Chapter

Zebrafish Photoreceptor Degeneration and Regeneration Research to Understand Hereditary Human Blindness

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Abstract

Humans with mutations in photoreceptor-related genes develop forms of retinal degeneration, such as retinitis pigmentosa, cone dystrophy, or Leber congenital amaurosis. Similarly, numerous photoreceptor mutant animal models present phenotypes that resemble retinal degeneration. Zebrafish retina manifests anatomical organization and development remarkably conserved in humans, making these fish a good model to study photoreceptor development and disease. Zebrafish are ideal for forward genetic screens to isolate mutants with visual defects. More recently, CRISPR/Cas system-mediated genome editing has enabled establishment of specific zebrafish photoreceptor mutants. Here, I review zebrafish models of inherited retinal diseases, focusing on rod versus cone photoreceptor mutants. Because zebrafish possess robust regeneration capacity to replace the lost photoreceptors, here I review the current understanding of molecular mechanisms underlying this response.

Keywords: photoreceptor, degeneration, genetic mutant, regeneration, Müller glia, genome editing, CRISPR/Cas9 system, zebrafish

1. Introduction

Photoreceptor degeneration includes a heterogeneous group of diseases characterized by death of photoreceptors and progressive loss of vision. Photoreceptor degeneration is a major cause of blindness in developed countries, for which there is currently no effective treatment [1]. Zebrafish are a good model to study photoreceptor development and disease, because the anatomical organization and development of the retina are remarkably conserved among vertebrates. In contrast to the mammalian retina, which is rod-rich, zebrafish have cone- and rod-rich retinas, facilitating the study of cones. Cone visual acuity can be evaluated by simple optokinetic response (OKR), even at very early stages of development. In this review, I discuss why the zebrafish model is useful to unravel mechanisms of photoreceptor loss. In addition, unlike mammals, zebrafish have the capacity to fully regenerate dead photoreceptors, raising the hope of future treatments for this disorder. Here I summarize our current understanding of this regeneration response.

1.1 Photoreceptors in the zebrafish retina

Zebrafish neural retina, like those of other vertebrates, comprises three nuclear layers, separated by two synaptic layers (**Figure 1**). The outer nuclear layer (ONL) comprises rod and cone photoreceptors, the light-sensing neurons. The inner nuclear layer (INL) consists of bipolar, horizontal, and amacrine neurons, the second-order neurons. The ganglion cell layer is formed by ganglion cells, axons of which exit the retina, forming the optic nerve, which connects with the tectum. These neurons interconnect by synapses in the plexiform layers. The neural retina is located adjacent to the retinal pigment epithelium (RPE), which supports general homeostasis of photoreceptors, such as recycling 11-cis retinal for visual pigment regeneration [2, 3].

Photoreceptors are polarized neurons with characteristic morphology. They display very specialized cell regions, including outer segments (OSs), connecting cilia, cell bodies, and terminal synapses. OS structure is important for phototransduction. The cell bodies possess the machinery to support all cell functions, and their synaptic termini transduce signals to bipolar neurons. OSs are formed by hundreds of cell membrane discs stacked horizontally and associated with a high concentration of proteins for phototransduction. These proteins are synthetized in cell bodies, and then are transported to the OS through connecting cilia.

Photoreceptors are sensory neurons that produce electrical responses when stimulated by light. In the OS, photons are captured by photopigment molecules to initiate phototransduction cascades [4]. Phototransduction is a complex signaling process that results in closing of voltage-gated ion channels, producing a change in membrane potential. Then, this electrical signal is amplified by other cell types in the inner retina and conducted to the brain. Although this signaling pathway is common to both rods and cones, signaling proteins are mostly encoded by distinct sets of rod- and cone-specific genes. Cone and rod photoreceptors have different sensitivity to light. Rods are extremely sensitive to low-intensity light, while cones function at higher light intensity, and enable color discrimination. In zebrafish, four different subtypes of cones are organized in a precise mosaic pattern. Cones are

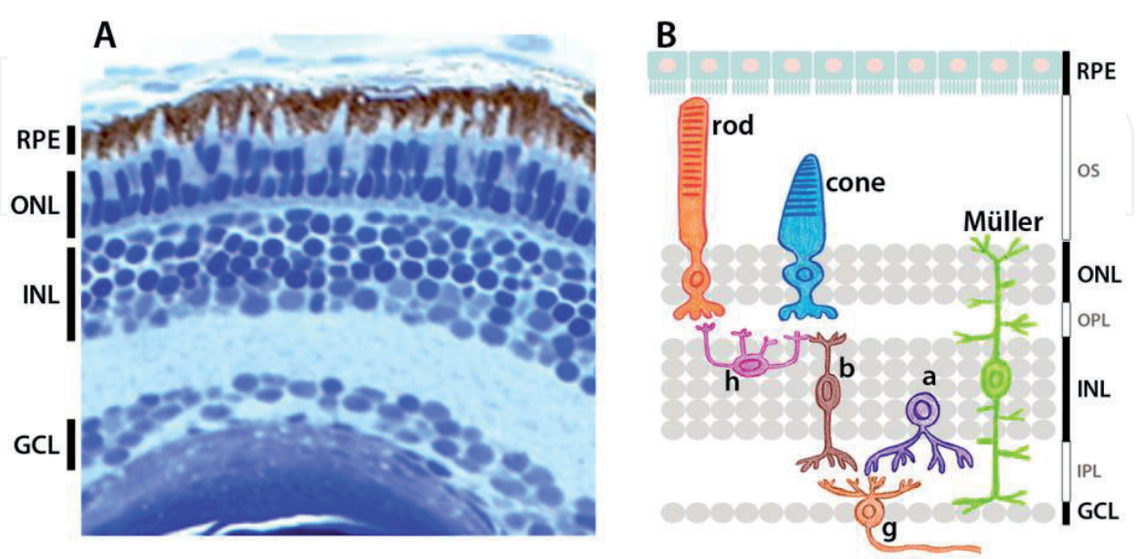

Figure 1.
Zebrafish retinal organization. (A) Semi-thin section of a zebrafish retina at 7 days post-fertilization (dpf). The neural retina is organized into 3 layers. The ONL is formed of cone and rod photoreceptors. (B) Schematic representation of all components of the retina. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer; RPE: retinal pigmentary epithelium; h: horizontal; b: bipolar; a: amacrine; g: ganglion neuron.

abundant throughout the zebrafish retina, unlike that of humans, in which cones are at high density in the fovea. Like humans, zebrafish are diurnal.

1.2 Zebrafish as a model to study hereditary eye diseases in humans

Hereditary diseases of the retina involve heterogeneous mutations that result in progressive photoreceptor death, leading to blindness. Mutations in over 200 genes are currently known to be associated with retinal disorders [Retinal Information Network (RetNet): https://sph.uth.edu/retnet]. Retinal degeneration includes multifactorial diseases such as retinitis pigmentosa, Leber congenital amaurosis (LCA), cone-rod dystrophy, and age-related macular degeneration (AMD). In many cases, these diseases are similar in morphological pathogenicity, whereas their genetic origins may be due to mutations affecting different proteins, such as opsins, proteins of the transduction cascade, ciliary protein, or metabolic proteins.

Retinal degeneration can affect rods, cones, or both. Retinitis pigmentosa is a remarkable disease, caused by a mutation in rod photoreceptors that progresses to affect wild-type cone photoreceptors. It is characterized by progression from night blindness due to rod photoreceptor death, to dysfunction and degeneration of cones concentrated in the fovea at the center of the retina [5]. Retinitis pigmentosa is the most common inherited retinal dystrophy (IRD), affecting approximately 1 in 4000 people [6]. AMD is a multi-factorial disease that affects RPE, and leads to the loss of central vision, sustained by cone photoreceptors. AMD is the leading cause of blindness in industrialized countries [7]. Age and a positive family history of AMD are the two strongest risk factors for AMD. This disease is characterized by pigmentation changes at the level of the RPE and deposition of extracellular deposits called drusen, between the basal surface of the RPE and Bruch's membrane in the macula [8].

Leber congenital amaurosis is a group of monogenic, inherited, retinal degenerative disorders that typically show early onset and severe visual dysfunction, with progressive degeneration [9]. At least 25 genes involved in the retinoid cycle and phototransduction, photoreceptor morphogenesis, and protein trafficking in the connecting cilia are associated with LCA. Cone and cone-rod dystrophies are a clinically and genetically heterogeneous group of inherited retinal diseases, involving as many as 30 genes. Initially cone photoreceptors degenerate, followed by rod photoreceptor loss. These disorders typically present progressive loss of central vision, color vision disturbances, and photophobia [10].

Why use zebrafish to research inherited retinal dystrophy? Zebrafish are small tropical fish that are easy to maintain, and that produce many eggs. They have transparent embryos that develop very rapidly, with a 3–4-month generation time. Zebrafish are also easy to modify genetically [11]. The visual system is highly conserved. It is already functional just 5 days post-fertilization (dpf), and it can be assessed by OKR [12]. The fish retina is cone-rich. Because several genes have extra paralogs caused by gene duplication in teleost fish, several genes are cone and rod-specific, making them suitable to study both types of photoreceptors independently [13]. The pioneering work of Streisinger, which produced mutants using UV-irradiated sperm, hydrostatic pressure, heat shock, or gamma irradiation, proved that genetics could be studied using the zebrafish [14, 15]. Soon after that, wide-ranging mutant collections that develop retinal degeneration were isolated, propelling zebrafish into ophthalmologic research.

1.2.1 Forward genetic screens to isolate mutants with visual defects

A special issue of *Development* in 1996 published 37 papers coming mainly from Nüsslein-Volhard's lab in Tübingen and Wolfgang Driever's lab in Boston. These

groups performed large-scale mutagenesis screens from founder fish chemically mutagenized with N-ethyl-N-nitrosourea (ENU) [16–18] to provide researchers with thousands of mutants (**Figure 2**). This special issue described roughly 1500 mutations in more than 400 genes involved in processes that govern development and organogenesis [19]. These initial screens were based on evaluations of phenotype by stereomicroscope, without staining or complex microscopy. Additionally, behavioral screens to isolate visual mutants were carried out [12, 20–23]. In these cases, OKR and optomotor responses (OMR) of mutagenized larvae were measured during a three-generation screen for recessive mutations (**Figure 2**). Other screening used F1 generation fish (8–10 months old) derived by ENU mutagenesis, and evaluated their escape responses to a threatening object in order to isolate dominant mutants [24, 25].

The zebrafish genome has been sequenced, providing targets for reverse genetics through use of morpholinos [26]. The use mutants identified from screening in combination with morpholino knock-down has been a widely employed strategy to understand mechanisms underlying many biological processes, including vision. Morpholinos are antisense oligonucleotides designed to temporarily downregulate gene function by blocking translation or splicing [27]. However, morpholinos limit

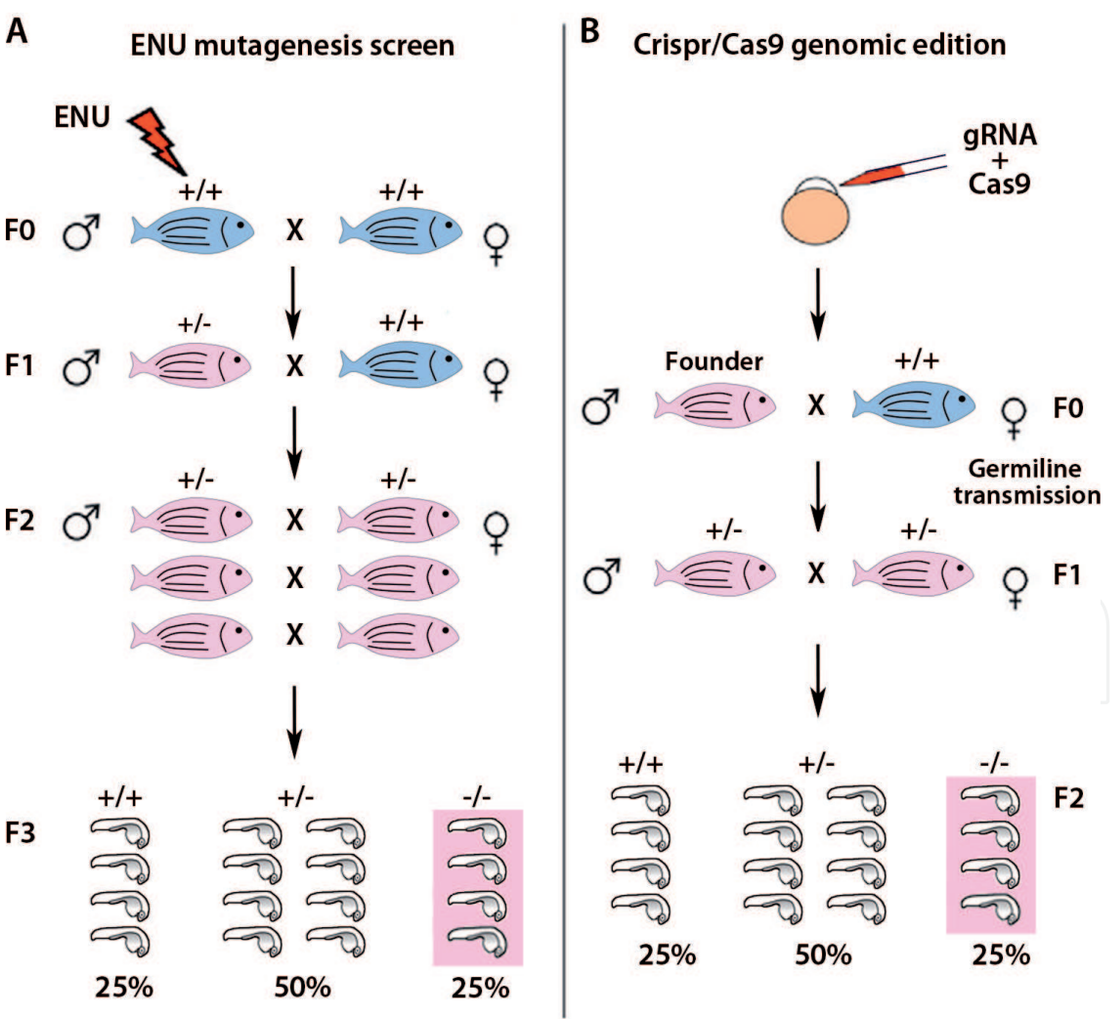

Figure 2.
Genetic strategies to isolate zebrafish visual mutants. (A) Three-generation forward screening. Male zebrafish are treated with the mutagenic drug, ENU, followed by 3 generations of crossing to isolate homozygous recessive mutants. Larvae are usually screened using visual behavior tests. (B) Reverse genetic screening based on Crispr/Cas9 technology. 1–4 cell eggs are microinjected with gRNA and Cas9 mix to target a specific gene of interest. Founders need to be screened in the next generation according to the inheritance of the mutation. Homozygous recessive mutants are evaluated to find abnormal phenotypes.

embryonic development. Usually 1 to 4-cell embryos are injected and effects can be studied up to 4–5 dpf. Mutagenic screening of zebrafish revealed conserved functions of numerous genes across vertebrate lineages and identified zebrafish orthologs for 82% of known human retinal disease genes.

1.2.2 Discerning specific signaling pathways between cone or rod photoreceptors

Genetic screens in zebrafish have shed light on the molecular bases of photoreceptor functions by isolation of visual mutants. Photoreceptor mutants have been isolated, characterized, and mapped. Cone function can be evaluated by OKR and OMR under normal light in 5–7-dpf larvae. A breakthrough discovery in retinal dystrophy was the identification of mutants of phosphodiesterase 6c (pde6c), a novel cone-specific phototransduction gene [12, 28]. *Pde6c*$^{-/-}$ was identified as a blind zebrafish mutant with rapid degeneration of cone photoreceptors having secondary, but transitory degeneration of rod photoreceptors. These two achromatopsia zebrafish mutants were the first visual disorders linked to cone-specific degeneration, and helped to identify human PDE6c mutations in patients [29, 30]. These findings indicated that like zebrafish pde6c mutants, cone-specific degeneration also occurs in humans.

The maturation and functional integrity of PDE6 depends on aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) [31]. We reported an *aipl1* mutant that was blind and developed cone photoreceptor degeneration accompanied by rod degeneration only at an early stage of development [32]. Retinal phenotypes of *aipl1* mutants are very similar to those of zebrafish hypomorphic *pde6c* mutants. Such results were not surprising, given the role of aipl1 in supporting pde6c functions. We confirm the absence of pd6c protein in the *aipl1* mutant. Unexpectedly, the level of guanylate cyclase-3 (gc-3), another important cone-specific phototransduction molecule, was also affected. At the moment, the molecular mechanism underlying the coupling of Pde6 and Gc3 maintenance in photoreceptors remains unknown. *gc3* mutants have been isolated by OKR and OMR screening and they present abnormal visual behavior, although their retinal morphology is normal during larval stages [22]. It would be interesting to study *gc3* mutants to elucidate the relationship between pde6c and gc3.

Though mutants isolated through OKR or OMR behavioral screening evaluate cone function, several mutants exhibited rod degeneration as well. Since rod and cone photoreceptor OSs are continually phagocytosed by the covering RPE, they need to be renewed actively by transport of molecules from the cell body to the OS through connecting cilia. This is called Intraflagellar Transport (IFT) [33]. Several genes have been identified as components of the IFT, such as ift52, ift57, ift80, ift88, and ift172 [23, 34]. In *ift88* zebrafish mutants, cilia are generated, but not maintained, causing an absence of photoreceptor OSs [35]. *ift57* mutant zebrafish had short OSs whereas *ift172* mutants lack OSs completely at 5 dpf [23, 36]. Degeneration of mutant photoreceptors is partly caused by ectopic accumulation of opsins. These results illustrate the unique mechanism of IFT, which is very different from cytosolic transport, and is important for OS formation and maintenance.

Intracellular vesicular transport is important for cytosolic distribution and recycling of molecules. *β-SNAP* cooperates with N-ethylmaleimide-sensitive factor (NSF) to recycle the SNAP receptor (SNARE) by disassembling the cis-SNARE complex generated during the vesicular fusion process. *β-SNAP*$^{-/-}$ presents photoreceptors degeneration, in which photoreceptors undergo apoptosis in a BH3- only, protein BNip1-dependent mechanism due to failure to disassemble the SNARE. *β-SNAP* mutant was the first zebrafish mutant to link photoreceptor degeneration to vesicular transport defects [37].

Unlike photopic cone-mediated vision mutants, which can easily be isolated by behavioral tests from genetic screens, rod mutant screens are much more laborious and time consuming. Scotopic vision needs to be evaluated under dim light, and rod maturation takes up to 3 weeks post-fertilization (wpf). The escape response has been used to screen adult male F1-generation zebrafish treated with ENU, looking for dominant inherited retinal mutants [24, 25, 38, 39]. When a fish is swimming in a circular container and is threatened, it reacts by turning away from the threat. Individuals that failed to show the escape response under dim light illumination were isolated, and named *night blindness a-g*. A spectrum of retinal phenotypes was observed, from photoreceptor degeneration in a patchy array ($nba^{+/-}$; $nbe^{+/-}$), thinner OSs or degenerated OSs ($nbc^{+/-}$; $nbd^{+/-}$; $nbg^{+/-}$), to absence of photoreceptor degeneration ($nbb^{+/-}$). Homozygous mutant embryos of the F3 generation in most cases died after several days of development, indicating that these mutations are not photoreceptor-specific.

1.2.3 CRISPR/Cas editing genome technology to produce photoreceptor mutants

Forward genetic screens have proven very powerful for isolating mutants. However, they do not allow specific genes or pathways to be investigated. Programmable nucleases have revolutionized genetics by allowing precise targeted genome modifications to produce mutants. There are several types of tools for genome editing, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR) systems. These tools have facilitated widespread DNA editing in various organisms, including zebrafish. High specificity and efficiency, flexible design and simple methodology are the most relevant features. CRISPR/Cas enzyme stands out for its extremely utility based on RNA–DNA interaction, while ZFNs and TALENs recognize specific DNA sequences through protein–DNA interactions. Any researcher with basic skill in molecular biology can easily implement CRISPR technology. CRISPR/Cas can target virtually any gene of interest with a customizable short RNA guide to produce knock-out of individual genes [40, 41]. CRISPR requires two key components, a nuclease, most commonly Cas9, and sgRNA (single-guide RNA) which targets the nuclease to a specific DNA location (**Figure 2**) [42]. By simply designing the sgRNA, CRISPR can be targeted to different genome locations. By expressing several sgRNAs, the system also enables multiplex genome editing with high efficiency [43].

1.2.4 Zebrafish photoreceptor-specific genes edited to model human retinal pathologies

Genome editing technologies have been employed in zebrafish for retinal studies. While it is quite easy to produce knock-outs, creating knock-in zebrafish remains challenging. Several photoreceptor knock-out mutants have been recovered in zebrafish that showed the involvement of several genes in photoreceptor development and survival. Mutations in over 50 genes, such as RP2, have been identified as causes of retinitis pigmentosa. RP2 is a GTPase activator protein for ARL3 and participates in trafficking of ciliary proteins. Fei Liu et al. generated an *RP2* knock-out zebrafish line using TALEN technology to understand the RP2 degeneration mechanism [44]. *RP2* knock-out zebrafish display progressive retinal degeneration, with a degeneration of rod OSs, followed by degeneration of cone OSs. RP2 knock-down by morpholinos resulted in abnormal retinal localization of GRK1 and rod transducin subunits, GNAT1 and GNB1. Furthermore, distribution of farnesylated proteins in zebrafish retina was also affected by

RP2 ablation. The same lab also produced *cerkl* knock-out zebrafish, a model for a rod-cone dystrophy [45]. Progressive degeneration of rods and cones, with an accumulation of shed OSs in the interphotoreceptor matrix was observed, suggesting that cerkl may regulate phagocytosis of OSs by the RPE. In addition, the phagocytosis-associated protein, MERTK, was significantly reduced in *cerkl* mutants. Despite a number of genes that have been implicated in retinitis pigmentosa pathogenesis, the mechanism of the disease remains unknown. Zebrafish have proven useful for modeling these ocular diseases.

Photoreceptor genesis requires precise regulation of progenitor cell competence, cell cycle exit, and differentiation. Several transcription factors that control photoreceptor-specific gene expression have been identified. The basic helix–loop–helix transcription factor NeuroD governs photoreceptor genesis, but the signaling pathway through which it functions is unknown. NeuroD was knocked-down with morpholinos, and knocked-out with CRISPR/Cas9 [46]. NeuroD induces cell cycle exit and photoreceptor maturation through cell–cell signaling. NeuroD knock-down resulted in failure to exit the cell cycle, but did not affect expression of photoreceptor lineage markers, Nr2e3 and Crx. NeuroD increased Notch gene expression. Notch inhibition rescued the cell cycle exit, but not photoreceptor maturation. The nuclear receptor transcription factor, Nr2e3, is expressed in photoreceptors. It forms a complex with Crx, which enhances expression of rod-specific genes and represses expression of cone-specific genes in rods [47]. CRISPR-edited *Nr2e3* knock-out animals displayed rod precursors undergoing terminal mitoses, but failed to differentiate into rods. They did not express rod-specific genes and the OS fails to develop. Cone differentiation was normal; however, later, progressive degeneration of OS of double cones began, with a reduction of phototransduction proteins. Nr2e3 acts synergistically with Crx and Nrl to enhance rhodopsin gene expression, without affecting cone opsin expression [47].

A large number of genetic defects can disrupt OS morphology to impair photoreceptor function and viability. Kinesin family members and IFT motors are important for trafficking proteins to photoreceptor OSs [33]. Edited-knock-out *osm-3/kif17* and *cos2/kif7* mutants have comparable OS developmental delays, although via different mechanisms [48]. *Cos2/kif7* mutant dysfunction depends on Hedgehog signaling, which leads to generalized, non-photoreceptor-specific delay of retinal neurogenesis, while *osm-3/kif17* OS morphogenesis delays are associated with initial disc morphogenesis of photoreceptors. The ciliary protein C2orf71a/pcare1 is almost exclusively expressed in photoreceptors, and modulates the ciliary membrane through recruitment of an actin assembly module. Embryos and adult retinas of *C2orf71a/pcare1*$^{-/-}$ zebrafish display disorganization of photoreceptor OSs [49]. This mutant shows visual impairment assessed by OKR and OMR in larvae. Lack of *pcare1* in zebrafish causes similar retinal phenotype to that in humans and indicates that the function of the pcare gene is conserved across species. When mutated, Eye shut homolog (EYS), another ciliary protein, causes retinitis pigmentosa and cone-rod dystrophy. Since eys is absent from several rodent genomes, including mice, zebrafish hold promise as a model for EYS-deficient patients. Several groups established an *eys* knock-out zebrafish model using CRISPR/Cas9 and TALEN technology [50–52]. Embryos and adult retinas showed disorganization of photoreceptor OSs. *eys*$^{-/-}$ zebrafish presented mislocalization of several OS proteins, such as rhodopsin, opn1lw, opn1sw1, GNB3 and PRPH2, and disruption of actin filaments in photoreceptors [50–52]. All these new zebrafish mutants present phenotypes that mimic clinical manifestations of patients, suggesting the utility of these animal models for studying the etiology of these retinopathies.

Gene target editing technologies enable production of rod-specific photoreceptor mutants, which were challenging to isolate using behavioral screening.

Mutations in rhodopsin are the most common cause of retinitis pigmentosa in humans [53]. The human rhodopsin mutation Q344X was expressed in zebrafish to study photoreceptor degeneration. Early mislocalization of hRho Q334X led to rod apoptosis, without affecting cone survival. Activation of phototransduction signaling through transduction and adenylyl cyclase increased photoreceptor loss [54]. Recently, CRISPR/Cas9-induced mutations were used to target the major zebrafish Rho locus, rh1–1, and several mutants were recovered [55]. These mutants were characterized by rapid degeneration of rod photoreceptors, but not of cones. These novel lines will provide badly needed *in vivo* models to study pathology of retinitis pigmentosa.

All these examples of mutants recovered by reverse genetic approaches have been used to identify key molecular pathways required for photoreceptor development and function. Nonetheless, they are generally limited in terms of the number of targets that can be evaluated. Reverse genetic screening techniques have been used with invertebrate animal models and cell culture systems to identify genes and pathways involved in various biological processes; however, their use with *in vivo* vertebrate model systems has been challenging [56]. Recently, several zebrafish labs have shown the feasibility of CRISPR/Cas-based mutagenesis assays to isolate high numbers of mutants focused on synapsis [56], thyroid morphogenesis and function [57], and the Fanconi Anemia pathway, which is involved in genomic instability syndrome, resulting in aplastic anemia [58]. One study screened 54 ciliary genes and isolated 8 mutants that were required for retinal development [59].

In summary, these descriptions of zebrafish phenotypic models isolated from forward mutagenesis screens and reverse genetic approaches targeting genes important in retinal biology have shown how it is possible to advance studies of retinal degeneration through zebrafish research. There still remain several unknown genes associated with retinal degeneration. Their eventual exploration will provide a deeper understanding of molecular mechanisms underlying photoreceptor degeneration and death.

1.3 Therapeutic treatments for retinal degeneration in humans

Several photoreceptor diseases such as retinitis pigmentosa, Leber congenital amaurosis, and macular degeneration produce photoreceptor cell death which leads to blindness. Current treatments of such diseases are ineffective; thus, several different strategies to treat them are being pursued. Neuroprotective approaches with drugs have been evaluated with different degrees of success. These include cGMP analogue treatment [60], calcium-channel blockers [61], and rod-derived cone viability factor [62]. These strategies aim to treat patients during early stages of retinal degeneration, since the disease cannot be reversed. On the other hand, neuroprotective strategies do not depend on any specific mutation and may provide a longer time window for other treatments [63]. Gene therapy has been applied to improve vision in patients with LCA caused by mutations in RPE65. In 2008, three groups reported success in delivering a healthy RPE65 gene using an AAV2 vector to the retina of three LCA patients [64–66], but the improvement may not persist [67]. External devices have been used to electrically stimulate neurons in the inner part of the retina. High visual acuity cannot be achieved, but face and object recognition, and orientation in unknown environments are possible [63].

The most promising therapies are cell transplantation and regeneration based on Müller cells. Using induced-pluripotent stem cells (iPSCs) it is possible to produce eyecup-like structures [68]. These eyecups present a layered structure similar to a retina, with photoreceptor-like cells that contain outer segments, express phototransduction proteins, and some light response [69]. When photoreceptors

are transplanted, they need to integrate into and establish synaptic connections with the remaining retina. Animal experiments showed that few photoreceptors integrate to produce functional recovery of vision [70, 71]. Unexpectedly, recently research demonstrated that the improvement in vision is due to exchange of cytoplasmic material (RNA and/or proteins) between donor cells and the host retina, and not to integration of transplanted photoreceptors [70, 72, 73], making it unclear through which mechanism restoration of vision is achieved. The goal of regeneration is to replace photoreceptors through induction of endogenous progenitor cells. Unfortunately, neurons in the mammalian central nervous systems cannot be replaced. In contrast, lower vertebrates such as reptiles, amphibians, and fish, have the capacity to regenerate lost neurons in brain, spinal cord, and sensory organs, such as the retina and ear [74]. Defining mechanisms of zebrafish retinal repair may offer a key to regenerative medicine.

1.3.1 Müller cell response to retinal injury

In response to injury, mammalian Müller cells exhibit signs of reactive gliotic, featuring cell hypertrophy and upregulation of glial fibrillary acidic protein (GFAP) [75, 76]. Initially this reactive gliosis is neuroprotective, but eventually leads to loss of retinal neurons and causes scarring. Unlike mammals, zebrafish retina responds to neuronal damage by proliferation of Müller glia, which can replace all neuron types, including photoreceptors. Müller glia are the major glial cell type in the retina and contribute to retinal structure and homeostasis [77]. Nuclei are located in the inner nuclear layer, and these cells present apical and basal projections that extend all through the retina (**Figure 1**). Apical feet form the outer limiting membrane. Müller glia are located so that they can monitor the entire retina and contribute to retinal structure and function.

When loss of neurons occurs, Müller glia respond by dedifferentiating, re-entering the cell cycle and producing neuronal progenitor cells (**Figure 3**). These progenitors amplify their numbers, and then migrate to the injured region. Then neuronal progenitor cells exit the cell cycle and differentiate into replacement neurons. All types of retinal neurons can be produced and replaced in injured zebrafish retina to achieve morphological and functional recovery of the retina [78–81]. Identifying molecular signals and pathways that drive this regeneration response is the focus of regenerative medicine.

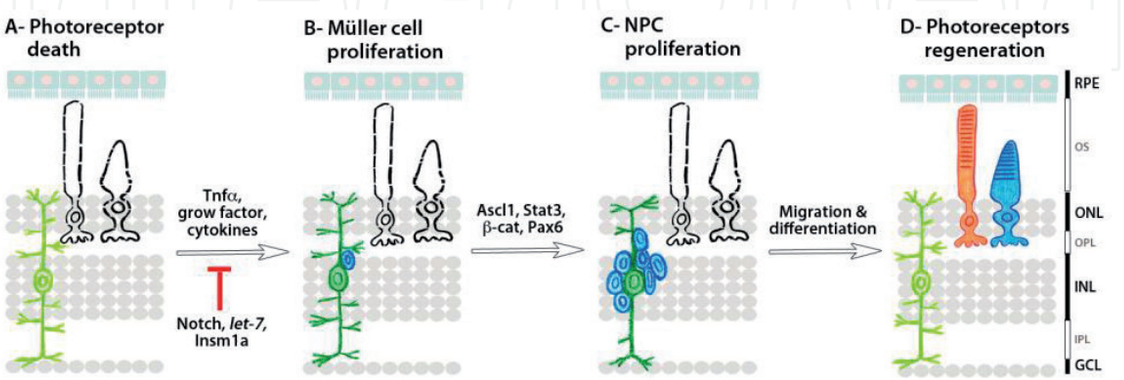

Figure 3.
Photoreceptor degeneration induces a regenerative response based on Müller cell activity. (A) Photoreceptor loss and/or other cells produce stress signals such as TNFα, growth factors, etc. to induce cell Müller activation. (B) Müller cells dedifferentiate to stem cells, and divide asymmetrically to produce one NPC and one Müller glial cell. Müller cells express Ascl1 and Stat3. (C) NPCs proliferate to increase the number of progenitor cells. (D) NPCs exit the cell cycle and differentiate into photoreceptor lineage cells to reverse loss of these neurons. NPC: neuronal proliferative cells.

1.3.2 Positive and negative signaling to modulate Müller cell proliferation in the regenerative response

Understanding mechanisms by which zebrafish can regenerate injured retina may provide strategies for stimulating retinal regeneration in mammals. Given the similarity of anatomy, cell types, and gene conservation between teleost fish and mammals, the regenerative approach offers hope for clinical treatment. Both species present Müller cells that are the primary cell type responsible for regeneration.

Acute injury models have been useful for dissecting many signaling pathways, and great progress has been achieved. Several secreted signaling molecules that participate in Müller glia dedifferentiation and proliferation have been identified. These include TNFα [82], HB-EFG [83], Wnt [84], TFGb [85], insulin, and Fgf2 [86] (**Figure 3**). In addition, Müller cells activate different transcription factors, and initiate signaling essential to differentiation and/or proliferation, such as Ascl1b [87], Stat3 [88, 89], Pax6 [90, 91], PCNA [90], Lin-28 [87]. Interestingly, it still has not been confirmed that these transcription factors and signaling molecules are also expressed and activated in genetic mutants with slow degeneration.

When a regeneration response occurs, around 50% of Müller cells dedifferentiate and proliferate in the injured region, while the other Müller cells remain as differentiated glia. Let-7, Notch and Insm1a [87, 92, 93] are involved in this quiescent Müller cell population. It may be important that some Müller cells remain quiescent to avoid an excessive neurogenesis and remodeling of the retina, as well to maintain homeostasis of healthy neurons.

Important results have come from uninjured retinas in relation to the external delivery of activation signals or transcription factors that are able to generate a regenerative response. For example, Tnfα intravitreal injection into adult fish induced a moderate proliferative response [82]. Tnfα combined with repressing Notch (γ-secretase inhibitor) via intravitreal injection produced a much stronger proliferative response [92]. These results suggest that identifying key molecules for the regenerative response, and modulating them can induce a proliferative response by Müller cells.

Recently, some exciting results came from studies in adult mice [94]. NMDA-damaged retinas, with injury to the inner part of the retina, were treated with a histone deacetylase inhibitor and overexpressed Ascl1. Under these conditions, Müller glia were induced to produce functional neurons via a trans differentiation mechanism. *Gnat1*^{rd17}*Gnat2*^{cpfl3} double mutant mice, a model of congenital blindness, were treated by gene transfer of β-catenin, and subsequent gene transfer of transcription factors essential for rod cell fate specification and determination. Müller glia-derived rods restored visual responses [95].

1.3.3 Genetic mutant models to investigate regeneration mechanisms

Most current studies use acute approaches to injure adult retinas, like light damage [79, 96], retinal puncture [80], chemical injection [81], or loss of specific cell populations due to activation of a toxic transgene (nitroreductase: NTR) [97, 98]. Light damage and toxic transgene NTR induce photoreceptor death, while retinal puncture kills specific neurons. These animal models use powerful, rapid damage that resembles traumatic injury in human patients. To model inherited photoreceptor degeneration diseases, better models need to be used.

Only a few studies have employed zebrafish photoreceptor genetic mutants [32]. Iribarne et al. used cone- or rod-specific mutants with a very rapid loss of photoreceptors, and observed that regeneration started as early as 1 wpf. Cone-specific mutant regeneration relied on Müller cell proliferation, while rod

photoreceptor-specific mutant regeneration was based on rod progenitor proliferation [99]. Another cone mutant, *Aipl1*$^{-/-}$, which developed a slower and progressive degeneration, surprisingly did not show an increase in Müller cell or rod progenitor proliferation, even though cell death was detected [32]. Both studies used larval animals (1 and 2 wpf) and lacked the information of later stages and the adult regenerative response. Further investigation during development and adulthood should be performed to gain a better understanding of the response of Müller cells to damage.

Interestingly, all these injury models elicited a Müller cell response that was similar overall. However, some molecules revealed injury-dependent induction. Hbegf was necessary for retinal regeneration following a mechanical injury, but it was not necessary for regeneration following photoreceptor damage by light [82, 83]. These adult acute models have proven to be powerful in revealing many of the molecular signals that drive the regenerative response. However, for modeling human photoreceptor genetic diseases, which usually proceed from embryogenesis or childhood to adulthood to completely degenerate, a more specific model needs to be used.

1.3.4 CRISPR/Cas system screening to isolate defective regenerative retinal processes

The high efficiency and multiplexing capabilities of CRISPR enable high-throughput, forward screening of "genotype to phenotype" functions in various model systems [43]. So far, several zebrafish labs have utilized CRISPR/Cas system screening (check Section 2.2.4 in this chapter). However, few screening studies have focused on regeneration. A screening method for hair cell regeneration identified 7 genes involved in this response [100]. To evaluate genes important for retinal regeneration, large-scale, reverse genetic screening has been established by applying a multiplexed gene disruption strategy [101]. This screening used an automated reporter quantification-based assay to identify cellular regeneration-deficient phenotypes in transgenic fish. Over 300 regeneration genes were targeted, and so far, data have been obtained from 120 targeted genomic sites. This screening is ongoing, and regeneration-defective mutants still have not been published. It will be interesting to see what types of new genes are associated with the regeneration response.

2. Conclusions

Inherited retinal degenerative diseases are characterized by photoreceptor death that leads to blindness. The underlying genetic causes of these disorders are numerous and diverse, and most involve photoreceptor-specific genes. Zebrafish are amenable to large-scale genetic manipulation and genome editing technology, which as I have illustrated, can generate a great mutant collection. These mutants are helping to uncover molecular mechanisms underlying retinal degeneration disorders. Currently, there are no effective treatments for these diseases in humans to reduce or impede the progression of degeneration; thus, different approaches have been investigated to develop medical interventions for the patients. Zebrafish exhibit extraordinary neuronal regeneration, including retinal photoreceptors, making them an excellent model to develop regenerative therapies to treat photoreceptor degeneration.

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Conflict of interest

The author has no competing interests.

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