Published in final edited form as:

Dev Neurobiol. 2013 April ; 73(4): 259–273. doi:10.1002/dneu.22053.

Protocadherin-17 Function in Zebrafish Retinal Development

Yun Chen¹, Richard Londraville¹, Sarah Brickner¹, Lana El-Shaar^{1,§}, Kelsee Fankhauser^{1,§}, Cassandra Dearth¹, Leah Fulton¹, Alicja Sochacka¹, Sunil Bhattarai¹, James A. Marrs², and Qin Liu^{1,*}

Yun Chen: chenyun08@gmail.com; Richard Londraville: londraville@uakron.edu; Sarah Brickner: seb58@zips.uakron.edu; Lana El-Shaar: lne3@zips.uakron.edu; Kelsee Fankhauser: klf30@zips.uakron.edu; Cassandra Dearth: ckd4@zips.uakron.edu; Leah Fulton: fultonl@kenyon.edu; Alicja Sochacka: sochacka.alicja@gmail.com; Sunil Bhattarai: sunilbhattarai@gmail.com; James A. Marrs: jmarrs@iupui.edu; Qin Liu: qliu@uakron.edu

¹Department of Biology and Integrated Bioscience Program, University of Akron, Akron, OH 44325

²Department of Biology, Indiana University-Purdue University Indianapolis, 723 West Michigan Street, Indianapolis, IN 46202

Abstract

Cadherin cell adhesion molecules play crucial roles in vertebrate development including the development of the retina. Most studies have focused on examining functions of classic cadherins (e.g. N-cadherin) in retinal development. There is little information on the function of protocadherins in the development of the vertebrate visual system. We previously showed that protocadherin-17 mRNA was expressed in developing zebrafish retina during critical stages of the retinal development. To gain insight into protocadherin-17 function in the formation of the retina, we analyzed eye development and differentiation of retinal cells in zebrafish embryos injected with protocadherin-17 specific antisense morpholino oligonucleotides (MOs). Protocadherin-17 knockdown embryos (*pcdh17* morphants) had significantly reduced eyes due mainly to decreased cell proliferation. Differentiation of several retinal cell types (e.g. retinal ganglion cells) was also disrupted in the *pcdh17* morphants. Phenotypic rescue was achieved by injection of protocadherin-17 mRNA. Injection of a vivo-protocadherin-17 MO into one eye of embryonic zebrafish resulted in similar eye defects. Our results suggest that protocadherin-17 plays an important role in the normal formation of the zebrafish retina.

Keywords

cell adhesion molecules; eye; retinal cells differentiation; optic nerve

Introduction

The cadherins are a family of Ca²⁺-dependent cell adhesion molecules that play important roles in tissue and organ development and maintenance of adult structures (Takeichi, 1990; Kemler, 1993; Yagi and Takeichi, 2000; Gumbiner, 2005). Classic cadherins (e.g. cadherin-1 and cadherin-2) are known to hold cells and tissues together through strong homophilic interactions (Takeichi, 1990; Halbleib and Nelson, 2006). These cadherins have been demonstrated to play crucial roles in development of visual structures (Matsunaga et al., 1988; Bixby and Zhang, 1990; Doherty et al., 1991; Paradies and Grunwald, 1993; Riehl

et al., 1996; Stone and Sakaguchi, 1996; Lele et al., 2002; Treubert-Zimmermann et al., 2002). In zebrafish cadherin-2 mutants *parachute* (*pac*, Masai et al., 2003) and *glass onion* (*glo*, Malicki et al., 2003), retinal structure (e.g. lamination) and differentiation of retinal cells (e.g. retinal ganglion cells) was severely disrupted.

The protocadherins (pcdhs) are the largest cadherin subfamily containing about 80 members in mammals (Yagi, 2003). Pcdhs have been demonstrated to play important roles in the development of the vertebrate nervous system (Bononi et al., 2008; Piper et al., 2008; Emond et al., 2009; Biswas et al., 2010; Lefebvre et al., 2008; Prasad et al., 2008; Fernandez-Monreal et al., 2009; Garret and Weiner, 2009; Katori et al., 2009; Ying et al., 2009; see Frank and Kemler, 2002 and Junhans et al., 2005 for reviews of studies published earlier). Similar to the classic cadherins, pcdhs control tissue development by regulating cell-cell adhesion and/or intracellular signaling pathways (Frank and Kemler, 2002; Redies et al., 2005). Pcdhs are divided into α -, β -, γ - and d-pcdhs. Genes of the first three types (α -, β -, γ -pcdhs) are organized into clusters, while the d-pcdhs genes are non-clustered. Similar to the classic cadherins, the d-pcdhs are transmembrane proteins consisting of extracellular, transmembrane and cytoplasmic domains. Based on the number of homologous repeats in their extracellular domains (EC) and the number of conserved motifs (CM) in their cytoplasmic domains, the d-pcdhs can be further grouped into d1-pcdhs (having 7 EC repeats and 3 CM, e.g. pcdh1, 7 and 9) and d2-pcdhs (having 6 EC repeats and 2 CM, e.g. pcdh17, 18 and 19) (Redies et al., 2005; Vanhalst et al., 2005). Compared to mammals, zebrafish has similar pcdhs except lacking β -pcdhs (Noonan et al., 2004; Wu, 2005). d-pcdhs show distinct expression patterns in both developing mammals and zebrafish brains (Vanhalst et al., 2005; Gaitan and Bouchard, 2006; Kim et al., 2007; Aamar and Dawid, 2008; Kubota et al., 2008; Neudert et al., 2008; Emond et al., 2009; Liu et al., 2009a,b).

Protocadherin-17 (pcdh17) is a member of the $\delta 2$ -pcdhs. There is little information on pcdh17 expression during the vertebrate development (Redies et al., 2005). Embryonic zebrafish and rat (day 3) showed similar pcdh17 mRNA (*pcdh17*) expression in the brain and retina (Kim et al., 2007; Biswas and Jontes, 2009; Liu et al., 2009a). There is no published report, to the best of our knowledge, on pcdh17 function in the vertebrate retinal development.

Methods

Zebrafish

Wild-type adult zebrafish (*Danio rerio*) were maintained as described in the Zebrafish Book (Westerfield, 2007). Zebrafish embryos were obtained from breeding of the adult zebrafish. Embryos for whole-mount immunohistochemistry, in situ hybridization or TUNEL labeling were raised in PTU (1-phenyl-2-thiourea, 0.003%) supplemented water (1:1 filtered fish tank water: egg water) to prevent melanization. All animal-related procedures were approved by the Care and Use of Animals in Research Committee at the University of Akron.

MO injections and mRNA synthesis

One translation blocking morpholino antisense oligonucleotide (MO; *pcdh17*atgMO: 5'-TGC ATC CCT TTC AGT GAG AGT GCC T-3'), a splice-blocking *pcdh17*MO (*pcdh17*smMO, designed to bind to the exon 1 and intron 1 boundary (Biswas and Jontes, 2009), with the exon 1 containing the entire EC domain and the transmembrane domain: 5'-ATA TAA GTT GTC GCT CCT ACC TGT A-3'), and a control 5-bp mismatch MO for the splice-blocking MO (5-misMO: 5'-ATA aAA cTT cTC cCT ACC TcT A-3'), from Gene

Tools (Philomath OR) were used as described (Nasevicius and Ekker, 2000) in the study. The *pcdh17* MOs sequences showed no significant similarities to any sequences (using BLAST) other than zebrafish *pcdh17* (GenBank accession number: XM 684743). MOs were microinjected into one- to four-cell stage embryos (1.5-3 ng/embryo) in Daneau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES pH 7.6).

The zebrafish *pcdh17* coding region was amplified with primers containing EcoRI (5') and XbaI (3') restriction sites and cloned first into pCR2.1-TOPO vector (Life Technologies, Carlsbad, CA), followed by cloning into pCS2+MT vector (Dr. Pamela Raymond, the University of Michigan). The pCS2+MT/*pcdh17* was verified by restriction digestion and sequencing (MacroGen, Rockville, MD). Capped *pcdh17* mRNA was synthesized from the pCS2+MT/*pcdh17* vector using a SP6 mMessage mMachine kit (Life Technologies). Purified *pcdh17* mRNA (95 to 190 pg/embryo) was injected alone or with the *pcdh17* MO into one- to four-cell stage embryos as described above.

Injected embryos were allowed to develop at 28.5°C until the embryos reached desired stages (e.g. 49 hpf), anesthetized in 0.02% MS-222 and fixed in 4% paraformaldehyde and processed as described below.

To assess a more direct *pcdh17* function on zebrafish retinal development, a vivo *pcdh17* MO (vivo-*pcdh17* MO, with the same sequence as the splice-blocking *pcdh17* MO, purchased from Gene Tools) was injected into the right eye of 25-26 hpf embryos (with the embryos held in position in 1% low melting agarose gel dissolved in the egg water supplemented with 0.01% MS-222). After the injection, the embryos were removed from the agarose and allowed to develop at 28.5°C until 49-50 or 72-73 hpf. The embryos were anesthetized and fixed as described above. Control embryos included injected (vivo 5-misMO with the same sequence as the 5-mis*pcdh17* MO, or phenol red injected) and uninjected control embryos. All control and morphant embryos were processed side by side.

Tissue processing

To prepare embryos for whole mount *in situ* hybridization or immunohistochemistry, the fixed embryos/larvae were rinsed in 0.1 M phosphate buffered saline (PBS, pH 7.4), followed by placing the embryos in increasing concentrations of methanol, and stored in 100% methanol at -20°C. Embryos prepared for immunohistochemistry on tissue sections were raised in PTU-free water (1:1 filtered fish tank water/egg water). Fixed embryos were washed in PBS, processed through a graded series of increasing sucrose concentrations, and placed in 20% sucrose in PBS overnight at 4°C. The embryos were then embedded and frozen in a mixture of OCT embedding compound and 20% sucrose (1:1, v/v). A cryostat was used to obtain 10-12 µm sections collected on pretreated glass slides (Fisher Scientific, Pittsburgh, PA), dried at room temperature and stored at -80°C.

In situ hybridization

Procedures for the synthesis of digoxigenin-labeled cRNA probes and whole mount *in situ* hybridization were described in detail previously (Liu et al., 1999; Westerfield, 2007). cDNAs used to generate other cRNA probes were kindly provided by Dr. Pamela Raymond at the University of Michigan (for *crx*, *otx5* and *rx1* genes), and Dr. Deborah Stenkamp at the University of Idaho (for *neuroD*). For each cRNA probe, control embryos (uninjected or embryos injected with the 5-misMO) and embryos injected with one of the *pcdh17* MOs (*pcdh17* morphants) were processed at the same time, side by side. For immunocytochemical detection of the digoxigenin-labeled cRNA probes, anti-digoxigenin Fab fragment antibodies conjugated to alkaline phosphatase were used, followed by an NBT/BCIP color reaction step (Roche Molecular Biochemicals, Indianapolis, IN).

Immunohistochemistry and TUNEL labeling

Detailed procedures for whole mount immunohistochemistry and immunostaining on tissue sections were described previously (Liu et al., 1999; Babb et al., 2005). Primary antibodies used were anti- β -catenin (1:500, Sigma), anti-HuC/HuD (1:2000; Molecular Probes/Invitrogen, Carlsbad, CA), anti-histone H3 (1:500; Chemicon International, Inc.), zn5 (1:1,500, Zebrafish International Resource Center, University of Oregon, Eugene, OR), and zpr-1 antibodies (1:500; Zebrafish International Resource Center). For immunofluorescent microscopy, an anti-rabbit or anti-mouse secondary antibody conjugated with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) was used. A biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was used for immunoperoxidase methods, and visualization of the reaction was achieved by using a DAB kit (Vector Laboratories).

Terminal dUTP nick-end labeling (TUNEL) was performed on whole-mount embryos using the Roche *in situ* cell death detection kit (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Data collecting and analysis

Stained whole mount embryos or tissue sections were observed with an Olympus BX51 compound microscope equipped with a SPOT digital camera. Quantitative data from each embryo processed for the histone H3 immunostaining was obtained from three alternate sections (to avoid counting the same positive cells twice) through the central retina, using the size and presence of the lens as reference points. The numbers of histone H3-positive cells from the three retinal sections were averaged to produce a value for that retina. Due to differences in eye sizes, numbers of histone H3-positive cells per unit eye area (1000 Sm^2) were used for comparison. Cell densities (number of labeled cells/area in square micrometer) in the retinal ganglion cell layer and outer nuclear layer (most histone H3-positive cells are located) were measured in anti-HuC/HuD and zpr-1 labeled central retinal sections from morphants and control embryos at 72 hpf. TUNEL-positive cells were collected from the entire eye of each whole mount embryo. Unpaired Student t-test was used to determined statistical significance ($p < 0.05$).

Results

Effects of *pcdh17*MOs injections on zebrafish body and eye development

Morpholino antisense oligonucleotide (MO) techniques effectively and selectively block gene function in vertebrates such as *Xenopus* and zebrafish (Egger, 2000; Nasevicius and Egger, 2000). Injection of either the translation blocking *pcdh17* MO (*pcdh17*atgMO) or splice-blocking *pcdh17* MO (*pcdh17*sMO) into one- to four-cell stage zebrafish embryos (*pcdh17* morphants) resulted in most embryos that had similar body shape and size as uninjected control embryos or embryos injected with the 5-misMO, but with obviously reduced eye size (Fig. 1; Table 1). Embryos injected with the *pcdh17*sMO (Fig. 1C) were indistinguishable from those injected with the *pcdh17*atgMO (Fig. 1E) in gross morphology and eye size (Fig. 1I and J). Efficacy of the *pcdh17*sMO was demonstrated by RT-PCR experiment showing that *pcdh17* mRNA was altered in *pcdh17*sMO injected embryos with inclusion of intron 1 (Fig. 1M; Morcos, 2007) in the mRNA leading to a premature stop codon 123 nucleotides downstream of the exon 1. The included *pcdh17* intron 1 sequence in the *pcdh17* morphants was verified by sequencing (Macrogen). Injection of a lower dosage of *pcdh17*MOs (1.5 ng/embryo) resulted in most embryos with slight to moderate defects (e.g. small eyes but no noticeable body defects). Injection of a higher amount of *pcdh17* MOs (3.0 ng/embryo) resulted in most embryos with moderate to severe phenotypes (Table 1). The severely defective embryos (19.6% and 19.2% for *pcdh17*sMO and *pcdh17*atgMO, respectively, Table 1) had smaller eyes and heads, slightly smaller and/or curved bodies).

The gross morphological defects were not obvious at 24 hpf, but became apparent at 49–50 hpf. The morphant eye defect was partially (Fig. 1F and L, Table 1) or completely rescued (Fig. 1D and K, Table 1) in embryos co-injected with the synthetic zebrafish *pcdh17* mRNA (95–190 pg/embryo) and *pcdh17*MO (3.0 ng/embryo). Embryos injected with *pcdh17* mRNA alone (190 pg/embryo) were indistinguishable in gross morphology and eye size from uninjected control embryos (Table 1, images not shown). To make analysis and interpretation of results more consistent, eye development and retinal cell differentiation were examined in moderately affected embryos injected with either *pcdh17*MO or *pcdh17*atgMO (3.0 ng/embryo).

Measurements of the eye size (area in square microns) of live embryos at 49 hpf and 72 hpf revealed that *pcdh17* morphant eyes were significantly smaller ($p < 0.001$) than uninjected control embryo eyes (Table 2). Moreover, embryos injected with the control 5-misMO had similar eye size as the uninjected control embryos or completely *pcdh17* mRNA rescued embryos (Table 2). Embryos injected with the *pcdh17*MO had similar eye size as those injected with the *pcdh17*atgMO. Partially *pcdh17* mRNA rescued embryos had eye sizes smaller than the control embryos, but larger than the morphants (Table 2). Cell density measurements on eye tissue sections ($n = 10$ sections/group, 2–3 alternate sections from the central retina/embryo) processed for anti-HuC/HuD and zpr-1 immunostaining (see below) revealed that there were similar number of labeled cells per 1,000 Sm^2 in the morphant retinal ganglion cell layer (gcl, 34.7 ± 3.1) and outer nuclear layer (onl, 20.6 ± 1.6) as in the control gcl (34.0 ± 2.5) and onl (19.8 ± 1.3).

Analysis of apoptosis and cell proliferation in *pcdh17* morphants

The small eyes in *pcdh17* morphants could be due either to increased cell death as in *cadherin-4* morphants retinæ (Babb et al., 2005) or reduced cell proliferation as in *cadherin-6* morphants retinæ (Liu et al., 2008). TUNEL labeling was performed to examine apoptosis in whole mount embryos (Fig. 2), while histone H3 immunostaining was used to determine retinal cell proliferation (Fig. 3; Adams et al., 2001). There were very few apoptotic cells in the retina of both young (25 hpf; 1 TUNEL-positive cell in 12 eyes) and older (50 hpf; 3 TUNEL-positive cells in 22 eyes) *pcdh17* morphants (Fig. 2D and E), which was similar to uninjected control embryos (Fig. 2A and B; 1 TUNEL-positive cell in 12 eyes at 25 hpf, 4 TUNEL-positive eyes in 26 eyes at 50 hpf). These low numbers of TUNEL-positive cells in the eye were unlikely due to experimental errors, because numerous labeled cells were detected in the trunk and tail regions of these embryos (Fig. 2C and F).

Mitotic nuclei in the control and *pcdh17* morphant retinæ were revealed using the histone H3 immunostaining (Fig. 3). As in the retina of control embryos (Fig. 3A), most labeled cells in morphant retinæ (Fig. 3B) were detected in the peripheral region at 50 hpf. However there were significantly more ($p < 0.001$) histone H3-positive cells in the control retina than *pcdh17* morphant retina (Fig. 3C).

Expression of transcription factors and in the *pcdh17* morphant retina

To determine whether *pcdh17* regulates retinal differentiation, four transcription factors that are known markers for vertebrate retinal differentiation (*rx1* (Chuang et al., 1999; Chuang and Raymond, 2001), *NeuroD* (Morrow et al., 1999; Inoue et al., 2002; Yan et al., 2005), *crx* (Furukawa et al., 1997; Blackshaw et al., 2001; Liu et al., 2001; Shen and Raymond, 2004) and *otx5* (Gamse et al., 2002)) were examined in control and *pcdh17* morphant retinæ (Fig. 4). Blocking *pcdh17* function appeared to have little effect on early development of the eye as *rx1* expression was similar between the control and morphant retinæ (Fig. 4A and E; Table 3). *neuroD*, *crx* and *otx5* were strongly expressed throughout the control retina at 49 hpf (Fig. 4B–D), but their expression (judging by staining intensity), was greatly reduced in

pcdh17 morphant retinæ (Fig. 4F-H; Table 3). *crx* expression was especially diminished, almost absent in the posterior half of the morphant retina (Fig. 4G). In contrast, *crx* and *otx5* expression in the morphant pineal gland was similar to control embryos (inserts in Fig. 4C, D, G and H).

Differentiation of retinal cells was disrupted in the *pcdh17* morphant retina

Differentiation of retinal ganglion cells (RGCs) was examined at 49 hpf using zn5 antibody (labeling both RGC body and axons; Malicki et al., 2003; Masai et al., 2003). A well-labeled retinal ganglion cell layer (gcl) and optic nerve were seen in the retina of control embryos (Fig. 5A), embryos injected with the 5-misMO (Fig. 5B), or embryos co-injected with the *pcdh17*sMO and *pcdh17* mRNA (completely rescued, Fig. 5C). In these embryos each optic nerve exited the retina, crossed the other optic nerve at the base of the diencephalon and projected toward their brain targets (Fig. 5A-C). Similar zn5 labeling pattern was observed in *pcdh17* morphants (Fig. 5D and E), except that the gcl was much reduced in size, and the optic nerve was much thinner compared to the control embryos. Partially *pcdh17* mRNA rescued embryos had gcl and optic nerve labeling between the morphants and control embryos (Fig. 5F). Reduced gcl in *pcdh17* morphants was confirmed using anti-HuC/HuD (labeling RGC body; Table 4; Malicki et al., 2003; Masai et al., 2003). Differentiation of retinal cells in 3-day old *pcdh17* morphants was examined by anti-HuC/HuD (labeling cell bodies of both RGCs and amacrine cells at this stage) and *zpr-1* (labeling double cones) immunostaining (Fig. 5G-L; Table 4; Malicki et al., 2003; Masai et al., 2003). In control embryos (Fig. 5G), embryos injected with the 5-misMO (Fig. 5H), or embryos co-injected with *pcdh17*sMO and *pcdh17* mRNA (completely rescued, Fig. 5I), there were well-formed gcl, inner nuclear layer (inl; amacrine cells are located in the inner half of the inl) and outer nuclear layer (onl). Moreover, in these embryos there was a distinct unlabeled region between the gcl and inl, where RGCs, amacrine cells and bipolar cells synapse (i.e. the inner plexiform layer). In *pcdh17* morphant retinæ (Fig. 5J and K; Table 4), regions expressing those markers, especially *zpr-1*, were much reduced, and the inner plexiform layer was barely detectable. Partially *pcdh17* mRNA rescued embryos had similar labeling as in controls, despite smaller eye sizes (Fig. 5L). To further examine *pcdh17* function on retinal lamination, we used β -catenin immunostaining (labeling cell membrane of retinal cells and retinal synaptic layers) and found that retinal layer organization was not much affected in most 3-day *pcdh17* morphants, but the inner plexiform layer was much reduced compared to that in control embryos (Table 4). Moreover, β -catenin staining intensity in the morphant eye was reduced compared to the control eye (images not shown).

Using vivo-*pcdh17*sMO to study *pcdh17* function on zebrafish retinal development

Vivo-MOs are morpholino oligos with covalently linked delivery moieties, allowing oligos to enter cells in older embryos and adult organisms (Morcos et al., 2008). Vivo-MOs technology has been successfully used to study gene function in several vertebrates including zebrafish (Carrillo et al., 2010; Guo et al., 2011; Kizil and Brand, 2011). To determine if *pcdh17* is directly involved in retinal cell differentiation, we injected a vivo-*pcdh17*sMO into the right eye of 25–26 hpf embryos (vivo-morphants), followed by examining the gross morphology, size, and differentiation of selective retinal cells using approaches described above. The vivo-morphants (Fig. 6B) had similar gross morphology as uninjected control embryos, phenol red injected (to the right eye) embryos or embryos injected (to the right eye) with the vivo-5-mis MO (Fig. 6A and B), but the vivo-morphants had smaller eyes compared to the control embryos (Fig. 6C-E; Table 5). At 49 and 72 hpf, both the right eye (injected) and the left eye (uninjected) were significantly smaller than those of the control embryos, and the vivo-morphant right eye was also significantly smaller than the left eye (Fig. 6 and Table 5). Effectiveness of the vivo-*pcdh17*sMO application was assessed by RT-PCR (using total RNA prepared from entire embryos (n=39 vivo-morphants

at 72–75 hpf, with the vivo-*pcdh17*MO injected into either the right or left eye at 25–27 hpf (Fig. 6F).

Differentiation of selective retinal cells in the vivo-morphants was analyzed using immunostaining on whole mount embryos and retinal sections as described above. At 49 hpf, zn5 immunostaining revealed that there was no detectable differences among uninjected control embryos, phenol red injected embryos and embryos injected (to the right eye) with the vivo-5-misMO (Fig. 7A). Labeling of the vivo-morphant gcl and/or the optic nerve was obviously changed (i.e. smaller zn5-positive gcl and/or thinner optic nerve) in less than half (43.4%) of the left eye (uninjected), but in the vast majority (87.5%) of the right eye (injected) (Fig. 7B and C; Table 6). At 72 hpf, again vivo-5-misMO injected embryos (Fig. 7G) had similar anti-HuC/HuD and zpr-1 labeling as uninjected control embryos or embryos injected with phenol red. For the vivo-morphant eyes, similar percentages (41.6% and 91.7% for the left and right eye, respectively) had altered anti-HuC/HuD and zpr-1 staining, with reduced immunoreactive regions in the gcl, inner half of the inl (amacrine cells reside), onl (photoreceptors reside), and narrower inner plexiform layer (Fig. 7F, H and I). As in the eye size and zn5 staining in 49 hpf vivo-morphants, the retinal defects in the 72 hpf vivo-morphants were more severe in the injected right eye (Fig. 7F and I) than the uninjected left eye (Fig. 7E and H).

Discussion

Pcdh17 is involved in zebrafish eye development

The expression pattern of *pcdh17* in embryonic and larval zebrafish (Liu et al., 2009a) suggests that *pcdh17* plays a role in retinal cells differentiation. This is confirmed by the current study. The smaller eye phenotype in *pcdh17* morphants is likely due mainly to reduced retinal cell proliferation, which is similar to *cadherin-6* morphants (Ruan et al., 2006; Liu et al., 2008). Disrupted development of RGCs, amacrine cells and photoreceptors in *pcdh17* morphants suggests that *pcdh17* participates in differentiation of these cells. Moreover, *pcdh17* may be more directly involved in RGCs and amacrine cells differentiation than photoreceptor development, because *pcdh17* is expressed throughout the retina of 34–72 hpf, except in the outer nuclear layer (photoreceptors reside) where its expression is weaker at 50 hpf when this layer is becoming distinguishable, and its expression is much reduced at 72 hpf (Liu et al., 2009a). Reduced photoreceptor differentiation may, at least partially, due to *pcdh17* effect on differentiation of retinal cells located in the inl such as the horizontal cells (located in the outer portion of the inl where *pcdh17* expression remains strong at 50–72 hpf), which then affects photoreceptor development. Horizontal cells differentiation was not examined in *pcdh17* morphants mainly due to lack of reliable horizontal cell markers. It is also possible that *pcdh17* is directly involved in development of the earliest formed photoreceptors (e.g. blue cones and double cones) that begin to express photoreceptor specific markers as early as 40–44 hpf, but is less involved in differentiation of later formed photoreceptors (e.g. red cones and rods that start to form between 48 and 60 hpf) (Larison and Bremiller, 1990; Robinson et al., 1995; Schmitt and Dowling, 1996; Raymond et al., 1996).

Similar eye (smaller) and retinal cells defects (e.g. smaller gcl and thinner optic nerve) were observed in embryos whose right eye was injected with the vivo-*pcdh17*MO at 25–26 hpf, suggesting that *pcdh17* function directly on zebrafish retinal cell differentiation. The results that the uninjected eye (left eye) of these embryos was also smaller compared to control embryos suggest that the vivo-*pcdh17*MO must have reached to the other eye via blood circulation. Zebrafish optic vessels (e.g. optic artery and optic vein) become distinguishable around 26–28 hpf (Isogai et al., 2001). Vivo-MOs have been successfully used to study gene function in developing and adult organisms by intraperitoneal or intravenous injections to

deliver MOs systemically (Carrillo et al., 2010; Notch et al., 2011; Para et al., 2012), and stronger MOs effects have been obtained from localized injection to specific tissue/organ such as cerebroventricular, intraotic, and intravitreal injections (Moulton and Jiang, 2009; Kizil and Brand, 2011; Kowalik and Hudspeth, 2011; Liu et al., 2012), which may explain, at least partially, the more severe retinal defects in the injected right eye compared to the uninjected left eye.

Compared to zebrafish embryos with disrupted classic cadherins expression (e.g. cadherin-2, cadherin-4 and cadherin-6; Lele et al., 2002; Malicki et al., 2003; Masai et al., 2003; Babb et al., 2005; Liu et al., 2008), *pcdh17* morphants show defects that are mainly confined to the eye with less severe retinal defects. Despite similar cadherin-2 and *pcdh17* expression (throughout the retina) in the retina of 34–50 hpf embryos, *cadherin-2* morphants and mutants showed disrupted retinal lamination (Malicki et al., 2003; Masai et al., 2003), while *pcdh17* morphants have normal appearing retinal lamination. It is possible that cadherin-2 compensates for *pcdh17* function in retinal lamina formation, or later expressed molecules (e.g. *pcdh17* see above) have less effect on the formation of the retinal lamination than earlier expressed molecules (e.g. cadherin-2 throughout the retina at 24 hpf, Liu et al., 2001a). Although cadherin-4 and cadherin-6 are expressed later (after 30 hpf) and have more restricted expression (confined mainly to the inner portion of the inner nuclear layer, Liu et al., 1999; 2001a, b), differentiation of RGCs, amacrine cells and photoreceptors is much more severely affected in these morphants (Babb et al., 2005; Liu et al., 2008) than in *pcdh17* morphants. This may partially due to wider expression of these classical cadherins in other parts of the CNS and/or other tissues. For example, cadherin-6 is expressed in both the CNS and selective mesodermal structures (e.g. cartilages, kidney). Embryos with disrupted cadherin-6 function also show apparent head and body defects (e.g. enlarged pericardial cavity and abnormally developed kidney, Kubota et al., 2007; Liu et al., 2008). Again, the less severe retinal defects observed in the *pcdh17* morphants may due to compensatory function of other cadherins (e.g. cadherin-2, -4 and -6) expressed in the developing retina.

Piper and colleagues (Piper et al., 2008) showed that *Xenopus* NF-protocadherin (the *Xenopus* ortholog of protocadherin-7) plays an essential role in retinal ganglion cell differentiation. Blocking NF-protocadherin function using an extracellular domain deleted dominant-negative construct in *Xenopus* embryos resulted in retinal ganglion cells with severely disrupted axonal initiation and elongation, which is somewhat similar to RGCs in *pcdh17* morphants. RGC dendrite genesis is also disrupted in those *Xenopus* embryos (Piper et al., 2008). Although *pcdh17* morphant RGC dendrites were not examined in this study, reduced morphant inner plexiform layer, where RGCs dendrites synapse with retinal cells from the INL, suggests that RGC dendrite genesis is also affected when *pcdh17* function is disrupted.

Possible mechanisms of *pcdh17* function in retinal development

It is possible that *pcdh17* function in retinal cell development through controlling expression of regulatory molecules known to be involved in the vertebrate retinal differentiation. Expression of *crx*, *neuroD* and *otx5* in the morphant retina is reduced compared to control retina, which is similar to results obtained from embryos with disrupted cadherin-2, cadherin-4, or cadherin-6 function (Babb et al., 2005; Liu et al., 2007; 2008). Also similar to cadherin-2 *glo* mutants (Liu et al., 2007), *crx* expression appears to be more reduced in the posterior half of the *pcdh17* morphant retina. But *otx5* expression in the *glo* mutant is almost absent in the posterior half of the retina, whereas *otx5* expression is only slightly reduced compared to the anterior portion of the retina in the *pcdh17* morphant. Like *cadherin-6* morphants (24 hpf, Liu et al., 2008), *rx1* expression was unchanged in the 24 hpf *pcdh17* morphant retina. But unlike the *cadherin-6* morphant retina where *neuroD* and *crx* expression is barely detectable, while *otx5* expression is mainly confined to the anterior ¼ of

the retina (Liu et al., 2008), expression of these molecules is less affected in the *pcdh17* morphant retina. The above discussion is based on *in situ* hybridization staining intensity differences between morphants and control embryos, therefore needs to be confirmed by a quantitative technique (e.g. quantitative PCR). In summary, reducing cadherin function appears to diminish expression of most of these transcription factors, but each cadherin seems to have differential effects on expression of these regulatory molecules.

Pcdhs (e.g. *pcdh24*) were shown to regulate cell motility, growth and differentiation through β -catenin-T-cell factor transcription factor (TCF/lef) signaling pathway (Yu and Malenka, 2003; Rosso et al., 2005; Ose et al., 2009). β -catenin expression appears to be reduced in *pcdh17* morphant retina. Pcdhs also control tissue development by regulating tyrosine kinase Fyn, the alpha isoform of protein phosphatase 1, or adaptor protein Disabled 1 (binding to protocadherins cytoplasmic domains), and/or by mediating weak hemophilic adhesion (reviewed by Frank and Kemler, 2002). *Pcdh19* was recently shown to regulate tissue development by interacting with a classic cadherin (cadherin-2, Biswas et al., 2010; Emond et al., 2011). Although our results suggest that *pcdh17* plays an important role in zebrafish retinal development, exactly how *pcdh17* function to regulate retinal cell differentiation is unclear. *Pcdh17* may affect retinal cell differentiation by interacting with its intracellular partners, which in turn affects gene expression, such as NF-protocadherin interacting with its cofactor TAF1 to control RGC differentiation in *Xenopus* (Piper et al., 2008). It is also possible that *pcdh17* exerts its effect by interacting with other types of cadherin molecules to mediate cell-cell adhesion, which is known to be essential for axonal initiation, outgrowth, fasciculation and pathfinding (Bixby and Zhang, 1990; Riehl et al., 1996; Stone et al., 1996; Treubert-Zimmermann et al., 2002; Biswas et al., 2010). We are currently using proteomics and genomics approaches to identify potential molecular players involved in *pcdh17* function in zebrafish retinal development.

Acknowledgments

NIH; grant numbers: R15 EY13879

We thank Drs. Pamela Raymond (University of Michigan) and Deborah Stenkamp (University of Idaho) for providing the expression vector and cDNAs of the retinal genes. This study was supported by NIH EY13879 (Q. Liu) and NIH DC006436 (J.A. Marrs).

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Figure 1.

Gross morphological defects in *pcdh17* morphants were mainly confined to the eye. Panels A-L show lateral views of live embryos with anterior to the left and dorsal up. Eyes in whole embryos (panels A-F) are indicated by asterisks. Embryos injected with the splice-blocking *pcdh17* MO (*pcdh17*sMO, panel C) and those injected with the translation blocking *pcdh17* MO (*pcdh17*atgMO, panel E) had similar body shape and size as uninjected control embryos (control, panel A) or embryos injected with the 5-misMO (5-mis, panel B), but had smaller eyes. Panels G-L show higher magnification of the head region focusing on the eye. The control eye from an uninjected embryo (panel G) is indistinguishable in appearance and size from the eye of an embryo injected with the 5-misMO (panel H), while the *pcdh17*sMO morphant eye (panel I) looks similar to the *pcdh17*atgMO morphant eye (panel J). Embryos co-injected with *pcdh17*sMO and *pcdh17* mRNA were indistinguishable in appearance (complete rescue, CmRNAs; panels D and K) from control embryos, or showed milder eye defects (partial rescue, PmRNAs; panels F and L). Scale bar = 200 μ m for panels A-F, and 100 μ m for panels G-L. Panel M shows diagnostic RT-PCR confirming effect of the splice-blocking MO (*pcdh17*sMO) on *pcdh17* transcription of 50 hpf embryos. Lane 1 is 1 kb DNA ladder. Lane 2 (morphants cDNA as template) shows a PCR band of the correct size due to inclusion of intron 1, using PCR primers bracketing part of the exon 1 (forward primer starting at nucleotide 2222) and part of intron 1 (45 nucleotides downstream of the end of the exon 1). Lane 3 (the same morphant cDNA as used in lane 2) is a PCR product using primers bracketing part of zebrafish *pcdh17* exon 1 (nucleotides 1506–1958). Lanes 4 (control embryo cDNA) and 5 (adult brain cDNA) show a faint band (asterisk) of the size seen in lane 2, likely due to unspliced or aberrantly spliced transcripts. Lanes 6 (control embryo cDNA) and 7 (adult brain cDNA) are loading controls showing a PCR band using the same pair of primers as in lane 3.

Figure 2.

Apoptosis analysis using TUNEL staining. All panels are lateral views of whole mount embryos with anterior to the left and dorsal up. Panels A, B, D and E are lateral views of the head region focusing on the eye, while panels C and F are from posterior trunk and tail regions of the same embryos shown in panels B and E, respectively. There are no TUNEL positive nuclei detected in either the control or morphant eye, but numerous in the trunk and tail regions of the same respective embryos. Abbreviations: le, lens; nr, neural retina. Scale bars = 100 μ m for all panels.

Figure 3.

Histone-H3 immunostaining in control retina (panel A) and *pcdh17sMO* morphant retina (panel B). The images are cross sections (10 μm) from central retina (dorsal to the right). Retinal areas are outlined by the dashed line. Arrows indicate a few labeled nuclei in focus. The data in panel C is from 20 eyes (10 for each group). Asterisks indicate highly significant differences ($p < 0.0001$) between the control and morpholino treatment. Abbreviations are the same as in Figure 2. Scale bar = 50 μm for panels A and B.

Figure 4.

Expression of transcription factors in the control and *pcdh17* morphant retinas. Panels A and E are in-face views (dorsal up) of embryo heads from embryos processed for whole mount *in situ* hybridization. The remaining panels are lateral views (anterior to the left and dorsal up) of eyes and/or heads of whole mount embryos processed for *in situ* hybridization. All except *rx1* expression was reduced in the morphant retina. Arrows in the insert in panels C, D, G and H point to the pineal gland that show similar transcription factor expression between control embryos and morphants. Abbreviations are the same as in Figure 2. Panels A and E are of the same magnification, while the remaining panels have the same magnification as panel B. Scale bars = 100 Sm.

Figure 5.

Immunostaining analysis of retinal cell differentiation in control embryos (panels A and G), embryos injected with the 5-mis MO (panels B and H), embryos co-injected with the *pcdh17s*MO and *pcdh17*mRNA (panels C, F, I and L) and *pcdh17*morphants (panels D, E, J and K). Panels A-F show ventral views (anterior up) of the head region of 49 hpf whole mount embryos processed for zn5 peroxidase immunostaining. Panels G-L show cross retinal sections (dorsal to the left, from central retina) from 72 hpf embryos labeled with anti-HuC/HuD (labeling retinal ganglion cell layer, gcl, and the inner portion of the inner nuclear layer, inl) and zpr-1 (labeling double cones in the outer nuclear layer, onl) antibodies (immunofluorescent methods). Arrows in panels H and J indicate location of the optic nerve. Other abbreviations: ipl, inner plexiform layer; le, lens; on, optic nerve; te, telencephalon. Remaining abbreviations are the same as in Figure 1. Scale bars = 100 Sm for panels A-F, and 50 Sm for panels G-L.

Figure 6.

Gross body (panels A and B) and eye (panels C-E) morphology in live embryos injected with the vivo-5-misMO (vivo-5-mis, panels A and C) or embryos injected with the vivo splice-blocking *pcdh17* MO (vivo-*pcdh17*sMO, panels B, D and E), to the right eye at 25-26 hpf. The upper panels show lateral views (anterior to the right and dorsal up) of the right side of the embryos, while panels C-E show ventral views (anterior to the top) of the head region. In some vivo-morphant embryos (e.g. panel D), only the right eye was obviously smaller than eyes of vivo-5-mis embryos, while in others (e.g. panel E), both eyes were apparently smaller than control eyes, and the right eye was the smallest. Scale bars = 250 Sm for panels A and B, and 50 Sm for panels C-E. Panel F shows diagnostic RT-PCR confirming effect of the vivo splice-blocking MO (vivo-*pcdh17*sMO) on *pcdh17* transcription of 72-75 hpf embryos. Lane 1 is 1 kb DNA ladder. Lanes 2 and 4 are morphants and control embryos, respectively, using the same pair of primers as in Fig. 1M (lanes 2, 4 and 5, the reverse primer is from intron 1). Lanes 3 and 5 are vivo morphants and control embryos loading controls, respectively using primers bracketing part of zebrafish *pcdh17* exon 1 (nucleotides 1506–1958). The faint band in lane 4 is likely due to unspliced or aberrantly spliced transcripts.

Figure 7.

Immunostaining analysis of retinal cell differentiation in vivo-morphant retinæ. Panels A-C are ventral views (dorsal up) of the head region of 49 hpf whole mount embryos processed for zn5 immunoperoxidase staining. Panels D-I show cross retinal sections (dorsal to the left, from central retina) of 72 hpf embryos processed for anti-HuC/HuD and zpr-1 immunofluorescent labeling. In some embryos (e.g. panel B), the right optic nerve was apparently thinner than the left optic nerve, while in others (e.g. panel C) both optic nerves seemed to be of the same thickness. For retinal section images, a more obviously affected example (panels H and I) and less affected example (panels E and F) are shown for the uninjected left eye and injected right eye. Arrows in panels D, E, G and I point to location of the optic nerve. Abbreviations are the same as in Figures 2 and 6. Scale bars = 100 Sm for panels A-C, 50 Sm for the remaining panels.

Table 1Effects of *pcdh17* MOs injections on zebrafish (48–50 hpf) development

	Number of Embryos with slight gross defects	Number of embryos with moderate- gross defects (%)	Number of embryos with severe gross defects (%)	Number of embryo examined (% wild type)
Uninjected control	16 * (2.8%)	6 * (1.1%)	10 * (1.8%)	563 (94.3%)
<i>pcdh17</i> sMO (1.5 ng)	153 (47.1%)	124 (38.2%)	25 (7.7%)	325 (7.1%)
<i>pcdh17</i> sMO (3.0 ng)	61 (12.5%)	311 (63.6%)	96 (19.6%)	489 (4.3%)
<i>pcdh17</i> atgMO (1.5 ng)	150 (45.7%)	110 (33.5%)	29 (8.8%)	328 (11.9%)
<i>pcdh17</i> atgMO (3.0 ng)	79 (15.6%)	298 (58.9%)	97 (19.2%)	506 (6.9%)
5-mis <i>pcdh17</i> sMO (3.0 ng)	5 * (2.8%)	3 * (1.7%)	3 * (1.7%)	178 (93.8%)
<i>pcdh17</i> sMO (3.0 ng) + <i>pcdh17</i> mRNA (95 pg)	52 (67.5%)	16 ** (20.8%)	4 * (5.2%)	77 (6.5%)
<i>pcdh17</i> sMO (3.0 ng) + <i>pcdh17</i> mRNA (190 pg)	27 (26.5%)	6 * (5.9%)	4 * (3.9%)	102 (63.7%)
<i>pcdh17</i> mRNA (190 pg)	4 * (4.7%)	1 * (1.2%)	2 * (2.4%)	85 (91.8%)

Gross morphological defects in the morphants are mainly seen in the eye (i.e. eye size).

* The defects (e.g. smaller, curled, truncated bodies and/or much smaller or no heads and eyes) are different from those of morphants.

** Morphological defects in those embryos appeared to be between the slightly affected and moderately affected *pcdh17* morphants.

Table 2

Effects of *pcdh17*MOs injections on eye size (region area in μm^2)

	Uninjected Control	<i>pcdh17</i> sMO	<i>pcdh17</i> atgMO	5-mis	PmRNAres	CmRNAres
49 hpf	49,872 $\pm$ 3,825	34,457 $\pm$ 3,185 [*]	33,500 $\pm$ 2,825 [*]	49,740 $\pm$ 2,196	42,462 $\pm$ 2,065 [*]	49,025 $\pm$ 2,282
72 hpf	57,301 $\pm$ 2,469	43,875 $\pm$ 3,082 [*]	43,591 $\pm$ 2,905 [*]	ND	ND	ND

n = 20 for embryos injected with *pcdh17*sMO. n = 10 eyes for each remaining group of embryos. Measurements were taken from the lateral side of the eye of live embryos.

^{*} The morphant eyes and partially mRNA rescued embryo eyes (PmRNAres) are significantly smaller ($p < 0.01$) than either the uninjected control embryo eyes, those of embryos injected with the 5-bp mismatched MO (5-mis), or those completely rescued embryo eyes (CmRNAres). The partially rescued eyes are also significantly ($p < 0.01$) larger than the morphant eyes. The eyes of 72 hpf morphants are significantly smaller ($p < 0.01$) than those of 49 hpf control embryos. Other abbreviation: ND, analysis not done.

Table 3Effects of *pcdh17* knockdown on expression of transcription factors and in the retina

	<i>rx1</i>	<i>neuroD</i>	<i>crx</i>	<i>otx5</i>
24 hpf				
Uninjected control (n=20)	0% (n=24)	ND	ND	ND
<i>pcdh17</i> MO (n=14)	0% (n=16)	ND	ND	ND
<i>pcdh17</i> atgMO (n=18)	0% (n=18)	ND	ND	ND
48-50 hpf				
Uninjected control	ND	0% (n=16)	0% (n=14)	0% (n=20)
<i>pcdh17</i> MO (n=10)	ND	100% (n=12)	ND	100% (n=12)
<i>pcdh17</i> atgMO (n=10)	ND	100% (n=12)	100% (n=16)	100% (n=14)
5-misMO	ND	0% (n=10)	ND	0% (n=10)

n, number of eyes examined for each probe; %, percentages of greatly reduced or altered staining (staining area and/or staining intensity), compared to the majority of control embryos. Abbreviation: ND, analysis not done.

Table 4Effects of *pcdh17* knockdown on zebrafish retinal development revealed by immunostaining

	Hu	Hu&zpr-1	zn5	beta-catenin
48-50 hpf				
Uninjected control	0% (n=20)	ND	12.5% (n=40)	ND
<i>pcdh17</i> MO	100% (n=20)	ND	100% (n=30)	ND
<i>pcdh17</i> atgMO	ND	ND	100% (n=24)	ND
5-mis <i>pcdh17</i> MO	8.3% (n=24)	ND	13.3% (n=30)	ND
<i>pcdh17</i> MO (3.0 ng) + <i>pcdh17</i> mRNA (190 pg)	ND	ND	20% (n=50)	ND
72 hpf				
Uninjected control	ND	8.0% (n=25)		
<i>pcdh17</i> MO	ND	85% (n=20)	ND	16.7% (n=6)
<i>pcdh17</i> atgMO	ND	100% (n=12)	ND	ND
5-misMO	ND	0% (n=8)	ND	ND
<i>pcdh17</i> MO (3.0 ng) + <i>pcdh17</i> mRNA (190 pg)	ND	16.7% (n=12)	ND	ND

n, number eyes examined; %, percentages of greatly reduced, altered staining (staining area and/or intensity), and reduced inner plexiform layer, compared to the majority of control embryos. Abbreviations: Hu, anti-HuC/HuD immunostaining; Hu & zpr-1, anti-HuC/HuD and zpr-1 double immunostaining; ND, analysis not done; zn5, zn5 immunostaining.

Table 5Effects of vivo-*pcdh17*sMO injection on eye size (region area in μm^2)

	Uninjected control	vivo-5-mis	phenol-red injected	vivo- <i>pcdh17</i> sMO
49 hpf				
Left eye	49,858 $\pm$ 961 (n=6)	ND	ND	45,107 $\pm$ 2,433 (n=12) *
Right eye	50,358 $\pm$ 1,534 (n=6)	ND	ND	40,001 $\pm$ 2,561 (n=12) *
72 hpf				
Left eye	ND	57,644 $\pm$ 2,724 (n=10)	57,924 $\pm$ 2,420 (n=12)	53,390 $\pm$ 3,555 (n=12) *
Right eye	ND	58,003 $\pm$ 3,244 (n=10)	58,763 $\pm$ 1,701 (n=12)	46,098 $\pm$ 2,838 (n=12) *

n, number of eyes examined. Measurements were taken from the lateral side of the eye.

* The vivo-morphant eyes are significantly smaller ($p < 0.01$) than the uninjected control, phenol red injected control or vivo-5-misMO injected embryo eyes.

Table 6Effects of vivo-*pcdh1*7sMO injection on zebrafish retinal development revealed by immunostaining

	Zn5	Hu&zpr-1
49 hpf		
Uninjected control	9.4% (n=32)	ND
Morphant left eye	43.4% (n=16)	ND
Morphant right eye	87.5% (n=16)	ND
Vivo-5-misMO	15.0% (n=20)	ND
Phenol red injected control	12.5% (n=16)	ND
72 hpf		
Uninjected control	ND	0% (n=10)
Morphant left eye	ND	41.6% (n=12)
Morphant right eye	ND	91.7% (n=12)
Vivo-5-misMO	ND	12.5% (n=16)
Phenol red injected control	ND	10% (n=10)

n, number eyes examined; %, percentages of greatly reduced, altered staining (staining area and/or intensity) and reduced inner plexiform layer compared to the majority of control embryos, Abbreviations: Hu & zpr-1, anti-HuC/HuD and zpr-1 double immunostaining; ND, analysis not done; zn5, zn5 immunostaining.