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Neurogenin1 Defines Zebrafish Cranial Sensory Ganglia Precursors

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Cells delaminate from epithelial placodes to form sensory ganglia in the vertebrate head. We describe the formation of cranial neurogenic placodes in the zebrafish, *Danio rerio*, using bHLH transcription factors as molecular markers. A single *neurogenin* gene, *neurogenin1* (*ngn1*), is required for the development of all zebrafish cranial ganglia, which contrasts with other described vertebrates. Expression of *ngn1* delineates zebrafish ganglionic placodes, including trigeminal, lateral line, and epibranchial placodes. In addition, *ngn1* is expressed in a subset of cells within the otic vesicle that will delaminate to form the octaval (statoacoustic) ganglion. The trigeminal placode is the first to differentiate, and forms just lateral and adjacent to the neural crest. Expression of *ngn1* is transient and prefigures expression of a related bHLH transcription factor, *neuroD*. Interfering with *ngn1* function using a specific antisense morpholino oligonucleotide blocks differentiation of all cranial ganglia but not associated glial cells. Lateral line sensory neuromasts develop independently of *ngn1* function, suggesting that two derivatives of lateral line placodes, ganglia and migrating primordia, are under separate genetic control.

Key Words: **trigeminal placode; lateral line; epibranchial placode; otic placode; neurogenin; bHLH transcription factor; morpholino oligonucleotides.**

INTRODUCTION

In the vertebrate head, neurons of the peripheral ganglia are derived from two sources: neural crest and placodes. The development of cranial placodes, along with neural crest, has been suggested to be a critical feature in vertebrate evolution (Northcutt and Gans, 1983). Neurogenic neural crest precursors migrate from the dorsal neural tube to positions in the periphery, where they coalesce to form ganglia (Hall, 1999; Le Douarin and Kalcheim, 1999). In contrast, placodal neuronal precursors delaminate from localized ectodermal thickenings (placodes) and form adjacent ganglia (Baker and Bronner-Fraser, 2001; Begbie and Graham, 2001a; Webb and Noden, 1993). While the development of neurons from the neural crest has been extensively studied, much less is known about how the neurogenic placodes are specified.

Neurogenic placodes can be distinguished by their location and by the types of neurons they form (Northcutt, 1992). The profundal and trigeminal placodes are positioned posterior to the eye and generate neurons of the profundal and trigeminal ganglia. In many vertebrates, including zebrafish, these placodes and ganglia are fused and are often just designated as the trigeminal placode and ganglion. In addition, two series of placodes also give rise to cranial ganglia: the dorsolateral and the epibranchial (or ventrolateral) placodes. Dorsolateral placodes include the otic placode and the lateral line placodes, which are situated anterior and posterior to the otic vesicle and form the sensory structures of the mechanosensory lateral line. The otic placode forms the otic vesicle, which will generate structural elements of the inner ear and sensory hair cells. In addition, cells delaminate from the otic vesicle to form the neurons of the VIII nerve (octaval or statoacoustic) ganglion. The development of the zebrafish otic placode has been described in detail, and delamination of neurons has been documented by lineage tracing (Haddon and Lewis, 1996). The epibranchial placodes are positioned at the dorsal aspect of the posterior pharyngeal pouches and generate visceral sensory neurons that innervate these struc-

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tures. These placodes form the sensory ganglia of the facial, glossopharyngeal, and vagal nerves. Although not examined in this study, the olfactory placode is also considered neurogenic, forming the olfactory epithelium.

In addition to sensory ganglia, the lateral line placodes give rise to migrating primordia that deposit sensory neuromasts along the head and body (Gompel *et al.*, 2001a; Metcalfe *et al.*, 1985; Stone, 1922). These neuromasts contain mechanosensory hair cells that detect water flow. As the lateral line primordia migrate, they remain in close contact with growth cones of the ganglia that will innervate the neuromasts they deposit (Gompel *et al.*, 2001b; Metcalfe, 1985). Little is known about how these different derivatives of lateral line placodes are specified.

Some of the earliest genes expressed in placodes are the *atonal*-related bHLH transcription factors *neurogenin1* (*ngn1*), *neurogenin2* (*ngn2*), and *neuroD*. *ngn1* and *ngn2* are expressed in different subsets of placodes, although the exact pattern differs in each organism (Abu-Elmagd *et al.*, 2001; Schlosser and Northcutt, 2000; Sommer *et al.*, 1996). *neuroD* is expressed after *neurogenin* and is found in all placodes. Functional analysis of these genes suggests that they may be necessary and sufficient for neurogenesis. Overexpression of these genes drives formation of ectopic neurons (Blader *et al.*, 1997; Kim *et al.*, 1997; Lee *et al.*, 1995; Ma *et al.*, 1996; Perron *et al.*, 1999). Targeted inactivation of *ngn1* results in loss of ganglia derived from the trigeminal placode, otic placode, and neural crest (Ma *et al.*, 1998), while inactivation of *ngn2* results in loss of ganglia derived from the epibranchial placodes (Fode *et al.*, 1998). Disruption of *neuroD* function results in loss of sensory neurons associated with the inner ear (Kim *et al.*, 2001).

We describe here the formation of cranial neurogenic placodes in the zebrafish, *Danio rerio*. We present evidence that, in contrast to the situation in other described vertebrates, a single *neurogenin* gene, *ngn1*, is required for the development of all zebrafish cranial ganglia. *ngn1* is expressed in all zebrafish neurogenic placodes, including the trigeminal, lateral line, and epibranchial placodes, and is expressed in cells within the otic vesicle that will delaminate to form the octaval (acoustic) ganglion. Expression of *ngn1* in placodes is transient and prefigures the expression of *neuroD* in the same structures. Finally, we show that interfering with *ngn1* translation using a specific antisense morpholino oligonucleotide blocks the differentiation of all zebrafish cranial ganglia as well as trunk dorsal root ganglia. In contrast, sensory neuromasts do not depend on *ngn1* to develop, suggesting that the two derivatives of the lateral line placodes, ganglia and migrating primordia, are under separate genetic control.

MATERIALS AND METHODS

Antibody Staining

Antibody staining was performed as described previously (Raible and Kruse, 2000). Embryos were anesthetized in tricaine (10 mg/ml) in embryo medium and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 2 h at room temperature. They were then rinsed three times for 5 min in PBS/0.1% Triton X-100, washed three times for 30 min in distilled H_2O, and incubated for 2 h in blocking solution [2% goat serum, 1% bovine serum albumin (BSA), 1% dimethylsulfoxide (DMSO), 0.1% Triton X-100 in PBS]. Embryos were then incubated overnight at room temperature in anti-Hu antibody (16A11; Marusich *et al.*, 1994) or anti-acetylated tubulin (Sigma, St. Louis, MO) diluted 1:1000 in blocking solution. Embryos were rinsed three times for 30 min in PBS/0.1% Triton X-100 and then incubated overnight at room temperature in Alexa488-or Alexa568-conjugated secondary antibodies (Molecular Probes, Eugene, OR) diluted 1:500 in blocking solution. They were then rinsed three times for 30 minutes in PBS/0.1% Triton X-100 and transferred to 50% glycerol in PBS. In some cases, embryos were counterstained with Sytox Green (Molecular Probes). Stained embryos were examined on a Zeiss LSM Pascal confocal microscope, and image stacks were processed in Adobe Photoshop.

In Situ Hybridization

Embryos for *in situ* hybridization were collected from timed matings, raised at 28.5°C, and carefully staged before fixing. Embryos collected at stages older than 24 h were treated with 0.003% phenylthiourea to prevent melanin pigment formation. Embryonic stages are presented as hours postfertilization (h) following the staging series of Kimmel *et al.* (1995). Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. *In situ* hybridization was performed by using standard methods. Probes were synthesized for *ngn1* (Blader *et al.*, 1997; Kim *et al.*, 1997; Korzh *et al.*, 1998), *neuroD* (Blader *et al.*, 1997; Korzh *et al.*, 1998), *foxd3* (Odenthal and Nusslein-Volhard, 1998), or *eya1* (Sahly *et al.*, 1999). Plasmids were digested with restriction enzymes and probes synthesized with polymerase as follows: *ngn1*, *Xho*I and T7; *neuroD*, *Not*I and T3; *foxd3*, *Not*I and T7; and *eya1*, *Not*I and T7. Fast Red product was detected by fluorescence. After processing and refixation, embryos were split down the middle with fine needles before mounting and photographing on a Nikon Microphot microscope using a Spot digital camera. Composite images of several focal planes were generated with Adobe Photoshop. For sectioning, embryos were first processed for *in situ* hybridization, dehydrated, and then embedded in Araldite resin (Polysciences, Warrington, PA).

Antisense Morpholino Injection

Antisense morpholino oligonucleotides were synthesized by GeneTools (Corvallis, OR). The sequences of the oligonucleotides used were 5′-ACG ATC TCC ATT GTT GAT AAC CTG G-3′ (MO#1), 5′-ACC TTA TTG GTG GGC TGG GAG ATG C-3′ (MO#2), and 4-bp mismatch 5′-TAT tCG AaC TCC ATT GTT cAT AtC C-3′ (mismatches shown in lower case). These oligonucleotides are identical to those used by Cornell and Eisen (2002). Embryos were injected at the one-cell stage by using an ASI pressure injection apparatus (Eugene, OR). Unless otherwise noted, 5 ng of morpholino oligonucleotide was injected into each embryo.

RESULTS

ngn1 and neuroD Expression Delineate Neurogenic Placodes

Placodes are conventionally defined as distinct ectodermal thickenings. In the zebrafish embryo, neurogenic placodes were not distinguishable using this criterion, possibly due to the small numbers of cells involved and the rapid schedule of development. Instead, the expression patterns of the bHLH genes *ngn1* and *neuroD* were used as molecular determinants of placode formation. These genes have been shown to be reliable markers for placode formation in Xenopus (Schlosser and Northcutt, 2000). In the following sections, we describe the developmental schedules of trigeminal, dorsolateral, and epibranchial placodes.

Trigeminal placode. The trigeminal placodes can be identified by 9 h as two lateral patches of *ngn1* expression at the lateral edge of the neuroepithelium (Fig. 1). Expression of *ngn1* in the trigeminal placode begins at the same time as expression in other early zones within the neural plate (Fig. 1A). Even at the earliest stages, the trigeminal placode is not recognizable as a distinct thickening but rather as a loosely packed cluster of cells more than one cell diameter thick (Fig. 1B). After gastrulation is complete, the trigeminal placodes move medially, remaining at the edge of the developing central nervous system as it narrows, thickens, and elongates through convergence extension movements (Fig. 1C). Expression of *neuroD* begins at this stage (11 h, Fig. 1D).

The trigeminal placode is positioned adjacent to neural crest cells forming at the edge of the neural plate (Figs. 1E and 1F). Neural crest precursors form as two lateral stripes of cells that will converge toward the midline, forming a lateral mass adjacent to the developing neural keel before undergoing a mesenchymal transformation and migrating into the periphery. At 11.5 h, neural crest cells express the winged helix transcription factor *foxd3*. Trigeminal placode cells are located just lateral to the neural crest. While there may be overlap between these two expression domains, the double *in situ* method does not allow this question to be resolved.

Expression of *ngn1* and *neuroD* in the trigeminal placode and associated delaminated neuroblasts is transient. Expression of *ngn1* is reduced by 14 h (Fig. 2A), and *neuroD* is diminished by by 18 h (Fig. 2F). However, neural crest cells that will contribute to the trigeminal ganglion subsequently express these genes (e.g., Figs. 2E and 2H).

Dorsolateral placodes. The dorsolateral series is composed of the otic and lateral line placodes (Fig. 2). The otic placode is distinguishable by DIC optics at 14 h, and lateral line placode cells are detected just anterior and posterior to it (Figs. 2A and 2B). *ngn1* is only transiently expressed in the posterior lateral line placode, in a scattering of cells (Figs. 2A and 2C). Posterior lateral line cells appear to almost immediately express *neuroD* (Figs. 2B and 2D) and retain expression through 41 h (Fig. 4F). Scattered posterior lateral line cells coalesce by 20 h (Fig. 2F).

The anterodorsal and anteroventral placodes form from a common region at the anterior edge of the otic placode. Scattered cells in this region express *ngn1* and *neuroD* at 14 h (Figs. 2A and 2B), and over time, a subset of cells extends anteriorly (Figs. 2C–2H). As the otic placode forms a vesicle, some cells begin to express *ngn1* and *neuroD*, delaminate, and extend medially and posteriorly (Figs. 2E and 2F). By 24 h, the anterior lateral line placodes are separate from the otic vesicle and from each other (Figs. 2I and 2J). Finally, by 26 h they resolve into anterodorsal and anteroventral components and separate from the anteriormost epibranchial placode, the facial placode (Fig. 3). The position of each placode is distinct relative to the underlying anterodorsal lateral line ganglion, revealed by the anti-Hu antibody (Fig. 3B). The anterodorsal placode extends anteriorly, above the trigeminal ganglion. The anteroventral placode is just posterior to the nascent anterior lateral line ganglion. The facial epibranchial placode extends anteriorly and ventrally below the anterior lateral line and trigeminal ganglia.

The middle lateral line placode is the last to form, just posterior and ventral to the otic vesicle (Fig. 4A). The middle placode forms adjacent to the vagal epibranchial placode and cannot be recognized as distinct from this placode by *ngn1* expression. A separate group of middle lateral line placode cells expressing *neuroD* is found adjacent to the otic vesicle, just superior to a cluster of vagal cells (Fig. 4B).

Epibranchial placodes. Epibranchial placodes form at the dorsoanterior edge of each pharyngeal arch except for the first, the mandibular arch. The epibranchial placode of the second arch, the facial placode, appears to express *ngn1* by 24 h (Fig. 2I), and is recognizable as a distinct entity by 26 h (Fig. 3A). The epibranchial placode of the third arch, the glossopharyngeal placode, and of the posteriormost arches, the vagal placodes, also are apparent by 24 h. Over time, the initial vagal placode separates into several distinct epibranchial placodes associated with each arch (Fig. 4).

neuroD Expression follows That of ngn1 and Persists in Delaminated Neuroblasts

In each placode region, *ngn1* expression precedes the expression of *neuroD*. As shown above, *ngn1* is expressed in the trigeminal placode prior to *neuroD* expression (Fig. 1) and decreases in trigeminal precursors prior to when *neuroD* expression decreases (Fig. 2). The relationship between these two genes is best exemplified by expression in two long-lasting neurogenic regions, the otic placode and the vagal placode (Fig. 5). Cells delaminate from the otic vesicle to form neurons of the octaval, or statoacoustic, ganglion positioned medial to the developing ear. These cells express *ngn1* before delaminating, but only transiently, downregulating expression soon after they leave the vesicle (Fig. 5A). In contrast, cells rarely express *neuroD* before delaminating, but continue ex-

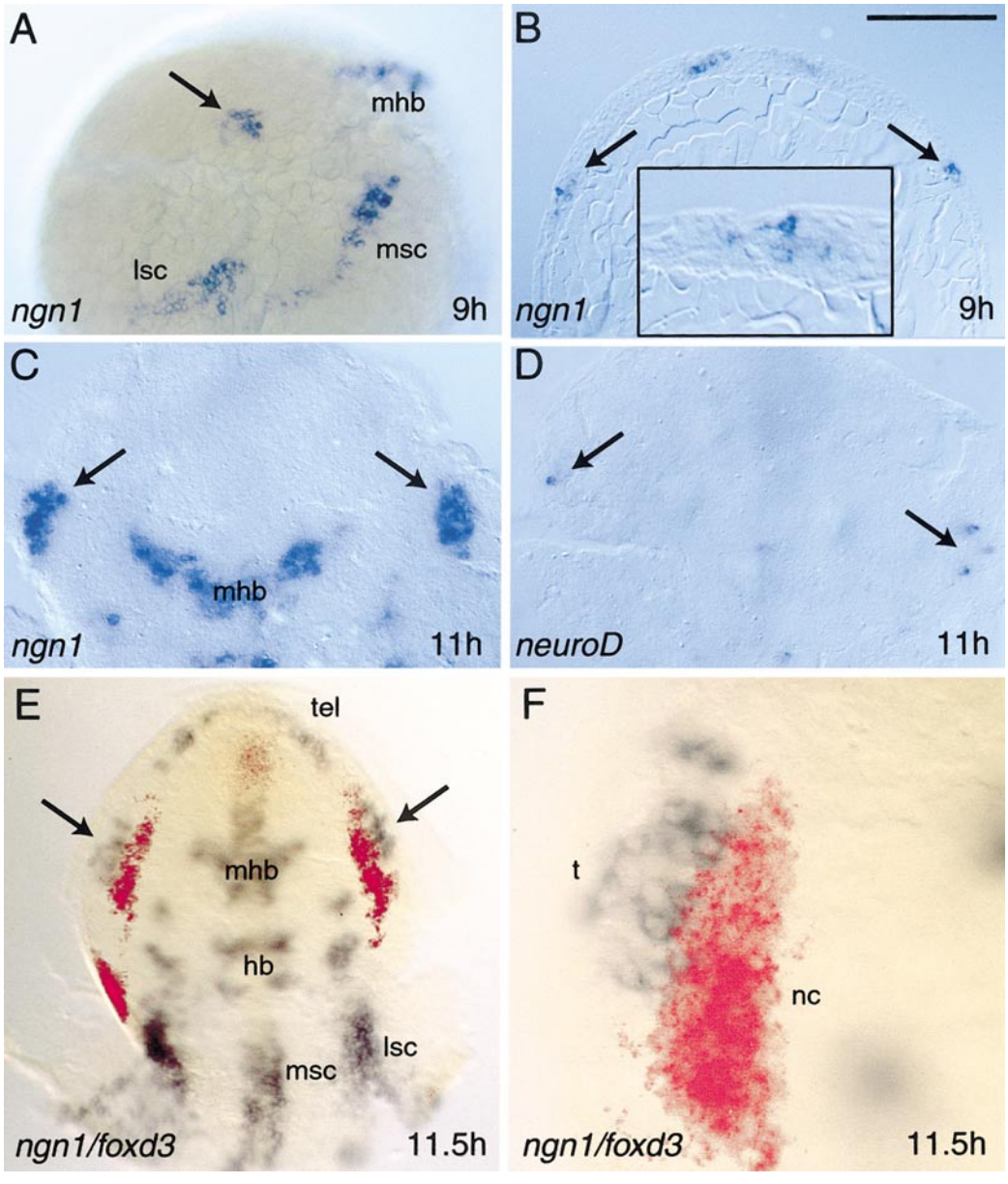
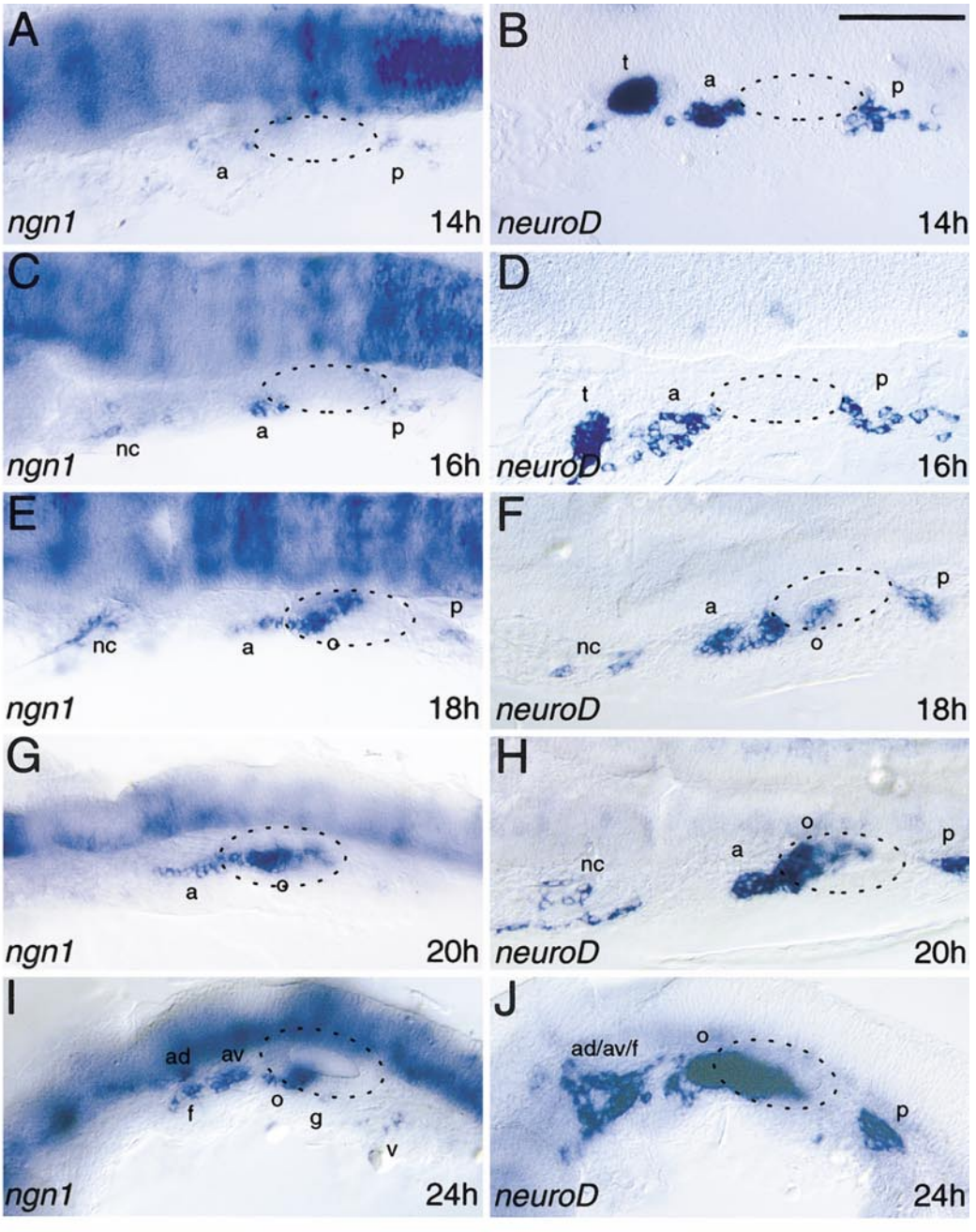


FIG. 1. Development of trigeminal placodes. Trigeminal placodes first express *ngn1* at 9 h (A, B). *ngn1* expression appears nearly simultaneously within the trigeminal placode (arrows), and in patches of cells that will contribute to the mid/hindbrain (mhb), medial/ventral spinal cord (msc), and lateral/dorsal spinal cord (lsc). In (A), the embryo is shown in lateral view with anterior to the top and dorsal to the right. At this stage, the epiblast is several cell diameters thick and shows no distinct layering (B). *ngn1*-positive cells are found throughout the epiblast layer (B, inset). By 11 h, the trigeminal placodes have converged toward the midline (C) and begin to express *neuroD* (D). Embryos are flat-mounted dorsal view in (C, D) with anterior to the top. Relationship of the trigeminal placode with cranial neural crest (E, F). At 11.5 h, the trigeminal placode (arrows, t) expresses *ngn1* (blue) and is located just lateral to the earliest differentiating neural crest cells (nc) marked with *foxd3* (red). In (F), individual trigeminal cells can be distinguished adjacent to the neural crest. Embryo is shown in flat-mount dorsal view with anterior to the top. Bar = 200 μm (A, B, E); 50 μm, (B, inset, F); 100 μm (C, D).

pression as they coalesce into a ganglion (Fig. 5B). In the vagal placode, cells express *ngn1* in the epithelial layer, but downregulate expression soon after delamination (Fig. 5C). Fewer cells express *neuroD* than *ngn1* in the epithelium, but *neuroD* expression remains high in the nascent ganglion (Fig. 5D).



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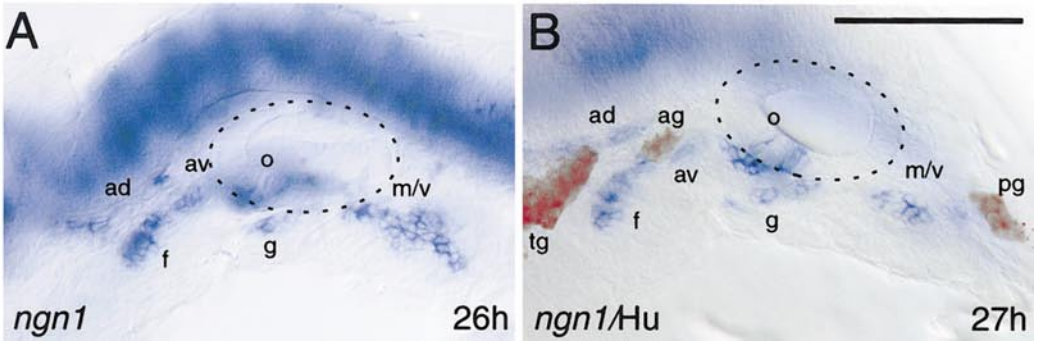


3

ngn1 Antisense Morpholino Oligonucleotides Block Formation of Peripheral Ganglia

To test the function of *ngn1,* we injected morpholino oligonucleotides complementary to the 5′ end of the *ngn1* mRNA. Morpholino oligonucleotides efficiently block translation of mRNAs to which they bind and have been used successfully to disrupt function in Xenopus (Heasman *et al.,* 2000), zebrafish (Nasevicius *et al.,* 2000), and avian (Kos *et al.,* 2001) systems. After injection of 5 ng of the *ngn1*-specific morpholino oligonucleotide (MO1), no overt morphological differences occur and embryos are indistinguishable from controls at all stages examined. However, disruption of cranial ganglion formation is clearly apparent after embryos are fixed and stained for specific markers (Fig. 6). After *ngn1* morpholino injection, *neuroD* expression is completely abolished in neurogenic placodes and associated neuroblasts in comparison to controls (Figs. 6A and 6B), while expression in the central nervous system and retina is largely unchanged. When embryos are processed for immunohistochemistry using the anti-Hu antibody, which delineates neuronal cell bodies, cranial ganglion neurons are absent (Figs. 6C and 6D). Ganglia formation is almost completely disrupted by morpholino injection; at 96 h, only a few scattered neurons are occasionally observed. In addition to cranial ganglia, *ngn1* is necessary for the formation of trunk sensory neurons (Figs. 6E and 6F); after injection of *ngn1* morpholino oligonucleotides, dorsal root ganglia do not form. Injected embryos are also touch-insensitive. We saw no effects on either cranial or dorsal root ganglia by anti-Hu staining after injection of 10 ng of a 4-bp mismatch oligonucleotide. These results demonstrate that *ngn1* is necessary for the formation of all zebrafish sensory ganglia.

The results of morpholino oligonucleotide injections are quantified in Table 1. Effects of different doses of morpholino oligonucleotides were assessed at 36 h by *in situ* hybridization for *neuroD* expression and at 72 h by immunohistochemistry for acetylated tubulin. Formation of cranial placodes/ganglia and dorsal root ganglia was disrupted by morpholino injection at 36 h. Expression is eliminated in dorsal root ganglia by injection of 0.5 ng *ngn1* morpholino oligonucleotide (MO1) per embryo. Higher doses are needed to completely eliminate expression in cranial placodes and ganglia, although some staining persisted in a few cells in a minority of embryos after injection of 10 ng. Development of the posterior lateral line ganglia was assessed at 72 h by examining the posterior lateral line nerve. The nerve was eliminated in a majority of embryos after injection of 0.5 ng of MO1 oligonucleotide per embryo, and was almost completely eliminated after injection of 5.0 ng. No effects were observed in any assay after injection of 10 ng of a morpholino oligonucleotide with a 4-bp mismatch, demonstrating specificity. A second nonoverlapping *ngn1* morpholino oligonucleotide (MO2) showed similar effects as MO1 at 0.5 ng per embryo; however, at higher doses this oligonucleotide proved to be toxic.

Lateral Line Primordia and Neuromasts Are Not Dependent on ngn1 Function

In addition to ganglia, the lateral line placodes give rise to the hair cells they innervate. However, blocking neurogenin function with morpholino oligonucleotides does not alter the formation or migration of the hair cell-generating lateral line primordia (Fig. 7). Lateral line primordia and sensory ridges can be identified by expression of the zebrafish *eyes absent* homologue *eya1* (Sahly *et al.,* 1999). Anterodorsal and anteroventral primordia/sensory ridges (Fig. 7A) and posterior lateral line primordium (Fig. 7B) in injected embryos are indistinguishable from uninjected controls (not shown). Even without innervation, neuromasts can still be found at 96 h (Figs. 7C and 7D). The neuromasts shown are those at the tip of the tail, indicating that the posterior primordium migrated completely to the caudal end of the embryo. Stereocilia are particularly prominent in Fig. 7D, demonstrating that hair cells differentiate in the absence of innervation.

Effect of ngn1 Morpholinos on PNS Glial Development

In the central nervous system, *ngn1* both promotes progenitor cells to acquire neuronal fates and inhibits glial cell fates (Nieto *et al.,* 2001; Sun *et al.,* 2001). We therefore examined whether blocking zebrafish *ngn1* function increased the differentiation of glial cells in the peripheral

FIG. 2. *ngn1* and *neuroD* expression reveals the positions of the dorsolateral placodes. Panels show lateral views of zebrafish heads, just posterior to the eye, with anterior left and dorsal up. Neurogenic dorsolateral placodes form at the edges of the otic placode/vesicle, outlined with a dashed line. a, anterior lateral line placode area; ad, anterodorsal lateral line placode/ganglion; av, anteroventral lateral line placode/ganglion; f, facial epibranchial placode/ganglion; g, glossopharyngeal epibranchial placode/ganglion; m, middle lateral line placode/ganglion; nc, neural crest; o, octaval/statoacoustic ganglion precursors; p, posterior lateral line placode/ganglion; t, trigeminal placode/ganglion; v, vagal epibranchial placode/ganglion. Bar, 100 μm.

FIG. 3. Spatial separation of anterior placodes. Anterior to the otic vesicle, *ngn1*-expressing anterodorsal, anteroventral, and facial placodes (blue) separate from an initially common area (see Fig. 2). The relationship of the placodes is apparent when embryos are stained with the anti-Hu antibody (red), which recognizes ganglion neurons. The otic vesicle is outlined with a dashed line. ad, anterodorsal lateral line placode; ag, anterodorsal lateral line ganglion; av, anteroventral lateral line placode; f, facial epibranchial placode/ganglion; g, glossopharyngeal epibranchial placode/ganglion; m, middle lateral line placode/ganglion; o, octaval/statoacoustic ganglion precursors; pg, posterior lateral line ganglion; tg, trigeminal ganglion; v, vagal epibranchial placode/ganglion. Bar, 100 μm.

TABLE 1
Effects of ngn1 Morpholino Oligonucleotide Injections

			Dorsal root ganglia		Cranial placodes and ganglia		
Oligo	Dose	*N*	Normal	Missing	Normal	Reduced	Missing
36 h *neuroD*							
Uninjected		238	97.8	2.2	100	0	0
4 bp mismatch	10 ng	124	97.6	2.4	100	0	0
MO1	0.5 ng	71	0	100	100	0	0
	10 ng	79	0	100	0	24.0	76.0
MO2	0.5 ng	90	7.8	92.2	100	0	0

			Lateral line nerve	
Oligo	Dose	*N*	Present	Absent
72 h Anti-acetylated tubulin				
Uninjected		125	100	0
4 bp mismatch	10 ng	97	100	0
MO1	0.5 ng	146	29.5	70.5
	5.0 ng	126	0.8	99.2
MO2	0.5 ng	38	39.5	60.5

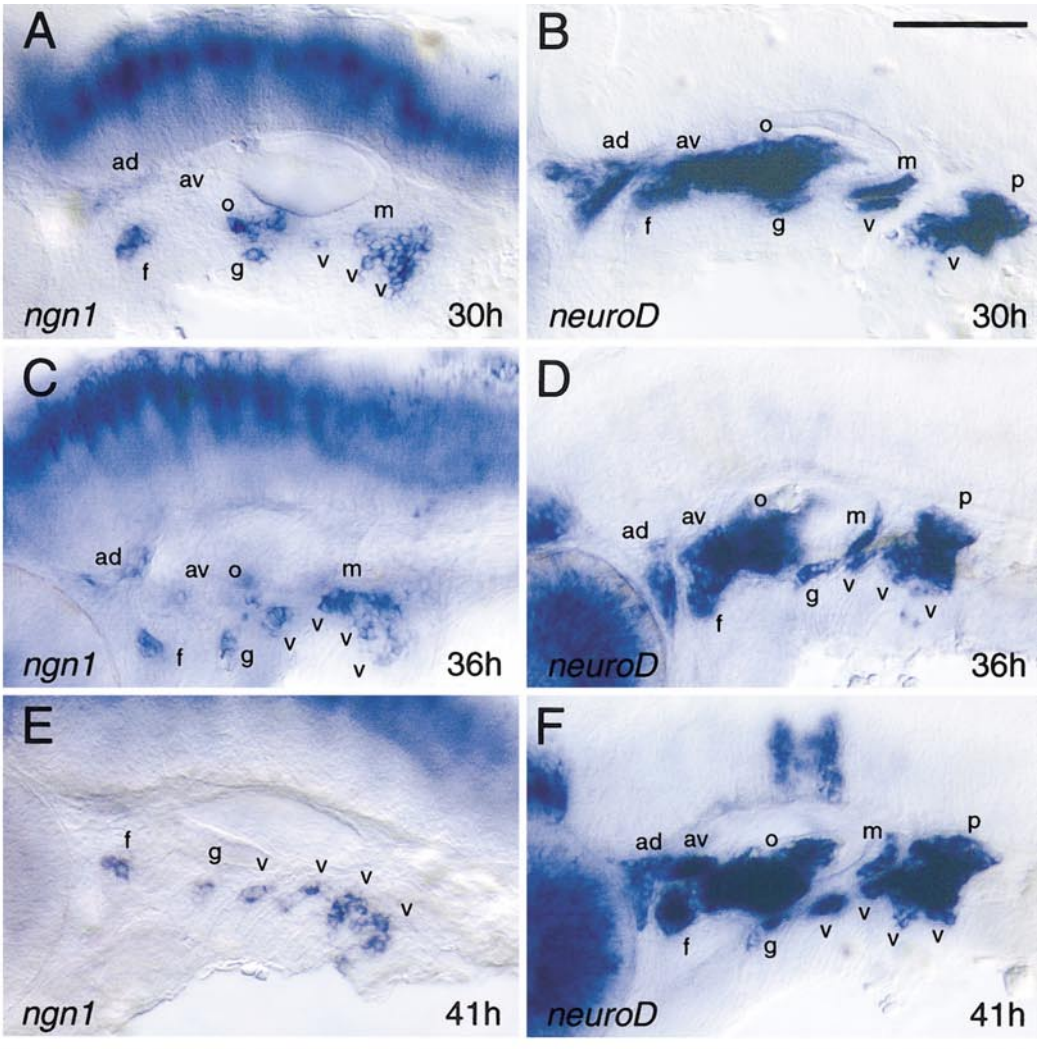
Note. Oligonucleotides were injected at the dose indicated. Some embryos were fixed at 36 h, processed for *in situ* hybridization with the *neuroD* probe, and scored for the presence of dorsal root ganglia or cranial ganglia. Other embryos were fixed at 72 h, stained with anti-acetylated tubulin antibody, and scored for the presence of the lateral line nerve. For each condition, the phenotypes are recorded as the percentage of the total number of embryos (*N*).

nervous system. The winged helix transcription factor *foxd3* (*fkd6*) is a good early marker for zebrafish neural crest-derived glial cells (Kelsh *et al.*, 2000). Blocking *ngn1* function did not increase the numbers of glial cells that initially differentiate, and instead there was consistently less *foxd3* staining anterior to the ear (Figs. 8A and 8B). After injection of *ngn1* morpholino oligoncleotides, glial cells cannot be found associated with the migrating primordium (Figs. 8C and 8D). Glial cells are also absent from the vicinity of the lateral line nerve, presumably because these nerve fibers are missing.

DISCUSSION

In the present study, we have used the expression of the bHLH transcription factors *ngn1* and *neuroD* to define the positions of the developing zebrafish neurogenic placodes. Zebrafish placodes are not reliably defined by using the conventional criterion of ectodermal thickening. At the initial stages of trigeminal placode formation, there appears to be no distinct layer from which cells delaminate; rather cells begin to express *ngn1* as a cluster that is several cells thick. It should be noted that the very early differentiation of the zebrafish trigeminal placode may use mechanisms different from that of other placodes since these events occur before the neuroectodermal border is morphologically defined. Cells expressing *ngn1* in the lateral line placodes form a loose association of scattered cells. The posterior vagal epibranchial placode forms at a time when a distinct layer of overlying ectoderm is present, but it is not distinguished as a region of thickening. We believe that expression of the bHLH genes is a better marker for placode formation than traditional morphological criteria. In addi-

FIG. 4. Development of epibranchial placodes. Each of the posterior pharyngeal pouches has an associated epibranchial placode, revealed by *ngn1* expression. Cells continue to express *neuroD* after *ngn1* is extinguished. ad, anterodorsal lateral line placode/ganglion; av, anteroventral lateral line placode/ganglion; f, facial epibranchial placode/ganglion; g, glossopharyngeal epibranchial placode/ganglion; m, middle lateral line placode/ganglion; o, octaval/statoacoustic ganglion precursors; v, vagal epibranchial placode/ganglion. Bar, 100 μm.
FIG. 5. Relationship between *ngn1* and *neuroD* expression in otic and vagal placodes. Transverse sections through the otic vesicle (A, B) and vagal placode (C, D) at 30 h are shown. *ngn1* is expressed in the otic vesicle (arrowheads, A) and surface ectoderm (arrowheads, C), but down-regulated in cells soon after they delaminate (arrows). In contrast, fewer cells express *neuroD* in the otic vesicle (arrowhead, B) and surface ectoderm (arrowheads, D), but continue to express *neuroD* as cells coalesce into ganglia (arrows). Bar, 100 μm.



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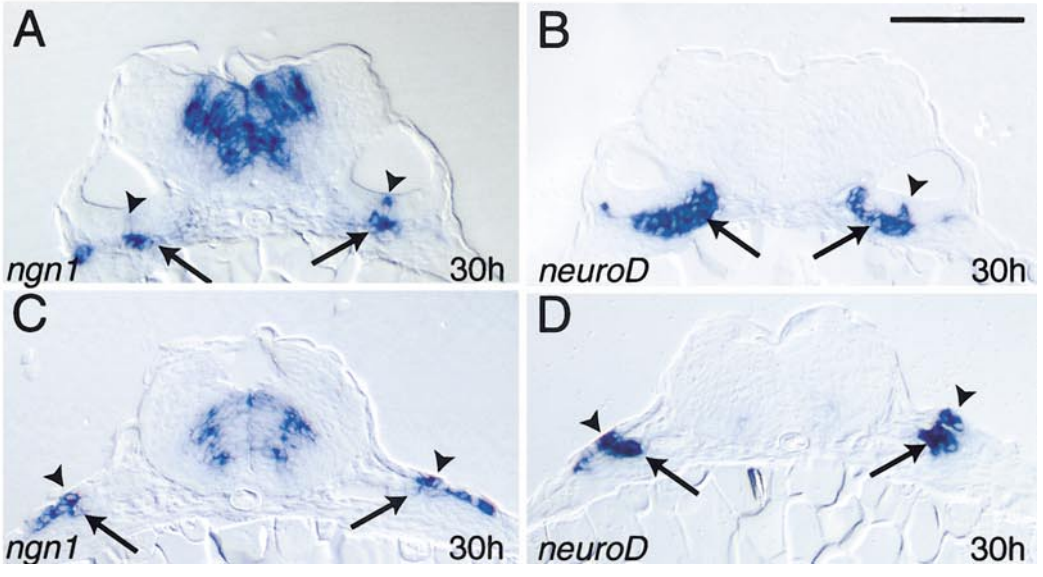

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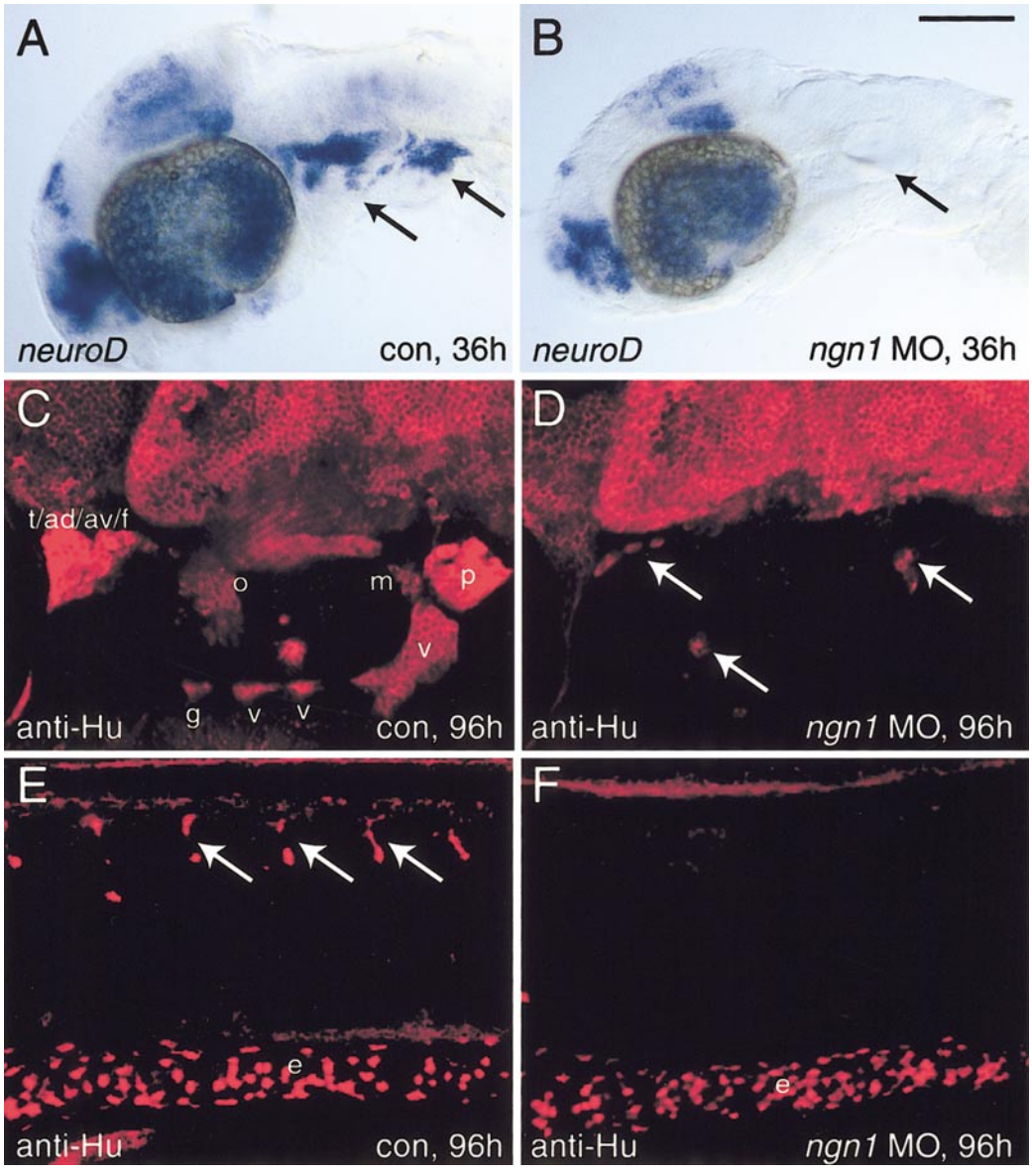


FIG. 6. *ngn1* morpholino oligonucleotides block differentiation of all cranial ganglia. Control embryos are shown in (A, C, E); *ngn1* morpholino injected embryos in (B, D, F). (A, B) Lateral flat mounts of embryos expressing *neuroD* at 36 h. After *ngn1* morpholino injection, *neuroD* expression is abolished in cranial ganglia (arrows) but not in other regions. (C, D) Lateral view of Hu expression in cranial ganglia at 96 h. In most cases, cranial ganglia are absent, but in some cases, a few neurons remain (arrows). (E, F) Lateral view of Hu expression in the zebrafish trunk at 96 h. The dorsal root ganglia (arrows) are eliminated by *ngn1* morpholino injection, while enteric neurons are unaffected. ad, anterodorsal lateral line ganglion; av, anteroventral lateral line ganglion; e, enteric neurons; f, facial ganglion; g, glossopharyngeal ganglion; m, middle lateral line ganglion; o, octaval/statoacoustic ganglion; p, posterior lateral line ganglion; v, vagal ganglia. Bar, 100 μm (A, B) and (E, F); 30 μm (C, D).

tion, the elimination of all placode-derived sensory neurons after morpholino oligonucleotide injection supports the use of *ngn1* expression as an accurate placode marker.

As observed in other systems (Fode *et al.*, 1998; Ma *et al.*, 1998), *ngn1* appears to regulate *neuroD* expression in zebrafish neurogenic placodes. Expression of *ngn1* always precedes *neuroD* expression in the same structure, sometimes by several hours. Placode expression of *neuroD* is also completely blocked by injection of the *ngn1* morpholino oligonucleotide. However, *neuroD* expression persists in several structures long after *ngn1* expression is normally extinguished, suggesting that other factors may be involved in its maintenance.

Placodes identified by expression criteria match the previously described organization of cranial ganglia. In this study, there is no evidence for a profundal placode associated with the trigeminal; although cells of the trigeminal placode are often loosely packed, we never observed two distinct clusters of cells. These results are consistent with the observation that in zebrafish a single ganglion gives rise to the nerves equivalent to the profundal and trigeminal (Metcalfe *et al.*, 1990). Lateral line placodes also appear to correspond to previously identified lateral line ganglia (Raible and Kruse, 2000). In zebrafish, two lateral line ganglia develop anterior to the ear, the anterodorsal and anteroventral, and two posterior to the ear, the middle and posterior. In some vertebrates, two additional placodes and ganglia are found: an otic lateral line placode anterior to the ear and a supratemporal placode posterior to the ear (Northcutt, 1992). Although lateral line structures equivalent to those derived from the otic and supratemporal placodes are formed in zebrafish, they appear to be innervated by rami of the anterodorsal and posterior nerves, respectively (Raible and Kruse, 2000). However, fusion of ganglia is common, and it is still possible that these structures were initially derived from separate placodes. While it should be noted that it would be difficult to distinguish a separate otic lateral line placode in the anterior area, a supratemporal placode would have been easy to identify. We have no evidence for the formation of these additional lateral line placodes.

Spatial Relationships among Placodes

The existence of a common placodal primordium from which cranial placodes are derived has been the subject of debate (for review, see Baker and Bronner-Fraser, 2001; Begbie and Graham, 2001a; Schlosser and Northcutt, 2000). The existence of a common primordium would suggest a two-step model for placode formation: the initial induction of the primordium and subsequent delineation of individual placodes. In zebrafish, expression of genes such as *dlx3* (Akimenko *et al.*, 1994), *eya1* (Sahly *et al.*, 1999), and *six4.1* (Kobayashi *et al.*, 2000) support the idea of a common placodal primordium: these genes are first expressed in a continuous horseshoe along the border of the anterior neural plate and then subsequently in placodes and their derivatives. Some aspects of placode development observed in the present study also support the idea of common placode origins. Subsets of placodes appear to form from common regions. The anterodorsal, anteroventral, and facial placodes appear to derive from a common region that includes the neurogenic region of the otic placode. The middle lateral line and vagal placodes cannot be distinguished as distinct via *ngn1* expression (although appear as such with *neuroD*). In addition, the distinct vagal epibranchial placodes extend from an initially contiguous patch of cells. However, different schedules of placode appearance may be used to argue against the two-step model. The timing of trigeminal placode formation also does not agree with the idea of a common primordium; this placode expresses *ngn1* significantly before a presumptive placode primordium is revealed by *eya1* expression. Additional embryological and genetic studies are needed to test the primordium model.

The apposition of neural crest and trigeminal placode may suggest developmental relationships between these cell types. Fate-mapping studies have demonstrated that the most lateral cells within the premigratory neural crest are those that will contribute neurons to the trigeminal ganglion (Schilling and Kimmel, 1994); thus, neurogenic crest cells may be in direct contact with placode cells. Interactions between these two cell populations may regulate their development; however, in avians, removal of one structure does not prevent formation of neurons derived from the other (Hamburger, 1961; Stark *et al.*, 1997), suggesting that if such interactions occur, they are not absolutely necessary for initial ganglion development. The adjacent location of neural crest and placode may also be the result of common environmental signals, such as BMPs that are involved in the development of both cell types (Barth *et al.*, 1999; Nguyen *et al.*, 1998, 2000). Interactions among crest- and placode-derived cells are important in later stages for proper axon pathfinding (Begbie and Graham, 2001b; Hamburger, 1961; Moody and Heaton, 1983). In addition, the results in this study are consistent with the idea that placode-derived axons are necessary for proper migration of neural crest-derived glial cells (Gilmour *et al.*, 2002).

Independence of Lateral Line Neuromasts and Ganglia

Our results demonstrate that lateral line neuromasts, derived from migrating lateral line primordia, can differentiate and persist for several days even in the absence of innervation from lateral line ganglia. Although the posterior lateral line nerve is closely associated with differentiating neuromasts and growth cones follow the migrating primordium (Metcalfe *et al.*, 1985; Gilmour *et al.*, 2002), interactions with the nerve are not necessary for neuromast differentiation. These results also suggest that placode-derived neuromast precursors develop in the absence of *ngn1* function, and thus that the two derivatives of the lateral line placodes, ganglia and migrating primordia, are under separate genetic control. However, it has been reported that the lateral line receives contributions from both placodes and neural crest (Collazo *et al.*, 1994), and it is thus possible that the neuromasts that differentiate in the absence of *ngn1* function form from neural crest and not from placode. The distribution of neuronal and neuromast precursors within the lateral line placodes and the resolution of this question will require additional fate-mapping experiments.

A Single Neurogenin Gene Is Required for All Zebrafish Sensory Ganglia Precursors

A single zebrafish *neurogenin* gene is necessary for the formation of all sensory ganglia, while in contrast, two genes are used in other vertebrates. In placode development, the function of a single zebrafish *neurogenin* appears to consist of the sum of functions of the two mouse *neurogenin* genes. In mouse, *ngn1* is required for the development of proximal ganglia, while *ngn2* is necessary for the development of the epibranchial ganglia (Fode *et al.*, 1998; Ma *et al.*, 1998). In chick, *ngn2* is expressed in the trigeminal ganglion, while *ngn1* is found in the epibranchials (Abu-Elmagd *et al.*, 2001). In *Xenopus*, *XNGNR1* (which is probably the orthologue of mouse *ngn2*; Sommer *et al.*, 1996) is expressed in profundal, trigeminal, and epibranchial placodes but not lateral line placodes (Schlosser and Northcutt, 2000). Presumably, a second unidentified *Xenopus neurogenin* family member is expressed in these structures. In zebrafish, *ngn1* appears to perform all the functions of the two neurogenins found in other vertebrates. Zebrafish *ngn1* is expressed in all cranial ganglion placodes, including those that in other animals would express *ngn2*. Furthermore, disruption of zebrafish *ngn1* function with antisense morpholino oligonucleotides blocks the differentiation of all the placode-derived ganglia. Zebrafish dorsal root ganglia development is also blocked by injection of *ngn1* morpholinos (Fig. 5; see also Cornell and Eisen, 2002), while in mouse, both *ngn1* and *ngn2* must be inactivated for dorsal root ganglion development to be fully disrupted (Ma *et al.*, 1999). We have not been able to isolate a zebrafish orthologue of *ngn2* despite substantive efforts, nor identify a *ngn2* orthologue in publicly available fugu sequences.

Other zebrafish cell types whose precursors express *ngn1* appear to be unaffected. Although dorsal root ganglia and Rohon–Beard sensory neurons in the dorsal spinal cord are also affected by morpholino injection, ventral motor neurons are not (Cornell and Eisen, 2002; J. U., unpublished results). In addition, while *neuroD* expression is completely blocked in cranial ganglia after morpholino injection, other aspects of its expression pattern remain unchanged. These results suggest that, during some embryonic processes outside the cranial ganglia, loss of *ngn1* function is compensated for by other gene(s).

The occurrence of a single gene in zebrafish where in other vertebrates there are two genes is unusual. Often two teleost genes correspond to a single tetrapod gene; by some estimates, 20–30 percent of zebrafish genes have duplicates (Postlethwait *et al.*, 2000; Robinson-Rechavi *et al.*, 2001). It has been hypothesized that the duplicates resulted from an ancient whole genome duplication that occurred soon after separation of ray-finned and lobe-finned fish lineages (Amores *et al.*, 1998; Taylor *et al.*, 2001). If a second *ngn1* gene arose from a wholesale genome duplication in ancestral ray-finned fishes, it was subsequently lost in zebrafish.

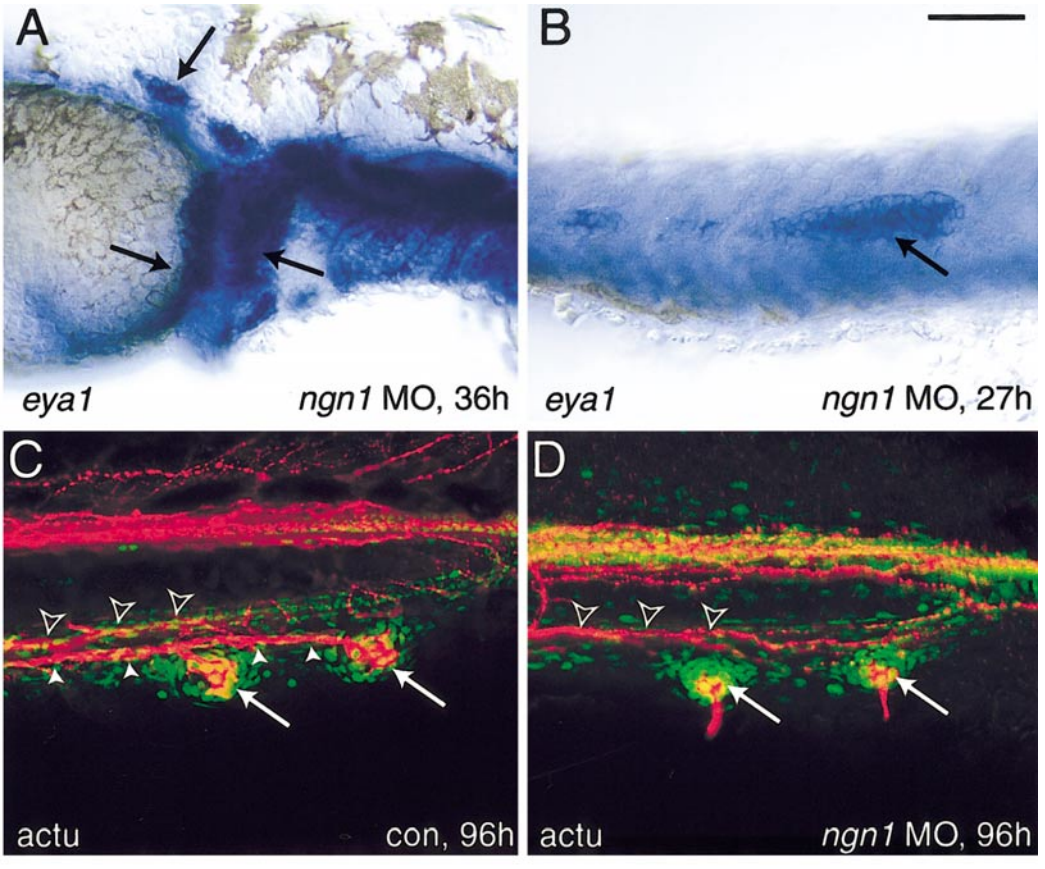
Separate Developmental Mechanisms for Lateral Line Ganglia and Sensory Neuromast Formation

The lateral line placodes give rise to both migrating primordia that lay down sensory neuromasts as well as the sensory ganglia that innervate them (Metcalfe *et al.*, 1985; Stone, 1922). The work presented here demonstrates that the lateral line structures are under separate genetic control: lateral line ganglia require *ngn1* function, while primordia and derived neuromasts do not. These results also demonstrate that although zebrafish migrating primordia are normally innervated by lateral line nerves (Gompel *et al.*, 2001b; Metcalfe, 1985; Gilmour *et al.*, 2002), formation of sensory neuromasts does not require this association. These results are in agreement with classical experiments demonstrating that neuromasts still form after denervation (Harrison, 1924; Stone, 1937; see Wright, 1951 for further discussion).

The expression of *ngn1* in the lateral line placodes may serve to select cells to delaminate from the placode to form ganglia, while the remaining cells coalesce into a migrating primordium. As noted above, *ngn1* is expressed in a loose association of cells in the lateral line placodes, and suggests that adjacent, *ngn1*-negative cells might form the migrating primordia. This differentiation scheme is analogous to that of the developing ear, where a small number of *ngn1*-positive cells delaminate from the otic vesicle to form the octaval/statoacoustic ganglion (Fig. 5; Haddon and Lewis,

FIG. 7. Lateral line development in the absence of *ngn1* function. Migrating anterior lateral line (A) and posterior lateral line (B) primordia (arrows) form normally after injection of *ngn1* morpholinos. Lateral line primordia are revealed by *in situ* hybridization for *eya1*. Injected embryos are indistinguishable from controls (not shown). Posterior lateral line primordia migrate to the tail and form normal neuromasts (C, D). The positions of neuromasts (arrows) are revealed by staining with anti-acetylated tubulin antibody (red) and sytox green (nuclear counterstain, green). Neuromasts are innervated by the posterior lateral line nerve (solid arrowheads) that in the tail is located just ventral and lateral to the ventral lateral fasiculus (outline arrowheads; Kuwada *et al.*, 1990). Bar, 100 μm.

FIG. 8. Glial cell differentiation after *ngn1* morpholino injection. (A, B) Neural crest-derived glial cells initially differentiate despite the lack of cranial ganglia. In control embryos (A), glial cells found in association with cranial ganglia (arrows) on either side of the otic vesicle (ot) express *foxd3*. In injected embryos, glial cells are found in approximately the same positions, although sometimes *foxd3* expression is lower. (C) Glial cells (arrows) are closely associated with the lateral line nerve and migrating primordium (outlined by arrowheads), but are missing after *ngn1* morpholino injection. Bar, 100 μm.



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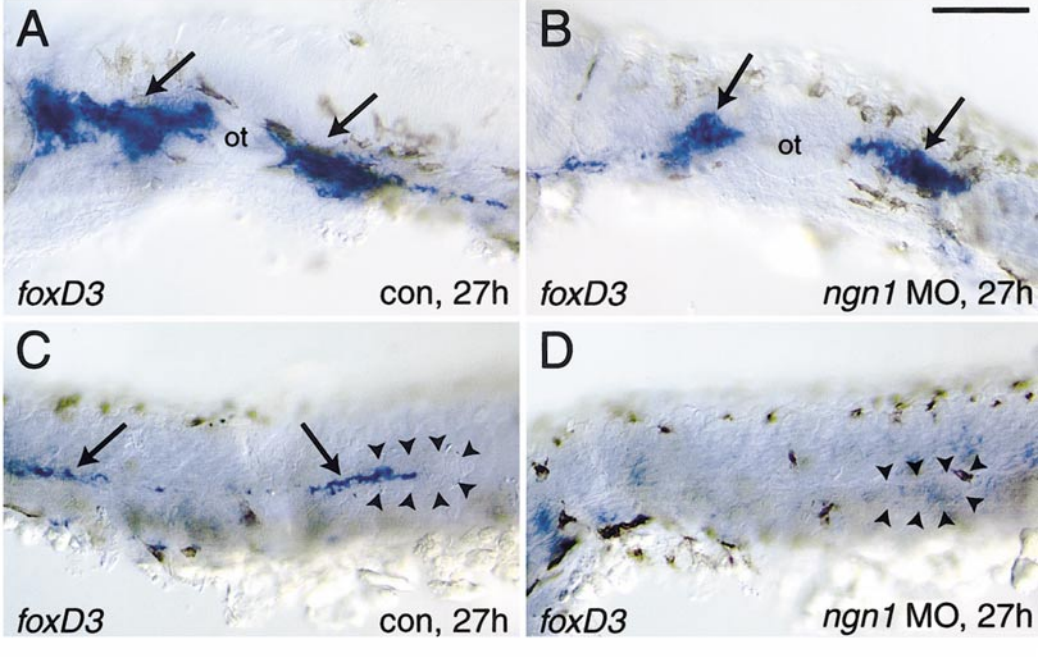

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1996). Understanding the regulation of *ngn1* expression will shed light on how different placode cells are distinguished.

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