderm. We therefore performed tissue transplants to test where fgf3+8 is required. To maximize the efficiency of these experiments, we transplanted wild-type cells into embryos injected with fgf3+8 morpholino oligonucleotides, rather than into fgf3;fgf8 double mutants. Because endoderm-derived Fgf3 is required for later stages of EB placode neurogenesis (Nechiporuk et al., 2005), we first determined a dose of fgf3-MO that alone allowed normal neurogenesis, but in conjunction with fgf8-MO phenocopied fgf3;fgf8 mutants (see Fig. S4 in the supplementary material).

We injected host zebrafish embryos at the one-cell stage with fgf3+8-MO, and wild-type donors were injected with a fluorescein-dextran lineage tracer. At early gastrula stages (30-40% epiboly), we transplanted 25-30 cells into the margin of fgf3+8 morphants. EB

<table>
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<th>Table 1. Fgf signaling is required cell-autonomously in EB placodes1</th>
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<td><strong>Total number of</strong></td>
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<td>hsp70::dn-fgfr1–wt</td>
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<tr>
<td>wt–hsp70::dn-fgfr1 (transplant side)</td>
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<td>wt–hsp70::dn-fgfr1 (contralateral control)</td>
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125-30 donor cells were transplanted into the presumptive placodal domain of shield-staged host embryos (Fig. 3), resulting embryos were heat-shocked at 38.5°C for 30 minutes (bud-3s for hsp70::dn-fgfr1–/– wt transplants and 9-14s for wt–hsp70::dn-fgfr1 transplants), collected at 24 hpf and analyzed for Pax2 expression.

P < 0.014; t-test.

P = 0.004, paired t-test.
ganglia were assessed at 54 hpf for *phox2b* mRNA expression (Fig. 6A) or at 72 hpf for *phox2b::egfp* transgene expression (Fig. 6B). The *phox2b::gfp* strain carries a stably integrated BAC clone with *egfp* recombined into the endogenous *phox2b* start site. Hindbrain transplant alone did not rescue EB ganglia (Fig. 6A and Table 2), but were capable of rescuing the Fgf requirement for locus coeruleus development (Guo et al., 1999). In contrast, mesodermal transplants alone or together with hindbrain efficiently rescued EB ganglia (Fig. 6A and Table 2). We did not observe any differences in the efficiency of the rescue between mesoderm and hindbrain transplants versus mesoderm transplants alone (Table 2). Although in some cases transplants also included pharyngeal endoderm, donor endoderm did not appear to be necessary for EB ganglia rescue in other cases (Fig. 6B; Table 2). When visualized at 72 hpf, it is apparent that donor cells contributed to the various facial muscles in the rescued embryos. From these data we conclude that the cephalic mesoderm, but not the neural tube, is sufficient to rescue EB ganglia in *fgf3*+*8* morphants.

**Fgf3 or Fgf8 are sufficient to induce EB placodes**

To test whether ectopic expression of Fgf is sufficient to induce *foxi1*-positive EB placode precursors or *phox2b*-positive EB neurons we expressed Fgf from an ectopic source. In the first series of experiments, we mosaically expressed Fgf3 or Fgf8 using heat-shock promoter constructs in wild-type embryos. Embryos were injected with *hs-fgf3myc* or co-injected with *hs-fgf8* and *hs-gfp* plasmids, heat shocked between 10 and 13 hpf, and assayed for *foxi1* expression at 19-20 hpf (Fig. 7A). As controls, we injected embryos with a *hs-gfp* plasmid alone. Injection of a plasmid results in a mosaic DNA distribution in zebrafish embryo, leading to randomly distributed *fgf* - and *gfp*-expressing clones (usually on one side of an embryo) upon heat shock. A significant number of the injected wild-type embryos exhibited ectopic *foxi1* foci, 31% (n=148) and 33% (n=228) for *hs-fgf3myc* and *hs-fgf8* injections, respectively (compare with only 4%, n=218, for *hs-gfp* alone) (Fig. 7A). Ectopic *foxi1* staining often colocalized with Myc-positive cells surrounded by punctate antibody staining, presumably recognizing secreted Myc-tagged Fgf3 (Fig. 7A).

To introduce Fgf from a more localized source, we performed bead implantation experiments. The 20-μm beads were soaked in mouse recombinant Fgfb and placed under the ectoderm just lateral to the neural plate in wild-type embryos between 11 and 13 hpf. Resulting embryos were analyzed for *foxi1* expression at 19 hpf (Fig. 7B). Control embryos that received BSA-soaked beads showed no changes in *foxi1* expression (Table 3 and data not shown).
shown). In contrast, we observed ectopic foxi1 expression foci in the vicinity of the Fgf8b-coated beads in 40% of the analyzed embryos (n=15; Fig. 7B and Table 3). Fig. 7C summarizes the location of ectopic foxi1 expression from all ectopic Fgf overexpression experiments. We observed ectopic foci throughout the ectoderm located ventrally to the endogenous foxi1 expression domain, but found no ectopic foci dorsal to the endogenous domain or in trunk ectoderm.

In chick embryos, placodal precursors are initially specified as lens prior to acquiring their definitive fates, and subsequently the lens fate is restricted by Fgf8 signals derived from the anterior neural plate (Bailey et al., 2006). To determine whether anterior expansion of foxi1 by ectopic Fgf expression similarly affects lens specification, we assayed hs-fgf3myc-injected embryos for a lens marker Prox1 (Tomarev et al., 1998; Wigle et al., 1999). Almost all embryos with rostral expansion of foxi1 expression displayed either reduction or absence of lens Prox1 expression (15/17; Fig. 7D). We also observed a similar result using Fgf8b-coated beads (3/4; data not shown). This result implies that more anterior PPE can be respecified to form EB placode precursors, at the expense of lens precursors.

To confirm that the ectopically generated foxi1-positive precursors could give rise to EB neurons, we assayed wild-type embryos after bead implantation for phox2b expression at 48 hpf. Most embryos displayed ectopic phox2b-positive foci (6/10; Fig. 7E and Table 3). Similarly, activation of hs-fgf3myc or hs-fgf8 at 12-13 hpf also resulted in formation of ectopic foci. Importantly, a number of ectopic phox2b-positive foci were located on the ventral side of the head and belly ectoderm, away from the endogenous phox2b-expression sites and pharyngeal endoderm (Fig. 7F). Analyses of phox2b::egfp embryos revealed that the ectopic cells extended peripheral projections, confirming their neuronal identity. When ectopic phox2b-positive foci were located adjacent to the eye, we often observed either reduction or complete loss of lens tissue (Fig. 5).
To determine whether Fgf is sufficient to restore foxi1 expression in Fgf-deficient embryos, we also injected hs-fgf3myc and hs-fgf8 plasmids into fgf3+8 morphants. Either construct efficiently restored the extent of the endogenous foxi1 expression domain (Fig. 8A). A total of 33% of hs-fgf3myc injected embryos (n=161) and 20% of hs-fgf8-injected embryos (n=135) had one side of foxi1 expression domain restored when compared with the contralateral control. Moreover, induction of Fgf3 at the 10 hpf stage restored EB ganglia in 27% (n=72) of embryos (compare with 6% in fgf3+8-MO, n=80) (Fig. 8B). We also performed bead implantation experiments in fgf3+8 morphants. When beads were placed in the tissue lateral to the neural plate we observed restoration of the foxi1 expression domain (12/15; Fig. 8C and Table 3). We often observed foxi1 expression a few cell diameters away from the bead, suggesting that Fgf acts over a distance. In contrast, we did not observe rescue of foxi1 expression when beads were placed in the hindbrain (0/5; Table 3). These results are consistent with our previous finding that a hindbrain source is not sufficient as an Fgf-inducing signal.

**DISCUSSION**

Taken together, our data demonstrate a requirement for mesodermally-derived fgf3 and fgf8 for generation of EB placodes. We propose that a subset of cells within the PPE are induced by Fgf signaling to form a foxi1 expression domain, and it is from within this domain that pax2a+ EB placodes are derived. Once a foxi1-positive field of precursors is established, pax2a expression appears in an anterior-to-posterior sequence: first, in the facial placode at 16-18 hpf and then in the glossopharyngeal and vagal placodes at 18-22 hpf. Expression of pax2a is more restricted than that of foxi1 and pax2a is never expressed in the foxi1-positive ectoderm between facial and glossopharyngeal placodes, a region that is devoid of neurogenesis. Thus, pax2a expression within this foxi1-positive field of precursors might indicate the commitment of these cells to neurogenic EB placode fate. Further studies will be necessary to test this hypothesis.

Although mesodermal Fgf signals are necessary for both the expansion of foxi1 and the induction of pax2a, our analysis suggests that the critical event is the establishment of foxi1 expression. The timing of expansion of foxi1 expression to include the presumptive EB placode domain corresponds to the anterior and posterior spread of fgf3 and fgf8 expression in cranial mesoderm between 10 and 14 hpf, precisely the critical period delineated by inhibitor studies and, significantly, before the initiation of pax2a expression at 16 hpf. Interestingly, pax2a has been similarly suggested to act downstream of Fgf and Foxi1 signals in otic placode formation (Hans et al., 2004).

**Table 2. Cephalic mesoderm restoration in fgf3;fgf8 morphants rescues EB ganglia**

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>gVII</th>
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<th>Small gX</th>
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<tr>
<td>Mesoderm alone</td>
<td>8</td>
<td>3</td>
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<td>8</td>
</tr>
<tr>
<td>Mesoderm and hindbrain</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Hindbrain alone</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total rescued</td>
<td>15</td>
<td>4 (14)</td>
<td>3 (2)</td>
<td>5 (2)</td>
<td>14 (4)</td>
</tr>
</tbody>
</table>

1 Out of 226 embryos that received the transplant, 15 (7%) displayed rescue of at least one EB ganglion.

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7G, top panels). We confirmed that formation of ectopic phox2b-positive neurons did not require the presence of endodermal pouch tissue, as assayed by an endodermal pouch marker nkx2.3 (Lee et al., 1996). This result is consistent with previous observations demonstrating that a subset of EB placodal cells did not require endoderm-derived signals to undergo neurogenesis (Holzschuh et al., 2005; Nechiporuk et al., 2005). Overall our data show that Fgf-induced ectopic foxi1-positive precursors could differentiate into phox2b-positive epibranchial neurons.
What is the role of Pax2a in the EB placode development? Targeted inactivation of mouse Pax2 demonstrated that it is required for ear patterning and acoustic ganglion development (Burton et al., 2004; Torres et al., 1996), but its function in EB placode development was not determined. Pax2 expression has been described in EB placodes in chick and *Xenopus* (Baker and Bronner-Fraser, 2000; Schlosser and Ahrens, 2004), but not previously in zebrafish. We found that, in zebrafish *pax2a* mutants, the development of some but not all EB neurons was disrupted. It is possible that other *pax* genes may play redundant roles during EB placode development, similar to the situation for the otic placode. Whereas *pax2a* mutants have a very mild reduction of the otic placode, injection of *pax8* morpholino results in its almost complete absence (Hans et al., 2004; Mackereth et al., 2005). Interestingly, *pax8* expression has been reported in the EB placode in *Xenopus* (Schlosser and Ahrens, 2004). Pax2b, a zebrafish ortholog of Pax2a (Pfeffer et al., 1998), may also play a redundant role during EB placode development.

**Model of EB placode induction in zebrafish**

To summarize data presented here and by others, we suggest the following model for EB placode induction (Fig. 9). Bmp, Wnt and Fgf signals are responsible for the initial specification and positioning of the PPE domain (Ahrens and Schlosser, 2005; Brugmann et al., 2004; Litsiou et al., 2005). After the PPE is formed, the ventral part of the competent ectoderm receives Fgf3 and Fgf8 signals from the cephalic mesoderm (Fig. 9A). Importantly, Fgf signals are not required for the initial induction of *foxi1* expression, as *foxi1* message is still present in Fgf-deficient embryos. Residual *foxi1* expression may represent a subset of cells that will not
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Table 3. Fgf8 over-expression is sufficient to induce foxi1-positive EB precursors and phox2b-positive EB neurons

<table>
<thead>
<tr>
<th>Bead–host</th>
<th>Stage (hpf)/ marker</th>
<th>n1</th>
<th>Ectopic/ rescued</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA–wt</td>
<td>19/fox1</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Fgf8b–wt</td>
<td>19/fox1</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>BSA–wt</td>
<td>48/phox2b</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Fgf8b–wt</td>
<td>48/phox2b</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>BSA–fgf3+8–MO</td>
<td>19/fox1</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Fgf8b–fgf3+8–MO</td>
<td>19/fox1</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Fgf8b–fgf3+8–MO (hindbrain)</td>
<td>19/fox1</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

1One to three BSA- or Fgf8b-coated beads were implanted into wild-type or fgf3;fgf8 morphants at 11-13 hpf (Fig. 7).
2Resulting embryos were collected at either 19 or 48 hpf and analyzed for foxi1 or

normally contribute to EB placodes, or may indicate cells that are stalled in the early stages of their development. Although our misexpression experiments demonstrated that most of the PPE and ventral embryonic ectoderm are competent to express foxi1, only the ventral portion of the PPE normally expresses it. While the foxi1 expression domain remains broad, pax2a expression is refined over the course of development: pax2a is more restricted than foxi1 and is expressed only in the individual placodes during neurogenesis. Our observations support a multi-step model of EB placode induction where a first step is to induce a broad field of precursors marked by foxi1 expression, which is further refined by other signals during later steps of development. This multi-step model might explain how otic and EB precursors interpret the same Fgf signals. Close proximity of cephalic mesoderm to the ventral part of competent ectoderm might help to restrict foxi1 + EB precursors. Alternatively, additional signal(s), possibly from a different source, might distinguish EB and otic precursors. In support of this idea, a recent study demonstrated that in addition to inductive Fgf signals, Wnt signals, possibly from hindbrain, help refine otic placode positioning in mouse embryos (Ohyama et al., 2006).

In addition to their roles in EB placode induction, Fgf3 and Fgf8 derived from the cephalic mesoderm and neural tube pattern the pharyngeal pouches (Fig. 9A) (Crump et al., 2004). We suggest dual roles for Fgf3 and Fgf8 in the initial development of these tissues to ensure the subsequent developmental coordination of EB neurons and their target of innervation. Signals from pharyngeal pouch endoderm, including fgf3, bmp2b and bmp5, are required to initiate expression of the early proneural genes such as ngn1 and neuroD (nrd) in the individual EB placodes (Fig. 9B) (Holzschuh et al., 2005; Nechiporuk et al., 2005). ngn1 is also transiently expressed in migrating and condensing neuroblasts, and is required for the subsequent formation of phox2-positive neuronal precursors (Andermann et al., 2002; Nechiporuk et al., 2005). Eventually, phox2-positive precursors condense together with neural-crest-derived glia to form cranial ganglia. Further studies will be necessary to determine what cues guide EB neurons to their final destination.

It has been suggested previously that pharyngeal endoderm is required for EB placode induction in avian embryos (Begbie et al., 1999). Therefore, one might interpret our results showing a role for mesodermal Fgf signaling in zebrafish placode induction as simply a reflection of the Fgf requirement for pharyngeal pouch development (Crump et al., 2004). However, there are several arguments against this explanation. First, in zebrafish, pharyngeal pouches do not begin to form until 22 hpf (Holzschuh et al., 2005), well after pax2a expression is established in the thickened EB ectoderm (this study). Second, loss of pharyngeal endoderm in zebrafish casanova mutants results not in elimination of EB placodes but rather reduction in neurogenesis; expression of ngn1, phox2b and the Hu antigen is lost, whereas foxi1, pax2a and morphological thickening of the ectoderm is retained (Holzschuh et al., 2005; Nechiporuk et al., 2005) (this study). Although this result may reflect differences between fish and avian species, it is

![Fig. 8. Fgf3 or Fgf8 is sufficient to induce foxi1-positive EB precursors and phox2b-positive EB neurons in fgf3+8 morphants.](imageA)

(A,C) Dorsal views; (B) lateral views. (A,B) Zebrafish embryos were injected with hs-fgf3myc or co-injected with hs-fgf8 and hs-gfp plasmids at one-cell stage, heat shocked at 10-13 hpf and then assayed for foxi1 or phox2b expression (blue) and Myc or GFP expression (red and green, respectively). Fgf3myc or Fgf8 efficiently rescued foxi1 (brackets in A) or phox2b (arrows in B) expression in fgf3+8 morphants. Note the difference between foxi1 expression domain adjacent to Myc or GFP-expressing cells and contralateral side. (C) fgf3+8 morphant

![Fig. 9. A model of EB placode induction in zebrafish.](imageB)

See text for details.
important to note that only late markers of neurogenesis (Phox2a, NF-M) were analyzed in the previous avian study (Begbie et al., 1999), leaving open the possibility that mechanisms might be conserved. Third, our mosaic analysis demonstrates that Fgf signaling is required directly within the ectoderm, suggesting that the mesodermal Fgf signal is received directly by placodal precursors. Finally, we show that ectopic expression of Fgf results in formation of new phox2b-positive neurons in the absence of nkx2.3+ pharyngeal endoderm. Although we cannot rule out that pharyngeal endoderm supplies additional EB-promoting signals, our results strongly support a role for mesodermal Fgf directly acting upon placodal ectoderm.

Role of Fgf signaling in specification and induction of EB placodes

FGFs play a critical role in morphogenesis of multiple organ systems by regulating cell proliferation, differentiation, cell migration and cell survival. Thus, Fgf signals could regulate expansion of foxi1-positive precursors by any of the above mechanisms. Fgf signals can act as chemotaectants during both invertebrate and vertebrate development. In Drosophila, Fgf plays the role of a chemotactic signal during tracheal morphogenesis (Sutherland et al., 1996), whereas in worms, the Fgf signal guides sex myoblasts to the gonads (Burdine et al., 1998; Burdine et al., 1997). Fgf4 and Fgf8 act as chemotactic signals to directly coordinate cell movements during gastrulation in the chick embryo (Yang et al., 2002). We observed that foxi1 expression could be induced a few cell diameters away from an Fgf source, arguing that Fgf might function as a long-range signal to recruit EB placode precursors. This is consistent with recent work in zebrafish revealing that Fgf ligand could travel as far as 16-cell diameters away from an Fgf source (Scholpp and Brand, 2004). Although it is attractive to speculate that Fgf signals could promote migration, further studies are necessary to define the exact role of Fgf3 and Fgf8 in the recruitment of EB placode precursors.

Conservation of the inductive signals in the cranial placodes

It has been argued that once the PPE domain is established, local signal(s) promote the fate of the individual placodes (Streit, 2004). The initial step common to all placodes include signals from mesendoderm (Ahrens and Schlosser, 2005; Litsiou et al., 2005), whereas later inductive signals for specific placodes would originate from distinct tissues. Dorsolateral (trigeminal, lateral line and otic) placodes are in close proximity to the neural tube, whereas ventrolateral (EB) placodes are in close contact with the endoderm and cephalic mesoderm. Indeed, Fgf signals from the neural tube are required during otic placode development (Leger and Brand, 2002), and neural tube signal(s) is sufficient to induce trigeminal placode markers in the head ectoderm, although the nature of this signal(s) is still unknown (Stark et al., 1997).

There are surprising similarities between otic and EB placode development. Both otic and EB placodes require eya1 and six1 (Ozaki et al., 2004; Xu et al., 1999; Xu et al., 2003; Zheng et al., 2003; Zou et al., 2004), and their precursors are intermingled in the PPE (Streit, 2002). Mesodermal Fgfs are involved in the initial induction of each placode from PPE (Kil et al., 2005; Wright and Mansour, 2003), and induction is regulated by foxi1 to induce expression of pax2a (Mackereth et al., 2005; Nissen et al., 2003; Solomon et al., 2003; Solomon et al., 2004). Finally, Fgf signals subsequently regulate neurogenesis from both EB and otic placodes upstream of ngn1 (Alsina et al., 2004; Nechiporuk et al., 2005). Although it is not surprising that the six/eya/dach network is conserved in various ectodermal placodes, as these transcription factors are expressed during PPE formation and are important for PPE development, it is remarkable that the same Fgf inductive signals are conserved in the EB and otic placodes.

However, it is important to note that although Fgf3 and Fgf8 signals are necessary during both otic and EB placode induction, we were able to separate the timing and Fgf requirement during these two processes. First, we defined a separate time-window requirement for Fgf signaling during EB placode development. During the otic placode induction, Fgf signaling is not required beyond 11 hpf, whereas it is essential until 16.5 hpf during EB placode development. Second, ectopic activation of Fgf signaling at 13 hpf, well after the otic placodes had been induced, promotes formation of the foxi1-positive precursors and EB-specific phox2b-positive neurons. Finally, analyses of MZoep mutants, which completely lack EB placodes while their otic vesicles are only reduced, support the idea that Fgf signaling plays distinct roles during initial stages of the otic and EB placode development. Alternatively, because EB and otic precursors are initially intermingled within PPE (Schlosser and Ahrens, 2004; Streit, 2002), it is possible that the same signals, including Fgfs, might control initial specification of common precursors. Interestingly, inhibition of Fgf signaling during gastrulation in zebrafish blocks expression of the early otic placode markers (Marrow et al., 2002; Phillips et al., 2001). It will be interesting to investigate whether this early Fgf block affects EB placode development as well.

The preservation of the molecular cascades involved in each type of placode development might be even more conserved than had previously been anticipated. A recent report suggests that Fgfs promote olfactory placode at the expense of lens, the ground state for cranial placodes (Bailey et al., 2006); we found similarly that Fgfs promote formation of foxi1+ placode precursors while simultaneously blocking lens formation. Fgfs are also involved in neurogenesis from olfactory epithelium (DeHamer et al., 1994; Kawachi et al., 2005), suggesting that recurrent roles for Fgfs are also a common theme in placode formation. Surprisingly, Fgf signaling is also important during inductive phases of the lens placode, although the identity of Fgf ligands involved remains unknown (Faber et al., 2001). Overall, our findings are consistent with the idea that new placodes have arisen by co-option of similar molecular mechanisms, while the tissue origin of the inducing signal diverged over the course of evolution.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/3/611/DC1

References


Induction of epibranchial placodes in zebrafish


