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Differential Expression of Photoreceptor-Specific Genes in the Retina of a Zebrafish Cadherin2 Mutant *glass onion* and Zebrafish Cadherin4 Morphants

Q. Liu^{1,*}, R. A. Frey², S. G. Babb-Clendenon³, B. Liu¹, J. Franci¹, A. L. Wilson¹, J. A. Marrs³, and D. L. Stenkamp²

¹Department of Biology, University of Akron, Akron, Ohio

²Department of Biological Sciences, University of Idaho, Moscow, Idaho

³Department of Medicine, Indiana University Medical Center, Indianapolis, Indiana

Abstract

Cadherins are Ca^{++} -dependent transmembrane molecules that mediate cell-cell adhesion through homophilic interactions. Cadherin2 (also called N-cadherin) and cadherin4 (also called R-cadherin), members of the classic cadherin subfamily, have been shown to be involved in development of a variety of tissues and organs including the visual system. To gain insight into cadherin2 and cadherin4 function in differentiation of zebrafish photoreceptors, we have analyzed expression patterns of several photoreceptor-specific genes (*crx*, *gnat1*, *gnat2*, *irbp*, *otx5*, *rod opsin*, *rx1*, and *uv opsin*) and/or a cone photoreceptor marker (*zpr-1*) in the retina of a zebrafish cadherin2 mutant, *glass onion* (*glo*) and in zebrafish embryos injected with a *cadherin4* specific antisense morpholino oligonucleotide (*cdh4MO*). We find that expression of all these genes, and of *zpr-1*, is greatly reduced in the retina of both the *glo* and *cadherin4* morphants. Moreover, in these embryos, expression of some genes (e.g. *gnat1*, *gnat2* and *irbp*) is more affected than others (e.g. *rod opsin* and *uv opsin*). In embryos with both cadherins functions blocked (*glo* embryos injected with the *cdh4MO*), the eye initially formed, but became severely and progressively disintegrated and expressed little or no *crx* and *otx5* as development proceeded. Our results suggest that cadherin2 and cadherin4 play important roles in the differentiation of zebrafish retinal photoreceptors.

Keywords

retina; rods and cones; development; cell adhesion molecules; *Danio rerio*

1.Introduction

Zebrafish has become an important model system to study animal development due to its many experimental advantages including ease of care and maintenance, external development, routine production of large clutches of eggs from single females, transparency of embryos, and its demonstrated utility as a genetic model. Similar to other vertebrate retinas, the zebrafish retina is a well-laminated structure consisting of alternating cellular and plexiform layers. There are five types of photoreceptors in the zebrafish retina: rods, short single cones, long

*To whom correspondence should be addressed., Phone: 330-972-7558; Fax: 330-972-8445; E-mail: qliu@uakron.edu

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single cones, and principal and accessory members of a double cone pair (reviewed by Malicki, 1999). These photoreceptors can be distinguished by their different morphologies as well as by their differential expression of photoreceptor-specific genes. These include rod cell-specific genes *rod opsin* and *transducing α -subunit* for rod (*gnat1*), cone cell-specific genes *transducing α -subunit* for cones (*gnat2*) (Brockhoff et al., 2003), *blue opsin* (by long single cones), *uv opsin* (by short single cones), *red opsin* and *zpr-1* (double cones) (Larison and BreMiller, 1990; Raymond et al., 1993; Hisatomi et al., 1996). All photoreceptor cells express *interphotoreceptor retinoid-binding protein gene* (*irbp*) (Stenkamp et al., 1998). During zebrafish retinal development, photoreceptors begin to become postmitotic between 43 and 48 hpf, starting in the ventral retina. Some of the earliest generated photoreceptors begin to express visual pigments by 48 hpf (Robinson et al., 1995), and by 72 hpf, photoreceptors throughout the retina express most of the photoreceptor-specific genes (Raymond et al., 1995; Stenkamp et al., 1998; Brockhoff et al., 2003).

Cadherins are transmembrane molecules that mediate cell adhesion mainly through homophilic interactions (Takeichi, 1991; Gumbiner, 1996). Cadherin2 and cadherin4 are members of the classic cadherin subfamily (Redies, 1995; Nollet et al., 2000). Functional studies of cadherin2 in the visual system of a variety of vertebrate species have demonstrated that this molecule plays a crucial role in the formation of the visual system (Matsunaga et al., 1988; Riehl et al., 1996; Stone and Sakaguchi, 1996; Inoue and Sanes, 1997; Treubert-Zimmermann et al., 2002). Results from several recent studies using zebrafish cadherin2 mutants *parachute* (*pac*) and *glass onion* (*glo*) convincingly demonstrated that cadherin2 is essential for retinal histogenesis in general, and development of retinal ganglion cells in particular (Lele et al., 2002; Erdmann et al., 2003; Malicki et al., 2003; Masai et al., 2003). Cadherin4, most similar to cadherin2 in amino acid sequence among the classic cadherins (Redies, 1997), has been shown to play a role in the formation of specific brain circuits including the visual circuit (Treubert-Zimmermann et al., 2002). We previously showed that cadherin2 is expressed by all retinal layers, while cadherin4 is expressed by retinal ganglion cell layer and the inner most layer of the inner nuclear layer, and that this expression persists during periods of photoreceptor differentiation (Liu et al., 2001a). The purpose of this study was to test the hypothesis that cadherin2 and cadherin4 play important roles in the differentiation of photoreceptors. Our findings are consistent with this hypothesis, and suggest targeted functions in photoreceptor development for cadherin2 and cadherin4.

2. Materials and Methods

2.1. Zebrafish

Zebrafish (*Danio rerio*) were maintained at 28°C as described in the Zebrafish Book (Westerfield, 2000). The *glo* heterozygous mutant carriers and their wild-type siblings from a single breeding, obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, OR) as embryos, were raised to reproductive maturity in the animal care facility at the University of Akron. Pairwise breeding was performed to identify *glo* heterozygous mutant carriers, and the *glo* mutant embryos were identified by gross morphological phenotype. Their wildtype and heterozygous siblings were used as controls. Embryos for cadherin4 morpholino oligonucleotides (MO) experiments were obtained from breeding of wildtype adult zebrafish. Embryos for whole mount *in situ* hybridization were raised in PTU (1-phenyl-2-thiourea, 0.003%). All animal-related procedures were approved by the Care and Use of Animals in Research Committee at the University of Akron and Indiana University.

2.2. MO Injections

Either a cadherin4 translation blocking morpholino oligonucleotide (5'-AAGGAG GCA GAT GTT TGT TAT TCA C-3', Babb et al., 2005) or a control morpholino oligonucleotide (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'), purchased from Gene Tools, Corvallis OR, was microinjected into either blastomeres and/or the yolk immediately below the blastomeres of 1-8 cell stage embryos at 4.2 µg/µL (0.5 mM) in Danieau 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). Total volume injected was 1-2 nl (4.2-8.4 ng) per embryo. Injected embryos were allowed to develop at 28.5°C until they were 60 hours post fertilization (hpf), 3 days (74-78 hpf) or 4 days (96-100 hpf) post fertilization. The *cadherin4* morpholino was confirmed as having no significant homology to any region of the *cadherin2*, or any other zebrafish gene (Babb et al., 2005). To obtain embryos whose *cadherin2* and *cadherin4* functions were simultaneously blocked, we injected *cdh4*MO into 1-8 cell stage embryos obtained from breeding heterozygous *glo* mutant carriers. *glo* mutant embryos injected with the *cdh4*MO (double knockdowns) were readily identified by their gross morphological defects (e.g. clubbed tail, Pujic and Malicki, 2001) that can be recognized under a dissecting scope as early as 20 hpf.

2.3. Tissue processing

Zebrafish embryos and larvae were anesthetized in 0.02% MS-222, and fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) overnight at 4°C. To prepare tissue for whole mount *in situ* hybridization or immunohistochemistry, the tissue was rinsed in PBS, followed by 70% methanol and 100% methanol. The tissue was stored in 100% methanol at -20°C. Preparation of tissues for *in situ* hybridization, immunohistochemistry or TUNEL staining on sections was performed according to Barthel and Raymond (1990). Briefly, the fixed tissue was processed through a graded series of increasing sucrose concentrations, placed in 20% sucrose in PBS overnight, then embedded and frozen in a mixture of OCT embedding compound and 20% sucrose (1:2, v/v). A cryostat was used to obtain 3 µm sections. Some 3-day *glo* and control embryos processed for *zpr-1* whole mount immunostaining, and some 3-day and 4-day *cadherin4* morphants processed for whole mount *in situ* hybridization using *irbp* or *rod opsin* cRNA probes were embedded as described above and sectioned at 12 µm and 8 µm, respectively. Tissue sections were collected on pretreated glass slides (Fisher Scientific, Pittsburgh, PA), dried at room temperature and stored at -80°C.

2.4. In situ hybridization

Full length cDNAs used to generate the cRNA probes were kindly provided by Pamela Raymond at the University of Michigan and Thomas Vihtelic at the University of Notre Dame. Synthesis of digoxigenin-labeled cRNA probes, procedures for whole mount *in situ* hybridization and *in situ* hybridization on tissue sections were described previously (Liu et al., 1999; Stenkamp et al., 2002). For whole mount *in situ* hybridization, *glo* mutant embryos, *cadherin4* morphants, or double knockdowns were processed in the same reaction tubes as their respective control ones, while for *in situ* hybridization on tissue sections, tissues from the *glo* mutant embryos were processed at the same time, side by side, with their respective control tissues. Anti-digoxigenin Fab fragment antibodies conjugated to alkaline phosphatase were used for immunocytochemical detection of the cRNA probes, and this was followed by an NBT/BCIP color reaction step (Roche Molecular Biochemicals, Indianapolis, IN).

2.5. Immunohistochemistry

Procedures for whole mount immunohistochemistry and immunostaining on tissue sections were described in detail previously (Stenkamp et al., 2002; Babb et al., 2005). Primary antibody used was *zpr-1* (Zebrafish International Resource Center, University of Oregon, Eugene, OR)

at 1:1000. A biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was used at 1:200. Visualization of the reaction was achieved by using a DAB kit (Vector Laboratories).

2.6. TUNEL staining

Cryosections were blocked in 3% H₂O₂ in MeOH, permeabilized in 0.1% Triton X-100, and then processed for terminal dUTP nick-end labeling (TUNEL) using the Roche *in situ* cell death detection kit, according to the manufacturer's instructions. Labeling was amplified by using immunohistochemistry with a peroxidase-based color reaction (Stenkamp et al., 2002).

2.7. Microscopy and Photography

Stained whole mount embryos or tissue sections were analyzed with either an Olympus BX51 or a Leica DMR compound microscope, equipped with a SPOT digital camera. Photomicrographs were processed and formatted with Adobe Photoshop (Mountain View, CA). To quantify retinal cells expressing a specific marker, processed whole embryos (in 100% glycerol) were placed (ventral up, because most labeled cells in the mutant or *cadherin4* morphants were confined to the ventral region of the eye) on a glass slide with a viewing chamber (Westerfield, 2000). The slide was coverslipped, and labeled retinal cells were recorded and counted using the Olympus compound microscope under 40X objective lens. To quantify TUNEL labeling, sections were viewed at 40X magnification on the Leica compound microscope, in brightfield and Nomarski optics. Statistics were performed in Excel and SASS.

3. Results

3.1. Expression of photoreceptor-specific genes is greatly and differentially reduced in *glo* mutants

The zebrafish *glass onion* (*glo*)^{m117} is a T-to-G point mutation in *cadherin2* that converts the second amino acid tryptophan in the first ectodomain of cadherin2 to glycine (Malicki et al., 2003). This tryptophan is highly conserved in vertebrate classic cadherins and has been shown to play a crucial role in adhesive properties of cadherin molecules (Tamura et al., 1998; Pertz et al., 1999). Cadherin2 function is suggested to be completely missing in the *glo*^{m117/m117} homozygotes (Malicki et al., 2003). To determine cadherin2 function in the differentiation of the photoreceptors, we first examined expression of three transcription factors that are known to regulate photoreceptor differentiation in vertebrate retina and/or pineal organ: *crx* (Furukawa et al., 1997; Blackshaw et al., 2001; Shen and Raymond, 2004); *otx5* (Gamse et al., 2002); and *rx1* (Chuang and Raymond, 2001), in the retinas of 2-day and 3-day old *glo* embryos/larvae. In control embryos/larvae (wildtype or heterozygous for *glo* mutation), expression of *crx* (Fig. 1A-C) and *otx5* (Fig. 1I and K) was restricted to the outer layers of the retina, and expression of *rx1* was restricted to the photoreceptor layer and to retinal progenitor cells at the retinal margin (Fig. 1D; Chuang et al., 1999; Liu et al., 2001b; Games et al., 2002). Expression of these genes was greatly altered in the *glo* mutant retina. At 50 hpf, *crx* expression in the *glo* mutant retina was much reduced (Fig. 1E) compared to control retinas (Fig. 1A). By 3 days, expression of *crx* in the *glo* mutant retina had increased, but only in the anterior half of the retina (Fig. 1F). *In situ* hybridization on tissue sections revealed that in 3-day old control retinal, *crx* expression was confined to the outer half of the retina (outer half of the inner nuclear layer and the outer nuclear layer) (Fig. 1C), while *crx* expression was detected throughout the thickness of the *glo* mutant retina (Fig. 1G). Moreover, numerous *crx* expressing cells were also detected outside the retina, in the ventral forebrain region, in the mutant embryos (Fig. 1G). Similar to *crx* expression in the *glo* mutant retina, *otx5* expression was reduced in the posterior half of the mutant retina (Fig. 1J and L). *rx1* expression in the control 3-day old retina was confined mainly to the photoreceptor layer (Fig. 1D), but it was found throughout the thickness of the *glo* mutant retina (Fig. 1H). Unlike the *crx* expression in the mutant embryos

(within and outside the retinal regions), *rx1* expression appeared to be more limited to the retinal region (Fig. 1H).

Crx has been shown to regulate gene expression in photoreceptors of both mice (Furukawa et al., 1997; Blackshaw et al., 2001) and zebrafish (Shen and Raymond, 2004), and *rx1* has been implicated in early eye specification (Chuang and Raymond, 2001), as well as later in photoreceptor differentiation, in zebrafish (Stenkamp et al., 2002). To further characterize the differentiation of the photoreceptors in the *glo* mutants, we examined expression of the photoreceptor-specific gene *irbp*, the rod-specific genes *gnat1* and *rod opsin*, and the cone-specific genes *gnat2*, *uv opsin*, and the double cone-specific marker *zpr-1*, in the retina of the *glo* mutants. In control fish, these markers were expressed throughout the developing photoreceptor layer by 3 days (Figs. 2 and 3; Larison and BreMiller, 1990; Raymond et al., 1995; Stenkamp et al., 1998; Brockerhoff et al., 2003). In 3-day old *glo* mutants, expression of *irbp* (Fig. 2A-C), *gnat1* or *gnat2* (Fig. 2D-I), was found in only a few cells (Fig. 4) in the ventral retina (Fig. 2B, E and H). This reduced gene expression in the 3-day old *glo* mutant retina was unlikely due to a delay in general eye development, because their expression, although increased a little in 4-day old *glo* mutant larvae (Fig. 2C, F and I; Fig. 4), remained low.

Expression of *rod opsin* or *uv opsin* in the 3-day old *glo* mutant retina (Fig. 3B and E) was greatly reduced compared to control retinas (Fig. 3A and D), similar to our findings for the genes mentioned above. In the 4-day old mutant embryos, expression of *rod opsin* or *uv opsin* was still confined to the ventral 1/3 of the retina and was still much reduced compared to control retinas (Fig. 3C and F). The number of cells expressing *rod opsin* or *uv opsin* increased (Fig. 4), and was greater than the number of *irbp* expressing retinal cells of the same stage mutant embryos (Fig. 4).

Intensely labeled *zpr1*-positive cells were found throughout the photoreceptor layer of 3-day old control embryos (Fig. 3G), while there were only a few *zpr-1* stained cells ($n = 18.8 \pm 5.5$) in the ventral region of the 3-day old *glo* mutant retina (Fig. 3H). This number was similar to that of *rod opsin* or *uv opsin* expressing cells, but larger than that of *gnat1*, *gnat2* or *irbp* expressing cells (Fig. 4). By 4 days, *zpr-1* staining became increased in the *glo* mutant retina (Fig. 3I), but still much reduced compared to the control retina (Fig. 3G), and it was confined mainly to the ventral half of the retina.

Interestingly, a one-way ANOVA indicated highly significant ($p < 0.0001$) differences among numbers of cells expressing the different photoreceptor-specific markers, in both the 3-day and 4-day *glo* mutants. A post-hoc Fisher's Least Significant Difference test revealed that, at 3 days, the number of cells expressing *uvo*, *rho*, or *zpr-1*, was significantly greater than that expressing *gnat1*, *gnat2*, or *irbp*. At 4 days, the number of cells expressing *rho* was significantly greater than that expressing *uvo*, which in turn was significantly greater than that expressing *irbp*. These statistical findings suggest differential effects of the loss of cadherin2 function on the different photoreceptor-specific genes over developmental time.

To determine if initiation of photoreceptor differentiation is affected in the *glo* mutants, we examined *rod opsin* and *uv opsin* expression in the retina of 60 hpf mutant embryos, a time when expression of all opsin genes has recently commenced in ventral retina of zebrafish (Raymond et al., 1995). In control fish, both *rod opsin* and *uv opsin* were expressed by many photoreceptors in the ventral retina (Fig. 3J and K; Raymond et al., 1995). In the *glo* mutant retina, no expression was found in 1/3 and 1/2 of the embryos for the *rod opsin* and *uv opsin*, respectively ($n = 6$ embryos for each probe), while there were only a couple of *rod opsin*- or *uv opsin*-positive cells in the ventral retina of the remaining mutant embryos (Fig. 3L and M).

These results suggest that the initiation of the photoreceptor differentiation in the ventral retina is impaired in the absence of cadherin2 function.

During the time of early retinal histogenesis (18-24 hpf), the *glo* mutant eye experiences an increased rate of cell death as compared to wildtype eyes (Pujik and Maliki, 2001), and this may, in part, explain the paucity of cells expressing photoreceptor-specific genes. To determine whether this increased rate of cell death continues during retinal cell differentiation, we performed TUNEL staining in 3-day old *glo* and control embryos. There were very few TUNEL labeled cells in the control eyes (approximately 1 TUNEL+ cell/section), and TUNEL labeling was only slightly increased in the *glo* mutant eyes (approximately 2.5 TUNEL+ cells/section; data not shown).

The teleost pineal organ is known to contain photoreceptors that express many of the genes expressed by retinal photoreceptors (Herwig, 1980; Östholm et al., 1987; Robinson et al., 1995; Masai, et al., 1997). To determine if cadherin2 also plays a role in photoreceptor differentiation in this structure, we examined *crx*, *gnat1*, *gnat2*, *irbp*, *otx5* and *rod opsin* in the pineal organ of *glo* mutant embryos/larvae. Interestingly, unlike in the retina where expression of these genes was greatly reduced, their expression in the pineal organ of the *glo* mutant fish appeared to be identical to that of control fish throughout the stages examined (2-4 days, Fig. 5). This finding supports the idea that differentiation of photoreceptors is differentially regulated in the zebrafish retina and pineal gland (Gamse et al., 2002).

3.2. Expression of photoreceptor-specific genes is greatly and differentially reduced in cadherin4 morphants

We previously showed that cadherin4 was expressed by the retinal ganglion cell layer and the inner most layer of the inner nuclear layer where amacrine cells reside (Liu et al., 1999; 2001a). Morpholino antisense oligonucleotide techniques have been successfully used to study gene function in zebrafish (Ekker, 2000; Andermann et al., 2002; Kerstetter et al., 2004). We recently showed that interfering with cadherin4 function in zebrafish using cadherin4 specific morpholino antisense oligonucleotides (*cdh4*MOs) disrupted differentiation of retinal cells including the photoreceptors as judged by *zpr-1* staining (Babb et al., 2005). This was a surprise given the non-photoreceptor expression pattern of cadherin4. To further study this indirect function of cadherin4 in photoreceptor differentiation and compare that with the more likely direct function of cadherin2, we examined expression of the other photoreceptor-specific genes in the *cadherin4* morphants.

Injection of a zebrafish *cadherin4* specific morpholino antisense oligonucleotide into 1-8 cell stage embryos resulted in morphants with phenotypes ranging from slightly affected to severely affected, whereas embryos injected with a control morpholino were indistinguishable from wildtype embryos (Babb et al., 2005). After 3-days of development, the severely affected embryos (74.9% of 347 injected in this study) had eyes that were much smaller than control eyes due mainly to increased cell death in the eye (Babb et al., 2005), with much reduced (restricted to the ventral 1/3 of the retina) or no retinal lamination and opaque lenses. The slightly affected (6.9%) morphant eyes were largely indistinguishable from control embryos, except that the inner plexiform layer was thinner and disorganized, while the moderately affected (18.2%) morphants had intermediate eye phenotypes (Babb et al., 2005). These various phenotypes likely represent a hypomorphic series that resulted from variable levels of cadherin4 suppression, with the severely affected likely representing a null phenotype, as supported by immunoblotting on individual embryos demonstrating that the severity of the phenotypes correlates with cadherin4 protein levels (Babb et al., 2005).

Similar to the *glo* mutants, expression of *gnat1* (Fig. 6B), *gnat2* (Fig. 6E), *uv opsin* (Fig. 6H), *irbp* (Fig. 6K), and *rod opsin* (data not shown), in severely affected 3-day old *cadherin4*

morphants was either non-detectable or restricted to a small patch in the ventral retina, although these genes appeared to be normally expressed in their pineal organs (data not shown). As in the *glo* mutants, the number of retinal cells expressing *rod opsin* or *uv opsin* was larger than the number of cells expressing *gnat2* (Fig. 7). A one-way ANOVA indicated highly significant ($p < 0.0001$) differences among numbers of cells expressing the different photoreceptor-specific markers in the *cadherin4* morphants. A post-hoc Fisher's Least Significant Difference test revealed that the number of cells expressing *uvo* or *rod opsin* was significantly greater than that expressing *gnat2*. It is possible that the reduced gene expression might have resulted from a delay in general eye development in the *cadherin4* morphants, so we examined 4-day old *cadherin4* morphants for the expression of the photoreceptor-specific genes. Similar to the 3-day old morphants, gene expression in the severely affected 4-day old *cadherin4* morphants was greatly reduced compared to control embryos, and expression of these genes was differentially affected (Fig. 6C, F and I). Expression of *gnat1* or *gnat2* continued to be confined to the ventral retina (Fig. 6C and F), although their expression domains were larger than 3-day old morphants (Fig. 6B and E). In contrast, expression of *uv opsin* (Fig. 6I) and *rod opsin* (data not shown) in 4-day old *cadherin4* morphants had expanded to almost the entire retina.

Expression of *irbp* in the zebrafish eye is unlike the other photoreceptor-specific genes in that it is expressed by both the photoreceptors and retinal pigmented epithelium (RPE, Fig. 6J; Stenkamp et al., 1998). Weak *irbp* expression in the RPE was detected in the eyes of some 3-day old *cadherin4* morphants (data not shown), but there was no *irbp* expression in the photoreceptors (Fig. 6K). *irbp* expression was increased in 4-day old *cadherin4* morphant eyes (Fig. 6L and M), but it was still much reduced compared to *irbp* expression in the control retina (Fig. 6G). Careful examination of the *irbp* expression in the morphant eyes showed a hexagonal pattern consistent with localization to the RPE, while *irbp* expression in the photoreceptor layer was confined to a small patch in the ventral retina (Fig. 6L and M). Initiation of photoreceptor development must have also been disrupted in *cadherin4* morphants because at 60 hpf there was no (in severely affected eyes) or very little (in moderately affected eyes) *rod opsin* or *uv opsin* staining (data not shown).

3.3. Blocking both *cadherin2* and *cadherin4* functions severely disrupts zebrafish eye development

To determine the combined function of *cadherin2* and *cadherin4* in zebrafish eye development in general, and photoreceptor development in particular, we studied *glo* mutant embryos injected with the *cdh4*MO (double knockdowns). In double knockdowns, the eye initially formed, and was indistinguishable morphologically from the *glo* mutant eyes at 26 hpf (Fig. 8). This is not surprising because *Cadherin4/cadherin4* expression in zebrafish retina begins late (about 29 hpf). As development proceeded, the eye tissue in double knockdowns became more difficult to recognize. In 36 hpf control embryos, *crx* and *otx5*, markers of differentiating neural retina (see above), expression was found throughout the retina (Fig. 9A and D; Liu et al., 2001b), expression of these genes was confined to the anteroventral retina in the *glo* mutants (Fig. 9B and E), while in the double knockdowns, there was very little or no labeling, except in the dorsal thalamic region where the pineal organ resided (Fig. 9C and F). This lack of *crx* or *otx5* staining was unlikely due to a delay in general eye development in the double knockdowns, because similar results were obtained from 50 hpf double knockdowns (Fig. 9H and J).

We monitored cell death in the *cdh2/cdh4* double knockdowns at 36 hpf by using the TUNEL procedure on cryosections. This developmental stage was chosen because *cadherin4* is expressed in the zebrafish retina at about 29 hpf, while the retinal tissue became difficult to distinguish in older embryos (e.g. 48-50 hpf). Control embryos showed very little cell death in the retina over this time of development (Fig. 10A, E; Cole and Ross, 2001). The retinas of

cdh4 MO-injected embryos contained higher numbers of TUNEL+ cells, approximately 3-7 apoptotic cells/section at each time point (Fig. 10B and E), consistent with previous findings (Babb et al., 2005). The retinas of *glo* (*cdh2*) mutants had similar numbers of TUNEL+ cells (Fig. 10C and E), also consistent with earlier reports of cell death during retinal neurogenesis (Malicki et al., 2003). Retinas of double knockdowns contained large numbers of TUNEL+ cells, approximately twice as many apoptotic cells/section than those of single knockdowns; however, retinas were especially difficult to recognize, and lacked any histological boundary with the brain (Fig. 10D, and E). The double knockdown eyes therefore experienced more extensive cell loss during development than did those of either the *glo* mutants or the *cdh4* morphants. The additive nature of this effect suggests that cadherin2 and cadherin4 have independent effects on cell survival.

4. Discussion

4.1. Reduced expression of photoreceptor-specific genes in *glo* and cadherin4 morphants retinae suggests cadherins function in photoreceptor differentiation

In the present study, examination of expression of several photoreceptor-specific genes has revealed severe abnormalities in the differentiation of retinal photoreceptors in the *glo* mutant embryos and *cadherin4* morphants. Cadherin2 function in zebrafish retinal development was previously studied in *parachute* (*pac*) (Masai et al., 2003) and *glo* mutants (Pujic and Malicki, 2001; Malicki et al., 2003). *pac* mutants are another group of cadherin2 mutants in which mutations result in stop codons, and therefore premature termination of the transcript, or mutations affect splice donor sites in introns, causing incorrect splicing of the precursor mRNA (Lele et al., 2002; Masai et al., 2003). Among the retinal cell types in *pac* mutants, differentiation of the photoreceptors appeared to be the least affected as judged by either *zpr-1* staining (Masai et al., 2003) or by photoreceptor ultra-structure (Erdmann et al., 2003). However, Masai et al. (2003) noted that the degree of disruption in the photoreceptor layer varied greatly among *pac* mutants with different *pac* alleles, with the least affected in weaker *pac* alleles (e.g. *pac^{rw95}*) where small amount of functional cadherin2 protein may still be produced, and much more disruption in stronger *pac* alleles (e.g. *pac^{fr7}*). The *glo* homozygotes, displaying even more severe phenotypes throughout the body than the strong *pac* alleles, have been suggested to display a null phenotype (Malicki et al., 2003). This is consistent with our observation that photoreceptor differentiation is severely affected in the *glo* mutants.

Our results also show that cadherin4 plays an important role in photoreceptor differentiation. This finding was unexpected given the differential expression patterns of these two cadherin genes. The outer nuclear layer, where differentiating photoreceptors reside, expresses cadherin2 (Liu et al., 2001a), and so it is not surprising that photoreceptor differentiation is severely perturbed when cadherin2 function is blocked, as in the *glo* mutants. However, it is less clear why photoreceptor development is also severely disrupted in the *cadherin4* morphants, since the photoreceptors do not express cadherin4. Cadherin4 is strongly expressed by the retinal ganglion cells and the amacrine cells (Liu et al., 2001a) whose differentiation was severely affected in the *cadherin4* morphants (Babb et al., 2005). Therefore, cadherin4 may affect photoreceptor differentiation indirectly through its control on the differentiation of other classes of retinal cells.

4.2. Expression of photoreceptor-specific genes in *glo* and cadherin4 morphants retinae is differentially regulated

Evaluation of photoreceptor-specific gene expression in the *glo* mutants and *cadherin4* morphants has also revealed that the effects of the cadherins on photoreceptor differentiation are highly targeted, in that the expression of some genes is more severely affected than the expression of others. For example, in wildtype retina, rod photoreceptors express *rod opsin*,

gnat1, and *irbp*, while UV opsin-sensitive cone photoreceptors express *uv opsin*, *gnat2* and *irbp*. However, in the *glo* mutant and *cadherin4* morphant retinæ, there were far more cells expressing *rod opsin* or *uv opsin*, than those expressing *gnat1*, *gnat2*, or *irbp*. These results suggest that different aspects of photoreceptor differentiation are affected when cadherins functions are disrupted, and the majority of the *uv opsin* (or *rod opsin*) positive cells in *glo* mutants or *cadherin4* morphants are likely not fully differentiated. Our results are consistent with findings from *in vitro* study on chicken retinal cells in which expression of photoreceptor specific genes is selectively and independently regulated by specific extracellular factors (Bradford et al., 2005).

Results from recent studies on vertebrate photoreceptor differentiation suggest that regulation of photoreceptor specific genes may not be controlled by common regulatory mechanisms (Johnson et al., 2001; Bradford et al., 2005). Expression of some genes (e.g. *rod opsin*) is likely controlled mainly by intracellular determinants (Johnson et al., 2001; Cook and Desplan, 2001), while expression of other genes (e.g. transducin subunits) may require additional intrinsic as well as extrinsic factors including signals from the microenvironment (Bradford et al., 2005). Here we have extended this important finding to an *in vivo* situation, through the use of loss-of-function strategies. Our results suggest that expression of a subset of photoreceptor-specific genes (e.g. *gnat1*, *gnat2* and *irbp*) is more dependent on cadherin-mediated cell-cell adhesion than expression of other genes (e.g. *rod opsin* and *uv opsin*), and that normal cadherin2 and cadherin4 functions are required for complete differentiation of retinal photoreceptors. Moreover, our results support the idea that differentiation of photoreceptors, as well as that of other cell types, can be more thoroughly assessed by a combination of several biochemical, morphological, and/or physiological criteria (Bradford et al., 2005).

4.3. Combined function of cadherin2 and cadherin4 is crucial for zebrafish eye development

Subpopulations of photoreceptors continue to differentiate when either of cadherin2 or cadherin4 function is blocked (Pujic and Malicki, 2001; Masai et al., 2003; Malicki et al., 2003; results from this study). It is possible that each of the two cadherins compensates for the loss of the other. Therefore it is not surprising that when both cadherins functions were interfered with simultaneously as in the double knockdowns, there was much more disruption in the zebrafish eye development. Unlike in mammals where *Crx* expression is confined primarily to the outer nuclear layer, and *Crx* is involved mainly in the development and maintenance of the photoreceptors (Freund et al., 1997, 1998; Jacobson et al., 1998; Furukawa et al., 1999; Swaroop et al., 1999; Rivolta et al., 2001), zebrafish *crx* is expressed in both retinal progenitor cells and differentiating retinal cells (Liu et al., 2001b), and *crx* is crucial for differentiation of retinal cells including photoreceptors (Shen and Raymond, 2004). The absence of *crx* expression in the double knockdowns indicates a lack of retinal cells destined to become photoreceptors. Since in zebrafish *cadherin2* is expressed earlier (5 hpf, Bitzur et al., 1994) than *crx* (17 hpf, Liu et al., 2001b), and *crx* expression is reduced in the *glo* mutant and *cadherin4* morphant retinæ (Babb et al., 2005 and this study), it is possible that cadherin2 and cadherin4 function in zebrafish eye formation is through, at least in part, regulation of *crx* expression. Moreover, since increased rates of cell death are observed in *glo* mutant and *cadherin4* morphant retinæ (Pujic and Malicki, 2001; Babb et al., 2005), in the retina of the double knockdowns (this study) and zebrafish embryos injected with *crx* specific morpholino antisense oligonucleotides (Shen and Raymond, 2004), promoting cell survival is likely a key mechanism through which cadherin2 and cadherin4 function in zebrafish eye development regardless of pathways involved.

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Figure 1.

Expression of *crx*, *otx5* and *rx1* was changed in 2 (50 hpf)- and 3-day (3-d) old *glass onion* (*glo*) mutant retina. Panels C, D, G and H are radial sections of the retina with dorsal up. The remaining panels are lateral views of whole mount retina with anterior to the left and dorsal up. Abbreviations: Fb, forebrain; gcl, retinal ganglion cell layer; inl, inner nuclear layer; le, lens; onl, outer nuclear layer.

Figure 2.

Expression of *irbp*, *gnat1* and *gnat2* was greatly reduced in the eye of 3- and 4-day (4-d) old *glo* mutants. All panels show ventral views of whole mount heads with the anterior to the top. Arrows point to pigmented tissue, and arrowheads indicate labeled retinal cells. Abbreviations are the same as in Figure 1.

Figure 3.

Expression of *rod opsin* (*rho*), *uv opsin* (*uvo*) and *zpr-1* in 3- and 4-day old, and 60 hpf control and *glo* mutant retinas. All panels show ventral views of whole mount heads with the anterior to the top. Inserts in panels D and E are *in situ* hybridization on radial sections of 3-day old tissue with dorsal up. Inserts in panels G and I are radial sections (dorsal up) of larval eyes processed for whole mount *zpr-1* immunostaining. Arrowheads point to labeled cells. p, pineal organ. Other abbreviations are the same as in Figure 1.

Figure 4.

Blocking cadherin2 function has differential effects on expression of photoreceptor specific genes/marker. Five zebrafish larvae (ten eyes) were used for collecting data from each gene/marker; bars represent means $\pm$ standard deviations. nd, not done.

Figure 5.

Photoreceptor-specific gene expression in the pineal organ (p) of control and *glo* mutant embryos. All panels are lateral views of the dorsal head region in 3-day old whole mount embryos with anterior to the left and dorsal up.

Figure 6.

Expression of *gnat1*, *gnat2*, *uvo* and *irbp* in the retina of 3- and 4-day old control embryos and *cadherin4* morphants (*cdh4MO*). Panels A-F are ventral views (anterior up) of whole mount heads. Panels G-I are lateral views (anterior to the left and dorsal up) of whole mount eyes. Panels J-M are ventral views of whole mount eyes (anterior up). Arrowheads point to labeled photoreceptors, while arrows indicate retinal pigmented epithelium. Abbreviations are the same as in Figures 1 and 3.

Figure 7.

Blocking cadherin4 function has differential effects on expression of photoreceptor specific genes. Five zebrafish larvae (ten eyes) were used for collecting data from each gene; bars represent means $\pm$ standard deviations

Figure 8.

Gross eye morphology of control (panel A), *cadherin4* morphant (panel B), *glo* mutants (panels C and D), and double knockdowns (panels E and F). All panels show lateral views of the head region of live embryos with anterior to the left and dorsal up. Abbreviations: h, hindbrain; ot, optic tectum. Other abbreviations are the same as in Figure 1.

Figure 9.

Expression of *crx* and *otx5* was greatly reduced or eliminated in 36 hpf and 50 hpf *glo* mutant and double knockdown (*glo/cdh4MO*) retinæ. All panels are ventral views of whole mount embryo heads with dorsal up. Arrows point to pigmented tissues while the arrowhead in panel F indicates a small cluster of cells expressing *otx5*. Abbreviations are the same as in Figures 1 and 3.

Figure 10.

Combined knockdown of cadherin2 and cadherin4 results in additive effects on cell death. Representative, TUNEL-labeled cryosections are shown for control (A), *cdh4*MO injected (B), *glo* mutant (C), and *cdh4*MO injected *glo* mutant (D) embryos 36 hpf. TUNEL+ cells are brown profiles. Abbreviations: d, dorsal, v, ventral, Other abbreviations are the same as in Figure 1. Panel E shows average numbers of TUNEL+ cells/retinal cryosection $\pm$ S.D.