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The Rod Photoreceptor Lineage of Teleost Fish

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Abstract

The retinas of postembryonic teleost fish continue to grow for the lifetime of the fish. New retinal cells are added continuously at the retinal margin, by stem cells residing at the circumferential germinal zone. Some of these retinal cells differentiate as Müller glia with cell bodies that reside within the inner nuclear layer. These glia retain some stem cell properties in that they carry out asymmetric cell divisions and continuously generate a population of transit-amplifying cells – the rod photoreceptor lineage – that are committed to rod photoreceptor neurogenesis. These rod progenitors progress through a stereotyped sequence of changes in gene expression as they continue to divide and migrate to the outer nuclear layer. Now referred to as rod precursors, they undergo terminal mitoses and then differentiate as rods, which are inserted into the existing array of rod and cone photoreceptors. The rod lineage displays developmental plasticity, as rod precursors can respond to the loss of rods through increased proliferation, resulting in rod replacement. The stem cells of the rod lineage, Müller glia, respond to acute damage of other retinal cell types by increasing their rate of proliferation. In addition, the Müller glia in an acutely damaged retina dedifferentiate and become multipotent, generating new, functional neurons. This review focuses on the cells of the rod lineage and includes discussions of experiments over the last 30 years that led to their identification and characterization, and the discovery of the stem cells residing at the apex of the lineage. The plasticity of cells of the rod lineage, their relationships to cone progenitors, and the applications of this information for developing future treatments for human retinal disorders will also be discussed.

Keywords

rod; photoreceptor; development; lineage; zebrafish; regeneration; Müller glia

1. Introduction

1.1. The retina of teleost fish

The retinas of teleost fish, like other vertebrate retinas, consist of three cellular layers separated by two synaptic, or plexiform, layers. The light-sensitive cells of the retina, the rod and cone photoreceptors, reside in the outer nuclear layer (ONL). Processing neurons – horizontal cells, bipolar cells, and amacrine cells – and the cell bodies of Müller glia occupy the inner nuclear layer (INL). Ganglion cells, which are the output cells of the retina, are found in the ganglion cell layer (GCL), along with displaced amacrine cells. General

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features of the anatomy and physiology of the teleost retina are conserved as compared to other vertebrates, making them a suitable model system.

There are specific features of the teleost retina that make this system especially valuable as a model system for vision science. Most teleosts have a duplex retina, with rod photoreceptors and four spectral classes of cone photoreceptors, maximally sensitive to long (red), middle (green), short (blue), and ultraviolet (UV) wavelengths. The cone photoreceptors are arranged in a precise mosaic, such that each cone type has a predictable spatial relationship to other cone types (Allison et al., 2011; Cameron and Easter, 1993; Raymond et al., 1993; Stenkamp and Cameron, 2002; Stenkamp et al., 2001). Rod photoreceptors occupy spaces between the cones, and a spatial order to the rod mosaic is appreciable in embryonic, larval, and adult retinas of teleosts (Fadool, 2003; Stenkamp *et al.*, 1996).

Teleosts in general, and zebrafish in particular, excel as model systems for the study of retinal development (Easter and Malicki, 2002; Stenkamp, 2007). Fish develop externally and relatively rapidly, and many are optically transparent, facilitating experimental manipulations and observations. Genomic information is available for the zebrafish, excellent technologies have been developed for creating stable transgenics, and both large- and small-scale mutagenesis/screening has permitted the identification and characterization of developmentally important genes (Easter and Malicki, 2002; Fadool and Dowling, 2008). In addition to these advantages, the teleost retina experiences persistent neurogenesis as the animal continues to grow, offering the opportunity to study the process of neurogenesis in postembryonic animals, as well as the process of retinal regeneration in response to acute damage (Hitchcock et al., 2004; Stenkamp, 2007).

1.2. Persistent neurogenesis in the teleost fish retina

The postembryonic teleost retina continues to grow, with new neurons arising from two spatially distinct populations of stem cells. One of these stem cell populations is multipotent and resides at the retinal periphery, near the ciliary margin of larval and juvenile fish, and is referred to as the circumferential germinal zone (CGZ) or ciliary marginal zone (CMZ) (Hitchcock et al., 2004; Johns, 1977; Kock, 1982). The CGZ resembles a pseudostratified neuroepithelium, and continues to proliferate throughout the lifetime of the fish, generating postmitotic neurons that migrate radially, and differentiate to generate new, concentric annuli of properly laminated retina immediately adjacent to the CGZ (Johns, 1977). This process allows the teleost retina to expand in size in concert with growth of the animal (Johns, 1977; Johns and Easter, 1977).

The second major population of stem cells in teleost retina is a population of slowly dividing cells that reside in the INL (Johns, 1977; Julian et al., 1998; Otteson et al., 2001). These cells, recently identified as Müller glia (Bernardos et al., 2007), generate progeny that continue to divide, and migrate to the ONL, where they undergo terminal mitoses and differentiate as rod photoreceptors (Johns, 1977). The new rods are inserted into the existing array of cone and rod photoreceptors. Therefore, as a fish grows, the ratio of rods to cones within the retina increases (Johns and Easter, 1977; Kock, 1982; Sandy and Blaxter, 1980). This process allows retinal rod density to remain relatively constant, and visual sensitivity to remain stable (Powers et al., 1988) as the retina expands in a balloon-like manner during fish growth (Johns, 1977; Johns and Easter, 1977). The stem cell population of the INL, along with the transit-amplifying cells of the INL and ONL, will collectively be referred to as the rod lineage in the remainder of this review.

2. The rod lineage of teleost fish

2.1. Description of the rod lineage

A persistent source of proliferative cells dedicated to the ongoing production of new rod photoreceptors in fish was initially reported 30 years ago (Johns, 1982; Johns and Fernald, 1981; Sandy and Blaxter, 1980). In these studies, adult goldfish, adult cichlids, or larval herring were injected intraocularly with ^3H -thymidine to label proliferative cells. Retinal cryosections processed at short, post-treatment survival times showed ^3H -thymidine incorporation into the ONL, in nuclei having ultrastructural appearances resembling those of germinal cells. Retinal sections processed at longer, post-treatment survival times following ^3H -thymidine exposure showed clear evidence of ^3H -thymidine incorporation into rod photoreceptor nuclei, again defined by their ultrastructure. Following these remarkable studies were further reports, confirming the presence of rod precursors in larval and adult retinas of a variety of teleost fish, and providing a deeper understanding of their characteristics. For example, rod precursors are most abundant in peripheral retina, and the time between terminal mitosis and expression of visual pigment protein is rapid as compared to the corresponding interval for cone photoreceptors (Knight and Raymond, 1990; Stenkamp et al., 1997; Wan and Stenkamp, 2000). In cichlids, rod precursor mitoses take place near the outer limiting membrane, and their postmitotic, undifferentiated progeny migrate along Müller cell processes to deeper regions of the ONL, where they take up residence and differentiate as rods (Chiu *et al.*, 1995; Knight and Raymond, 1990; Kwan *et al.*, 1996; Raymond and Rivlin, 1987; Stenkamp *et al.*, 1997). Numerous growth factors have been evaluated for their effects on rod precursor proliferation, with the general consensus that insulin-like growth factor (IGF), regulated by systemic growth hormone, controls the production of new rods in correspondence with the regulation of body growth (Boucher and Hitchcock, 1998a; Boucher and Hitchcock, 1998b; Mack and Fernald, 1993; Otteson et al., 2002; Zygar et al., 2005). It is perhaps not surprising then, that adult, wild-type zebrafish, in which body growth comes to a virtual standstill, have only minimal proliferative activity within the retina's ONL (Marcus et al., 1999).

What is the source of rod precursors? The question of whether rod precursors represent a self-maintaining pool of stem cells, vs. a pool of transit-amplifying cells replenished from sources outside the ONL, was challenging to address directly. The first hint that the latter situation was most likely the case came from painstaking reconstruction of electron micrographs of ^3H -thymidine-exposed goldfish retinas (Raymond and Rivlin, 1987). These reconstructions verified the presence of labeled cells in ONL and, in lesser numbers, within the INL. The labeled INL cells displayed a fusiform morphology, and were associated with Müller glial processes such that they appeared to be migrating toward the ONL. Evidence for a lineage relationship between the proliferative cells in the INL and the rod precursors came 10 years later. A study utilizing systemic exposure to BrdU as a means to cumulatively label dividing cells in retinas of rapidly growing juvenile rainbow trout revealed radial columns of proliferative cells, spanning the thickness of the INL and continuing into the ONL (Julian et al., 1998). The kinetic measures of BrdU incorporation were consistent with the movement of proliferative cells from the INL into the ONL. A subsequent study in adult goldfish confirmed and extended these observations, identifying a population of slowly-proliferating cells with spherical nuclei at the base of the INL as the putative, unipotent stem cells, the “apex” of the teleost rod lineage (Otteson et al., 2001). These stem cells, recently identified as Müller glia (Bernardos et al., 2007; Nelson et al., 2008) [discussed further in section 2.3] are seeded into a growing retina by the proliferative cells at the CGZ. The progeny of these stem cells have a fusiform shape, and migrate toward the ONL. These cells are generally referred to as rod progenitors. Once in the ONL, cells of the rod lineage, at this point referred to as rod precursors, divide again and their progeny differentiate as rods. The overall characteristics of this model (Figure 1) have been supported in zebrafish by a

number of additional studies, using a variety of approaches (Bernardos *et al.*, 2007; Morris *et al.*, 2008a; Morris *et al.*, 2008b; Nelson *et al.*, 2008; Ochocinska and Hitchcock, 2007). The lineage is most readily recognized in the larval zebrafish, when the retina is growing rapidly and many new rods are generated (Bernardos *et al.*, 2007), and in a genetic model displaying upregulated rod production (Morris *et al.*, 2008b).

We have recently extended this model of the rod lineage to include the late embryonic period of development of wild-type zebrafish (Nelson *et al.*, 2008). A spatially distinctive, proliferative lineage associated exclusively with rod neurogenesis can be identified as early as 60 hpf in the zebrafish. At this time the embryonic INL has already been seeded with stem cells for the generation of rod photoreceptors, presumably put in place, along with postmitotic neurons, by the late embryonic proliferative retinal neuroepithelium. The rod lineage of late embryonic zebrafish retina shows proliferation kinetics consistent with the existence of 1) slowly dividing stem cells in the INL, 2) a population of transit-amplifying cells that migrate from the INL to the ONL, and 3) rod precursors that undergo terminal mitosis within the ONL and differentiate as rod photoreceptors (Nelson *et al.*, 2008). Interestingly, the existence of a distinct rod lineage prior to 60 hpf has not been demonstrated (Nelson *et al.*, 2008; Ochocinska and Hitchcock, 2007), although rod neurogenesis takes place at this time. Indeed, in goldfish and zebrafish embryos, a small population of rod photoreceptors differentiates before the first cone photoreceptors differentiate (Kljava, 1987; Raymond *et al.*, 1995; Stenkamp *et al.*, 1996). This population of precocious rod photoreceptors, along with other retinal cell types present in a “ventral patch” of early-differentiating cells in the zebrafish retina, have been fate-mapped as arising from the rostralmost region of the optic vesicle near the optic stalk (Li *et al.*, 2000). Differentiation of retinal cells in this precocious ventral patch is remarkably resistant to genetic, molecular, or pharmacological perturbations that disrupt neurogenesis and differentiation of the remainder of the embryonic retina (Kashyap *et al.*, 2007; Nelson *et al.*, 2009; Stenkamp *et al.*, 2002), suggesting that distinct or earlier factors regulate their development [see (Kay *et al.*, 2005; Masai *et al.*, 2000; Stenkamp and Frey, 2003)]. At any rate, the relationships of the precocious rods of the ventral patch to Müller glia, or to the early-differentiating cones of the ventral patch, remain unresolved.

2.2. Gene expression within the rod lineage

A “candidate gene” approach has been successful in identifying the transcription factors expressed by cells of the rod lineage, as well as their respective roles in regulating lineage development. For example, the paired homeodomain gene *pax6* is expressed by retinal progenitor cells in all vertebrates (Hitchcock *et al.*, 1996), and by cells of the CGZ in fish (Hitchcock *et al.*, 1996; Otterson *et al.*, 2001; Raymond *et al.*, 2006) and frogs (Perron *et al.*, 1998), and is considered an identifier for retinal stem cells. The stem cells of the rod lineage also express *pax6* (Bernardos *et al.*, 2007; Nelson *et al.*, 2008; Otterson *et al.*, 2001). These slowly-proliferating, *pax6*⁺ cells have a confirmed identity as Müller glia in larval zebrafish (Bernardos *et al.*, 2007), and, at minimum, have a confirmed lineage relationship with Müller glia in embryonic zebrafish (Nelson *et al.*, 2008). The role of Pax6 for the rod lineage has not been directly evaluated. However, because the fusiform INL rod progenitors and ONL rod precursors *do not* express *pax6* (Nelson *et al.*, 2008; Otterson *et al.*, 2001), it is likely that Pax6 is important for maintaining stem cell identity (i.e. the capacity for self-replacement), while the lack of Pax6 in the remainder of the lineage is consistent with their identities as transit-amplifying cells, committed to the generation of a single cell type. Further roles for Pax6 expression in response to retinal damage are discussed later, in section 3.2.

Most (though not all) of the rod progenitor cells of the INL in teleost fish express the bHLH transcription factor *NeuroD* (Nelson *et al.*, 2008; Ochocinska and Hitchcock, 2007).

NeuroD is also expressed in numerous postmitotic neurons of the retina, including developing rod and cone photoreceptors. The likely role of NeuroD in the retina is cell cycle regulation within populations of rod progenitors and cone progenitors. Overexpression of *NeuroD* results in reduced proliferation, while knockdown of *NeuroD* expression results in increased proliferation (Ochocinska and Hitchcock, 2009). It is possible that the sporadic expression of NeuroD in the INL cells of the rod lineage (Nelson et al., 2008) reflects this function in that expression may be associated with a specific phase of the cell cycle. This possibility has not been explicitly investigated. *NeuroD* is not coexpressed with *pax6* in the stem cells of the rod lineage (Ochocinska and Hitchcock, 2007).

The *retinal homeobox (rx1)* gene is also expressed in cells of the rod lineage, and like *NeuroD*, is not coexpressed with *pax6* (Nelson et al., 2008). Interestingly, *rx1* and *pax6* are coexpressed in retinal progenitors and in cells of the CGZ (Chuang et al., 1999; Mathers et al., 1997), making the unipotent stem cell of the rod lineage (in an undamaged retina), molecularly distinctive from these other retinal stem cells. Also noteworthy is that expression of *rx1*, like that of *NeuroD*, is not seen in all rod progenitors of the INL, but appears to be expressed sporadically (Nelson et al., 2008). We have tested a role for *rx1* in the rod lineage through temporally-selective gene knockdown experiments (Nelson et al., 2009). In these experiments, reduced *rx1* (and *rx2*) expression resulted in reduced photoreceptor differentiation, but no changes in proliferation of the rod lineage were detected. The function of *rx1* specifically within the rod lineage therefore remains obscure.

The *cone-rod homeobox (crx)* gene encodes a transcription factor that regulates expression of photoreceptor-specific genes, and, in mammals, *crx* is expressed exclusively in photoreceptors (Hennig et al., 2008). In the zebrafish, *crx* is expressed in retinal progenitors, in differentiating rods and cones, and throughout the outer half of the INL (Liu et al., 2001; Nelson et al., 2008; Ochocinska and Hitchcock, 2007). Although many of the *crx*⁺ cells in the INL correspond to differentiated neurons, some of them are cells of the rod lineage, and all of the cells of the rod lineage found in this region are *crx*⁺ (Nelson et al., 2008). Interestingly, rod progenitor cells undergoing M-phase, identified by an antibody directed against MPM-2, contain Crx protein (Nelson et al., 2008). While this does not necessarily indicate a role for Crx in regulating the cell cycle within the rod lineage, it does demonstrate that a mitotic cell can express a “photoreceptor-specific” transcription factor.

An additional “photoreceptor-specific” transcription factor found within the zebrafish rod lineage is the orphan nuclear receptor, **Nr2e3** (also referred to as PNR, photoreceptor cell-specific nuclear receptor). In mammals, Nr2e3 promotes the expression of rod-specific genes and suppresses the expression of cone-specific genes (Chen et al., 2005). In the zebrafish, *Nr2e3* mRNA is expressed in newly-generated cells of the ONL (nascent rods and cones, and rod precursors), and sporadically within the rod lineage (Nelson et al., 2008). A function in rod neurogenesis is likely, as this gene is upregulated within the lineage in a genetic model for rod loss and replacement (Morris et al., 2008b).

The *nrl* gene, encoding a leucine zipper type transcription factor, is also rod-specific (and rod-promoting) in mammals (Hennig et al., 2008), but is more widely expressed in the zebrafish. We localized *nrl* mRNA to the ONL of embryonic zebrafish retina, in nascent cones as well as in rod precursors and developing rods (Nelson et al., 2008). Zebrafish *nrl* is also expressed in lens of the eye (Nelson et al., 2008; Vihtelic et al., 2005).

Through a series of BrdU incorporation and colabeling experiments, we have unraveled a preliminary genetic hierarchy within the rod lineage of wild-type zebrafish embryos (Nelson et al., 2008) (Fig. 2). The apex of the rod lineage is a *pax6*-expressing stem cell with its cell body positioned deep within the INL (the “proximal INL”). In larval retina these stem cells

correspond to Müller glia (Bernardos et al., 2007), and in embryonic retina, at minimum they have a lineage relationship with Müller glia (Nelson et al., 2008). Their progeny are fusiform cells that migrate to the medial region of the INL and cease expression of *pax6* while initiating expression one or both of the transcription factors *rx1* and *NeuroD*. As the cells of the lineage migrate through the distal INL, they begin to express *crx* as well, but remain heterogeneous with respect to *rx1* and *NeuroD* expression. In addition, some rod progenitors in the distal INL express *Nr2e3*. When progenitors emerge into the ONL, they become molecularly homogeneous, as all rod precursors express *NeuroD*, *rx1*, *crx*, and *Nr2e3*. Postmitotic rod photoreceptors then express all of these transcription factors, at least transiently, as well as *nrl*, and markers of terminal differentiation such as rod opsin and rod transducin (*gnat1*). We speculate that the shift to molecular homogeneity upon entrance into the ONL is mediated by extracellular signaling molecules with a differential distribution in the ONL as compared to the INL. However, the coordinated expression of *NeuroD*, *rx1*, *crx*, and *Nr2e3* mRNA is seen in rod precursors as well as in differentiating rods, indicating that their coordinated activity is likely not sufficient to drive rod differentiation. Because expression of the transcription factor *Nrl* is found infrequently in ONL cells of the rod lineage (Nelson et al., 2008), and because the mouse *nrl* has a key role in promoting rod differentiation, it is tempting to speculate further that transient *nrl* expression is the last piece of the transcriptional network required for rod development in the zebrafish. The presence of *nrl* mRNA in zebrafish cone progenitors (Nelson et al., 2008), and the presence of *Nrl* response elements in an *in vivo* enhancer of both rod and cone photoreceptor gene expression (Morrissey et al., 2011) argue against this possibility. However, *Nrl* may be required in nascent cones as a transcriptional repressor (Hennig et al., 2008).

It is noteworthy that, in zebrafish, the suite of photoreceptor transcription factors expressed by cells of the rod lineage is no different than the corresponding set expressed by nascent cone photoreceptors. *NeuroD* (Ochocinska and Hitchcock, 2007), *crx* (Bernardos et al., 2007), *Nr2e3* (Chen et al., 2005), *rx1* (Nelson et al., 2008), and *nrl* (Nelson et al., 2008) are all found in cells fated to become rods, and in those fated to become cones. In addition, the transcription factor *pax6* is downregulated in both rod and cone progenitors (Hitchcock et al., 1996). This issue will be revisited in the context of photoreceptor progenitor plasticity (Section 3). This issue has also presented us with particular challenges in labeling of the rod lineage, as there currently are no truly selective markers.

2.3. Stem cells of the rod lineage: Müller glia

An intricate relationship between Müller glia and cells of the teleost rod lineage was recognized shortly after the lineage was first described (Raymond and Rivlin, 1987). It was initially characterized as a spatial relationship, in which the elaborate radial processes of Müller glia serve as a scaffold upon which rod progenitors migrate from the INL to the ONL. Rod progenitors, labeled by the incorporation of BrdU or ³H-thymidine, are indeed physically associated with glial processes (Mack et al., 2003; Raymond and Rivlin, 1987), and are arranged in radial arrays matching the morphology and distribution of Müller glia (Julian et al., 1998; Otteson et al., 2001), supporting this view. The role of Müller glia as the apex of the rod lineage was conclusively demonstrated through the use of transgenic zebrafish that reported the activity of the glial fibrillary acidic protein (GFAP) gene, with the long-lasting reporter molecule GFP (*GFAP:GFP*) (Bernardos et al., 2007). In this transgenic line, Müller glia express the transgene, and as they proliferate, their progeny also contain GFP. In rapidly-growing larval zebrafish, where proliferation within the rod lineage is vigorous, this transgene is found in Müller glia as well as in radial arrays of fusiform cells spanning the INL; these cells also incorporate BrdU, confirming proliferative activity. The GFP⁺ Müller glia within the transgenic larval zebrafish all express the transcription factor Pax6, providing a further, very strong indication that Müller glia and the stem cells that

reside at the apex of the rod lineage represent the same cell population. Finally, some of the GFP+ cells derived from proliferating Müller glia were observed to enter the ONL, where they express photoreceptor markers, including Crx (Bernardos et al., 2007). Collectively these results reveal the critical and complex roles for Müller glia in providing for the existence of the rod lineage, as well as providing the structural apparatus upon which rod progenitors migrate to reach the ONL.

Within the embryonic retina, a lineage relationship has been shown to exist between the first Müller glia and cells of the rod lineage that are generated (Nelson et al., 2008). Through a series of colabeling experiments, we observed Müller glia (glutamine synthetase, GS+) that had incorporated BrdU, as well as *pax6*+ cells that incorporated BrdU, and in each case the cells were located within the proximal INL (Nelson et al., 2008). However, the numbers of *pax6*+/BrdU+ cells greatly exceeded those of GS+/BrdU+ cells, suggesting incomplete overlap, and potentially distinct cell populations. Alternatively, the glial markers available, such as GS (but also GFAP and carbonic anhydrase), are expressed too late in glial differentiation to make definitive conclusions from these experiments.

Thirty years after the rod lineage was first described, a consensus model of the teleost rod lineage now emerges. In this model, Müller glia are seeded into the retina by the stem cells of the CGZ as the fish retina grows. The Müller glia, particularly those that have been most recently generated, remain proliferative, albeit at a markedly lower rate than cells of the CGZ. The proliferative glia are *pax6*+ and can be considered modified stem cells, as they undergo asymmetric divisions that replace themselves as well as generate progeny with a different fate. They are distinct from the textbook definition of a stem cell (Gilbert, 2010) in that they display differentiated properties, consistent with their identity as Müller glia. Their immediate progeny (rod progenitors) downregulate *pax6*, and begin to express photoreceptor transcription factors as they migrate along glial processes toward the ONL. Once in the ONL, cells of the lineage (rod precursors) continue to proliferate, and coordinately express multiple photoreceptor transcription factors. Upon terminal mitosis, rod photoreceptors begin to differentiate. The capacity of this lineage to respond to changing requirements for the production of rod photoreceptors, as well as the plasticity of this lineage to replace other retinal cell types in response to damage, will be discussed in the next section.

3. Plasticity of the rod lineage

3.1. Regulation of rod neurogenesis

The production of new rods in teleosts is regulated by the growth factor/IGF axis (Boucher and Hitchcock, 1998a; Boucher and Hitchcock, 1998b; Mack and Fernald, 1993; Otteson et al., 2002; Zygier et al., 2005), and proliferative activity of the lineage is highest when the animal is rapidly growing (Bernardos et al., 2007; Julian et al., 1998; Marcus et al., 1999). An interesting line of inquiry has now established that the rate of rod neurogenesis can also change in response to rod photoreceptor damage and death (Braisted and Raymond, 1993; Montgomery et al., 2011; Morris et al., 2008b; Morris et al., 2005). For example, selective destruction of a large fraction of rod photoreceptors using the pharmacological agent tunicamycin in goldfish results in significant increases in BrdU incorporation into the ONL, but not into cells of the INL, suggesting that the rod precursor population can sense and respond to rod photoreceptor death (Braisted and Raymond, 1993). More recently, a zebrafish model for genetic rod photoreceptor degeneration has been used to address a similar question. The model is a transgenic zebrafish in which the transgene consists of the *Xenopus* rod opsin promoter driving expression of membrane-associated GFP (*XOPS:mCFP*). Developing rods express the transgene at approximately the same time they express native rod opsin, but they degenerate shortly thereafter [the mCFP may cause protein trafficking errors that are incompatible with rod survival] (Morris et al., 2005). The

XOPS:mCFP zebrafish retina displays a high rate of proliferation within the rod lineage, and this proliferation is limited to the ONL, consistent with the hypothesis that rod precursors can change the rate of rod neurogenesis in response to rod death. Results from alternative genetic models for rod photoreceptor degeneration support and extend this hypothesis (Montgomery et al., 2011). In these transgenic models, the transgene consists of a zebrafish rod opsin promoter driving expression of *E. coli* nitroreductase fused to GFP. When fish are treated with a “prodrug,” metronidazole, the nitroreductase enzyme (expressed exclusively in rod photoreceptors) generates a cytotoxin, causing rods to die. A transgenic line in which a subset of rods were selectively destroyed by prodrug treatment displayed increased proliferation of rod precursors in the ONL, providing an acute injury counterpart to the more chronic degenerative model of the *XOPS:mCFP* zebrafish. However, another transgenic line in which all rods were destroyed by prodrug treatment induced proliferation of Müller glia, suggesting that catastrophic loss of rods can influence the activity of the stem cell as well as the transit-amplifying population (Montgomery et al., 2011). The rod lineage therefore has a mechanism not only for responding to rod death through upregulation of proliferation and rod production, but also for sensing the extent of rod death.

3.2. The rod lineage and the retinal response to injury

Persistent neurogenesis in the teleost retina, including that related to rod production, has long been considered important for the fish retina’s capacity to regenerate following damage. This capacity is distinct from the damage response of the mammalian retina, which is dominated by glial hypertrophy and does not result in the production of new neurons (Lamba et al., 2009). The multipotent stem cell source of regenerated retinal neurons in the zebrafish is now known to be the Müller glial cell (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007; Yurco and Cameron, 2005), the same cell type that represents the apex of the rod lineage. Following retinal damage, Müller glia increase their rate of proliferation, dedifferentiate, and begin to act as multipotent stem cells. Their progeny migrate to appropriate positions within the regenerating retinal tissue and differentiate into mature retinal cell types. Regenerated teleost retina is functional, and can restore and support visually-mediated behaviors that were lost due to retinal damage (Mensinger and Powers, 1999; Mensinger and Powers, 2007; Sherpa et al., 2008).

The lineage of cells that gives rise to rods in the undamaged teleost retina is therefore plastic, as the stem cell otherwise dedicated to the production of rod photoreceptors has the capacity to become multipotent. There is therefore great interest in identifying the factors that regulate the status of Müller glia in the teleost retina. Müller glia exist within a local environment, or niche, that is suited for the support of stem cells. Features of this environment include proximity to blood vessels, and access, via the radial processes of Müller glia, to the apical epithelial surface (Raymond et al., 2006). Upon retinal injury, Müller glia acquire phenotypic characteristics of the multipotent stem cells of the CGZ, including activation of the Notch-Delta signaling pathway (Raymond et al., 2006) and upregulation of the proneural gene *ascl1a*. (Fausett et al., 2008; Ramachandran et al., 2011; Yurco and Cameron, 2007). In addition, the Müller glia at the site of retinal injury upregulate their expression of glial fibrillary acidic protein (GFAP) (Raymond et al., 2006). A number of additional genes have recently been identified that are regulated within Müller glia as they respond to acute injury. The transcription factor *Ascl1a* promotes the expression of the pluripotency factor *Lin-28*, which in turn suppresses expression of *let-7* microRNA (Ramachandran et al., 2011). The role of *let-7* miRNA in undamaged retina is likely to promote the degradation of numerous mRNAs required for glial pluripotency (*ascl1a*, *hspdl*, *pax6b*, *c-myc*, and others). Retinal regeneration in zebrafish does not take place, or is severely limited, when expression of any of the following factors is experimentally or genetically altered: the mitotic checkpoint regulator *mps1*, the mitochondrial chaperone

hspd1 (Qin et al., 2009), or *pax6b* (Thummel et al., 2011). In addition, glial proliferation, and expression of *hspd1* and *pax6b*, are reduced in the absence of *ascl1a* and *lin-28* (Ramachandran et al., 2011). Interestingly, although there is evidence that a regulatory network including these factors is involved in triggering the Müller glial response to retinal damage, roles for components of this network in regulating proliferation of the rod lineage in undamaged retina have not been tested.

How do Müller glia sense damage and engage this regulatory network? The search for environmental signals regulating Müller glial pluripotency remains an ongoing process in many laboratories. One approach utilizes a comparative evaluation of the retinal response to ongoing rod loss, vs. the response to loss of cones (Morris et al., 2008b). Cone death clearly results in glial activation while death of rods generally does not (but see (Montgomery et al., 2011)). There is evidence that Müller glia must participate in phagocytosis of apoptotic bodies in damaged retina in order to proliferate (Bailey et al., 2011). However, in a zebrafish model for chronic retinal cell stress (the *lrp2*^{-/-} fish, *bugeye*), extensive cell death is not required for a robust proliferative response (Sherpa et al., 2011). A current approach in our laboratory utilizes a comparative evaluation of the retinal response to damage in zebrafish vs. the retinal response to damage in mouse. Interestingly, many of the signaling factors speculated as important for regulating the regenerative response, including extracellular signals Lgals112 and Prgn1 (Craig et al., 2008; Craig et al., 2011), intracellular signaling regulator SOCS3 (Kassen et al., 2007), and GFAP (Raymond et al., 2006; Thummel et al., 2008) are also upregulated in damaged mouse retina (Stenkamp and Hunter, unpublished results), indicating that these factors are not specifically associated with regeneration, but instead may participate in a conserved retinal damage response.

While the mechanisms that control multipotency in Müller glia in zebrafish in response to acute damage have yet to be firmly established, the developmental genetics of retinal regeneration itself has been well-documented. Regeneration recapitulates development to some degree, in that genes required for embryonic retinal neurogenesis are re-deployed in stereotyped temporal pattern (Fimbel et al., 2007; Hitchcock et al., 1996; Sherpa et al., 2008). However, regenerated retina contains lamination errors (Fimbel et al., 2007; Hitchcock et al., 1992; Sherpa et al., 2008) as well as errors in the planar mosaic array of new retinal neurons (Cameron and Carney, 2000; Cameron and Easter, 1995; Stenkamp and Cameron, 2002; Stenkamp et al., 2001), suggesting that at least some mechanistic aspects of the retinal developmental process are not reconstituted during regeneration. This is perhaps not surprising, as complete retinal regeneration demands the production of a far larger number of neurons over an immense area as compared to the process of embryonic retinal development and the comparatively small retinal volume.

3.3. Diversion of cone progenitors to the rod fate?

The lineage of cells that gives rise to rod photoreceptors in teleost fish can clearly respond to changing conditions within the retina by changing the rate of rod production, and by adjusting the status of the Müller glia stem cell at the apex of the rod lineage. But can other 'lineages' of retinal progenitors adjust their fates to generate rods? Our analysis of gene expression within the rod lineage indicated that, at the molecular level, rod progenitors and cone progenitors are virtually identical, expressing the photoreceptor transcription factor-encoding mRNAs *rx1*, *crx*, *NeuroD*, *Nr2e3*, and *nrl* (Nelson et al., 2008). This similarity suggests the potential for cone-rod plasticity within the zebrafish retina, although the two photoreceptor populations arise from spatiotemporally distinct lineages. Suggestive evidence that cone progenitors may be diverted to the rod fate comes from a recent study utilizing genetic manipulations that favor the production of rods over that of cones (Alvarez-Delfin et al., 2009).

Mutations in the gene encoding the t-box transcription factor Tbx2b (*lor*, and *fby*) result in the generation of numerous additional rod photoreceptors at the expense of the UV cone photoreceptor population (Alvarez-Delfin et al., 2009). The spatial pattern of rods in the *lor* and *fby* mutants resembles that of the native pattern of UV cones in wild-type zebrafish, suggesting that the UV cone progenitors in the mutant may have altered their fate to differentiate as rods. In genetic chimeras, retinal clones derived from the mutant lacked UV cones, while those derived from the wild-type donor contained UV cones (Alvarez-Delfin et al., 2009). As Tbx2b is a transcriptional repressor, the findings are consistent with Tbx2b acting in UV cones to suppress the rod fate. However, *tbx2b* is expressed in the retinal neuroepithelium from 20–28 hpf, a time in retinal development well before the terminal mitoses of embryonic cones or rods (Hu and Easter, 1999). Therefore, it is possible that the function of Tbx2b is to repress potential fates of progenitor lineages. There is evidence supporting other forms of transcriptional repressor-mediated lineage restriction during retinal neurogenesis in zebrafish (Vitorino et al., 2009).

The question of plasticity of rod vs. cone neurogenesis requires a re-evaluation of the relationship between rod progenitors and cone progenitors early in embryonic retinal neurogenesis [see also (Adler and Raymond, 2008), and the earlier discussion in section 2.1 of this review]. While there is compelling evidence that the rod and cone lineages are spatially distinct in adult (Stenkamp et al., 1997), larval (Bernardos et al., 2007), and late embryonic (Nelson et al., 2008) teleost retinas, rod and cone progenitors are molecularly similar (Nelson et al., 2008). A common progenitor within the early embryonic retina, when rods and cones are first generated, cannot be ruled out.

4. Future Directions

4.1. Rod lineage identification and imaging strategies

Our current understanding of the rod lineage in teleost fish retina is based predominantly upon the capacity to label rod progenitor cells with specific markers, most notably those that indicate proliferation, and those that indicate expression of a photoreceptor-specific gene (Nelson et al., 2008). This approach carries limitations that will soon be overcome by the ability to identify and follow retinal progenitor cells through developmental time using recently developed imaging strategies (Baye and Link, 2007; Del Bene et al., 2008; Poggi et al., 2005; Vitorino et al., 2009; Williams et al., 2011). *In vivo* time-lapse imaging techniques, in conjunction with the use of transgenes that are expressed in specific progenitor lineages or progenitor types, have already uncovered novel mechanisms for the regulation of retinal neurogenesis and photoreceptor differentiation.

The following two examples of selective progenitor cell labeling and imaging are particularly relevant for guiding future studies aimed at specific analysis of the rod lineage. The first example provides evidence supporting lineage restriction during embryonic retinal neurogenesis, such that ongoing expression vs. downregulation of the transcription factor *Vsx2* determines progenitor fate (Vitorino et al., 2009). The *vsx2*–expressing cells were tracked *in vivo* through their expression of a *vsx2*:GFP transgene. Because Müller glia are one of two major retinal cell populations that continue to express *vsx2*:GFP, the rod lineage is inferred as arising from *Vsx2*⁺ progenitors. However, *vsx2* (or *vsx2*:GFP) expression has not been observed in rods or in cells of the rod lineage, suggesting that downregulation of *vsx2* is important for rod neurogenesis. In the second example, nascent cones were visualized by their expression of a transgene consisting of the promoter region for the nuclear hormone receptor TR β 2 driving expression of tdTomato (Williams et al., 2011). This is noteworthy because it represents an approach for identifying cone photoreceptors prior to opsin expression; similar transgene-related strategies for identifying and tracking the rod lineage are likely to be developed. Use of the GFAP:GFP transgenic zebrafish may

address the question of Müller glia as the apex of the rod lineage in embryos; however, the transgene is diffusely expressed during embryonic development, and becomes definitively restricted to cells with specific morphologies as the fish transition to the larval stage (Bernardos and Raymond, 2006). The lengthy interval required for the production of new rods from a Müller glial stem cell within a larval retina [7 days; (Bernardos et al., 2007)] presents further challenges for time-lapse imaging. Nevertheless, the current pace at which new genetic and imaging tools are developed suggests that these challenges soon will be met.

4.2. Lineage manipulation and gene expression

A mechanistic understanding of rod neurogenesis is under pursuit through the use of model systems in which the output of the rod lineage is modified. One recent approach was to describe differences in global gene expression within the retinas of wild-type zebrafish as compared to those of the *XOPS:mCFP* zebrafish, in which chronic rod loss and upregulated rod production take place (Morris et al., 2011). This study identified three potential markers of rod precursors, the transcription factor-encoding genes *sox11b*, *insml*, and *cmyb*. Similar approaches using zebrafish subjected to damage of selected retinal cell types (Kassen et al., 2007; Montgomery et al., 2011; Morris et al., 2008b) or other manipulations that result in changes in rod neurogenesis (Alvarez-Delfin et al., 2009) are likely to identify additional selective markers and potentially reveal the genetic networks engaged in rod vs. cone production. Ongoing studies in our laboratory suggest that an extracellular signal, retinoic acid (RA) influences retinal progenitor cells such that rod neurogenesis is favored over cone neurogenesis, resulting in disrupted photoreceptor spatial arrangements (Kashyap, 2011; Stevens et al., unpublished results). Manipulation of the RA signaling system (and perhaps others) will likely reveal mechanisms underlying plasticity in the developmental trajectory of photoreceptor progenitors. Finally, transcriptome information may also be used to creatively design new transgenic lines in which rod precursors or other cellular components of the rod lineage are selectively labeled and/or tracked.

4.3. Photoreceptor/glia relationship in other models

Perhaps the most exciting new direction related to the study of the rod lineage is the application of this information for the treatment of retinal diseases involving the loss of rod photoreceptors. The identification of the stem cells of the rod lineage (and of the multipotent stem cells of regenerated retinal neurons in general) as Müller glia in zebrafish (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007) has re-invigorated the pursuit of endogenous regenerative approaches to human retinal disorders (Fischer and Bongini, ; Karl and Reh, 2011). There are already preliminary indications that mammalian Müller glia may have neurogenic potential (Bhatia et al., 2011; Karl et al., 2008; Phillips and Otteson, 2011). Can the gliotic response to damage in mammals be manipulated to generate new rod photoreceptors? Such a strategy would be of outstanding benefit, as over 1 in 4000 people are affected with inherited retinal dystrophies such as retinitis pigmentosa (RP; (Huang et al., 2011). RP involves progressive degeneration of rod photoreceptors, resulting in reduced scotopic visual function, and this is followed by degeneration of cone photoreceptors, resulting in blindness. While a regenerative intervention will not correct the genetic lesion causing rod degeneration in the first place, ongoing production of new rods may maintain the health of cone photoreceptors, and thus maintain photopic visual function. Furthermore, such a strategy in conjunction with gene therapy (Simonelli et al., 2011) may restore or maintain scotopic visual function as well.

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Figure 1.

Model of retinal neurogenesis and the generation of rod photoreceptors in the retinas of teleosts. Upper left: Cartoon of the retina depicted as a hemisphere with the sites of neurogenesis in red. Below is a photomicrograph of a radial section through circled region of retina showing growth zones and their characteristic patterns of cellular proliferation. Mitotically active, Pax6-expressing retinal progenitors (shown in red) form the CGZ and give rise to all retinal neurons, except rod photoreceptors. Stem cells are also “seeded” into the INL from the CGZ. Within the region immediately adjacent to the CGZ, mitotically active cells, daughters of resident Pax6 expressing stem cells (shown in red), migrate from the INL to the ONL to produce the first generation of rod photoreceptors. This process continues, but at a slower pace, in the mature retina. On the right is a lineage model for rod genesis [adapted from (Otterson *et al.*, 2001)]. Within the differentiated retina, stem cells (Müller glia) in the INL proliferate, renewing the stem cell population and giving rise to Pax6-negative INL progenitors (shown in white). Progenitors proliferate as they migrate to the ONL, where they become rod precursors and ultimately differentiate into new rod photoreceptors. INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer; MR, mature retina; CGZ, circumferential germinal zone. Modified from (Otterson and

Hitchcock, 2003), with permission of the author and with permission of Elsevier Science, Ltd.

Figure 2.

Gene expression in the late embryonic rod lineage of zebrafish. Gene expression summary at right shows the genetic hierarchy of the rod lineage; darkness of font reflects the extent to which the transcription factor is expressed within the identified cells of the lineage. *Pax6*-positive Müller glia/stem cells are shown in blue with their cell bodies in the inner region of the inl. As rod progenitors proliferate and migrate toward the onl, some become *NeuroD*-positive and *rx1*-positive (green cells), but none remain *pax6*-positive. All of these progenitors later express *crx* (yellow). Prior to entering the onl, some rod progenitors express *NeuroD*, *rx1*, and *Nr2e3*, while all continue to express *crx* (orange). All rod precursors of the onl (red) are *NeuroD*, *rx1*, *crx*, and *Nr2e3*-positive but do not express *nrl*, *rod opsin*, or *gnat 1*. Postmitotic rod photoreceptors (purple) express *NeuroD*, *rx1*, *crx*, *Nr2e3*, *nrl*, *rod opsin*, and *gnat 1*. onl, outer nuclear layer; p-inl, m-inl, d-inl are proximal, middle, and distal compartments of the inl, respectively, and are delineated by dotted lines. Modified from (Nelson *et al.*, 2008) with permission of Wiley and Sons, Inc.

Figure 3.

A model for persistent and injury- or degeneration-induced neurogenesis in the zebrafish central retina. In a wild-type, uninjured retina, slowly dividing stem cells in the INL (orange), which are derived from neurogenic Müller glia, give rise to committed rod progenitor cells in the INL (green, pathway A). The INL progenitors migrate to the ONL where they are further amplified as rod precursors (purple) and differentiate into mature rods (red). In response to rod photoreceptor degeneration alone, the rod precursors of pathway A increase their proliferation in the absence of proliferation in the INL. In response to degeneration of the cone photoreceptors, damage to both rods and cones, or death of other inner retinal neurons, Müller glia re-enter the cell cycle to produce neurogenic clusters

(green, pathway B) in the INL that migrate to the correct laminar position and differentiate into multiple retinal cell types (red). CGZ, circumferential germinal zone; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Modified from (Morris *et al.*, 2008b) with permission of the author, and with permission of John Wiley & Sons, Inc.

Figure 4. Genetic manipulations reveal plasticity of photoreceptor progenitors in zebrafish retinas. A–B. Increased rod number and fewer UV cones in the *lor^{p25bbl}* mutants. Confocal images of eyes from 5-dpf WT (A), and *lor^{p25bbl}* (B) larvae, immunolabeled for UV opsin (green) and rods (red). Modified from (Alvarez-Delfin *et al.*, 2009), with permission from the author; copyright © by the National Academy of Sciences.