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Epibranchial and otic placodes are induced by a common Fgf signal, but their subsequent development is independent

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Abstract

The epibranchial placodes are cranial, ectodermal thickenings that give rise to sensory neurons of the peripheral nervous system. Despite their importance in the developing animal, the signals responsible for their induction remain unknown. Using the placodal marker, *sox3*, we have shown that the same Fgf signaling required for otic vesicle development is required for the development of the epibranchial placodes. Loss of both Fgf3 and Fgf8 is sufficient to block placode development. We further show that epibranchial *sox3* expression is unaffected in mutants in which no otic placode forms, where *dlx3b* and *dlx4b* are knocked down, or deleted along with *sox9a*. However, the forkhead factor, Foxi1, is required for both otic and epibranchial placode development. Thus, both the otic and epibranchial placodes form in a common region of ectoderm under the influence of Fgfs, but these two structures subsequently develop independently. Although previous studies have investigated the signals that trigger neurogenesis from the epibranchial placodes, this represents the first demonstration of the signaling events that underlie the formation of the placodes themselves, and therefore, the process that determines which ectodermal cells will adopt a neural fate.

Keywords: Sox3; Zebrafish; Epibranchial placodes; Fgf signaling; *foxi1*

Introduction

The mechanisms underlying the decision of ectoderm to adopt a neural fate have been a central area of developmental studies for many decades. While research into the origins of the CNS have been abundant, their success has been restricted by the early stage of development when these events occur, and their association with other fundamental processes to which neural induction is linked, such as gastrulation and the specification of the mesendoderm. The neurogenic placodes, on the other hand, appear later after such basic aspects of the embryonic body plan are established, making them a useful alternative system in which to study the mechanisms controlling neural fate. These placodes include the nasal, otic and also the epibranchial placodes, which give rise to many of the neurons associated with the sensory ganglia.

Placodes are, by definition, patches of thickened ectoderm (Webb and Noden, 1993). They arise outside of the axial ectoderm from which the CNS and neural crest are formed. As such they are the only regions outside of the CNS and the neural crest (derived from the margins of the CNS) that give rise to cells of the nervous system. Understanding the mechanisms by which placodes arise is therefore an important issue in developmental biology. Although identified many years ago, a general lack of studies has meant that understanding of the basic processes underlying the formation and development of the epibranchial placodes has lagged behind the CNS and other placodal structures.

Classical embryological studies carried out in the latter part of the 20th century led to a generally accepted picture of the inductive processes underlying the formation of these structures. These studies appeared to demonstrate that formation of all placodes except the epibranchial placodes involves signals

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from the CNS, although a role for hindbrain derived neural crest cells in later migration of neurons from the epibranchial placodes has been shown (Baker and Bronner-Fraser, 2001; Begbie and Graham, 2001; Graham and Begbie, 2000; Yntema, 1944). Formation of the epibranchial placodes therefore required an alternative source of inductive signals to be invoked. To date the exact nature of these signals and inducing tissues remains unclear, with the mesoderm or endoderm as the most likely candidate source. Endoderm and endoderm-derived Fgfs and Bmps have been shown to be required for neurogenesis in the epibranchial placodes (Begbie et al., 1999; Holzschuh et al., 2005; Nechiporuk et al., 2005). However, despite showing that these factors could induce the formation of neurons from the ectoderm, they did not determine which signals actually induced the early placodal ectoderm itself.

Our recent studies in chick (Abu-Elmagd et al., 2001; Ishii et al., 2001), and the work of others in *Xenopus laevis* (Penzel et al., 1997), have identified the transcription factor, Sox3, as one of the earliest markers of placode development. In chick, *Sox3* is expressed in a broad domain near to the developing ear and only later does this domain become segmented to give rise to the final, four epibranchial placodes (Ishii et al., 2001). Here we show the same pattern of *sox3* expression in the epibranchial placodes of the zebrafish, where it represents a unique marker for the early events in placode induction. This has allowed us to re-examine the signals underlying epibranchial placode formation. In particular, based on the location of the early placodal domain adjacent to the developing otic placodes, and data implicating hindbrain derived Fgfs in otic development (Alsina et al., 2004; Ladher et al., 2000; Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001; Vendrell et al., 2000; Wright and Mansour, 2003), we have shown that the same signals, Fgf3 and Fgf8, are required for development of the epibranchial placodes. Although these data suggest that Fgfs normally act by maintaining *sox3* expression from within a larger earlier domain, we show that Fgfs are capable of re-inducing *sox3* expression if signaling restarts after an initial block. In addition, we demonstrate that the gene *foxi1*, which is expressed in both the otic and epibranchial ectoderm and is required for formation of epibranchial placode-derived neurons (Lee et al., 2003), is required for *sox3* expression.

Our experiments further show that the epibranchial placode domain was unaffected in mutants in which no otic placode forms or when several genes associated with otic development are inhibited. Thus, the initial induction of both otic and epibranchial placodes share common signals, but the subsequent development of these two structures is independent.

Methods

Maintenance of fish

Breeding zebrafish were maintained and embryos were raised (Westerfield, 2000) and staged according to Kimmel et al. (1995). The *fgf8*/*ace* mutant has been previously described (Reifers et al., 1998). Homozygous B380 mutant embryos were a kind gift from Monty Westerfield (Liu et al., 2003).

Morpholino injections

Morpholino antisense oligonucleotides (Gene Tools, LLC, Corvallis) and controls were as previously described: *fgf3* (Phillips et al., 2001), *fgf8* (Araki and Brand, 2001), *dlx3b* and *dlx4b* (Solomon and Fritz, 2002), and *foxi1* (Solomon et al., 2003). Embryos were injected at the 1–4 cell stage at concentrations of 1–8 ng/embryo.

Whole-mount in situ hybridisation

Whole-mount *in situ* hybridization on zebrafish embryos was carried out as previously described (Jowett, 2001). For double *in situ* hybridizations, riboprobes were synthesized either with digoxigenin (DIG) or fluorescein-labelled nucleotides (Roche). Detection of DIG/fluorescein antibody–alkaline phosphatase conjugate was performed using BM-purple (Roche) for dark blue/purple stain, Fast Red (Sigma-Aldrich) for red stain, or BCIP (Roche) for green/pale blue stain. The following probes were derived from previously described cDNA clones: *pax2a* (Krauss et al., 1991), *pax8* (Pfeffer et al., 1998), *dlx3b* (Ekker et al., 1992) *krox20* (Yi-Chuan Cheng, Taipei). Other clones were obtained as ESTs from RZPD: *sox3* (GenBank Accession Number: AI959362), *foxi1* (GenBank Accession Number: CF997841) *neurogenin1* (GenBank Accession Number: CA496091), *neuroD* (GenBank Accession Number: CD757273). After *in situ* hybridization, embryos were re-fixed in 4% paraformaldehyde, transferred into 80% glycerol and photographed. Embryos that were to be sectioned were rehydrated, transferred into ethanol and embedded in JB4 methacrylate (Agar Scientific, UK) for microtome (Leica RM2265) sectioning.

SU5402 and retinoic acid treatments

SU5402 (Calbiochem; in DMSO to give a 2 mg/ml stock solution) was used at a final concentration of 20–50 μM in fish water containing methyl blue. Retinoic acid (Sigma; in DMSO to give a stock concentration of 10 mM) was diluted to a working concentration of 10 μM in fish water containing methyl blue. For both treatments, embryos were treated in their chorions at 28 °C, in the absence of light, for specific time periods as required.

Results

Expression of sox3 defines the developing, epibranchial placodes

In zebrafish, as has been described in chick (Rex et al., 1997b) and mouse (Wood and Episkopou, 1999), *sox3* is initially expressed throughout the epiblast (Okuda et al., 2006). As embryos undergo neural induction, its expression becomes restricted to the prospective CNS (Kudoh et al., 2004; Penzel et al., 1997; Rex et al., 1997a and Fig. 1A). At about the time that the first somites are formed, a small domain of *sox3* expression, flanking either side of the prospective hindbrain, also becomes apparent (arrow, Fig. 1B). This domain becomes more pronounced with time (Figs. 1C, D). Initially the domain is symmetrical, but it rapidly takes on an arced shape, with a *sox3*-negative region most medially, at the same rostrocaudal level as the prospective otic placode (Fig. 1D). As we have shown previously in chick (Abu-Elmagd et al., 2001; Ishii et al., 2001), this domain of *sox3* expression is subsequently divided into a series of patches (Figs. 1E, F, K, L), which are coincident with later neurogenesis as shown by expression of the neurogenic genes, *neurogenin1* (Figs. 1G, H, M, N) and *neuroD* (Figs. 1I, J). Expression rostral to the otic vesicle is lost by 60 hpf, and