CADHERIN4 FUNCTION IN THE DEVELOPMENT OF ZEBRAFISH CRANIAL GANGLIA AND LATERAL LINE SYSTEM

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CADHERIN4 FUNCTION IN THE DEVELOPMENT OF ZEBRAFISH CRANIAL GANGLIA AND LATERAL LINE SYSTEM

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Thesis

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CHAPTER I

BACKGROUND

Zebrafish as a Model Organism

Zebrafish is an ideal model organism to study early vertebrate development because of its experimental advantages. The zebrafish produces large clutches of eggs that quickly develop (e.g. most organs become functional by the end of the third day, and the larval fish begin to swim soon afterward) and have a high degree of similarity with other vertebrates (e.g. molecular function and body organization). The transparency of the zebrafish embryo makes following the morphogenesis and pattern formation of embryonic structures easy to examine in both in vivo and in vitro. The zebrafish model has been used to examine the role of cadherin molecules in sensory system development and patterning of the central nervous system (Metcalfe, et al., 1985; Henion et al., 1996; Raible and Kruse, 2000; Bricaud et al., 2001; Liu, et al., 2001; Babb et al., 2005)

Cadherin Molecules

Members of cadherin superfamily are a large group of calcium-dependent cell adhesion molecules (Takeichi et al., 1995; Gumbiner et al., 1996; Fuchs, et al. 2004). Typical cadherin molecules consist of a calcium binding extracellular domain, an anchoring transmembrane domain, and a cytoplasmic domain that interacts with actin filaments via a group of proteins called catenins (Takeichi, et al., 1991; Appendix B).
Cadherins are classified into several subfamilies based on their sequences and domain structures, and to this date, there are seven distinct subfamilies (Appendix C). Classical type I cadherins are the most studied and best understood members of the cadherins. Binding preferentially to their own type of cadherins, each variety of cadherins exhibits distinct expression patterns during development. In addition to mediating cell-cell adhesion, cadherins control cell differentiation through β-catenin-T-cell factor pathway (the Wnt pathway) or p120 catinin-GTPases (Rac or Rho) pathway (reviewed by Wheelock & Johnson, 2003; Junghans et al., 2005).

In addition to the classical type I cadherins, there are several other types of cadherins including atypical (classical type II) cadherins, protocadherins, and desmosomal cadherins. Desmosomal cadherins, found in desmosomal junctions, are more closely related to the classical cadherins in their structure (Nollet et al., 2000). Protocadherins, the largest of the subgroups, have a much larger extracellular domain and are expressed during synapse formation and differentiation along with the cadherins (Junghans et al., 2005). The type II/atypical subfamily is a diverse subclass whose molecular functions are still largely unknown, although their expression patterns during embryogenesis suggest a role in tissue and organ formation (Nollet et al., 2000). The functional information that is known about type II/ atypical cadherins is that they are more similar to type I cadherins than any other type. The type II/atypical cadherins differ mainly from the type I cadherins in their tripeptide His-Ala-Val (HAV) sequence in their extracellular domain one (Appendix B). This tripeptide is crucial for homophilic cell adhesion (Nollet et al., 2000). Recently cadherin6 and cadherin10, expression patterns in
the cranial ganglia and lateral line system was examined in the embryonic zebrafish (Liu et al., 2006a; Liu et al., 2006b).

Previous studies have shown that cadherin2 (Cdh2) and cadherin4 (Cdh4), two classical type I cadherins also known as N-cadherin and R-cadherin, respectively, are differentially expressed in the vertebrate central nervous system (CNS) (Redies, 1993; Treubert-Zimmerman et al., 2002; Liu et al., 2004). In the CNS, these cadherins have been demonstrated to play important roles in differentiation of major brain regions including the telencephalon and visual structures (Redies, 1992; Treubert-Zimmerman et al., 2002; Lele et al., 2002; Masai et al., 2003; Malicki et al., 2003; Babb et al., 2005).

There are a variety of vertebrate species, including zebrafish (see below), *Xenopus*, chicken, and mouse, which have been used to study Cdh4 expression and/or function (Redies et al., 1992; Matsunami and Takeichi, 1995; Tashiro et al., 1995, Liu et al., 1999; Treubert-Zimmermann et al., 2002; Babb et al., 2005).

Similar to their expression in the CNS, these two cadherins exhibit different distribution patterns in the peripheral nervous system (PNS). Cdh2 is expressed throughout the cranial ganglia and the lateral line system of developing zebrafish during the first three days of zebrafish development, while Cdh4 is expressed later (1.5-3 days) in only a subset structures of the cranial ganglia and lateral line ganglia system (Liu et al., 2003). Cdh2 function in zebrafish cranial ganglia and lateral line system development was recently studied (Kerstetter et al., 2004), but there is no published report on Cdh4 function in the vertebrate peripheral nervous system formation.
Organization and Development of the Cranial Ganglia and the Lateral Line System

The vertebrate cranial ganglia originate from the neurogenic neural crest cells and cranial epidermal placodes (Hall, 1999; Kelsh and Raible, 2002). Similar to other vertebrates, the zebrafish cranial ganglia consist of the trigeminal (gV), facial (gVII), statoacoustic (gVIII), glossopharygeal (gIX), and vagal (gX) ganglia that are located lateral to the hindbrain. Of these ganglia, the gV, gVIII, and gX express cadherin4 (Liu et al., 2003). The first zebrafish cranial ganglion that begins to differentiate is gV occurring at 9 hours post fertilization (hpf) (Raible and Kruse, 2000). The gV begins to express Cdh4 around 32 hpf (Liu et al., 2003). The precursor of gVIII can be identified by 16-19 hpf using molecular markers such as NeuroD and Neurogenin I (Andermann et al., 2002). Cdh4 is detected in this ganglion by 29 hpf with increased expression as development proceeds (Liu et al., 2003). The gX develops later (around 46-48 hpf) in zebrafish, and it is Cdh4-positive as soon as it becomes recognizable (Liu et al., 2003).

Unlike the cranial ganglia, the lateral line system originates mainly from the lateral line placodes (Metcalf, 1985). The lateral line system has four main ganglia with sets of neuromasts on the head and in the lateral lines of the body. The lateral line system consists of the anterodorsal (gAD) and the anteroventral ganglia (gAV) which are located anterior to the otic vesicle (inner ear), and the middle lateral line ganglion (gM) and the posterior lateral line ganglion (gP) located posterior to the otic vesicle in developing zebrafish (Metcalf, 1989; Raible and Kruse, 2000). The lateral line ganglia or their precursors are visible using specific markers such as NeuroD as early as 14 hpf (Raible and Kruse, 2000; Liu et al., 2006). These ganglia are Cdh4 positive by 32 hpf and they
continue expressing Cdh4 as development proceeds (Liu et al., 2003). The neuromasts located on the head, jaw, and opercula develop from the anterior lateral line (ALL) primordium, while neuromasts located caudal to the inner ear migrate out of the posterior lateral line primordium (PLLp) (Raible and Kruse, 2000). The PLLp first appears immediately caudal to the otic vesicle. It migrates to the tip of the tail by 48 hpf (Gompel et al., 2001). As it migrates, it deposits neuromast clusters in regular intervals (Gompel et al., 2001) that allow the organism to perceive approaching predators, prey, or environment obstacles that surround the organism. All neuromasts are innervated by lateral line nerves that are connected to the brain via the lateral line ganglia. It is important to note that neither the PLLp, nor the neuromasts express Cdh4. The neuromasts of the lateral line system contain mechanosensory hair cells (Metcalf, 1986; Gompel et al., 2001). The mechanosensory hair cells are closely related to the inner ear hair cells in their placodal origin, organization, and function (Bricaud et al., 2004; Haddon and Lewis, 1996).

Hypothesis

Cdh4 is expressed in the majority of the cranial and lateral line ganglia during critical periods of their development, therefore I hypothesize that interfering with Cdh4 function will disrupt the development of the cranial ganglia and lateral line system structures.
CHAPTER II

METHODS AND MATERIALS

Zebrafish and Tissue Preparation

Adult zebrafish were obtained from Dr. Liu’s inbred colony and kept in 10-gallon tanks at 25°C with a 12-hour light/dark cycle. The adult fish were bred to obtain embryos. The eggs were collected and placed in 120-150 ml fish tank water in 500ml plastic beakers maintained at 28.5°C in water baths. Ages of the embryos are expressed in hours post fertilization (hpf). All animal related procedures were conducted in accordance with protocols approved by the University of Akron Committee on care and use of animals IACUC# 05-12A.

Morpholino Oligonucleotides

Morpholino antisense oligonucleotides (MO’s) have been shown to effectively block specific gene function in several model organisms including zebrafish (Ekker, 2004). MO’s are modified DNAs (replacing the sugar ring with a synthetic morpholino ring) with important advantages over other types of antisense oligonucleotides such as high specificity, high efficiency, long lasting effect (not degraded by endogenous Rnases) and less toxicity (Summerton, 1999). MO technology has become one of the most used and powerful methods to reduce or block gene functions in zebrafish. Our lab has
employed this approach to study cadherin function in the development of the zebrafish CNS and PNS (Liu et al., 2004; Kerstetter, et al., 2004; Babb et al., 2005).

**Morpholino Antisense Oligonucleotide Injection**

Two Cdh4 translation blocking antisense MO’s (cd4MphA, 5’-AAG GAG GCA GAT GTT TGT TAT TCA C-3’, cd4MphB, 5’ TTC CTG TGA GAT GTG CTG TCG TGG TCA C-3’) were designed to be complimentary mRNA sequences of translation initiation site of zebrafish Cdh4, and a 5-mismatched control MO (5’, AAc GAc GCA cTT TcT TAT TgA C-3’) (all purchased from Gene Tools, Covalis, OR) were microinjected into the blastomere(s) or regions of yolk directly below the blastomere(s) of 1-4 cell stage embryos at 2.1 ng/μl and 1.0 ng/μl (0.25 mM cd4MphA and 5-mis MO, and 0.12 mM cd4MphB) using a microinjection system. The embryos were allowed to develop to specific developmental times (e.g. 34, 48-50, and 72 hpf) at 28.5°C. The embryos were anesthetized in 0.02% methane tricane sulfonate (MS-222), followed by fixation in 4% paraformaldehyde in phosphate buffered saline (PBS). The embryos were rinsed with PBS and stored in 100% methanol at -20°C until use.

**DAPSEI Staining**

Developing neuromasts can be labeled using DAPSEI (4-diethylaminostyryl)-N-methylpyridinium iodide) staining. Live embryos were placed in a DAPSEI solution (100 μM DAPSEI from Sigma dissolved in filtered fish tank water) for one hour in the dark. After the staining, the embryos were washed three times for approximately five minutes each in fish tank water. The embryos were then immobilized using MS-222, placed into a viewing chamber on a glass slide, and viewed under a fluorescent
microscope (Olympus BX51). Pictures were taken using a SPOT digital camera system (SPOT Diagnostic Instruments, Inc, Sterling Heights, Michigan) attached to the microscope. Results from the \textit{cdh4} morphants were compared to non-injected and 5-mis MO injected embryos.

\textbf{In Situ Hybridization}

In situ hybridization (ISH) was used to study expression patterns of neural markers in the cranial ganglia, lateral line primordia and neuromasts. ISH is a three-day procedure. The embryos taken from 100% methanol, were gradually rehydrated with 1X PBS with 10% Tween 20 (PBST). The embryos were treated with 0.01 mg/ml Protinase K for 15-20 minutes, followed by brief PBST washes. The embryos were re-fixated with 4% paraformaldehyde in PBS for 20 minutes at room temperature. The embryos were rinsed five times in PBST for 5 minutes each at room temperature, then transferred to a new tube. The embryos were incubated for 2 hours at 59°C in a prehybridization solution to reduce nonspecific staining. Specific cRNA probes (dioxigenin-labeled) were added to fresh prehybridization solution, and the embryos were placed in the solution overnight at 59°C in a hybridization oven with constant movement. The next day, after rinsing in 2X SSC at room temperature, the embryos were washed in a 1:1 formamide/ 2X SSC solution for an hour at the hybridization temperature. The embryos were rinsed twice with 2X SSCT (2X SSC with Tween 20) for 10 minutes each at 59°C. This was followed by two 30-minute stringent washes (0.2X SSCT at 68°C). The embryos were incubated in increasing concentrations of PBST, followed by incubating in a blocking solution (BSA, normal goat serum, DMSO, and PBST) for 2 hours at room temperature. An anti-
digoxigenin antibody (Roche, Indianapolis, IN) was added to the blocking solution and was left to incubate overnight at 4 °C with constant agitation. By day three, the embryos were ready to be washed 8 times, for 15 minutes each, with PBST. The embryos were rinsed twice with alkaline phosphate buffer (also known as genius buffer 3), followed by an incubation in the dark in a NBT/BCIP solution (Roche, Indianapolis, IN). The embryos were then rinsed twice with the genius buffer 3 and PBST at room temperature, followed by refixation in 4 % paraformaldehyde for 20 minutes at room temperature. The embryos were washed with PBST, and placed in 100% glycerol overnight at 4°C before microscopy and data collecting. The processed embryos can be stored in 100% glycerol at -20°C.

Immunohistochemistry

Embryos in 100% methanol were rehydrated gradually with PBST and methanol washings ending with 100% PBST washing. Older embryos (>48 hpf) were incubated in a Protinase K solution (2 μg/ml in PBST) at room temperature, followed by two ten minute washings with PBST. The embryos were fixed again with 4% paraformaldehyde for 20 minutes in room temperature, ending with three more PBST washes that are 10 minutes each. A 15 minute PBST with supplements (PBS with DMSO added) rinse was followed by 2 hours of incubation in a blocking solution (3 drops of normal horse serum from Vector kit (Vector Laboratories, Burlingame, CA) in 10 ml PBST with supplement at room temperature). The primary antibodies, anti-Hu (which labels cell bodies of differentiating neurons), anti-acetylated tubulin (that labels α-tubulin), or Zn5 (labels differentiating neurons and their processes) were purchased from Molecular Probes.
(Eugene, OR), Sigma, and the Zebrafish International Resource Center, respectively. The antibodies were used at 1:1500-2000 for the immunofluorescent method, and 1:3000-6000 for the peroxidase method. An overnight incubation with these primary antibodies at 4°C was followed by four 30-minute washes in PBST with supplement. For immunofluorescence method, an anti-mouse IgG conjugated to FITC (Jackson Laboratories, West Grove, PA) at 1:100, was used for an overnight incubation at 4°C. After the secondary antibody solution was removed, four 30-minute each washes with PBST were used to rinse the embryos. The embryos were placed in Vectashield (Vector Laboratories, Burlingame, CA), a fluorescent-mounting medium, for viewing under the fluorescent microscope or storage at -20°C.

For the immunoperoxidase method, following the primary antibody incubation and washes the embryos were placed in a solution containing a biotinylated secondary antibody (Vector Laboratories), and incubated for overnight at 4°C. After the secondary antibody was removed from the embryos, and washed for four times, 30 minutes each in PBS, the embryos were incubated in an ABC complex (Vector Laboratories, Burlingame, CA) for five hours at room temperature. The embryos were washed again four times, 30 minutes each with PBST. Visualization of the signal was achieved by incubating the embryos in a DAB solution (Vector Laboratories). Finally, the embryos were washed in PBST and stored in 100% glycerol at -20°C.
Cadherin4 Immunocytochemistry

To assess effectiveness of \textit{cdh4} MO’s knocking down of Cdh4 protein, some injected embryos were processed for cryosectioning. Briefly, after fixation and washing, the embryos were placed in 20% sucrose PBS solution overnight at 4°C. The next day, the embryos were moved to an embedding solution (1:1 20% sucrose and OCT embedding medium) and incubated for one hour at room temperature. The embryos were placed in a mold made of tinfoil and embedded by freezing in the same solution. The embryo blocks were sectioned at 12-14 \(\mu\)m using a cryostat. Tissue sections were placed on pretreated glass slides (Fisher Scientifics) and processed for Cdh4 immunostaining using an affinity-purified zebrafish Cdh4 antibody. Briefly the tissue sections were rehydrated in PBS, blocked in normal goat serum before incubating with Cdh4 antibody solution (6 \(\mu\)g/ml) overnight at 4°C. After 3 washes in PBS for 10 minutes each, the sections were reacted with a secondary antibody solution (Cy3 anti-rabbit IgG) for 2 hours in the dark at room temperature. Finally, the sections were washed 3 times, 10 minutes each, in PBS, cover slipped using Vectashield (Vector lab), and observed under the fluorescent microscope.

Image Acquisition, Processing, and Data Organization

Processed embryos and larvae were viewed under the fluorescent compound microscope equipped with the digital camera system (SPOT). Images were processed using Adobe Photoshop. Statistics were performed using Student’s t-test.
CHAPTER III

RESULTS

Cdh4 is Involved in the Formation of the Cranial and Lateral Line Ganglia Affected in cdh4 Morphants

The injection of cdh4 specific antisense morpholino oligonucleotides (cdh4 MOs: cd4MphA, or cd4MphB), into one-to-four cell stage embryos greatly reduced Cdh4 protein levels in the injected embryos at 50-55 hpf (see Appendix D; also see Babb et al., 2005) while injection of the 5-mis MO resulted in Cdh4 staining similar to uninjected control embryos. At 24 hours post fertilization (hpf), cdh4 morphants were similar in body size and shape in comparison to control (uninjected) embryos or 5-mis MO injected embryos. As the embryos developed to 50-55 hpf, the majority of cdh4 morphants (86%) showed a similar gross morphology to the control embryos, except that the morphants eye sizes were slightly or moderately reduced and some had smaller bodies with slightly ventrally curved tails. cdh4 morphants that had been injected with lower concentrations MOs (0.125 mM, 1.0 ng/embryo for cd4MphA, 0.06 mM, 0.5 ng/embryo for cd4MphB) were largely indistinguishable from the control embryos. Injection with the 5-mis 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 showed that Cdh4 expression levels were greatly reduced throughout the embryos, specifically in the brain, cranial and lateral line ganglia at 55 hpf (Appendix D, Fig. 3C) compared to the control embryos (Appendix D, Fig. 3A) and embryos injected with the 5-mis MO (Appendix D Fig. 3B). Using multiple markers, I analyzed organization of these structures in cdh4 morphants, and compared it with the control embryos and those injected with the 5-mis MO.

Anti-Hu immunostaining demonstrated that cdh4 morphant cranial and lateral line ganglia had similar appearances to those of control embryos at 30 hpf (Appendix E, Fig. 4A and B; Table 1), except that the morphant gVIII was slightly smaller in size than the control gVIII. However, by 50-55 hpf, there was morphologic disruption in the vast majority of cdh4 morphants (Appendix J). In the cdh4 morphants, ganglia were smaller in size, had altered shapes, and/or became fragmented compared to the control group or the 5-mis MO injected embryos (Appendix E, Fig. 4C –E). The Zn5 antibody strongly labels the gV, gAD, and gVIII at 36-40 hpf (Appendix E, Fig. 4F-K). A smaller Zn5 positive region and reduced staining were found in the gVIII of cdh4 morphants at 40 hpf, while the staining in the gV/AD was only slightly to moderately altered (e.g. change of shape and/or reduced staining; Appendix E, Fig. 4F-K; Appendix J). At 40-55 hpf, the gX and gP are marked with NeuroD (Andermann et al., 2002), while the gX, gM, and gP are cadherin6 positive (Liu et al., 2006). In cdh4 morphants, NeuroD and/or cadherin6 expression in gX and gP was reduced (Appendix F, Fig. 5B and D) compared to the control embryos (Appendix F, Fig. 5A and C), while there was no apparent difference in
cadherin6 expression in the gM between the control embryos and morphants (Appendix F, Fig. 5C and D).

To examine developing zebrafish cranial and lateral line nerves, I used anti-acetylated tubulin immunostaining (Appendix G; Raible and Kruse, 2000). Control embryos at 50-55 hpf had distinct axonal bundles that exit the gV/AD and project anterodorsally (the superior ophthalmic ramus of the anterodorsal lateral line nerve, nADso and dorsolateral nerve of the trigeminal ganglion nVD1, Appendix G, Fig. 6A), or anteroventrally (the buccal ramus of the anteroventral lateral line nerve, nADb) and the mandibular ramus of the anterventral lateral (nAVm, Appendix G, Fig. 6A, Raible and Kruse, 2000). The cdh4 morphants had those nerves present, but they had thinner process size and featured weaker staining. In addition, the distance between nADb and nAVm was larger in the morphants than in the control embryos (Appendix G, Fig. 6A and C). The gX is a conspicuous ganglion with a thick central projection, the vagus root, and several peripheral nerves projected ventrally and ventrolaterally in control embryos at 50-55 hpf (Appendix G, Fig. 6B). In contrast, the gX of cdh4 morphants was smaller in size and had reduced numbers of peripheral nerves, while the vagus roots were similar to the control (Appendix G, Fig. 6D; Appendix K).

Organization of the Lateral Line System Disrupted in cdh4 Morphants

Growth cones of zebrafish posterior later line nerve (nP) develop 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. By 46-48 hpf, the nP has reached the tail (Metcalf, 1985, 1989). Anti-acetylated tubulin immunostaining showed that the nP
reached the tail region of all control and 5-mis MO injected embryos at 50-55hpf (Appendix H, Fig. 7A; Appendix K). Though the nP was straight like the control embryos in the majority of cdh4 morphants, the length was much shorter (ending at the level of the posterior end of the yolk ball or the anus region). The nP remained short in about 2/3 (13/20) of the older morphants (72-74 hpf). Those morphants in which the nP reached the tail, the nerve was thinner than the control embryos.

The development of cdh4 morphant neuromasts was assessed using DAPSEI staining, which labels neuromasts in live embryos and larvae. In control embryos or 5-mis MO injected embryos at 50-55 hpf, the posterior lateral line system features five to six neuromasts on each side of the trunk and tail. The cdh4 morphants had significantly reduced neuromast numbers on the trunk and/or tail (Appendix M). In one third of the cdh4 morphants (n = 13), there were one or two neuromasts on each side of the trunk and in the remaining morphants (n = 24), only three or four neuromasts were seen on each side of the trunk and/or tail. In all of the morphants except for two, neuromasts were not seen in the tail region. In older morphants (72-74 hpf, n = 24), I found that their neuromasts numbers were significantly reduced compared to the control embryos (n = 11,) (Appendix M). The average neuromast number found in the 72-74 hpf morphants (3.7 ± 1.3) were significantly lower (p<0.001) than the younger controls (50-55 hpf) (5.4 ± 0.4) or 5-mis MO injected embryos (5.4 ± 0.5). Ten of the cdh4 morphants were allowed to develop from 55 hpf to 74 hpf (with each morphant kept in a separate container). Monitoring their development, I found that 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 six on the right side of the trunk and tail. Once again, no neuromasts were found in the tail region in the majority of these morphants (n = 7, 70%).

Neuromasts in the head region were also different between control and morphants. In 72 hpf control embryos (Raible and Kruse, 2000) or 5-mis MO injected embryos (Appendix I, Fig. 8D; Appendix M ), there were fourteen to fifteen neuromasts on each side of the head. In comparison, the morphant embryos had only five to seven neuromasts on each side of the head (Appendix I, Fig. 8E; Appendix M). Again, the younger control (50-55 hpf) or 5-mis MO injected embryos had significantly more neuromasts (p<0.001) in the head than those of older morphants (72-74 hpf).
CHAPTER IV
DISCUSSION

Morpholino antisense oligonucleotide technology has been shown to be a powerful tool in zebrafish to study gene functions in various tissues and organs including the cranial and lateral line systems (Ekker, 2000; Nasevicius and Ekker, 2000; Andermann et al., 2002; Kerstetter et al., 2004; Knaut et al., 2005). cdh4 MOs greatly reduced Cdh4 protein expression levels in the zebrafish cranial ganglia and lateral line structures. The specificity of the cdh4 MOs was also previously demonstrated using immunoblotting methods (Babb et al., 2005). The defects observed in embryos injected with the cdh4 MOs are likely due to specific blockage of Cdh4 protein expression.

Cdh4 expression in the zebrafish cranial ganglia and the lateral line system is distinct from other cadherins expression in these structures. Cadherin2 is expressed early, as soon as these structures are visible, and in all of the cranial and lateral line structures (Liu et al., 2003). cadherin6 expression is early too (14 hpf), but it is detected in only a subset of the structures (e.g. gVIII and gP, but not in gV) (Liu, et al., 2006). In contrast, Cdh4 is expressed later (after 26 hpf), also in a subset of these structures (e.g. gV, gVIII and gP) (Liu et al., 2003). Differential expression of these cadherins in the cranial and lateral line system suggests differential function of these cadherins in the formation of these peripheral nervous structures.
The cdh4 morphant defects are unlikely due to a general delay in zebrafish development for the following reasons. First, cdh4 morphants are indistinguishable in gross morphology from control or 5-mis MO injected embryos from 24-30 hpf, when there is little or no Cdh4 expression in the developing zebrafish nervous system. Second, cdh4 morphants are similar in body size and shape to control embryos at later stages (e.g. 40 to 50 hpf), except for staining of neural markers such as Anti-Hu and Zn5 in structures (e.g. gV, gVIII and gP) that contain Cdh4. Third, the cdh4 morphant defects (e.g. shortened gP and reduced number of neuromasts) persisted in older (e.g. 72-74 hpf) embryos. For example, older cdh4 morphants (72-74 hpf) had smaller number of neuromasts than that of younger control embryos (50 hpf).

As mentioned above, the cranial and lateral line ganglia were similar in appearance, as judged by anti-Hu immunostaining, to control embryos at 30 hpf, except the morphant gVIII was slightly smaller than the control gVIII (Appendix E, Fig. 4A and B; Appendix J). This is likely due to Cdh4 not being expressed in the other ganglia until 32 hpf (Liu et al., 2003), but can be detected in the gVIII as early as 26 hpf (Liu, unpublished observation). The severity of gV/AD, gVIII and gP defects in cdh4 morphants was not as severe as that in cdh2 morphants or mutants, while the severity of gX defects were similar between these two morphants/mutants (Kerstetter et al., 2004). This may be explained by differential expression of these two cadherins in these ganglia (e.g. Cdh2 is expressed early, while Cdh4 is expressed later, and both cadherins are expressed in the gX throughout its development).
The Anti-Acetylated tubulin immunostaining showed that the posterior lateral line nerve (nP) was straight, but much shorter in cdh4 morphants compared to control or 5-mis MO injected embryos. The nP has reached to about 1/3 to 1/2 of the body length when the gP becomes Cdh4-positive (30-36 hpf), therefore, Cdh4 is likely involved in the extension, but not initiation of the nP. Moreover, the cdh4 morphant nP defects are different from cdh2 morphant/mutant defects in which the nerve was not shorter, but curved or doubled back, suggesting a pathfinding problem when Cdh2 function is blocked.

Pathfinding of nP depends on the migrating of the posterior lateral line primordium (PLLp) (Gilmour et al. 2004). The PLLp begins migrating caudally by 20 hpf (Metcalf, 1989; Gompel et al., 2001). As the PLLp migrates to the tail end, it deposits in regular intervals along the horizontal myoseptum, six to eight pairs of proneuromasts (L1 to L7) which are innervated by the nP (Metcalf, 1989; Gompel et al., 2001). The migration reaches the zebrafish tail by 46-48 hpf and stops migration by forming terminal neuromasts (Gompel et al., 2001). Due to this close association of the neuromasts and the nP, the neuromast defects (e.g. reduced number) seen in the cdh4 morphants, as revealed by DAPSE staining, are likely an indirect one: Cdh4 affect development of the neuromasts via its effects on nP extension. Using other neuromasts markers (e.g. cdh2 and cxcr4b), we obtained supporting results for this idea.

In summary, an earlier study showed that Cdh4 is expressed in a subset of zebrafish cranial ganglia and lateral line system during critical periods of their development, interfering with Cdh4 function using cdh4 specific MOs greatly disrupted
differentiation of these structures. My results suggest that Cdh4 is crucial for the formation of the zebrafish cranial ganglia and lateral line system.
REFERENCES


21


APPENDICES
### APPENDIX A

### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>gAD</td>
<td>anterodorsal lateral line ganglion</td>
</tr>
<tr>
<td>nADso</td>
<td>superior opthalmic ramus of the anterodorsal lateral line nerve</td>
</tr>
<tr>
<td>gAV</td>
<td>anteroventral lateral line ganglion</td>
</tr>
<tr>
<td>nVDI</td>
<td>dorsal lateral nerve line</td>
</tr>
<tr>
<td>gM</td>
<td>middle lateral line ganglion</td>
</tr>
<tr>
<td>nAVm</td>
<td>mandibular ramus of the anteroventral lateral line nerve</td>
</tr>
<tr>
<td>gP</td>
<td>posterior lateral line ganglion</td>
</tr>
<tr>
<td>nIX</td>
<td>glossopharyngeal nerve</td>
</tr>
<tr>
<td>gV</td>
<td>trigeminal ganglion</td>
</tr>
<tr>
<td>nP</td>
<td>posterior lateral line nerve</td>
</tr>
<tr>
<td>gX</td>
<td>vagus ganglion</td>
</tr>
<tr>
<td>nx</td>
<td>vagus nerve</td>
</tr>
<tr>
<td>H</td>
<td>hindbrain</td>
</tr>
<tr>
<td>rX</td>
<td>vagus root</td>
</tr>
<tr>
<td>nADb</td>
<td>buccal ramus of the anterodorsal lateral line nerve</td>
</tr>
</tbody>
</table>
The cadherin superfamily domain organization. The classic cadherin subfamilies feature a catenin-binding site on their C terminal end. The Type I and type II cadherins are very similar in structure, however Type I has a distinct His-Ala-Val sequence on the N-terminal (see the black circle). Non-chordate classic cadherins feature multiple cadherin repeats on the N-terminal. The non-classical cadherins lack catenin-binding sites. Figure adapted from Truong and Ikura, 2002.
APPENDIX C

FIGURE 2

<table>
<thead>
<tr>
<th>Superfamily</th>
<th>Subfamily</th>
<th>Class</th>
<th>Type</th>
<th>Sub-type</th>
<th>Variety</th>
</tr>
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<tbody>
<tr>
<td>Classic</td>
<td></td>
<td>Chordate</td>
<td>Type I</td>
<td>Neuronal</td>
<td>1,2,4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-chordate</td>
<td>Type II</td>
<td>Non-neuronal</td>
<td>1,3,4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6,7,10</td>
</tr>
</tbody>
</table>

Cadherins

- Flamingo

Fat-like

RET

Desmosomal

- Desmocollins
- Desmogleins

Dsc1, Dsc2

Dsg1, Dsg2

Truncated

Protocadherin

- α
- β
- λ
- λ

PcdhλA

PcdhλB

PcdhλC

Cadherin superfamily group organization. Classical cadherins are grouped into a chordate and non-chordate class. Cadherin4, a type I cadherin can be expressed in both neuronal and non-neuronal tissue. The classical cadherin subfamily have a conserved cytoplasmic domain (see Appendix B). Flow chart made with information from Nollet, et al. (2000).
APPENDIX D

FIGURE 3

Cross sections of the hindbrain showing Cadherin immunostaining that demonstrates the reduction of Cdh4 protein expression in a cdh4 morphant injected with RcadMphA (cdh4MO, panel C) compared to a control embryo (panel A) or a 5-mis MO injected embryo (5-mis, panel B). All panels are cross-sections of the hindbrain region, with the dorsal end being up, at the level of posterior lateral line ganglion and vagal ganglion (55 hpf). The arrows point to the structures of cdh4 immunoreactive fiber tracts in the ventral hindbrain. Abbreviations: gP, posterior lateral line ganglion; gX, vagal ganglion; H, hindbrain; Nc, notochord; rX, vagal root; Tm, trunk muscles.
Cranial and lateral line ganglion marked by anti-Hu immunostaining (Hu, panels A-E) and Zn5 immunostaining (panels F-K). All panels are lateral views of whole mount zebrafish embryos with anterior to the left and dorsal up. Panels A-H are from immunofluorescent methods, while panel I-K are from immunoperoxidase methods. The cdh4 morphant in panel H was from RcadMphB injections, while the remaining morphants were from RcadMphA injections. The trigeminal (gV) and anterodorsal lateral line ganglia (gAD) in panels D and E were out of focus, their focused images are shown in their respective inserts in the panels. Abbreviations: gVIII, statoacoustic ganglion; gM medial lateral line ganglion; other abbreviations are the same as Appendix D.
APPENDIX F

FIGURE 5

Lateral views of the hindbrain region of whole mount embryos (anterior is to the left and dorsal is up) processed by in situ hybridization using NeuroD (panels A and B) or cadherin6 (cdh6, panels C and D) cRNA probes. The cdh4 morphants were from RcadMph4 injections. Abbreviations are the same as in Appendix D and Appendix E.
Cranial and lateral line ganglion nerves in a control embryo (panels A and B) and an embryo injected with RcadMphA (panels C and D), as demonstrated by anti-acetylated tubulin immunostaining (an-tub). All panels are lateral views of the hindbrain region with the anterior to the left and dorsal up. Panels A and B, C and D are from the same embryos, respectively, with panels A and C focusing on the gV/AD nerves, while panels B and D focusing on the gX and gP nerves. Abbreviations: nAdb, buccal ramus of the anterodorsal lateral line; 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; nVDI, dorsolateral nerve of the trigeminal ganglion; nX, vagus nerve; rX, vagus root; other abbreviations are the same as in Appendix D and Appendix E.
APPENDIX H

FIGURE 7

The posterior lateral line system in a 5-mis MO injected embryo (panel A) and embryos injected with RcadMphA (panels B-D) as revealed by anti-acetylated tubulin immunostaining. Panels A-C show lateral views of whole embryos (50 hpf, anterior to the left and dorsal up) of the same magnification. Panel D is 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 arrow indicates the terminus of the lateral line.
DAPSEI staining of neuromasts in a 5-mis MO-injected embryo (panels A and C), and embryos injected with RcadMphA (cdh4 MO, panels B and D). Panels 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. Panels C and D show higher magnifications of the lateral views of the head region with anterior to the left and dorsal up. Abbreviations: 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.
APPENDIX J

TABLE 1

Effects of cdh4 MOs injection on cranial and lateral line ganglia development.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>gV/AD</th>
<th>gVIII</th>
<th>gX</th>
<th>gP</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 hpf anti-Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants (n=20)</td>
<td>10%</td>
<td>65%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>control (n=20)</td>
<td>0%</td>
<td>5%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>40 hpf zn5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n1=20, n2=12)</td>
<td>70% (75%)</td>
<td>85% (83.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (n=20)</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-55 hpf anti-Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n1=42, n2=20)</td>
<td>85.7% (80%)</td>
<td>81% (85%)</td>
<td>100% (95%)</td>
<td>85.7% (95%)</td>
</tr>
<tr>
<td>control (n=20)</td>
<td>0%</td>
<td>10%</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>5-mis MO (n=40)</td>
<td>7.5%</td>
<td>5%</td>
<td>5%</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

n represents number of ganglia examined. n1 and n2 are the numbers of embryos injected with RcadMphA and RcadMphB, respectively. % represents 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 parenthesis are from RcadMphB injected embryos.
APPENDIX K

TABLE 2

Effects of cdh4 MOs injection on cranial and lateral line nerves development.

<table>
<thead>
<tr>
<th></th>
<th>gV/AD nerves</th>
<th>gX nerves</th>
<th>nP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>anti-acetylated tubulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-55 hpf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants (n1=38, n2=18)</td>
<td>71% (83.3%)*</td>
<td>100% (100%)</td>
<td>94.7% (100%)</td>
</tr>
<tr>
<td>control (n=20)</td>
<td>0%</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>5-mis MO (n=20)</td>
<td>5%</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>72-74 hpf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants (n1=20)</td>
<td>85%</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td>control (n=20)</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

n represents number of ganglia nerves (or collection of nerves for gV/AD) examined. n1 and n2 are the numbers of embryos injected with RcadMphA and RcadMphB, respectively. % represents 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 parenthesis are from RcadMphB injected embryos. *The defects in the gV/AD nerves are milder compared to those of gX.
APPENDIX M

TABLE 3

Effects of cdh4 MO (RcadMphA) injection on neuromasts development

<table>
<thead>
<tr>
<th></th>
<th>average number of neuromasts in the PLL system</th>
<th>average number of neuromasts in the ALL system</th>
</tr>
</thead>
<tbody>
<tr>
<td>DASPEI staining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-55 hpf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants (N=37)</td>
<td>2.8 ± 0.8</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>control (N=15)</td>
<td>5.4 ± 0.4*</td>
<td>6.6 ± 0.4*</td>
</tr>
<tr>
<td>5-mis MO (N=15)</td>
<td>5.4 ± 0.5*</td>
<td>6.6 ± 0.5*</td>
</tr>
<tr>
<td>72-74 hpf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdh4 morphants (N=24)</td>
<td>3.7 ± 1.3</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>control (N=11)</td>
<td>7.7 ± 0.5*</td>
<td>14.5 ± 0.4*</td>
</tr>
</tbody>
</table>

N represents number of embryos examined. The averages are from both sides of the embryos. * indicates that the number is significantly larger (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 larger (p<0.001) than those of older cdh4 morphants. Abbreviations: PLL, posterior lateral line system; ALL, anterior lateral line system.
December 19, 2005

Drs. Qir Liu, Londaville, Ms. Wilson, Ms. Zheng
Department of Biology
The University of Akron
Akron, OH 44325

Dear Dr. Liu:

The Institutional Animal Care and Use Committee reviewed your protocol titled:

Dr. Liu, Dr. Londaville, Amy Wilson, Bin Zheng. “Cadherin-4 and cadherin-6 function in development of zebrafish cranial and lateral line ganglia”

IACUC # 05-12A
30 adult and 2,500 embryos Zebrafish (Danio rerio)
Faculty/Graduate student research starting date 7-1-06

The committee voted and your proposal received unanimous approval.

You must provide the committee with documentation of updated risk information, or serious adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Sincerely,

James Holda
IACUC Chair