Cadherin-4 Plays a Role in the Development of Zebrafish Cranial Ganglia and Lateral Line System

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We previously reported that cadherin-4 (also called R-cadherin) was expressed by the majority of the developing zebrafish cranial and lateral line ganglia. Cadherin-4 (Cdh4) function in the formation of these structures in zebrafish was studied using morpholino antisense technology. Differentiation of the cranial and lateral line ganglia and lateral line nerve and neuromasts of the *cdh4* morphants was analyzed using multiple neural markers. We found that a subset of the morphant cranial and lateral line ganglia were disorganized, smaller, with reduced staining, and/or with altered shape compared to control embryos. Increased cell death in the morphant ganglia likely contributed to these defects. Moreover, *cdh4* morphants had shorter lateral line nerves and a reduced number of neuromasts, which was likely caused by disrupted migration of the lateral line primordia. These results indicate that Cdh4 plays a role in the normal formation of the zebrafish lateral line system and a subset of the cranial ganglia. *Developmental Dynamics 236:893–902, 2007.*

Key words: zebrafish; development; lateral line nerve; neuromasts; cell adhesion molecules; cranial nerves

Accepted 9 January 2007

INTRODUCTION

The vertebrate cranial ganglia and lateral line systems originate from neural crest and epidermal placodes (Northcutt and Gans, 1983; Hall, 1999). The zebrafish cranial ganglia, located ventrolateral to the hindbrain, consist of the trigeminal (gV), facial (gVII), statoacoustic (gVIII), glossopharyngeal (gIX), and vagal (gX) ganglia (Raible and Kruse, 2000). The gV becomes recognizable as early as 9 hr post fertilization (hpf), the earliest of the cranial ganglia (Andermann et al., 2002). The gVIII is formed from delaminating otic epithelium between 22–30 hpf (Haddon and Lewis, 1996). The remaining cranial ganglia (gVII and gX) are formed later (after 36 hpf). The zebrafish lateral line system contains four ganglia and sets of neuromasts (Metcalfe, 1985, 1989; Raible and Kruse, 2000). The posterior lateral line ganglion (gP), caudal to the otic vesicle, can be recognized as early as 14 hpf (Andermann et al., 2002). The anterodorsal ganglion (gAD) and anteroventral ganglion (gAV), located anterior to the otic vesicle, develop from the anterior lateral line placode area around 24 hpf, while the middle lateral line ganglion (gM), located anteromedial to the gP, develops after 26 hpf (Andermann et al., 2002). The gAD and gAV partially fuse with gV and the gVII, respectively, during development (Higashijima et al., 2000; Raible and Kruse, 2000). The neuromasts on the head, jaw, and opercle originate from the anterior lateral line (ALL) primordium, while the neuromasts caudal to the otic vesicle develop from the posterior lateral line primordium (PLLp) (Metcalfe, 1985, 1989; Raible and Kruse, 2000).

Despite the detailed anatomical knowledge of the developing zebrafish cranial ganglia and lateral line sys-

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Grant sponsor: NIH; Grant numbers: RO1 DC006436, RO1 DC05939, DC04184, R15 EY13879, T32 DC00011.
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DOI 10.1002/dvdy.21085
Published online 6 February 2007 in Wiley InterScience (www.interscience.wiley.com).

tem, the molecular mechanisms underlying the formation of these structures are still largely unknown. Transcription factors neurogenin, foxd3 and NeuroD (Fode et al., 1998; Ma et al., 1998; Kim et al., 2001; Andermann et al., 2002), chemokine guidance receptor Cxcr4b (Knaut et al., 2005; Haas and Gilmour, 2006), and cell adhesion molecule cadherin-2 (Kerstetter et al., 2004) have been implicated in the development of the cranial ganglia and/or lateral line system.

Cadherins are cell surface molecules that mediate cell adhesion mainly through homophilic interactions (Takeichi, 1991; Gumbiner, 1996). Cadherin-4 (Cdh4), also called R-cadherin, is a member of the type-I classical cadherin subfamily (Redies, 1995; Nollet et al., 2000). Cdh4 expression and/or function in the vertebrate nervous system has been studied in a variety of vertebrate species including zebrafish, *Xenopus*, chicken, and mouse (Inuzuka et al., 1991; Redies et al., 1992; Ganzler and Redies, 1995; Matsunami and Takeichi, 1995; Tashiro et al., 1995; Liu et al., 1999; Wohrn et al., 1998; Gerhardt et al., 2000; Honjo et al., 2000; Treubert-Zimmermann et al., 2002; Andrews and Mastick, 2003; Babb et al., 2005). We previously showed that Cdh4/*cdh4* is expressed in the majority of the cranial and lateral line ganglia and their nerves in the developing zebrafish (Liu et al., 2003). We hypothesize that interfering with Cdh4 function disrupts formation of these structures. This study was designed to test this hypothesis.

RESULTS AND DISCUSSION

Cdh4 Is Involved in the Formation of the Cranial and Lateral Line Ganglia

Morpholino antisense oligonucleotide techniques have been successfully employed in zebrafish to study gene function in various tissues and organs including the cranial and/or lateral line system (Ekker, 2000; Nasevicius and Ekker, 2000; Andermann et al., 2002; Kerstetter et al., 2004; Knaut et al., 2005). Injection of *cdh4*-specific antisense morpholino oligonucleotides (*cdh4* MOs: RcadMphA, 0.25 mM, 2.1 ng/embryo, or RcadMphB, 0.12 mM, 1.0 ng/embryo), into one- to four-cell stage zebrafish embryos greatly reduced Cdh4 protein levels in the injected embryos at 50–55 hpf (Fig. 1; also see Babb et al., 2005). At 24 hr post fertilization (hpf), the injected embryos (*cdh4* morphants) were similar in body shape and size to control (uninjected) embryos. By 50–55 hpf, the majority of the *cdh4* morphants (716/867, 86.6%) showed a similar gross morphology to the control embryos, except that the *cdh4* morphants had eyes of reduced size, although some were only slightly smaller, and some had slightly ventrally curled tails. These embryos were similar to the moderately to severely affected embryos shown in our previous study (Babb et al., 2005). Moreover, zn5 (an antibody that labels differentiating neurons and their processes) or acetylated tubulin antibody (that labels α-tubulin) immunostaining demonstrated that these embryos had a much reduced retinal ganglion cell layer and retinal axons, as reported in our study of Cdh4 function in zebrafish visual system development (Babb et al., 2005). Embryos injected with lower concentrations of the MOs (0.125 mM, 1.0 ng/embryo for RcadMphA, 0.06 mM, 0.5 ng/embryo for RcadMphB) were largely indistinguishable from control embryos. Injection with a 5-mismatch control MO (5-mis MO) at similar concentrations to *cdh4* MOs (e.g., 0.25 mM, 2.1 ng/embryo) resulted in embryos that were morphologically indistinguishable from uninjected embryos.

Cdh4 immunostaining of *cdh4* morphants demonstrated that Cdh4 expression levels were greatly reduced throughout the embryos, specifically in the brain, cranial, and lateral line ganglia at 55 hpf (Fig. 1C) compared to the control embryos (Fig. 1A) or embryos injected with the 5-mis MO (Fig. 1B). Expression of cadherin-2 (N-cadherin), a closely related cadherin molecule, in *cdh4* morphants appeared to be normal (data not shown).

In our previous study of cadherin expression in the cranial ganglia and lateral line system of developing zebrafish, we found that the trigeminal (gV), anterodorsal ganglia (AD), and posterior lateral line ganglion (gP) began to express *cdh4* by 32 hpf, the statoacoustic ganglion (gVIII) became *cdh4*-positive by 29 hpf (our most recent study showed that the gVIII expressed *cdh4* at an earlier stage, 26 hpf; Q. Liu and A.L. Wilson, unpublished observation), while the vagal ganglion (gX) was Cdh4 immunoreactive at 45 hpf (Liu et al., 2003). Using multiple markers, we analyzed organization of these structures in *cdh4* morphants, and compared it with that of control embryos and those injected with the 5-mis MO.

Anti-Hu immunostaining (which labels cell bodies of differentiating neurons, Raible and Kruse, 2000) showed that in *cdh4* morphants cranial and lateral line ganglia had a similar appearances to those of control embryos at 30 hpf (Fig. 2A and B; Table 1), except that the morphant gVIII was slightly smaller than control gVIII. This was not surprising since *cdh4* expression is detected in the gVIII of slightly younger embryos (26 hpf), and not observed in other ganglia until 32 hpf (Liu et al., 2003). In contrast, disruption of cadherin2 expression (expressed throughout the development of most of the cranial and lateral line ganglia; Liu et al., 2003) in developing zebrafish resulted in detectable abnormalities in these ganglia as early as 25 hpf (Kerstetter et al., 2004). However, by 50–55 hpf, development of the ganglia was disrupted in the vast majority of *cdh4* morphants (Table 1). In the *cdh4* morphants, ganglia were smaller, altered in shape, and/or became a little fragmented compared to control or 5-mis MO injected embryos (Fig. 2C–E). The zn5 antibody strongly labels the gV, gAD, and gVIII at 36–40 hpf (Fig. 2F–K). Zn5 staining was evidently reduced (smaller zn5 positive region and/or reduced staining) in the gVIII of *cdh4* morphants at 40 hpf, while the staining in the gV/AD was only slightly to moderately altered (we observed a changed shape and/or reduced staining) (Fig. 2F–K; Table 1). At 40–55 hpf, the gX and gP are well-labeled with *NeuroD* (Andermann et al., 2002), while the gX, gM (the middle lateral line ganglion), and gP are *cadherin6* positive (Liu et al., 2006). Similar to the anti-Hu staining, *NeuroD* or *cadherin6* expressing gX and gP were smaller and/or less distinct in *cdh4* morphants (Fig. 3B and D), than in control embryos (Fig. 3A and C), although there was no

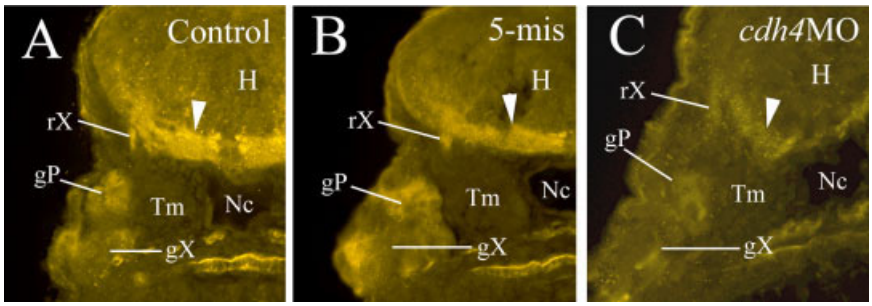

Fig. 1. Cdh4 immunostaining showing that Cdh 4 protein expression levels are greatly reduced in a *cdh4* morphant injected with RcadMphA (*cdh4*MO, **C**) compared to a control embryo (**A**) or a 5-mis MO-injected embryo (5-mis, **B**). All panels are cross-sections in the hindbrain region (dorsal is up) at the level of posterior lateral line ganglion and vagal ganglion (55 hpf). The arrowhead points to Cdh4 immunoreactive fiber tracts in the ventral hindbrain. gP, posterior lateral line ganglion; gX, vagal ganglion; H, hindbrain; Nc, notochord; rX, vagal root; Tm, trunk muscles.

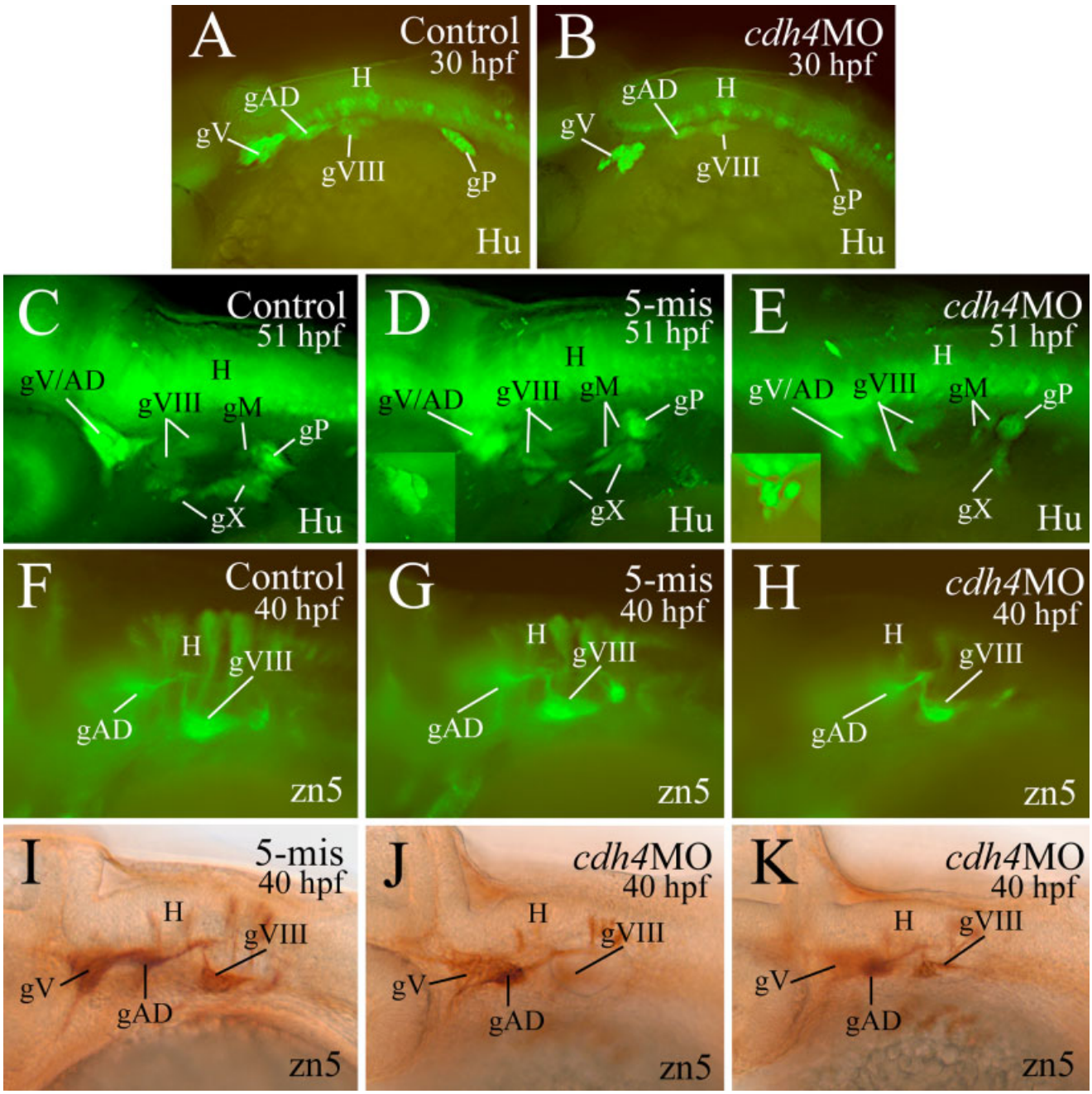

Fig. 2. Cranial and lateral line ganglia as revealed by anti-Hu immunostaining (Hu, **A–E**) and zn5 immunostaining (**F–K**). All panels are lateral views of whole mount zebrafish embryos with anterior to the left and dorsal up. A–H are from immunofluorescent methods, while panel I–K are from immunoperoxidase methods. The *cdh4* morphant in H was from RcadMphB injections, while the remaining morphants were from RcadMphA injections. The trigeminal (gV) and anterodorsal lateral line ganglia (gAD) in D and E were out of focus, and their focused images are shown in their respective insets. J and K are from the same embryo with the former panel focusing on gV and gAD, while the latter one focuses on the statoacoustic ganglion (gVIII). gM, medial lateral line ganglion. Other abbreviations are the same as in Figure 1.

TABLE 1. Effects of *cdh4* MOs Injection on Cranial and Lateral Line Ganglia Development[a]

	gV/AD (%)	gVIII (%)	gX (%)	gP (%)
30 hpf anti-Hu *cdh4* morphants (n_1 = 20)	10	65		0
Control (n = 20)	0	5		0
40 hpf zn5 *cdh4* morphants (n_1 = 20, n_2 = 12)	70 (75)	85 (83.3)		
Control (n = 20)	0	0		
50–55 hpf anti-Hu *cdh4* morphants (n_1 = 42, n_2 = 20)	85.7 (80)	81 (85)	100 (95)	85.7 (95)
Control (n = 20)	0	10	5	5
5-mis MO (n = 40)	7.5	5	5	7.5

[a]n, Number of ganglia examined; n_1 and n_2, the numbers of embryos injected with RcadMphA and RcadMphB, respectively. %, percentages of abnormally formed ganglia (e.g., smaller size, altered shape, and/or reduced staining compared to the majority of control embryos). The percentages in parentheses are from RcadMphB-injected embryos.

TABLE 2. Effects of *cdh4* MOs Injection on Cranial and Lateral Line Nerves Development[a]

	gV/AD nerves (%)	gX nerves (%)	nP (%)
Anti-acetylated tubulin 50–55 hpf *cdh4* morphants (n_1 = 38, n_2 = 18)	71 (83.3)[b]	100 (100)	94.7 (100)
Control (n = 20)	0	5	0
5-mis MO (n = 20)	5	5	0
72–74 hpf *cdh4* morphants (n_1 = 20)	85	100	90
Control (n = 20)	0	0	0

[a]n, Number of ganglion nerves (or collection of nerves for gV/AD) examined; n_1 and n_2, the numbers of embryos injected with RcadMphA and RcadMphB, respectively. %, percentages of abnormally formed nerves (e.g., thinner, shorter, reduced staining, and/or missing branches compared to the majority of control embryos). The percentages in parentheses are from RcadMphB-injected embryos.
[b]The defects in the gV/AD nerves are milder compared to those of gX.

obvious difference in *cadherin6* staining of the gM between the control embryos and morphants (Fig. 3C and D).

To visualize developing zebrafish cranial and lateral line nerves, we used anti-acetylated tubulin immunostaining (Fig. 4, Raible and Kruse, 2000). At 50–55 hpf, several distinct axonal bundles were seen to exit the gV/AD and projected anterodorsally (the superior ophthalmic ramus of the anterodorsal lateral line nerve (nADso) and dorsolateral nerve of trigeminal ganglion (nVDl, Fig. 4A), or anteroventrally (the buccal ramus of the anterodorsal lateral line nerve (nADb) and the mandibular ramus of the anteroventral lateral line nerve (nAVm, Fig. 4A, Raible and Kruse, 2000). Those nerves were present in *cdh4* morphants, but they were thinner in process size and weaker in staining. Moreover, the distance between nADb and nAVm was larger in the morphants than in the control (Fig. 4A and C). The gX of control embryos at 50–55 hpf was a conspicuous ganglion with a thick central projecting vagus root and several peripheral nerves (Fig. 4B). In contrast, the gX of *cdh4* morphants was smaller and had much reduced peripheral nerves, while the vagus roots appeared similar to that of the control (Fig. 4D; Table 2). The above defects were unlikely due to a general developmental delay in *cdh4* morphants, because most of the morphants were similar in size and shape to control or 5-mis MO injected embryos. Moreover, these defects persisted in older morphants (72–74 hpf, Table 2).

To assess whether or not increased cell death in *cdh4* morphants contributed to the cranial and lateral line ganglia defects, we analyzed cell death in both control (uninjected) embryos and *cdh4* morphants at 40 hpf by using whole mount TUNEL staining. This developmental stage was chosen because Cdh4/*cdh4* is expressed in these ganglia between 26 and 40 hpf. Quantitative data were obtained from two ganglia, the gV and gP, because they were easier to identify in the embryos processed for TUNEL staining at this stage. Control embryos showed very little cell death in these ganglia, while *cdh4* morphant ganglia contained significantly higher numbers of TUNEL-positive cells (Fig. 5). Large variations in the number of TUNEL-positive cells in the ganglia of both control embryos and *cdh4* morphants likely resulted from the small sizes of the cranial and lateral line ganglia. Increased cell death was also detected in the *cdh4* morphant retina (Babb et al., 2005).

In *cadherin2* morphants or in a *cadherin2* mutant *glass onion*, the gV/AD and their nerve defects are much more severe, with highly fragmented ganglia, defasciculated, missing, or unrecognizable nerves (Kerstetter et al., 2004), compared to *cdh4* morphants. On the other hand, the severity of gX defects in *cdh4* morphants, *cadherin2* morphants, and *glass onion* mutants is similar. The differences may be due, at least in part, to differences in the timing of ganglion development: the zebrafish gV can be recognized as early as 9 hpf (before *cdh4* expression), and by 32 hpf, when *cdh4* is beginning to be expressed, some of the

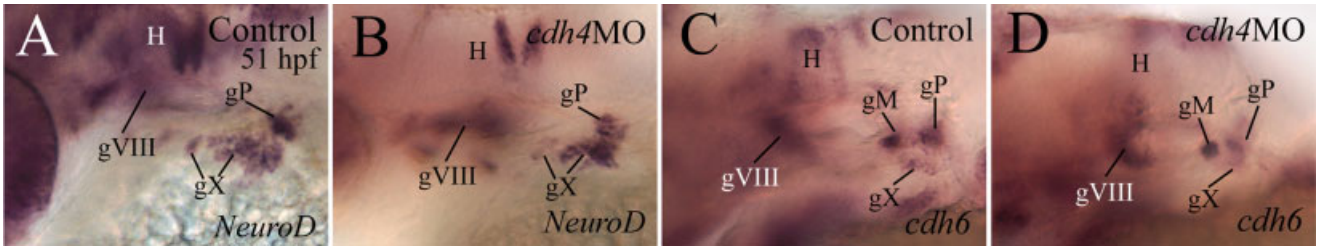

Fig. 3. Lateral views of the hindbrain region of whole mount embryos (anterior is to the left and dorsal is up) processed for in situ hybridization using *NeuroD* (**A,B**) or *cadherin6* (*cdh6*, **C,D**) cRNA probes. The *cdh4* morphants were from RcadMphA injections. Abbreviations are the same as in Figures 1 and 2.

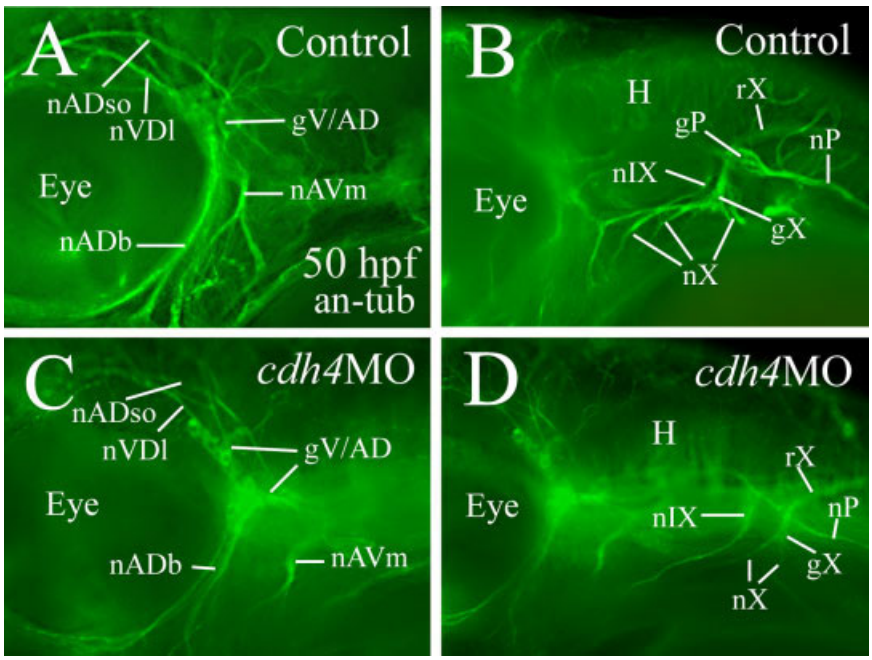

Fig. 4. Cranial and lateral line ganglion nerves in a control embryo (**A,B**) and an embryo injected with RcadMphA (**C,D**), as demonstrated by anti-acetylated tubulin immunostaining (an-tub). All panels are lateral views of the hindbrain region with anterior to the left and dorsal up. A and B, C and D are from the same embryos, respectively, with A and C focusing on the gV/AD nerves, and B and D focusing on the gX and gP nerves. nADb, buccal ramus of the anterodorsal lateral line nerve; nADso, superior ophthalmic ramus of the anterodorsal lateral line nerve; nAVm, mandibular ramus of the anteroventral lateral line nerve; nIX, glossopharyngeal nerve; nP, posterior lateral line nerve; nVDl, dorsolateral nerve of the trigeminal ganglion; nX, vagus nerve; rX, vagus root. Other abbreviations are the same as in Figures 1 and 2.

major axonal bundles are established. In contrast, the gX does not become a distinct ganglion until after one and a half days post fertilization (Higashijima et al., 2000; Raible and Kruse, 2000; Andermann et al., 2002). In addition, the differences are probably due to the differential expression and functioning of these two cadherins.

Cdh4 Is Involved in the Organization of the Lateral Line System

Growth cones of the zebrafish posterior lateral line nerve (nP) begin to emerge from the posterior lateral line ganglion (gP) around 20 hpf, and reach approximately 1/3 and 2/3 of the body trunk by 26 hpf and 30 hpf, respectively. They arrive in the tail by 46–48 hpf (Metcalfe, 1985, 1989). The expression pattern of Cdh4/*cdh4* in the gP and nP of developing zebrafish (Liu et al., 2003) suggests that Cdh4 participates in the formation of the ganglion (see above) and extension of the nerve. Anti-acetylated tubulin immunostaining showed that the nP reached the tail region of all control and 5-mis MO-injected embryos at 50–55 hpf (Fig. 6A; Table 2), but was much shorter (e.g., it ended at the level of the posterior end of the yolk ball, or the anus), although it was straight as in the control, in the majority of the *cdh4* morphants. This was unlikely due to a general developmental delay in the morphants because the control and 5-miss MO-injected embryos and *cdh4* morphants were similar in shape and size (Fig. 6), and the nP remained short in about 2/3 (13/20) of older morphants (72–74 hpf). Moreover, even in those morphants in which the nP reached the tail region, the nerve was thinner than in the control embryos. The nP defect in the morphants correlates well with Cdh4/*cdh4* expression in the system (Liu et al., 2003), suggesting that Cdh4 is involved in the extension of the nerve. Moreover, the nP defects in the *cdh4* morphants are different from those of embryos with disrupted cadherin-2 function, in which the nP had greatly altered trajectories (curved or turned around), instead of reduced length (Kerstetter et al., 2004).

The growth cones of nP in developing zebrafish are always found to be associated with the primordium of the posterior lateral line (PLLp) (Metcalfe, 1989), and pathfinding of the nP depends on the migrating PLLp (Gilmour et al., 2004). The PLLp begins to migrate caudally around 20 hpf (Metcalfe, 1989; Gompel et al., 2001), and as it migrates, it deposits along the horizontal myoseptum six to eight pairs of proneuromasts (L1 to L7), all innervated by the nP, that are spaced at regular intervals (Metcalfe, 1989; Gompel et al., 2001). The PLLp reaches the tip of the tail by 46–48 hpf, where it stops migration and constitutes the terminal neuromasts (Gompel et al., 2001).

Because of the close association between the nP and neuromasts, the defects in the *cdh4* morphant nP suggest that the organization of the neuromasts in these morphants might also be disrupted. Using DASPEI staining, which labels neuromasts in live embryos/larvae (Bricaud et al., 2001), we examined the organization of the *cdh4* morphant neuromasts. In the posterior lateral line system of control embryos or 5-mis MO-injected embryo at 50–55 hpf, there were five to six neuromasts on each side of the trunk and tail. The neuromast numbers in the posterior lateral line system were significantly reduced in all *cdh4* morphants (Table 3). In about one third of the morphants (n = 13), there were only one or two neuromasts on each side of the trunk, and in the remaining morphants (n = 24), there were three or four neuromasts on each side of the trunk and/or tail. Moreover, in all but two morphants the neuromasts were not detected in the tail region. To determine if the reduced number of neuromasts was mainly due to a general developmental delay, we examined older morphants (72–74 hpf, n = 24), and found that their neuromast numbers were significantly smaller than control embryos (n = 11) (Table 3). Moreover, the average neuromast numbers found in 72–74 hpf morphants (3.7 ± 1.3) was significantly lower ($P < 0.001$) than that of either younger (50–55 hpf), control (5.4 ±

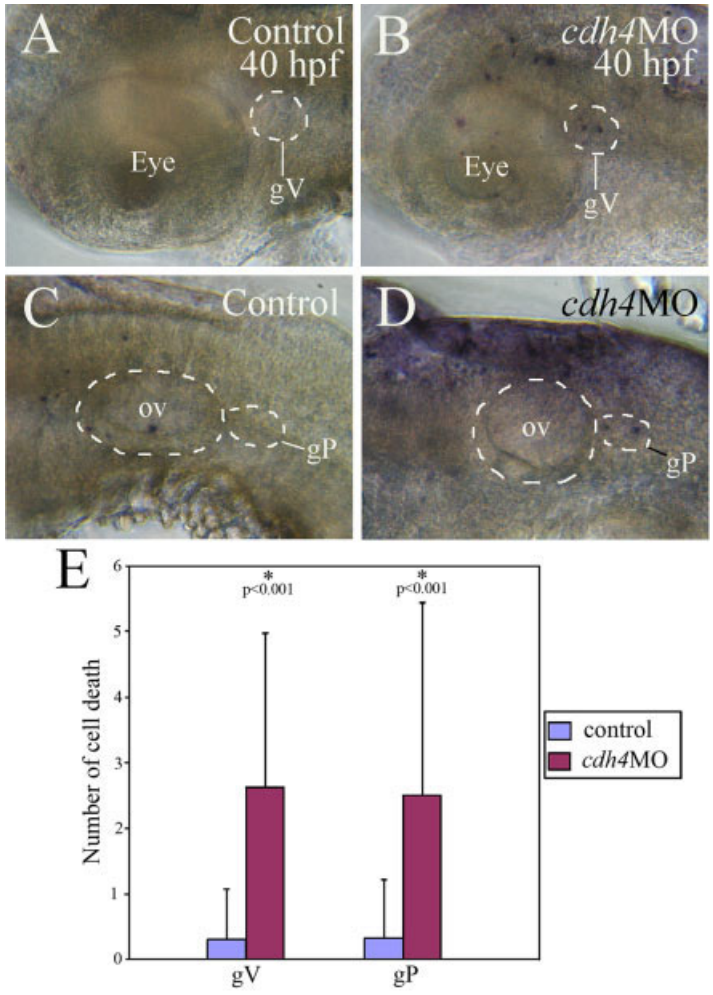

Fig. 5.

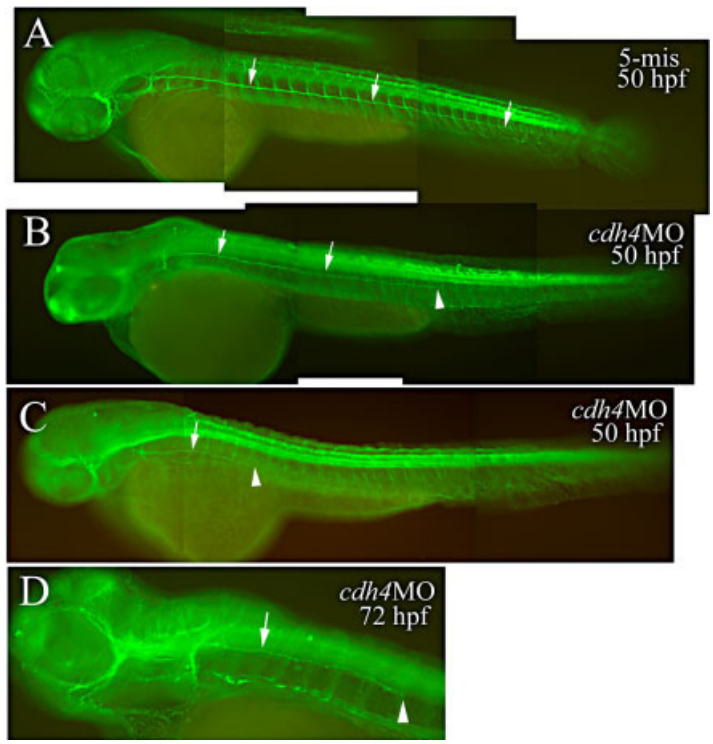

Fig. 6.

Fig. 5. TUNEL staining of whole mount embryos demonstrates increased cell death in *cdh4* morphants trigeminal ganglion (gV) and posterior lateral line ganglion (gP). **A–D:** Lateral views of the embryo head (anterior to the left and dorsal up), with A and B focusing on the eye and gV, and C and D focusing on the otic vesicle (ov) and gP. **E:** Cell death was significantly greater ($P < 0.001$) in both the gV and gP of *cdh4* morphants (RcadhMphA injected, n = 26) than those of control embryos (n = 37).

Fig. 6. The posterior lateral line system in a 5-mis MO-injected embryo (A) and embryos injected with RcadMphA (B–D) as revealed by anti-acetylated tubulin immunostaining. **A–C:** Lateral views of whole embryos (50 hpf, anterior to the left and dorsal up) of the same magnification. **D:** A higher magnification of lateral view of the anterior half of an older *cdh4* morphant (72 hpf, anterior to the left and dorsal up). In all panels, arrows point to the lateral line nerve, while the arrowhead indicates the terminus of the lateral line nerve.

TABLE 3. Effects of *cdh4* MO (RcadMphA) Injection on Neuromasts Development

	Average number of neuromasts in the PLL system	Average number of neuromasts in the ALL system
DASPEI staining 50–55 hpf *cdh4* morphants (N = 37)	2.8 ± 0.8	1.9 ± 0.5
Control (N = 15)	5.4 ± 0.4*	6.6 ± 0.4*
5-mis MO (N = 15) 72–74 hpf	5.4 ± 0.5*	6.6 ± 0.5*
cdh4 morphants (N = 24)	3.7 ± 1.3	5.5 ± 1.3
Control (N = 11)	7.7 ± 0.5*	14.5 ± 0.4*

[a]N, Number of embryos examined. The averages are from both sides of the embryos. ALL, anterior lateral line system; PLL, posterior lateral line system.

*The number is significantly greater ($p < 0.001$) than that of *cdh4* morphants of the same stage. Moreover, the numbers of younger control or 5-mis-injected embryos were significantly greater ($p < 0.001$) than those of older *cdh4* morphants.

0.4), or 5-mis MO-injected embryos (5.4 ± 0.5). Development of 10 of the *cdh4* morphants was monitored from 55 hpf to 74 hpf (each of the morphants was kept in a separate container). During this period, the neuromast numbers were either unchanged (n = 3, 30%) or increased only by one (n = 6, 60%) in these morphants. The remaining morphant had four pairs of neuromasts at 55 hpf, which increased to five on the left side, and to six on the right side of the trunk and tail. Similar to the younger morphants, no neuromasts were found in the tail region in the majority of these morphants (n = 7, 70%).

We also examined the neuromasts in the head region, which develop from the anterior lateral line primordium. There were 14 to 15 neuromasts on each side of the head by 72 hpf in control (Raible and Kruse, 2000) or 5-mis MO-injected embryos (Fig. 7C; Table 3), but there were only about 5 to 7 neuromasts on each side of the morphant head (Fig. 7D; Table 3). Similar to the posterior lateral line system, there were significantly more neuromasts ($P < 0.001$) in the head of younger (50–55 hpf) control or 5-mis MO-injected embryos than those of older morphants (72–74 hpf).

To determine whether the reduced numbers of neuromasts in the posterior lateral line system of *cdh4* morphants were due mainly to disrupted formation, migration of the posterior lateral line primordium (PLLp), and/or deposition of the PLLp, we analyzed PLLp formation and migration in younger (26 and 34 hpf) and older (46 hpf) embryos. *cadherin2*, which labels both PLLp and differentiating neuromasts (Liu et al., 2003), or *cxcr4b*, which labels PLLp (Chong et al., 2001), were used in these experiments. Formation, migration, and deposition of the PLLp appeared to be similar in control embryos and *cdh4* morphants in younger embryos (26 and 34 hpf, Fig. 8). As development proceeds, the PLLp in control embryos continues migrating caudally, and depositing neuromasts as it migrates. By 46 hpf, the PLLp reached to the tail and gave rise to the terminal neuromasts in the control embryos (Fig. 8C), but the PLLp in 46 hpf *cdh4* morphants remained in the same region or migrated slightly caudal compared to its position in the younger embryos (Fig. 8F). These results suggest that disrupted lateral line primordium migration is mainly responsible for the reduced numbers of neuromasts and shortened *nP* in the *cdh4* morphants.

It is not surprising to find reduced neuromast numbers in zebrafish embryos with disrupted cadherin-2 function, because both the lateral line primordium and neuromasts express high levels of cadherin-2 (Liu et al., 2003; Kerstetter et al., 2004). Neither the PLLp nor the neuromasts was Cdh4-positive (data not shown); therefore, Cdh4 may function in neuromast development by influencing differentiation of other structures.

EXPERIMENTAL PROCEDURES

Zebrafish embryos were maintained as described in the Zebrafish Book (Westerfield, 2000) in accordance with University of Akron, Indiana University, and University of Michigan policies on animal care and use. Embryos for whole-mount immunocytochemistry or in situ hybridization were raised in PTU (1-phenyl-2-thiourea, 0.003%) to prevent melanization.

Morpholino oligonucleotides (MOs) were purchased from Gene Tools (Corvalis, OR). Two translation blocking antisense MOs (RcadMphA 5′-AAG GAG GCA GAT GTT TGT TAT TCA C-3′, RcadMphB 5′-TTC CTG TGA GAT GTG CTG TCG GTA G-3′, designed according to Gene Tools targeting guidelines, Babb et al., 2005), and a MO with five-mismatched nucleotides (5-mis RcadMphA 5′-AAc GAc GCA GAT cTT TcT TAT TgA C-3′, designed by Gene Tools) were used as described (Nasevicius and Ekker, 2000). Compared with databases using BLAST, the MOs sequences showed no significant similarities, other than zebrafish *cdh4* (GenBank accession number: DQ018999). MOs were microinjected into one- to four-cell stage embryos at 2.1 μg/μL (0.25 mM, for RcadMphA and 5-mis MO), or 1.0 μg/μL (0.12 mM, for RcadMphB) in Daneau buffer. Injected embryos were allowed to develop at 28.5°C until the embryos reached the desired stage (e.g., 50 hpf), and then embryos were either processed for DASPEI (4-(4-diethylaminostyryl)-N-methylpyridinium iodide; no. D-3418, Sigma, St. Loius, MO) staining (see below) or anesthetized in 0.02% MS-222 and fixed in 4% paraformaldehyde and processed as described below.

DASPEI labeling of the neuromasts in live embryos was performed accord-

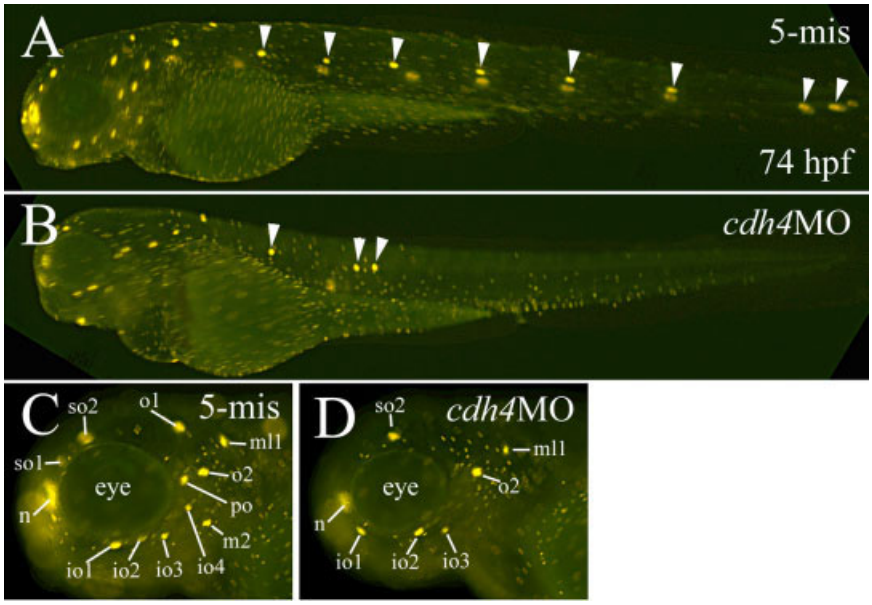

Fig. 7. DASPEI labeling of neuromasts in a 5-mis MO-injected embryo (**A,C**), and embryos injected with RcadMphA (*cdh4*MO, **B,D**). A and B are lateral views of whole live embryos (anterior to the left and dorsal up) with the same magnification. The neuromasts in the body and tail on the same side are indicated by arrowheads in these panels. C,D: Higher magnifications of the lateral view of the head region with anterior to the left and dorsal up. io1-4, infraorbital line neuromasts 1-4; n, nasal organ; m2, middle lateral line neuromast 2; ml1, middle line neuromast 1; o1 and o2, otic lateral line neuromasts 1 and 2; po, postorbital neuromast; so1 and so2, supraorbital line neuromasts 1 and 2.

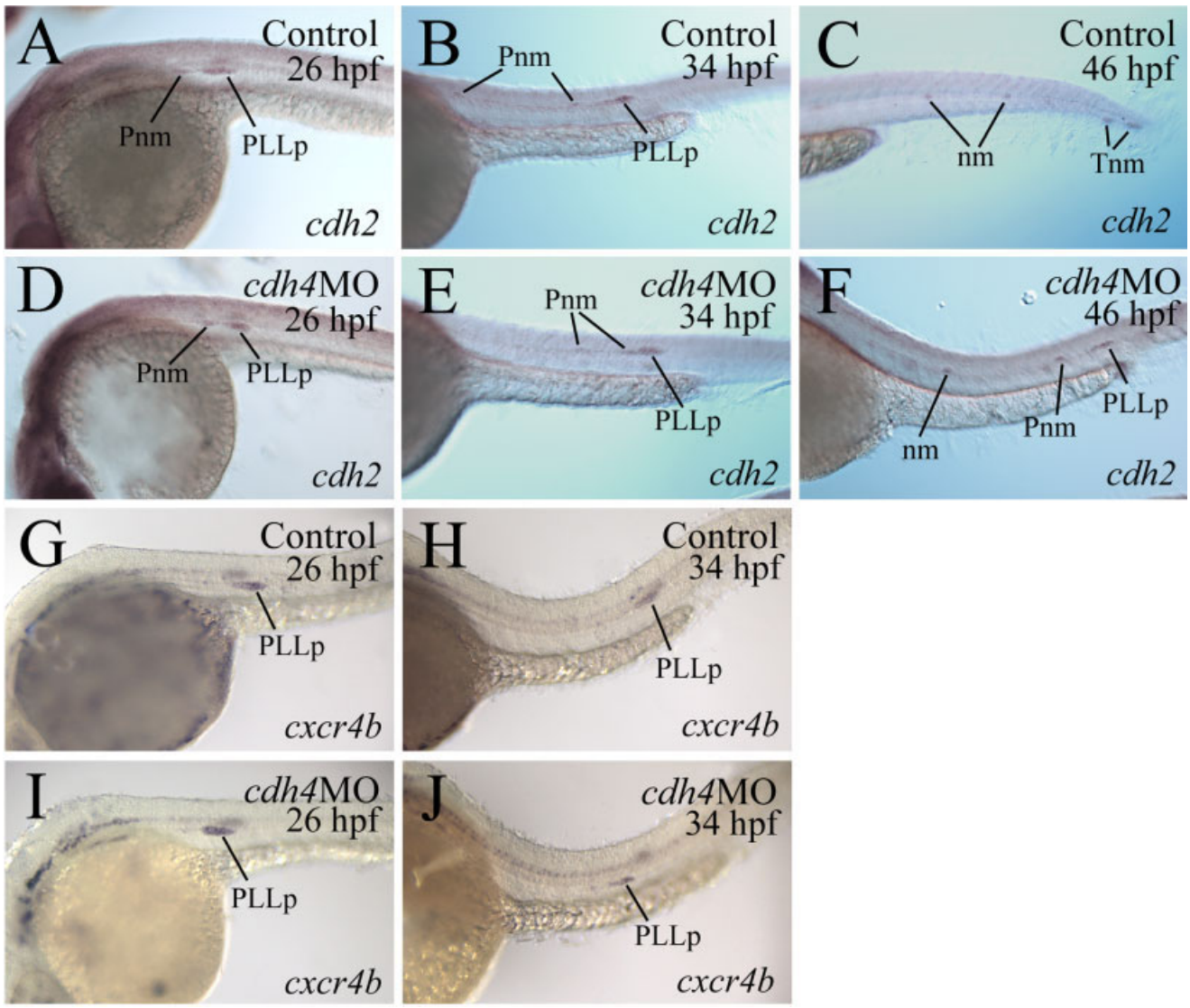

Fig. 8. Formation and migration of posterior lateral line primordium (PLLp), and deposition of proneuromasts (Pnm) and neuromasts (nm) in control embryos and embryos injected with RcadMphA as shown by whole mount in situ hybridization using *cadherin2* (*cdh2*, **A–F**) or *cxcr4b* (**G–J**) cRNA probes. C: Lateral view of the mid trunk and tail region of a control embryo, while the remaining panels show lateral views of the mid trunk region of control embryos and *cdh4* morphants, with anterior to the left and dorsal up.

ing to procedures described by Bricaud et al. (2001). Anti-Hu antibody (Molecular Probe, Eugene, OR) and anti-acetylated tubulin antibody (Sigma) were used at 1:1,500. Zebrafish Cdh4 antibody (affinity purified polyclonal antibody, Liu et al., 2001) was used at 1:150. The secondary antibodies were either FITC-labeled or Cy3-labeled anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Procedures for synthesis of digoxigenin-labeled *cadherin2, cadherin6*, and *NeuroD* cRNA probes for in situ hybridization were described previously (Liu et al., 1999, 2003, 2006). A DNA template for making the *cxcr4b* probe was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA isolated from whole larvae of 8 days post fertilization and zebrafish *cxcr4b* specific primers (forward primer: 5′-GGT GGC ATT TTG GGG GAT TTC T-3′; reverse primer: 5′-ACC AGG ATG CCG GCA CAG TGG-3′). The resulting PCR product, a 496-bp fragment corresponding to the nucleotides 342–837 of zebrafish *cxcr4b* gene (GenBank accession number AY057094), was cloned into pCRII-TOPO vector (Invitrogen Corp., Carlsbad, CA). *EcoRV* was used to digest the plasmid and Sp6 RNA polymerase was used for the synthesis of the digoxigenin-labeled *cxcr4b* probe. Detailed procedures for whole mount in situ hybridization and immunocytochemistry were reported previously (Liu et al., 1999; Westerfield, 2000). TUNEL staining was performed using an in situ cell death detection kit (Roche Applied Science, Indianapolis, IN). Statistical analysis was performed using a two-tail unpaired Student's *t*-test.

ACKNOWLEDGMENTS

We thank Dr. Deborah Stenkamp (University of Idaho) for providing a cDNA coding for zebrafish NeuroD. This study was supported by grants from the NIH to J.A.M., K.F.B., and Q.L. (RO1 DC006436), to K.F.B. (RO1 DC05939 and DC04184), to Q.L. (R15 EY13879), as well as NIH training grant support to Y.C.S. (T32 DC00011, University of Michigan).

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