Drug Screening to Treat Early-Onset Eye Diseases: Can Zebrafish Expedite the Discovery?

Liyun Zhang[1,3], Leelyn Chong[1,3], Jin Cho[1], Pin-Chao Liao[1], Feichen Shen[1], Yuk Fai Leung[1,2,4]

[1]Department of Biological Sciences, Purdue University, 915 W. State Street, West Lafayette, IN, 47907, USA

[2]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine Lafayette, 625 Harrison Street, West Lafayette, IN 47907, USA

[3]Contributed Equally

[4]Corresponding author

Running head: Drug screening to improve vision with zebrafish

Key words: eye diseases, zebrafish, traditional Chinese medicines, naturally-derived chemicals, nutrition, drug screening

Disclosures: The authors have no funding or conflicts of interest to declare

Corresponding author:
Yuk Fai Leung
Department of Biological Sciences, Purdue University, LILY 2-236, 915 W. State Street
West Lafayette, IN 47907
Telephone number: 765-496-3153
Fax number: 765-494-0876
Email: yfleung@purdue.edu

Abstract

The molecular basis of many early-onset eye diseases has been uncovered but the number of available drug treatments for improving deteriorated vision is still scarce. Consequently, there is a high demand for new drugs to treat these diseases. This review first provides a brief synopsis of the utility of zebrafish model for screening drugs with vision benefits. In particular, visual-motor response (VMR), the activity response of larvae to a change in light stimuli, is proposed to serve as a simple and efficient tool for screening drugs that may improve vision in various zebrafish visual mutants. The second part of the review discusses the identification of novel drug candidates, with particular emphasis on naturally-derived chemicals including traditional Chinese medicines (TCMs) and nutritional therapies on retinal degenerative diseases. Many of these chemicals have been used in neuroprotection and/or have been consumed by many populations for good health and vision; thus, the screening of these chemicals with various zebrafish visual mutants would expedite the development of novel drugs for treating early-onset eye diseases.

Introduction

Early-onset eye diseases, including photoreceptor dystrophy, retinitis pigmentosa (RP) glaucoma, cataract and corneal dystrophy, generally lead to visual impairment in children. These young patients will have a lifelong inconvenience and an expensive healthcare cost ahead of them. For example, it is estimated that patients with RP consumes $7317 more in annual expenditure than people without RP[1]. Even though surgery can treat some of these diseases such as cataract, there are not many effective treatments available for retinal disorders, as the damaged retina has no ability to regenerate itself once injured. Nonetheless, some retinal degenerative diseases are progressive and the visual loss is a gradual process; thus, this has opened a valuable therapeutic window for intervention. In particular, any drugs or therapies that may slow the progression of disease and preserve the residual vision can potentially improve the quality of life of patients and alleviate the financial burden on the health care system. Unfortunately, the process of drug discovery and development involves a significant amount of time and cost[2]; therefore, it would be particularly helpful to establish new approaches that can identify novel drug candidates in an economical and convenient manner.

Traditionally, high-throughput drug screening involves assay plate preparation and reaction observation in solution and in cultured cells[3,4]. Large libraries of tens to hundreds of thousands of chemicals are applied to the testing system[5]. A number of assays can be done to reveal and determine the effects of a drug on protein sub-cellular localization, protein-protein interaction and signal transduction[6]. Combined with automated microscopy and advanced computational analysis, cell-based screening can rapidly reveal the possible function(s) of novel drug candidates[7,8]. Nonetheless, analyzing drug's effect *in vitro* cannot entirely replace the need

of *in vivo* screening. This is due to a drug's action and metabolism are not only far more complicated in a multicellular organism, but also the efficacy of a drug depends on its capacity to restore and/or improve an impaired function. The latter issue is particularly true for screening drugs to improve vision, which is the output of multiple cell types in the eye in response to light stimuli. Therefore, it is essential to utilize *in vivo* models to screen drugs which can have a therapeutic value in vision improvement. Research that involves traditional laboratory animal models for drug screening is often costly but the zebrafish model can potentially bridge this gap and expedite drug discovery and development[9].

Zebrafish model for rapid drug discovery

There are six major reasons for supporting zebrafish as a good vertebrate model for drug screening, specifically on visual problems: 1) zebrafish's eye, particularly the retina, is anatomically similar to many vertebrates including human[10]. In fact, zebrafish is a diurnal animal and thus possesses richer color vision than the other classical models such as mice and rats. This feature facilitates research that is focused on diseases affecting cones; 2) zebrafish vision develops early during embryogenesis[10] and thus visual problems can be examined rapidly; 3) zebrafish can be easily raised in the laboratory at a large scale in an economical manner[11]; 4) the fecundity of zebrafish is high compared with other animal models and each pair of fish can lay up to 100-200 embryos at a weekly interval. These two reasons allow a large number of embryos to be obtained for drug screening; 5) the embryo size is small and this enables the analysis of individual drug response in a 96-well plate; and 6) various drugs can be simply added to the water and absorbed by the larvae through their skin[12]. These last two reasons indicate that a large number of drugs can be rapidly administered to the zebrafish embryos.

The feasibility of high-throughput drug screening with zebrafish was first demonstrated by a screening of 1100 small chemicals that could affect various aspects of embryogenesis[13], including heart patterning defects[14]. There was also a screening of 5000 small molecules that successfully revealed two suppressors of congenital aortic coarctation in a zebrafish *grl* mutant[15]; demonstrating the possibility of finding novel drugs to treat disease through the use of zebrafish mutants in high-throughput screening.

Visual behavior of zebrafish as a means to study visual impairment and find treatment

The behavior of animal will change when there is a visual impairment. Thus, the characterization of visual behavior is an effective way to assess the presence of visual problem and visual improvement after drug treatment. To identify drugs that can improve vision in zebrafish mutants, it is necessary to identify a robust visual behavior that can be reliably characterized in a high-throughput manner. As a highly visual animal, zebrafish's visual behaviors have been studied and applied frequently for genetic analysis of vision[16,17]. However, many of these behaviors are not suitable for high-throughput drug screening. For example, optokinetic response (OKR), a stereotypic eye movement in response to movement in the environment[18–20], has been used in four genetic studies[21–24] that identified many mutants with visual problems. Nonetheless, OKR comes with a drawback as it requires frequent transfers of larvae to viscous methylcellulose for immobilization. Even though the contemporary implementation of computer-assisted analysis[25] may speed up the analyzing process, it is still not versatile enough for a high-throughput drug screening that requires the examination of different drugs at different concentrations, let alone the analysis of multiple fish lines. Recently, a photo-

motor response (PMR) was used to screen 14000 drugs in 30 hours post-fertilization (hpf) embryos to identify neuroactive drugs[26]. PMR consists of a stereotypic series of motor behaviors elicited by a high-intensity light stimulus. However, the retina of 30 hpf embryos is immature and the first detectable visual response will not occur until at least 68 hpf[27]. Thus, PMR is less likely to be compatible for screening zebrafish mutants that have visual impairments.

A novel visual-behavior assay has recently been utilized in a screen of 4000 drugs for identifying candidates that could alter sleeping behavior of fish larvae[28]. In this assay, zebrafish larvae were placed individually in each well of a 96-well plate, treated with different drugs, and monitored concurrently by ZebraBox (ViewPoint Life Sciences), an automated video-tracking system[29]. The embryos treated with different drugs elicited different locomotor behaviors in the light and dark phases. Remarkably, it was observed that a drastic response could be induced during the transition between light-on and off. This led to the subsequent development of visual-motor response (VMR)[30], a special adaption of the aforementioned assay, which is particularly suitable for high-throughput screening of drugs to improve vision.

Potential application of VMR for high-throughput screening of drugs that can improve vision in larval zebrafish

In the VMR assay, larvae that are at least 5 days post-fertilization (dpf) would be first acclimatized to the machine environment for three hours, before being subjected to 30 minutes of light on and 30 minutes of light off for a total of three trials[31]. Different variations of the assay have been reported since the initial development of VMR[32,33]. While they were all based on the same light-on and off principle, there were differences in the acclimatization time, duration of

the light on and off, intensity of light, length of the whole assay and definition of activity. The VMR of visual mutants has been shown to be different from wild-type (WT) zebrafish[30]. In particular, the VMR of an eyeless *chk* mutant was completely abolished, suggesting that the response was mediated by vision. The VMR in another *nrc* mutant, which had defects in the ON ganglion cells, was attenuated. By testing the VMR in enucleated larvae, Fernandes and colleagues has recently demonstrated that there were two additional photosensitive brain regions that contributed to this response in addition to the eye[32]. These included the pineal gland and a domain in the hypothalamus that is specified by the Orthopedia transcription factor. Intriguingly, this study found that the *chk* mutant elicited a VMR by the light-off stimulus, even though it was substantially lower than the normal siblings. While it was proposed that the difference in the VMR parameters, including the activity definition and the length of the assay, might have contributed to the observed difference, a few interpretations about VMR can be drawn: 1) There is a vision-mediated component and non-vision-mediated component of VMR; and 2) The vision-mediated component mediates a distinctive fast response, including a large-angle turn (O-bend) that is the most prominent in the first two minutes; while the non-vision-mediated components mediate another slower response, including a smaller-angle routine turn (R-turn) that is sustained for at least ten minutes.

The vision-mediated component of the VMR is also supported by an early enucleation experiment that studied the development of larval startle response, defined as an increase in body twitch in two seconds after the interruption of light illumination[27]. This is essentially the early light-off phase of the VMR. Specifically, the enucleated embryos in this study did not show any startle response despite having an intact pineal photoreceptor and brain. It should be noted that

the light illumination in this study was substantially lower (~ 7 μW cm^{-2}) than the ones utilized Zebrabox (69-83[30] and 90[32] μW cm^{-2}), suggesting that the vision-mediated component of the startle response/VMR is more sensitive than the other non-vision-mediated components. Together, these observations indicate that analyzing the immediate part of the VMR and/or using a lower light intensity may maximize the difference in VMR that is mediated by the vision-mediated component.

The usage of this VMR assay for screening drugs to improve vision of visual mutants has several advantages: 1) it measures the improvement in an activity output that is substantially mediated by vision and hence directly shows the potential therapeutic value of a drug candidate; 2) it does not require a prior knowledge of disease mechanism; 3) it alleviates the need to implement and use advance microscopy for screening histological improvement; and 4) it is scalable and can be conducted in a high-throughput manner, which can facilitate testing of multiple drug libraries with numerous visual mutants. Our laboratory is currently using the VMR assay to screen drugs that can potentially improve the altered response in a visual mutant (Figure 1) with photoreceptor degeneration (Figure 2). This mutant has been specifically chosen because it possesses a point mutation that is also found in human patients suffering from early-onset photoreceptor degeneration. While the photoreceptors first develop normally in this mutant, they degenerate by 5 dpf.

Despite its advantages, it should be noted that the VMR assay is not suitable for screening drugs for every type of eye diseases. Since the current format of the VMR assay requires the usage of larvae, it may not be applicable to screening drugs for eye diseases in which

the histological and VMR defects are late-onset. Specifically, zebrafish requires feeding to survive after 9 dpf; thus, the high-throughput application of VMR is not easily amenable after 9 dpf and is the most compatible with drugs that may show a fast response. Even though it is possible to extend the screen beyond 9 dpf in a smaller-scale study by feeding the larvae; one has to consider the confounding factor caused by the possible variation in nutrients of the feed. A standardized diet may help alleviate this problem[34] and may potentially extend the use of VMR to later larval stages. Nonetheless, this does not negate the utility of the zebrafish model for studying late-onset eye diseases and determining the long-term protective effects of the identified drugs in a smaller scale or the possible development of comparable high-throughput visual-behavioral assay for adult fish in the future. Since it was also described above that there are non-vision-mediated components in the VMR, it is essential to further characterize the promising drug candidates by additional methods (see conclusions). Together, these suggest that VMR is very suitable for a large-scale drug screening on visual mutants that manifest a visual defect at an early stage.

Generation of zebrafish models of early-onset eye diseases for drug screening

The establishment of the VMR assay allows for the screening of drugs that can improve vision with visual mutants of zebrafish. A number of these mutants have been generated through ethylnitrosourea (ENU)[11] and retroviral[35] mutagenesis. As discussed above, many mutants have been discovered by OKR and other visual-behavioral screens[21,23,24]. In addition, the same mutagenesis approaches have generated a great number of mutants that have morphological defects in the visual systems[36–38]. Many of these mutants affect early eye development and have detectable defects by 5 dpf; thus, they serve as valuable models for drug screening with the VMR

assay and studying the underlying mechanisms for various eye diseases[39,40]. There is an ongoing effort by the Zebrafish Mutation Project (http://www.sanger.ac.uk/Projects/D_rerio/zmp/) to knockout every gene in the zebrafish genome. The newly generated mutants have been deposited in the Zebrafish International Resource Center (ZIRC; http://zebrafish.org), a public repository of reagents. Therefore, it is anticipated that more visual mutants to become publicly available for translational research in the future. It is believed the first type of mutants that is useful for drug screening would be those that carry mutations in genes that cause human visual impairment and have a similar histological defects and alteration in visual behavior.

In the meantime, there are two excellent approaches for generating targeted gene perturbation if a mutant is not available. The first one is TALEN (transcription activator-like effector (TALE) nuclease)[41] which can be used to generate targeted disruption in the zebrafish genome[42,43] and the second one is Tol2 transgenesis which can be utilized to drive the expression of an exogenous gene by various promoters[44]. The transgenesis is made efficient by the *Tol2* transposon element originally isolated from medaka[45]. Thus, the former approach allows for a rapid targeted disruption of the disease-causing genes identified from human genetics research, while the latter enables the generation of transgenic fish carrying a transgene that contains the same disease-causing mutation as in human and/or the mutated gene isolated from patients. Nonetheless, it should be noticed that the transgenic fish still contain two normal zebrafish alleles in the genome, which may complicate the downstream analysis; hence, this type of mutants requires careful histological confirmation to show clear signs of ocular impairments before their usage in drug screening.

Finding novel drugs, especially from naturally-derived chemicals, for treating eye disorders

As described above, one major advantage of VMR-based drug screening is that it is possible to identify new drugs that can improve vision prior to the dissection of disease mechanism and drug function. Hence, this assay can be applicable to screening the established Western drug/chemical libraries and a plethora of naturally-derived chemicals from Eastern medical literature and nutrition sources. The contemporary high-throughput screening with the large Western drug/chemical libraries has identified a number of drugs that have been approved by the Food and Drug Administration for treating different diseases over the years[5]. It is very likely that new drugs for eye disorders will be discovered from these libraries.

A number of failures of western drugs after years of research[46] have prompted the search of additional new drug leads. Another promising group of chemicals that may benefit the eyes is naturally-derived compounds that have been consumed by many populations for good vision and/or have proven therapeutic values based on Eastern medical literature and nutritional concepts. This category of compounds includes traditional Chinese medicines (TCMs), herbal medicines, and nutritional therapies/supplements. They present as a novel source of chemicals for drug development[47,48]. While many of these naturally-derived chemicals have been characterized due to their ability to act as antioxidants (See Box 1), it should be noted that their therapeutic values reported in the Eastern medical literature involved other possible properties or actions exerted *in vivo.* In particular, many TCMs and nutritional therapies aim at achieving a balance of the living system, so that desired health effects can be reached. Moreover, effective TCMs from the literature are often administered in complex formulations. The characterization and treatment philosophy of this type of chemicals constitute a whole body of knowledge and

challenges[49] that is out of the scope of this review. Nonetheless, VMR-based screening, which does not require prior knowledge of drug action, can potentially be an excellent tool to expedite the characterization of these compounds.

In the following, we will provide a brief review of several promising candidates, which we plan to screen and determine their capability on vision improvement with zebrafish mutants of retinal degeneration.

I. Antioxidants recognized with protective effects on photoreceptors

There are at least three dietary antioxidants that have been investigated and established for their ability to protect photoreceptors from damages including oxidative stress in rat cell culture and whole animal. These include docosahexaenoic acid (DHA), Lutein (LUT) and zeaxanthin (ZEA). DHA is the major polyunsaturated fatty acid in the retina, which makes up 35-60 percent of the photoreceptor outer segment[50]. It has been demonstrated to protect rat retinal photoreceptors from oxidative stress[51] and ceramide-induced apoptosis[52] *in vitro.* This anti-apoptotic effect was shown to be mediated by the ERK/MAPK pathway[53]. Interestingly, DHA not only was found to prevent photoreceptors from undergoing apoptosis but also was shown to enhance their survival during development[54] and promotes differentiation[55–57]. Notably, DHA has been shown to enhance opsin expression and axonal outgrowth without affecting the expression of Crx, a transcription factor that determines the fate of rods and cones[56]. This supports DHA's role in late stage differentiation, which was also shown to be mediated by the ERK/MAPK pathway[53]. Thus, this further suggests that photoreceptors use the same signal transduction pathway for controlling differentiation and apoptosis. For clinical

applications, DHA supplementation on patients with RP receiving vitamin A treatment was reported to slow the disease progression for two years[58], while dietary intake of eicosapentaenoic acid (EPA) and DHA was demonstrated to decrease the likelihood of age-related macular degeneration (AMD)[59].

LUT and ZEA are two major components in the macula that possess antioxidant capability[60,61]. Treatment with LUT, ZEA, and DHA has been indicated to protect rat photoreceptors from apoptosis induced by oxidative stress[61]. Long-term dietary supplementation with ZEA has also been shown to reduce photoreceptor death in a light-damaged Japanese quail model[62]. Furthermore, ZEA and LUT promoted photoreceptor differentiation by increasing opsin expression and promoting development of outer segment[61]. There exists epidemiological evidence of intakes of LUT and ZEA can protect patients from acquiring various eye disorders such as AMD and cataracts[63,64]. It appears that a dietary supplement of LUT/ZEA could decrease the likelihood of AMD[65].

II. Vitamin C and Vitamin E

Vitamin C or ascorbic acid serves as a cofactor in eight important enzymatic reactions, including collagen synthesis, carnitine synthesis, biosynthesis of norepinephrine from dopamine, addition of amide groups to peptide hormones, and modulation of tyrosine metabolism[66]. It also has the ability to perform antioxidant activities against oxidative stress since it possesses the capability to donate its electrons to prevent other compounds from oxidation. Vitamin E is the term for eight lipophilic compounds that consist of four tocopherols and four tocotrienols[67]. It is mainly known for its ability to act as a chain-breaking antioxidant that terminates the

propagation of lipid peroxidation. Vitamin C and E may directly interact with each other under conditions of oxidative stress as studies have indicated that ascorbic acid can either save α-tocopherol from undergoing oxidation[68–70] or allow α-tocopherol to regenerate from its oxidized form of α-tocopheroxyl radical[71]. Due to their antioxidant capabilities, vitamin C and E perhaps can be utilized simultaneously to improve the conditions of photoreceptor death. In fact, in two recent *in vivo* studies, treatment with a mixture of α-tocopherol, ascorbic acid, Mn(III) tetrakis porphyrin (MnTBAP) and α-lipoic acid was found to reduce death of photoreceptors and preserve function of cones in the retinal degeneration 1 (*rd1*) mice[72] and both rods and cones in the retinal degeneration 10 (*rd10*) mice[73]. When a single treatment of α-tocopherol was applied, cone survival was still promoted in the *rd1* mice[72]. Therefore, combining various antioxidants together as a treatment may provide protective advantages for the photoreceptors. Despite these positive results in the laboratory research, it should be noted that there are clinical trials and epidemiological studies that reported different outcomes. These include a positive[74] and no effect[65,75–77] with vitamin A and C supplement on AMD progression and a deleterious effect with vitamin E supplement on RP progression[78]. (See Box 1 for a thorough discussion). These clinical observations indicate that not all antioxidants will necessarily act on all retinal diseases in the same manner.

III. Resveratrol

Resveratrol (RSV) is a compound present abundantly in the Japanese medicinal plant *Polygonum Capsidatum* and grapevines[79]. It is also found in peanuts, pines, and red wines. It possesses antioxidant properties that may offer health benefits, including reducing the risk of cardiovascular disease and eye disorders. For example, treatment of retinal pigment epithelium

(RPE) cell culture by RSV has been shown to protect these cells from H_2O_2-induced cell death[80]. This protection was mediated through the inhibition of MAPK. In the meantime, the same treatment also reduced RPE cell proliferation. In a study of light-induced retinal degeneration in mice, it has been demonstrated that oral-administration of RSV suppressed the deleterious effects on retinal structure and function by light damage[81]. Furthermore, intraocular injection of RSV has been indicated to suppress retinal vascular degeneration caused by ischemia-reperfusion (I/R) injury in mice, while orally-administrated RSV could reduce capillary degeneration induced by endoplasmic reticulum stress[82]. Interestingly, two recent studies have also shown that RSV could inhibit endothelial cell proliferation *in vitro*[83] and pathologic retinal neovascularization in very low-density lipoprotein receptor mutant mice[84]. These findings suggest that RSV played multiple roles on maintaining the health of retinal vasculature.

IV. Schisandrin A & B (Sch A & B)

Sch A & B are two active components of *Fructus Schisandrae*, a fruit that is commonly consumed by the Chinese for sustaining health and vision. In Chinese medicine, the fruit is believed to provide nourishment as well as therapeutic actions. For example, Sch B has been demonstrated to possess antioxidant activity and protect heart cells against oxidative damage in hypoxia/reoxygenation-induced apoptosis[85] and in rats suffered from I/R injury[86]. This protective effect was mediated by the activation of glutathione antioxidant[87] and heat shock[88] responses. The activation of the protective genes in the glutathione antioxidant response has also been determined to be regulated by the ERK/Nrf2 pathway[89]. Despite these good protective effects against oxidative stress, the extent to which these schisandrins inhibit retinal degeneration is

unclear. Our group is currently conducting a preliminary investigation of Sch B on improving the histology and visual behavior of the retinal degeneration mutant as shown in Figures 1 and 2.

V. Lycium

Lycium barbarum is a plant that produces a fruit that is commonly referred to as goji/ gouqi berry or wolfberry. The fruit is harvested for health food and supplement purposes, specifically for good vision. It has been known for its powerful antioxidant properties and potential benefits for cardiovascular system and inflammation. Furthermore, its extract has been extensively characterized to have neuroprotective, neurogenic, and antioxidative effects in both retinal and non-retinal systems[90–95]. In the retinal system, it has been tested that oral administration of *Lycium* promoted the survival of retinal ganglion cells (RGCs), the target cell type that is affected by glaucoma, in an ocular-hypertension rat model[90]. Notably, the RGCs were protected even though the elevated intraocular pressure was not significantly altered. This protective effect on RGCs was mediated by the up-regulation of βB2-crystallin[92]. Pretreatment with *Lycium* before I/R injury has also been demonstrated to protect the retinas from oxidative damage and apoptosis[93].

VI. Flavonoids

Isoliquiritigenin (ISL) is a flavonoid existing in Licorice. It exhibits many desirable properties such as antioxidant, anti-inflammatory, anti-bacterial, anti-viral and anti-tumor activities[96]. It has been shown to suppress neovascularization in experimental ocular angiogenesis models[97] and further proposed to work as a plausible therapy for the wet form of AMD, which exists an exuberant growth of blood vessels. Catechins are flavonoids that present

richly in green tea leaves. They have been confirmed to possess anti-oxidative property in eye research[83,98]. In particular, epigallocatechin gallate (EGCG) is a commonly used catechin in research and there are a number of reports demonstrating its protective role on retina. It produced a protective effect on RGCs after optic nerve axotomy[99], optic nerve crush[100] and intraocular pressure increase[101] when administered via intraperitoneal, intraperitoneal and oral, and oral route respectively. Moreover, orally-administered EGCG has been demonstrated to attenuate photoreceptor damage induced by a light insult and preserve the electrophysiological properties of the retina[102]. A similar protective effect against light-induced damage has also been uncovered in RGCs[101]. Intraperitoneally-administrated EGCG also protected the retina after I/R injury[103,104], which could be mediated through a suppression of nitric oxide synthase expression[104]. Furthermore, EGCG also protected retina[105] and RGCs[103] against oxidative stress induced *in vivo* and *in vitro,* respectively. There is also a report on EGCG's protective effect on Müller cells against catechol-induced toxicity *in vitro*[106]**.** In humans, a short-term oral administration of EGCG for three months has been reported to improve the pattern-evoked electroretinogram (ERG) in open-glaucoma patients[107]. Thus, these studies have established EGCG as a good candidate for retinal protection. In addition to EGCG, other catechin isoforms have been demonstrated to reach a higher concentration in the eye *in vivo*[98]. These isoforms are potentially useful therapeutic agents for further characterizations.

Bioavailability of drugs and therapeutic window in zebrafish

Drug absorption and distribution are two important issues to consider in drug development and screening. Eye drugs are usually delivered by topical or intravenous administration, or intravitreal injection[108]. The first two routes are more amenable in drug

screening and are the likely mechanisms through which the zebrafish larvae absorb the drugs into the eyes. The drugs that are dissolved in water can potentially diffuse through the larval body and get into the blood circulation and/or through the ocular surface as if it is applied topically. In the latter case, they may diffuse into the eye through the permeation of cornea, conjunctiva and sclera, which are exposed to the water continuously.

While it is currently not clear about the bioavailability of drugs in larval eye, which has to be determined case by case, a few investigations have laid down important foundations for its characterization. First, there are studies that have attempted to determine the bioavailability of the drugs in the whole embryo[109,110]. In particular, these studies showed that the hydrophobicity of the drug, as calculated by the logarithm of the octanol:water partition coefficient (LogP), could be a good indicator of the general bioavailability. Specifically, drugs with LogP value > 1 have been proposed to be used in general screen[111] because they were readily absorbed by fish embryos and showed specific response in a screen of drugs that cause bradycardia[110]. The drugs that had a LogP below 1, even though were bioactive, required microinjection to elicit the effect. It can be speculated that these hydrophobicity rules can potentially be applicable to the drugs that are diffusing into the eye directly. Second, drugs that diffuse into the body and get into the general circulation have to pass through blood-retinal barrier (BRB) and/or blood-aqueous barrier (BAB) before they can elicit their effect. Zebrafish have functional BRB[112,113] and blood-brain barrier (BBB)[112–114] that are analogous to the other mammals and begin to function at around 3 dpf; while the BAB has not been characterized in fish yet. It should be noted that a drug that does not show an alteration in VMR can be a false negative caused by a lack of diffusion and/or a problem in getting through the BRB and BAB. If necessary, the specific bioavailability

in the eye can be determined by methods such as liquid chromatography-mass spectroscopy (LC-MS)[109].

With regards to the therapeutic window, it has to be determined case by case as well. For example, the study of retinal degeneration has to take into account of the process of photoreceptor development. Zebrafish photoreceptors begin to differentiate at 48-50 hpf[10,115] and the first visual behavior is detected at 68 hpf[27]. To avoid unnecessary drug exposure during early embryogenesis, treatment starting at around 2 or 3 dpf is a logical choice. Alternatively, treatment can begin at the stage when the first detectable degeneration occurs. For the visual mutant that we are currently characterizing, 5 dpf would be a reasonable choice.

Conclusions and outlook

The VMR assay of zebrafish is potentially a powerful approach for screening drugs that can affect visual behavior. It also has a huge potential in characterizing naturally-derived chemicals, in particular TCMs, for improving vision for various eye disorders. Since it has become feasible to generate targeted knock-out in zebrafish, mutants of human eye diseases can be created for rapid characterization of potential drug therapies. While zebrafish is certainly not easily amenable to the ultra-high throughput screening that involves hundreds of thousands of compounds, it can be envisioned that the zebrafish *in vivo* drug screening could complement with the *in vitro* biochemical and/or cellular-based screening by following up leads that are identified from these other faster screens. In the meantime, a screen with a few thousand chemicals with the zebrafish model is definitely feasible[28].

In addition, the effect of drugs that shows a positive effect in the zebrafish VMR screening can be further characterized at different levels: 1) At the behavioral level, the drug-treated larvae can be tested by OKR; 2) At the physiological level, the drug-treated retinas can be analyzed by ERG[116,117], a measurement of the electrical activity of various retinal cell type under light stimulus, as this can help localize the functional improvement to specific cell type(s); 3) At the cellular level, the drug-treated eyes can be analyzed by various histological and immunohistochemical methods[118–120]; 4) At the pharmacological level, the bioavailability of the drug *in vivo* can be determined by LC-MS[109]; and 5) At the molecular level, the components in the disease-causing gene network that are affected by the drug treatment can potentially be identified by expression studies. Our group has not only developed unique micro-dissection and expression analysis approaches for retinas and RPE[121–123] but also successfully utilized them to analyze a retinal dystrophic mutant[118,119,124]. Together, they have created an efficient pipeline to discover and analyze novel drugs for better vision in zebrafish.

Box 1 – Antioxidative therapies for retinal degenerative diseases

Due to the constant exposure of photoreceptors to light, which can induce free radical formation and a high oxygen-tension environment, oxidative stress/damage has been proposed as one of the theories for photoreceptor death in retinal degeneration[72,125–127]. In fact, hyperoxia has been shown to cause photoreceptor death in the P23H retinal-degeneration rats[128] and normal mice[129]. Additionally, oxidative damage has been reported to be the underlying cause of photoreceptor death in the Pro347Leu-rhodopsin transgenic pigs[130]. This oxidative stress/damage theory has led to the proposal of antioxidative treatment could delay late-stage photoreceptor dystrophy[126]. As described in the review, there have been promising successes in using

antioxidants on improving vision of two models of RP mice[72,73]. Nonetheless, the clinical trials of different types of antioxidants on treating human retinal degeneration have mixed outcomes. For example, a number of long-term clinical trials have indicated that vitamin E supplement did not decrease the incidence and/or slow the progression of AMD[75–77]. One of these studies has also suggested that vitamin C supplement had no effect on AMD incidence[75]. The failure of preventing the progression of AMD by vitamin E and C has also been indicated by an epidemiology study on dietary intake[65]. However, a combination supplement of vitamin C, E, carotene and zinc reduced the development of advanced AMD in another trial[74]. In addition, DHA supplemented with EPA and dietary LUT/ZEA intake have been reported to decrease the likelihood of AMD in two epidemiological studies[59,65]. This type of mixed outcomes has also been illustrated by a classical clinical trial of RP[78], in which vitamin A supplement slowed the progression of disease while vitamin E had the opposite effect. A subsequent trial conducted by the same group of authors on DHA supplement in conjunction with vitamin A initially showed no effect[131]; however, a careful analysis of the subgroups indicated that if the patient had not been taking vitamin A beforehand, addition of DHA slowed the course of disease for two years[58]. All these studies have indicated that not all antioxidants would work for all diseases. The efficiency depends on the types of disease, dosage of the drugs, time of drug administration and the underlying mechanism of the antioxidative property. The latter also suggests that a combinatorial therapy is necessary to achieve some protective effects, an idea that has been supported by the aforementioned animal studies[72,73] and clinical trials[58,59,65,74]. Therefore, it would be critical to test multiple drugs on various disease models in an efficient manner as offered by zebrafish.

Acknowledgements

This study was supported by a Pediatric Ophthalmology Research Grant from the Knights Templar Eye Foundation and a Charles D. Kelman, M.D. Scholar award from the International Retinal Research Foundation to LZ, and awards from Hope for Vision & Showalter Research Trust to YFL. The authors also thank Robert Ko, Calvin Pang, Kwok-Fai So, Raymond Chang, Amy Lo for sharing their thoughts and experience in studying TCMs for good vision; David Prober and Jason Rihel for sharing their experience on using VMR of zebrafish for high-throughput drug screening; and Viewpoint Life Sciences on their technical support.

References

1. Frick KD, Roebuck MC, Feldstein JI, McCarty CA, Grover LL. Health services utilization and cost of retinitis pigmentosa. *Arch. Ophthalmol.* 2012;130(5):629–34.

2. Edwards L. *Principles and practice of pharmaceutical medicine*. 3rd ed. Oxford: Wiley-Blackwell; 2010.

3. An WF, Tolliday N. Cell-based assays for high-throughput screening. *Mol. Biotechnol.* 2010;45(2):180–6.

4. Sundberg S. High-throughput and ultra-high-throughput screening: solution- and cell-based approaches. *Curr. Opin. Biotechnol.* 2000;11(1):47–53.

5. Macarron R, Banks MN, Bojanic D, et al. Impact of high-throughput screening in biomedical research. *Nat. Rev. Drug Discov.* 2011;10(3):188–95.

6. Michelini E, Cevenini L, Mezzanotte L, Coppa A, Roda A. Cell-based assays: fuelling drug discovery. *Anal. Bioanal. Chem.* 2010;398(1):227–38.

7. Perlman ZE, Slack MD, Feng Y, Mitchison TJ, Wu LF, Altschuler SJ. Multidimensional drug profiling by automated microscopy. *Science*. 2004;306(5699):1194–8.

8. Loo L-H, Wu LF, Altschuler SJ. Image-based multivariate profiling of drug responses from single cells. *Nat. Methods*. 2007;4(5):445–53.

9. Lessman CA. The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries. *Birth Defects Res. C. Embryo Today*. 2011;93(3):268–80.

10. Fadool JM, Dowling JE. Zebrafish: a model system for the study of eye genetics. *Prog Retin Eye Res*. 2008;27(1):89–110.

11. Patton EE, Zon LI. The art and design of genetic screens: zebrafish. *Nat Rev Genet*. 2001;2(12):956–966.

12. Rihel J, Schier AF. Behavioral screening for neuroactive drugs in zebrafish. *Dev. Neurobiol.* 2012;72(3):373–85.

13. Peterson RT, Link BA, Dowling JE, Schreiber SL. Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc. Natl. Acad. Sci. U. S. A.* 2000;97(24):12965–9.

14. Peterson RT, Mably JD, Chen J-N, Fishman MC. Convergence of distinct pathways to heart patterning revealed by the small molecule concentramide and the mutation heart-and-soul. *Curr. Biol.* 2001;11(19):1481–1491.

15. Peterson RT, Shaw SY, Peterson TA, et al. Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nat. Biotechnol.* 2004;22(5):595–9.

16. Fleisch VC, Neuhauss SCF. Visual behavior in zebrafish. *Zebrafish*. 2006;3(2):191–201.

17. Orger MB, Gahtan E, Muto A, Page-McCaw P, Smear MC, Baier H. Behavioral screening assays in zebrafish. *Methods Cell Biol.* 2004;77:53–68.

18. Brockerhoff SE. Measuring the optokinetic response of zebrafish larvae. *Nat Protoc*. 2006;1(5):2448–2451.

19. Huang Y-Y, Neuhauss SCF. The optokinetic response in zebrafish and its applications. *Front. Biosci.* 2008;13:1899–916.

20. Easter SS, Nicola GN. The development of eye movements in the zebrafish (Danio rerio). *Dev. Psychobiol.* 1997;31(4):267–76.

21. Brockerhoff SE, Hurley JB, Janssen-Bienhold U, Neuhauss SC, Driever W, Dowling JE. A behavioral screen for isolating zebrafish mutants with visual system defects. *Proc. Natl. Acad. Sci. U. S. A.* 1995;92(23):10545–9.

22. Brockerhoff SE, Hurley JB, Niemi GA, Dowling JE. A new form of inherited red-blindness identified in zebrafish. *J. Neurosci.* 1997;17(11):4236–42.

23. Neuhauss SC, Biehlmaier O, Seeliger MW, et al. Genetic disorders of vision revealed by a behavioral screen of 400 essential loci in zebrafish. *J. Neurosci.* 1999;19(19):8603–15.

24. Muto A, Orger MB, Wehman AM, et al. Forward genetic analysis of visual behavior in zebrafish. *PLoS Genet*. 2005;1(5):e66.

25. Mueller KP, Schnaedelbach ODR, Russig HD, Neuhauss SCF. VisioTracker, an innovative automated approach to oculomotor analysis. *J. Vis. Exp.* 2011;(56).

26. Kokel D, Bryan J, Laggner C, et al. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat. Chem. Biol.* 2010;6(3):231–237.

27. Easter Jr. SS, Nicola GN, Easter Jr. SS. The development of vision in the zebrafish (Danio rerio). *Dev Biol*. 1996;180(2):646–663.

28. Rihel J, Prober DA, Arvanites A, et al. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science*. 2010;327(5963):348–51.

29. Rihel J, Prober DA, Schier AF. Monitoring sleep and arousal in zebrafish. *Methods Cell Biol.* 2010;100:281–94.

30. Emran F, Rihel J, Adolph AR, Wong KY, Kraves S, Dowling JE. OFF ganglion cells cannot drive the optokinetic reflex in zebrafish. *Proc. Natl. Acad. Sci. U. S. A.* 2007;104(48):19126–31.

31. Emran F, Rihel J, Dowling JE. A behavioral assay to measure responsiveness of zebrafish to changes in light intensities. *J. Vis. Exp.* 2008;(20):pii:923.

32. Fernandes AM, Fero K, Arrenberg AB, Bergeron SA, Driever W, Burgess HA. Deep Brain Photoreceptors Control Light-Seeking Behavior in Zebrafish Larvae. *Curr. Biol.* 2012;null(null).

33. Maurer CM, Schönthaler HB, Mueller KP, Neuhauss SCF. Distinct retinal deficits in a zebrafish pyruvate dehydrogenase-deficient mutant. *J. Neurosci.* 2010;30(36):11962–72.

34. Kaushik S, Georga I, Koumoundouros G. Growth and body composition of zebrafish (Danio rerio) larvae fed a compound feed from first feeding onward: toward implications on nutrient requirements. *Zebrafish*. 2011;8(2):87–95.

35. Amsterdam A, Hopkins N. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet.* 2006;22(9):473–8.

36. Malicki J, Neuhauss SC, Schier AF, et al. Mutations affecting development of the zebrafish retina. *Development*. 1996;123:263–73.

37. Fadool JM, Brockerhoff SE, Hyatt GA, Dowling JE. Mutations affecting eye morphology in the developing zebrafish (Danio rerio). *Dev. Genet.* 1997;20(3):288–95.

38. Gross JM, Perkins BD, Amsterdam A, et al. Identification of zebrafish insertional mutants with defects in visual system development and function. *Genetics*. 2005;170(1):245–261.

39. Gross JM, Perkins BD. Zebrafish mutants as models for congenital ocular disorders in humans. *Mol. Reprod. Dev.* 2008;75(3):547–55.

40. Brockerhoff SE, Fadool JM. Genetics of photoreceptor degeneration and regeneration in zebrafish. *Cell. Mol. Life Sci.* 2011;68(4):651–9.

41. Miller JC, Tan S, Qiao G, et al. A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* 2011;29(2):143–8.

42. Sander JD, Cade L, Khayter C, et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat. Biotechnol.* 2011;29(8):697–8.

43. Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. Heritable gene targeting in zebrafish using customized TALENs. *Nat. Biotechnol.* 2011;29(8):699–700.

44. Kwan KM, Fujimoto E, Grabher C, et al. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn*. 2007;236(11):3088–3099.

45. Kawakami K. Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol.* 2007;8 Suppl 1(Suppl 1):S7.

46. Gershell LJ, Atkins JH. A brief history of novel drug discovery technologies. *Nat. Rev. Drug Discov.* 2003;2(4):321–7.

47. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. *Nat. Rev. Drug Discov.* 2005;4(3):206–20.

48. Mishra BB, Tiwari VK. Natural products: an evolving role in future drug discovery. *Eur. J. Med. Chem.* 2011;46(10):4769–807.

49. Gao H, Wang Z, Li Y, Qian Z. Overview of the quality standard research of traditional Chinese medicine. *Front. Med.* 2011;5(2):195–202.

50. Neuringer M, Connor WE. n-3 fatty acids in the brain and retina: evidence for their essentiality. *Nutr. Rev.* 1986;44(9):285–94.

51. Rotstein NP, Politi LE, German OL, Girotti R. Protective effect of docosahexaenoic acid on oxidative stress-induced apoptosis of retina photoreceptors. *Invest. Ophthalmol. Vis. Sci.* 2003;44(5):2252–9.

52. German OL, Miranda GE, Abrahan CE, Rotstein NP. Ceramide is a mediator of apoptosis in retina photoreceptors. *Invest. Ophthalmol. Vis. Sci.* 2006;47(4):1658–68.

53. German OL, Insua MF, Gentili C, Rotstein NP, Politi LE. Docosahexaenoic acid prevents apoptosis of retina photoreceptors by activating the ERK/MAPK pathway. *J. Neurochem.* 2006;98(5):1507–20.

54. Rotstein NP, Aveldaño MI, Barrantes FJ, Politi LE. Docosahexaenoic acid is required for the survival of rat retinal photoreceptors in vitro. *J. Neurochem.* 1996;66(5):1851–9.

55. Politi L, Rotstein N, Carri N. Effects of docosahexaenoic acid on retinal development: Cellular and molecular aspects. *Lipids*. 2001;36(9):927–935.

56. Garelli A, Rotstein NP, Politi LE. Docosahexaenoic acid promotes photoreceptor differentiation without altering Crx expression. *Invest. Ophthalmol. Vis. Sci.* 2006;47(7):3017–3027.

57. Rotstein NP, Politi LE, Aveldaño MI. Docosahexaenoic acid promotes differentiation of developing photoreceptors in culture. *Invest. Ophthalmol. Vis. Sci.* 1998;39(13):2750–8.

58. Berson EL, Rosner B, Sandberg MA, et al. Clinical trial of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment. *Arch. Ophthalmol.* 2004;122(9):1297–305.

59. SanGiovanni JP, Chew EY, Agrón E, et al. The relationship of dietary omega-3 long-chain polyunsaturated fatty acid intake with incident age-related macular degeneration: AREDS report no. 23. *Arch. Ophthalmol.* 2008;126(9):1274–9.

60. Landrum JT, Bone RA. Lutein, zeaxanthin, and the macular pigment. *Arch. Biochem. Biophys.* 2001;385(1):28–40.

61. Chucair AJ, Rotstein NP, Sangiovanni JP, During A, Chew EY, Politi LE. Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: relation with docosahexaenoic acid. *Invest. Ophthalmol. Vis. Sci.* 2007;48(11):5168–77.

62. Thomson LR, Toyoda Y, Delori FC, et al. Long Term Dietary Supplementation with Zeaxanthin Reduces Photoreceptor Death in Light-damaged Japanese Quail. *Exp. Eye Res.* 2002;75(5):529–542.

63. Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annu. Rev. Nutr.* 2003;23:171–201.

64. Carpentier S, Knaus M, Suh M. Associations between lutein, zeaxanthin, and age-related macular degeneration: an overview. *Crit. Rev. Food Sci. Nutr.* 2009;49(4):313–26.

65. SanGiovanni JP, Chew EY, Clemons TE, et al. The relationship of dietary carotenoid and vitamin A, E, and C intake with age-related macular degeneration in a case-control study: AREDS Report No. 22. *Arch. Ophthalmol.* 2007;125(9):1225–32.

66. Padayatty SJ, Katz A, Wang Y, et al. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* 2003;22(1):18–35.

67. Burton GW, Ingold KU. Vitamin E as an in vitro and in vivo antioxidant. *Ann. N. Y. Acad. Sci.* 1989;570:7–22.

68. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. U. S. A.* 1989;86(16):6377–81.

69. Huang J, May JM. Ascorbic acid spares alpha-tocopherol and prevents lipid peroxidation in cultured H4IIE liver cells. *Mol. Cell. Biochem.* 2003;247(1-2):171–6.

70. May JM, Qu ZC, Mendiratta S. Protection and recycling of alpha-tocopherol in human erythrocytes by intracellular ascorbic acid. *Arch. Biochem. Biophys.* 1998;349(2):281–9.

71. Bisby RH, Parker AW. Reaction of ascorbate with the alpha-tocopheroxyl radical in micellar and bilayer membrane systems. *Arch. Biochem. Biophys.* 1995;317(1):170–8.

72. Komeima K, Rogers BS, Lu L, Campochiaro PA. Antioxidants reduce cone cell death in a model of retinitis pigmentosa. *Proc. Natl. Acad. Sci. U. S. A.* 2006;103(30):11300–5.

73. Komeima K, Rogers BS, Campochiaro PA. Antioxidants slow photoreceptor cell death in mouse models of retinitis pigmentosa. *J. Cell. Physiol.* 2007;213(3):809–15.

74. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch. Ophthalmol.* 2001;119(10):1417–36.

75. Christen WG, Glynn RJ, Sesso HD, et al. Vitamins E and C and medical record-confirmed age-related macular degeneration in a randomized trial of male physicians. *Ophthalmology*. 2012;119(8):1642–9.

76. Christen WG, Glynn RJ, Chew EY, Buring JE. Vitamin E and age-related macular degeneration in a randomized trial of women. *Ophthalmology*. 2010;117(6):1163–8.

77. Taylor HR, Tikellis G, Robman LD, McCarty CA, McNeil JJ. Vitamin E supplementation and macular degeneration: randomised controlled trial. *BMJ*. 2002;325(7354):11.

78. Berson EL, Rosner B, Sandberg MA, et al. A Randomized Trial of Vitamin A and Vitamin E Supplementation for Retinitis Pigmentosa. *Arch. Ophthalmol.* 1993;111(6):761–772.

79. Pervaiz S. Resveratrol: from grapevines to mammalian biology. *FASEB J.* 2003;17(14):1975–85.

80. King RE, Kent KD, Bomser JA. Resveratrol reduces oxidation and proliferation of human retinal pigment epithelial cells via extracellular signal-regulated kinase inhibition. *Chem. Biol. Interact.* 2005;151(2):143–9.

81. Kubota S, Kurihara T, Ebinuma M, et al. Resveratrol prevents light-induced retinal degeneration via suppressing activator protein-1 activation. *Am. J. Pathol.* 2010;177(4):1725–31.

82. Li C, Wang L, Huang K, Zheng L. Endoplasmic reticulum stress in retinal vascular degeneration: protective role of resveratrol. *Invest. Ophthalmol. Vis. Sci.* 2012;53(6):3241–9.

83. Cao L, Liu H, Lam DS-C, Yam GH-F, Pang C-P. In vitro screening for angiostatic potential of herbal chemicals. *Invest. Ophthalmol. Vis. Sci.* 2010;51(12):6658–64.

84. Hua J, Guerin KI, Chen J, et al. Resveratrol inhibits pathologic retinal neovascularization in Vldlr(-/-) mice. *Invest. Ophthalmol. Vis. Sci.* 2011;52(5):2809–16.

85. Chiu PY, Luk KF, Leung HY, Ng KM, Ko KM. Schisandrin B stereoisomers protect against hypoxia/reoxygenation-induced apoptosis and inhibit associated changes in Ca2+-induced mitochondrial permeability transition and mitochondrial membrane potential in H9c2 cardiomyocytes. *Life Sci.* 2008;82(21-22):1092–101.

86. Chiu PY, Ko KM. Time-dependent enhancement in mitochondrial glutathione status and ATP generation capacity by schisandrin B treatment decreases the susceptibility of rat hearts to ischemia-reperfusion injury. *Biofactors*. 2003;19(1-2):43–51.

87. Yim TK, Ko KM. Schisandrin B protects against myocardial ischemia-reperfusion injury by enhancing myocardial glutathione antioxidant status. *Mol. Cell. Biochem.* 1999;196(1-2):151–6.

88. Chiu PY, Ko KM. Schisandrin B protects myocardial ischemia-reperfusion injury partly by inducing Hsp25 and Hsp70 expression in rats. *Mol. Cell. Biochem.* 2004;266(1-2):139–44.

89. Chiu PY, Chen N, Leong PK, Leung HY, Ko KM. Schisandrin B elicits a glutathione antioxidant response and protects against apoptosis via the redox-sensitive ERK/Nrf2 pathway in H9c2 cells. *Mol. Cell. Biochem.* 2011;350(1-2):237–50.

90. Chan H-C, Chang RC-C, Koon-Ching Ip A, et al. Neuroprotective effects of Lycium barbarum Lynn on protecting retinal ganglion cells in an ocular hypertension model of glaucoma. *Exp. Neurol.* 2007;203(1):269–73.

91. Yu M-S, Lai CS-W, Ho Y-S, et al. Characterization of the effects of anti-aging medicine Fructus lycii on beta-amyloid peptide neurotoxicity. *Int. J. Mol. Med.* 2007;20(2):261–8.

92. Chiu K, Zhou Y, Yeung S-C, et al. Up-regulation of crystallins is involved in the neuroprotective effect of wolfberry on survival of retinal ganglion cells in rat ocular hypertension model. *J. Cell. Biochem.* 2010;110(2):311–20.

93. Li S-Y, Yang D, Yeung C-M, et al. Lycium barbarum polysaccharides reduce neuronal damage, blood-retinal barrier disruption and oxidative stress in retinal ischemia/reperfusion injury. *PLoS One*. 2011;6(1):e16380.

94. Lau BW-M, Lee JC-D, Li Y, et al. Polysaccharides from wolfberry prevents corticosterone-induced inhibition of sexual behavior and increases neurogenesis. *PLoS One*. 2012;7(4):e33374.

95. Yang D, Li S-Y, Yeung C-M, et al. Lycium barbarum extracts protect the brain from blood-brain barrier disruption and cerebral edema in experimental stroke. *PLoS One*. 2012;7(3):e33596.

96. Yadav VR, Prasad S, Sung B, Aggarwal BB. The role of chalcones in suppression of NF-κB-mediated inflammation and cancer. *Int. Immunopharmacol.* 2011;11(3):295–309.

97. Jhanji V, Liu H, Law K, et al. Isoliquiritigenin from licorice root suppressed neovascularisation in experimental ocular angiogenesis models. *Br. J. Ophthalmol.* 2011;95(9):1309–15.

98. Chu KO, Chan KP, Wang CC, et al. Green tea catechins and their oxidative protection in the rat eye. *J. Agric. Food Chem.* 2010;58(3):1523–34.

99. Peng P-H, Chiou L-F, Chao H-M, et al. Effects of epigallocatechin-3-gallate on rat retinal ganglion cells after optic nerve axotomy. *Exp. Eye Res.* 2010;90(4):528–34.

100. Xie J, Jiang L, Zhang T, Jin Y, Yang D, Chen F. Neuroprotective effects of Epigallocatechin-3-gallate (EGCG) in optic nerve crush model in rats. *Neurosci. Lett.* 2010;479(1):26–30.

101. Zhang B, Rusciano D, Osborne NN. Orally administered epigallocatechin gallate attenuates retinal neuronal death in vivo and light-induced apoptosis in vitro. *Brain Res.* 2008;1198:141–52.

102. Costa BL da SA da, Fawcett R, Li G-Y, Safa R, Osborne NN. Orally administered epigallocatechin gallate attenuates light-induced photoreceptor damage. *Brain Res. Bull.* 2008;76(4):412–23.

103. Zhang B, Safa R, Rusciano D, Osborne NN. Epigallocatechin gallate, an active ingredient from green tea, attenuates damaging influences to the retina caused by ischemia/reperfusion. *Brain Res.* 2007;1159:40–53.

104. Peng P-H, Ko M-L, Chen C-F. Epigallocatechin-3-gallate reduces retinal ischemia/reperfusion injury by attenuating neuronal nitric oxide synthase expression and activity. *Exp. Eye Res.* 2008;86(4):637–46.

105. Zhang B, Osborne NN. Oxidative-induced retinal degeneration is attenuated by epigallocatechin gallate. *Brain Res.* 2006;1124(1):176–87.

106. Mansoor S, Gupta N, Luczy-Bachman G, Limb GA, Kuppermann BD, Kenney MC. Protective effects of memantine and epicatechin on catechol-induced toxicity on Müller cells in vitro. *Toxicology*. 2010;271(3):107–14.

107. Falsini B, Marangoni D, Salgarello T, et al. Effect of epigallocatechin-gallate on inner retinal function in ocular hypertension and glaucoma: a short-term study by pattern electroretinogram. *Graefes Arch. Clin. Exp. Ophthalmol.* 2009;247(9):1223–33.

108. Urtti A. Challenges and obstacles of ocular pharmacokinetics and drug delivery. *Adv. Drug Deliv. Rev.* 2006;58(11):1131–5.

109. Berghmans S, Butler P, Goldsmith P, et al. Zebrafish based assays for the assessment of cardiac, visual and gut function--potential safety screens for early drug discovery. *J. Pharmacol. Toxicol. Methods*. 2008;58(1):59–68.

110. Milan DJ, Peterson TA, Ruskin JN, Peterson RT, MacRae CA. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation*. 2003;107(10):1355–8.

111. Peterson RT, Fishman MC. Discovery and use of small molecules for probing biological processes in zebrafish. *Methods Cell Biol.* 2004;76:569–91.

112. Xie J, Farage E, Sugimoto M, Anand-Apte B. A novel transgenic zebrafish model for blood-brain and blood-retinal barrier development. *BMC Dev. Biol.* 2010;10:76.

113. Watanabe K, Nishimura Y, Nomoto T, et al. In vivo assessment of the permeability of the blood--brain barrier and blood-retinal barrier to fluorescent indoline derivatives in zebrafish. *BMC Neurosci.* 2012;13(1):101.

114. Jeong J-Y, Kwon H-B, Ahn J-C, et al. Functional and developmental analysis of the blood-brain barrier in zebrafish. *Brain Res. Bull.* 2008;75(5):619–28.

115. Hu M, Easter SS. Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. *Dev Biol*. 1999;207(2):309–321.

116. Fleisch VC, Jametti T, Neuhauss SCF. Electroretinogram (ERG) Measurements in Larval Zebrafish. *CSH Protoc.* 2008;2008:pdb.prot4973.

117. Wong KY, Gray J, Hayward CJC, Adolph AR, Dowling JE. Glutamatergic mechanisms in the outer retina of larval zebrafish: analysis of electroretinogram b- and d-waves using a novel preparation. *Zebrafish*. 2004;1(2):121–31.

118. Hensley MR, Emran F, Bonilla S, et al. Cellular Expression of Smarca4 (Brg1)-regulated Genes in Zebrafish Retinas. *BMC Dev Biol*. 2011;11(1):45.

119. Zhang Y, Yang Y, Trujillo C, Zhong W, Leung YF. The Expression of irx7 in the Inner Nuclear Layer of Zebrafish Retina Is Essential for a Proper Retinal Development and Lamination. Linden R, ed. *PLoS One*. 2012;7(4):e36145.

120. Li Z, Ptak D, Zhang LY, Walls EK, Zhong W, Leung YF. Phenylthiourea specifically reduces zebrafish eye size. *PLoS One*. 2012;7(6):e40132.

121. Leung YF, Dowling JE. Gene Expression Profiling of Zebrafish Embryonic Retina. *Zebrafish*. 2005;2(4):269–283.

122. Zhang L, Leung YF. Microdissection of zebrafish embryonic eye tissues. *J. Vis. Exp.* 2010;(40):pii: 2028.

123. Leung YF, Ma P, Dowling JE. Gene expression profiling of zebrafish embryonic retinal pigment epithelium in vivo. *Invest Ophthalmol Vis Sci*. 2007;48(2):881–890.

124. Leung YF, Ma P, Link BA, Dowling JE. Factorial microarray analysis of zebrafish retinal development. *Proc Natl Acad Sci U S A*. 2008;105(35):12909–12914.

125. Punzo C, Xiong W, Cepko CL. Loss of daylight vision in retinal degeneration: are oxidative stress and metabolic dysregulation to blame? *J. Biol. Chem.* 2012;287(3):1642–8.

126. Stone J. Mechanisms of photoreceptor death and survival in mammalian retina. *Prog. Retin. Eye Res.* 1999;18(6):689–735.

127. Travis GH, Sutcliffe JG, Bok D. The retinal degeneration slow (rds) gene product is a photoreceptor disc membrane-associated glycoprotein. *Neuron*. 1991;6(1):61–70.

128. Yu D-Y, Cringle S, Valter K, Walsh N, Lee D, Stone J. Photoreceptor death, trophic factor expression, retinal oxygen status, and photoreceptor function in the P23H rat. *Invest. Ophthalmol. Vis. Sci.* 2004;45(6):2013–9.

129. Yamada H, Yamada E, Ando A, et al. Fibroblast growth factor-2 decreases hyperoxia-induced photoreceptor cell death in mice. *Am. J. Pathol.* 2001;159(3):1113–20.

130. Shen J, Yang X, Dong A, et al. Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa. *J. Cell. Physiol.* 2005;203(3):457–64.

131. Berson EL, Rosner B, Sandberg MA, et al. Further evaluation of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment: subgroup analyses. *Arch. Ophthalmol.* 2004;122(9):1306–14.

Figure Legends

Figure 1. VMR is altered in a zebrafish mutant that suffers from retinal degeneration. The VMR of 24 WT (black trace) and 24 visual mutants (red trace) with photoreceptor degeneration (Figure 2) were recorded at 5 dpf. The activity is defined as the fraction of a second that a larva moves. Each activity trace is an average of the response of 24 larvae over three consecutive trials. In this example, light illumination was turned on (A) or off (B) at 60 s (illustrated by a white and a black bar above the graph, respectively). (A) The light-on stimulus elicited an immediate and intense response in WT; then, the larvae promptly adapted and returned to a baseline activity level. The visual mutant, on the other hand, only showed a minuscule immediate response to the light-on stimulus compared with WT. (B) The light-off stimulus again triggered an instantaneous and strong response that sustained for more than two minutes in WT. The activity gradually diminished and returned to the baseline level by the end of a 30-min light-off phase; and the first 60 s in (A) illustrates this baseline activity level immediately before the change of light stimulus at 60 s. On the contrary, the visual mutant did not respond to the light-off stimulus.

Figure 2. A zebrafish model of retinal degeneration. Immunohistological analysis of the visual mutant in Figure 1 demonstrated that the photoreceptors were degenerating. Top row: WT retinas stained with anti-zpr1 for red/green double cones (left, yellow) and anti-zpr3 for rods (right, yellow). Bottom row: retinas of the visual mutant stained with anti-zpr1 (left, yellow) and anti-zpr3 (right, yellow). The cell nuclei were stained with DAPI (blue). These immunostaining analyses were conducted with 8 dpf embryos with standard procedures[119]. The mutant embryos collected at 5 dpf revealed similar photoreceptor degeneration.

Figures

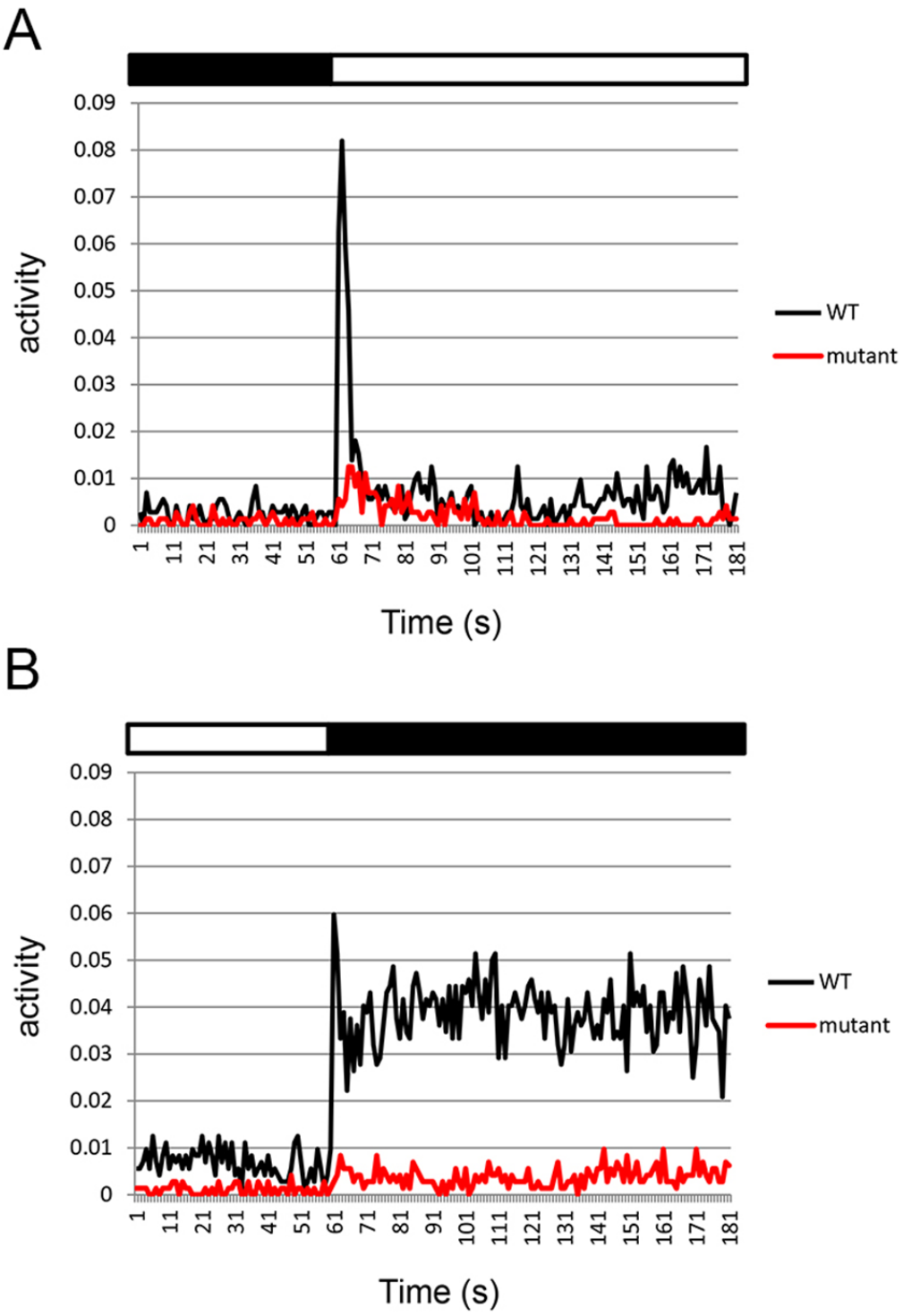

Figure 1

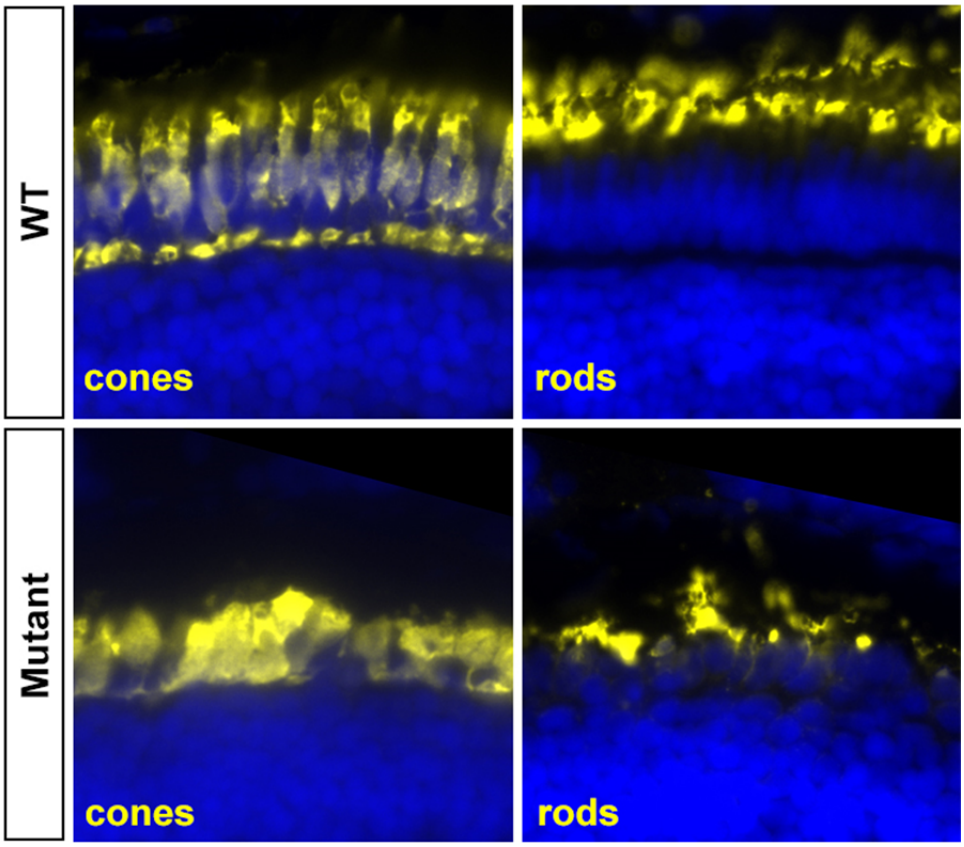

Figure 2