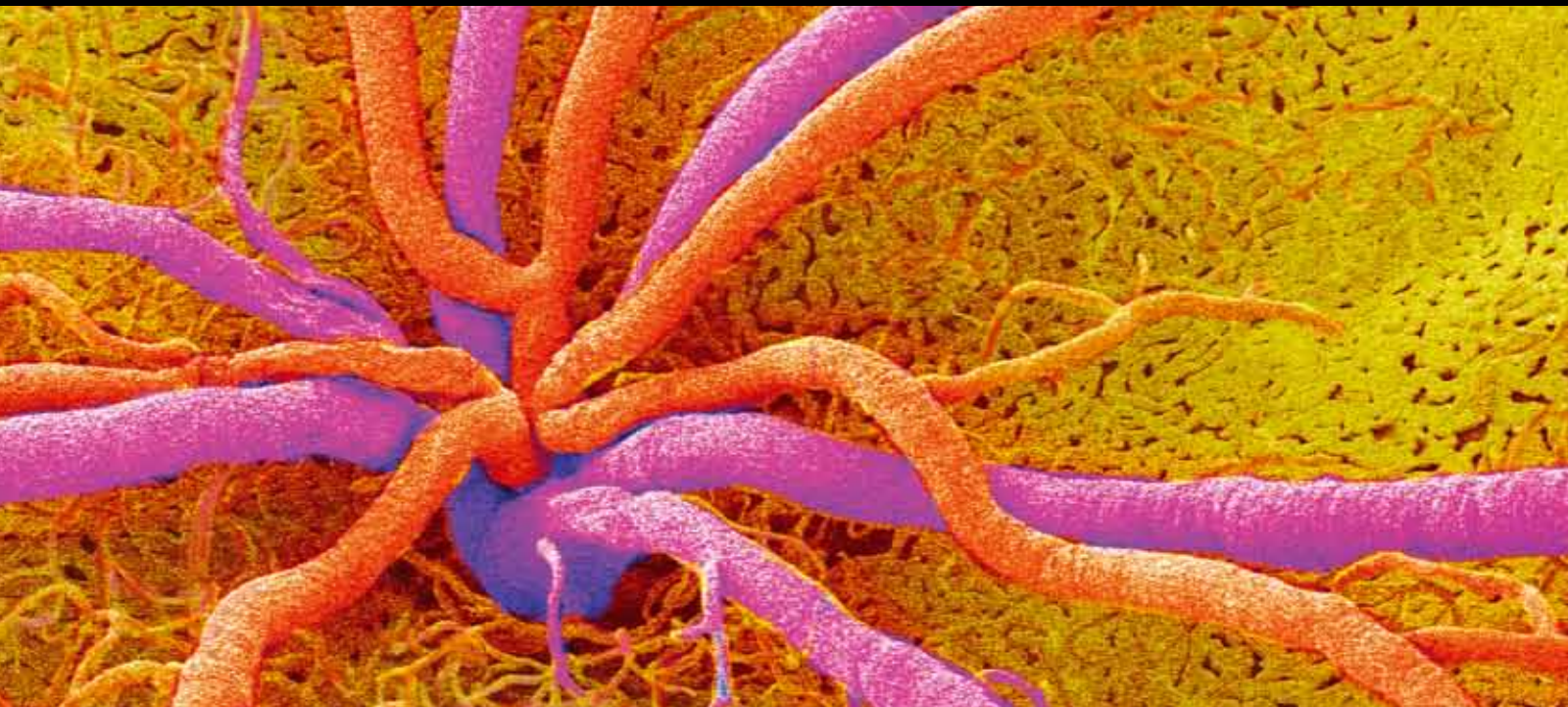# Retinal Degenerations: Genetics, Mechanisms, and Therapies

Guest Editors: Ian M. MacDonald, Muna I. Naash, and Radha Ayyagari

Retinal Degenerations: Genetics, Mechanisms, and Therapies

Retinal Degenerations: Genetics, Mechanisms, and Therapies

Guest Editors: Ian M. MacDonald, Muna I. Naash, and Radha Ayyagari

Editorial Board

Contents

Editorial

Retinal Degenerations: Genetics, Mechanisms, and Therapies

Ian M. MacDonald,[1] Muna I. Naash,[2] and Radha Ayyagari[3]

[1] *Department of Ophthalmology, University of Alberta, Royal Alexandra Hospital,*
 10240 Kingsway Avenue Room 2319, Edmonton, AB, Canada T5H 3V9
[2] *Department of Cell Biology, The University of Oklahoma Health Sciences Center,*
 940 Stanton L Young Boulevard, Oklahoma City, OK 73104, USA
[3] *Shiley Eye Center, University of California San Diego, 9415 Campus Point Drive, Room 206, La Jolla, CA 92093, USA*

Correspondence should be addressed to Radha Ayyagari, rayyagari@ucsd.edu

Received 17 July 2011; Accepted 17 July 2011

The cumulative results from genetic research and treatment protocols tested on cell and animal models have improved our understanding of pathobiology of inherited and degenerative eye diseases, and have lead to the development of a broad range of potential strategies to treat these conditions. In this special issue of the Journal of Ophthalmology, a series of articles provide a review of various areas of current eye research and clinical practice.

A retinal degeneration phenotype is observed in several nonsyndromic and syndromic disorders, which are genetically and phenotypically heterogeneous. Usher syndrome, the commonest cause of deaf-blindness, is characterized by retinal degeneration, hearing loss, and, in some cases, abnormal vestibular function. Usher syndrome has been classified into three forms based on the age of onset, severity of retinal degeneration, and hearing loss. So far, nine genes associated with Usher syndrome have been identified. In this issue, Millán et al. "*An Update on the Genetics of Usher Syndrome*" review the genetics of Usher syndrome, providing a composite picture of the complexity of genetic and phenotypic heterogeneity as associated with retinal degenerations.

As an introduction to basic mechanisms, Ozawa and colleagues "*Regulation of Posttranscriptional Modification as a Possible Therapeutic Approach for Retinal Neuroprotection*" discuss how the ubiquitin-proteosome system is recruited as part of chorioretinal inflammation and diabetic retinopathy. In patients with diabetic retinopathy, clinical electrophysiology will reveal an effect on the oscillatory potentials that can be explained by impaired synaptic function in retinal cells. We learn that two FDA-approved proteosome inhibitors, bortezomib and sorafenib, are now being tested in clinical trials, targeting the ubiquitin-proteosome system, and may have future applications in the treatment of retinal degenerations. More background on mechanisms is provided by Mitsura et al "*Effects of Calcium Ion, Calpains, and Calcium Channel Blockers on Retinitis Pigmentosa*" who discuss the central role that apoptosis plays in the pathways of retinal degeneration. Intracellular calcium increases with apoptosis, activating calpains that in turn trigger caspase-mediated events. They explain how calpain inhibitors and calcium channel antagonists may modulate photoreceptor degeneration and advocate for expanded human trials targeting this pathway.

Several advances have been made in developing therapeutic strategies to treat retinal degenerations, and the success of these approaches to restore vision in patients with retinal degeneration may depend on the functional integrity of retinal ganglion cells. Using animal models, Margolis and Detwiler "*Cellular Origin of Spontaneous Ganglion Cell Spike Activity in Animal Models of Retinitis Pigmentosa*" demonstrate ganglion cell spike discharge occurs in the absence of photoreceptors and explain the cellular origin of this activity. Loss of photoreceptors in retinal degeneration can alter the synaptic activity of ganglion cells and understanding this phenomenon is critical for developing successful therapies for retinal degenerations. An article by Musarella and MacDonald "*Current Concepts in the Treatment of Retinitis*

Pigmentosa" provides a synopsis of the current treatments of retinitis pigmentosa including the use of pharmacologic agents, nutritional therapies, stem cell approaches, artificial retinal implants, neuroprotective agents (CNTF, GDNF, and others), and gene therapy. Some of these approaches are exemplified in papers from this special issue, with more in depth discussion of the treatment of specific monogenic diseases such as Leber hereditary optic neuropathy (LHON) and animal models of human disease. Sullivan et al. "*Variables and Strategies in Development of Therapeutic Post-Transcriptional Gene Silencing Agents*" present an overview on various strategies used in the development of therapies by post-transcriptional gene silencing. These strategies are effective in silencing the mutant target mRNA in dominant hereditary conditions or a normal wildtype mRNA that is over expressed. You and colleagues "*Efficient Transduction of Feline Neural Progenitor Cells for Delivery of Glial Cell Line-Derived Neurotrophic Factor Using a Feline Immunodeficiency Virus-Based Lentiviral Construct*" demonstrate how feline neural progenitor cells can be transduced with a lentiviral construct to deliver the neurotrophic factor, GDNF. These cells might then have a potential application, after intravitreal delivery, in the treatment of retinal degeneration. In the paper by Koilkonda and Guy "*Leber's Hereditary Optic Neuropathy-Gene Therapy From Benchtop to Bedside*", we understand the importance of cell and animal models to test new therapies for mitochondrial disease before introducing them into the clinical setting. The challenges of finding a treatment for a sudden onset, rare mitochondrial disorder, such as LHON, are illustrated by the unsuccessful effect in LHON patients of brimonidine, a conventional agent that has potential neuroprotective effects. Further, we see the wonder and potential of gene therapy for LHON as they introduce the concept of "allotropic" expression of a gene; essentially, introducing the normal gene, which is usually encoded as a mitochondrial gene, into the nucleus and then adding a sequence that would allow the protein to be targeted to its normal site of expression in the mitochondrion. Genes that are expressed in multiple cell types can be involved in causing degeneration in selected cell types. The article by Le "*Conditional Gene Targeting: Dissecting the Cellular Mechanisms of Retinal Degenerations*" describes conditional targeting of genes by a cre-lox-based approach which will enable targeting genes in specific cell types to understand the pathobiology of retinal degeneration observed in selected retinal layers.

Animal models offer an excellent opportunity to study disease pathology and evaluate therapeutic strategies. Among the various animal models, the mouse is a primary model of choice for retinal degeneration studies as the retinal morphology and physiology of these animals is well understood, their life span is shorter and studies on mice can be carried out in a cost-effective manner. In an article by Won et al. "*Mouse Model Resources for Vision Research*" 160 mutant mouse lines with ocular diseases including cataracts, retinal degeneration, and abnormal blood vessel formation have been described in detail. In addition to mouse models, other naturally occurring animal models also contributed significantly to our knowledge of retinal degenerations. In

this issue, Narfström et al. "*The Domestic Cat as a Large Animal Model for Characterization of Disease and Therapeutic Intervention in Hereditary Retinal Blindness*" describe two feline models of human retinal dystrophies due to mutations in the *Cep290* and *Crx* genes. Cats with large eye size and a cone-rich, area centralis may serve as valuable models to study human retinal degeneration and evaluate therapeutic strategies.

Recent advances in retinal degeneration research and the advent of new therapies improve our understanding of these conditions and provide hope for patients and families with retinal degenerations.

Ian M. MacDonald
Muna I. Naash
Radha Ayyagari

Review Article

An Update on the Genetics of Usher Syndrome

**José M. Millán,[1, 2] Elena Aller,[1, 2] Teresa Jaijo,[1, 2] Fiona Blanco-Kelly,[2, 3]
Ascensión Gimenez-Pardo,[2, 3] and Carmen Ayuso[2, 3]**

[1] *Unidad de Genética, Instituto de Investigación Sanitaria-La Fe, 46009 Valencia, Spain*

[2] *Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), 46010 Valencia, Spain*

[3] *Departamento de Genética Médica, Instituto de Investigación Sanitaria, Fundación Jiménez Díaz, Avenida Reyes Católicos 2,
28040 Madrid, Spain*

Correspondence should be addressed to Carmen Ayuso, cayuso@fjd.es

Usher syndrome (USH) is an autosomal recessive disease characterized by hearing loss, retinitis pigmentosa (RP), and, in some
cases, vestibular dysfunction. It is clinically and genetically heterogeneous and is the most common cause underlying deafness
and blindness of genetic origin. Clinically, USH is divided into three types. Usher type I (USH1) is the most severe form and
is characterized by severe to profound congenital deafness, vestibular areflexia, and prepubertal onset of progressive RP. Type II
(USH2) displays moderate to severe hearing loss, absence of vestibular dysfunction, and later onset of retinal degeneration. Type
III (USH3) shows progressive postlingual hearing loss, variable onset of RP, and variable vestibular response. To date, five USH1
genes have been identified: *MYO7A* (USH1B), *CDH23* (USH1D), *PCDH15* (USH1F), *USH1C*(USH1C), and *USH1G*(USH1G).
Three genes are involved in USH2, namely, *USH2A* (USH2A), *GPR98* (USH2C), and *DFNB31* (USH2D). USH3 is rare except in
certain populations, and the gene responsible for this type is *USH3A*.

1. Introduction

Usher syndrome (USH) was first described by von Graefe in
1858 and is characterized by the association of sensorineural
hearing loss, retinitis pigmentosa (RP), and, in some cases,
vestibular dysfunction. Its heritability was established by
Charles Usher, a British ophthalmologist [1]. The syndrome
is inherited in an autosomal recessive pattern. The syndrome
is the most frequent cause of deaf-blindness, accounting for
more than 50% of individuals who are both deaf and blind
[2, 3], about 18% of RP cases [4], and 5% of all cases
of congenital deafness [5]. Its range of prevalence is 3.2–
6.2/100,000 depending on the study [2, 4, 6–8].

Usher patients present progressive photoreceptor degen-
eration in the retina called retinitis pigmentosa, which
leads to a loss of peripheral vision. This degeneration is
predominantly attributable to rod dysfunction, although
cones usually degenerate later in the course of the disease.

Clinical symptoms may vary and include night blind-
ness (nyctalopia) with elevated dark adaptation thresholds,
abnormal electroretinogram responses, visual field constric-
tion, abnormal retinal pigmentation including peripheral
bone spicules, arterial narrowing, and optic-nerve pallor, and
predisposition to myopia and posterior subcapsular cataracts
[9].

The human inner ear consists of the cochlea, a snail-
shaped organ which mediates sound transduction, and
the vestibular labyrinth, which detects gravitational force
and angular and linear accelerations. Both structures have
specialized hair cells which convert mechanical stimuli
into variations of intracellular potential, thus transmitting
afferent nerve signals toward the brain. On the apical surface
of these cells there is a mechanosensitive organelle, the
hair bundle, which consists of precisely organized actin-
filled projections known as stereocilia. In Usher syndrome
patients, alteration in the morphogenesis and stability of

stereocilia results in sensorineural hearing loss and may also cause balance defects [10].

The majority of patients with Usher syndrome usually fall into one of three clinical categories [11]. Of these, Usher syndrome type I (USH1) is the most severe form, consisting of profound hearing loss and vestibular dysfunction from birth. Moreover, onset of RP occurs earlier in USH1 than in Usher syndrome type II (USH2), which produces less severe congenital hearing loss and does not impair normal vestibular function. In most populations, USH1 accounts for approximately one-third of USH patients whereas two-thirds are classified as USH2. Usher syndrome type III (USH3) is a less common form except in such populations as Finns and Ashkenazi Jews. In this USH3 type, hearing loss is progressive and leads to variable vestibular dysfunction and onset of RP. Table 1 outlines the clinical characteristics of each type. Some cases are not easily classifiable under the aforementioned categories and could be categorized as atypical USH syndrome [12].

All subtypes are genetically heterogeneous and 12 loci have been described, namely, *USH1B-H*, *USH2A, C-D*, and *USH3A-B* (hereditary hearing loss homepage: http://hereditaryhearingloss.org). Nine genes have been identified through the discovery of a mouse homolog or by positional cloning. There are five USH1 genes that codify known products: myosin VIIA (*MYO7A*), the two cell-cell adhesion cadherin proteins cadherin-23 (*CDH23*) and protocadherin-15 (*PCDH15*), and the scaffold proteins harmonin (*USH1C*) and SANS (*USH1G*). The three identified USH2 genes are *USH2A*, which codes for the transmembrane protein usherin (*USH2A*); the G-protein-coupled 7-transmembrane receptor VLGR1 (*GPR98*), and whirlin (*DFNB31*), another scaffolding protein. The *USH3A* gene encodes clarin-1, which exhibits 4 transmembrane domains. Mutations in any one of these genes cause primary defects of the sensory cells in the inner ear and the photoreceptor cells of the retina, both being the source of the clinical symptoms of USH.

Many of these genes can also cause either nonsyndromic hearing loss (NSHL) or isolated RP. In fact, *MYO7A* causes DFNB2/DFNA11 [13, 14]; *USH1C* also causes DFNB18 [15, 16]; *CDH23* causes DFNB12 [17, 18]; *PCDH15* causes DFNB23 [19]; mutations in *DFNB31* also lead to DFNB31 [20, 21]. Moreover, some mutations in the *USH2A* gene cause isolated RP [22]. Table 2 shows the genetic classification of Usher syndrome, the implicated loci and responsible genes, as well as the involvement of USH in nonsyndromic hearing loss and RP.

2. Usher Syndrome Type I

2.1. Clinical Features. Usher syndrome type I is the most severe form. USH1 patients suffer from severe to profound congenital and bilateral sensorineural hearing loss. These individuals are either born completely deaf or experience hearing impairment within the first year of life and usually do not develop speech.

Constant vestibular dysfunction is present from birth; children manifest a delay in motor development and begin sitting independently and walking later than usual.

Onset of retinitis pigmentosa occurs during childhood, resulting in a progressively constricted visual field and impaired visual acuity which rapidly proceeds to blindness. Anomalies of light-evoked electrical response of the retina can be detected by electroretinography at 2-3 years of age, which allows for early diagnosis of the disease.

2.2. Genetic Findings. Seven loci (*USH1B–USH1H*) have been mapped and five causative Usher genes have been cloned: *MYO7A, USH1C, CDH23, PCDH15*, and *USH1G*, which are known to be implicated in USH1B, USH1C, USH1D, USH1F, and USH1G, respectively.

Several studies have investigated the *MYO7A* gene, identifying a wide range of mutations (reviewed in [23]). These reports reveal that the myosin VIIA gene bears the main responsibility for Usher type I. Its implication ranges from 29% to ~50% in different populations [24–27]. *CDH23* is probably the second most common mutated gene underlying USH1. Its prevalence accounts for 19%–35% of USH1 families [23, 25, 26, 28]. The next most frequent is *PCDH15*, reportedly involved in about 11%–19% of USH1 cases with and a significant proportion of cases due to large genomic rearrangements [25, 26, 29, 30]. The remaining genes show a minor implication in the disorder, with the *USH1C* gene accounting for 6%-7% [25, 26] and the *USH1G* for 7% as seen in USH1 populations from the United States and the United Kingdom [31]. However, in cohorts of USH1 patients from France and Spain screened for the *USH1G* gene, no pathological mutations have been identified [26, 32]. There are some exceptions to this distribution due to mutation founder effects in specific populations. As an example, the mutation c.216G>A in *USH1C* found in French Canadians of Acadian origin accounts for virtually all USH1 cases in this population [33] but has not been found in other populations; or the c.733C>T (p.R254X) in the *PCDH15* [34] gene, which is present in up to 58% of USH1 families of Ashkenazi origin.

3. Usher Syndrome Type II

3.1. Clinical Features. Firstly, RP symptoms manifest later in USH2 patients than in their USH1 counterparts, for whom onset occurs during or after puberty.

The degree of hearing impairment in patients diagnosed with USH2 increases from moderate in low frequencies to severe in high frequencies, tending to remain stable. Hearing loss is congenital but may be detected at later stages when it hinders communication.

Vestibular function in Usher type II patients is normal.

3.2. Genetic Findings. To date, three loci (*USH2A, USH2C-2D*) have been proposed as being responsible for USH2, and three causative genes have been identified: *USH2A* (USH2A), *GPR98* (USH2C), and *DFNB31* (USH2D).

Mutational screenings performed on the long isoform of the *USH2A* gene exons have shown that *USH2A* is involved in 55%–90% of USH2 cases [35–39]. Of the high number of mutations detected in this huge gene, the c.2299delG mutation is the most prevalent and accounts for 45%–15%

TABLE 1: Clinical features of Usher syndrome types.

	USH1	USH2	USH3
Hearing loss	Severe to profound Congenital Stable	Moderate to severe Congenital Stable	Moderate to severe Progressive
Vestibular function	Altered	Normal	Variable
RP onset	Usually prepubertal	Around pubertyor postpubertal	Around puberty or postpubertal
Language	Unintelligible	Intelligible	Intelligible

TABLE 2: Genetic classification of Usher syndrome.

Locus	Location	Gene/protein	Function
USH1B/DFNB2/DFNA1	11q13.5	MYO7A/myosin VIIA	IE and R: transport
USH1C/DFNB18	11p15.1	USH1C/harmonin	IE and R: scaffolding
USH1D/DFNB12	10q22.1	CDH23/cadherin 23	IE: tip link formation; R: periciliary maintenance
USH1E	21q21	−/−	Unknown
USH1F/DFNB23	10q21.1	PCDH15/protocadherin 15	IE: tip link formation; R: periciliary maintenance
USH1G	17q25.1	USH1G/SANS	IE and R: scaffolding and protein trafficking
USH1H	15q22-23	−/−	Unknown
USH2A/RP	1q41	USH2A/usherin	IE: ankle links formation and cochlear development; R: periciliary maintenance
USH2C	5q14.3	GPR98/VLGR1	IE: ankle links formation Cochlear development; R: periciliary maintenance
USH2D/DFNB31	9q32-34	DFNB31/whirlin	IE: scaffolding and cochlear development; R: scaffolding
USH3A	3q25.1	USH3A/clarin-1	IE and R: probable role in synapsis transport*
USH3B	20q	−/−	Unknown

USH: usher syndrome; DFNB: autosomal recessive deafness; DFNA: autosomal dominant deafness; RP: retinitis pigmentosa; IE: inner ear; R: retina.
*A role in the retinal and inner ear synapses as been proposed for all the USH proteins. This remains to be elucidated.

of all mutated alleles [37, 40]. The c.2299delG mutation appears to be an ancestral mutation of European origin which spread from Europe to other regions of the world during colonization, and it shows a particular distribution decreasing in frequency from Northern to Southern Europe [40]. Again, a founder effect has been identified for the c.4338_4339delCT deletion (p.C1447QfsX29) in the USH2A gene which accounts for 55.6% of the USH2 alleles among Quebec French-Canadians [41].

To date, few mutation screenings have been published on GPR98, although based upon the results available, mutations in GPR98 do not seem to be responsible for a large proportion of USH2 cases, approximately 3%–5.6% [39, 42].

Ebermann et al. found two DFNB31 mutations in a German family suffering from USH2 [21]. Later, in a transnational study, Aller et al. failed to find any pathological mutation in a series of 195 USH patients [43]. DFNB31 mutations appear to be a rare cause of recessive hearing loss and Usher syndrome.

4. Usher Syndrome Type III

4.1. *Clinical Features.* The onset of RP symptoms (nyctalopia, progressive constriction of visual field, and reduction of central visual acuity) is variable though usually occurs by the second decade of life.

Sensorineural hearing loss is postlingual and progressive and can appear between the first and third decade of life. In its initial stages, the degree of hearing impairment is similar to that seen in USH2, with major impairment seen in high frequencies. The progression rate is variable but, in most cases, hearing loss becomes profound. Nevertheless, hearing levels during the first stages of development are good enough to permit well-developed speech. Thus, successive audiometric examinations are needed in USH3 patients in order to obtain an accurate clinical diagnosis. The vestibular responses are also variable, with 50% of cases experiencing impairment.

4.2. *Genetic Findings.* Although the USH3A gene was initially described as being responsible for USH3 cases, recent studies have demonstrated that mutations in USH3A can also produce clinical forms of Usher that are similar to USH1 and USH2 [44, 45]. Usher syndrome type III is the least common clinical type of the syndrome in the general population. However, in some populations like the Finns or the Ashkenazi Jews, the syndrome accounts for over 40% of USH cases due to the mutation founder effect of c.300T>C (p.Y176X; known as the Finn mayor mutation) and c.143T>C (p.N48K), respectively, [46, 47].

The existence of a second locus for this clinical type (USH3B) was suggested by Chaïb et al. in 1997, although these findings have yet to be confirmed in [48].

5. The Usher Interactome

The proteins encoded by the identified USH genes belong to different protein classes. Myosin VIIA (*USH1B*) is an actin-based motor protein; harmonin (*USH1C*), SANS (*USH1G*), and whirlin (*USH2D*) are scaffolding proteins [20, 49, 50]; cadherin 23 (*USH1D*) and protocadherin 15 (*USH1F*) are cell-adhesion molecules [15, 51]; usherin (*USH2A*) and VLGR1 (*USH2C*) are transmembrane proteins with very large extracellular domains [42, 52]. Finally, clarin-1 (*USH3A*) is a protein with four transmembrane domains [53]. All these proteins have one or several protein-protein interaction domains.

USH1 and USH2 proteins are integrated in a protein network known as Usher "interactome."

The central core of the interactome is formed by the PDZ domain containing the homologues harmonin and whirlin and the microtubule-associated protein SANS, with the remaining USH proteins attached to this core (Figure 1).

Many of the USH proteins also interact with other proteins that are present in the inner ear and retina. These additional interacting proteins may cause Usher syndrome, nonsyndromic hearing loss, or retinal dystrophies.

Recently, one of these proteins, the protein encoded by the *PDZD7* gene, has been shown to be involved in the pathogenesis of Usher syndrome. Mutations in *PDZD7* act as negative modifiers of the phenotype [54].

The localization of the Usher proteins in the hair cells of the organ of Corti and in the photoreceptor cells suggests that they play an important role in the neurosensorial function of both the inner ear and the retina.

5.1. The USH Interactome in the Inner Ear.
The main sites of colocalization of Usher proteins are the stereocilia and the synaptic regions of hair cells.

Usher proteins are essential for the correct development and cohesion of the hair bundle of hair cells in the cochlea and vestibular organ (reviewed in [56–58]).

In murine models, hair cells in the developing inner ear, known as stereocilia, maintain their cohesion by interstereocilia fibrous links and links with the kinocilium. There are several types of links depending on the stage of hair-cell development. In the mouse, transient lateral links appear at very early stages of stereocilia formation, but while other links appear at the base of stereocilia (ankle links), these lateral links diminish progressively throughout development. Later, ankle links diminish, and tip and horizontal links appear and are preserved in adulthood [10].

The large extracellular domains of the cell adhesion proteins cadherin-23 and protocadherin-15 and the transmembrane proteins usherin and VLGR1 are part of these links. The proteins are anchored to the intracellular scaffolding proteins harmonin and/or whirlin, which connect, via myosinVIIa and possibly other interactome proteins, to the actin core of the stereocilia [55, 56, 59, 60].

The role of the different proteins in the links probably depends on the spatiotemporal stage of the links. It has been proposed that protocadherin-15 and cadherin-23 in the tip link play an essential role in triggering the mechanotransduction cascade [61]. McGee et al. proposed that usherin and VLGR1 are expressed in the transient ankle links [62].

Usher proteins also take part in the transport of vesicles from the cuticular plate to the growing apical tip of stereocilia [56].

Besides this, the presence of many of these proteins in the synaptic regions of inner and/or outer hair cells suggests that the Usher interactome might play a role in the neurotransmission of the mechanotransduction signal [58, 60, 63].

5.2. The USH Interactome in the Retina.
There is evidence that myosin VIIa plays a role in the transport of opsin from the inner segment to the outer segment of the photoreceptors through the connecting cilium. Such evidence appears in studies in shaker-1, the mouse model defective for myosin VIIA, since shaker-1 accumulates opsin in the ciliary plasma membrane of photoreceptor cells [64, 65].

Further studies have proven that both USH1 and USH2 proteins interact in the ciliary/periciliary region of cone and rod photoreceptors. The proteins usherin, VLGR1b, and SANS are associated with the periciliary ridge complex, which is thought to be the docking side for cargo loaded post-Golgi vesicles [66]. In mammals, this specialized domain extends over the plasma membrane of the proximal part of the calycal process, which is connected via extracellular fibrous links to the plasma membrane of the connecting cilium. In the extracellular space between the membranes of the inner segment and the connecting cilium, the extracellular domains of usherin and VLGR1b may be part of these links, perhaps by means of homomeric, heteromeric, or both interactions together. Furthermore, the short intracellular domains of usherin and VLGR1b anchor to whirlin in the cytoplasm. Finally, whirlin would link to SANS and myosin VIIa, which directly interact with the cytoskeleton microtubules and F-actin filaments [67]. Cadherin-23, vezatin, and maybe other partners of the multiprotein complex that bind myosin VIIa may serve as anchors for this molecular motor at the periciliary membrane (reviewed in [57, 68]). Thus, the Usher protein network should provide mechanical support to the membrane junction between the inner segment and the connecting cilium, participating in the control of vesicle docking and cargo handover in the periciliary ridge.

Usher proteins also localize in the photoreceptor synapse, as they do in the hair cells in the organ of Corti, where they could form a complex involved in the trafficking of the synaptic vesicles [57]. However, some researchers do not support this idea since there are no mouse models with photoreceptor synaptic dysfunction [69].

In the retinal pigment epithelium (RPE) the absence of myosin VIIa causes a significant decrease in phagocytosis of outer segment disks by the pigment epithelial cells [70], suggesting a role for myosin VIIa in the shedding and phagocytosis of the distal outer segment disks by the RPE. A role involving the intracellular transport of melanosomes in the RPE cells has also been proposed for myosin VIIa [56]. The same authors suggested that protocadherin-15, together with cadherin-23 or other cadherins, could ensure
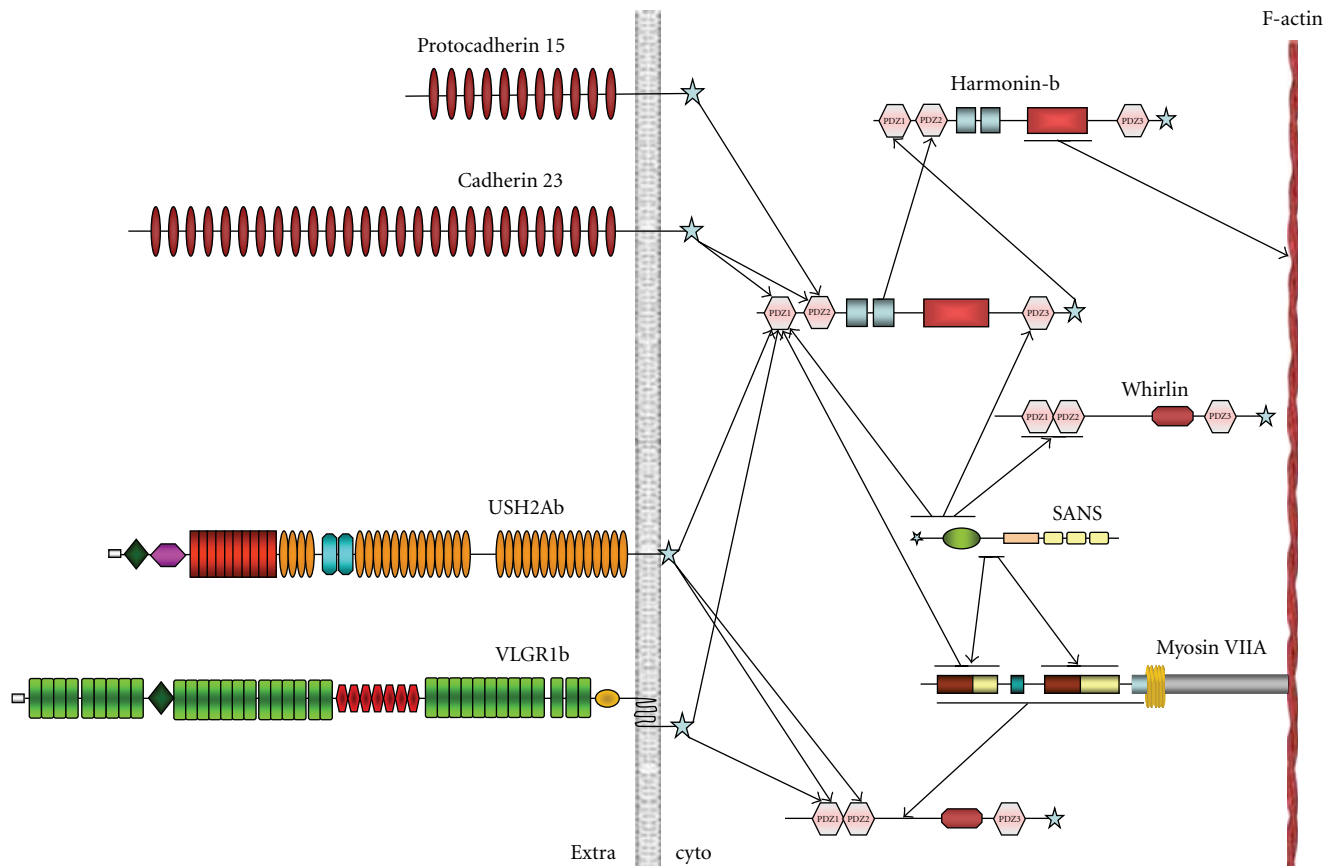
FIGURE 1: Schematic diagram illustrating the deciphered interactions within the USH protein network interactome adapted from van Wijk et al. [55].

proper alignment of outer segment disks of photoreceptors and apical microvilli of RPE cells through interactions with harmonin. However, none of the USH2 proteins have been shown to be present in the RPE.

Most of the USH genes are responsible not only for Usher syndrome but also for nonsyndromic hearing loss. To date, however, only one gene *(USH2A)* is known to be responsible for isolated RP, which suggests that usherin plays a main role for the photoreceptor or that the rest of the Usher proteins are not essential in the photoreceptor function.

6. Conclusion

6.1. Diagnosis. Usher syndrome is a clinically and genetically heterogeneous disorder which is important from a public health viewpoint because of the social isolation which Usher patients must endure. The first step towards correct diagnosis is proper differential diagnosis of the syndrome.

Initially, USH manifests as a sensorineural hearing impairment, sometimes with vestibular dysfunction, with RO onset occurring later in life. Several syndromes may exhibit clinical signs which are similar to USH. Differential diagnosis should take into account the presence of endocrine abnormalities such insulin resistance, type 2 diabetes, hypertriglyceridemia, hepatic dysfunction, and/or renal failure, all of them would indicate Alström syndrome or the presence

of obesity, mental retardation or cognitive impairment, and postaxial polydactly and hypogenitalism, which may be indicators of a Bardet-Biedl syndrome (BBS). If a family history of X-linked inheritance is observed, or if signs of dystonia or ataxia are detected, Mohr-Tranebjaerg syndrome should be suspected.

Genetic tests could be a very powerful tool in differential diagnosis of USH patients. However, there are many factors that make the genetic study of this disease a complicated difficult one. As explained in this paper, the genes identified to date do not explain all the USH cases (this is true for BBS and Alström syndromes as well), and the variable nature of the proteins involved in USH and the complexity of the USH interactome make identifying novel genes a difficult task. This is due to genetic and allelic heterogeneity, which contribute to the low rate of mutation detection, together with the possible presence of large deletions, mutations in noncoding regions, or isoforms in low concentration only present in the affected tissues. Moreover, other complex inheritance forms could modify the phenotype and its expression, as recently shown by Ebermann et al. [54]. All of these factors make the use of traditional techniques for mutation detection difficult.

Application of new technologies based on DNA chips could solve this problem; in fact, the recent creation of a specific microchip for this disease [71, 72] permits the

identification of mutations in 30%–50% of the affected patients and requires only a very small DNA sample, and the technique is both cheap and fast [71–73, 72]. Advances in massive sequencing technologies will certainly change the approaches to molecular diagnosis of Usher syndrome.

Gene characterization and mutation screening will unravel the functional aspects and allow a phenotype-genotype correlation to be established.

6.2. Therapy. Currently, there is no treatment available for Usher syndrome. The hearing-loss problem can be solved by the use of hearing aids and cochlear implantation, but the retinal problem remains unsolved. Therapeutic strategies to treat retinal degeneration target the specific genetic disorder (gene therapy), slowing or stopping photoreceptor degeneration or apoptosis (e.g., growth factors or calcium blocker applications, vitamin supplementation, and endogenous cone viability factors) or even the replacement of lost cells (e.g, transplantation, use of stem or precursor cells) (reviewed in [73]). However, before these strategies can be applied to humans, animal models, pre clinical studies, and appropriately designed human clinical trials are needed to test different treatments and provide information on their safety and efficacy.

Acknowledgments

References

[1] C. H. Usher, "Bowman's lecture: on a few hereditary eye affections," *Transactions of the Ophthalmological Societies of the United Kingdo*, vol. 55, pp. 164–245, 1935.

[2] M. Vernon, "Sociological and psychological factors associated with hearing loss," *Journal of Speech and Hearing Research*, vol. 12, no. 3, pp. 541–563, 1969.

[3] H. M. Fortnum, A. Q. Summerfield, D. H. Marshall, A. C. Davis, and J. M. Bamford, "Prevalence of permanent childhood hearing impairment in the United Kingdom and implications for universal neonatal hearing screening: questionnaire based ascertainment study," *British Medical Journal*, vol. 323, no. 7312, pp. 536–539, 2001.

[4] J. A. Boughman, M. Vernon, and K. A. Shaver, "Usher syndrome: definition and estimate of prevalence from two high-risk populations," *Journal of Chronic Diseases*, vol. 36, no. 8, pp. 595–603, 1983.

[5] M. L. Marazita, L. M. Ploughman, B. Rawlings, E. Remington, K. S. Arnos, and W. E. Nance, "Genetic epidemiological studies of early-onset deafness in the U.S. school-age population," *American Journal of Medical Genetics*, vol. 46, no. 5, pp. 486–491, 1993.

[6] C. I. Hope, "Usher syndrome in the city of Birmingham—prevalence and clinical classification," *British Journal of Ophthalmology*, vol. 81, no. 1, pp. 46–53, 1997.

[7] T. Rosenberg, M. Haim, A. M. Hauch, and A. Parving, "The prevalence of Usher syndrome and other retinal dystrophy-hearing impairment associations," *Clinical Genetics*, vol. 51, no. 5, pp. 314–321, 1997.

[8] C. Espinos, J. M. Millan, M. Beneyto et al., "Epidemiology of Usher syndrome in Valencia and Spain," *Community Genet*, vol. 1, pp. 223–228, 1998.

[9] M. Kalloniatis and E. L. Fletcher, "Retinitis pigmentosa: understanding the clinical presentation, mechanisms and treatment options," *Clinical and Experimental Optometry*, vol. 87, no. 2, pp. 65–80, 2004.

[10] G. I. Frolenkov, I. A. Belyantseva, T. B. Friedman, and A. J. Griffith, "Genetic insights into the morphogenesis of inner ear hair cells," *Nature Reviews Genetics*, vol. 5, no. 7, pp. 489–498, 2004.

[11] S. L. H. Davenport and G. S. Omenn, "The heterogeneity of Usher's syndrome," in *Proceedings of the 5th International Conference of Birth Defects*, 1977, abstract 215.

[12] C. R. Otterstedde, U. Spandau, A. Blankenagel, W. J. Kimberling, and C. Reisser, "A new clinical classification for Usher's syndrome based on a new subtype of Usher's syndrome type I," *Laryngoscope*, vol. 111, no. 1, pp. 84–86, 2001.

[13] X. Z. Liu, J. Walsh, P. Mburu et al., "Mutations in the myosin VIIA gene cause non-syndromic recessive deafness," *Nature Genetics*, vol. 16, no. 2, pp. 188–190, 1997.

[14] X. Z. Liu, J. Walsh, Y. Tamagawa et al., "Autosomal dominant non-syndromic deafness caused by a mutation in the myosin VIIA gene," *Nature Genetics*, vol. 17, no. 3, pp. 268–269, 1997.

[15] Z. M. Ahmed, T. N. Smith, S. Riazuddin et al., "Nonsyndromic recessive deafness DFNB18 and usher syndrome type IC are allelic mutations of USHIC," *Human Genetics*, vol. 110, no. 6, pp. 527–531, 2002.

[16] X. M. Ouyang, X. J. Xia, E. Verpy et al., "Mutations in the alternatively spliced exons of USH1C cause non-syndromic recessive deafness," *Human Genetics*, vol. 111, no. 1, pp. 26–30, 2002.

[17] L. M. Astuto, J. M. Bork, M. D. Weston et al., "CDH23 mutation and phenotype heterogeneity: a profile of 107 diverse families with Usher syndrome and nonsyndromic deafness," *American Journal of Human Genetics*, vol. 71, no. 2, pp. 262–275, 2002.

[18] J. M. Bork, L. M. Peters, S. Riazuddin et al., "Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23," *American Journal of Human Genetics*, vol. 68, no. 1, pp. 26–37, 2001.

[19] Z. M. Ahmed, S. Riazuddin, S. L. Bernstein et al., "Mutations of the protocadherin gene PCDH15 cause usher syndrome type 1F," *American Journal of Human Genetics*, vol. 69, no. 1, pp. 25–34, 2001.

[20] P. Mburu, M. Mustapha, A. Varela et al., "Defects in whirlin, a PDZ domain molecule involved in stereocilia elongation, cause deafness in the whirler mouse and families with DFNB31," *Nature Genetics*, vol. 34, no. 4, pp. 421–428, 2003.

[21] I. Ebermann, H. P. N. Scholl, P. Charbel Issa et al., "A novel gene for Usher syndrome type 2: mutations in the long isoform of whirlin are associated with retinitis pigmentosa and sensorineural hearing loss," *Human Genetics*, vol. 121, no. 2, pp. 203–211, 2007.

[22] C. Rivolta, E. A. Sweklo, E. L. Berson, and T. P. Dryja, "Missense mutation in the USH2A gene: association with

recessive retinitis pigmentosa without hearing loss," *American Journal of Human Genetics*, vol. 66, no. 6, pp. 1975–1978, 2000.

[23] Z. M. Ahmed, S. Riazuddin, S. Riazuddin, and E. R. Wilcox, "The molecular genetics of Usher syndrome," *Clinical Genetics*, vol. 63, no. 6, pp. 431–444, 2003.

[24] A. K. Bharadwaj, J. P. Kasztejna, S. Huq, E. L. Berson, and T. P. Dryja, "Evaluation of the myosin VIIA gene and visual function in patients with usher syndrome type I," *Experimental Eye Research*, vol. 71, no. 2, pp. 173–181, 2000.

[25] X. M. Ouyang, D. Yan, LI. L. Du et al., "Characterization of Usher syndrome type I gene mutations in an Usher syndrome patient population," *Human Genetics*, vol. 116, no. 4, pp. 292–299, 2005.

[26] A. F. Roux, V. Fougère, S. Le Guédard et al., "Survey of the frequency of USH1 gene mutations in a cohort of Usher patients shows the importance of cadherin 23 and protocadherin 15 genes and establishes a detection rate of above 90%," *Journal of Medical Genetics*, vol. 43, no. 9, pp. 763–768, 2006.

[27] T. Jaijo, E. Aller, S. Oltra et al., "Mutation profile of the MYO7A gene in Spanish patients with Usher syndrome type I," *Human Mutation*, vol. 27, no. 3, pp. 290–291, 2006.

[28] A. Oshima, T. Jaijo, E. Aller et al., "Mutation profile of the CDH23 gene in 56 probands with Usher syndrome type I," *Human Mutation*, vol. 29, no. 6, pp. E37–E46, 2008.

[29] S. Le Guédard, V. Faugère, S. Malcolm, M. Claustres, and A. F. Roux, "Large genomic rearrangements within the PCDH15 gene are a significant cause of USH1F syndrome," *Molecular Vision*, vol. 13, pp. 102–107, 2007.

[30] E. Aller, T. Jaijo, G. García-García et al., "Identification of large rearrangements of the PCDH15 gene by combined MLPA and oligonucleotide CGH-array: large duplications can be responsible for Usher syndrome," *Investigative Ophthalmology & Visual Science*, vol. 51, pp. 5480–5485, 2010.

[31] X. M. Ouyang, J. F. Hejtmancik, S. G. Jacobson et al., "USH1C: a rare cause of USH1 in a non-Acadian population and a founder effect of the Acadian allele," *Clinical Genetics*, vol. 63, no. 2, pp. 150–153, 2003.

[32] E. Aller, T. Jaijo, M. Beneyto et al., "Screening of the USH1G gene among Spanish patients with usher syndrome. Lack of mutations and evidence of a minor role in the pathogenesis of the syndrome," *Ophthalmic Genetics*, vol. 28, no. 3, pp. 151–155, 2007.

[33] I. Ebermann, I. Lopez, M. Bitner-Glindzicz, C. Brown, R. K. Koenekoop, and H. J. Bolz, "Deafblindness in French Canadians from Quebec: a predominant founder mutation in the USH1C gene provides the first genetic link with the Acadian population," *Genome Biology*, vol. 8, no. 4, article R47, 2007.

[34] T. Ben-Yosef, S. L. Ness, A. C. Madeo et al., "A mutation of PCDH15 among Ashkenazi Jews with the type 1 Usher syndrome," *The New England Journal of Medicine*, vol. 348, no. 17, pp. 1664–1670, 2003.

[35] E. Aller, T. Jaijo, M. Beneyto et al., "Identification of 14 novel mutations in the long isoform of USH2A in Spanish patients with Usher syndrome type II," *Journal of Medical Genetics*, vol. 43, no. 11, article e55, 2006.

[36] D. Baux, L. Larrieu, C. Blanchet et al., "Molecular and in silico analyses of the full-length isoform of usherin identify new pathogenic alleles in usher type II patients," *Human Mutation*, vol. 28, no. 8, pp. 781–789, 2007.

[37] B. Dreyer, V. Brox, L. Tranebjaerg et al., "Spectrum of USH2A mutations in Scandinavian patients with Usher syndrome type II," *Human Mutation*, vol. 29, no. 3, p. 451, 2008.

[38] T. L. McGee, B. J. Seyedahmadi, M. O. Sweeney, T. P. Dryja, and E. L. Berson, "Novel mutations in the long isoform of the USH2A gene in patients with Usher syndrome type II or non-syndromic retinitis pigmentosa," *Journal of Medical Genetics*, vol. 47, no. 7, pp. 499–506, 2010.

[39] T. Besnard, C. Vaché, and L. Larrieu, "Implication of GPR98 in Usher syndrome type 2," in *International Symposium on Usher Syndrome and Related Diseases*, Valencia, Spain, May 2010.

[40] E. Aller, L. Larrieu, T. Jaijo et al., "The USH2A c.2299delG mutation: dating its common origin in a Southern European population," *European Journal of Human Genetics*, vol. 18, no. 7, pp. 788–793, 2010.

[41] I. Ebermann, R. K. Koenekoop, I. Lopez, L. Bou-Khzam, R. Pigeon, and H. J. Bolz, "An USH2A founder mutation is the major cause of Usher syndrome type 2 in Canadians of French origin and confirms common roots of Quebecois and Acadians," *European Journal of Human Genetics*, vol. 17, no. 1, pp. 80–84, 2009.

[42] M. D. Weston, M. W. J. Luijendijk, K. D. Humphrey, C. Möller, and W. J. Kimberling, "Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II," *American Journal of Human Genetics*, vol. 74, no. 2, pp. 357–366, 2004.

[43] E. Aller, T. Jaijo, E. van Wijk et al., "Sequence variants of the DFNB31 gene among Usher syndrome patients of diverse origin," *Molecular vision*, vol. 16, pp. 495–500, 2010.

[44] R. J. E. Pennings, A. F. Deutman, R. R. Fields, W. J. Kimberling, P. L. M. Huygen, and C. W. R. J. Cremers, "Usher syndrome type III can mimic other types of Usher syndrome," *Annals of Otology, Rhinology and Laryngology*, vol. 112, no. 6, pp. 525–530, 2003.

[45] E. Aller, T. Jaijo, S. Oltra et al., "Mutation screening of USH3 gene (clarin-1) in Spanish patients with Usher syndrome: low prevalence and phenotypic variability," *Clinical Genetics*, vol. 66, no. 6, pp. 525–529, 2004.

[46] T. Joensuu, R. Hämäläinen, B. Yuan et al., "Mutations in a novel gene with transmembrane domains underlie usher syndrome type 3," *American Journal of Human Genetics*, vol. 69, no. 4, pp. 673–684, 2001.

[47] S. L. Ness, T. Ben-Yosef, A. Bar-Lev et al., "Genetic homogeneity and phenotypic variability among Ashkenazi Jews with Usher syndrome type III," *Journal of Medical Genetics*, vol. 40, no. 10, pp. 767–772, 2003.

[48] H. Chaïb, J. Kaplan, S. Gerber et al., "A newly identified locus for Usher syndrome type I, USH1E, maps to chromosome 21q21," *Human Molecular Genetics*, vol. 6, no. 1, pp. 27–31, 1997.

[49] E. Verpy, M. Leibovici, I. Zwaenepoel et al., "A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies Usher syndrome type 1C," *Nature Genetics*, vol. 26, no. 1, pp. 51–55, 2000.

[50] D. Weil, A. El-Amraoui, S. Masmoudi et al., "Usher syndrome type I G (USH1G) is caused by mutations in the gene encoding SANS, a protein that associates with the USH1C protein, harmonin," *Human Molecular Genetics*, vol. 12, no. 5, pp. 463–471, 2003.

[51] H. Bolz, B. Von Brederlow, A. Ramírez et al., "Mutation of CDH23, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D," *Nature Genetics*, vol. 27, no. 1, pp. 108–112, 2001.

[52] E. Van Wijk, R. J. E. Pennings, H. Te Brinke et al., "Identification of 51 novel exons of the Usher syndrome type 2a (USH2A) gene that encode multiple conserved functional

domains and that are mutated in patients with Usher syndrome type II," *American Journal of Human Genetics*, vol. 74, no. 4, pp. 738–744, 2004.

[53] A. Adato, S. Vreugde, T. Joensuu et al., "USH3A transcripts encode clarin-1, a four-transmembrane-domain protein with a possible role in sensory synapses," *European Journal of Human Genetics*, vol. 10, no. 6, pp. 339–350, 2002.

[54] I. Ebermann, J. B. Phillips, M. C. Liebau et al., "PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome," *Journal of Clinical Investigation*, vol. 120, no. 6, pp. 1812–1823, 2010.

[55] E. van Wijk, B. van der Zwaag, T. Peters et al., "The DFNB31 gene product whirlin connects to the Usher protein network in the cochlea and retina by direct association with USH2A and VLGR1," *Human Molecular Genetics*, vol. 15, no. 5, pp. 751–765, 2006.

[56] A. El-Amraoui and C. Petit, "Usher I syndrome: unravelling the mechanisms that underlie the cohesion of the growing hair bundle in inner ear sensory cells," *Journal of Cell Science*, vol. 118, no. 20, pp. 4593–4603, 2005.

[57] H. Kremer, E. van Wijk, T. Märker, U. Wolfrum, and R. Roepman, "Usher syndrome: molecular links of pathogenesis, proteins and pathways," *Human Molecular Genetics*, vol. 15, no. 2, pp. R262–R270, 2006.

[58] J. Reiners, K. Nagel-Wolfrum, K. Jürgens, T. Märker, and U. Wolfrum, "Molecular basis of human Usher syndrome: deciphering the meshes of the Usher protein network provides insights into the pathomechanisms of the Usher disease," *Experimental Eye Research*, vol. 83, no. 1, pp. 97–119, 2006.

[59] B. Boëda, A. El-Amraoui, A. Bahloul et al., "Myosin VIIa, harmonin and cadherin 23, three Usher I gene products that cooperate to shape the sensory hair cell bundle," *The EMBO Journal*, vol. 21, no. 24, pp. 6689–6699, 2002.

[60] J. Reiners, E. van Wijk, T. Märker et al., "Scaffold protein harmonin (USH1C) provides molecular links between Usher syndrome type 1 and type 2," *Human Molecular Genetics*, vol. 14, no. 24, pp. 3933–3943, 2005.

[61] P. Kazmierczak, H. Sakaguchi, J. Tokita et al., "Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells," *Nature*, vol. 449, no. 7158, pp. 87–91, 2007.

[62] J. McGee, R. J. Goodyear, D. R. McMillan et al., "The very large G-protein-coupled receptor VLGR1: a component of the ankle link complex required for the normal development of auditory hair bundles," *Journal of Neuroscience*, vol. 26, no. 24, pp. 6543–6553, 2006.

[63] A. Adato, G. Lefèvre, B. Delprat et al., "Usherin, the defective protein in Usher syndrome type IIA, is likely to be a component of interstereocilia ankle links in the inner ear sensory cells," *Human Molecular Genetics*, vol. 14, no. 24, pp. 3921–3932, 2005.

[64] X. Liu, I. P. Udovichenko, S. D. M. Brown, K. P. Steel, and D. S. Williams, "Myosin VIIa participates in opsin transport through the photoreceptor cilium," *Journal of Neuroscience*, vol. 19, no. 15, pp. 6267–6274, 1999.

[65] D. S. Williams, "Transport to the photoreceptor outer segment by myosin VIIa and kinesin II," *Vision Research*, vol. 42, no. 4, pp. 455–462, 2002.

[66] D. S. Papermaster, "The birth and death of photoreceptors: the Friedenwald lecture," *Investigative Ophthalmology & Visual Science*, vol. 43, no. 5, pp. 1300–1309, 2002.

[67] T. Maerker, E. van Wijk, N. Overlack et al., "A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells," *Human Molecular Genetics*, vol. 17, no. 1, pp. 71–86, 2008.

[68] R. Roepman and U. Wolfrum, "Subcellular proteomics," in *Protein Networks and Complexes in Photoreceptor Cilia*, E. Bertrand and M. Faupel, Eds., chapter 10, pp. 209–235, Springer, New York, NY, USA, 2007.

[69] X. Liu, O. V. Bulgakov, K. N. Darrow et al., "Usherin is required for maintenance of retinal photoreceptors and normal development of cochlear hair cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 11, pp. 4413–4418, 2007.

[70] D. Gibbs, J. Kitamoto, and D. S. Williams, "Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the usher syndrome 1B protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 11, pp. 6481–6486, 2003.

[71] F. P. M. Cremers, W. J. Kimberling, M. Külm et al., "Development of a genotyping microarray for Usher syndrome," *Journal of Medical Genetics*, vol. 44, no. 2, pp. 153–160, 2007.

[72] T. Jaijo, E. Aller, G. García-García et al., "Microarray-based mutation analysis of 183 Spanish families with Usher syndrome," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 3, pp. 1311–1317, 2010.

[73] C. Ayuso and J. M. Millan, "Retinitis pigmentosa and allied conditions today: a paradigm of translational research," *Genome Medicine*, vol. 2, article 34, 2010.

Review Article

Regulation of Posttranscriptional Modification as a Possible Therapeutic Approach for Retinal Neuroprotection

Yoko Ozawa,[1, 2, 3] Toshihide Kurihara,[1, 2] Kazuo Tsubota,[2] and Hideyuki Okano[3, 4]

[1] *Laboratory of Retinal Cell Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[2] *Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[3] *Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[4] *Solution Oriented Research for Science and Technology (SORST), Japan Science and Technology Corporation (JST),*
  4-1-8 Honcho, Kawaguchi, Saitaima 332-0012, Japan

Correspondence should be addressed to Yoko Ozawa, yoko-o@sc.itc.keio.ac.jp

Understanding pathogenesis at the molecular level is the first step toward developing new therapeutic approaches. Here, we review the molecular mechanisms of visual dysfunction in two common diseases, innate chorioretinal inflammation and diabetic retinopathy, and the role of the ubiquitin-proteasome system (UPS) in both processes. In innate chorioretinal inflammation, interleukin-6 family ligands induce STAT3 activation in photoreceptors, which causes UPS-mediated excessive degradation of the visual substance, rhodopsin. In diabetic retinopathy, angiotensin II type 1 receptor (AT1R) signaling activates ERK in the inner layers of the retina, causing UPS-mediated excessive degradation of the synaptic vesicle protein, synaptophysin. This latter effect may decrease synaptic activity, in turn adversely affecting neuronal survival. Both mechanisms involve increased UPS activity and the subsequent excessive degradation of a protein required for visual function. Finally, we review the therapeutic potential of regulating the UPS to protect tissue function, citing examples from clinical applications in other medical fields.

1. Introduction

Recent progress in molecular biology has revealed the molecular basis in the pathogenesis of various diseases. Molecular targeting therapies have been developed, primarily in the field of vascular biology. One such therapy is antivascular endothelial growth factor (anti-VEGF) therapy, which is now widely used to treat age-related macular degeneration (AMD) and cancer. Its role in treating AMD is to regulate ocular vascular lesions and prevent secondary damage to the neural retinal cells, which are critical for visual function.

The first research into VEGF was reported in the 1970s [1], and in 2004 the FDA approved the first anti-VEGF drug for clinical use in human eyes [2]. Basic research on neurotrophic regulation also began in the 1970s [3], but clinical trials started only recently [4]. Molecular-targeting therapies for retinal neuroprotection are on the horizon, and further studies are needed to understand the molecular

mechanisms in retinal diseases and to explore new treatment approaches.

In the treatment of retinal diseases, developing neuroprotective therapies for neural retinal cells deserves special emphasis; these cells have a very limited regenerative capacity and are critical to vision. The neural retinal cells derive from the monolayer of the neural tube during embryogenesis and are part of the central nervous system. Damage to these cells occurs in common diseases such as chorioretinal inflammation and diabetic retinopathy, as well as in less-common conditions, like retinitis pigmentosa, a hereditary retinal degeneration with mutated genes in the retinal cells. Severe chorioretinal inflammation acutely disturbs visual function [5]. Diabetes chronically affects it, even in the absence of obvious microangiopathy [6–8]: patients experience a gradual loss of visual function even when diabetic neovascularization is well regulated by vitreous surgery and/or anti-VEGF therapy. In AMD, local retinal inflammation is involved in the process of vision loss;
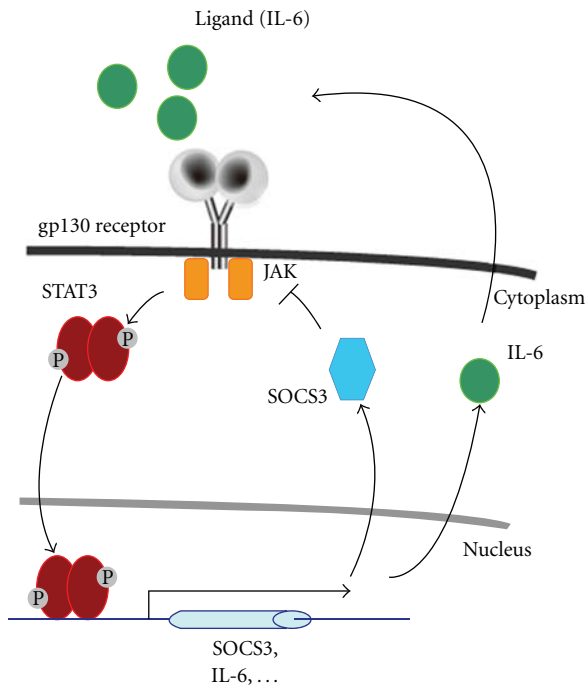
FIGURE 1: *Model of the gp130-STAT3-SOCS3 pathway*. IL-6 family ligands activate the gp130 receptor, which subsequently phosphorylates and activates STAT3 through JAK. Activated and dimerized STAT3 translocates into the nucleus to promote the transcription of various molecules, including SOCS3 and IL-6. SOCS3 inhibits JAK and STAT3 activation. IL-6 is secreted and further activates STAT3.

association of inflammatory molecules is reported in both early and late stage AMD [9]. Inflammatory cytokines can play a role in most of these changes.

However, the investigation of the molecular mechanisms of retinal neuropathogenesis is in its early stages. Here, we describe the molecular mechanism of neurodegeneration that we recently reported in animal models of innate chorioretinal inflammation (endotoxin-induced uveitis) and diabetic retinopathy, and compare our findings with studies from other fields to obtain additional clues to the pathogenesis of retinal diseases.

2. Retinal Neuronal Changes in Innate Chorioretinal Inflammation

Inflammatory cytokines such as interleukin-6 (IL-6) are closely connected to retinal diseases. Clinical reports show that IL-6 in the vitreous fluid increases not only in uveitis [10] but also in diabetic retinopathy [11, 12], retinal vein occlusion [13], and retinal detachment [14].

2.1. IL-6 Family Ligands and STAT3/SOCS3 Pathway in the Retina. Research with experimental animals has shown that diffusible factors, IL-6 and other proteins in the IL-6 family, such as leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), are expressed in the retina. Both IL-6 [15] and LIF [16] are found in Müller glial

cells, and CNTF is found in the retinal ganglion cells and astrocytes around the vessels [17]. These endogenous IL-6 family proteins are upregulated during inflammation and function to promote pathogenesis of the vascular system [18].

IL-6 family proteins use cytokine-specific receptors to activate a transmembrane receptor, gp130 [19], which then recruits Janus kinase (JAK) to activate transcription factor signal transducer and activator of transcription 3 (STAT3). STAT3 then regulates various molecules at the transcriptional level, including suppressor of cytokine signaling 3 (SOCS3). SOCS3 acts as a negative feedback modulator of STAT3 by inhibiting JAK and subsequent STAT3 activation [20] (Figure 1). In the retina, SOCS3 is expressed in the photoreceptor cells, Müller glial cells, and retinal ganglion cells, and it inhibits STAT3 activation in these cells [21, 22]. Since STAT3 activation induces further STAT3-activating factors, such as the IL-6 family ligands [23], the balance between STAT3 activation and SOCS3 level is one of the key determinants of an inflammatory reaction [23, 24].

2.2. STAT3/SOCS3 Pathway in the Developing Retina. This balance between STAT3 and SOCS3 also plays an important role during the development of the retina; activated STAT3 inhibits the photoreceptor-specific transcription factor crx at the transcriptional level, which in turn inhibits downstream photoreceptor-specific markers such as rhodopsin [25]. Retina-specific conditional knockout mice of *SOCS3*, α-Cre SOCS3 flox/flox mice (SOCS3CKO), induce increase in the endogenous STAT3 activation, and show delay in the initiation of rhodopsin expression at the transcriptional level [22]. STAT3 is activated in the embryonic retina but is shut down by SOCS3 which appears in the neonatal retina, thereby allowing rod photoreceptor cell differentiation. Therefore, the timing of rod photoreceptor cell differentiation is fine-tuned by the initiation of SOCS3 expression and downregulation of STAT3 activation. Although STAT3 activation in the SOCS3CKO retina is still upregulated in the adulthood, the rhodopsin level in the SOCS3CKO mice is compensated for by as-yet-unknown mechanisms and matches that of wild-type mice.

2.3. STAT3/SOCS3 Pathway in the Adult Retina with Inflammation. A murine model of accelerated innate immunity, the endotoxin-induced uveitis model (EIU model), was used to further analyze the role of the gp130-STAT3-SOCS3 loop in adult retinal inflammation. In this model, uveitis is induced by administering lipopolysaccharide (LPS). Inflammatory cytokine, IL-6 is upregulated [18], leading to STAT3 activation [21, 26, 27] in the retina. This induction does not cause retinal cellular apoptosis, but it does reduce visual function [21, 26, 27]: rhodopsin protein levels decrease, and the rod photoreceptor outer segments (OSs), where rhodopsin is concentrated, are shortened. The scotopic electroretinogram (ERG) a-wave amplitude, which represents rod photoreceptor cell function, also decreases. STAT3 activation correlates with the rhodopsin reduction in the adult retina, as in the developing retina. Surprisingly, though,

(a)

(b)

(c)

(d)
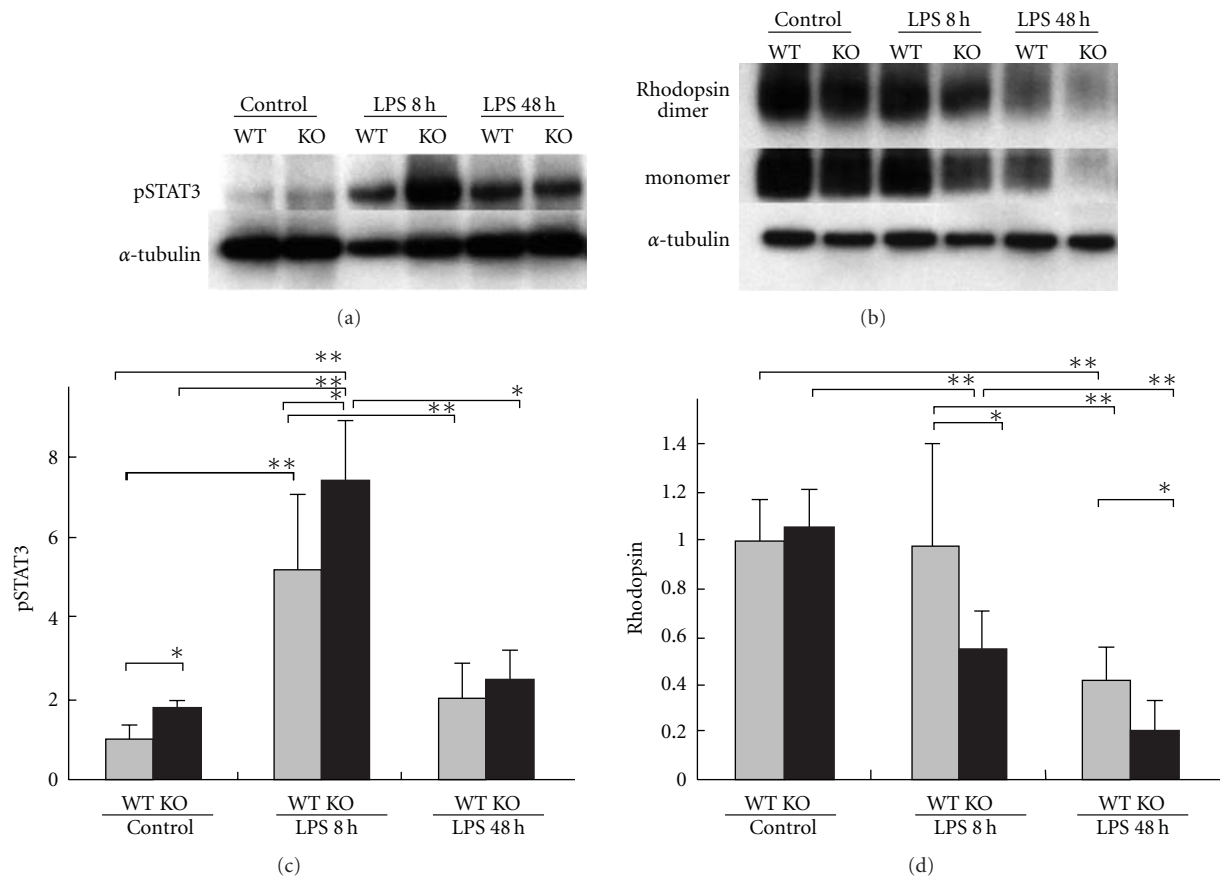
FIGURE 2: *STAT3 activation and rhodopsin protein levels in the retina of EIU model generated in SOCS3CKO mice.* Immunoblot analyses. In the SOCS3CKO retina, phosphorylated and activated STAT3 are upregulated in the control condition, and more greatly increased in the EIU model induced by LPS (a, c). Although the rhodopsin protein level in the adult SOCS3CKO retina is almost the same as wild-type retina under control condition, its level was more severely reduced in the EIU model induced by LPS (b, d). WT: wild-type; KO, SOCS3CKO; LPS: lipopolysaccharide; pSTAT3: phosphorylated STAT3; EIU: endotoxin-induced uveitis. This research was originally published in *J Biol Chem*. Ozawa Y, et al. Roles of STAT3/SOCS3 Pathway in Regulating the Visual Function and Ubiquitin-Proteasome-dependent Degradation of Rhodopsin during Retinal Inflammation. *J Biol Chem*. 2008; 283(36):24561–24570. the American Society for Biochemistry and Molecular Biology.

neither rhodopsin mRNA nor its upstream regulator, crx, decreases in the adult retina during inflammation. This suggests that a different mechanism is involved in rhodopsin and crx regulation in the adult retina than in the developing retina [22, 25].

The role of activated STAT3 in retinal dysfunction during inflammation has been analyzed using SOCS3CKO mice [21]. In these *SOCS3*-deficient mice, STAT3 activation can increase greatly in the retina (Figures 2(a) and 2(c)). Thus, we have hypothesized that the mechanism of rhodopsin reduction during inflammation might be enhanced in these cells.

As expected, the EIU models generated in the adult SOCS3CKO mice showed a relative depletion of rhodopsin protein (Figures 2(b) and 2(d)), followed by OS shortening. The subsequent rod photoreceptor cell dysfunction, as measured by scotopic ERG, was prolonged. This model also revealed that during inflammation, rhodopsin reduction is not regulated at the transcriptional level, but by a post-transcriptional inhibitory mechanism. The reduction in

rhodopsin protein levels is rapid and global, starting only several hours from the onset of inflammation.

2.4. STAT3-Induced Rhodopsin Degradation through UPS. Under stress conditions, massive protein degradation through the ubiquitin-proteasome system (UPS) is known to increase [28]. A genetically abnormal rhodopsin protein that causes autosomal dominant retinitis pigmentosa, P23H, interacts with the UPS and forms aggresomes when over-expressed in a cell line [29, 30]. Aggresomes are inclusion bodies of accumulated waste proteins, formed when cellular degradation machinery is impaired or overwhelmed, and they are a pathologic finding in neurodegenerative diseases. In the case of P23H, the rhodopsin protein folds abnormally and accumulates rather than following the normal process of elimination from the cell. This finding hinted that genetically normal rhodopsin protein might also be degraded extensively following the excessive induction of the UPS by inflammation. Moreover, ubiquitin is present in the rod OS

<Innate inflammation>

IL-6 family ligands

↓

gp130 receptor

↓

STAT3 activation

↓

UPS (via UBR1?)

↓

Rhodopsin degradation

↓

"Visual dysfunction"

FIGURE 3: *Model of the molecular mechanism in retinal inflammation.* IL-6 family ligands induce STAT3 activation, which promotes excessive UPS-mediated rhodopsin protein degradation and subsequent visual dysfunction.

FIGURE 4: *Impairment of visual function and the protective effect of ARB in diabetic mice.* OPs in ERG from diabetic or nondiabetic mice. Amplitude and implicit time of OPs are impaired in the diabetic mice, but these changes were avoided by administrating ARB (Telmisartan). ERG: electroretinogram; Ops: oscillatory potentials; ARB: Angiotensin II type 1 receptor blocker.
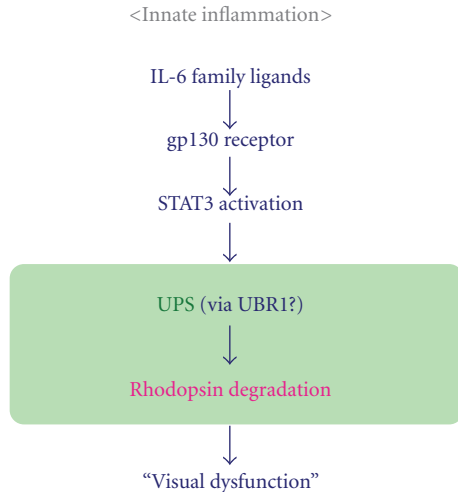
under control conditions [31], thus it can rapidly degrade rhodopsin as needed. This hypothesis has been clearly verified *in vivo* and *in vitro* [21]. Elevated levels of ubiquitin-conjugated rhodopsin are followed by rhodopsin depletion in the SOCS3CKO EIU mouse model. The same process occurs in wild-type mice, but it is more rapid and more severe in the SOCS3CKO mice, in which STAT3 activation is increased. Therefore, the activated STAT3 level correlates with the ubiquitination and degradation of rhodopsin.

This has been confirmed *in vitro* by using JAK inhibitor to inhibit IL-6-induced STAT3 activation. It is illustrated by the preservation of rhodopsin levels under IL-6-induced STAT3 activation, when a STAT3-dependent ubiquitin E3 ligase, ubiquitin-protein ligase E3 component n-recognin 1 (UBR1), is inhibited through the small inhibitory RNA (siRNA) system. UBR1 is expressed in the OSs. We propose that it contributes to rhodopsin protein degradation during inflammation, especially given that inflammatory cytokines, including IL-6, LIF, and CNTF, induce the ubiquitin-conjugation of rhodopsin protein and UBR1 expression in the rod photoreceptor cells, resulting in excessive rhodopsin degradation and disturbed visual functioning (Figure 3).

2.5. Other Molecules Related to STAT3 Activation during Inflammation. This process lasts as long as STAT3 is activated. STAT3 can be activated not only through the gp130 receptor, but also through an inflammatory diffusible factor, angiotensin II. An angiotensin II type 1 receptor blocker (ARB) suppresses STAT3 activation [26] during inflammation directly, or indirectly, inhibiting IL-6 production, thereby preserving rhodopsin levels and visual function. Angiotensin II also induces oxidative stress, which can induce the ubiquitination of specific proteins [32]. The antioxidant lutein, which suppresses oxidative stress and the induction of reactive oxygen species (ROS) during inflammation, also

reduces STAT3 activation, thus preserving the rhodopsin level and visual function during inflammation [27].

Therefore, the mechanism of visual dysfunction is, at least in part, explained by the excessive degradation of the essential protein during inflammation.

3. Retinal Neuronal Changes in Diabetes

In diabetic retinopathy, the main findings include microangiopathy [33, 34] and neurodegeneration [35, 36]. Visual dysfunction begins before vascular abnormalities become obvious [6–8], and inner retinal dysfunction is reflected in changes in ERG oscillatory potentials (OPs) (Figure 4). However, little is known about the molecular mechanism of diabetes-related neuronal degeneration. Our recent analyses using a streptozotocin- (STZ-) induced type 1 diabetes model shed light on this critical issue [35, 36].

3.1. Angiotensin II and Its Type1 Receptor Signaling. Both diabetes and hypertension are involved in metabolic syndrome, in which angiotensin II signaling plays an important role. ARBs have been approved for treatment of not only high blood pressure, but also diabetes-related renal failure [37]. Angiotensin II is converted from angiotensinogen in a stepwise fashion by enzymes, including renin, angiotensin converting enzyme, and others. Angiotensin II can bind to either the angiotensin II type 1 receptor (AT1R) or type 2 receptor (AT2R) on the cell surface, which in turn activates several contextually-dependent intracellular signals. These components of the rennin angiotensin system (RAS) are all

present physiologically in the retina, and are upregulated in pathological conditions, as we have shown in the murine model retina of STZ-induced diabetes [35].

AT1R is coexpressed with the major synaptic vesicle protein synaptophysin in the inner layers of the retina [26]. This is consistent with several previous reports showing the synaptic expression of AT1R in the brain [38, 39]. Synaptophysin, a synapse marker, is reduced in the postmortem brains of patients who had had neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [40]. Given that the OPs in ERG originate from inner retinal neurons bearing AT1R, these ERG changes may represent angiotensin II-induced synaptophysin dysregulation and the resulting damage to visual function.

We have verified this hypothesis by administering ARB (either telmisartan or valsartan) to STZ-induced diabetic mice [35]. ARBs protect the expression of synaptophysin protein and OPs in the diabetic retina (Figure 4). Interestingly, synaptophysin mRNA is not reduced in the diabetic retina, indicating that the protein's reduction is regulated post-transcriptionally.

3.2. AT1R-Mediated Synaptophysin Degradation through UPS. Post-transcriptional synaptophysin reduction caused by angiotensin II exposure was reproduced in a rat neuronal cell line, PC12 [35]. In this system, synaptophysin protein degradation is inhibited by the proteasome inhibitor MG132, but not the lysosome inhibitor E64. AT1R and its downstream extracellular signal-related protein kinase (ERK) activation induce synaptophysin degradation, and AT1R increases the ubiquitin-conjugated synaptophysin protein levels. Angiotensin II signaling activates ERK in the diabetic retina *in vivo*, suggesting that the AT1R-ERK pathway is responsible for diabetes-induced pathogenic protein degradation through the UPS.

Synaptophysin protein may be degraded by the mammalian homolog of *Drosophila* seven in absentia (sina), an E3-ligase selective for synaptophysin named seven in absentia homologue (Siah). Since *Drosophila* sina is regulated by ERK signaling [41], the Siah may also be regulated by ERK activation, which is increased in the diabetic retina. ERK activation and the resulting reduction of synaptophysin in the diabetic retina is also inhibited by an antioxidant, lutein [36], which indicates that angiotensin II signaling and oxidative stress may share a role in the pathogenesis of diabetic retinopathy.

3.3. Influence of Synaptophysin Depletion. Not only that the reduction of synaptophysin, a synaptic protein, impairs the transmission of neuronal and visual signals, but impairment of synaptic activity itself inhibits neuronal cell survival [34, 35]. Synaptic activity, that is, the neuronal electric stimuli, directly increases the levels of intracellular calcium ion in neurons, which promotes cell survival. Moreover, brain-derived neurotrophic factor, BDNF, a neuronal survival factor, is regulated by neuronal synaptic activity [37, 38]. Taken together, these findings suggest that the synaptophysin levels and the related neuronal synaptic activity function
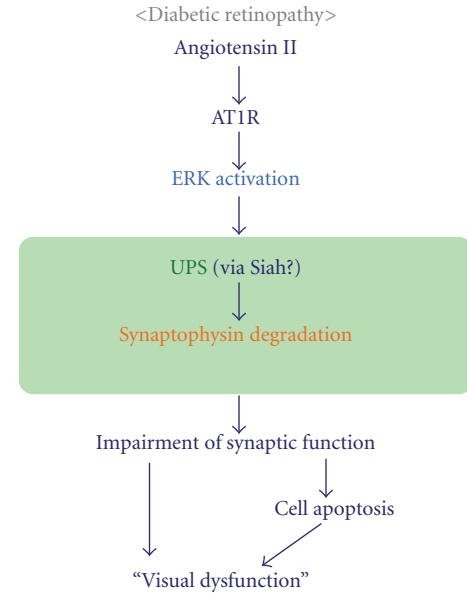
FIGURE 5: *Model of the molecular mechanism in diabetic retinopathy.* Angiotensin II binds to AT1R and induces ERK activation, resulting in excessive UPS-mediated synaptophysin protein degradation. This impairs synaptic activity, which is critical for visual function and neuronal cell survival.

together to influence neuronal survival and neuronal network activity. The reduction of synaptophysin levels and neuronal activity, observed 1 month after the onset of diabetic retinopathy, is later followed by the apoptosis of retinal ganglion cells and inner retinal cells [36]. Therefore, one part of the neurodegenerative mechanism in the diabetic retina is explained by the excessive degradation of a protein that is essential for visual function (Figure 5).

4. Dysregulation of UPS in Pathogenesis

The UPS is a rapid and effective method of degrading specific proteins, and in many cases a protein is degraded only in response to a particular cellular signal or event [42]. Ubiquitin molecules are attached to targeted proteins and variably elongated. This process involves the coordinated actions of three enzymes—a generally distributed E1 ubiquitin-activating enzyme, several more specific E2 ubiquitin-conjugating enzymes, and highly specific E3 ubiquitin ligases for the targeted protein.

4.1. Excessive Degradation of Proteins Essential for Tissue Function. The UPS involvement in pathogenesis has led to interest in targeting proteasomes as a therapeutic approach in several fields. UPS is involved in cardiomyocyte cell pathogenesis: oxidized and ubiquitinated proteins are observed in rat hearts after cardiac ischemia/reperfusion injury [43, 44]. This may indicate the excessive degradation of proteins that are needed in muscle contraction. Muscle wasting due to UPS activation is also reported in cases of chronic kidney disease, diabetes, high angiotensin II levels, and sepsis, all of which

cause inflammation, inhibit insulin signaling, and promote glucocorticoid expression to induce protein degradation [45–47]. This pathway can be blocked by overexpressing IGF-1, which inhibits atrogin-1, an E3-ligase acting for muscle atrophy, through PI3K/AKT [46, 48].

In the retina, innate inflammation activates the IL-6-STAT3 pathway, and diabetes activates the angiotensin II-ERK pathway. Both pathways induce the UPS, most likely through inducing a specific E3-ligase. Moreover, both pathogenic conditions induce oxidative stress [27, 36], which oxidizes and unfolds proteins, after which they are easily ubiquitinated and pushed into the UPS pathway.

4.2. Insufficient Degradation of Proteins and Tissue Dysfunction. In contrast, modification of the 20S proteasome subunits by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE), which occurs in cardiac ischemia/reperfusion, results in the selective inactivation of 20S activity [49]. Thus, modified and ubiquitinated proteins may accumulate to induce cell death in some pathological conditions. The UPS degrades numerous proteins, including apoptotic proteins, and regulates multiple signaling pathways. In human-dilated cardiomyopathy, the increased expression of the proapoptosis regulator p53 has recently been associated with UPS dysregulation and accumulation of polyubiquitinated proteins [50].

4.3. Dysregulation of UPS and Tissue Dysfunction. Therefore, the above findings show that the UPS, a selective and bulk protein degradation system, may be modified through multiple pathways. This system excessively degrades proteins necessary for tissue-specific function and/or cell survival, causing tissue pathogenesis. However, if this system is overwhelmed and/or dysregulated, modified proteins can accumulate and damage the cells. In inflammation, post-transcriptional molecular regulation involves several pathways that induce tissue dysfunction.

5. Potential Treatments of Diseases through UPS Regulation

Protein degradation damages tissue function, while it may also protect tissue from pathogenic protein accumulations. Since the UPS acts on specific proteins, regulating it may improve the prognosis. Bortezomib, a dipeptide boronic acid, is the first FDA-approved proteasome inhibitor for the treatment of multiple myeloma [51]. Bortezomib directly induces cell-cycle arrest and apoptosis, and it targets the tumor microenvironment. Combination chemotherapy regimens using Bortezomib have been developed that provide high rates of long-lasting remissions. Interestingly, in patients treated with Bortezomib, proteasome inhibition improves myocardial ischemia/reperfusion injuries, prevents postischemic ventricular tachyarrhythmias, promotes cardiac hypertrophy regression, and reverses diabetes-induced vascular endothelial dysfunction [52, 53]. Proteasome inhibition can be also applied locally. In a balloon injury model of the rat carotid artery, a locally administered proteasome

inhibitor, MG132 [54] or lactacystin [55], significantly reduces atherosclerotic changes. In addition to Bortezomib, another proteasome inhibitor, Sorafenib has been also approved by FDA for advanced cancer therapy, and another candidate reagent is now under trial. In view of the potential therapeutic benefit of UPS regulation, its application to retinal diseases deserves further study.

6. Conclusions

Inflammatory retinal diseases, including diabetic retinopathy, induce inflammatory cytokines that influence protein metabolism. UPS-mediated protein degradation is a significant source of tissue dysfunction. Excessive degradation of tissue function essential proteins is an important factor in retinal neuronal dysfunction. Further analyses of the mechanisms that impair visual function may lead us to new therapeutic approaches for retinal neuroprotection.

Acknowledgments

References

[1] J. Folkman, E. Merler, C. Abernathy, and G. Williams, "Isolation of a tumor factor responsible or angiogenesis," *Journal of Experimental Medicine*, vol. 133, no. 2, pp. 275–288, 1971.

[2] E. S. Gragoudas, A. P. Adamis, E. T. Cunningham Jr., M. Feinsod, and D. R. Guyer, "Pegaptanib for neovascular age-related macular degeneration," *The New England Journal of Medicine*, vol. 351, no. 27, pp. 2805–2816, 2004.

[3] D. B. Drachman and D. M. Johnston, "Neurotrophic regulation of dynamic properties of skeletal muscle: effects of botulinum toxin and denervation," *Journal of Physiology*, vol. 252, no. 3, pp. 657–667, 1975.

[4] P. A. Sieving, R. C. Caruso, W. Tao et al., "Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 10, pp. 3896–3901, 2006.

[5] H. Ikeda, A. Franchi, G. Turner, J. Shilling, and E. Graham, "Electroretinography and electro-oculography to localize abnormalities in early-stage inflammatory eye disease," *Documenta Ophthalmologica*, vol. 73, no. 4, pp. 387–394, 1989.

[6] J. Kizawa, S. Machida, T. Kobayashi, Y. Gotoh, and D. Kurosaka, "Changes of oscillatory potentials and photopic negative response in patients with early diabetic retinopathy," *Japanese Journal of Ophthalmology*, vol. 50, no. 4, pp. 367–373, 2006.

[7] Y. Shirao and K. Kawasaki, "Electrical responses from diabetic retina," *Progress in Retinal and Eye Research*, vol. 17, no. 1, pp. 59–76, 1998.

[8] D. Yonemura, T. Aoki, and K. Tsuzuki, "Electroretinogram in diabetic retinopathy," *Archives of Ophthalmology*, vol. 68, pp. 19–24, 1962.

[9] X. Ding, M. Patel, and C.-C. Chan, "Molecular pathology of age-related macular degeneration," *Progress in Retinal and Eye Research*, vol. 28, no. 1, pp. 1–18, 2009.

[10] J. H. De Boer, M. A. C. Van Haren, W. A. E. J. De Vries-Knoppert et al., "Analysis of IL-6 levels in human vitreous fluid obtained from uveitis patients, patients with proliferative intraocular disorders and eye bank eyes," *Current Eye Research*, vol. 11, supplement, pp. 181–186, 1992.

[11] A. M. A. El Asrar, D. Maimone, P. H. Morse, S. Gregory, and A. T. Reder, "Cytokines in the vitreous of patients with proliferative diabetic retinopathy," *American Journal of Ophthalmology*, vol. 114, no. 6, pp. 731–736, 1992.

[12] H. Funatsu, H. Yamashita, T. Ikeda, T. Mimura, S. Eguchi, and S. Hori, "Vitreous levels of interleukin-6 and vascular endothelial growth factor are related to diabetic macular edema," *Ophthalmology*, vol. 110, no. 9, pp. 1690–1696, 2003.

[13] H. Noma, H. Funatsu, M. Yamasaki et al., "Pathogenesis of macular edema with branch retinal vein occlusion and intraocular levels of vascular endothelial growth factor and interleukin-6," *American Journal of Ophthalmology*, vol. 140, no. 2, pp. 256–261, 2005.

[14] B. Kenarova, L. Voinov, C. Apostolov, R. Vladimirova, and A. Misheva, "Levels of some cytokines in subretinal fluid in proliferative vitreoretinopathy and rhegmatogenous retinal detachment," *European Journal of Ophthalmology*, vol. 7, no. 1, pp. 64–67, 1997.

[15] M. Nakatani, T. Seki, Y. Shinohara et al., "Pituitary adenylate cyclase-activating peptide (PACAP) stimulates production of interleukin-6 in rat Müller cells," *Peptides*, vol. 27, no. 7, pp. 1871–1876, 2006.

[16] C. Neophytou, A. B. Vernallis, A. Smith, and M. C. Raff, "Muller-cell-derived leukaemia inhibitory factor arrests rod photoreceptor differentiation at a postmitotic pre-rod stage of development," *Development*, vol. 124, no. 12, pp. 2345–2354, 1997.

[17] A. Müller, T. G. Hauk, M. Leibinger, R. Marienfeld, and D. Fischer, "Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF," *Molecular and Cellular Neuroscience*, vol. 41, no. 2, pp. 233–246, 2009.

[18] N. Nagai, Y. Oike, K. Noda et al., "Suppression of ocular inflammation in endotoxin-induced uveitis by blocking the angiotensin II type 1 receptor," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 8, pp. 2925–2931, 2005.

[19] X. Wang, P. Lupardus, S. L. LaPorte, and K. C. Garcia, "Structural biology of shared cytokine receptors," *Annual Review of Immunology*, vol. 27, pp. 29–60, 2009.

[20] A. Yoshimura, "Regulation of cytokine signaling by the SOCS and Spred family proteins," *Keio Journal of Medicine*, vol. 58, no. 2, pp. 73–83, 2009.

[21] Y. Ozawa, K. Nakao, T. Kurihara et al., "Roles of STAT3/SOCS3 pathway in regulating the visual function and ubiquitin-proteasome-dependent degradation of rhodopsin during retinal inflammation," *Journal of Biological Chemistry*, vol. 283, no. 36, pp. 24561–24570, 2008.

[22] Y. Ozawa, K. Nakao, T. Shimazaki et al., "SOCS3 is required to temporally fine-tune photoreceptor cell differentiation," *Developmental Biology*, vol. 303, no. 2, pp. 591–600, 2007.

[23] H. Ogura, M. Murakami, Y. Okuyama et al., "Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction," *Immunity*, vol. 29, no. 4, pp. 628–636, 2008.

[24] B. J. Baker, L. N. Akhtar, and E. N. Benveniste, "SOCS1 and SOCS3 in the control of CNS immunity," *Trends in Immunology*, vol. 30, no. 8, pp. 392–400, 2009.

[25] Y. Ozawa, K. Nakao, T. Shimazaki et al., "Downregulation of STAT3 activation is required for presumptive rod photoreceptor cells to differentiate in the postnatal retina," *Molecular and Cellular Neuroscience*, vol. 26, no. 2, pp. 258–270, 2004.

[26] T. Kurihara, Y. Ozawa, K. Shinoda et al., "Neuroprotective effects of angiotensin II type 1 receptor (AT1R) blocker, telmisartan, via modulating AT1R and AT2R signaling in retinal inflammation," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 12, pp. 5545–5552, 2006.

[27] M. Sasaki, Y. Ozawa, T. Kurihara et al., "Neuroprotective effect of an antioxidant, lutein, during retinal inflammation," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 3, pp. 1433–1439, 2009.

[28] O. A. J. Adegoke, N. Bédard, H. P. Roest, and S. S. Wing, "Ubiquitin-conjugating enzyme E214k/HR6B is dispensable for increased protein catabolism in muscle of fasted mice," *American Journal of Physiology*, vol. 283, no. 3, pp. E482–E489, 2002.

[29] M. E. Illing, R. S. Rajan, N. F. Bence, and R. R. Kopito, "A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system," *Journal of Biological Chemistry*, vol. 277, no. 37, pp. 34150–34160, 2002.

[30] R. S. Saliba, P. M. G. Munro, P. J. Luthert, and M. E. Cheetham, "The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation," *Journal of Cell Science*, vol. 115, no. 14, pp. 2907–2918, 2002.

[31] C. E. Remé, U. Wolfrum, C. Imsand, F. Hafezi, and T. P. Williams, "Photoreceptor autophagy: effects of light history on number and opsin content of degradative vacuoles," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 10, pp. 2398–2404, 1999.

[32] T. Jung and T. Grune, "The proteasome and its role in the degradation of oxidized proteins," *IUBMB Life*, vol. 60, no. 11, pp. 743–752, 2008.

[33] N. Nagai, K. Izumi-Nagai, Y. Oike et al., "Suppression of diabetes-induced retinal inflammation by blocking the angiotensin II type 1 receptor or its downstream nuclear factor-κB pathway," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 9, pp. 4342–4350, 2007.

[34] S. Satofuka, A. Ichihara, N. Nagai et al., "(Pro)renin receptor-mediated signal transduction and tissue renin-angiotensin system contribute to diabetes-induced retinal inflammation," *Diabetes*, vol. 58, no. 7, pp. 1625–1633, 2009.

[35] T. Kurihara, Y. Ozawa, N. Nagai et al., "Angiotensin II type 1 receptor signaling contributes to synaptophysin degradation and neuronal dysfunction in the diabetic retina," *Diabetes*, vol. 57, no. 8, pp. 2191–2198, 2008.

[36] M. Sasaki, Y. Ozawa, T. Kurihara et al., "Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes," *Diabetologia*, vol. 53, no. 5, pp. 971–979, 2010.

[37] B. M. Brenner, M. E. Cooper, D. De Zeeuw et al., "Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy," *The New England Journal of Medicine*, vol. 345, no. 12, pp. 861–869, 2001.

[38] D.-P. Li, S.-R. Chen, and H.-L. Pan, "Angiotensin II stimulates spinally projecting paraventricular neurons through presynaptic disinhibition," *Journal of Neuroscience*, vol. 23, no. 12, pp. 5041–5049, 2003.

[39] C. Sun, H. Li, L. Leng, M. K. Raizada, R. Bucala, and C. Sumners, "Macrophage migration inhibitory factor: an intracellular inhibitor of angiotensin II-induced increases in

neuronal activity," *Journal of Neuroscience*, vol. 24, no. 44, pp. 9944–9952, 2004.

[40] S.-S. Zhan, K. Beyreuther, and H. P. Schmitt, "Quantitative assessment of the synaptophysin immuno-reactivity of the cortical neuropil in various neurodegenerative disorders with dementia," *Dementia*, vol. 4, no. 2, pp. 66–74, 1993.

[41] R. W. Carthew, T. P. Neufeld, and G. M. Rubin, "Identification of genes that interact with the sina gene in Drosophila eye development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 24, pp. 11689–11693, 1994.

[42] J. Pines and C. Lindon, "Proteolysis: anytime, any place, anywhere?" *Nature Cell Biology*, vol. 7, no. 8, pp. 731–735, 2005.

[43] A.-L. Bulteau, K. C. Lundberg, K. M. Humphries et al., "Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion," *Journal of Biological Chemistry*, vol. 276, no. 32, pp. 30057–30063, 2001.

[44] S. R. Powell, P. Wang, H. Katzeff et al., "Oxidized and ubiquitinated proteins may predict recovery of postischemic cardiac function: essential role of the proteasome," *Antioxidants and Redox Signaling*, vol. 7, no. 5-6, pp. 538–546, 2005.

[45] W. E. Mitch, J. L. Bailey, X. Wang, C. Jurkovitz, D. Newby, and S. Russ Price, "Evaluation of signals activating ubiquitin-proteasome proteolysis in a model of muscle wasting," *American Journal of Physiology*, vol. 276, no. 5, pp. C1132–C1138, 1999.

[46] Y.-H. Song, Y. Li, J. Du, W. E. Mitch, N. Rosenthal, and P. Delafontaine, "Muscle-specific expression of IGF-1 blocks angiotensin II-induced skeletal muscle wasting," *Journal of Clinical Investigation*, vol. 115, no. 2, pp. 451–458, 2005.

[47] G. Tiao, S. Hobler, J. J. Wang et al., "Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle," *Journal of Clinical Investigation*, vol. 99, no. 2, pp. 163–168, 1997.

[48] S. W. Lee, G. Dai, Z. Hu, X. Wang, J. Du, and W. E. Mitch, "Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase," *Journal of the American Society of Nephrology*, vol. 15, no. 6, pp. 1537–1545, 2004.

[49] L. Farout, J. Mary, J. Vinh, L. I. Szweda, and B. Friguet, "Inactivation of the proteasome by 4-hydroxy-2-nonenal is site specific and dependant on 20S proteasome subtypes," *Archives of Biochemistry and Biophysics*, vol. 453, no. 1, pp. 135–142, 2006.

[50] E. J. Birks, N. Latif, K. Enesa et al., "Elevated p53 expression is associated with dysregulation of the ubiquitin-proteasome system in dilated cardiomyopathy," *Cardiovascular Research*, vol. 79, no. 3, pp. 472–480, 2008.

[51] J. Sterz, I. von Metzler, J.-C. Hahne et al., "The potential of proteasome inhibitors in cancer therapy," *Expert Opinion on Investigational Drugs*, vol. 17, no. 6, pp. 879–895, 2008.

[52] O. Enrico, B. Gabriele, C. Nadia et al., "Unexpected cardiotoxicity in haematological bortezomib treated patients," *British Journal of Haematology*, vol. 138, no. 3, pp. 396–397, 2007.

[53] A. Hacihanefioglu, P. Tarkun, and E. Gonullu, "Acute severe cardiac failure in a myeloma patient due to proteasome inhibitor bortezomib," *International Journal of Hematology*, vol. 88, no. 2, pp. 219–222, 2008.

[54] S. Meiners, M. Laule, W. Rother et al., "Ubiquitin-proteasome pathway as a new target for the prevention of restenosis," *Circulation*, vol. 105, no. 4, pp. 483–489, 2002.

[55] K. G. Barringhaus and M. E. Matsumura, "The proteasome inhibitor lactacystin attenuates growth and migration of vascular smooth muscle cells and limits the response to arterial injury," *Experimental and Clinical Cardiology*, vol. 12, no. 3, pp. 119–124, 2007.

Review Article

Effects of Calcium Ion, Calpains, and Calcium Channel Blockers on Retinitis Pigmentosa

Mitsuru Nakazawa

Department of Ophthalmology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8561, Japan

Correspondence should be addressed to Mitsuru Nakazawa, mitsuru@cc.hirosaki-u.ac.jp

Recent advances in molecular genetic studies have revealed many of the causative genes of retinitis pigmentosa (RP). These achievements have provided clues to the mechanisms of photoreceptor degeneration in RP. Apoptosis is known to be a final common pathway in RP and, therefore, a possible therapeutic target for photoreceptor rescue. However, apoptosis is not a single molecular cascade, but consists of many different reactions such as caspase-dependent and caspase-independent pathways commonly leading to DNA fractionation and cell death. The intracellular concentration of calcium ions is also known to increase in apoptosis. These findings suggest that calpains, one of the calcium-dependent proteinases, play some roles in the process of photoreceptor apoptosis and that calcium channel antagonists may potentially inhibit photoreceptor apoptosis. Herein, the effects of calpains and calcium channel antagonists on photoreceptor degeneration are reviewed.

1. Introduction

Retinitis pigmentosa (RP) represents a group of hereditary retinal degenerations principally characterized by progressive rod-dominant photoreceptor degeneration in the initial stage and eventual cone photoreceptor degeneration in later stages. Patients with RP mainly complain of night blindness and photophobia in the early stage, followed by gradual constriction of the visual field, decreased visual acuity, and color blindness in later stages. The prevalence of RP is roughly 1 in 4,000-5,000 people, and the condition is common in both Asian and Western countries. Significant features of RP include heterogeneity in both clinical and genetic characteristics. For instance, the severity and progression of RP vary from patient to patient even in the same family, despite affected members presumably sharing the same causative gene mutation. Heredities are also heterogeneous, characterized by at least 3 different modes of inheritance, such as autosomal-dominant, autosomal-recessive, and X-linked patterns. Since a mutation in the rhodopsin gene was first identified as causing one type of autosomal-dominant RP [1], at least 48 different causative genes have been identified (Ret-Net: http://www.sph.uth.tmc.edu/retnet/disease.htm); however, many other putative causative genes and mutations

have yet to be identified. Molecular genetic studies have also demonstrated that a primary lesion in RP involves photoreceptor and/or retinal pigment epithelial cells in which many causative genes are specifically expressed under physiological conditions. Photoreceptor or retinal pigment epithelial cells are known to degenerate mostly through apoptosis [2], which is now understood as a final common pathway for RP at the cellular level. As the mechanisms of photoreceptor degeneration have been gradually elucidated, studies on therapeutic approaches have dramatically increased, including pharmacotherapy, cellular transplantation, gene therapy, regenerative therapy, and retinal prosthesis. This paper mainly focuses on studies examining the effects of calcium ions and calpains on photoreceptor apoptosis, as well as pharmacological treatments for RP using calcium channel antagonists.

2. Genetic Background of RP

One of the most important breakthroughs in RP research was the identification of a point mutation (P23H) in the rhodopsin gene as a causative gene mutation for one form of autosomal-dominant RP [1, 3]. Since then, using a candidate gene approach, various mutations in the rhodopsin gene and

many other genes have been identified in several RP families. These include mutations in the genes encoding β- and α-subunits of rod cGMP-phosphodiesterase for autosomal-recessive RP [4, 5] and peripherin/RDS (RDS: retinal degeneration slow) for autosomal-dominant RP [6, 7]. These findings in the early 1990s suggested to many researchers that RP is caused by a single or one allelic pair of mutations in one of the genes specifically or dominantly expressed in photoreceptor cells. The candidate gene approach was also relatively easy to perform once researchers suspected genes already known to be retina specific as possible candidates for RP. Many other genes and mutations in these genes were then found to cause RP (Table 1). However, the candidate gene approach is limited in that screening can only be performed for known genes and involvement could not be ascertained for previously unknown genes that might be expressed not only in the retina, but also in other organs or tissues in a ubiquitous fashion. For these reasons, genetic linkage and/or association analyses have been performed in combination with a candidate gene approach to identify many other previously unpredictable genes as causative genes for RP. This group includes *PRPF31* [8], *PRPF3* [9], *PRPF8* [10], *IMPDH1* [11], *Mertk* [12, 13], and *CA4* [14] which are expressed in other tissues besides retina (Table 1). These findings indicate that photoreceptors and retinal pigment epithelium are much more active in protein synthesis than any other tissues and show high levels of gene expression and protein metabolism. In addition, molecular genetic studies have disclosed that RP is genetically more heterogeneous than it used to be considered and that the genetic heterogeneity may be one explanation for the clinical heterogeneity.

3. Photoreceptor Apoptosis as a Common Mechanism in RP

Despite the clinical and genetic heterogeneity, RP demonstrates common features derived from rod-predominant degeneration. This essential phenomenon allowed researchers to suspect some common mechanisms leading to photoreceptor cell death once the patient carries a single or one allelic pair of many causative gene mutations. Apoptosis is a genetically programmed mechanism that leads cells to death, and RP has been known to be initiated by photoreceptor apoptosis as a final common pathway at the cellular level, irrespective of gene mutations. For instance, apoptosis was detected in retinal degeneration 1 (rd1), rds, and rhodopsin mutant mice [2]. To date, many pathways have been found for apoptosis itself, involving caspases, cathepsins, calpains, apoptosis-inducing factor (AIF), Fas, and more. Once abnormal and/or insufficient structural or metabolic stresses induced by a certain gene mutation exceed predetermined thresholds that a cell can tolerate, mechanisms of apoptosis are initiated that lead to nuclear DNA fragmentation and subsequent cell death. Many experimental studies have supported that caspase-dependent or -independent apoptotic pathways are activated during experimental retinal degeneration models [15, 16].

TABLE 1: List of causative genes of RP: retina specific and nonspecific.

Category	ADRP (20)	ARRP (25)	XLRP (2)
Retina specific	CRX	ABCA4	RP2
	FSCN2	CERKL	
	GUCA1B	CNGA1	
	NRL	CNGB1	
	NR2E3	CRB1	
	PRPH2	EYES	
	RDH12	IDH3B	
	RHO	LRAT	
	ROM1	NR2E3	
	RP1	NRL	
	RP9	PDE6A	
	SEMA4A	PDE6B	
		PRCD	
		PROM1	
		RBP3	
		RGR	
		RHO	
		RLBP1	
		RP1	
		RPE65	
		SAG	
		SPATA7	
		TUP1	
		USH2	
Retina nonspecific	CA4	MERTK	RPGR
	IMPDH1		
	KHLH7		
	PRPF3		
	PRPF8		
	PRPF31		
	SNRNP200		
	TOPORS		

Abbreviations are listed in Ret:Net: http://www.sph.uth.tmc.edu/retnet/disease.htm.

Apoptosis can thus be considered as a therapeutic target as it plays many roles in retinitis pigmentosa [17, 18].

Calpains [EC 3.4.22.17], a group of calcium-dependent cysteine proteases, play some important roles in caspase-independent photoreceptor apoptotic pathways with light-induced retinal damage [19] and in rd1 mice [20, 21] and Royal College of Surgeons (RCS) rats [22] as models of retinal degeneration. Calpains are also involved in calcium-induced cell death in a murine photoreceptor-derived cell line [23, 24]. There is little doubt that intracellular concentrations of calcium ion were elevated in apoptosis [25–28]. As calcium influx is actually elevated in degenerating rd1 rod photoreceptors [20, 29], calpains are suspected to play important roles in photoreceptor apoptosis in RP. In addition, calpain inhibitors and calcium channel blockers appear to offer reasonable candidates at least in part as

pharmacotherapeutic agents for RP. Transient inhibitory effects of calpain inhibitors on photoreceptor apoptosis in RCS rats have recently been described by Mizukoshi et al. [22].

4. Effects of Calcium Ion on Photoreceptor Apoptosis

As mentioned above, intracellular concentrations of calcium ion are increased in apoptosis [20, 25–29]. Intracellular calcium ions are provided through several types of calcium channels and transporters located on cell membranes, endoplasmic reticulum, and mitochondria. Cyclic-nucleotide-gated cation channels (CNGCs) are located in the outer segment and closed by depletion of cGMP as a result of the phototransduction reaction triggering hyperpolarization. L-type voltage-gated calcium channels (VGCCs) are located in the cell body and synaptic terminal and are closed by hyperpolarization of the cell membrane induced by phototransduction. Steele Jr. et al. [30] suggested that the average concentration of calcium in the terminal ranges from ∼350 nM in hyperpolarized light-adapted cells to more than $39\,\mu M$ in cells depolarized to dark potentials in salamander rods and cones. In addition to CNGC and VGCC, intracellular concentrations of calcium ions are regulated by many other factors, such as plasma membrane calcium ATPase, store-operated calcium entry, calcium stores in the endoplasmic reticulum, and mitochondria (Figure 1). Under pathological conditions, like those in rd1 mice, intracellular calcium levels significantly increase in rods, even before the detection of apoptotic cells [29]. The marked elevation of intracellular concentrations of calcium ions activates downstream reactions, including hydrolytic enzymes like calpains, and eventually leads to cell death [25]. Excessive calcium influx is initiated in the cytosol and subsequently in mitochondria in rd1 mouse [29], suggesting that increased calcium ions may affect many biochemical cascades and reactions not only in the cytosol but also in the mitochondria [31, 32]. As mentioned above, increased intracellular calcium concentrations activate calpains, leading to the activation of both caspase-dependent and -independent apoptotic pathways. First, as a caspase-dependent pathway, calpains activate caspase 12, which sequentially activates caspases 9, 3, 4, and 7 and finally apoptosis is upregulated. Second, cytosolic calpains further activate cathepsins and mitochondrial calpains activate AIF, which subsequently translocates from mitochondria to the nucleus [22]. This reaction has been speculated to represent one of the caspase-independent pathways of apoptosis [33, 34].

5. Ca²⁺ Channel Antagonists for Photoreceptor Apoptosis in Animal Experiments

Since Frasson et al. [35] first reported the effects of D-cis-diltiazem, a benzothiazepin calcium channel antagonist which blocks both CNGC and VGCC, on photoreceptor protection in rd1 mice, several investigators have reported positive and negative effects of calcium channel blockers

on animal models of RP [19–21, 36–43]. Since rd1 is caused by a mutation in the gene encoding the β-subunit of rod cGMP-phosphodiesterase, one of the key enzymes in the phototransduction pathway, CNGCs located in the outer segment cannot be closed despite light stimulation in the rod photoreceptor cells. Inhibition of light-induced hyperpolarization, caused by a mutation in the rod cGMP-phosphodiesterase gene, also does not close VGCC. These phenomena increase calcium influx in both outer and inner segments in rd1 mice. The intracellular concentration of calcium ions is subsequently elevated, leading to photoreceptor apoptosis [35], possibly by upregulation of calpains and other proteins [28]. Sanges et al. [20] demonstrated that systemic administration of D-cis-diltiazem reduced intracellular concentrations of calcium, downregulating calpains and photoreceptor apoptosis in rd1 mice. Direct inhibitory effects of D-cis-diltiazem on CNGC [44] or L-type VGCC [39] have been reported, and D-cis-diltiazem effectively blocks photoreceptor light damage in mouse models by inhibiting photoreceptor apoptosis [19]. In contrast, L-cis isomer inhibits L-type VGCC similarly to D-cis isomer [45]. The difference in action between D-cis- and L-cis-diltiazems on photoreceptor neuroprotection [35] suggests that CNGC might also be important for photoreceptor neuroprotection [44]. Read et al. [46] also reported that the β-subunit of VGCC knock-out rd1 mice showed retardation of photoreceptor degeneration, suggesting that blockage of calcium influx may partially contribute to photoreceptor rescue in these animal models although it did not prevent photoreceptor degeneration. Despite these studies, however, Pawlyk et al. [36] and Takano et al. [41] found no rescue effects of D-cis-diltiazem on retinal degeneration in rd1 mice, and Bush et al. [42] also reported that D-cis-diltiazem was ineffective for photoreceptor rescue in rhodopsin P23H transgenic rats.

While the effects of diltiazem on animal models of retinal degeneration remain controversial, another type of calcium channel blocker, nilvadipine, a member of the dihydropyridine derivatives, is another candidate therapeutic agent for RP. Nilvadipine has low-voltage-activated calcium blocking actions in addition to L-type high-voltage calcium blocking actions. The hydrophobic nature induced by the chemical structure of nilvadipine allows high permeability to the central nervous system, including the retina [47]. Systemic administration of nilvadipine has been shown to be effective for protecting photoreceptors in RCS rats [37, 40], rd1 mice [41], and heterozygous rd2 (rds) mice [43]. In addition to direct effects of calcium channel blockers on intracellular concentrations of calcium ion in photoreceptor cells, other indirect effects are expected such as increased expression of fibroblast growth factor (FGF) 2 [40, 41] and ciliary neurotrophic factor (CNTF) [43] in the retina, and increased choroidal blood flow [48]. Since FGF2 and CNTF are known to exert photoreceptor-protective effects [49–56], upregulating such intrinsic neurotrophic factors by nilvadipine may demonstrate beneficial effects against RP. CNTF has also been applied as a clinical trial for RP [57]. In addition, oxidative stress may be involved in photoreceptor death in RP [58–63], and nilvadipine has
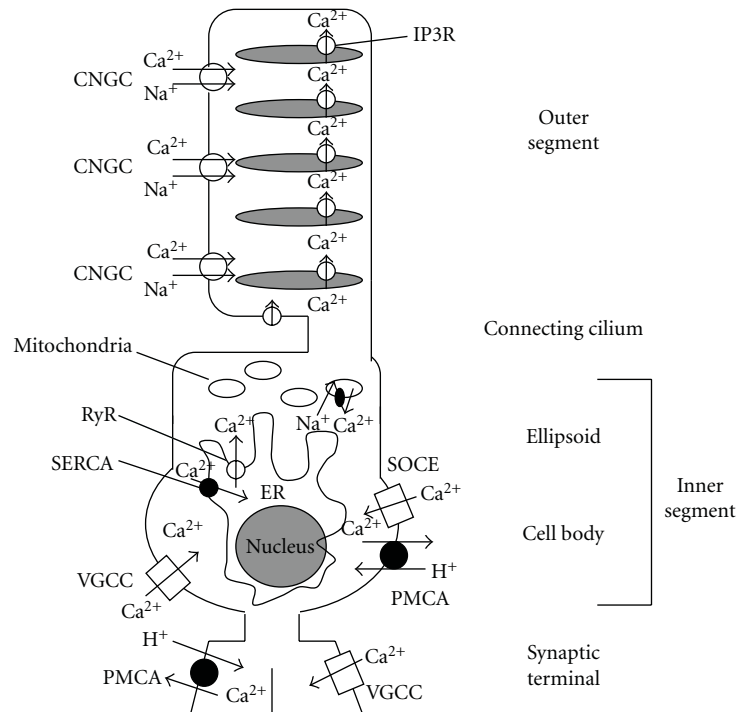
FIGURE 1: Schematic view of calcium channels and transporters in the rod-photoreceptor. Abbreviations: CNGC, cyclic-nucleotide-gated cation channel; VGCC: voltage-gated calcium channel; PMCA: plasma membrane calcium ATPase; SOCE: store-operated calcium entry; ER: endoplasmic reticulum; SERCA: sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase; IP3R: inositol 1, 4, 5-triphoaphate receptor; RyR: ryanodine receptor. Ca^{2+} stock and release from the mitochondria are mediated by Ca^{2+} uniporter channels and Na^+/Ca^{2+} transporters.

the highest antioxidant potency among calcium channel blockers [64]. The direct effects of calcium channel blockers on photoreceptor calpains have not yet been studied. Studies involving calcium channel antagonists are listed in Table 2. As the effects of calcium channel blockers on photoreceptor rescue remain controversial, further biochemical studies are required in order to facilitate our understanding of the mechanisms of photoreceptor degeneration induced by various types of gene mutations, the effects of intracellular calcium ions on downstream reactions, and the effects of calcium channel blockers on both concentrations of calcium ions and downstream reactions in various types of heterogeneous conditions of RP. Although human RP is caused by various kinds of heterogeneous causative gene mutations, our understanding regarding photoreceptor degeneration in RP is still limited to relatively small numbers of experimental models of RP.

6. Human Trials

Although human RP is genetically heterogeneous, possible rescue effects of calcium channel blockers on photoreceptor degeneration in certain animal models of RP, such as rd1 and rds mice and RCS rats, have encouraged researchers to expect therapeutic effects of calcium channel blockers for RP. Pasantes-Morales et al. [65] reported that a combination of D-cis-diltiazem, taurin, and vitamin E has beneficial effects on the visual field progression, although the study did

not clarify whether diltiazem alone demonstrated beneficial effects. Ohguro [66] reported the photoreceptor rescue effects of nilvadipine in a small patient group. We expanded his nilvadipine study for RP patients to confirm the results. Although both treated and control groups are still small, our results have shown significant retardation of the mean deviation (MD) slope as calculated by the central visual field (Humphry Visual Field Analyzer, 10-2 Program) after a mean of 48 months of observation [67]. As these pilot studies are small-sized and cannot completely exclude possible biases, a large-scale, randomized, multicenter human trial of calcium channel blockers is required in order to evaluate their efficacy as therapeutic agents for RP.

7. Future Insights

As pharmacotherapeutic agents for RP, vitamin A [68, 69] and lutein [70] are reportedly effective in slowing RP, and carbonic anhydrase inhibitors appear effective for reducing chronic cystoid macular edema [71, 72], although the basic molecular mechanisms underlying these actions remain unclear. Effects of calcium channel blockers have been speculated based on the molecular mechanisms in RP identified in recent molecular genetic [4] and animal studies [20, 35] of RP and also research on neuroprotection for glaucoma [73]. In addition to previous pilot studies, large-scale human trials to examine the effects of calcium channel blockers in the progression of RP are needed to

TABLE 2: Photoreceptor rescue by calcium channel antagonists.

	Authors	Year	Ref.
Supportive			
Diltiazem on rd1 mouse	Frasson et al.	1999	[35]
Diltiazem on rd1 mouse	Sanges et al.	2006	[20]
Diltiazem on light damage	Vallaza-Deschamps et al.	2005	[19]
Calcium channel knockout on rd1	Read et al.	2002	[46]
Nilvadipine on rd1 mouse	Takano et al.	2004	[41]
Nilvadipine on RCS rat	Yamazaki et al.	2002	[37]
Nilvadipine on RCS rat	Sato et al.	2003	[40]
Nilvadipine on rd2 (rds) mouse	Takeuchi et al.	2008	[43]
Negative			
Diltiazem on rd1 mouse	Pawlyk et al.	2002	[36]
Diltiazem on rd1 mouse	Takano et al.	2004	[41]
Diltiazem on pde β knock-out dog	Pearce-Kelling et al.	2001	[21]
Diltiazem on rhodopsin P23H rat	Bush et al.	2000	[42]

obtain solid evidence-based results. Since calcium channel blockers may not effectively block enough calcium influx to rescue degenerating photoreceptors depending on the kinds of gene mutations, downstream reactions like calpains should be considered when planning therapy. Effects of calpain inhibitors on human RP patients should also be examined in the future. As other modern technologies have advanced, new therapeutic modalities including gene therapy, retinal prostheses, and regenerative medicine have become increasingly developed, and some applications of these technologies are now commercially available. Of note is the fact that pharmacotherapeutic agents aimed at photoreceptor rescue can be used in combination with gene therapy and regenerative medicine.

References

[1] T. P. Dryja, T. L. McGee, E. Reichel et al., "A point mutation of the rhodopsin gene in one form of retinitis pigmentosa," *Nature*, vol. 343, no. 6256, pp. 364–366, 1990.

[2] G. Q. Chang, Y. Hao, and F. Wong, "Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice," *Neuron*, vol. 11, no. 4, pp. 595–605, 1993.

[3] P. McWilliams, G. J. Farrar, P. Kenna et al., "Autosomal deminant retinitis pigmentosa (ADRP): location of an ADRP gene to the long arm of chromosome 3," *Genomics*, vol. 53, pp. 619–622, 1989.

[4] M. E. McLaughlin, M. A. Sandberg, E. L. Berson, and T. P. Dryja, "Recessive mutations in the gene encoding the β-subunit of rod phosphodiesterase in patients with retinitis pigmentosa," *Nature Genetics*, vol. 4, no. 2, pp. 130–134, 1993.

[5] T. P. Dryja, D. E. Rucinski, S. H. Chen, and E. L. Berson, "Frequency of mutations in the gene encoding the α subunit of rod cGMP- phosphodiesterase in autosomal recessive retinitis pigmentosa," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 8, pp. 1859–1865, 1999.

[6] G. J. Farrar, P. Kenna, S. A. Jordan et al., "A three-base-pair deletion in the peripherin-RDS gene in one form of retinitis pigmentosa," *Nature*, vol. 354, no. 6353, pp. 478–480, 1991.

[7] K. Kajiwara, L. B. Hahn, S. Mukai, G. H. Travis, E. L. Berson, and T. P. Dryja, "Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa," *Nature*, vol. 354, no. 6353, pp. 480–483, 1991.

[8] E. N. Vithana, L. Abu-Safieh, M. J. Allen et al., "A human homolog of yeast pre-mRNA splicing gene, *PRP31*, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (*RP11*)," *Molecular Cell*, vol. 8, no. 2, pp. 375–381, 2001.

[9] C. F. Chakarova, M. M. Hims, H. Bolz et al., "Mutations in *HPRP3*, a third member of pre-mRNA splicing factor genes, implicated in autosomal dominant retinitis pigmentosa," *Human Molecular Genetics*, vol. 11, no. 1, pp. 87–92, 2002.

[10] A. B. McKie, J. C. McHale, T. J. Keen et al., "Mutations in the pre-mRNA splicing factor gene *PRPC8* in autosomal dominant retinitis pigmentosa (RP13)," *Human Molecular Genetics*, vol. 10, no. 15, pp. 1555–1562, 2001.

[11] S. J. Bowne, L. S. Sullivan, S. H. Blanton et al., "Mutations in the inosine monophosphate dehydrogenase 1 gene (IMPDH1) cause the RP10 form of autosomal dominant retinitis pigmentosa," *Human Molecular Genetics*, vol. 11, no. 5, pp. 559–568, 2002.

[12] P. M. D'Cruz, D. Yasumura, J. Weir et al., "Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat," *Human Molecular Genetics*, vol. 9, no. 4, pp. 645–651, 2000.

[13] A. Gal, Y. Li, D. A. Thompson et al., "Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa," *Nature Genetics*, vol. 26, no. 3, pp. 270–271, 2000.

[14] G. Rebello, R. Ramesar, A. Vorster et al., "Apoptosis-inducing signal sequence mutation in carbonic anhydrase IV identified in patients with the RP17 form of retinitis pigmentosa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 17, pp. 6617–6622, 2004.

[15] C. Jomary, M. J. Neal, and S. E. Jones, "Characterization of cell death pathways in murine retinal neurodegeneration implicates cytochrome c release, caspase activation, and Bid cleavage," *Molecular and Cellular Neuroscience*, vol. 18, no. 4, pp. 335–346, 2001.

[16] F. Doonan, M. Donovan, and T. G. Cotter, "Caspase-independent photoreceptor apoptosis in mouse models of

retinal degeneration," *Journal of Neuroscience*, vol. 23, no. 13, pp. 5723–5731, 2003.

[17] F. Doonan and T. G. Cotter, "Apoptosis: a potential therapeutic target for retinal degenerations," *Current Neurovascular Research*, vol. 1, no. 1, pp. 41–53, 2004.

[18] S. Cottet and D. F. Schorderet, "Mechanisms of apoptosis in retinitis pigmentosa," *Current Molecular Medicine*, vol. 9, no. 3, pp. 375–383, 2009.

[19] G. Vallazza-Deschamps, D. Cia, J. Gong et al., "Excessive activation of cyclic nucleotide-gated channels contributes to neuronal degeneration of photoreceptors," *European Journal of Neuroscience*, vol. 22, no. 5, pp. 1013–1022, 2005.

[20] D. Sanges, A. Comitato, R. Tammaro, and V. Marigo, "Apoptosis in retinal degeneration involves cross-talk between apoptosis-inducing factor (AIF) and caspase-12 and is blocked by calpain inhibitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 46, pp. 17366–17371, 2006.

[21] S. E. Pearce-Kelling, T. S. Aleman, A. Nickle et al., "Calcium channel blocker D-cis-diltiazem does not slow retinal degeneration in the PDE6B mutant rcd1 canine model of pigmentosa," *Molecular Vision*, vol. 7, pp. 42–47, 2001.

[22] S. Mizukoshi, M. Nakazawa, K. Sato, T. Ozaki, T. Metoki, and S. I. Ishiguro, "Activation of mitochondrial calpain and release of apoptosis-indusing factor from mitochondria in RCS rat retinal degeneration," *Experimental Eye Research*, vol. 91, no. 3, pp. 353–361, 2010.

[23] N. Sanvicens, V. Gómez-Vicente, I. Masip, A. Messeguer, and T. G. Cotter, "Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6," *Journal of Biological Chemistry*, vol. 279, no. 38, pp. 39268–39278, 2004.

[24] V. Gómez-Vicente, M. Donovan, and T. G. Cotter, "Multiple death pathways in retina-derived 661W cells following growth factor deprivation: crosstalk between caspases and calpains," *Cell Death and Differentiation*, vol. 12, no. 7, pp. 796–804, 2005.

[25] P. Nicotera and S. Orrenius, "The role of calcium in apoptosis," *Cell Calcium*, vol. 23, no. 2-3, pp. 173–180, 1998.

[26] D. A. Fox, A. T. Poblenz, and L. He, "Calcium overload triggers rod photoreceptor apoptotic cell death in chemical-induced and inherited retinal degenerations," *Annals of the New York Academy of Sciences*, vol. 893, pp. 282–285, 1999.

[27] M. N. Delyfer, T. Léveillard, S. Mohand-Saïd, D. Hicks, S. Picaud, and J. A. Sahel, "Inherited retinal degenerations: therapeutic prospects," *Biology of the Cell*, vol. 96, no. 4, pp. 261–269, 2004.

[28] F. Paquet-Durand, L. Johnson, and P. Ekström, "Calpain activity in retinal degeneration," *Journal of Neuroscience Research*, vol. 85, no. 4, pp. 693–702, 2007.

[29] F. Doonan, M. Donovan, and T. G. Cotter, "Activation of multiple pathways during photoreceptor apoptosis in the rd mouse," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 10, pp. 3530–3538, 2005.

[30] E. C. Steele Jr., X. Chen, P. M. Iuvone, and P. R. MacLeish, "Imaging of Ca2+ dynamics within the presynaptic terminals of salamander rod photoreceptors," *Journal of Neurophysiology*, vol. 94, no. 6, pp. 4544–4553, 2005.

[31] T. Szikra and D. Krizaj, "Calcium signals in inner segments of photoreceptors," in *The Visual Transduction Cascade: Basic and Clinical Principles*, J. Tombran-Tink and C. Barnstable, Eds., pp. 197–223, Jumana Press, Totowa, NJ, USA, 2009.

[32] P. Barabas, C. C. Peck, and D. Krizaj, "Do calcium channel blockers rescue dying photoreceptors in the *Pde6b^{rd1}* mouse?" *Advances in Experimental Medicine and Biology*, vol. 664, pp. 491–499, 2010.

[33] S. A. Susin, H. K. Lorenzo, N. Zamzami et al., "Molecular characterization of mitochodrial apoptosis-inducing factor," *Nature*, vol. 397, no. 6718, pp. 441–446, 1999.

[34] P. Widlak, L. Y. Li, X. Wang, and W. T. Garrard, "Action of recombinant human apoptotic endonuclease G on naked DNA and chromatin substrates: cooperation with exonuclease and DNase I," *Journal of Biological Chemistry*, vol. 276, no. 51, pp. 48404–48409, 2001.

[35] M. Frasson, J. A. Sahel, M. Fabre, M. Simonutti, H. Dreyfus, and S. Picaud, "Retinitis pigmentosa: rod photoreceptor rescue by a calcium-channel blocker in the rd mouse," *Nature Medicine*, vol. 5, no. 10, pp. 1183–1187, 1999.

[36] B. S. Pawlyk, T. Li, M. S. Scimeca, M. A. Sandberg, and E. L. Berson, "Absence of photoreceptor rescue with D-cis-diltiazem in the rd mouse," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 6, pp. 1912–1915, 2002.

[37] H. Yamazaki, H. Ohguro, T. Maeda et al., "Preservation of retinal morphology and functions in Royal College Surgeons rat by nilvadipine, a Ca2+ Antagonist," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 4, pp. 919–926, 2002.

[38] M. Donovan and T. G. Cotter, "Caspase-independent photoreceptor apoptosis in vivo and diffrential expression of apoptotic protease activating factor-1 and caspase-3 during retinal development," *Cell Death and Differentiation*, vol. 9, no. 11, pp. 1220–1231, 2002.

[39] J. Hart, M. F. Wilkinson, M. E. M. Kelly, and S. Barnes, "Inhibitory action of diltiazem on voltage-gated calcium channels in cone photoreceptors," *Experimental Eye Research*, vol. 76, no. 5, pp. 597–604, 2003.

[40] M. Sato, H. Ohguro, I. Ohguro et al., "Study of pharmacological effects of nilvadipine on RCS rat retinal degeneration by microarray analysis," *Biochemical and Biophysical Research Communications*, vol. 306, no. 4, pp. 826–831, 2003.

[41] Y. Takano, H. Ohguro, M. Dezawa et al., "Study of drug effects of calcium channel blockers on retinal degeneration of rd mouse," *Biochemical and Biophysical Research Communications*, vol. 313, no. 4, pp. 1015–1022, 2004.

[42] R. A. Bush, L. Kononen, S. Machida, and P. A. Sieving, "The effect of calcium channel blocker diltiazem on photoreceptor degeneration in the rhodopsin *Pro23His* rat," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 9, pp. 2697–2701, 2000.

[43] K. Takeuchi, M. Nakazawa, and S. Mizukoshi, "Systemic administration of nilvadipine delays photoreceptor degeneration of heterozygous retinal degeneration slow (rds) mouse," *Experimental Eye Research*, vol. 86, no. 1, pp. 60–69, 2008.

[44] K. W. Koch and U. B. Kaupp, "Cyclic GMP directly regulates a cation conductance in membranes of bovine rods by a cooperative mechanism," *Journal of Biological Chemistry*, vol. 260, no. 11, pp. 6788–6800, 1985.

[45] D. Cia, A. Bordais, C. Varela et al., "Voltage-gated channels and calcium homeostasis in mammalian rod photoreceptors," *Journal of Neurophysiology*, vol. 93, no. 3, pp. 1468–1475, 2005.

[46] D. S. Read, M. A. McCall, and R. G. Gregg, "Absence of voltage-dependent calcium channels delays photoreceptor degeneration in rd mice," *Experimental Eye Research*, vol. 75, no. 4, pp. 415–420, 2002.

[47] Y. Tokuma, T. Fujiwara, and H. Noguchi, "Absorption, distribution and excretion of nilvadipine, a new dihydropyridine

calcium antagonist, in rats and dogs," *Xenobiotica*, vol. 17, no. 11, pp. 1341–1349, 1987.

[48] N. Koseki, M. Araie, A. Tomidokoro et al., "A placebo-controlled 3-year study of a calcium blocker on visual field and ocular circulation in glaucoma with low-normal pressure," *Ophthalmology*, vol. 115, no. 11, pp. 2049–2057, 2008.

[49] E. G. Faktorovich, R. H. Steinberg, D. Yasumura, M. T. Matthes, and M. M. LaVail, "Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor," *Nature*, vol. 347, no. 6288, pp. 83–86, 1990.

[50] M. Cayouette, D. Behn, M. Sendtner, P. Lachapelle, and C. Gravel, "Intraocular gene transfer of ciliary neurotrophic factor prevents death and increases responsiveness of rod photoreceptors in the retinal degeneration slow mouse," *Journal of Neuroscience*, vol. 18, no. 22, pp. 9282–9293, 1998.

[51] F. Q. Liang, N. S. Dejneka, D. R. Cohen et al., "AAV-mediated delivery of ciliary neurotrophic factor prolongs photoreceptor survival in the rhodopsin knockout mouse," *Molecular Therapy*, vol. 3, no. 2, pp. 241–248, 2001.

[52] F. Q. Liang, T. S. Aleman, N. S. Dejneka et al., "Long-term protection of retinal structure but not function using rAAV.CNTF in animal models of retinitis pigmentosa," *Molecular Therapy*, vol. 4, no. 5, pp. 461–472, 2001.

[53] W. Tao, R. Wen, M. B. Goddard et al., "Encapsulated cell-based delivery of CNTF reduces photoreceptor degeneration in animal models of retinitis pigmentosa," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 10, pp. 3292–3298, 2002.

[54] D. Bok, D. Yasumura, M. T. Matthes et al., "Effects of adeno-associated virus-vectored ciliary neurotrophic factor on retinal structure and function in mice with a P216L rds/peripherin mutation," *Experimental Eye Research*, vol. 74, no. 6, pp. 719–735, 2002.

[55] F. C. Schlichtenbrede, A. MacNeil, J. W. B. Bainbridge et al., "Intraocular gene delivery of ciliary neurotrophic factor results in significant loss of retinal function in normal mice and in the Prph2 model of retinal degeneration," *Gene Therapy*, vol. 10, no. 6, pp. 523–527, 2003.

[56] R. Wen, Y. Song, S. Kjellstrom et al., "Regulation of rod phototransduction machinery by ciliary neurotrophic factor," *Journal of Neuroscience*, vol. 26, no. 52, pp. 13523–13530, 2006.

[57] P. A. Sieving, R. C. Caruso, W. Tao et al., "Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 10, pp. 3896–3901, 2006.

[58] R. J. Carmody and T. G. Cotter, "Oxidative stress induces caspase-independent retinal apoptosis in vitro," *Cell Death and Differentiation*, vol. 7, no. 3, pp. 282–291, 2000.

[59] D. Y. Yu, S. Cringle, K. Valter, N. Walsh, D. Lee, and J. Stone, "Photoreceptor death, trophic factor expression, retinal oxygen status, and photoreceptor function in the P23H rat," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 6, pp. 2013–2019, 2004.

[60] J. Shen, X. Yang, A. Dong et al., "Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa," *Journal of Cellular Physiology*, vol. 203, no. 3, pp. 457–464, 2005.

[61] K. Komeima, B. S. Rogers, L. Lu, and P. A. Campochiaro, "Antioxidants reduce cone cell death in a model of retinitis pigmentosa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 30, pp. 11300–11305, 2006.

[62] M. Tuson, A. Garanto, R. Gonzàlez-Duarte, and G. Marfany, "Overexpression of CERKL, a gene responsible for retinitis pigmentosa in humans, protects cells from apoptosis induced by oxidative stress," *Molecular Vision*, vol. 15, pp. 168–180, 2009.

[63] S. Usui, K. Komeima, S. Y. Lee et al., "Increased expression of catalase and superoxide dismutase 2 reduces cone cell death in retinitis pigmentosa," *Molecular Therapy*, vol. 17, no. 5, pp. 778–786, 2009.

[64] H. Sugawara, K. Tobise, and K. Kikuchi, "Antioxidant effects of calcium antagonists on rat myocardial membrane lipid peroxidation," *Hypertension Research*, vol. 19, no. 4, pp. 223–228, 1996.

[65] H. Pasantes-Morales, H. Quiroz, and O. Quesada, "Treatment with taurine, diltiazem, and vitamin E retards the progressive visual field reduction in retinitis pigmentosa: a 3-year follow-up study," *Metabolic Brain Disease*, vol. 17, no. 3, pp. 183–197, 2002.

[66] H. Ohguro, "New drug therapy for retinal degeneration," *Nippon Ganka Gakkai zasshi*, vol. 112, no. 1, pp. 7–21, 2008.

[67] M. Nakazawa, H. Ohguro, K. Takeuchi, Y. Miyagawa, T. Ito, and T. Metoki, "Effect of nilvadipine on central visual field in retinitis pigmentosa: a 30-month clinical trial," *Ophthalmologica*, vol. 225, no. 2, pp. 120–126, 2010.

[68] E. L. Berson, B. Rosner, M. A. Sandberg et al., "A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa," *Archives of Ophthalmology*, vol. 111, no. 6, pp. 761–772, 1993.

[69] E. L. Berson, "Long-term visual prognoses in patients with retinitis pigmentosa: the Ludwig von Sallmann lecture," *Experimental Eye Research*, vol. 85, no. 1, pp. 7–14, 2007.

[70] E. L. Berson, B. Rosner, M. A. Sandberg et al., "Clinical trial of lutein in patients with retinitis pigmentosa receiving vitamin A," *Archives of Ophthalmology*, vol. 128, no. 4, pp. 403–411, 2010.

[71] G. A. Fishman, L. D. Gilbert, R. G. Fiscella, A. E. Kimura, and L. M. Jampol, "Acetazolamide for treatment of chronic macular edema in retinitis pigmentosa," *Archives of Ophthalmology*, vol. 107, no. 10, pp. 1445–1452, 1989.

[72] S. Grover, G. A. Fishman, R. G. Fiscella, and A. E. Adelman, "Efficacy of dorzolamide hydrochloride in the management of chronic cystoid macular edema in patients with retinitis pigmentosa," *Retina*, vol. 17, no. 3, pp. 222–231, 1997.

[73] M. Araie and C. Yamaya, "Use of calcium channel blockers for glaucoma," *Progress in Retinal and Eye Research*. In press.

Review Article

Cellular Origin of Spontaneous Ganglion Cell Spike Activity in Animal Models of Retinitis Pigmentosa

David J. Margolis[1, 2] and Peter B. Detwiler[1]

[1] *Program in Neurobiology and Behavior, Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195, USA*

[2] *Department of Neurophysiology, Brain Research Institute, University of Zurich, 8057 Zurich, Switzerland*

Correspondence should be addressed to Peter B. Detwiler, detwiler@u.washington.edu

Here we review evidence that loss of photoreceptors due to degenerative retinal disease causes an increase in the rate of spontaneous ganglion spike discharge. Information about persistent spike activity is important since it is expected to add noise to the communication between the eye and the brain and thus impact the design and effective use of retinal prosthetics for restoring visual function in patients blinded by disease. Patch-clamp recordings from identified types of ON and OFF retinal ganglion cells in the adult (36–210 d old) *rd1* mouse show that the ongoing oscillatory spike activity in both cell types is driven by strong rhythmic synaptic input from presynaptic neurons that is blocked by CNQX. The recurrent synaptic activity may arise in a negative feedback loop between a bipolar cell and an amacrine cell that exhibits resonant behavior and oscillations in membrane potential when the normal balance between excitation and inhibition is disrupted by the absence of photoreceptor input.

1. Introduction

Retinitis pigmentosa (RP) refers to a number of related diseases that result in the death of rod and cone photoreceptors causing blindness in about one in 3,500 people, nearly 2 million people worldwide. Not surprisingly, PubMed lists more than 7,000 papers on RP that provide an abundant source of information about the genetic, biochemical, physiological, and therapeutic characteristics of the disease. The goal of much recent work on RP has been to develop methods to restore vision by resuscitating the retina using gene therapy to repair the mutation that gives rise to the dystrophy [1] or by driving it artificially using neural prosthetics that are based on either electrical stimulation via implanted retinal electrodes [2] or optical stimulation via light activation of ectopically expressed photosensitive proteins [3–9]. The success of any of these approaches ultimately depends on the functional integrity of retinal ganglion cells (RGCs), the output cells of the retina whose axons carry spike-encoded information to the visual centers in the central nervous system. To make optimal use of ganglion cells for communicating with the brain, it is necessary to know how they are affected by the degenerative loss of photoreceptors and the accompanying changes in the cellular architecture of the retina [10–15].

2. RP Increases Spontaneous Spike Activity in Ganglion Cells

Out of the several thousand publications on RP, less than a dozen have addressed questions about the effects of retinal degeneration on RGC firing properties. The responses of individual cells cannot be evaluated using the electroretinogram (ERG), which is the widely employed standard method for assessing the functional changes in the retina resulting from loss of photoreceptor input. An early study by Drager and Hubel [16] based on extracellular single unit recordings from either optic nerve, superior colliculus (SC), or visual cortex reports an increase in spontaneous spike activity with maintained rhythmic firing in *rd1* mice that was not present in normal animals. The patterned spike activity was reversibly abolished by temporarily occluding blood flow to the eye, providing evidence of its retinal origin. The frequency of the persistent discharge was dependent on the

anesthetic and ranged between 9–14 Hz. These findings were confirmed subsequently using autocorrelgrams to demonstrate the rhythmicity of maintained spike activity in units recorded from the SC in dystrophic but not nondystrophic Royal College of Surgeons (RCS) rats [17]. There are also reports of increased *c-fos-like* immunoreactivity in the superior colliculus and lateral geniculate nucleus in *rd1* mice and RCS rats that is eliminated by intraocular injection of TTX or optic nerve transection [18, 19]. The increase in *c-fos* expression was attributed to the generation of rhythmic input from retinal ganglion cells.

The changes in RGC spike activity during the progression of photoreceptor degeneration has been documented more directly using extracellular single RGC recording in the RCS rat [20] as well as multielectrode array recordings in retina from the *rd1* mouse [21] and the P23H rat, an animal model of human autosomal dominant RP [22]. In agreement with the earlier accounts the single cell and multielectrode recordings showed a marked increase in the frequency of maintained spontaneous spike activity with rhythmic bursts [9, 21] in adult animals that have lost their ability to respond to light. An increase in glutamate-mediated excitatory signaling has also been observed in rodent models of RP using organic cations and immunoreactivity to map neuronal activity [11]. Taken together, the overall conclusion of these studies is that photoreceptor death due to degenerative disease leads to hyperactivity in ganglion cells.

It is important to understand the properties of the ongoing spike activity that is present in RP because it represents an undesirable noise source that degrades the communication between the eye and the brain that the aforementioned strategies to restore vision in patients blinded by degenerative disease depend upon. Here we review experiments designed to investigate the cellular mechanisms responsible for the increase in maintained spike activity and explore the retinal circuitry that may give rise to it.

3. RP-Induced Changes in Spike Activity in Identified Retinal Ganglion Cells

To determine whether RGC hyperactivity was caused by changes in the intrinsic properties of RGCs, such as ion channel function or distribution, or by altered synaptic input, intracellular recording was used to study the effect of photoreceptor loss on the electrophysiological properties of selected types of ganglion cells in *rd1* retina [23]. RGCs with the soma diameters ($\geq 20\,\mu$m)—which, by virtue of their large size, are referred to here as alpha cells [24]—were targeted for whole cell current or voltage clamp recording and filled by internal dialysis with an intracellular fluorescent indicator. Images obtained by 2-photon laser scanning fluorescent microscopy [25] were used to classify recorded RGCs as either ON, OFF transient, or OFF-sustained alpha cells, based on their dendrite stratification depth in the inner plexiform layer [24]. The use of morphological criteria to reliably identify RGC subtypes in blind animals is made possible by the fact that the dendritic morphology of ganglion cells is not affected by photoreceptor degeneration [23, 26].

Unlike ganglion cells from normal animals, which generate resting spike activity with no obvious temporal periodicity, the rate of spontaneous spike discharge in alpha RGCs from animals blinded by degeneration is increased and consists of continuous rhythmic bursts of spikes (Figure 1) with a beat frequency of ~10 Hz; the same frequency as the persistent discharge was reported by Drager and Hubel [16]. The clockwork firing of the alpha RGCs is maintained 24/7 in adult animals ranging in age from 36 to 210 days; experiments were not done on older animals. During this time the intrinsic network and electrophysiological properties of the cells were remarkably stable [23]. More specifically *rd1* alpha RGS retained the characteristic differences in the weights of excitatory and inhibitory synaptic inputs that ON and OFF cell types receive. They also continued to generate rebound excitation in OFF cells and gave rise to voltage-evoked dendrite calcium signals that were similar to those recorded from the dendrites of RGCs in non-dystrophic retina [27]. The rhythmic bursts of spikes that are a hallmark of *rd1* alpha RGC activity are triggered by oscillatory synaptic inputs as shown by the fact that they persist under voltage clamp recording conditions and are eliminated by CNQX, a glutamatergic blocker (Figure 1).

4. Source of Enhanced Synaptic Inputs

The presynaptic source of the synaptic inputs that give rise to rhythmic firing is not known. That *rd1* ON and OFF RGCs retain their normal distinguishing differences in the strengths of the excitatory and inhibitory inputs they receive, in spite of the ongoing oscillations in maintained synaptic activation, suggests that the organization and distribution of RGC contacts with presynaptic neurons have not been remodeled. The extensive changes in retina morphology that have been reported in this and other models of RP [28, 29] emerge in animals that are more than twice as old as the oldest animals used by Margolis [23]. While the slow onset of retinal remodeling makes it clearly important to document the accompanying changes in the cellular physiology of identified retinal neurons in older (P500) animals, this has not been done for purely practical reasons having to do with the required investments of time (nearly two years) and money (cost of maintaining a geriatric mouse colony). Hence the following discussion pertains to P36 to P210 *rd1* animals where it appears that functional changes have occurred but massive remodeling of the inner retina has not taken place.

Single cell recordings from bipolar cells isolated from dissociated *rd1* retina show no evidence of having intrinsic pacemaker activity that gives rise to spontaneous fluctuations in membrane potential [30]. This indicates that the rhythmic synaptic input to RGCs does not originate in bipolar cells suggesting instead that it first arises in a subset of amacrine cells. The underlying circuitry must, however, also include bipolar cells, since amacrine cell synaptic output is inhibitory and mediated by release of either GABA or glycine while the rhythmic synaptic input that drives ganglion hyperactivity is blocked by CNQX and is thus glutamatergic (Figure 1). A retinal circuit (Figure 2) that could give rise to the observed rhythmic spike discharge in ON and OFF RGCs begins with

(a)

(b)
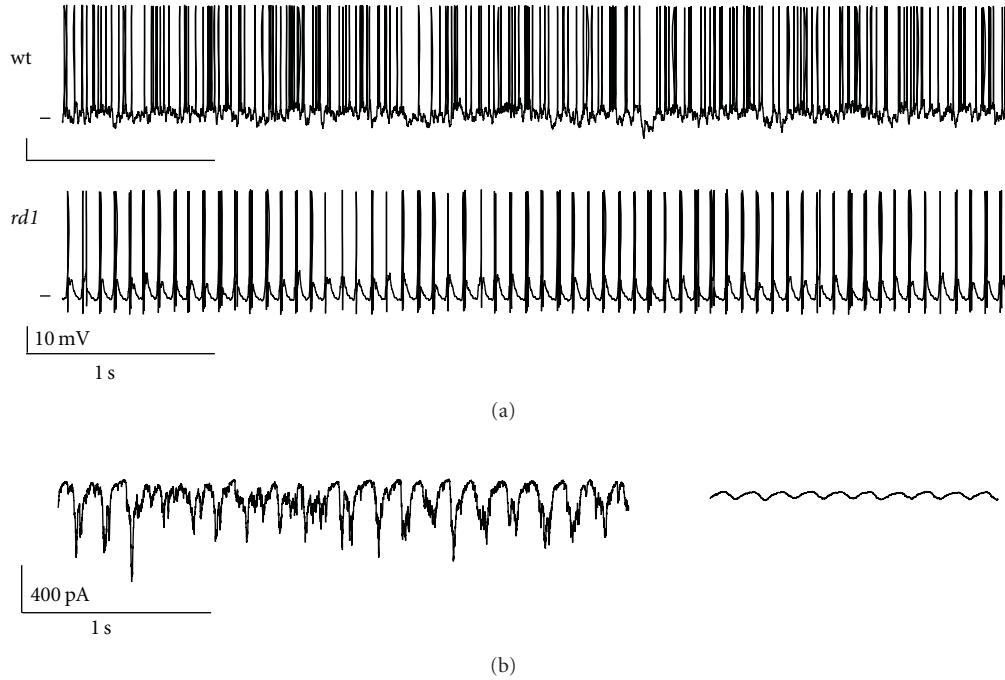
Figure 1: Spontaneous activity in *rd1* alpha ganglion cell. (a) Whole-cell current clamp recordings of ongoing spiking activity in wild-type (top) and *rd1* (bottom) ON-type retinal ganglion cells. Horizontal tick mark at left indicates −60 mV for wt and −70 mV for rd1 cells, respectively. (b) Whole-cell voltage clamp recording of ongoing synaptic currents in an *rd1* ON ganglion cell before (left) and after (right) bath application of CNQX.
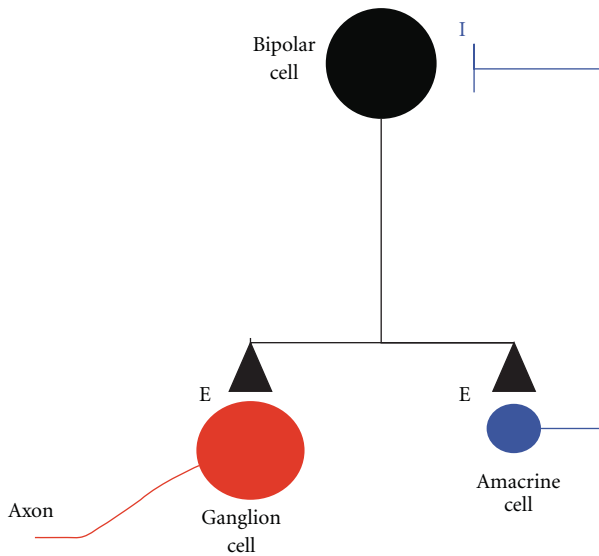
Figure 2: Retinal circuit that may give rise to spontaneous ganglion cell spike activity. The membrane potential of the amacrine cell oscillates spontaneously due to resonance (see text) which drives oscillatory release of inhibitory transmitter on to the bipolar cell causing oscillations in bipolar voltage triggering pulsatile release of excitatory transmitter on to the ganglion cell, causing rhythmic spike discharge, and the amacrine cell with negative feedback to the bipolar. The reverberating input to the ganglion cell arises from the presence of a negative feedback loop that includes a resonant oscillator.

an amacrine cell having the necessary intrinsic combination of ion conductances to produce resonant oscillations in membrane voltage [31]. This is not an unusual property to ascribe to a member of the amacrine cell population where spontaneous oscillations in current and voltage have been reported to occur in starburst [32], wide field [33] and dopaminergic [34, 35] amacrines. In this speculative circuit, subthreshold oscillations in amacrine cell voltage, with or without amplification by voltage-gated conductances, are postulated to trigger oscillatory changes in inhibitory transmitter release, which, in turn, generate oscillations in the membrane potential of the bipolar cells they are synaptically coupled to. The resulting periodic variation in bipolar cell potential gives rise to pulsatile glutamate release and rhythmic excitatory synaptic input to RGCs.

This hypothesized mechanism for oscillatory spike discharge could be tested by recording from bipolar cells in an intact dystrophic retina to determine if rhythmic changes in membrane voltage are present and sensitive to inhibitory synaptic blockade. Note that the proposed circuit cannot be rejected solely on the basis of finding no evidence of periodic fluctuations in baseline voltage in recordings from bipolar cells in retinal slices [36]. In such a preparation, there are affiliated uncertainties about whether the cellular connections required for rhythmic synaptic interactions have been disrupted in the process of slicing the retina. If the proposed feedback circuitry drives rhythmic spike activity in the *rd1* retina, it might be expected that oscillations in spike

(a)   (b)
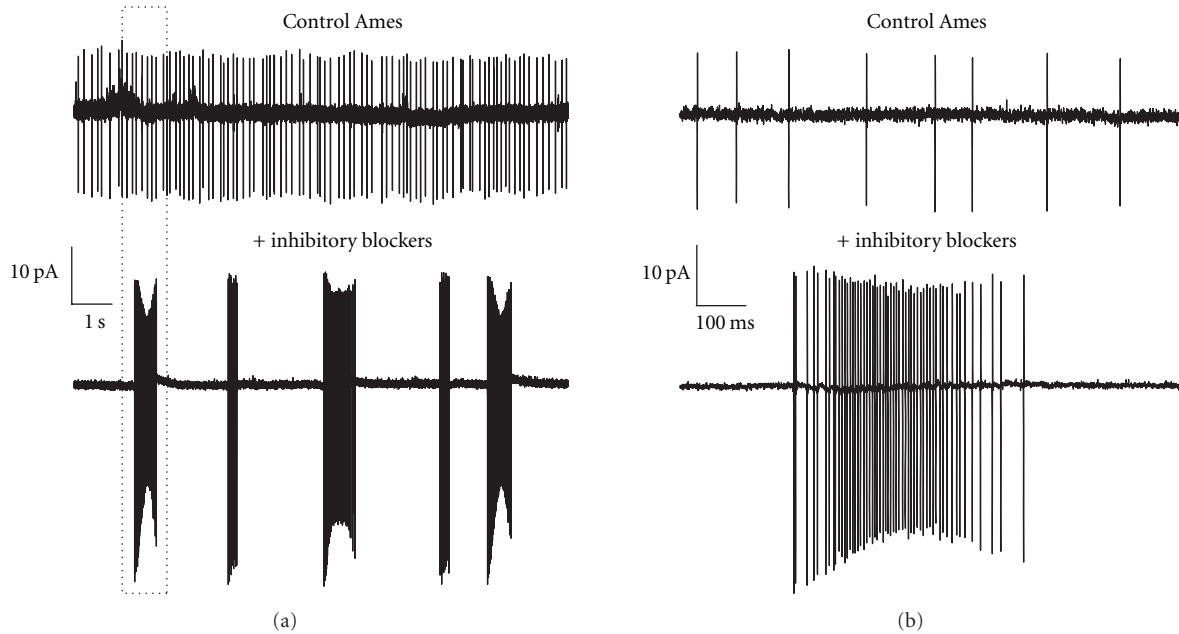
Figure 3: Loose patch extracellular recording of spontaneous spike activity from wild-type (non-dystrophic) retinal ganglion cell perfused with Control Ames solution without (top traces (a) and (b)) and with (lower traces (a) and (b)) the addition of mixture of inhibitory synaptic blockers containing 40 uM Gabazine, 50 uM TPMPA, and 1 uM Strychnine. Boxed region outlined by the dash lines in (a) are shown on a faster time in (b). The trace shown on an expanded time scale in control Ames (b) was taken from a region of trace in (a) that was shifted to the right to avoid the period of baseline instability.

discharge would not be confined to local spatial areas, but would instead be rather widespread. Stasheff [21], however, did not find evidence of correlations in spiking between pairs of ganglion cells. This either suggests that ganglion cells are in fact independent, or that correlations exist but only on a spatial scale smaller than the $200\,\mu$m spacing of the electrode array that the study made use of.

5. Oscillations Arising from Resonance in a Feedback Loop

In the proposed circuit the oscillations that give rise to rhythmic RGC spike discharge originate in an unidentified amacrine cell as a result of photoreceptor death and deafferentation. In this scenario, it is the loss of photoreceptor synaptic input that unbalances the circuitry of the normal retina and in so doing exposes the resonant membrane properties of an amacrine cell that is normally held in check in the functionally intact retina. Resonance is a consequence of the interactions between the active and passive membrane properties of a cell [24] that effectively combines a high-pass filter, arising from the presence of an active, that is, voltage-dependent, conductance [37], and a low pass filter that is an inherent consequence of the cell's passive membrane properties. The interplay between the two filters produces the equivalent of a notch filter that passes inputs with a select frequency band and rejects inputs with frequencies outside its band-pass. Changes in the input to the cell may influence the expression of resonant behavior and the generation of

oscillations in two ways by changing the active and passive membrane properties that set the resonant frequency and by shifting the frequency of the input relative to the band-pass of the resonant filter, which under the right conditions can generate reverberating activity in a negative feedback loop. As anyone who has attempted to build an electronic feedback amplifier knows, the output of a circuit like the one we have proposed is much more likely to be oscillatory than stationary. Similarly, the output of a neural network, with multiple synaptic feedback loops, such as the retina, is particularly prone to oscillations. This notion is supported by recent results in non-dystrophic mouse retina showing that in the presence of a mixture of inhibitory synaptic blockers RGCs generate spontaneous bursts of spikes (Figure 3) that are eliminated by addition of CNQX, showing that the periodic bursts of activity are produced by excitatory synaptic input (Newkirk and Detwiler unpublished observations). These observations suggest that the synaptic circuitry in the healthy retina is critically tuned to establish a balance between excitation and inhibition in a way that minimizes resonance and optimizes the dynamic range and response properties of the output cells, that is, the RGCs. Unbalanced synaptic interactions may also be the mechanistic explanation for the marked increase in spontaneous ganglion cell spike activity in transplanted retina [38].

6. Conclusions

The RP retina retains functional connections with the brain as shown originally by Drager and Hubel [16] who found

that the 10 Hz rhythmic spike discharges they recorded from the optic track of *rd1* mice were also present in single unit recordings in the visual cortex. Thus it is likely that the increased level of spontaneous activity that has been described in animals models of RP is also be present in patients with degenerative retinal disease and may participate in the generation of the phantom visual images that are reported by some RP patients [39–41]. These sensations are not continuous, as one might expect they would be if produced by sustained rhythmic spike activity. They are described as being intermittent, as if produced by "lights" that twinkle, flash, or shimmer. This, however, does not rule out the possibility that spontaneous RGC spike activity is the underlying substrate for this phenomenon that when processed by normal or *rd1*-modified CNS circuitry gives rise to discontinuous visual sensations. In any case uncontrolled spontaneous spike activity would be expected to degrade the action potential encoded messages RGCs send to the brain and thus hinder attempts to restore vision using electrical or optical prosthetics designed to directly evoke RGC spike trains that the brain can interpret as meaningful visual information.

Research designed to evaluate the treatment of RP using electronic or optical retinal prosthetics has not considered the influence that increased spontaneous RGC spike discharge might have on the successful use of prosthetics. While electrical stimulation of the retina in blind subjects can evoke the sensation of light and provide a rudimentary means of detecting motion, it has not been possible to use this approach to elicit the complex pattern percepts that are associated with more robust visual function [2]. Whether this has to do with the degradation of the retinal output signal by increased "noise" due to maintained rhythmic spike activity is not known but worthy of further investigation. Thus far studies focused on optical prosthetics have demonstrated that genetic incorporation of light-sensitive proteins, which included either melanopsin [5] or channelrhodopsin-2 alone [3, 4, 9] or coexpressed with halorhodopsin [7, 8], can restore light-evoked spike production in RGCs in animal models of RP; they have not been attempted in human subjects. Here again, however, the influence of increased spontaneous activity has not been addressed, but will need to be considered in order for treatments based on optical prosthetics to be optimized.

Acknowledgments

References

[1] A. N. Bramall et al., "The genomic, biochemical, and cellular responses of the retina in inherited photoreceptor degenerations and prospects for the treatment of these disorders," *Annual Review of Neuroscience*, vol. 33, pp. 441–472, 2010.

[2] J. D. Weiland, W. Liu, and M. S. Humayun, "Retinal prosthesis," *Annual Review of Biomedical Engineering*, vol. 7, pp. 361–401, 2005.

[3] A. Bi, J. Cui, Y.-P. Ma et al., "Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration," *Neuron*, vol. 50, no. 1, pp. 23–33, 2006.

[4] P. S. Lagali, D. Balya, G. B. Awatramani et al., "Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration," *Nature Neuroscience*, vol. 11, no. 6, pp. 667–675, 2008.

[5] B. Lin, A. Koizumi, N. Tanaka, S. Panda, and R. H. Masland, "Restoration of visual function in retinal degeneration mice by ectopic expression of melanopsin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 41, pp. 16009–16014, 2008.

[6] H. Tomita, E. Sugano, H. Yawo et al., "Restoration of visual response in aged dystrophic RCS rats using AAV-mediated channelopsin-2 gene transfer," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 8, pp. 3821–3826, 2007.

[7] Y. Zhang, E. Ivanova, A. Bi, and Z.-H. Pan, "Ectopic expression of multiple microbial rhodopsins restores ON and OFF light responses in retinas with photoreceptor degeneration," *Journal of Neuroscience*, vol. 29, no. 29, pp. 9186–9196, 2009.

[8] V. Busskamp, J. Duebel, D. Balya et al., "Genetic reactivation of cone photoreceptors restores visual responses in retinitis pigmentosa," *Science*, vol. 329, no. 5990, pp. 413–417, 2010.

[9] S. Thyagarajan, M. Van Wyk, K. Lehmann, S. Löwel, G. Feng, and H. Wässle, "Visual function in mice with photoreceptor degeneration and transgenic expression of channelrhodopsin 2 in ganglion cells," *Journal of Neuroscience*, vol. 30, no. 26, pp. 8745–8758, 2010.

[10] B. W. Jones, C. B. Watt, J. M. Frederick et al., "Retinal remodeling triggered by photoreceptor degenerations," *Journal of Comparative Neurology*, vol. 464, no. 1, pp. 1–16, 2003.

[11] R. E. Marc, B. W. Jones, J. R. Anderson et al., "Neural reprogramming in retinal degeneration," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 7, pp. 3364–3371, 2007.

[12] R. E. Marc, B. W. Jones, C. B. Watt, and E. Strettoi, "Neural remodeling in retinal degeneration," *Progress in Retinal and Eye Research*, vol. 22, no. 5, pp. 607–655, 2003.

[13] E. Strettoi, V. Pignatelli, C. Rossi, V. Porciatti, and B. Falsini, "Remodeling of second-order neurons in the retina of rd/rd mutant mice," *Vision Research*, vol. 43, no. 8, pp. 867–877, 2003.

[14] E. Strettoi, V. Porciatti, B. Falsini, V. Pignatelli, and C. Rossi, "Morphological and functional abnormalities in the inner retina of the rd/rd mouse," *Journal of Neuroscience*, vol. 22, no. 13, pp. 5492–5504, 2002.

[15] E. Strettoi and V. Pignatelli, "Modifications of retinal neurons in a mouse model of retinitis pigmentosa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 20, pp. 11020–11025, 2000.

[16] U. C. Drager and D. H. Hubel, "Studies of visual function and its decay in mice with hereditary retinal degeneration," *Journal of Comparative Neurology*, vol. 180, no. 1, pp. 85–114, 1978.

[17] Y. Sauvé, S. V. Girman, S. Wang, J. M. Lawrence, and R. D. Lund, "Progressive visual sensitivity loss in the Royal College of Surgeons rat: perimetric study in the superior colliculus," *Neuroscience*, vol. 103, no. 1, pp. 51–63, 2001.

[18] B. Lu, P. Coffey, and R. Lund, "Increased c-Fos-like immunoreactivity in the superior colliculus and lateral geniculate nucleus of the rd mouse," *Brain Research*, vol. 1025, no. 1-2, pp. 220–225, 2004.

[19] B. Lu, R. D. Lund, and P. J. Coffey, "Basal increase in c-Fos-like expression in superior colliculus of Royal College of Surgeons dystrophic rats can be abolished by intraocular injection of tetrodotoxin," *Neuroscience*, vol. 107, no. 1, pp. 109–115, 2001.

[20] M. Pu, L. Xu, and H. Zhang, "Visual response properties of retinal ganglion cells in the royal college of surgeons dystrophic rat," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 8, pp. 3579–3585, 2006.

[21] S. F. Stasheff, "Emergence of sustained spontaneous hyperactivity and temporary preservation of OFF responses in ganglion cells of the retinal degeneration (rd1) mouse," *Journal of Neurophysiology*, vol. 99, no. 3, pp. 1408–1421, 2008.

[22] C. Sekirnjak, C. Hulse, L. H. Jepson et al., "Loss of responses to visual but not electrical stimulation in ganglion cells of rats with severe photoreceptor degeneration," *Journal of Neurophysiology*, vol. 102, no. 6, pp. 3260–3269, 2009.

[23] D. J. Margolis, G. Newkirk, T. Euler, and P. B. Detwiler, "Functional stability of retinal ganglion cells after degeneration-induced changes in synaptic input," *Journal of Neuroscience*, vol. 28, no. 25, pp. 6526–6536, 2008.

[24] D. J. Margolis and P. B. Detwiler, "Different mechanisms generate maintained activity in ON and OFF retinal ganglion cells," *Journal of Neuroscience*, vol. 27, no. 22, pp. 5994–6005, 2007.

[25] T. Euler, S. E. Hausselt, D. J. Margolis et al., "Eyecup scope-optical recordings of light stimulus-evoked fluorescence signals in the retina," *Pflugers Archiv European Journal of Physiology*, vol. 457, no. 6, pp. 1393–1414, 2009.

[26] F. Mazzoni, E. Novelli, and E. Strettoi, "Retinal ganglion cells survive and maintain normal dendritic morphology in a mouse model of inherited photoreceptor degeneration," *Journal of Neuroscience*, vol. 28, no. 52, pp. 14282–14292, 2008.

[27] D. J. Margolis, A. J. Gartland, T. Euler, and P. B. Detwiler, "Dendritic calcium signaling in on and off mouse retinal ganglion cells," *Journal of Neuroscience*, vol. 30, no. 21, pp. 7127–7138, 2010.

[28] B. W. Jones and R. E. Marc, "Retinal remodeling during retinal degeneration," *Experimental Eye Research*, vol. 81, no. 2, pp. 123–137, 2005.

[29] R. E. Marc and B. W. Jones, "Retinal remodeling in inherited photoreceptor degenerations," *Molecular Neurobiology*, vol. 28, no. 2, pp. 139–147, 2003.

[30] C. Varela, I. Igartua, E. J. De La Rosa, and P. De La Villa, "Functional modifications in rod bipolar cells in a mouse model of retinitis pigmentosa," *Vision Research*, vol. 43, no. 8, pp. 879–885, 2003.

[31] B. Hutcheon and Y. Yarom, "Resonance, oscillation and the intrinsic frequency preferences of neurons," *Trends in Neurosciences*, vol. 23, no. 5, pp. 216–222, 2000.

[32] J. Petit-Jacques, B. Völgyi, B. Rudy, and S. Bloomfield, "Spontaneous oscillatory activity of starburst amacrine cells in the mouse retina," *Journal of Neurophysiology*, vol. 94, no. 3, pp. 1770–1780, 2005.

[33] J. Vigh, E. Solessio, C. W. Morgans, and E. M. Lasater, "Ionic mechanisms mediating oscillatory membrane potentials in wide-field retinal amacrine cells," *Journal of Neurophysiology*, vol. 90, no. 1, pp. 431–443, 2003.

[34] A. Feigenspan, S. Gustincich, B. P. Bean, and E. Raviola, "Spontaneous activity of solitary dopaminergic cells of the retina," *Journal of Neuroscience*, vol. 18, no. 17, pp. 6776–6789, 1998.

[35] M. A. Steffen, C. A. Seay, B. Amini et al., "Spontaneous activity of dopaminergic retinal neurons," *Biophysical Journal*, vol. 85, no. 4, pp. 2158–2169, 2003.

[36] T. Puthussery, J. Gayet-Primo, S. Pandey, R. M. Duvoisin, and W. R. Taylor, "Differential loss and preservation of glutamate receptor function in bipolar cells in the rd10 mouse model of retinitis pigmentosa," *European Journal of Neuroscience*, vol. 29, no. 8, pp. 1533–1542, 2009.

[37] P. B. Detwiler, A. L. Hodgkin, and P. A. McNaughton, "Temporal and spatial characteristics of the voltage response of rods in the retina of the snapping turtle," *Journal of Physiology*, vol. 300, pp. 213–250, 1980.

[38] W. Radner, S. R. Sadda, M. S. Humayun, S. Suzuki, and E. De Juan Jr., "Increased spontaneous retinal ganglion cell activity in rd mice after neural retinal transplantation," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 9, pp. 3053–3058, 2002.

[39] J. F. Amos, "Differential diagnosis of common etiologies of photopsia," *Journal of the American Optometric Association*, vol. 70, no. 8, pp. 485–504, 1999.

[40] F. E. Lepore, "Spontaneous visual phenomena with visual loss: 104 patients with lesions of retinal and neural afferent pathways," *Neurology*, vol. 40, no. 3, part 1, pp. 444–447, 1990.

[41] T. Murtha and S. F. Stasheff, "Visual dysfunction in retinal and optic nerve disease," *Neurologic Clinics*, vol. 21, no. 2, pp. 445–481, 2003.

Review Article

Current Concepts in the Treatment of Retinitis Pigmentosa

Maria A. Musarella[1] and Ian M. MacDonald[2]

[1] *Department of Ophthalmology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA*
[2] *Department of Ophthalmology, Royal Alexandra Hospital, University of Alberta, 10240 Kingsway Avenue, Rm. 2319, Edmonton, Canada AB T5H 3V9*

Correspondence should be addressed to Ian M. MacDonald, macdonal@ualberta.ca

Inherited retinal degenerations, including retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA), affect 1 in 4000 individuals in the general population. A majority of the genes which are mutated in these conditions are expressed in either photoreceptors or the retinal pigment epithelium (RPE). There is considerable variation in the clinical severity of these conditions; the most severe being autosomal recessive LCA, a heterogeneous retinal degenerative disease and the commonest cause of congenital blindness in children. Here, we discuss all the potential treatments that are now available for retinal degeneration. A number of therapeutic avenues are being explored based on our knowledge of the pathophysiology of retinal degeneration derived from research on animal models, including: gene therapy, antiapoptosis agents, neurotrophic factors, and dietary supplementation. Technological advances in retinal implant devices continue to provide the promise of vision for patients with end-stage disease.

1. Retinitis Pigmentosa

Retinitis pigmentosa (RP) describes a heterogeneous group of inherited retinal dystrophies characterized by progressive photoreceptor cell degeneration that affects approximately 1 in 4000 in a general population [1]. The genetics of RP is varied; nonsyndromic cases may be inherited as an autosomal dominant (30%), autosomal recessive (20%), X-linked recessive (15%), or sporadic/simplex traits (30%), and 5% may be early-onset and grouped as part of Leber congenital amaurosis [2]. Rarer forms also exist: X-linked dominant, mitochondrial, and digenic (due to mutations in two different genes). While RP is a disease usually limited to the eye, it may occur as part of a syndrome; as examples, Usher syndrome and Bardet-Biedl syndrome. Approximately 20%–30% of patients with RP have an associated nonocular disease and would be classified as having syndromic RP. A list of nonsyndromic and syndromic RP is maintained through RetNet (http://www.sph.uth.tmc.edu/retnet/). A majority of the genes associated with RP are expressed in either the photoreceptors or the retinal pigment epithelium (RPE). There is considerable variation in the severity of these conditions; the most severe being recessively inherited conditions generally resulting in loss of function of an important protein in a pathway.

RP is characterized by progressive degeneration of the retina usually starting in the midperiphery of the fundus and advancing towards the macula and fovea. The most common form of RP is a rod-cone dystrophy in which night blindness is the first symptom, followed by progressive loss of peripheral visual field. Classic clinical findings include: bone spicule pigmentation or pigment clumping, retinal arteriolar narrowing, waxy pallor of the optic nerve, epiretinal membrane formation, atrophy of the RPE and choriocapillaris (starting at the midperiphery of the retina with preservation of the RPE in the macula until late in the disease), posterior subcapsular cataract, epiretinal membrane formation, and cystoid macular edema (CME) [1].

Potentially important findings can be obtained from ERG recordings. The term rod-cone dystrophy, commonly used to describe RP, denotes the predominant system affected by retinal degeneration (rod versus cone) and is reflected by the rod-driven responses of the ERG being more severely affected than cone-driven responses. Early in the disease, the rod ERG amplitude is affected more than the cones; and with progression, the rod and cone responses are "extinguished".

Visual field testing often reveals a mid-peripheral ring scotoma which enlarges peripherally and centrally as the disease progresses.

In the majority of cases, RP is an isolated disorder, but infrequently is associated with other systemic conditions for which treatment strategies have been implicated; for example, abetalipoproteinemia (MIM no. 200100) and Refsum disease (MIM no. 266500). Adult-onset Refsum disease is an autosomal recessive disorder of lipid metabolism caused by a deficiency of phytanic acid hydroxylase. Clinically, patients present in early childhood with cardiomyopathy, ichthyosis, neurologic diseases (polyneuritis, spinocerebellar ataxia, hearing loss, and loss of smell), and odd-shaped red blood cells. The ocular findings include: nystagmus, strabismus, pupillary abnormalities, cataract, and RP. Treatment requires dietary restriction of plant foods and milk which are sources of phytanic acid.

Abetalipoproteinemia or Bassen-Kornzweig syndrome (MIM no. 200100) is an autosomal recessive disorder in which there is abnormal absorption of fat and fat-soluble vitamins, A, D, E, and K. The signs and symptoms of abetalipoproteinemia appear in the first few months of life with failure to thrive, steatorrhea and acanthocytosis. Vitamin A deficiency may result and lead to retinal degeneration that is treatable with vitamin supplementation.

Leber congenital amaurosis (LCA), (MIM no. 204000) was first described in 1869 by Theodore Leber as a congenital form of RP [3, 4]. LCA is an autosomal recessive disorder that is genetically and clinically heterogeneous. LCA is the most severe inherited retinopathy and the most common cause of congenital blindness in children, accounting for 10%–18% of cases [3, 5, 6]. LCA has several phenotypes; symptoms or fundus findings within the first year of life may suggest a particular genotype [3, 7, 8]. Clinical features include: nyctalopia, photoaversion, eye poking (oculodigital sign), nystagmus, hyperopia, an abnormal fundus, and an abnormal ERG [3, 7].

At least 14 genes are associated with LCA and involve various pathways including: retinal development (*CRB1* and *CRX*), phototransduction, (*GUCY2D* and *AIPL1*), vitamin A metabolism (*RPE65*, *LRAT*, and *RDH12*), protein transport (*TULP1*, *RPGRIP1*, and *CEP290*), and RPE phagocytosis (*MERTK*) [8]. Together LCA and juvenile-onset retinal degeneration constitute 70% of cases of severe retinal degeneration or retinal dystrophy. Several of these genes have also been implicated in nonsyndromic or syndromic retinal diseases such as RP and Joubert syndrome, respectively. *CEP290* (15%), *GUCY2D* (12%), and *CRB1* (10%) are the most frequently genes found to be mutated in cases of LCA.

2. Gene Therapy

Gene therapy holds promise for a wide variety of inherited human disease. To date, ocular gene therapy (OGT) has been tried with success in mice, dogs, and now in some humans. OGT requires genetic modification of mutant ocular cells to produce a therapeutic effect. Retinal diseases are excellent targets of OGT as in many cases, the genetic etiology is understood, and there is access to the photoreceptors or the retinal pigment epithelium (RPE) by subretinal injection. In addition, both transgenic and knockout animal models are available that provide preclinical evidence of safety and efficacy. OGT requires first identifying the genetic cause of the RP, and then genotyping patients for mutations in that gene prior to enrolment in gene therapy trials.

Gene therapy strategies differ greatly depending on the inheritance of the disease or more accurately the type of mutation targeted. Some forms of RP are due to loss-of-function mutations (usually autosomal and X-linked recessive). For OGT to be effective, the therapy must replace the missing or insufficient gene product. For example, Tan and colleagues used adenoviral vectors to transduce two mouse models of RP/LCA due to aryl hydrocarbon receptor protein-like 1 (*Aipl1*) deficiency (hypomorphic mutant) and absence (null mutant), establishing the potential of gene replacement therapy in the human condition [9].

Human OGT is most advanced for the form of LCA associated with mutations in *RPE65* [10–14]. Preliminary studies in the Briard dog, a naturally occurring model of LCA (*rpe65-/-*), helped make clinical trials possible. A similar degeneration is seen in the Swedish-Briard/Briard-beagle due to a 4-base pair deletion in the *rpe65* gene [15]. The initial study of OGT in dogs was done in the USA [5, 6, 16] and later in France [17]. Surgical delivery of recombinant adenovirus associated vectors (AAV) carrying the wild type *rpe65* cDNA into the subretinal space of three affected dogs demonstrated efficacy as measured by improved ERG responses. The dogs' vision improved in the treated eye and has been stable after five years. More than fifty dogs have since been tested for their response to OGT. Successful gene therapy has also been demonstrated in mice with mutations in the *rpe65* gene [16]. The treatment rescued photoreceptors and also retinal function as measured by the ERG.

The results of separate human trials in the USA, UK, and Italy enrolling patients with mutations in the *RPE65* gene have been reported with encouraging results [11–14]. Bainbridge et al. [11] and Maguire et al. [14] first described separate clinical trials investigating the short-term safety and preliminary efficacy of OGT for LCA in humans. Both groups initially presented short-term data (12 and 5 months, resp.) on three LCA patients enrolled in trials of recombinant AAV delivery of the human *RPE65* gene into the subretinal space. In both studies, patients had severe vision loss documented by visual acuity testing and the ERG. Both studies showed some improvement in navigational testing in at least one patient. This outcome measure has yet to be accepted as a measure of functional visual improvement.

Bainbridge and colleagues studied their patients with microperimetry (which measured retinal sensitivity at precise locations in light-adapted conditions) and observed an improvement after gene therapy in one patient [11]. Maguire et al. observed visual field improvement using Goldmann perimetry and decreased nystagmus after treatment in all their three patients [14] whereas Bainbridge et al. only noted improvement in the dark-adapted perimetry of one patient [11]. Bainbridge et al. [11] showed no change in patients'

visual acuity whereas Maguire et al. [14] recorded a gain in visual acuity in all three patients in their study. These outcomes must be replicated with additional subjects and patients' function assessed long term. Further, if safety can be demonstrated, patients with better visual function at baseline should be included in future trials.

Maguire et al. employed the pupillary light reflex as an objective measure of retinal function and found improvement in each of the treated eyes [14]. The pupillary light reflex is a consensual response; a light stimulus to either eye will normally cause both pupils to contract. Fundamentally, it is a measure of the amount of signal input from the photoreceptors, interneurons, and ganglion cells, conveyed through an afferent arc to the brain, with the output driving bilateral pupil constriction. The pupillary response of patients with LCA is significantly diminished, consistent with decreased photoreceptor input to the afferent arc of the reflex [14, 18–20]. In a report of a total of 12 patients (age 8–44) who had undergone OGT for LCA, all had an improvement in the pupillary response, with the greatest effect seen in children [21].

Optical coherence tomography (OCT) allows a non-invasive measure of photoreceptor layer thickness in the central retina of LCA patients [22]. The topography of the photoreceptor layer based on OCT scans, with superimposed retinal landmarks, should be available to the retinal surgeon to guide the subretinal injection of AAV gene vectors. The response to treatment may also be measured with OCT. Photoreceptor loss in the fovea and extrafoveal retina has been shown to be prominent, even in the youngest LCA patient studied. As disease severity in LCA has a broad spectrum, detailed retinal imaging and mapping with OCT should be conducted in all candidates for LCA-*RPE 65* clinical trials, independent of age [23].

The ERG responses were extremely low or undetectable in patients in both studies at baseline and remained unchanged after treatment. Whether the improvements in retinal function are reproducible and persistent in subjects remain as questions along with whether retinal degeneration is delayed or averted. Systemic or ocular complications may yet be encountered as additional patients are treated with higher doses of vector and followed for longer periods.

Alternates to OGT for the treatment of LCA are also being pursued; for example, oral administration of a reti-noid, QLT091001 (NCT00765427, NCT01014052, see: http://www.clinicaltrials.gov/). Preliminary results, presented at the Association for Research on Vision in Ophthalmology meeting in May, 2010, suggested improved function in three LCA patients with *RPE65* and *LRAT* mutations.

For autosomal dominant RP, caused by gain-of-function mutations, effective therapy must either prevent the mutant protein from being produced or counter the expression of the protein. Ribozymes catalyze enzymatic reactions that break down RNA [24, 25]. Conceptually, it would, therefore, be possible to use ribozymes to treat autosomal dominant RP by blocking the gene product from the mutant allele, thereby halting or slowing the progression of the disease. In 1998, Drenser et al. [26] showed that ribozyme could be used to decrease the amount of mutant rhodopsin

messenger RNA. Later, the same group used recombinant AAV to transduce photoreceptor cells of rhodopsin mutant (pro23his) transgenic rats with ribozyme and an opsin promoter, demonstrating that ribozyme could slow photoreceptor degeneration. They showed that treatment was effective at age 1 month and 1.5 months when 40%–45% of photoreceptors would have normally degenerated [27, 28]. The pro23his mutation in rhodopsin represents a change from proline to histidine at position 23 and is the most common rhodopsin mutation in humans. By targeting only the mutant RNA sequence, ribozyme therapy is mutation-dependent and therefore limited in its application. Autosomal dominant RP is genetically heterogeneous; 25% of cases are caused by different mutations in rhodopsin and the remaining cases are not linked to rhodopsin. Unique gene therapies with a large number of ribozymes would have to be developed for each of these disorders.

RNA interference (RNAi) is mutation-independent and a powerful method for posttranslational gene silencing. In mammalian systems, small interfering RNAs (siRNAs) are introduced directly into the cell or processed in the cell from translated short hairpin RNA (shRNA) [29] and then assembled into an RNA-induced silencing complex known as RISC. RISC allows the antisense strand to form a duplex with the target messenger RNA which is then degraded by an enzyme, and then rendered inactive. Compared to ribozyme therapy, RNAi is at least as potent, less dependent on RNA secondary structure and does not require a particular sequence motif. RNAi has been used to identify genes that promote apoptosis or oxidative damage in retinal cells and could provide new avenues for treatment of photoreceptor degenerations [30, 31].

3. Retinal Implants

The treatment of RP patients with severe visual loss using either epiretinal or subretinal implants was reviewed recently by Margalit et al. [32]. Humayun et al. reported direct retinal stimulation using epiretinal implants in RP patients [33]. Using a 16-electrode array, patients saw spots of light that were usually colored (yellow/blue/yellow-green) and the direction of movement (http://www.artificial-retina.energy.gov/ and Second Sight Medical Products, Inc. Sylmar, CA). Resolution in this model is believed to be up to 1.8 degrees of visual field. At the 2009 annual meeting of the Association for Research on Vision in Ophthalmology, the Artificial Retina Project released an update on the Argus II, a 60-electrode retinal prosthesis. As of March 31, 2009, 21 people with RP had been implanted with the device; this number continues to rise as more subjects are enrolled in a Phase II, three year clinical trial. Although the Argus II prosthesis consists of an array of 60 electrodes attached to the retina, the project aims to increase the number of electrodes beyond 200.

Caspi et al. [34] used a 16 electrode retinal prosthesis in a totally blind subject with RP. The implant was controlled wirelessly by an external computer and head mounted video camera. Spatial vision was assessed by measuring the

subject's response to direct stimulation patterns and by comparing the ability of the subject to identify the orientation of gratings with the system on and off. Results showed that synchronized stimulation of different retinal locations could produce spatial vision long term with an acuity level determined by the distance between the electrodes.

Yanai et al. [35] assessed visual task performance in three subjects blinded by RP. An epiretinal prosthesis was implanted in the eye with worse vision and the input was wirelessly controlled by a computer or head-worn video camera. Subjects scored better in 8 of 9 computer-controlled experiments. This study, although small in size, suggested that a low-resolution, epiretinal prosthesis could provide visual information to perform simple tasks that were impossible with only light perception vision.

Subretinal electrodes have been attempted in animal models and the results indicate that cortical activity can be induced [36, 37]. Similar experiments have since been initiated in humans [38]. The long-term effect of the implants has not been assessed, nor has the effect of the electrodes placed between the neuroretina and the retinal pigment epithelium on retinal metabolic function.

4. Neurotrophic Factors

Several neuorotrophic factors have been shown to protect photoreceptors from degeneration, including ciliary neurotrophic factor (CNTF). In different animal models of retinal degeneration, CNTF was shown to delay photoreceptor degeneration [27, 39–42]. A thicker outer nuclear layer was observed in treated animals, reflecting preservation of the photoreceptors and anatomical rescue. Electrophysiological recordings performed to evaluate retinal function demonstrated an improvement in the scotopic and photopic responses recorded from CNTF-treated eyes compared to untreated eyes [39, 41]. Glial cell line-derived neurotrophic factor (GDNF) has also been shown to have a neuroprotective effect on degenerating photoreceptors by slowing down the degeneration of rods while preserving visual function [42].

While neuroprotective factors may offer promising results in the treatment of RP in animal models, effective treatment strategies need to be developed for clinical delivery. Direct intravitreal or subretinal neurotrophic factor injections have been performed in animal models with therapeutic effect; [43] however, an implantable device allows for long term delivery avoiding repeated injections with the risk of mechanical or infectious complications.

Ex vivo gene therapy is a promising approach whereby genetically engineered and encapsulated human retinal pigment epithelial cells are implanted into the vitreous in a device [44]. A Phase I safety trial of the delivery of CNTF through encapsulated cell therapy was completed on patients with RP (one of which had choroideremia) without serious adverse event and some suggestion of improvement in visual acuity. A Phase II trial are currently underway and designed to show efficacy in treating atrophic macular degeneration and RP [45].

5. Retinal Transplantation

Retinal transplantation places sheets of developing retina and retinal pigment epithelial cells into the subretinal space [46]. Whereas adult transplants have been performed in humans with RP and age-related macular degeneration (AMD); [47] the transplants have not caused harm but there is no evidence that the cells of the transplanted tissue mingle with or develop synaptic connections. Radtke and his group reported efficacy and safety in implanting fetal retina with accompanying RPE in AMD and RP patients with vision of 20/200. Seven of the ten patients showed improved visual acuity, corroborating results in animal models of retinal degeneration [48].

An alternate approach may be the transplantation of photoreceptor precursors. MacLaren and colleagues demonstrated that the timing of the harvest of the donor cells must be at the correct stage of rod morphogenesis, when they have exited the cell cycle and are in the first stages towards becoming mature photoreceptors [49]. If the cells were isolated just a couple of days too early or too late, they would not integrate into the retina. When successful, the treated eye showed an improved pupillary light response suggesting that the transplanted cells were responsive to light and had integrated into the retinal circuitry connecting to the central nervous system.

Lamba et al. incubated human embryonic stem cells in a complex cocktail that coaxed cells into becoming photoreceptor progenitors [50]. These progenitors, like the *in vivo* derived progenitors described by MacLaren et al., were able to integrate into degenerated mouse retinas [49].

It may be possible to prepare unlimited numbers of progenitor cells that are suitable for transplantation regardless of whether donor progenitor cells are isolated from adult tissue or from embryonic stem cells. How can one ensure a sufficient number of stem cells that are available for an effective graft? MacLaren et al. showed that it is not necessary to integrate each precursor cell with each secondary neuron to achieve a therapeutic effect [49]. Also, it may not be necessary to treat the entire retina; treatment of the macula alone may suffice.

6. Stem Cells

Enzmann and colleagues have reviewed the use of stem cells, their plasticity, their ability to give rise to specialized cells, and their capacity for self-renewal [51]. Lund and coworkers have derived RPE cells that are critical to the health of photoreceptors from human embryonic stem cells [52]. The RPE cells were then transplanted into rats with retinal degenerative disease. The investigators reported that the improvement in vision of treated rats was 100% over untreated controls. Although the RPE cells were not sufficiently developed to completely replace the damaged RPE, they were able to rescue vision by the long-term production of growth factors beneficial to the health of the retina. Lund and his collaborators are proceeding to produce entirely functional RPE and photoreceptors from stem cells to replace and repair degenerated retinas in humans.

7. Light Protection

Clinical evidence and data from animal studies suggest that some pigmentary retinopathies are particularly susceptible to light damage [53]. Patients with RP are advised to wear dark glasses outdoors. The use of amber spectacles should block ultraviolet rays and visible wavelengths up to about 527 nm. Outdoors, it is ideal to use spectacles that block ultraviolet rays and light up to approximately 550 nm to filter blue light.

8. Vitamin Therapy

Vitamin A may protect the photoreceptors by trophic and antioxidant effects. Long-term (5 to 15 year) vitamin A supplementation in doses of 15,000 IU per day slowed down the loss of ERG amplitudes [54]. Vitamin E at 4,000 IU had an adverse effect [54]. Clinicians continue to debate the conclusions of these studies [55]. There is no consensus about the utility of vitamin A treatment. Vitamin A should not be given to patients with RP caused by mutations in the *ABCA4* gene. In another study, RP patients were given docosahexaenoic acid (DHA) supplementation at 1200 mg/day in addition to vitamin A [56]. This study showed that the disease course was initially slowed by the addition of DHA; however, the beneficial effect did not last beyond two years. Berson and colleagues have reported on the benefits to RP patients of a diet rich in omega-3 fatty acids [57]. RP patients taking vitamin A palmitate, but not DHA capsules, benefited from an omega-3 rich diet (equivalent to eating salmon, tuna, mackerel, herring, or sardines, once to two times a week). Recently, Berson and colleagues reported on patients taking Vitamin A randomly assigned to either lutein supplementation (12 mg/da) or placebo over a four year period [58]. Lutein appeared to slow the decline in the mean rate of sensitivity loss as measured by the Humphrey visual field 60-test. An accompanying article in the same journal discussed carefully the merits of all these studies [59].

A study of the potential benefit of DHA in patients with X-linked RP is ongoing [60]. Patients between the ages of 8 and 32 who have X-linked RP are enrolled in a four year, Phase II, clinical trial studying the effect of nutritional supplementation with DHA. DHA is a component of cell membranes throughout the body, and most highly concentrated in the retina and the brain where it plays a role in phototransduction and synaptic transmission [47].

9. Drug Delivery

A group of international experts in drug delivery are studying the treatment of retinal degenerative diseases by long-term, sustained drug delivery through the sclera [61–64]. They are also investigating a range of delivery devices such as microneedles, collagen gels, and the use of an electric field. Better methods of drug delivery could be crucial for future therapies to save or restore sight.

10. Macular Edema

Cystoid macular edema (CME), which occurs frequently in RP patients, is often chronic and may not improve with carbonic anhydrase inhibitors [65, 66]. A trial of therapy over two months may instituted, and if effective, should be continued indefinitely; if no response is seen with treatment, it should be discontinued. Care must be taken when considering long-term acetazolamide as it has been shown to depress the ERG responses in mice [67]. In a small trial of 20 treated RP patients and 20 matched untreated RP controls, intravitreal triamcinolone (4 mg) did not result in a statistically significant improvement in best corrected visual acuity [68].

11. Conclusion

Therapies are becoming available to restore vision or stop the progressive loss of visual function caused by pigmentary retinopathies. The psychological boost to researchers, patients, and families from the results of LCA gene therapy trials is very evident. Therapeutic strategies are being designed and applied to slow down the degenerative process, to treat ocular complications, and to help with the social and psychological impact of blindness resulting from RP. Approaches to therapy for RP now include: gene therapy, neurotrophic growth factors, anti-apoptotic agents, ribozyme therapy, RNAi, retinal transplantation, dietary supplementation, retinal prostheses, and stem cell therapy. We hope that, in the future, discoveries from the laboratory will be brought into the clinical setting.

12. Method of Literature Search

References for this paper were identified through a comprehensive English-language literature search of the electronic Medline database (1993–2009), using the Medline search service. Search of other databases did not add to the search of Medline. The following key words were used alone or in combination: retinitis pigmentosa, rod-cone dystrophy, RP, RNAi, neurotrophic growth factors, encapsulated cellular therapy, ciliary neurotrophic factor, anti-apoptosis, genes, bionic eye, precursor photoreceptor transplantation, optical aids, cystoid macular edema, Leber congenital amaurosis, retinal cell transplantation, precursor photoreceptors, treatment, and Vitamin A.

Conflict of Interests

The authors have no proprietary or commercial interest in any product mentioned or perhaps discussed in this paper.

Websites

(1) Artificial Retina Project, US Department of Energy Office of Science. http://www.artificialretina.energy .gov/

(2) Daiger, S. RetNet. http://www.sph.uth.tmc.edu/retnet/sum-dis.htm/

(3) Clinical trials: http://www.clinicaltrials.gov/

Acknowledgments

References

[1] C. Hamel, "Retinitis pigmentosa," *Orphanet Journal of Rare Diseases*, vol. 1, no. 1, article no. 40, 2006.

[2] S. P. Daiger, S. J. Bowne, and L. S. Sullivan, "Perspective on genes and mutations causing retinitis pigmentosa," *Archives of Ophthalmology*, vol. 125, no. 2, pp. 151–158, 2007.

[3] R. K. Koenekoop, "An overview of leber congenital amaurosis: a model to understand human retinal development," *Survey of Ophthalmology*, vol. 49, no. 4, pp. 379–398, 2004.

[4] T. Leber, "Ueber retinitis pigmentosa angehorene Amaurixe," *Albrecht von Greferarch Ophthol*, vol. 15, pp. 1–25, 1869.

[5] G. M. Acland, G. D. Aguirre, J. Ray et al., "Gene therapy restores vision in a canine model of childhood blindness," *Nature Genetics*, vol. 28, no. 1, pp. 92–95, 2001.

[6] G. M. Acland, G. D. Aguirre, J. Bennett et al., "Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness," *Molecular Therapy*, vol. 12, no. 6, pp. 1072–1082, 2005.

[7] D. C. Chung and E. I. Traboulsi, "Leber congenital amaurosis: clinical correlations with genotypes, gene therapy trials update, and future directions," *Journal of AAPOS*, vol. 13, no. 6, pp. 587–592, 2009.

[8] A. I. den Hollander, R. Roepman, R. K. Koenekoop, and F. P. M. Cremers, "Leber congenital amaurosis: genes, proteins and disease mechanisms," *Progress in Retinal and Eye Research*, vol. 27, no. 4, pp. 391–419, 2008.

[9] M. H. Tan, A. J. Smith, B. Pawlyk et al., "Gene therapy for retinitis pigmentosa and Leber congenital amaurosis caused by defects in AIPL1: effective rescue of mouse models of partial and complete *Aipl1* deficiency using AAV2/2 and AAV2/8 vectors," *Human Molecular Genetics*, vol. 18, no. 12, pp. 2099–2114, 2009.

[10] E. Ahmed and J. Loewenstein, "Leber congenital amaurosis: disease, genetics and therapy," *Seminars in Ophthalmology*, vol. 23, no. 1, pp. 39–43, 2008.

[11] J. W. B. Bainbridge, A. J. Smith, S. S. Barker et al., "Effect of gene therapy on visual function in Leber's congenital amaurosis," *New England Journal of Medicine*, vol. 358, no. 21, pp. 2231–2239, 2008.

[12] S. G. Jacobson, S. L. Boye, T. S. Aleman et al., "Safety in nonhuman primates of ocular AAV2-RPE65, a candidate treatment for blindness in Leber congenital amaurosis," *Human Gene Therapy*, vol. 17, no. 8, pp. 845–858, 2006.

[13] W. W. Hauswirth, T. S. Aleman, S. Kaushal et al., "Treatment of Leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial," *Human Gene Therapy*, vol. 19, no. 10, pp. 979–990, 2008.

[14] A. M. Maguire, F. Simonelli, E. A. Pierce et al., "Safety and efficacy of gene transfer for Leber's congenital amaurosis," *New England Journal of Medicine*, vol. 358, no. 21, pp. 2240–2248, 2008.

[15] A. Veske, S. E. G. Nilsson, K. Narfström, and A. Gal, "Retinal dystrophy of Swedish briard/briard-beagle dogs is due to a 4-bp deletion in RPE65," *Genomics*, vol. 57, no. 1, pp. 57–61, 1999.

[16] J. Bennicelli, J. F. Wright, A. Komaromy et al., "Reversal of blindness in animal models of leber congenital amaurosis using optimized AAV2-mediated gene transfer," *Molecular Therapy*, vol. 16, no. 3, pp. 458–465, 2008.

[17] M. Weber, J. Rabinowitz, N. Provost et al., "Recombinant adeno-associated virus serotype 4 mediates unique and exclusive long-term transduction of retinal pigmented epithelium in rat, dog, and nonhuman primate after subretinal delivery," *Molecular Therapy*, vol. 7, no. 6, pp. 774–781, 2003.

[18] T. S. Aleman, A. V. Cideciyan, A. Sumaroka et al., "Retinal laminar architecture in human retinitis pigmentosa caused by Rhodopsin gene mutations," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 4, pp. 1580–1591, 2008.

[19] T. S. Aleman, S. G. Jacobson, J. D. Chico et al., "Impairment of the transient pupillary light reflex in Rpe65$^{-/-}$ mice and humans with leber congenital amaurosis," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 4, pp. 1259–1271, 2004.

[20] J. W. Miller, "Preliminary results of gene therapy for retinal degeneration," *New England Journal of Medicine*, vol. 358, no. 21, pp. 2282–2284, 2008.

[21] A. M. Maguire, K. A. High, A. Auricchio et al., "Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial," *The Lancet*, vol. 374, no. 9701, pp. 1597–1605, 2009.

[22] S. G. Jacobson, T. S. Aleman, A. V. Cideciyan et al., "Identifying photoreceptors in blind eyes caused by RPE65 mutations: prerequisite for human gene therapy success," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 17, pp. 6177–6182, 2005.

[23] S. G. Jacobson, A. V. Cideciyan, T. S. Aleman et al., "Photoreceptor layer topography in children with Leber congenital amaurosis caused by RPE65 mutations," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 10, pp. 4573–4577, 2008.

[24] C. Hammann and D. M. J. Lilley, "Folding and activity of the hammerhead ribozyme," *ChemBioChem*, vol. 3, no. 8, pp. 690–700, 2002.

[25] C. Hammann and E. Westhof, "Searching genomes for ribozymes and riboswitches," *Genome Biology*, vol. 8, no. 4, article no. 210, 2007.

[26] K. A. Drenser, A. M. Timmers, W. W. Hauswirth, and A. S. Lewin, "Ribozyme-targeted destruction of RNA associated with autosomal-dominant retinitis pigmentosa," *Investigative Ophthalmology and Visual Science*, vol. 39, no. 5, pp. 681–689, 1998.

[27] M. M. LaVail, D. Yasumura, M. T. Matthes et al., "Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 21, pp. 11488–11493, 2000.

[28] A. S. Lewin, K. A. Drenser, W. W. Hauswirth et al., "Ribozyme rescue of photoreceptor cells in a transgenic rat model of

autosomal dominant retinitis pigmentosa," *Nature Medicine*, vol. 4, no. 8, pp. 967–971, 1998.

[29] T. R. Brummelkamp, R. Bernards, and R. Agami, "A system for stable expression of short interfering RNAs in mammalian cells," *Science*, vol. 296, no. 5567, pp. 550–553, 2002.

[30] P. A. Campochiaro, "Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders," *Gene Therapy*, vol. 13, no. 6, pp. 559–562, 2006.

[31] J. Shen, X. Yang, A. Dong et al., "Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa," *Journal of Cellular Physiology*, vol. 203, no. 3, pp. 457–464, 2005.

[32] E. Margalit, M. Maia, J. D. Weiland et al., "Retinal prosthesis for the blind," *Survey of Ophthalmology*, vol. 47, no. 4, pp. 335–356, 2002.

[33] M. S. Humayun and E. De Juan Jr., "Artificial vision," *Eye*, vol. 12, no. 3, pp. 605–607, 1998.

[34] A. Caspi, J. D. Dorn, K. H. McClure, M. S. Humayun, R. J. Greenberg, and M. J. McMahon, "Feasibility study of a retinal prosthesis: spatial vision with a 16-electrode implant," *Archives of Ophthalmology*, vol. 127, no. 4, pp. 398–401, 2009.

[35] D. Yanai, J. D. Weiland, M. Mahadevappa, R. J. Greenberg, I. Fine, and M. S. Humayun, "Visual performance using a retinal prosthesis in three subjects with retinitis pigmentosa," *American Journal of Ophthalmology*, vol. 143, no. 5, pp. 820–827, 2007.

[36] A. Y. Chow and V. Y. Chow, "Subretinal electrical stimulation of the rabbit retina," *Neuroscience Letters*, vol. 225, no. 1, pp. 13–16, 1997.

[37] E. Zrenner, K.-D. Miliczek, V. P. Gabel et al., "The development of subretinal microphotodiodes for replacement of degenerated photoreceptors," *Ophthalmic Research*, vol. 29, no. 5, pp. 269–280, 1997.

[38] D. Besch, H. Sachs, P. Szurman et al., "Extraocular surgery for implantation of an active subretinal visual prosthesis with external connections: feasibility and outcome in seven patients," *British Journal of Ophthalmology*, vol. 92, no. 10, pp. 1361–1368, 2008.

[39] D. Bok, D. Yasumura, M. T. Matthes et al., "Effects of adeno-associated virus-vectored ciliary neurotrophic factor on retinal structure and function in mice with a P216L rds/peripherin mutation," *Experimental Eye Research*, vol. 74, no. 6, pp. 719–735, 2002.

[40] F.-Q. Liang, N. S. Dejneka, D. R. Cohen et al., "AAV-mediated delivery of ciliary neurotrophic factor prolongs photoreceptor survival in the rhodopsin knockout mouse," *Molecular Therapy*, vol. 3, no. 2, pp. 241–248, 2001.

[41] F.-Q. Liang, T. S. Aleman, N. S. Dejneka et al., "Long-term protection of retinal structure but not function using rAAV.CNTF in animal models of retinitis pigmentosa," *Molecular Therapy*, vol. 4, no. 5, pp. 461–472, 2001.

[42] M. Frasson, S. Picaud, T. Léveillard et al., "Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 11, pp. 2724–2734, 1999.

[43] Y. Li, W. Tao, L. Luo et al., "CNTF induces regeneration of cone outer segments in a rat model of retinal degeneration," *PLoS ONE*, vol. 5, no. 3, article no. e9495, 2010.

[44] W. Tao, R. Wen, M. B. Goddard et al., "Encapsulated cell-based delivery of CNTF reduces photoreceptor degeneration in animal models of retinitis pigmentosa," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 10, pp. 3292–3298, 2002.

[45] P. A. Sieving, R. C. Caruso, W. Tao et al., "Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 10, pp. 3896–3901, 2006.

[46] S. Tao, C. Young, S. Redenti et al., "Survival, migration and differentiation of retinal progenitor cells transplanted on micro-machined poly(methyl methacrylate) scaffolds to the subretinal space," *Lab on a Chip*, vol. 7, no. 6, pp. 695–701, 2007.

[47] V. K. Gullapailli, M. Khodair, H. Wang, I. K. Sugino, S. Madreperla, and M. A. Zarbin, "Retinal pigment epithelium and photoreceptor transplantation frontiers," in *Retina*, S. J. Ryan, Ed., pp. 2597–2612, Elsevier-Mosby, Philadelphia, Pa, USA, 4th edition, 2006.

[48] N. D. Radtke, R. B. Aramant, H. M. Petry, P. T. Green, D. J. Pidwell, and M. J. Seiler, "Vision improvement in retinal degeneration patients by implantation of retina together with retinal pigment epithelium," *American Journal of Ophthalmology*, vol. 146, no. 2, pp. 172–182, 2008.

[49] R. E. MacLaren, R. A. Pearson, A. MacNeil et al., "Retinal repair by transplantation of photoreceptor precursors," *Nature*, vol. 444, no. 7116, pp. 203–207, 2006.

[50] D. A. Lamba, M. O. Karl, C. B. Ware, and T. A. Reh, "Efficient generation of retinal progenitor cells from human embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 34, pp. 12769–12774, 2006.

[51] V. Enzmann, E. Yolcu, H. J. Kaplan, and S. T. Ildstad, "Stem cells as tools in regenerative therapy for retinal degeneration," *Archives of Ophthalmology*, vol. 127, no. 4, pp. 563–571, 2009.

[52] R. D. Lund, S. Wang, I. Klimanskaya et al., "Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats," *Cloning and Stem Cells*, vol. 8, no. 3, pp. 189–199, 2006.

[53] M. Wang, T. T. Lam, M. O. M. Tso, and M. I. Naash, "Expression of a mutant opsin gene increases the susceptibility of the retina to light damage," *Visual Neuroscience*, vol. 14, no. 1, pp. 55–62, 1997.

[54] E. L. Berson, B. Rosner, M. A. Sandberg et al., "A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa," *Archives of Ophthalmology*, vol. 111, no. 6, pp. 761–772, 1993.

[55] E. L. Berson, B. Rosner, M. A. Sandberg et al., "Vitamin A supplementation for retinitis pigmentosa," *Archives of Ophthalmology*, vol. 111, pp. 1456–1493, 1993.

[56] E. L. Berson, B. Rosner, M. A. Sandberg et al., "Further evaluation of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment: subgroup analyses," *Archives of Ophthalmology*, vol. 122, no. 9, pp. 1306–1314, 2004.

[57] E. L. Berson, B. Rosner, M. A. Sandberg et al., "Clinical trial of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment," *Archives of Ophthalmology*, vol. 122, no. 9, pp. 1297–1305, 2004.

[58] E. L. Berson, B. Rosner, M. A. Sandberg et al., "Clinical trial of lutein in patients with retinitis pigmentosa receiving vitamin A," *Archives of Ophthalmology*, vol. 128, no. 9, pp. 403–411, 2010.

[59] R. W. Massof and G. A. Fishman, "How strong is the evidence that nutritional supplements slow the progression of retinitis pigmentosa?" *Archives of Ophthalmology*, vol. 128, no. 4, pp. 493–495, 2010.

[60] D. R. Hoffman, K. G. Locke, D. H. Wheaton, G. E. Fish, R. Spencer, and D. G. Birch, "A randomized, placebo-controlled clinical trial of docosahexaenoic acid supplementation for X-linked retinitis pigmentosa," *American Journal of Ophthalmology*, vol. 137, no. 4, pp. 704–718, 2004.

[61] D. H. Geroski and H. F. Edelhauser, "Drug delivery for posterior segment eye disease," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 5, pp. 961–964, 2000.

[62] D. Ghate, W. Brooks, B. E. McCarey, and H. F. Edelhauser, "Pharmacokinetics of intraocular drug delivery by periocular injections using ocular fluorophotometry," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 5, pp. 2230–2237, 2007.

[63] D. Ghate and H. F. Edelhauser, "Ocular drug delivery," *Expert Opinion on Drug Delivery*, vol. 3, no. 2, pp. 275–287, 2006.

[64] J. Jiang, D. H. Geroski, H. F. Edelhauser, and M. R. Prausnitz, "Measurement and prediction of lateral diffusion within human sclera," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 7, pp. 3011–3016, 2006.

[65] T. J. Wolfensberger, "The role of carbonic anhydrase inhibitors in the management of macular edema," *Documenta Ophthalmologica*, vol. 97, no. 3-4, pp. 387–397, 1999.

[66] S. Grover, G. A. Fishman, R. G. Fiscella, and A. E. Adelman, "Efficacy of dorzolamide hydrochloride in the management of chronic cystoid macular edema in patients with retinitis pigmentosa," *Retina*, vol. 17, no. 3, pp. 222–231, 1997.

[67] Y. Sauvé, G. Karan, Z. Yang, C. Li, J. Hu, and K. Zhang, "Treatment with carbonic anhydrase inhibitors depresses electroretinogram responsiveness in mice," *Advances in Experimental Medicine and Biology*, vol. 572, pp. 439–446, 2005.

[68] L. Scorolli, M. Morara, A. Meduri et al., "Treatment of cystoid macular edema in retinitis pigmentosa with intravitreal triamcinolone," *Archives of Ophthalmology*, vol. 125, no. 6, pp. 759–764, 2007.

Review Article

Variables and Strategies in Development of Therapeutic Post-Transcriptional Gene Silencing Agents

Jack M. Sullivan,[1, 2, 3, 4, 5, 6] **Edwin H. Yau,**[1, 2] **Tiffany A. Kolniak,**[1, 4] **Lowell G. Sheflin,**[1, 6] **R. Thomas Taggart,**[1] **and Heba E. Abdelmaksoud**[7]

[1] *Department of Ophthalmology, University at Buffalo SUNY, Buffalo, NY 14214, USA*
[2] *Department of Pharmacology and Toxicology, University at Buffalo SUNY, Buffalo, NY 14214, USA*
[3] *Department of Physiology and Biophysics, University at Buffalo SUNY, Buffalo, NY 14214, USA*
[4] *Neuroscience Program, University at Buffalo SUNY, Buffalo, NY 14214, USA*
[5] *Ross Eye Institute, University at Buffalo SUNY, Buffalo, NY 14209, USA*
[6] *Veterans Administration Western New York Healthcare System, Medical Research, Buffalo, NY 14215, USA*
[7] *Department of Neuroscience and Physiology, Upstate Medical University, Syracuse, NY 13215, USA*

Correspondence should be addressed to Jack M. Sullivan, jackmsullivanmdphd@yahoo.com

Post-transcriptional gene silencing (PTGS) agents such as ribozymes, RNAi and antisense have substantial potential for gene therapy of human retinal degenerations. These technologies are used to knockdown a specific target RNA and its cognate protein. The disease target mRNA may be a mutant mRNA causing an autosomal dominant retinal degeneration or a normal mRNA that is overexpressed in certain diseases. All PTGS technologies depend upon the initial critical annealing event of the PTGS ligand to the target RNA. This event requires that the PTGS agent is in a conformational state able to support hybridization and that the target have a large and accessible single-stranded platform to allow rapid annealing, although such platforms are rare. We address the biocomplexity that currently limits PTGS therapeutic development with particular emphasis on biophysical variables that influence cellular performance. We address the different strategies that can be used for development of PTGS agents intended for therapeutic translation. These issues apply generally to the development of PTGS agents for retinal, ocular, or systemic diseases. This review should assist the interested reader to rapidly appreciate critical variables in PTGS development and facilitate initial design and testing of such agents against new targets of clinical interest.

1. PTGS Technologies

The basic mechanisms of antisense (AS), ribozyme (Rz), and RNA interference (RNAi) approaches to PTGS will be presented here. A comparison of their properties is presented (Table 1).

1.1. Antisense. AS intended for clinical use is an oligodeoxynucleotide (ODN) string with bases chosen to form Watson Crick annealing pairs over an accessible region of the target mRNA or viral RNA. Various backbone formulations have been used with the intent of resisting nuclease degradation outside or inside cells, enhancing, the binding energy to the target RNA, reducing the strong electrostatic repulsive energies during annealing, and enhancing specificity of RNase H attack. Modifications to the intrinsic phosphodiester backbone chemistry include: phosphorothioate, methylphosphonoester, peptide nucleic acid, 2-ortho-methyl-deoxyribose, locked nucleic acid, and morpholino. Chemical modifications influence cellular uptake, and AS ODNs are provided to tissues directly rather than being expressed within cells from a genetic construct. Chemical modifications of ODNs and such engineered properties are not the focus here, and an interested reader should consult prior literature [1–4]. Single-stranded ODNs are transfected or transduced into cells where they diffuse and encounter target RNAs in either the nucleus or cytoplasm. Two generally accepted

TABLE 1: Comparison of the properties of antisense, ribozyme, and RNA$_i$.

Property	Antisense	Ribozyme	siRNA/shRNA/miRNA
Size	Small (15–20 nt)	Small (42–60 nt)	Small (19–22 nt)*
Crystal structure	No	Yes	Yes (RISC)
Mechanism of action	Known	Known	Known
Independence cell metabolism	No	Yes	No
Specificity	Moderate	High	Poor to moderate
Saturable	No	No	Yes
Cellular compartment	Cytoplasm	Nucleus/cytoplasm	Cytoplasm
Dependence on target structure	Yes	Yes	Yes
Proven *in vivo*	Yes	Yes	Yes

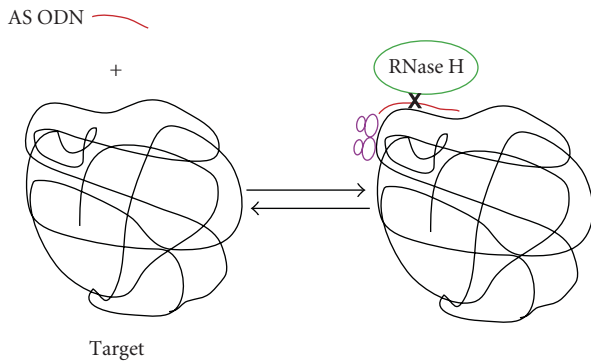* On one strand engaged in the RISC complex after cellular processing.

FIGURE 1: Antisense mechanism. A schematic representation is shown for two dominant mechanisms by which AS ODN molecules delivered into cells can suppress gene expression. The AS ODN must first anneal to an accessible region of the target mRNA. The first and likely dominant mechanism of inhibition is through recruitment of RNaseH (green) to cleave the RNA is the center of the ODN: Target RNA hybrid region. The second mechanism involves physical hindrance of biochemical processes operative on the mRNA such as ribosome- (violet-) mediated translation, 5′ decapping, and 3′ deadenylation. Here, the hybridized ODN is depicted blocking the progress of translating ribosomes on the mRNA.

mechanisms of AS ODN inhibition of gene expression are both dependent upon strong annealing to the target RNA (Figure 1). These include ODN catalysis of target RNA degradation by RNaseH and/or physical blockade mechanisms (e.g., translation block through ribosome stalling, blocking splicing, blocking polyadenylation) [1, 2, 4, 5]. The first mechanism can occur anywhere in the processed RNA, whereas the second mechanism must occur within the coding region of the target or at sites of splicing or polyadenylation. The translating ribosome can remove antisense ODNs due to its helicase function [6]. Therefore, AS conformational block may best be conducted at or in proximity of the translation initiation codon. AS inhibition of target gene expression can, therefore, occur at the post-transcriptional or cotranslational levels. The RNaseH-mediated mechanism of inhibition can occur with phosphodiester or phosphorothioate backbones of the ODN. The upstream cleavage product by RNaseH has a 3′ hydroxyl and the downstream cleavage product has a 5′ phosphate.

Information has accumulated that the RNaseH mechanism lacks great specificity with fully cleavable ODNs in that only a small number (≤ 5 nt) of annealing nucleotides (nt) are sufficient to support target phosphodiester cleavage [7, 8]. This results in substantial off-target effects and has sponsored the development of second-generation agents that have modified backbone and sugar chemistries. Many of these chemistries act to increase the affinity of the ODN to the target RNA. On the other hand, they do not allow RNaseH-mediated cleavage. In pure form, such agents may not have high efficacy when transduced into mammalian cells, indicating that the physical blockade mechanisms are not the most potent. Second-generation chimeric antisense molecules were then engineered that contained the modified chemistries for the backbones and sugars but also a central core of deoxynucleotides that permit RNaseH cleavage. Such chimeras have increased potency on the basis of catalyzing RNaseH attack on a target and specificity because of the strength of binding to the target [7, 9, 10]. During early development, RNaseH activity appeared to be the dominant mechanism of AS inhibition [11]. More recently, a combination of mechanisms is thought to be embraced depending upon the chemical nature of the ODN [12]. An effective AS PTGS agent requires an accessible region in the target RNA and especially strong binding energy of the ODN to the target RNA. The lifetime of the bound ODN: target state must be sufficiently long to embrace the natural kinetics of RNaseH and its stoichiometry-dependent kinetics or must be sufficiently long to impair translation of substantial numbers of cognate protein molecules. The AS reaction scheme can be simply represented as follows:

$$\text{ODN} + \text{RNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ODN} : \text{RNA}. \qquad (1)$$

The dissociation constant (K_d) is given by

$$K_d = \frac{[\text{ODN}][\text{RNA}]}{[\text{ODN} : \text{RNA}]} = \frac{k_{-1}}{k_1}. \qquad (2)$$

For AS, Rz, and RNA$_i$, on rates (k_1) of interaction between the PTGS agent and the target mRNA are limited by the expected rate of forming a nucleic acid double-stranded helix in solution from two (idealized) random coils (estimated at $5 \times 10^7 \, \text{M}^{-1} \, \text{min}^{-1}$), with the assumption of

preexisting regions of single-stranded accessibility able to support immediate base pairing [13]. However, measured AS annealing rates vary more than dissociation rates and appear responsible for the profound range of K_d values that span several orders of magnitude against a single-target mRNA [14–16]. The fact that the ON rates have such a wide variation is likely an index of the varying landscape of accessibility at different regions in a folded target mRNA or potential inaccessibility in the structure of the AS ligand that limits annealing (e.g., [15, 17–19]). OFF rates (k_{-1}) are typically many orders of magnitude smaller than on rates such that K_d can be approximated by the simple ratio of rates. The net energetic effects of the AS-binding process reflect the losses of potential inhibitory secondary structures in the target or AS ligand and the gain achieved by the annealing event. The strength of binding or the free energy (ΔG) of the AS dissociation reaction is represented as

$$\Delta G = -RT \ln K_d. \tag{3}$$

ΔG at a particular temperature can be calculated from nearest neighbor tabulations of ΔH and ΔS [20, 21]. ΔG can then be used to calculate K_d from which k_{-1} (dissociation rate) can be calculated. k_{-1} can then be used to calculate the lifetime (time constant) of the AS bound state

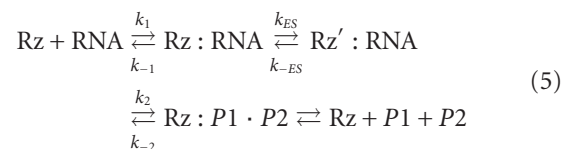$$\tau_{-1} = \frac{\ln 2}{k_{-1}} = \frac{0.693}{k_{-1}}. \tag{4}$$

Assume the lifetime of a cellular mRNA target that codes for a relatively abundant protein is 10 hours. In order to manifest significant target knockdown, an AS agent must remain stably bound to the target mRNA for a period at least as long as the target mRNA lifetime. The lifetime of the target: ODN complex allows RNaseH-mediated cleavage of the target mRNA or translation arrest. For an mRNA with a mean 10 hr lifetime, k_{-1} should be on the order of $1.2 \times 10^{-3} \, \text{min}^{-1}$, and K_d would be 23.1 picoMolar. AS-binding affinities can vary over several log orders depending upon the target sequence and are often not as strong as 23 pM [14–16, 22, 23]. The dominant factor in achieving a successful agent is to first identify the regions in the target mRNA that are indeed accessible to annealing (see [18, 19]). The length of the ODN and the backbone chemistry should be chosen appropriately to achieve a sufficiently negative ΔG, which can be calculated from nearest neighbor frequencies. Web databases for AS ODN effectiveness studies are available [24–26]. The *in vitro* binding capacity and affinity of AS agents to target mRNAs appears to correlate with knockdown potential in live cells [18, 19]. That local target accessibility is a major limiting variable *in vivo* has been shown by engineering a single AS annealing site into a reporter target mRNA in different local structural contexts and then testing knockdown by a single AS ODN relative to control [27]. There was marked changes in knockdown by the single AS ODN when its target sequence was present in different secondary structural contexts.

Vitrovene (fomiversen, Isis-2922) (Novartis, ISIS), currently the only FDA approved (August 1998) PTGS agent (antisense) for human use (CMV retinitis), is a 21-mer phosphorothioate AS ODN that anneals to the coding region of the mRNA transcribed from the major immediate-early (IE55) gene of the CMV genome [28–30].

1.2. Ribozymes. General reviews on the ribozyme are available [31–38]. A ribozyme is a catalytic RNA. The chemistry of RNA is sufficiently robust that it can fold into structures that permit specific phosphodiester bond cleavage in other target RNAs. There are several forms of ribozyme that have been identified. We focus on the hammerhead ribozyme (hhRz), because it has the most versatile set of cleavage sites (NUH↓, where N = G, C, U, A; H = C, U, A), because a large knowledge base is established for this RNA enzyme, and because the internal equilibrium of the reaction is strongly biased toward cleavage (k_2) as opposed to religation (k_{-2}) (>100 : 1). The hairpin ribozyme (hpRz) recognizes a broad set of target motifs, but has a religation rate that exceeds cleavage rate (10 : 1) such that religation is favored over cleavage [39, 40]. These issues complicate its potential for therapeutics, because there are fewer places to cleave a tightly compact target and cleaved target products can be relgated by the same agent unless they are displaced rapidly. For the hhRz there are an average of one NUH↓ cleavage site every twelve nts ($1/4 \times 1/1 \times 1/3 = 1/12$). Therefore, even an average size mRNA has a rich abundance of potential NUH↓ cleavage sites. This increases the probability for having a potential cleavage site in a rare region of target accessibility. Different NUH↓ cleavage sites demonstrate variation in the rate of cleavage with the two naturally occurring motifs (GUC↓, GUA↓) having the greatest intrinsic cleavage rates [41–44]. In addition to the NUH↓ cleavage motif hhRzs can be designed to cleave at NHH↓, but the catalytic rates are substantially reduced compared to high level GUC↓ motif [32]. All hhRzs cleave a phosphodiester bond to leave an upstream product terminated at the 3′ end with a cyclic 2′3′ phosphate and a downstream product terminated at the 5′ end with a hydroxyl group. Once the target mRNA is cleaved by the hhRz, the fragments are more readily degraded by exonucleases in the cell because of the loss of the polyadenylation signal at the 3′ end of the upstream fragment and the loss of the cap on the 5′ end of the downstream fragment.

A simplistic reaction schematic for the hhRz is shown (Figure 2). The hhRz folds into a conformation which is stabilized by Stem II. In its *trans* format, which is used for gene therapeutic purposes, the two antisense flanks form Stems I (5′ AS flank) and III (3′ AS flank) upon annealing to the target RNA. Annealing sets the stage for conformational changes (Rz′) that prepare and align the enzyme core with the phosphodiester bond at the target cleavage site. The H nt of the NUH↓ cleavage motif does not hydrogen bond to the hhRz. Upon cleavage the two products ($P1$, $P2$) must dissociate from the AS arms of the hhRz in order to free the hhRz to anneal to another target RNA and promote true catalytic turnover of substrate:

$$\text{Rz} + \text{RNA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{Rz} : \text{RNA} \underset{k_{-ES}}{\overset{k_{ES}}{\rightleftarrows}} \text{Rz}' : \text{RNA}$$

$$\underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{Rz} : P1 \cdot P2 \rightleftarrows \text{Rz} + P1 + P2 \tag{5}$$
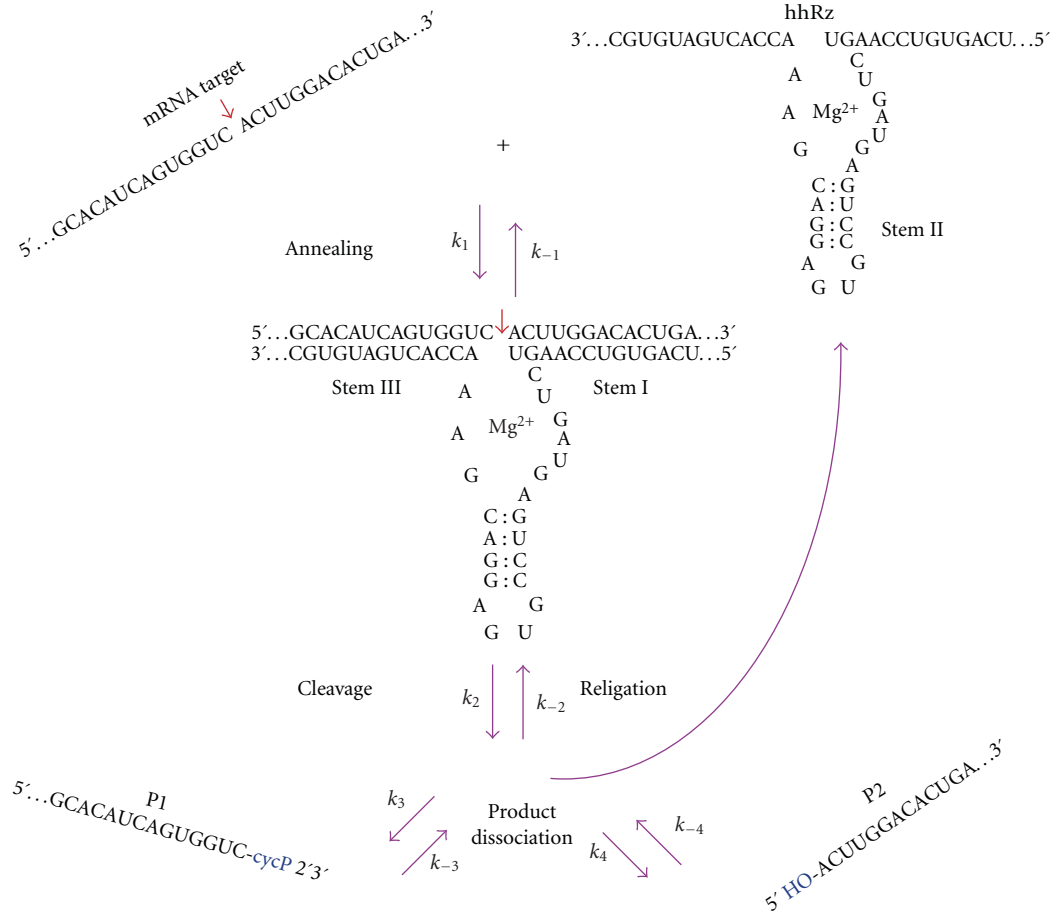
FIGURE 2: Ribozyme mechanism. A schematic reperesentation is shown with a simplified accessible region in a target mRNA with a cleavable hhRz NUH↓ motif, (GUC↓). The hhRz, drawn in an open enzymatically patent state, binds to the accessible target region by Watson Crick base pairing. Annealing precisely aligns the phosphodiester bond of the H residue (C here) with the enzymatic core of the catalytic RNA. The annealing reaction has an equilibrium specified by the ratio of rates k_1 and k_{-1}. Chemical cleavage (k_2) occurs to yield two products which remain bound to the AS flanks of the hhRz. Each product leaves with its own characteristic equilibrium determined by the strength of binding to the hhRz AS flanks. Product dissociation permits the enzyme to collide with another substrate and initiate subsequent rounds of catalysis as a Michaelis-Menten enzyme to achieve catalytic target turnover characterized by k_{cat}/K_m.

As for AS, the initial dissociation constant (K_d) is given by

$$K_d = \frac{[\text{hhRz}][\text{RNA}]}{[\text{hhRz}] : [\text{RNA}]} = \frac{k_{-1}}{k_1}. \qquad (6)$$

Like AS, Rz and RNA_i have ON rates (k_1) that are typically limited by the expected diffusion-limited rate of forming a nucleic acid double-stranded helix in solution from two (idealized) random coils (estimated at $5 \times 10^7 \text{ M}^{-1}\text{min}^{-1}$). Again, association rates are typically orders of magnitude lower than this index. OFF rates (k_{-1}) are typically many orders of magnitude smaller than ON rates such that K_d can be approximated by the simple ratio of rates. The free energy of the Rz dissociation reaction is represented as in (3) above. ΔG at a particular temperature can be calculated from nearest neighbor tabulations of ΔH and ΔS [20, 21]. ΔG can then be used to calculate K_d from which k_{-1} (dissociation rate) can be calculated. k_{-1} can then be used to calculate the lifetime (time constant) of the Rz bound state as in (4) above. The total AS flank lengths (Stem I + Stem III, H does not

hydrogen bond) of the Rz should be no more than 12–16 nts, depending upon the sequence context, in order to achieve a full annealing energy of between −12 to −16 kCal/mole [45]. HhRzs that bind too tightly to target RNA will have slow OFF rates prior to chemical cleavage (rate limiting for the ideal hhRz performance). Slow initial OFF rates could result in a loss of specificity for the intended target because chemical cleavage could occur if an NUH↓ site of an unintended target happened to be centrally placed within the AS flank span. The likelihood that an unintended target could precisely position itself on a given hhRz for cleavage at an NUH↓ site is, in fact, low, unless the unintended target had almost precise sequence identity to the intended target. This factor has been presented as a factor for hhRz specificity [46]. In addition, hhRz catalytic function is intolerant to base-pair mismatches near the core of the enzyme [47], which would act to decrease cleavage of bound nontarget mRNAs that do not have precise sequence specificity for annealing; in fact, this attribute of the hhRz can be used as a component of

a therapeutic strategy to suppress mutated versus normal target mRNAs in hereditary diseases (see below). Off-target effects with a hhRz would more likely result from pure AS effects independent of catalytic chemical cleavage. With an optimum total antisense flank length for catalysis on the order of 12–16 nt, the stable annealing of unintended targets with mismatches relative to the hhRz is expected to occur with low probability. The expected specificity of the hhRz is a considerable distinction from the mismatch tolerant AS or RNA_i processes.

Another challenge with the hhRz is the issue of product inhibition. If the two products cannot melt off of the antisense flanks of the hhRz after cleavage at physiological temperature, or one product is delayed in leaving, then the hhRz will be trapped in association with cleaved target and unable to recognize and anneal to subsequent target RNA molecules. This problem impacts catalytic turnover or enzyme efficiency (k_{cat}/K_m). A kinetic model exists for the hhRz that can greatly assist in the design of antisense flanks that permit energies of annealing sufficient to allow the hhRz to bind long enough to permit chemical cleavage ($\cong 1$/min) but not too long to promote product inhibition [13]. It is important to determine the extent to which target knockdown by a hhRz is due to catalytic, antisense, or catalytic antisense effects. There are several mutations that can be made at key residues in the enzymatic core of the hhRz, which are known to completely obviate catalysis (e.g., G5C, G8C, G12C [41, 44, 48]). Comparing the level of target knock down (RNA or protein or both) by a fully catalytic hhRz compared to a mutated hhRz should allow sufficient information to determine the extent to which the hhRz is performing catalytically, which is the desired outcome. A hhRz with true catalytic performance *in vivo* can knockdown significantly more target molecules in a given epoch of time than a hhRz that does not have this capacity (e.g., pure AS effect without cleavage or a catalytic antisense effect with annealing and cleavage but no product release and turnover). Hence, hhRzs that demonstrate catalytic turnover in live human cells require lower expression levels to achieve the same levels of target knockdown than those that do not have Michaelis-Menten turnover potential. Lower levels of PTGS agent expression are expected to decrease the potential for cellular toxicity and off target effects.

A relatively stable mRNA is a good target for gene silencing, because hhRzs are relatively slow enzymes. The intrinsic cleavage rate is maximal against small unstructured substrate RNAs and on the order of 1/min, which is several orders of magnitude slower than proteinaceous enzymes. Structured targets typically have slower cleavage rates. Because of the slow speed of catalytic RNAs, the intrinsic degradation kinetics of the target RNA (without the hhRz) and with the hhRz RNA must be considered. It is important to consider the lifetime of the target mRNA in its dominant locale within the cell. Targets that have short lifetimes (e.g., pulse transcribed mRNA with rapid turnover such a cell-cycle control genes) may be difficult to attack with current hhRzs, because the targets intrinsically degrade at a rate that cannot be practically impacted by a hhRz. One will want to choose targets carefully to insure that

there is sufficient time for enzymatic turnover within the cell at expression levels of the PTGS agent that are not toxic. We would recommend target mRNAs that have lifetimes on the order of several hours. Fortunately, most autosomal dominant disease genes and normal genes transcribe fairly stable mRNAs as potentially validated targets for PTGS therapeutics. These typically code for signaling, structural, or enzymatic proteins in photoreceptors and RPE cells. Any Rz acts kinetically by providing an additional component to the intrinsic degradation rate for a target RNA. The total rate of degradation of the target mRNA is the sum of the intrinsic and Rz-induced degradation rates ($k_{cat} = k_{int} + k_{PTGS}$). Clearly, if the intrinsic degradation rate is much faster than the rate of intracellular Rz catalysis, then $k_{cat} \cong k_{int}$, and there can be no significant knockdown of target RNA and protein mediated by the PTGS agent. An hhRz or an RNAi can be most effective if $k_{PTGS} \gg k_{int}$. On face value, this substantially restricts the types of mRNAs that can be suitable targets. RNAs with very short half-lives, such as those coding for transiently induced transcription factors, are unlikely to be viable targets because $k_{PTGS} \approx k_{int}$ or $k_{PTGS} < k_{int}$. Therefore, before embarking on the development of a PTGS agent for a particular target, it is prudent to have knowledge regarding the intrinsic degradation half life of the target mRNA in the cells in which gene therapy would need to be administered.

A ribozyme designed to cleave the mRNA for proliferating cell nuclear antigen [49] was recently tested in a Phase I clinical trial for proliferative vitreoretinopathy [50].

1.3. RNA_i Technology. Recent reviews will serve to orient the unfamiliar reader [51–53]. RNAi refers to an evolutionarily conserved phenomenon where double-stranded RNA (dsRNA) mediates the sequence-specific cleavage of target RNA using cellular machinery (Figure 3). In mammalian cells, RNAi is triggered by 21–23 nt RNA duplexes with symmetric 2 nt 3′ overhangs and 5′-phosphate termini called small interfering RNA (siRNA) [54–56]. These siRNA duplexes are processed from longer dsRNA by the ribonuclease III enzyme Dicer [57]. Dicer processed siRNA duplexes associate with a multiprotein complex known as the RNA-inducing silencing complex (RISC), and one strand of the duplex is loaded into RISC to serve as the AS guide strand. Within RISC, the guide RNA strand is bound by the Argonaute 2 protein that contains an amino-terminal Piwi Argonaute Zwille (PAZ) domain and a carboxy-terminal PIWI domain containing the catalytic RNA slicer site [58]. The PAZ domain recognizes and anchors the 3′ overhang of the duplex [59–61] while the PIWI domain anchors the 5′ end of the guide RNA [62]. The guide strand then adopts a A-form helix that extends along a channel in the PIWI domain, aligning the scissile phosphate of the target strand with the slicer catalytic site one helical turn away from the 5′ anchored end [63]. The PIWI domain is similar in structure to RNaseH. After RISC cleavage, the upstream product has a 3′ hydroxyl and the downstream product has a 5′ phosphate.

Despite the association of a cellular protein complex, effective gene silencing is still not realized with many siRNA or expressed short hairpin (shRNA) sequences [64]. Three
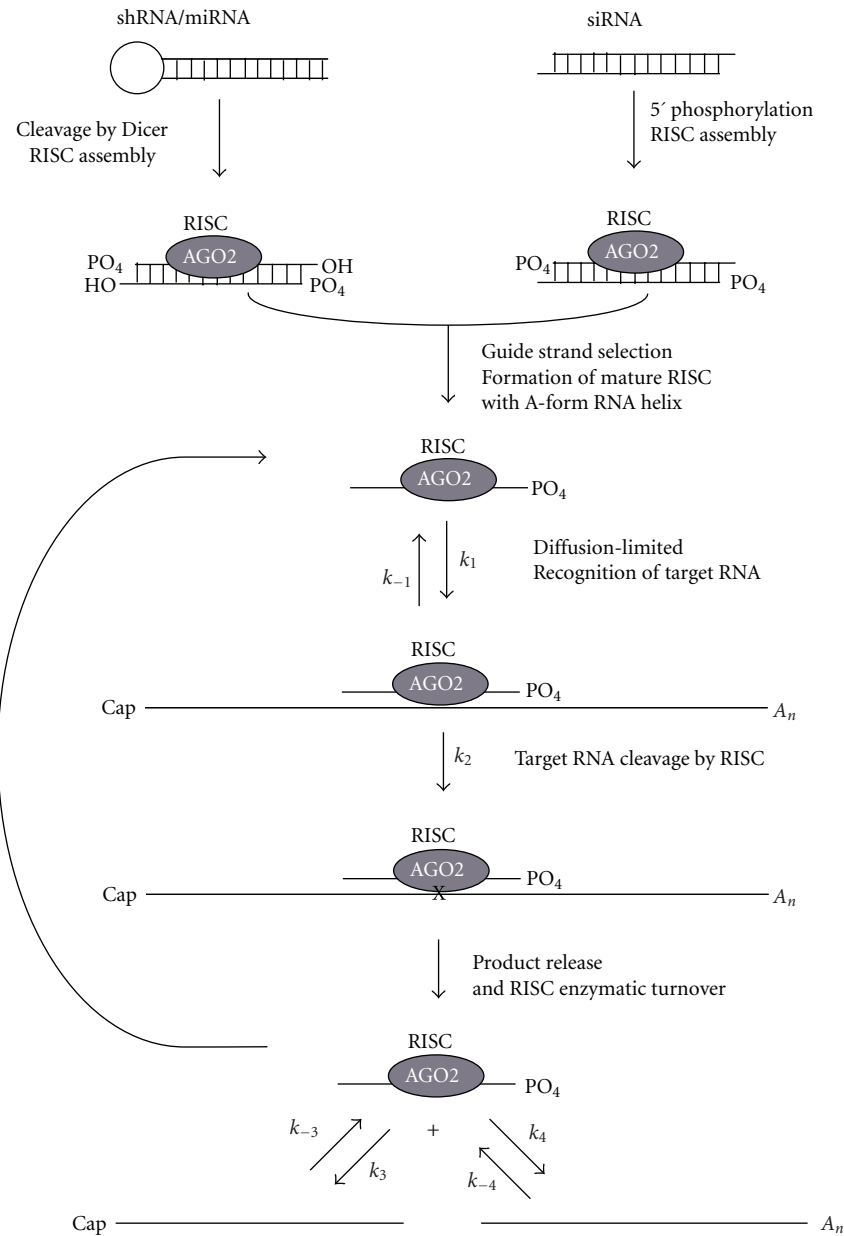
FIGURE 3: RNAi mechanism. An expressed RNA hairpin (shRNA) is cleaved first by Dicer III to a double-stranded RNA of 21 nt with 5′ phosphorylated ends. A pri-miRNA is processed in the nucleus into a pre-miRNA by Drosha, leaves the nucleus, and is further processed by Dicer in the cytoplasm or as part of RISC. Or a transfected or transduced siRNA is phosphorylated at each 5′ end. The short dsRNAs are incorporated into the RISC complex, and the antisense strand (guide strand) is selected on the basis of engineering weaker 5′ energy than 3′ energy. The passenger strand is displaced. The guide strand is organized into RISC as an A-form α-helix within Ago2, which is the RNA endonuclease of RISC. By diffusion limitations, loaded RISC searches for a complimentary partner to its antisense element in the transcriptosome. Upon collision, kissing complex formation and full annealing, the target RNA is positioned for endonuclease cleavage by Ago2. After cleavage, it is thought that ATP hydrolysis occurs, which provides helicase energy to strip the products from the Ago2 cavity in order to prevent product inhibition on RNAi. Product release then frees the charged RISC to seek other target mRNAs for subsequent rounds of Michaelis-Menten turnover.

crucial kinetic parameters are strongly implicated in the ability of a given siRNA sequence to effectively promote gene silencing in physiological conditions: the loading of the correct antisense RNA guide strand into RISC, target mRNA site annealing, and RISC reloading. These parameters are affected by sequence-specific problems. For the first parameter, loading of RISC, the thermodynamic stability of

the RNA ends has been shown to be the major determinant of which strand of the siRNA duplex is incorporated into RISC. Theoretically, either strand of the siRNA duplex can be incorporated into RISC, but only one strand will be AS for a given sense mRNA target. The discovery that the strand with the greater thermodynamic instability in the 5′ end is preferentially loaded into RISC has improved

the design of successful siRNAs or shRNAs [65, 66] by allowing the preferential loading of the correct antisense guide strand. While the loading of RNA guide strands into RISC is an RNAi-specific problem, the problem of the limits of target mRNA site accessibility and annealing that occurs for AS and ribozyme PTGS agents also affects efficacy of siRNA sequences [67–74]. Fundamentally, the Watson-crick base pairing that is required for all of these technologies profoundly limits the number of target mRNA regions that will support effective gene silencing. Like AS and ribozymes, target recognition for RNAi also seems to proceed by diffusion [71], with the guide strand of the RISC complex encountering sites nonspecifically until proper annealing with the target site forms the necessary geometry for RISC cleavage. Target recognition is dominated by the 5′ region of siRNA, which nucleates binding of target RNA with RISC and contributes to the overall strength of binding between the target RNA and RISC. The 5′ region of the siRNA (2–8 nt) has been called the "seed" sequence. The annealing of the central and 3′ regions are important for establishing the A-form helical geometry that is needed for efficient central cleavage [75, 76]. Although RISC proceeds with greatest activity when it anneals to a fully complementary target, it can still cleave RNA targets with mismatched bases, especially in the 3′ end. Even with such mismatches, the RNAi mechanism can also promote translational inhibition. The toleration of mismatches gives rise to the significant off-target effects of potential RNAi therapeutic agents (see below). In *D. melanogaster* embryo lysates, target annealing and cleavage by RISC are both ATP-independent steps. It is only the release of the target after cleavage that requires ATP [75]. The expected increased catalytic efficacy of RNAi compared to ribozymes is most likely due to the increased OFF rates of products that is facilitated by RISC. This step may be slower in humans as the Drosophila RISC enzyme seems to have a higher catalytic efficiency despite similar K_m values [77]. Recent studies indicate that the RISC complex can be saturationally inhibited by other competing siRNAs and that the loading (1 hr) and clearance (12 hrs) of the RISC complex have distinct kinetic rates [78]. While the suggestion that RNAi is more potent than AS or Rz modalities, RNAi still shares with all PTGS modalities the same major problem of the intrinsic limits of target inaccessibility, with the initial challenge being to identify rare accessible regions. Few studies have compared RNAi potency to other modalities at sites in target mRNAs that are predetermined to be accessible or inaccessible *in vivo*. Even if intrinsic potency is greater for RNA_i, the potential for off-site and toxic effects of RNA_i may make ribozyme or perhaps AS better choices for therapeutic PTGS development.

Recent discoveries continue to reveal the complexity of the machinery involved in the RNAi mechanism, and great care must be taken to evaluate potential RNAi therapeutic agents. For RNA therapeutics to be safe clinically, they must have specificity. There are an increasing number of reports about off-target knockdown effects by RNAi [79–83], some of which have induced toxic effects [84]. This likely results because of the tolerance of RISC to mismatches in bound target RNAs and a decrease in specificity for intended targets.

siRNA activation of interferon response genes has occurred [85] as well as activation of the immune system [86]. Recent serious concerns over RNAi safety were raised due to death of mice secondary to RNAi saturation of a nuclear exit pathway (exportin-5) used by micro-RNA [87–89]. Therapeutic interference with natural and essential functions of micro-RNAs, such as differentiation, cell-cycle control, and gene expression, could also cause serious deleterious consequences. These findings raise serious concern about potential toxicity of RNAi in human clinical trials. In addition to off-target knockdown concerns, a recent study also revealed the potential for sequence-independent knockdown of an RNAi target unrelated to off-target immune effects. Anti-angiogenic siRNAs were targeted to VEGF or its receptor for the treatment of choroidal neovascularization (CNV) in age-related macular degeneration. The siRNAs showed a suppression of CNV that was caused by a class effect of 21-nucleotide double-stranded RNA sequences stimulating cell-surface Toll-like receptor 3 that lead to an induction of interferon-gamma and interleukin-12 rather than a specific knockdown of VEGF or its receptor [90]. A siRNA database is available for the interested reader [91].

Micro-RNAs (miRNAs) are noncoding regulatory RNAs expressed in mammalian cells generally from RNA pol-II promoters as primary miRNAs (pri-miRNAs). Pri-miRNAs are processed in the nucleus by the endonuclease Drosha to form pre-miRNAs, which are derivative hairpin RNAs that are transported by Exportin-5 to the cytoplasm. There they are further processed by Dicer into 21–23 bp dsRNAs that enter the RISC processing pathway. miRNAs control development, gene expression, cellular differentiation, growth regulation, and many have been identified in human cells [92]. miRNAs appear to be the native substrates of the evolutionarily conserved RISC RNA_i pathway. miRNAs, like other RNA_i modalities, can promote cleavage of target mRNAs if there is full binding to the target mRNA, or translational inhibition when bound by seed sequences, but with mismatches, to 3′ UT sequences. Recent efforts have sought to create designer miRNAs in which a particular native human miRNA, which is expected to have its own intrinsic set of target mRNAs, is engineered to create potential for annealing to a disease target mRNA. Early data suggest that this approach may yield both potency and decreased potential for toxicity, because lower levels of expression of the miRNA are achieved [93, 94]. However, the design and embedding of PTGS agents as chimeras within usurped native human miRNAs that naturally interface through RISC to modulate critical cellular functions may create risk. More studies are needed to establish both effectiveness and safety of this approach. Clearly, miRNA evolution achieved specific RNA structures that were processed and reduced to functional siRNAs inside cells. The insertion of an alternative nonnative targeting sequence into a larger miRNA embraces substantial new biophysical constraints. How can one insure that the targeting siRNA is properly spliced from a larger RNA when multiple conformational states of the miRNA chimera can exist and these might affect how Drosha and Dicer process the expected target sequences?

2. Variables and Challenges in Therapeutic PTGS Development

2.1. Overview. A PTGS agent is designed to suppress the translation of a particular target mRNA into its cognate protein. This may occur through tight annealing of the PTGS agent to the target mRNA which stalls translation at the ribosome. Or, it may occur through annealing and cleavage of the target mRNA which promotes more rapid degradation of the target mRNA, to decrease the steady state concentration of the target mRNA and suppress translation at the ribosome and hence the steady state level of the cognate target protein. In the context of a therapeutic PTGS, the particular mRNA/protein targets must be validated for a given disease state, such as a retinal degenerative disease. Validation means that the expression of the specific target mRNA/protein has been strongly associated with the emergence of a particular disease state. For example, in an autosomal dominant form of hereditary retinal degeneration such as retinitis pigmentosa, the expression dose of mRNA from the mutated allele may generate a protein which has toxic gain of function for the cells in which it is expressed. This toxicity may promote stress and ultimately apoptosis. At least, early in the disease process, it is rational to select the mutated mRNA as the validated target for therapy of such a genetic disease. If the mutant mRNA and toxic protein can be reduced, this outcome is expected to ameliorate cellular stresses and reduce the probability of apoptosis and the coincident loss of cellular and visual function. Similarly, in certain retinal degenerative conditions, such as age-related macular degeneration, rational therapeutic PTGS strategies could potentially involve the reduction of levels of wild-type gene expression.

PTGS agents operate biophysically within the functional context of cellular housekeeping functions to reduce levels of specific target mRNAs and proteins. The critical *variables* in the design of efficacious potentially therapeutic PTGS agents are not specific to retinal or other ocular diseases and in fact, have largely emerged from research not specific to ocular disease states. Therefore, we have attempted here to represent to the reader the biocomplexity of these challenges garnered from the PTGS literature at large, because the rules identified are equally relevant and essential for development of such PTGS agents for human retinal or eye diseases. Hence, we have specifically not attempted here to review the emerging PTGS literature for retinal or ocular degenerations. Our focus here is on the variables that influence development and efficacy of a PTGS agent itself (the drug or R_x) rather than on the means of delivery of such an agent to the affected cells (e.g., through a vector or chemical design). When discussing the core *strategies* for therapeutic PTGS development, which did strongly emerge from early studies applying such agents to hereditary retinal degenerations, we touch on studies that lead to these strategies.

Successful design of a PTGS agent, be it AS, Rz, or RNAi, involves biocomplexity at the biophysical, biochemical, and cell biological levels. A target mRNA molecule is folded into dense secondary and tertiary structure, it is coated with heterogeneous proteins, it undergoes dynamic conformational fluctuations, and it resides in unique intracellular compartments with different lifetimes (nucleus, cytoplasm, ribosomes, etc.). These target mRNA factors *severely* constrain the locations in the RNA target that are accessible to the annealing of a colliding small PTGS agent and the range of timescales and spatial environments available for small PTGS ligand attack. In addition, the PTGS must be able to achieve a ground state conformation in which it is fully available to interact with and anneal to exposed regions of the target mRNA (molecular recognition). For Rz PTGS agents, the catalytic RNA bound to the target RNA must be able to undergo conformational transitions that promote RNA chemistry-based target cleavage. The structure-function properties of Rz-based PTGS agents become especially difficult when the Rz is embedded in a larger chimeric RNA to provide cell trafficking, stability, and high levels of expression. PTGS biocomplexity is a multivariate problem that is a major factor in the slow entry of nucleic acid knockdown agents into the pharmaceutical market despite obvious clinical potential. RNA structural biology greatly limits PTGS therapeutic strategies. In this paper we present the variables that must be understood for successful development of a PTGS agent. We present aspects of target RNA biology that will convince the reader about the biocomplexity of PTGS development. We present the different strategies and approaches of how PTGS agents can be used therapeutically for hereditary and degenerative diseases of the retina or eye and the relevant variables in the design of materials for such strategies. While RNA-directed drugs are still largely on the horizon, we briefly describe some high throughput screening (HTS) approaches that are expected to greatly influence further development of PTGS agents. Recent emergence of tools to address difficult scientific issues underlying the biocomplexity of the transcriptosome and RNA structure/function offer substantial hope that the dawn of clinical translation of RNA-directed drugs is visible in the near future. Use of HTS approaches to relieve bottlenecks in PTGS development is dealt with in detail in a separate review [95].

2.2. Common Variables That Affect Efficacy of PTGS Agents. There are five critical variables that are essential to understand in order to design PTGS agents that are efficacious *in vivo*. Limitation of any single property is sufficient to completely obviate functionality of the PTGS agent. *First*, the PTGS agent and its target RNA must be in the same cellular locale or compartment in order to allow for potential annealing interaction. *Second*, the PTGS agent must be in sufficient concentration to drive an adequate second-order collision frequency which is essential to secure annealing. *Third*, the target RNA must present an accessible and kinetically stable single-stranded platform at physiological temperature in order for the PTGS agent to anneal. *Fourth*, the PTGS agent itself must be in a conformational state that permits direct and full annealing to the target RNA. *Fifth*, for Rzs and RNAi, the cleavage products must dissociate rapidly from the enzyme to insure potential for enzymatic turnover (Michaelis-Menten kinetics).

2.2.1. Colocalization. In all cases, the PTGS ligand and the target RNA must colocalize in precisely the same spatial environment within the living cell, and on the same timescales, to support frequent collisional interactions that may result in kissing complex formation and full annealing [96–98]. RNAs (both target and PTGS agent) move along trafficking streams inside the human cell and have lifetimes at each stopping point along the way to their final destination(s). Hence, expressed RNAs may distribute among different spatial locales within the cell. Most mRNA targets for PTGS will spend the largest amount of their intrinsic lifetime in the cytoplasm, where they are diffusing, translated on the ribosome, or stored in RNA granules. While it is easy to appreciate that a PTGS agent that traffics to the nucleus will be unable to effect knockdown of a cytoplasmic mRNA target, more subtle issues are that both target mRNA and PTGS RNA agent could be in the same macroscopic compartment (e.g., cytoplasm) and yet not colocalize, because they do not occupy the same cellular RNA zip code, or that the lifetimes of the target and PTGS agent in a given locale are so disparate that meaningful second-order collision frequency is not probable. There are both gross macrocompartments and microcompartments within those in which mRNA targets and PTGS agents will need to colocalize for effective interactions. RNA zip codes are known to exist, and play a role in cellular RNA trafficking, storage, or to sponsor RNA: RNA interactions [99, 100]. The ideal situation for gene therapy is if the PTGS agent is engineered to occupy the same specific RNA zip code within cells as its cognate target, and that the lifetime or stability of the PTGS agent in the cell within the preferred locale is on the same order or greater than the lifetime of the target mRNA in the same spatial locale. In order to achieve colocalization with the target mRNA it may be beneficial to embed the PTGS agent into a carrier RNA to create a chimera. The carrier RNA (e.g., tRNA and VAI RNA) has established structure and function, is expressed to high levels in the cell, and has known trafficking properties that lend itself useful for colocalization of the PTGS agent with its target. Embedding a PTGS agent within a carrier RNA certainly adds to complexity of structure/function of the PTGS agent, which is an area that has not yet received much investigative effort.

2.2.2. PTGS Concentration and Diffusion Limitations. The PTGS agent must be present at sufficient concentrations in the same cellular locale as the target RNA to allow a fast and effective diffusion-limited second-order ON reaction rate (k_1) with the target RNA. While we normally think of enzyme reactions in macroscopic terms, with the substrate in substantial excess (Michaelis-Menten condition), it is prudent to consider the actual concentration of a target RNA inside a cell. Target RNAs are typically expressed in low numbers inside the cell. Even from a relatively strong promoter (e.g., human rod opsin) an estimated steady-state level of approximately 2500 mRNA molecules resides in a cytoplasm of total volume 1.75 picoliters (simplifying assumption of a spherical cell with 15 μm cell diameter and spherical nuclear diameter of 3 μm and with no excluded cytoplasmic volumes) would

yield a steady-state concentration of 2.4 nM. With weaker promoters, 250 mRNA molecules would yield a steady-state concentration of 240 pM, and 25 mRNA molecules could yield a concentration of only 24 pM. Even when the target mRNA is relatively abundant, these estimates indicate low cellular concentrations for the substrates (targets) of an initial PTGS annealing reaction. In addition to the target mRNA being in low concentration, it is also expected to be large with slow cellular diffusional coefficients [101]. Hence, the PTGS agent must be expressed or delivered in sufficient concentrations in the correct cellular compartment to promote an efficient collision rate with the target mRNA in order to promote a rapid second-order annealing reaction [102, 103]. The time scale needed for the functionality of the PTGS must also be embraced. For example, a rapidly degraded and intrinsically short lived mRNA that codes for a short lived protein involved in cell cycle regulation (e.g., a transcription factor), may have a half-life on the order of minutes and be expressed in low concentrations. It would be difficult for any PTGS technology to modulate the knockdown of such an mRNA and protein simply, because the kinetic action of the agent (e.g., Rz and RNAi) may be too slow to modulate an intrinsic process of mRNA degradation that is already rapid ($k_{cat} = k_{int} + k_{PTGS} \cong k_{int}$).

2.2.3. Target Accessibility. Regardless of the experimental approach, large bonafide regions of stable accessibility in target RNAs are rare in RNAs of any substantial size. The biocomplexity of the RNA target is the prime and profound limiting variable in the successful design of PTGS agents. It is the factor that limits successful PTGS design of any type (AS, ribozyme, RNAi) [104]. The secondary and tertiary structures of the folded mRNA impose a severe limitation to identifying suitable accessible regions for PTGS attack.

For any PTGS agent to be successful it must be able to collide with and anneal to accessible regions of the target mRNA. All successful PTGS technologies require bonafide regions of accessibility in the target RNA at 37°C for human therapeutics. Regions that are inaccessible, due to overriding RNA secondary and/or tertiary structure, or protein binding, will not permit rapid annealing, thus leading to delays in hybridization during the waiting time for local melting of secondary and tertiary structures at physiological temperature, if such melting is thermodynamically feasible (Figure 4). Any delays will decrease the overall observed catalytic rate (k_{cat}), and thereby reduce the amount of target mRNA that is cleaved within a given time interval such that target protein knockdown is limited. The capacity of the PTGS to anneal is not only dependent upon a kinetically stable accessible single-stranded platform, but also proper orientation for annealing of the accessible platform in the target mRNA to the approaching PTGS ligand in collisional reactions at physiological temperatures. The biophysical nature of the second-order annealing reaction between ligand and target RNA is critical to success but often unconsidered.

The biocomplexity of RNA target structure is the primary and dominant variable in the success of a therapeutic
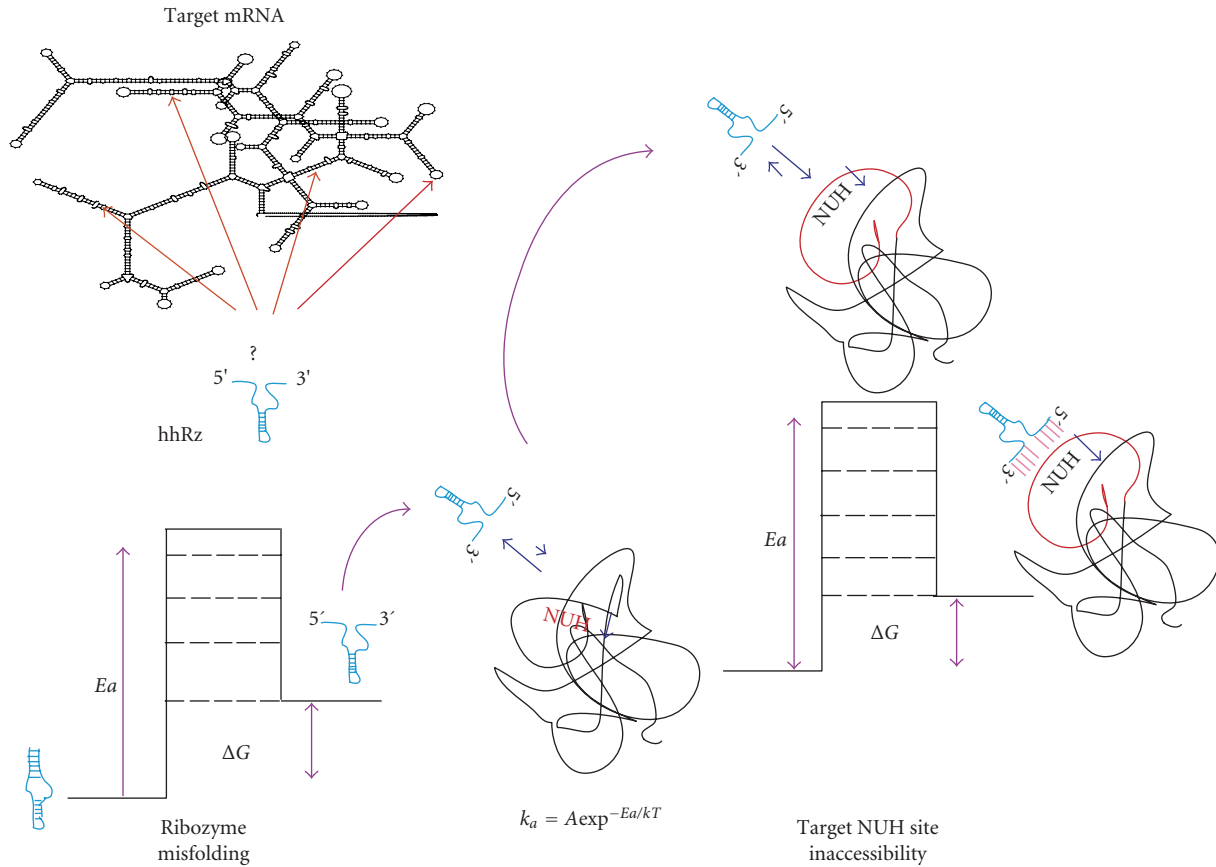
Figure 4: Secondary and tertiary structure as a limiting variable in PTGS efficacy. Energy diagrams are presented for both the target mRNA and the PTGS agent. The folded target mRNA has a site targeted for annealing which is buried in secondary or tertiary structure. The rate of unfolding of this region is determined by the activation energy required for conformational transition that leads to accessibility of the annealing platform. For the folded target RNA to be accessible it must present a single-stranded annealing platform(s) at its surface to allow annealing with the PTGS agent upon intermolecular collision. Buried regions of the RNA that are targeted must wait at physiological temperatures for relaxation of secondary and tertiary structure in order to present an annealing platform. Many regions are expected to never be exposed. The Arrhenius rate provides an estimate of how long it takes for a single-stranded platform to emerge at physiological temperature ($310°K = 37°C$) and is dependent upon the activation energy (Ea) of the transition. Likewise, any internal secondary structure of the PTGS agent itself can prevent annealing to target or slow catalysis and impact efficacy. Melting of inhibitory secondary or tertiary structure in the PTGS agent then can allow exposure of the antisense flanks to support annealing to the target mRNA.

PTGS agent. Large, accessible, and kinetically stable sites are rare and expected to follow Poisson distribution statistics. Therefore, initial efforts in any PTGS study should embrace the challenge to find the most accessible sites in the target mRNA. In any average size mRNA target, there are too many potential sites to try and attack with any PTGS technology and orders of magnitude insufficient resources to test them all. It is essential to be highly selective if one wants to achieve a PTGS agent that can be brought into preclinical trials in animal models. The first question is how to successfully identify accessibility in a target mRNA? We will discuss several possible means including emerging technologies. It is prudent to represent the complexity of the target RNA. For the sake of demonstration, we will consider two human mRNA disease targets for candidate therapies for autosomal dominant diseases. Human rod opsin (*RHO*) and human bestrophin (*BEST-1*) mRNAs are suitable examples. Both are the subject of over a hundred mutations that cause

human retinal degenerative diseases. Most mutations in the *RHO* gene cause autosomal dominant or autosomal recessive retinitis pigmentosa and less commonly autosomal dominant congenital stationary night blindness or retinitis punctata albescens [105, 106]. Most mutations in the *BEST-1* gene cause juvenile autosomal dominant vitelliform macular dystrophy (Best disease) and less commonly autosomal dominant adult vitelliform macular dystrophy or dominant bull's eye maculopathy [107–109]. The size of the dominant polyadenylated transcripts in the retina are 1.8 and 2.2 kB, respectively, [110, 111]. These mRNAs targets are of average size. The *RHO* gene is expressed exclusively in human rod photoreceptors in the retina that provide dim light (scotopic) vision, and the *BEST-1* gene is expressed exclusively in the retinal pigment epithelium.

There are several computational and experimental approaches that can be applied to the determination of accessible sites in RNA targets (Table 2). Computational

Table 2: Methods to identify accessible sites in target RNAs.

Method	Type	Properties	References
MFold	IS	Algorithm finds minimal free energy (MFE) structure and set of lower energy structures. Display as pictorial structures or output as a single-stranded frequency map vector (probability estimator).	[14–17, 22, 95, 112, 113, 124, 125, 128, 211, 223, 224]
SFold	IS	Algorithm searches all of folding space and samples on basis of free energy and determines probability of access directly.	[128, 221]
OligoWalk	IS	Algorithm takes output from MFold (.CT file) and uses this to determine local target unfolding energy, ligand binding energy, and net energy.	[25, 222]
mppRNA	IS	Uses MFold, SFold, OligoWalk, and in-house processing model to predict net probability of access in a region and to rank order the outcomes based on several parameters.	[95, 113, 128, 136, 211, 221]
ODN: RNaseH	EX	Search combinatorial ODN library for those entries able to bind to target RNA on basis of RNaseH of RNA: DNA hybrid, followed by primer extension analysis. Gel-based and cumbersome.	[14, 15, 31, 123–125, 225–227]
ODN arrays	EX	AS ODN sequence overlapping arrays are tiled onto silicon surfaces. Labeled target RNA is bound under defined conditions. Target binding to regions of the array identifies accessible regions.	[18, 228, 229]
Rz library	EX	Rich combinatorial library of hhRz sequences was used to cleave target RNA. First strand cDNA primed by Oligo-dT was followed by 3′ dG tailing, followed by PCR with a downstream gene-specific primerv and a poly-dC allowed amplification and sequencing to determine cleavage sites.	[136, 230–233]
RT-ROL	EX	Uses probe for reverse transcription that has 3′ randomized region to screen for accessibility and constant region for PCR. Gene-specific upstream primers allow agarose gel-based mapping of accessible sites for antisense or ribozymes. Requires concurrent sequencing analysis for mapping.	[234]
RT-TDPCR	EX	Cleavage by AS or Rzs is followed by RT, 3′cDNA tailing, and then PCR using a tail-specific primer and a downstream gene-specific primer. Very sensitive.	[235]
cMARS	EX	Uses probe for reverse transcription that has 3′antisense to all NUH↓ hhRz cleavage sites, followed by randomized region to screen for accessibility and 5′ constant region for PCR. Gene-specific upstream primers allow agarose gel-based mapping of accessible hhRz cleavage sites and their relative accessibility.	[95, 211]
MAST	EX	ODN with upstream and downstream constant regions embracing region of randomized sequence. Constant regions clamped by annealing complements. ssDNA region of MAST tags probes RNA target attached to beads. Annealing followed by washing, probe displacement, PCR, and sequencing. Little capacity to discriminate signal from noise.	[236]
gsMAST	EX	Refined version of MAST in which the library is gene-specific or sequence-specific MAST tags against a target RNA are evaluated in competitive hybridization assay.	[95, 211]

Notes: cMARS: cDNA mapping of accessible ribozyme sites; EX: experimental (method); IS: *in silico* (method); gsMAST: gene-specific MAST; MAST: mRNA accessible site tagging; mppRNA: multiple parameter prediction of RNA accessibility; RT-ROL: reverse transcription with random ODN libraries; RT-TDPCR: reverse transcription, terminal transferase-dependent PCR.

approaches are based upon algorithms that predict structures or energy, such as MFold, SFold, and OligoWalk [112]. The interested reader can explore the references associated with the different methods detailed in Table 2.

Computational algorithms are available to predict RNA secondary structure. Algorithms such as MFold [113] or the older version RNAFold can be used to obtain images of the secondary structure of a target mRNA. These specific

algorithms search for the most stable structure with the minimal (most negative) free energy (MFE). MFE secondary structures of human *RHO* and *BEST-1* mRNAs are shown (Figure 5). Both mRNAs demonstrate densely folded MFE secondary structures. There are rare single-stranded loops of significant size (e.g., >10 nt). This is a common appearance for all RNAs that we have folded computationally. There is always dense secondary structure present, and at best only rare regions containing large single-stranded platforms are present that appear suitable for rapid annealing. We have encountered RNAs that are even more densely folded than those presented here. These represent single-structure snapshots of the folding states of the mRNAs. There are many other potential structures and these MFE structures may not be the native structures in the cell. Nevertheless, even from this simple computational presentation target mRNA accessibility is clearly a dominant limiting variable in design of efficacious PTGS agents. Experimental approaches assessing accessibility support this perspective as well.

Cleavage with intended target site specificity requires that AS, hhRzs or RNAi be directed to accessible single-stranded regions where rapid annealing and generation of the enzyme: substrate complex equilibrium can occur [46, 75, 114–116]. Rapid binding/displacement equilibrium with a significant k_{OFF} rate allows mispaired mRNAs to be released before cleavage, which generates specificity for intended targets. Stabilized annealing complexes occurring at off-target mRNAs will decrease specificity. One wonders if the specificity problems reported for AS [117] and the promiscuous specificity of RNAi modalities somehow relates to cellular protein-based stabilization (e.g., through RNaseH or RISC) of mismatched target-PTGS ligand complexes.

The second-order annealing reaction appears to be the rate limiting step for PTGS agents in the living cell for long lived target mRNAs, and typically requires ten- to several hundred-fold molar excess of ligand over substrate mRNA to achieve good gene suppression [45, 96, 97, 114, 118]. This factor is likely due to the cellular reaction occurring under diffusion-limited conditions. While PTGS agent delivery, expression load, and colocalization with target RNA will affect collision encounter frequency, the major *initial* barrier to intracellular PTGS action is higher order target RNA structure that globally restricts the number of access sites [22, 119–125]. The secondary and tertiary structure obstacle is expected to pose the most serious limitation to the development of PTGS treatment strategies for diverse genetic or non-genetic diseases (see [126, 127]).

2.2.4. PTGS Conformation. The PTGS agent must itself be in a conformational state(s) supportive of the second order annealing reaction in order to achieve successful target knockdown [37]. Any abnormal intramolecular structure of the PTGS agent itself will create potentially unrecoverable annealing delays (Arrhenius rate of activation) and cause loss of efficacy for a constant ratio of PTGS agent to target mRNA (Figure 4). Regions of self-complementarity within a single-stranded AS ODN or hhRz RNA can occlude the antisense flanks from being freely available to interact with

the target mRNA. For a hhRz, it is also possible that the AS flanks intrude into the catalytic domain or into a structured domain (e.g., Stem II). Any perturbation of structure is expected to be potentially deleterious to both annealing and catalytic function. Waiting for such secondary structures to open at physiological temperatures implies an additional rate of reaction that acts to effectively slow the association rate with target mRNA. Since the minimal hhRz has only 4 bp of double stranded secondary structure expected for the entire active enzyme, it is not surprising that alternative conformational states of the hhRz can have marked impact on catalytic efficiency. Attempts to stabilize the hhRz into a proper secondary structure by extending Stem II or adding a stabilizing loop to cap this stem have not lead to improved function [128, 129], perhaps because extension of Stem II has negative impact on the catalytic cleavage rate of the enzyme. Proper structure as well as flexibility may be important for function. For the hhRz, the structure/function problem becomes more challenging with the recent identification of 5′ tertiary accessory elements that form pseudoknots with the Stem II loop and enhance the probability of achieving an enzymatically active state [130–135]. These considerations are focused on the ribozyme sequence itself. If the ribozyme is embedded in a chimeric RNA for strong expression, appropriate cellular trafficking for colocalization with target, and overall stability and lifetime in the cell, the potential for misfolded structures becomes much greater and requires careful rational design for the placement of the PTGS agent within the chimeric RNA (e.g., [136]).

2.2.5. Product Leaving. AS requires long hold times (high affinities) and hence must be highly stable once hybridized. For AS, a highly stable ES complex must form in order to attract RNaseH for cleavage, and it is necessary to identify stable, accessible regions for PTGS within this cellular milieu. Rzs must have sufficient holding time to permit cleavage but cannot bind too tightly or both loss of specificity for the intended target and product inhibition will occur. Upon cleavage, the upstream and downstream target products must be cleared rapidly from the AS flanks if the hhRz is to have substantial enzymatic turnover of additional target mRNA molecules. Similarly, RNAi requires sufficient hold time for RISC-mediated cleavage and must clear products to promote efficient multiturnover catalysis. For both hhRz and RNAi, too long a hold could be deleterious to specificity and contribute to off-target effects. RNAi is much more susceptible to off-target effects, because the RISC complex is highly tolerant of mismatches with target [75]. RISC in mammalian cells appears to use an ATP-dependent helicase to aid in stripping products, and this gives RNAi an advantage over native ribozymes which depend upon thermal solution properties (kT) for product release. In addition, the RISC complex is strongly dependent upon a relatively short "seed" sequence (6-7 nt) within the 5′ end of the guide RNA, which would be expected to interact with a larger set of targets when compared to the entire length of the siRNA (e.g., 19–23 nt). A Rz or RISC that cannot dissociate from the cleaved products of reaction becomes a catalytic

(a)

(b)
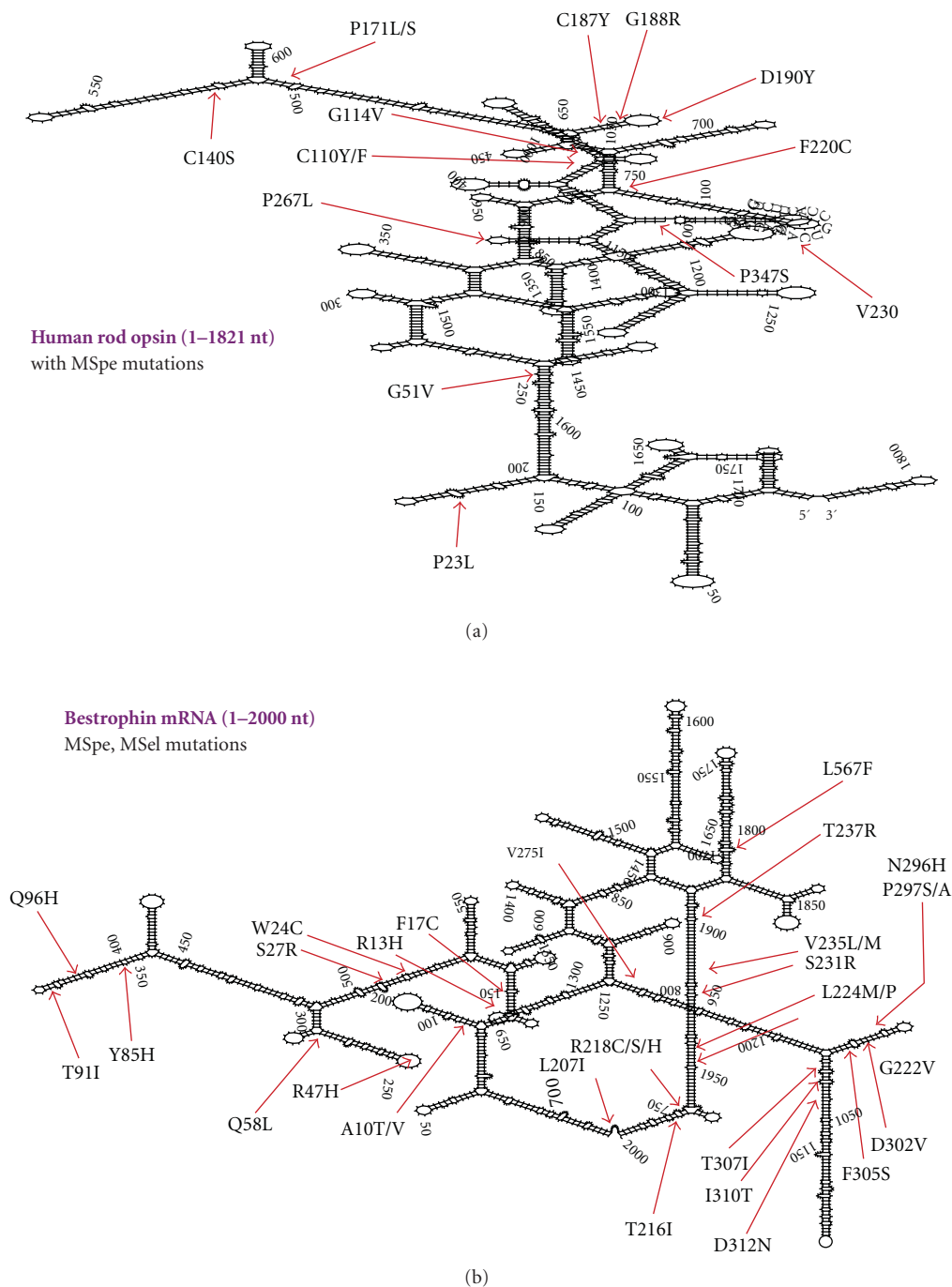
FIGURE 5: Predicted minimal free energy folding structures of human rod opsin and Bestrophin-1 mRNAs. GeneBank accession numbers for human rod opsin mRNA (NM000539.2) and Bestrophin-1 mRNA (NM004183) are indicated. (a) Human rod opsin mRNA (1–1820 nt) was folded *in silico* with RNA-Fold. The minimal free energy structure is shown. Note the dense secondary structure with only rare single-stranded annealing platforms of any substantial size. Also shown are the locations of human missense mutations that cause autosomal dominant retinitis pigmentosa and that generate *new* hhRz cleavage sites. These are mostly buried in secondary structure, where they would be poor targets for a mutation-specific (MSpe) approach to gene therapy as the PTGS agent would have limited capacity to anneal and cleave only the mutant target mRNA. (b) Human bestrophin-1 mRNA (1–2000 nt) was folded with RNA-fold and the MFE structure is shown. Again, secondary structure is dense with rare single-stranded annealing platforms larger than 10 nt. The locations of human mutations that cause autosomal dominant best macular dystrophy and that generate *new* hhRz cleavage sites for a mutation-specific strategy are shown (MSpe). Most are buried in dense secondary structure, where they would be expected to be inaccessible to annealing of a PTGS agent. Also shown are some mutations located in regions of WT cleavage sites, where they would permit a mutation-selective (MSel) approach to PTGS gene therapy.

antisense reagent without capacity for enzymatic turnover. Product leaving rates must be robust in order to clear the annealing or loading sites to promote next-target annealing. Product leaving rates can be predicted based upon nearest neighbor energetic analysis, which is prudent in early PTGS design (e.g., [13]).

2.3. Summary. The above variables lead to strong directives in approaches to PTGS design. The steady state level of a target mRNA is experimentally invariant as a defined characteristic of a particular target cell. The *only* way to influence the effective statistical collision frequency of the PTGS agent, whether in a deterministic or stochastic process, is to increase the numbers of PTGS ligands *immediately* within the local environment of the target and to keep those ligands relatively small such that they have substantial diffusional rate relative to the larger target RNA (expected to have a slower diffusional rate in the cell). Smaller size of the PTGS agents can also facilitate probing of targets in which the annealing site is found in recessed surface features of the tertiary structure. Successful knockdown of target requires both strong promoters and appropriate trafficking of the PTGS agents into the diffuse or specific microenvironments in which the target RNA resides inside the cell (e.g., knowledge of target RNA zip codes and the capacity to integrate this into the PTGS strategy). The PTGS agent must be stable, resist nucleases, and have a long cellular lifetime in the appropriate cellular compartment. The accessibility of the target mRNA must be rigorously determined if any successful target suppression is to occur. Regions of the target that present large, stable, single-stranded annealing platforms appear to be optimal, but these sites are typically rare in any target RNA. The PTGS agent must be able to appropriately sample necessary conformational transitions to achieve its activity, an issue which is especially challenging for an Rz and especially when the Rz is embedded in a chimeric RNA. For an Rz or RNAi the binding to the target must be sufficient but not too tight in order to achieve maximum specificity and to allow product release that is necessary to support enzymatic turnover. In aggregate, these variables create rational engineering and experimental challenges which must be embraced simultaneously to achieve efficacious PTGS agents for candidate therapeutics. This multivariable problem is a major reason why the entry of PTGS into the therapeutic landscape for human disease has been so slow.

3. Strategies and Approaches for PTGS Therapy

There are several types of therapeutic strategies that might be used for PTGS by any technological modality. The choice may depend upon whether the disease process is genetic in origin and whether the target mRNA or viral RNA codes for a normal or mutant protein. These strategies are (1) mutation-independent or knockdown, (2) RNA repair, (3) mutation-directed, and (4) combined therapy. By example, we will discuss here the different types of strategies as they might be applied to human retinal (or ocular) diseases, which is this

labs venue of interest. The generic knockdown approach may also be used to suppress wild-type mRNA expression [137].

3.1. Knockdown Therapeutic Approach. The mutation-independent or knockdown (KD) approach is the most straightforward. This strategy is used to suppress or knock down a target mRNA and its cognate protein. The target mRNA may be overexpressed from wild-type (WT) genes in particular clinical conditions or may be expressed in normal amounts but a therapeutic benefit can be envisioned from target suppression, or the target could be a viral RNA essential to a viral life cycle. KD may also be used as a component of combined PTGS therapy for genetic diseases (see below). The initial goal with KD is to identify the single most accessible site(s) of the WT target mRNA or viral RNA. Once this site(s) is identified then PTGS agents (AS, Rz, and RNAi) can be designed to anneal at these regions and promote target RNA knockdown within the live cell. AS ODNs are typically transfected into cells of a particular type, where the target is expressed and where the disease process is manifest. Rzs or siRNAs RNAs may also be directly transfected into cells. More commonly, Rzs, shRNAs, or miRNAs are transcribed from plasmid or vector constructs by RNA polymerases (Pol-II or Pol-III) within the cell harboring the target RNA. Expression constructs for the PTGS agent are delivered into cells by transfection agents, viruses, or nanoparticle systems (synthetic viruses).

3.2. Gene Therapies for Dominant Mutations. The molecular genetics of inherited retinal degenerations is very well developed and provides a suitable example. Many mutations in human genes that are expressed in the eye cause autosomal dominant disease patterns. Autosomal dominant hereditary retinal and macular degenerations are caused by mutations in genes expressed in specific cell types of the human retina [138, 139]. At least 204 retinal disease genes have been mapped, and 161 of these genes are cloned in part due to highly effective candidate gene approaches and the Human Genome Project and a database is available (RetNet, [140–142]). However, the RetNet database of disease genes underestimates the gene therapy challenge because multiple disease-causing mutations are commonly found in any given gene. The number of mutations can extend into the hundreds. Relevant examples are the many (>120) human rod opsin gene (*RHO*) mutations identified, since the P23H mutation was found causally associated with *adRP*. Opsin mutations are estimated to be responsible for 25%–30% of *all* cases of *ad*RP [105, 106, 138]. Mutations in the *RHO* gene cause *ad*RP, autosomal recessive RP, *ad* congenital stationary night blindness, and retinitis punctata albescens. The *RHO* gene, therefore, offers a robust *model* to investigate the extent to which PTGS therapies can be broadly applied as human gene therapies for *ad* retinal degenerations. The VMD2 or *Best-1* gene is another robust example. VMD2 is mutated in Best's vitelliform dystrophy and adult foveovitelliform dystrophy and has been found to harbor at least a hundred mutations, with almost all of these being dominant in nature. One can expect the general trend that the number of

mutations identified increases with time after identification of the disease gene.

3.2.1. Mutation-Independent or Allele-Independent Approach.

The mutation independent (MI) or KD approach in its stand alone format may be sufficient to suppress the disease process in a dominant genetic disease provided that no haploinsufficiency results. In a dominant hereditary condition, one can expect approximately 50% WT and 50% mutant protein expression from the two alleles. Often, in the normal case (no mutations), the WT protein is expressed in excess over that needed for cellular functions. In the autosomal dominant condition, the WT protein is already reduced by approximately 50%. This may already be insufficient to support cellular metabolism in the absence of the mutant protein (haploinsufficiency). In an MI or KD approach, the best PTGS agent is identified to target the most accessible site in the target mRNA to achieve the greatest degree of target mRNA/protein suppression. In this strategy, there is no specificity for the mutant mRNA versus the WT mRNA. Knockdown of mutant protein expression is expected to ameliorate the cellular toxicity that results from protein misfolding, or gain-of-function properties of the mutant protein, and thus relieve cellular stresses and permit longer cell vitality or normalization of cellular function. However, the MI or KD PTGS agent will also reduce WT protein expression below 50%, and this could promote haploinsufficiency and cell stresses and even cell death as a result. Thus, the MI or KD approach, as described above for WT targets, may possibly be used in autosomal dominant hereditary conditions, provided that the cell can resist haploinsufficiency due to further reduction of WT protein. If the relief of cellular stresses due to a highly toxic mutant protein can come about by relatively small reductions in mutant and WT protein, the impact of haploinsufficiency may not play as strong a role in cellular vitality.

3.2.2. mRNA Repair.

mRNA repair has been described with the use of the large trans-splicing Group I intron ribozyme of *Tetrahymena* that has been under development by the Sullenger and Haseloff labs [143–153] (Figure 6). The concept behind mRNA repair is that the guide sequence of the Group I Rz is engineered to anneal to a region of the target mRNA just upstream of the location of the mutation, cleave the RNA while using an available free guanosine as the nucleophile, release the downstream cleavage product, and finally trans-splice a normal 3′ exon onto the 3′ end of the upstream element of the target. All mutations in a target gene downstream of the cleavage splice site can thus be repaired. This makes mRNA repair an MI strategy. The engineered *Tetrahymena* Rz is actually a chimeric RNA and contains the Rz sequences, the guide sequence which is antisense to the target mRNA region, and an appended sequence which is the WT version of the target mRNA from just upstream of the site of mutation(s). The goal of mRNA repair is to cleave the target mRNA just above the site(s) of mutation and to splice onto the 3′ cleavage end an in-register copy of the downstream component of the WT mRNA. The

ribozyme splices itself out during this process. In this two-step process a WT mRNA is produced. This strategy could be used to repair all mutations downstream of the site of targeting. Therefore, just a few sites of targeting might be used to repair most or all known mutations in a given disease gene. A major disadvantage of this approach is that only a short guide sequence in the Rz (6 nt) is used to recognize the target RNA. This has resulted in lack of specificity regarding off-target mRNAs. Recently, the antisense region was extended to improve specificity against the intended target, and other elements of the Rz were optimized to generate better efficiency of trans-splicing. As for other PTGS strategies, the site of targeted annealing for *trans*-splicing has been found to be substantially affected by the secondary and tertiary structure of the mRNA in mammalian cells. Therefore, to be able to handle all or most mutations in a given gene, several accessible sites in the target will generally be necessary or a single accessible upstream target may be sufficient. While this specific MI approach by mRNA repair is more complex relative to other modalities (e.g., hhRz, RNAi), RNA repair still has potential to become clinically useful [147, 154]. One limit is that multiple agents will likely need to be developed to handle sets of mutations in a given gene, unless an upstream accessible region can be used for all mutations in a given gene. Another important issue is that the engineered Group I intron is spent for each mRNA that is repaired. There is no target turnover as one would have with either Rz or RNAi agents operating as Michaelis-Menten PTGS agents. Once the RNA repair enzyme operates on a single target that ribozyme no longer has a 3′ WT region to append to a subsequent target.

3.2.3. Mutation-Directed Strategies.

Mutation-directed (MD) therapeutic strategies target only the mutant mRNA with the intent of leaving the WT mRNA intact. MD PTGS agents have been shown to have therapeutic potential to stably rescue photoreceptors from toxic mutant opsin protein expression manifest in a transgenic *ad*RP rat model, albeit only a small fraction of mutant mRNA was suppressed [155, 156]. First, we detail the specific uses of a MD strategy, and then, we will present the advantages and substantial disadvantages which we expect will limit its use in gene therapy. There are two means by which a MD strategy might be realized, which depend upon the nature of the mutation and the nature of the PTGS technology utilized. In a mutation-specific strategy (MSpe) PTGS agents selectively inhibit *mutant* genes by targeting *only* mutant mRNA for cleavage. MSpe PTGS can easily be embraced by hhRz technology given the high degree of specificity of cleavage of the target mRNA at NUH↓ sites. To be susceptible to a MSpe strategy, the mutation in the gene must create a *new* cleavage site that is not present in the WT mRNA. For example, consider the human *RHO* mutation G51V (GGC → GUC↓). The mutation at the gene level is a transversion converting a G to a T residue (or U for RNA). This coding region mutation converts a glycine codon to a valine codon and generates a new hhRz cleavage site (GUC↓). This coding mutation results in a mutant protein, when expressed with WT protein, that
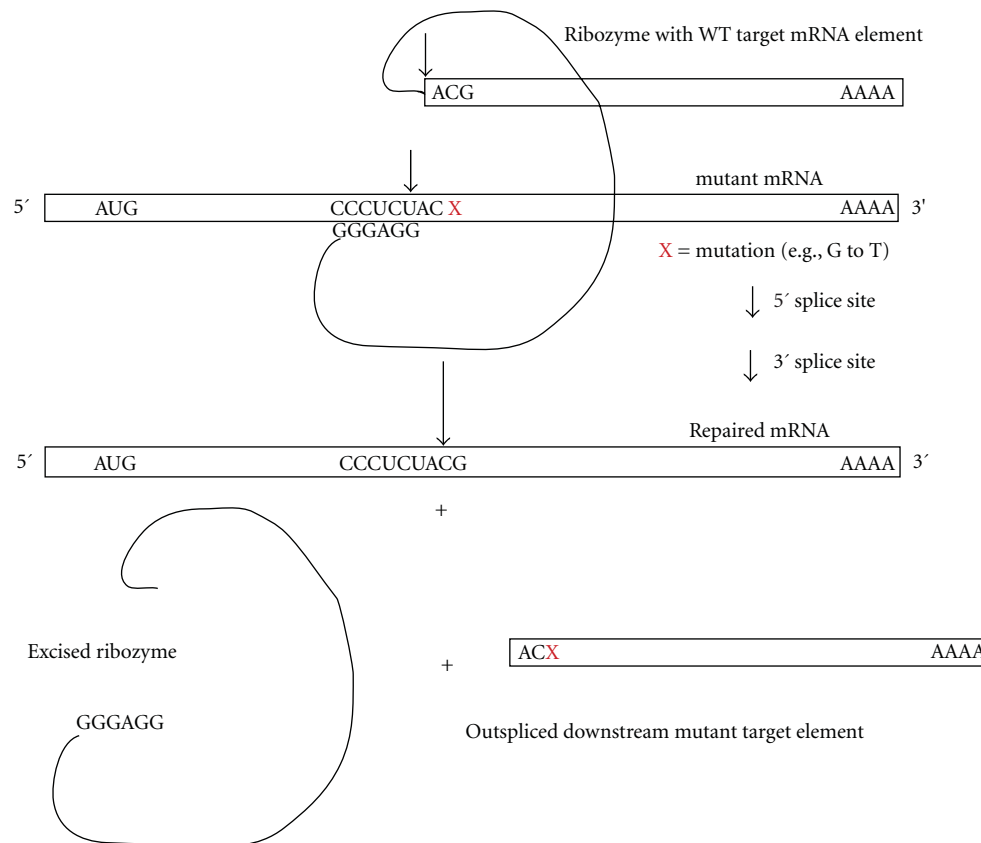
FIGURE 6: mRNA repair strategy for Dominant Mutations. The *trans*-splicing group I intron of Tetrahymena is engineered with an element that contains a WT mRNA sequence starting from just upstream of the mutation(s) in the target mRNA (labeled X). The Group I intron recognizes the region upstream of the target by way of complementary base pairing. It then cleaves the target using free guanosine as a nucleophile and then *trans*-splices a fresh downstream WT target mRNA element at the precise site of cleavage. All mutations in a given gene below the splice site could be treated with a single *trans*-splicing group I intron. One or several engineered group I introns could cover most mutations in a given human gene.

leads to photoreceptor stress and ultimately apoptotic cell death in *ad*RP [157–161]. The mutant protein could inhibit appropriate expression or trafficking of the WT protein (dominant negative mutation). It is possible that this specific mutation leads to a protein that is unstable in that it misfolds, is targeted for ubiquitination, and is then degraded by the protesome. Such a result in general could lead to haploinsufficiency as the assumed 50% of WT gene product that is made stably by the cell in a dominant hereditary condition may be insufficient to build the necessary multiprotein structure, create sufficient enzyme activity, or maintain the capacity of a signaling pathway. On the other hand, this mutation could promote a gain of function. Gain of function mutations could act at many levels. Such a mutation could create in the protein nonfunctional misfolded states that are not processed in large part for degradation, but rather become trapped in the endoplasmic reticulum, where they can accumulate and activate the unfolded protein response to exert toxicity and promote apoptosis [162–171]. On the other hand, the mutant protein might fold normally but mistraffic to the wrong compartment in the cell and exert signaling events that are a gain of function that is toxic to the cell (e.g., [172]). The mutant protein may fold and traffic correctly but have

some intrinsic instability that results in aberrant interactions with other proteins or aberrant signaling events that are also gains of function (e.g., [173]). Or, the mutant protein, in its interactions with other macromolecular components or the WT protein itself, affects the processing, trafficking, structure building and functional expression levels of the WT protein and thus have a dominant negative influence [174–176]. The possibility of the mutant protein creating a haploinsufficiency, gain of function, or dominant negative effects has substantial impact on PTGS strategies. Haploinsufficiency is treatable with a WT allele. Dominant negative effects might be treatable with a WT allele but may also require mutant protein knockdown. Gain of function effects certainly requires mutant protein suppression. It is appropriate to consider potential therapeutics in terms of whether they require WT protein reconstitution, or alterations in the relative ratio of WT to mutant mRNAs and proteins.

Returning to the RNA targets, the WT mRNA triplet GGC does not represent one of the classical NUH↓ sites for the hhRz, but the new GUC↓ motif is not only a classical triplet, but is also one of the two naturally occurring NUH↓ sites (GUC↓, GUA↓) with high intrinsic cleavage rates. The mutation creates a new hhRz cleavage site in the mutant

mRNA, while the WT mRNA has no cleavage site at the same position. This is the *necessary* and *sufficient* condition for use of the MSpe strategy. Such a mutation creates opportunity to design an MSpe hhRz intended to cleave *only* the mutant mRNA while leaving the WT mRNA intact, because it lacks the new NUH↓ site [177, 178]. The mutation could occur at any position in the gene (5'UT, coding, 3'UT) and still allow an MSpe strategy so long as a new NUH↓ cleavage site is created. Such a stand-alone strategy only makes sense for autosomal dominant mutations. On face value, the MSpe strategy seems ideal given that it allows a specific attack of a PTGS agent only on the mutant mRNA to suppress the mutant disease protein. However, there are a number of substantial limitations to the MSpe strategy. First, the MSpe strategy would only be indicated when there is a toxic gain of function of the mutant protein in the cell in which it is expressed. Second, only a fraction of mutations that occur in a given disease gene would create *new* hhRz cleavage NUH↓ motifs required for MSpe design. For example, of the 124 human rod opsin *a*dRP mutations that we have tabulated relatively few (∼21%) create new NUH↓ cleavage motifs for hhRzs. Similarly, of the 108 human VMD2 mutations that we have tabulated, only 15% create new NUH↓ cleavage motifs for hhRzs. Third, there is variation in the intrinsic rate of cleavage of NUH↓ motifs and some of these (e.g., AUA↓) have cleavage rates up to several orders of magnitude slower than those that occur in nature (GUC↓, and GUA↓) [43]. Fourth, even though the MSpe hhRz would be designed to cleave the new NUH↓ site created by mutation, most of the two antisense flank regions will precisely anneal to the WT target mRNA; this could result in a substantial antisense effect against the WT mRNA and contribute to an already preexisting haploinsufficiency effect due to the dominant mutation [178]. These factors alone would strongly limit the overall applicability of the MSpe approach for *ad* mutations in any gene except those that generate new *robust* hhRz cleavage sites. Fifth, random single-nucleotide mutations which constitute the bulk of human mutations are expected to mostly reside in regions of dense secondary structure, and be largely inaccessible for targeting. The expected lack of accessibility in the target mRNA around sites of most mutations is likely to be a single major factor that limits development of MSpe hhRz strategies. Sixth, each MSpe mutation requires an independent discovery and drug development process. The practical costs of such an effort are prohibitive.

A mutation-selective strategy (MSel) expands upon the limitations of the MSpe strategy [155, 179]. MSel hhRzs are designed to cleave at active NUH↓ sites (e.g. GUC↓, GUA↓, GUU↓, UUC↓, CUC↓, and AUC↓) that are *adjacent* to or in the immediate vicinity of the mutant codon. However, these cleavage sites are also present in the WT mRNA. This limits specificity (hence mutation selective), as some cleavage of WT mRNA will likely occur, in addition to the antisense (AS) effect on the WT mRNA that is expected to result from hhRz annealing [178]. The MSel rationale for development of an hhRz PTGS agent is that perfect hhRz annealing to mutant mRNA will lead to its selective cleavage, while mismatches between the hhRz and WT mRNA at N_1, N_2 or N_3 in the sequence $N_1N_2(\text{NUH↓})N_3$ (H does not base pair to hhRz)

will impair the cleavage rate for the WT mRNA (≥500-fold for an N_3 mismatch = *Strong MSel*; ≤10-fold for an N_1 or N_2 mismatch = *Weak MSel*) due to an expected hhRz structural perturbation [47, 180]. Strong MSel hhRzs can target ∼9% of opsin *ad*RP mutations, so we group them into a strong MD strategy (therapeutic potential for ∼24% of mutants). All of the other disadvantages seen with the MSpe approach are also expected for the MSel approach, the most significant being target mRNA structure which will severely limit annealing at most sites of human mutation. In all MD strategies, the Rz is *obligated* to anneal to local primary sequence around the site of a random *human* mutation associated with an NUH↓ motif. Most single nt *ad* mutations in any mRNA will predictably localize to hybridized secondary structure which is expected to limit or frankly block Rz annealing at the vast majority of potential cleavage sites (see Figures 5, 7). For example, attempts to develop mutation specific or selective ribozymes to the human rod opsin P347S mutant mRNA have failed *in vitro* [181].

All MD strategies of PTGS for genetic diseases require the design of the agent for a specific mutation or set of mutations (mRNA repair). The challenge to successfully build an efficacious PTGS agent has many pitfalls. This becomes greatly compounded when the design has to be repeated many times for a given disease gene, for example for those mutations where a strong MD strategy might possibly be feasible. The design and testing of a single PTGS agent requires extensive time, effort, and great expense when extending the development through the preclinical animal testing phase. And even if some such MD agents could be achieved, they likely would have varying efficacy to treat different mutations in a given gene. Yet, the development of such agents as drugs for orphan genetic diseases is clearly indicated for those suffering globally with such diseases. This need must ultimately be balanced by the fact that many human disease genes have substantial allelic heterogeneity or mutational diversity. Mutation frequency can vary from common with founder effects (e.g., P23H in human *RHO* gene) to rare (e.g., K296M in rod rhodopsin, [182]), where only a single family pedigree with two affected individuals has been identified globally to date. It is difficult to anticipate that rare genetic mutations would be targeted by a unique therapy that moves up through clinical approval and the many hundreds of millions of dollars that are needed to realize an effective and safe new drug. Research and development costs for PTGS gene therapy will be colossal if testing of many designs is needed to achieve optimized MD constructs for *each* mutant mRNA. Rather, what is rational to expect is that a single PTGS therapy directed to a single human disease gene might eventually come to fruition. While KD or MI PTGS therapy embraces a critical aspect of such an approach (one therapy for all/most dominant disease mutations in a single gene) the potential limitation of haploinsufficiency is already prompting development of combined therapeutic strategies.

3.3. Combined PTGS Therapeutic Strategies. A major advantage of the KD or MI strategy for autosomal dominant retinal or eye diseases is that the *best* hhRz or other PTGS

Mutation dependent

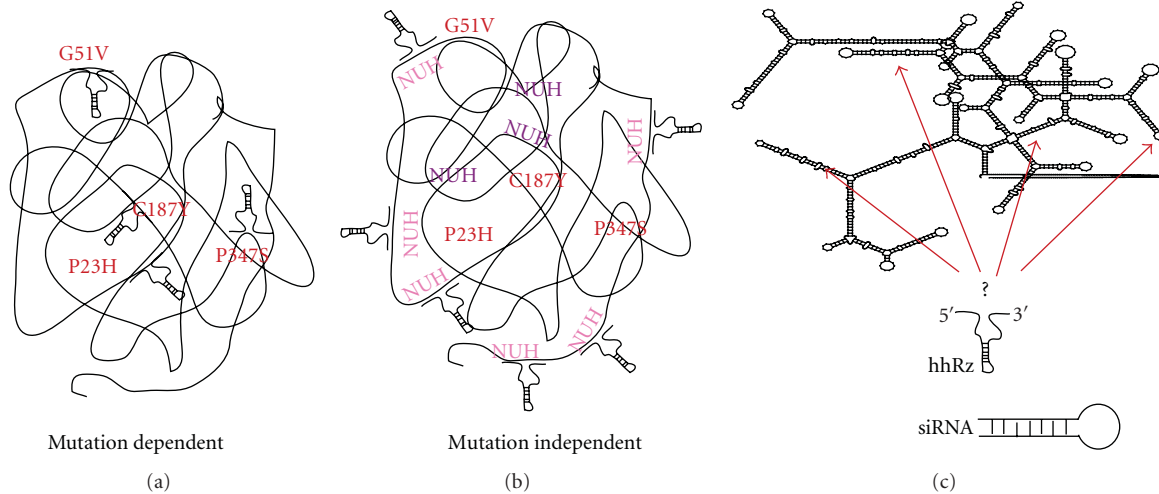(a)

Mutation independent

(b)

(c)

FIGURE 7: Comparison of mutation independent and mutation dependent strategies to PTGS therapy. The schematic representation shows two folded mRNAs, one in which a MD strategy is being used to attack discrete mutations which obligate the site of attack to regions that are likely to be buried, and the other is an MI strategy, where the best (most accessible) NUH↓ (lavender) or RNAi cleavage site is sought for use. This challenge applies to hhRz or RNAi type therapeutics.

agent can be sought to cleave the mutant (and WT) mRNA at the most accessible site and that a single KD agent can be used to cleave many or *all* mutations so long as the binding or NUH↓ cleavage motifs are not affected by mutation (probability <0.008). The KD strategy avoids repetitive and expensive R&D for each new mutant as per the MD strategy. One KD agent could provide therapy for all or most mutations in each disease allele. We, and others, have developed KD hhRzs that cut full-length mutant *human* rod opsin mRNA and could be used to target all currently known opsin *ad*RP mutations [128, 183–189]. Since the most optimal (accessible) cleavage site for the WT (and mutant) mRNAs is sought for attack, the critical limiting variable in development of PTGS agents, target RNA structure, is immediately embraced by this strategy. The single and substantial disadvantage of the KD strategy is that both mutant and WT mRNAs are expected to be equivalently cleaved by the PTGS agent. The cellular phenotypic outcome of expected equivalent knockdown of both WT and mutant mRNAs and proteins will depend critically upon the cell in which the gene is expressed, the function of the protein, and the resultant levels of expression induced by the MI PTGS agent. Let us consider *RHO* as a target of KD PTGS. Rod opsin is expressed in abundance in rod photoreceptors and is the visual pigment that subserves human scotopic vision. There is a plethora of biochemical, biophysical, cell biological, and genetic data on rhodopsin from over four decades of research. WT rod rhodopsin is expressed in great excess in photoreceptors to the levels over 2×10^8 copies/cell. Essentially, all of the apoprotein is trafficked to the outer segment, where over 98% is localized to topologically isolated disk membranes and under 2% is localized to the plasma membrane. The human rod photoreceptor has the capacity to detect and respond to the absorption of single photons of appropriate energy, in part due to an extremely low level

of electrophysiological noise in darkness and a high gain biochemical amplification pathway in light. Nevertheless, the dynamic physiological range of a human rod photoreceptor saturates upon approximately 200 photon absorptions [190]. Therefore, there are 99.9999% spare rhodopsin receptors in the rod photoreceptor to guarantee quantum catch when photon density is extremely low (dim starlight). There is substantial evidence that the rod photoreceptor autoregulates the amount of opsin that is expressed in order to maintain a constant daily absorption of photons in a process called photostasis [191]. Rhodopsin itself appears to be the sensor that drives this transcriptional regulation pathway. In rodents kept in dim light, the levels of opsin expression increase, and the outer segment length increases with no apparent change in diameter. In increasingly higher levels of light, the level of opsin expression decreases proportionally, and the outer segment shrinks in length. At sufficiently high levels of light, there is light damage and cell death. How much WT opsin is necessary to maintain the vitality and ideally the function of the rod photoreceptor? This is a systems biology question of critical relevance to the KD PTGS approach to gene therapy of opsin-based *ad*RP. We do not yet know the full answer, but studies have pointed to an understanding of gross limits on the range of normal rod opsin expression that are needed to maintain the structure and physiological vitality of the rod photoreceptor. There is substantial evidence that rhodopsin is in *at least* 50% excess for long-term structural maintenance and survival. A recessive human mutation, E249ter, causes 50% loss of WT rhodopsin but is phenotypically *silent* in the carrier state [192–194]. The heterozygous rod opsin mouse (50% rhodopsin/rod) is similar to human E249ter carriers in that very slow, if any, retinal degeneration occurs over 90–120 days [195–197]. With only 50% of normal WT rhodopsin being present, the outer segment lengths have shrunk to approximately 50% of their normal length while apparently

maintaining their diameters. When rhodopsin levels decrease to 25% of normal in rats exposed to moderate light intensity, *many* rods maintain vitality with shorter outer segments, but many rods also die [191]. One might, therefore, hypothesize that a significant reduction (between 50%–75%) of WT rod rhodopsin (rod sensitivity reduction by -0.3 to $-0.6 \log$) would not cause *rapid* retinal degeneration in mammals. Efficacious PTGS knockdown (knockout is unlikely) of 50% of *total* opsin protein would leave 25% WT and 25% mutant in *ad*RP rods. With severe mutants (e.g., C187Y), the benefits of reducing toxic gain of function mutant protein are expected to offset partial loss of WT opsin. However, WT opsin levels must be maintained at around 50% to permit rod survival *in mouse* [195, 196]. When the E249ter mutation is homozygous and no WT opsin is synthesized, affected patients have early onset autosomal recessive RP [192]. The mouse opsin knockout has a rapid retinal degeneration. Opsin expression is essential for stable elaboration of an outer segment and the formation of the phototransduction apparatus. A single WT allele slows degeneration in the presence of a single mutant opsin allele in mice [198]. This suggests that the WT allele is protective, at least under certain constraints. On the other hand there is a distinct limit on overexpression of the WT opsin protein. Tan et al. [199] showed in murine transgenic models that overexpression of WT rod opsin in rod photoreceptors beyond 125% of normal levels promotes retinal degeneration. Thus, it would appear that the tolerable limits of under and overexpression of WT rod opsin in the mammalian rod photoreceptor likely range from between 25% and 125%. This broad range indicates that the photoreceptor as a system is highly tolerant or capable of major fluctuations of one of its critical functional proteins. Many other phototransduction, structural, and metabolic proteins are expected to shift their expression levels in concert with opsin, as they are cotranscriptionally regulated. This may have substantial functional implications for cellular adaptations such as photostasis. If WT rhodopsin levels at 25% and above exceed a threshold supportive of rod photoreceptor vitality with an outer segment, then a relatively efficacious PTGS agent that knocks down 50% of total opsin protein (WT and mutant) would be expected to permit rod photoreceptor survival if the WT fraction was the only component for consideration. However, the impact of the mutant protein could be a toxic gain of function modality for the cell. The level of knockdown of the *mutant* fraction that is necessary to support vitality of the rod photoreceptor will likely prove to be dependent upon the nature of the mutation and the levels of photoreceptor systems biology that are impacted by any gain of function toxicity. At present, we can only anticipate that knockdown of mutant and WT protein levels will vary depending upon the PTGS agent that is used and its expression level in the appropriate cell type. It is prudent to expect that there will be a dynamic range of potential therapeutic outcomes from a single PTGS agent in a given cellular system. Any therapeutic rationale must embrace the intrinsic dynamic range of WT protein expression, varying toxicity of the mutant protein, varying therapeutic efficacy of the PTGS agent itself, and a means of transcriptionally regulating the PTGS agent both to tune the therapeutic effects or modulate against potential deleterious effects. These issues which tap into retinal systems biology make PTGS therapy a difficult but likely attainable goal in the road ahead.

3.3.1. Combined Knockdown: Reconstitution Therapy. The combined knockdown: reconstitution therapy (CKDRT) embraces both the knockdown potential of the PTGS agent and the protective effect of the WT allele. In CKDRT, both the native WT mRNA and the mutant mRNA are targeted for therapeutic KD PTGS attack, but the WT mRNA levels are reconstituted through expression of an engineered allele that transcribes a WT mRNA that is resistant or hardened to cleavage. Montgomery and Dietz [200] first reported that KD hhRzs embedded in an antisense sequence were able to efficiently cleave fibrillin-1 mRNA (disease gene in *ad* Marfan's syndrome). They suggested a general approach in treating a variety of *ad* genetic diseases by a knockdown hhRz/AS to suppress both mutant and WT mRNAs in association with a WT reconstitution construct, altered with respect to codon degeneracy, to reconstitute WT expression to appropriate levels in order to prevent intrinsic or therapeutic haploinsufficiency. It is this strategy that we call CKDRT. Later in the same year, Millington-Ward et al. [183] reported that ribozymes against opsin and peripherin mRNAs could potentially be used in a CKDRT strategy as a general approach for therapies of *ad* genetic diseases. CKDRT has also been applied to *ad* α-1 antitrypsin deficiency of liver [201, 202]. There is increasing utility of the CKDRT approach combined with a decreased frequency of reports on design of MD PTGS agents [128, 183–185, 187, 188, 203–206]. This is predictable given the severe constraints of the MD approach as presented above. Nevertheless, while CKDRT may be a suitable approach to clinical gene therapy, for an autosomal dominant disease, there remains many significant scientific hurdles to be overcome, some of which we will present here. The first goal beyond development of a potent KD or MI PTGS agent is to achieve a functional allelic variant WT (*a*WT) mRNA with full potential to translate sufficient levels of WT protein given normal levels of transcription.

3.3.2. Design of Allelic Variant WT Expression Constructs. A critical component of the CKDRT approach is that an *a*WT variant of the WT gene or cDNA must be engineered to express a processed mRNA that is resistant or hardened to cleavage by the specific KD or MI PTGS agent. We consider how target mRNA resistance can be engineered when the PTGS strategies use hhRz or shRNA modalities. The design of a cleavage-resistant mRNA for reconstituting WT protein expression can be simple or complex, depending upon the location and nature of the cleavage site in the mRNA relative to the reading frame of the protein. We will first consider the development of an hhRz resistant *a*WT variant for three attack sites in a given mRNA to indicate the potential complexities in *a*WT variant design. We will use the rod opsin (*RHO*) mRNA as a model in part, because there is a crystal structure available for the WT rhodopsin protein that

TABLE 3: Construction of allelic variant genes for combined knockdown reconstitute therapies.

Allelic variants for HhRz Therapeutics	
5′ UT Target Site	
5′…CCUGAGUGGCUGAG**CUC**↓AGGCCUU…	(5′ UT target site CUC↓)
5′…CCUGAGUGGCUGAG**CUG** AGGCCUU…	(aWT variant mRNA, CUG cannot be cleaved)

Coding V230 region	
5′…CUC GUC UUC ACC **GUC**↓ AAG GAG GCC…3′	(Coding region GUC↓)
L226 V227 F228 T229 V230 K231 E232 A233	(Amino acid triplets)
5′…CUC GUC UUC ACC **GUG** AAG GAG GCC…3′	(aWT variant mRNA,
L226 V227 F228 T229 V230 K231 E232 A233	GUG cannot be cleaved)
Single letter amino acid codes are used.	

Coding F293 region	
5′…AUC CCA GCG UU↓C UUU GCC AAG AGC…3′	(Coding region GUU↓)
I290 P291 A292 F293 F294 A295 K296 S297	
GUU↓ cleavage site occurs *within* the F293 codon rather than cutting at the end of a codon.	
5′…AUC CCA GCG UGC UUU GCC AAG AGC…3′	(aF293C variant mRNA)
I290 P291 A292 C293 F294 A295 K296 S297	
It is unclear whether or not the F293C mutation is an allelic variant WT or has a protein phenotype.	

Allelic variant for RNAi therapeutics in F293 region	
5′…AUC CCA GCG UUC UUU GCC AAG AGC…3′	(Coding region RNAi site)
I290 P291 A292 F293 F294 A295 K296 S297	
5′…AUA CCC GCA UUU UUC GCG AAA AGG…3′	(aWT variant mRNA)
I290 P291 A292 F293 F294 A295 K296 S297	
aWT variant generated by codon degeneracy across the region of designed RNAi antisense annealing.	

is encoded by this mRNA [207]. The protein crystal structure can guide decision making in complex aWT variant gene design, when the region of attack is in the protein-coding region and the cleavage site occurs within rather than at the end of a discrete codon. Consideration of WT protein structure is essential to maintain the WT structure/function and phenotype within the cell in which the aWT construct would be expressed, especially in the specific case when the aWT construct cannot be made silently with respect to the protein reading frame. We will assume three sites for hhRz attack with one in the 5′UT and the remaining two in the coding region of the mRNA (Table 3). Let us further assume that these three sites have equivalent and high levels of accessibility such that they would be sensible regions for PTGS attack by a KD, MI hhRzs, or shRNAs. The 5′UT and 3′UT regions of the processed mRNA are the easiest regions for design of a aWT construct because maintaining appropriate amino acid protein coding is not a variable.

aWT Construct Design for hhRz PTGS Agents. For a CUC↓ hhRz attack site in the 5′UT, it is relatively simple to obviate the proven efficacious hhRz cleavage at this site by a single nt change from CUC↓ to CUG (Table 3). An hhRz cleavage site is NUH↓ where N is any nt and H is any nt except G. CUG is representative of any NUG site (GUG, CUG, AUG, and UUG) that cannot be cleaved by a hhRz. Hence, any chosen NUH↓ site in an accessible region of the 5′UT (or 3′UT) could be converted to an NUG site which cannot be cleaved to generate an aWT construct. If the hhRz targeting this CUC↓ site has been shown to exert all of its KD on the basis of RNA catalysis, with the loss of all KD occurring through catalytic enzyme core mutations, then this simple mutation creates a sufficient aWT construct. One also wants to avoid the potential impact of significant antisense effects of the chosen PTGS hhRz agent on the aWT mRNA. Next, we consider two hypothetical accessible hhRz cleavage sites in the coding region of the opsin mRNA.
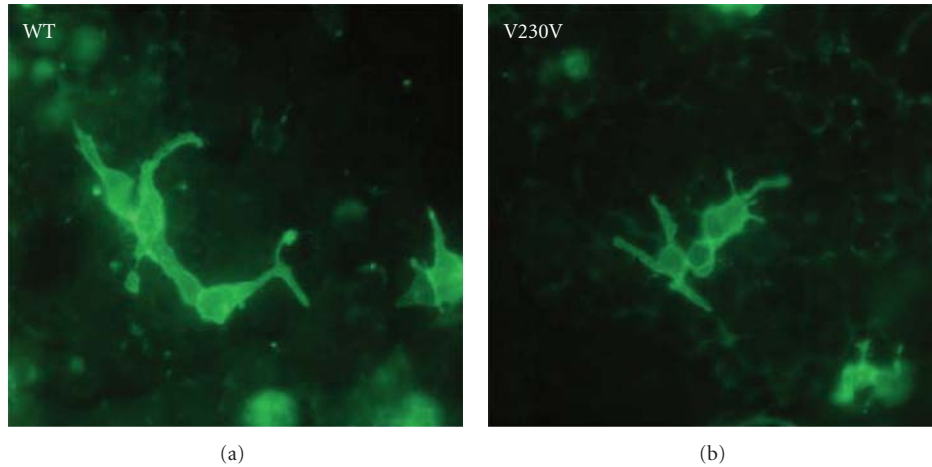
(a)

(b)

FIGURE 8: Allelic variant human opsin construct. The hhRz cleavage site at V230 (GUC↓) was mutated by site-specific mutagenesis to the degenerate human valine codon GUG. The V230V human opsin cDNA in a CMV expression vector (pCDNA3) was expressed in HEK293S cells along with control human WT opsin CMV expression vector. Immunocytochemistry with 1D4 opsin monoclonal and an FITC-labeled secondary antibody was conducted. Abundant human WT opsin expression and cell surface trafficking was noted in cells expressing WT (a) or V230V *a*WT proteins (b).
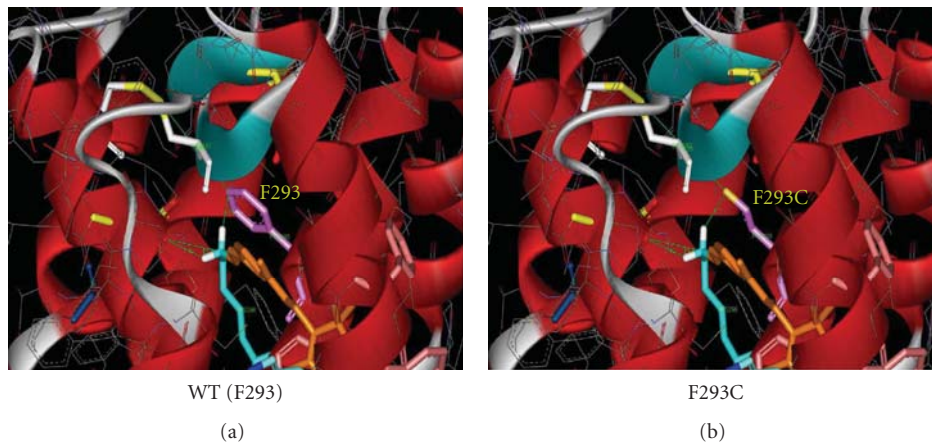

WT (F293)

F293C

(a)

(b)

FIGURE 9: Crystal structure analysis of allelic variant constructs. We analyzed the location of the F293C mutation in the bovine rod opsin crystal structure (1F88.pdb). WT protein (a). The image shows the cutout region around the protonated Schiff base linkage of 11-*cis*-retinal (orange) to K296 (blue sidechain). F293 is lavender in color. The disulfide bond between C110 and C187 is in yellow. F293 is within 5 Å of the Schiff base and within 10 Å of the disulfide bond. F293C Mutation (b). The C293 sidechain is lavender in color with a yellow tip indicating a free sulfhydryl group (-SH). The SH group is within 5 Å of the Schiff base and 10 Å of the disulfide bond.

First, let us consider the site at V230 which is encoded by a GUC↓ triplet. *In vitro* hhRzs were designed that were able to cleave at this site in the human *RHO* mRNA [185]. A single nt transversion leads to a GUG triplet which is no longer cleavable by the targeting hhRz. Moreover, GUG still codes for V230 due to degeneracy. *In vitro* hhRzs that successfully cleaved targets containing the GUC↓ site failed to cleave the *a*WT variant RNA containing the GUG triplet [185]. We engineered an *a*WT V230V (GUG) variant cDNA by site-specific mutagenesis and put this construct under the control of a strong CMV promoter in a cellular expression plasmid. When we expressed this *a*WT V230V (GUG) cDNA in HEK293S cells, we found, qualitatively, that the expression and cellular distribution of WT protein was

not different from otherwise equivalent V230 GUC↓ WT expression construct by immunocytochemistry (Figure 8). Assuming that a hhRz exerts full catalysis at this site without substantial pure AS effects, an *a*WT with potential for success has then been designed. Design of an *a*WT construct at the second coding site (F93) demonstrates the complexity that can arise, however, because an NUH↓ cleavage site within the coding region may not obey the serial order of protein coding triplets but rather overlap them. The assumed accessible targeted triplet GUU↓ overlaps two codons of human *RHO* mRNA at A292 and F293 (Table 3). To make an *a*WT mRNA that is resistant to hhRz attack the conversion of GUU↓ to noncleavable GUG results in a F293C (phenylalanine to cysteine) mutation in the opsin polypeptide. Moreover, the

location of this mutation at the protein level is one helical turn away from the side chain of K296, which is the site of covalent attachment of 11-*cis*-retinal to the opsin apoprotein. The replacement of a phenylalanine with a cysteine side chain in such a critical location in the protein as an *a*WT construct must be seriously considered. It is unclear whether the mutation represents an identified allelic variant WT or whether it is truly a mutation with a potential phenotypic effect on protein folding, function, or even potential toxicity (gain of function). The impact of such a mutation may be appreciated at a structural biological level if there is an available crystal structure. Also, expression of the mutant and normal proteins will be needed to compare functional profiles, if adequate assays are available (e.g. [208, 209]). A cellular expression to test for cell localization of the mutant versus WT protein clearly is indicated to insure that the variant protein has appropriate WT trafficking phenotype. Tests for expressed mutant protein structure-function relative to the native WT protein are also needed. An opsin F293C mutation replaces a hydrophobic aromatic ring sidechain with a polar and potentially reactive linear sidechain. The location of the normal and mutant sidechains in the rhodopsin crystal structure is shown (Figure 9). While the length of the mutant sidechain is similar to the native phenylalanine sidechain, the potentially reactive cysteine sulfhydryl group is approximately 4 Å from the Schiff base of K296 and 9 Å from the C187 or C110 sidechains. A cysteine sidechain residue in this position could alter the local environment important for 11-*cis*-retinal docking and covalent ligation, or it could impair or intrude upon disulfide bond formation between C110 and C187, which is essential to the tertiary structure of rhodopsin [210]. We found no biochemical structure function studies in rhodopsin that reported on mutations at F293, so it is unknown how well they might be tolerated, and coincident biochemical or biophysical structure function studies at the protein level could be important to assure that the allelic variant protein indeed has WT characteristics. While such a mutant protein that behaves like WT may be useful as an *a*WT variant, rigorous experimental proof will be necessary whenever development of an hhRz resistant mRNA requires the development of such a potential *a*WT variant. Clearly, if equivalently accessible regions present NUH↓ sites for targeting, where it would be easier to construct an *a*WT variant, it is prudent to consider further development of CKDRT PTGS agents for such sites.

aWT Construct Design for RNA_i PTGS Agents. The manner in which an *a*WT- or RNAi-resistant target needs to be designed is based strictly upon codon degeneracy within the coding region of the protein, with little apparent restriction elsewhere in the 5′UT and 3′UT regions, except for otherwise unknown protein-binding regions that might only be discovered empirically. For an *a*WT construct to be built for resistance of the mRNA to annealing and cleavage of charged RISC within the coding region of the protein, it is necessary to exploit codon degeneracy to preserve the amino acid sequence while substantially perturbing the

binding energy of the RISC to the target. Ideally, one will want to preserve, to the best extent possible, the use of the human codon bias (or animal codon bias in proof-of-principle studies) in the selection of alternative coding triplets, whenever these present so as not to potentially impact WT protein expression levels. We demonstrate an example of such a design (Table 3). As for the design of an *a*WT variant for hhRz resistance, it is necessary to empirically test for resistance to knockdown of the *a*WT expression construct mRNA in cultured cells relative to the original WT expression construct. The RISC complex of the antisense strand can tolerate several mismatches at the 5′ and 3′ ends and still be capable of cleavage [75]. While the energetic rules of nearest neighbor RNA: RNA binding of the guide sequence *within* RISC to a potential target are not yet well established, it is probably worthwhile to use nearest neighbor calculations to minimize the binding energy of the charged RISC to the *a*WT mRNA while preserving amino acid coding.

4. Conclusions and Outlook

The development of a successful PTGS agent for therapy is one of the more difficult tasks in molecular medicine today and is a task that is well described by the term biocomplexity. This biocomplexity is underscored by the fact that currently there is only a single PTGS agent that is FDA-approved for human use despite decades of academic and corporate research. Here, we have presented an overview of currently used PTGS technologies, the critical biophysical variables that impact efficacy, and the strategies that may be used for genetic or nongenetic retinal diseases, where PTGS agents are likely to have future therapeutic impact. We anticipate that ribozymes have substantially greater therapeutic potential than RNAi, because they can likely be as potent, given a predetermined accessible region in the target mRNA, yet they are not as fraught with the promiscuous off-target effects and toxicity that continues to be demonstrated with RNAi (shRNA, and siRNA). We have presented an overriding strategy used in this lab for development of hhRzs as therapeutic agents. We have tried to present not only a base of knowledge to begin work along this path, but also a view of the pitfalls so that other investigators may find it easier to proceed down these investigative paths in the interests of the patients.

There are several remaining issues that limit wide-scale development of RNA drugs [95]. First, one must have highly reliable and efficient tools to first solve the severe problem of identifying those rare regions of target accessibility for annealing of PTGS agents. Work in this lab has focused on this problem of target mRNA accessibility with the development of several HTS bioinformatics and experimental approaches ([211, 212], Taggart et al., *in preparation*). Work must be directed to efficient methods of searching for these rare sites when the target is presented in biologically complex mixtures such as the cell cytoplasm. Second, once such accessible regions are determined there will likely be a substantial number of PTGS agents that will need to be tested for cellular efficacy and toxicity. HTS approaches to

screening for cellular knockdown and toxicity by Rzs or other PTGS agents are needed to be able to quantitatively assess and rank order both efficacy and toxicity of sets of PTGS agents targeting given accessible regions (Yau and Sullivan, submitted; Kolniak and Sullivan, 2011; Butler et al., *in preparation* [213–215]). Third, without colocalization with target mRNA, any PTGS agent will fail at efficacy. Better tools to rapidly identify cellular target RNA trafficking routes, destination zip codes, and the sequence or structural motifs that effect such localization are needed. The later motifs could then be integrated into the PTGS agent constructs as complex chimeric RNAs with therapeutic, protective, and trafficking domains. Fourth, while it is generally accepted that Rz kinetic performance *in vivo* against native structure target RNAs is 100–1000 fold less effective than *in vitro* when measured against short unstructured model substrates, it has been difficult to evaluate the robust kinetic performance of ribozymes in mammalian cells. Approaches must be developed to determine the kinetic performance and rate limiting step(s) of a given PTGS agent *in vivo*, which is paramount to rational improvements for higher efficacy, and to understand the factors that influence intracellular failures of PTGS agents. Recent development of similar approaches in yeast might be beneficial to guide the way for kinetic Rz analysis in mammalian cells [216]. The engineering of *in vivo* cellular reporter systems for both the target mRNA and the PTGS agent might also be useful (Yau and Sullivan, *submitted*). Fifth, HTS assays to quantitatively assess the cellular levels of target mRNA and protein which will need to move beyond the classical gel-based approaches which are slow, complex, and semiquantitative and have high variability. We have developed a quantitative robotic imaging platform that is able to measure target proteins and mRNA in transfected cells that are fixed and permeabilized in 96-well format [213, 215] Butler et al., *in preparation*. Sixth, the power of macromolecular RNA as a drug must embrace rational and computational design for structure/activity assessments. Computational and biophysical approaches to this problem are emerging but will still currently require a fairly compact therapeutic RNA design to utilize such tools. Seventh, and finally, more efficient means of preclinical analysis of PTGS agents in appropriate animal models of disease are needed. Intraocular injections and certainly subretinal injections are complex multivariable surgical procedures when done on human eyes, let alone small mouse eyes [217]. Assessing the actual area of transduction as a normalization parameter to either histological or electrophysiological assays of rescue is paramount. In addition, for RNA drugs with potential to translate to the human condition, the target mRNA that drives the disease process in the animal models should be a full-length human mRNA that recapitulates both the target and the disease that will exist in future human clinical trials. Even though the primary sequence may be homologous over regions of PTGS targeting among mammalian cognate mRNA targets, it is the secondary and tertiary structure of the mRNA that governs accessibility and hence efficacy [212]. With human copies of the target mRNA in the animal model, there is more confidence that preclinical efficacy has hope of similar human clinical translation.

Currently, such animal models are rare [218]. This lab remains dedicated to the resolution of bottlenecks in RNA drug discovery and development of tools that will hasten the pace of development of efficacious and safe PTGS agents as candidate therapeutics for human retinal and macular degenerative diseases.

5. Materials and Methods

5.1. RNA Secondary Structure Prediction. The secondary structure of full length *RHO* and *BEST-1* mRNAs were subjected to analysis, using free energy minimization (RNA-Fold, MFold algorithm) [113], a Boltzmann-weighted sampling of all substructures (SFold algorithm) (not shown) [219–221], and local free energy analysis by OligoWalk [222] (not shown). RNA-fold was used with defaults to obtain MFE structures of the larger RNAs for Figure 5. MFold was used at 37°C with 10 kCal/mol window, for a maximum of 99 structures, and with a difference window of 3 bp. OligoWalk used the MFold output to obtain a LFE map along the mRNAs. A window of 15 nt, corresponding to symmetric 7/7 nt hhRz, was used to calculate the local free energy to break the target mRNA. Regions of low LFE (less negative or positive ΔG) indicate regions of low secondary structure or dynamic fluctuations.

5.2. Molecular Graphics. The bovine rod rhodopsin crystal structure (1F88.pdb) was visualized and annotated with ViewerPro (vers. 4.2) software (Accelrys). Changes in amino acid sidechains were made with the same software. There was no effort to minimize the energy around the local site of amino acid mutation (F293C) so the location of the sidechain is approximate.

5.3. Site Specific Mutagenesis, Stable Opsin Cell Line Generation, and Immunocytochemistry. These approaches and methods have been described in detail elsewhere [209].

Abbreviations

*a*WT:	Allelic variant wild type expression construct
AS:	Antisense
*a*WT:	Allelic variant wild type construct
BEST-1:	Best macular dystrophy gene
CKDRT:	Combined knockdown reconstitute therapy
dsRNA:	Double stranded RNA
ΔG:	Free energy (kCal/mol)
hhRz:	Hammerhead ribozyme
hpRz:	Hairpin ribozyme
HTS:	High throughput screening
KD:	Knockdown approach to therapy
MD:	Mutation dependent approach to PTGS therapy
MFE:	Minimum folding energy
MI:	Mutation independent approach to PTGS therapy
miRNA:	Micro-RNA
MSel:	Mutation selective approach (under MD)

MSpe: Mutation specific approach (under MD)
nt: Nucleotide
ODN: Oligodeoxynucleotide
PAZ: Piwi Argonaut Zwille
PTGS: Post-transcriptional gene silencing
RHO: Rod opsin
RISC: RNA-inducing silencing complex
RNAi: RNA interference
Rz: Ribozyme
shRNA: Short hairpin RNA
siRNA: Short interfering RNA
WT: Wild type.

Acknowledgments

References

[1] S. T. Crooke, "Molecular mechanisms of action of antisense drugs," *Biochimica et Biophysica Acta*, vol. 1489, no. 1, pp. 31–43, 1999.

[2] B. F. Baker and B. P. Monia, "Novel mechanisms for antisense-mediated regulation of gene expression," *Biochimica et Biophysica Acta*, vol. 1489, no. 1, pp. 3–18, 1999.

[3] J. Kurreck, "Antisense technologies: improvement through novel chemical modifications," *European Journal of Biochemistry*, vol. 270, no. 8, pp. 1628–1644, 2003.

[4] C. F. Bennett and E. E. Swayze, "RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform," *Annual Review of Pharmacology and Toxicology*, vol. 50, pp. 259–293, 2010.

[5] S. Agrawal, "Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides," *Biochimica et Biophysica Acta*, vol. 1489, no. 1, pp. 53–67, 1999.

[6] S. H. Shakin and S. A. Liebhaber, "Destabilization of messenger RNA/complementary DNA duplexes by the elongating 80 S ribosome," *The Journal of Biological Chemistry*, vol. 261, no. 34, pp. 16018–16025, 1986.

[7] B. P. Monia, E. A. Lesnik, C. Gonzalez et al., "Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression," *The Journal of Biological Chemistry*, vol. 268, no. 19, pp. 14514–14522, 1993.

[8] C. A. Stein, "Is irrelevant cleavage the price of antisense efficacy?" *Pharmacology and Therapeutics*, vol. 85, no. 3, pp. 231–236, 2000.

[9] B. Larrouy, C. Blonski, C. Boiziau et al., "RNase H-mediated inhibition of translation by antisense oligodeoxyribonucleotides: use of backbone modification to improve specificity," *Gene*, vol. 121, no. 2, pp. 189–194, 1992.

[10] R. V. Giles and D. M. Tidd, "Increased specificity for antisense oligodeoxynucleotide targeting of RNA cleavage by RNase H using chimeric methylphosphonodiester/phosphodiester structures," *Nucleic Acids Research*, vol. 20, no. 4, pp. 763–770, 1992.

[11] R. Y. Walder and J. A. Walder, "Role of RNase H in hybrid-arrested translation by antisense oligonucleotides," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 14, pp. 5011–5015, 1988.

[12] P. Sazani, M. M. Vacek, and R. Kole, "Short-term and long-term modulation of gene expression by antisense therapeutics," *Current Opinion in Biotechnology*, vol. 13, no. 5, pp. 468–472, 2002.

[13] T. K. Stage-Zimmermann and O. C. Uhlenbeck, "Hammerhead ribozyme kinetics," *RNA*, vol. 4, no. 8, pp. 875–889, 1998.

[14] S. P. Walton, G. N. Stephanopoulos, M. L. Yarmush, and C. M. Roth, "Prediction of antisense oligonucleotide binding affinity to a structured RNA target," *Biotechnology and Bioengineering*, vol. 65, no. 1, pp. 1–9, 1999.

[15] S.P. Walton, G. N. Stephanopoulos, M. L. Yarmush, and C. M. Roth, "Thermodynamic and kinetic characterization of antisense oligodeoxynucleotide binding to a structured mRNA," *Biophysical Journal*, vol. 82, no. 1, pp. 366–377, 2002.

[16] V. Patzel, J. Zu Putlitz, S. Wieland, H. E. Blum, and G. Sczakiel, "Theoretical and experimental selection parameters for HBV-Directed antisense RNA are related to increased RNA-RNA annealing," *Biological Chemistry*, vol. 378, no. 6, pp. 539–543, 1997.

[17] V. Patzel, U. Steidl, R. Kronenwett, R. Haas, and G. Sczakiel, "A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability," *Nucleic Acids Research*, vol. 27, no. 22, pp. 4328–4334, 1999.

[18] M. Sohail, H. Hochegger, A. Klotzbücher, R. Le Guellec, T. Hunt, and E. M. Southern, "Antisense oligonucleotides selected by hybridisation to scanning arrays are effective reagents *in vivo*," *Nucleic Acids Research*, vol. 29, no. 10, pp. 2041–2051, 2001.

[19] M. Sohail, "Antisense technology: inaccessibility and non-specificity," *Drug Discovery Today*, vol. 6, no. 24, pp. 1260–1261, 2001.

[20] M. J. Serra and D. H. Turner, "Predicting thermodynamic properties of RNA," *Methods in Enzymology*, vol. 259, pp. 242–261, 1995.

[21] S. M. Freier, R. Kierzek, and J. A. Jaeger, "Improved free-energy parameters for predictions of RNA duplex stability," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 24, pp. 9373–9377, 1986.

[22] V. Patzel and G. Sczakiel, "Theoretical design of antisense RNA structures substantially improves annealing kinetics and efficacy in human cells," *Nature Biotechnology*, vol. 16, no. 1, pp. 64–68, 1998.

[23] P. Schwille, F. Oehlenschlager, and N. G. Walter, "Quantitative hybridization kinetics of DNA probes to RNA in solution followed by diffusional fluorescence correlation analysis," *Biochemistry*, vol. 35, no. 31, pp. 10182–10193, 1996.

[24] M. C. Giddings, O. V. Matveeva, J. F. Atkins, and R. F. Gesteland, "ODNBase-A web database for antisense oligonucleotide effectiveness studies," *Bioinformatics*, vol. 16, no. 9, pp. 843–844, 2000.

[25] O. V. Matveeva, D. H. Mathews, A. D. Tsodikov et al., "Thermodynamic criteria for high hit rate antisense oligonucleotide design," *Nucleic Acids Research*, vol. 31, no. 17, pp. 4989–4994, 2003.

[26] X. Bo, S. Lou, D. Sun, J. Yang, and S. Wang, "AOBase: a database for antisense oligonucleotides selection and design," *Nucleic Acids Research*, vol. 34, pp. D664–D667, 2006.

[27] T. A. Vickers, J. R. Wyatt, and S. M. Freier, "Effects of RNA secondary structure on cellular antisense activity," *Nucleic Acids Research*, vol. 28, no. 6, pp. 1340–1347, 2000.

[28] R. M. Orr, "Technology evaluation: fomivirsen, Isis Pharmaceuticals Inc/CIBA Vision," *Current Opinion in Molecular Therapeutics*, vol. 3, no. 3, pp. 288–294, 2001.

[29] D. A. Jabs and P. D. Griffiths, "Fomivirsen for the treatment of cytomegalovirus retinitis," *American Journal of Ophthalmology*, vol. 133, no. 4, pp. 552–556, 2002.

[30] K. P. Anderson, M. C. Fox, V. Brown-Driver, M. J. Martin, and R. F. Azad, "Inhibition of human cytomegalovirus immediate-early gene expression by an antisense oligonucleotide complementary to immediate-early RNA," *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 9, pp. 2004–2011, 1996.

[31] K. R. Birikh, Y. A. Berlin, H. Soreq, and F. Eckstein, "Probing accessible sites for ribozymes on human acetylcholinesterase RNA," *RNA*, vol. 3, no. 4, pp. 429–437, 1997.

[32] A. R. Kore, N. K. Vaish, U. Kutzke, and F. Eckstein, "Sequence specificity of the hammerhead ribozyme revisited; The NHH rule," *Nucleic Acids Research*, vol. 26, no. 18, pp. 4116–4120, 1998.

[33] M. Amarzguioui and H. Prydz, "Hammerhead ribozyme design and application," *Cellular and Molecular Life Sciences*, vol. 54, no. 11, pp. 1175–1202, 1998.

[34] W. W. Hauswirth and A. S. Lewin, "Ribozyme uses in retinal gene therapy," *Progress in Retinal and Eye Research*, vol. 19, no. 6, pp. 689–710, 2000.

[35] A. S. Lewin and W. W. Hauswirth, "Ribozyme gene therapy: applications for molecular medicine," *Trends in Molecular Medicine*, vol. 7, no. 5, pp. 221–228, 2001.

[36] D. Castanotto, J. R. Li, A. Michienzi et al., "Intracellular ribozyme applications," *Biochemical Society Transactions*, vol. 30, no. 6, pp. 1140–1145, 2002.

[37] K. F. Blount and O. C. Uhlenbeck, "The structure-function dilemma of the hammerhead ribozyme," *Annual Review of Biophysics and Biomolecular Structure*, vol. 34, pp. 415–440, 2005.

[38] L. Q. Sun, M. J. Cairns, E. G. Saravolac, A. Baker, and W. L. Gerlach, "Catalytic nucleic acids: from lab to applications," *Pharmacological Reviews*, vol. 52, no. 3, pp. 325–347, 2000.

[39] L. A. Hegg and M. J. Fedor, "Kinetics and thermodynamics of intermolecular catalysis by hairpin ribozymes," *Biochemistry*, vol. 34, no. 48, pp. 15813–15828, 1995.

[40] M. J. Fedor, "Structure and function of the hairpin ribozyme," *Journal of Molecular Biology*, vol. 297, no. 2, pp. 269–291, 2000.

[41] D. E. Ruffner, G. D. Stormo, and O. C. Uhlenbeck, "Sequence requirements of the hammerhead RNA self-cleavage reaction," *Biochemistry*, vol. 29, no. 47, pp. 10695–10702, 1990.

[42] R. Perriman, A. Delves, and W. L. Gerlach, "Extended target-site specificity for a hammerhead ribozyme," *Gene*, vol. 113, no. 2, pp. 157–163, 1992.

[43] T. Shimayama, S. Nishikawa, and K. Taira, "Generality of the NUX rule: kinetic analysis of the results of systematic mutations in the trinucleotide at the cleavage site of hammerhead ribozymes," *Biochemistry*, vol. 34, no. 11, pp. 3649–3654, 1995.

[44] M. Zoumadakis and M. Tabler, "Comparative analysis of cleavage rates after systematic permutation of the NUX↓ consensus target motif for hammerhead ribozymes," *Nucleic Acids Research*, vol. 23, no. 7, pp. 1192–1196, 1995.

[45] E. Bertrand, R. Pictet, and T. Grange, "Can hammerhead ribozymes be efficient tools to inactivate gene function?" *Nucleic Acids Research*, vol. 22, no. 3, pp. 293–300, 1994.

[46] D. Herschlag, "Implications of ribozyme kinetics for targeting the cleavage of specific RNA molecules *in vivo*: more isn't always better," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 16, pp. 6921–6925, 1991.

[47] M. Werner and O. C. Uhlenbeck, "The effect of base mismatches in the substrate recognition helices of hammerhead ribozymes on binding and catalysis," *Nucleic Acids Research*, vol. 23, no. 12, pp. 2092–2096, 1995.

[48] K. J. Hertel, A. Pardi, O. C. Uhlenbeck et al., "Numbering system for the hammerhead," *Nucleic Acids Research*, vol. 20, no. 12, article 3252, 1992.

[49] N. Mandava, P. Blackburn, D. B. Paul et al., "Ribozyme to proliferating cell nuclear antigen to treat proliferative vitreoretinopathy," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 10, pp. 3338–3348, 2002.

[50] W. M. Schiff, J. C. Hwang, M. D. Ober et al., "Safety and efficacy assessment of chimeric ribozyme to proliferating cell nuclear antigen to prevent recurrence of proliferative vitreoretinopathy," *Archives of Ophthalmology*, vol. 125, no. 9, pp. 1161–1167, 2007.

[51] V. Mittal, "Improving the efficiency of RNA interference in mammals," *Nature Reviews Genetics*, vol. 5, no. 5, pp. 355–365, 2004.

[52] R. K. M. Leung and P. A. Whittaker, "RNA interference: from gene silencing to gene-specific therapeutics," *Pharmacology and Therapeutics*, vol. 107, no. 2, pp. 222–239, 2005.

[53] T. M. Rana, "Illuminating the silence: understanding the structure and function of small RNAs," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 1, pp. 23–36, 2007.

[54] S. M. Elbashir, W. Lendeckel, and T. Tuschl, "RNA interference is mediated by 21- and 22-nucleotide RNAs," *Genes and Development*, vol. 15, no. 2, pp. 188–200, 2001.

[55] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," *Nature*, vol. 411, no. 6836, pp. 494–498, 2001.

[56] N. J. Caplen, S. Parrish, F. Imani, A. Fire, and R. A. Morgan, "Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 17, pp. 9742–9747, 2001.

[57] E. Bernstein, A. A. Caudy, S. M. Hammond, and G. J. Hannon, "Role for a bidentate ribonuclease in the initiation step of RNA interference," *Nature*, vol. 409, no. 6818, pp. 363–366, 2001.

[58] J. J. Song, S. K. Smith, G. J. Hannon, and L. Joshua-Tor, "Crystal structure of argonaute and its implications for RISC slicer activity," *Science*, vol. 305, no. 5689, pp. 1434–1437, 2004.

[59] K. S. Yan, S. Yan, A. Farooq, A. Han, L. Zeng, and M. M. Zhou, "Structure and conserved RNA binding of the PAZ domain," *Nature*, vol. 426, no. 6965, pp. 469–474, 2003.

[60] A. Lingel, B. Simon, E. Izaurralde, and M. Sattler, "Nucleic acid 3′-end recognition by the Argonaute2 PAZ domain," *Nature Structural and Molecular Biology*, vol. 11, no. 6, pp. 576–577, 2004.

[61] J. B. Ma, K. Ye, and D. J. Patel, "Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain," *Nature*, vol. 429, no. 6989, pp. 318–322, 2004.

[62] J. S. Parker, S. M. Roe, and D. Barford, "Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex," *Nature*, vol. 434, no. 7033, pp. 663–666, 2005.

[63] F. V. Rivas, N. H. Tolia, J. J. Song et al., "Purified Argonaute2 and an siRNA form recombinant human RISC," *Nature Structural and Molecular Biology*, vol. 12, no. 4, pp. 340–349, 2005.

[64] T. R. Brummelkamp, R. Bernards, and R. Agami, "A system for stable expression of short interfering RNAs in mammalian cells," *Science*, vol. 296, no. 5567, pp. 550–553, 2002.

[65] A. Khvorova, A. Reynolds, and S. D. Jayasena, "Functional siRNAs and miRNAs exhibit strand bias," *Cell*, vol. 115, no. 2, pp. 209–216, 2003.

[66] D. S. Schwarz, G. Hutvágner, T. Du, Z. Xu, N. Aronin, and P. D. Zamore, "Asymmetry in the assembly of the RNAi enzyme complex," *Cell*, vol. 115, no. 2, pp. 199–208, 2003.

[67] E. A. Bohula, A. J. Salisbury, M. Sohail et al., "The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGF1R transcript," *The Journal of Biological Chemistry*, vol. 278, no. 18, pp. 15991–15997, 2003.

[68] R. Kretschmer-Kazemi Far and G. Sczakiel, "The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides," *Nucleic Acids Research*, vol. 31, no. 15, pp. 4417–4424, 2003.

[69] W. Wünsche and G. Sczakiel, "The activity of siRNA in mammalian cells is related to the kinetics of siRNA-target recognition *in vitro*: mechanistic implications," *Journal of Molecular Biology*, vol. 345, no. 2, pp. 203–209, 2005.

[70] K. Yoshinari, M. Miyagishi, and K. Taira, "Effects on RNAi of the tight structure, sequence and position of the targeted region," *Nucleic Acids Research*, vol. 32, no. 2, pp. 691–699, 2004.

[71] K. M. Brown, C. Y. Chu, and T. M. Rana, "Target accessibility dictates the potency of human RISC," *Nature Structural and Molecular Biology*, vol. 12, no. 5, pp. 469–470, 2005.

[72] S. Schubert, A. Grünweller, V. A. Erdmann, and J. Kurreck, "Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions," *Journal of Molecular Biology*, vol. 348, no. 4, pp. 883–893, 2005.

[73] M. Overhoff, M. Alken, R. K.-K. Far et al., "Local RNA target structure influences siRNA efficacy: a systematic global analysis," *Journal of Molecular Biology*, vol. 348, no. 4, pp. 871–881, 2005.

[74] B. S. Heale, H. S. Soifer, C. Bowers, and J. J. Rossi, "siRNA target site secondary structure predictions using local stable substructures," *Nucleic Acids Research*, vol. 33, no. 3, article e30, 2005.

[75] B. Haley and P. D. Zamore, "Kinetic analysis of the RNAi enzyme complex," *Nature Structural and Molecular Biology*, vol. 11, no. 7, pp. 599–606, 2004.

[76] Y. L. Chiu and T. M. Rana, "siRNA function in RNAi: a chemical modification analysis," *RNA*, vol. 9, no. 9, pp. 1034–1048, 2003.

[77] J. Martinez and T. Tuschl, "RISC is a 5′ phosphomonoester-producing RNA endonuclease," *Genes and Development*, vol. 18, no. 9, pp. 975–980, 2004.

[78] T. A. Vickers, W. F. Lima, J. G. Nichols, and S. T. Crooke, "Reduced levels of Ago2 expression result in increased siRNA competition in mammalian cells," *Nucleic Acids Research*, vol. 35, no. 19, pp. 6598–6610, 2007.

[79] A. L. Jackson, S. R. Bartz, J. Schelter et al., "Expression profiling reveals off-target gene regulation by RNAi," *Nature Biotechnology*, vol. 21, no. 6, pp. 635–637, 2003.

[80] A. L. Jackson, J. Burchard, J. Schelter et al., "Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity," *RNA*, vol. 12, no. 7, pp. 1179–1187, 2006.

[81] S. P. Persengiev, X. Zhu, and M. R. Green, "Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs)," *RNA*, vol. 10, no. 1, pp. 12–18, 2004.

[82] P. C. Scacheri, O. Rozenblatt-Rosen, N. J. Caplen et al., "Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 7, pp. 1892–1897, 2004.

[83] S. Qiu, C. M. Adema, and T. Lane, "A computational study of off-target effects of RNA interference," *Nucleic Acids Research*, vol. 33, no. 6, pp. 1834–1847, 2005.

[84] Y. Fedorov, E. M. Anderson, A. Birmingham et al., "Off-target effects by siRNA can induce toxic phenotype," *RNA*, vol. 12, no. 7, pp. 1188–1196, 2006.

[85] C. A. Sledz, M. Holko, M. J. de Veer, R. H. Silverman, and B. R. G. Williams, "Activation of the interferon system by short-interfering RNAs," *Nature Cell Biology*, vol. 5, no. 9, pp. 834–839, 2003.

[86] J. T. Marques and B. R. G. Williams, "Activation of the mammalian immune system by siRNAs," *Nature Biotechnology*, vol. 23, no. 11, pp. 1399–1405, 2005.

[87] D. Grimm, K. L. Streetz, C. L. Jopling et al., "Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways," *Nature*, vol. 441, no. 7092, pp. 537–541, 2006.

[88] S. Barik, "RNAi in moderation," *Nature Biotechnology*, vol. 24, no. 7, pp. 796–797, 2006.

[89] J. O. Snove Jr. and J. J. Rossi, "Toxicity in mice expressing short hairpin RNAs gives new insight into RNAi," *Genome Biology*, vol. 7, no. 8, article 231, 2006.

[90] M. E. Kleinman, K. Yamada, A. Takeda et al., "Sequence- and target-independent angiogenesis suppression by siRNA via TLR3," *Nature*, vol. 452, no. 7187, pp. 591–597, 2008.

[91] A. M. Chalk, R. E. Warfinge, P. Georgii-Hemming, and E. L. L. Sonnhammer, "siRNAdb: a database of siRNA sequences," *Nucleic Acids Research*, vol. 33, pp. D131–D134, 2005.

[92] R. T. Marquez and A. P. McCaffrey, "Advances in microRNAs: implications for gene therapists," *Human Gene Therapy*, vol. 19, no. 1, pp. 27–37, 2008.

[93] D. Boden, O. Pusch, R. Silbermann, F. Lee, L. Tucker, and B. Ramratnam, "Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins," *Nucleic Acids Research*, vol. 32, no. 3, pp. 1154–1158, 2004.

[94] J. L. McBride, R. L. Boudreau, S. Q. Harper et al., "Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 15, pp. 5868–5873, 2008.

[95] J. M. Sullivan, E. H. Yau, R. T. Taggart, M. C. Butler, and T. A. Kolniak, "Bottlenecks in development of retinal therapeutic post-transcriptional gene silencing agents," *Vision Research*, vol. 48, no. 3, pp. 453–469, 2008.

[96] E. Bertrand, D. Castanotto, C. Zhou et al., "The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization," *RNA*, vol. 3, no. 1, pp. 75–88, 1997.

[97] R. Hormes, M. Homann, I. Oelze et al., "The subcellular localization and length of hammerhead ribozymes determine efficacy in human cells," *Nucleic Acids Research*, vol. 25, no. 4, pp. 769–775, 1997.

[98] J. D. Thompson, D. F. Ayers, T. A. Malmstrom et al., "Improved accumulation and activity of ribozymes expressed from a tRNA-based RNA polymerase III promoter," *Nucleic Acids Research*, vol. 23, no. 12, pp. 2259–2268, 1995.

[99] Y. Oleynikov and R. H. Singer, "RNA localization: different zipcodes, same postman?" *Trends in Cell Biology*, vol. 8, no. 10, pp. 381–383, 1998.

[100] N. S. Lee, E. Bertrand, and J. Rossi, "mRNA localization signals can enhance the intracellular effectiveness of hammerhead ribozymes," *RNA*, vol. 5, no. 9, pp. 1200–1209, 1999.

[101] A. B. Fulton, "How crowded is the cytoplasm?" *Cell*, vol. 30, no. 2, pp. 345–347, 1982.

[102] S. Koseki, T. Tanabe, K. Tani et al., "Factors governing the activity *in vivo* of ribozymes transcribed by RNA polymerase III," *Journal of Virology*, vol. 73, no. 3, pp. 1868–1877, 1999.

[103] C. Echevería, K. Tucci, and R. Kapral, "Diffusion and reaction in crowded environments," *Journal of Physics Condensed Matter*, vol. 19, no. 6, Article ID 065146, 12 pages, 2007.

[104] K. R. Birikh, P. A. Heaton, and F. Eckstein, "The structure, function and application of the hammerhead ribozyme," *European Journal of Biochemistry*, vol. 245, no. 1, pp. 1–16, 1997.

[105] A. Gal, E. Apfelstedt-Sylla, A. R. Janecke, and E. Zrenner, "Rhodopsin mutations in inherited retinal dystrophies and dysfunctions," *Progress in Retinal and Eye Research*, vol. 16, no. 1, pp. 51–79, 1997.

[106] E. Souied, G. Soubrane, P. Benlian et al., "Retinitis punctata albescens associated with the Arg135Trp mutation in the rhodopsin gene," *American Journal of Ophthalmology*, vol. 121, no. 1, pp. 19–25, 1996.

[107] F. Krämer, K. White, D. Pauleikhoff et al., "Mutations in the VMD2 gene are associated with juvenile-onset vitelliform macular dystrophy (Best disease) and adult vitelliform macular dystrophy but not age-related macular degeneration," *European Journal of Human Genetics*, vol. 8, no. 4, pp. 286–292, 2000.

[108] J. M. Seddon, M. A. Afshari, S. Sharma et al., "Assessment of mutations in the Best macular dystrophy (VMD2) gene in patients with adult-onset foveomacular vitelliform dystrophy, age-related maculopathy, and bull's-eye maculopathy," *Ophthalmology*, vol. 108, no. 11, pp. 2060–2067, 2001.

[109] C. Hartzell, Z. Qu, I. Putzier, L. Artinian, L. T. Chien, and Y. Cui, "Looking chloride channels straight in the eye: bestrophins, lipofuscinosis, and retinal degeneration," *Physiology*, vol. 20, no. 5, pp. 292–302, 2005.

[110] J. Nathans and D. S. Hogness, "Isolation and nucleotide sequence of the gene encoding human rhodopsin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 15, pp. 4851–4855, 1984.

[111] K. Petrukhin, M. J. Koisti, B. Bakall et al., "Identification of the gene responsible for best macular dystrophy," *Nature Genetics*, vol. 19, no. 3, pp. 241–247, 1998.

[112] M. Amarzguioui, G. Brede, E. Babaie, M. Grøtli, B. Sproat, and H. Prydz, "Secondary structure prediction and *in vitro* accessibility of mRNA as tools in the selection of target sites for ribozymes," *Nucleic Acids Research*, vol. 28, no. 21, pp. 4113–4124, 2000.

[113] M. Zuker, "Mfold web server for nucleic acid folding and hybridization prediction," *Nucleic Acids Research*, vol. 31, no. 13, pp. 3406–3415, 2003.

[114] W. F. Lima, B. P. Monia, D. J. Ecker, and S. M. Freier, "Implication of RNA structure on antisense oligonucleotide hybridization kinetics," *Biochemistry*, vol. 31, no. 48, pp. 12055–12061, 1992.

[115] K. J. Hertel, D. Herschlag, and O. C. Uhlenbeck, "Specificity of hammerhead ribozyme cleavage," *EMBO Journal*, vol. 15, no. 14, pp. 3751–3757, 1996.

[116] K. J. Hertel, A. Peracchi, O. C. Uhlenbeck, and D. Herschlag, "Use of intrinsic binding energy for catalysis by an RNA enzyme," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 16, pp. 8497–8502, 1997.

[117] T. M. Woolf, D. A. Melton, and C. G. B. Jennings, "Specificity of antisense oligonucleotides *in vivo*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 16, pp. 7305–7309, 1992.

[118] K. Rittner, C. Burmester, and G. Sczakiel, "*In vitro* selection of fast-hybridizing and effective antisense RNAs directed against the human immunodeficiency virus type 1," *Nucleic Acids Research*, vol. 21, no. 6, pp. 1381–1387, 1993.

[119] P. J. L'Huillier, S. R. Davis, and A. R. Bellamy, "Cytoplasmic delivery of ribozymes leads to efficient reduction in α-lactalbumin mRNA levels in C1271 mouse cells," *EMBO Journal*, vol. 11, no. 12, pp. 4411–4418, 1992.

[120] Z. Xing and J. L. Whitton, "Ribozymes which cleave arenavirus RNAs: identification of susceptible target sites and inhibition by target site secondary structure," *Journal of Virology*, vol. 66, no. 3, pp. 1361–1369, 1992.

[121] D. Scarabino and G. P. Tocchini-Valentini, "Influence of substrate structure on cleavage by hammerhead ribozyme," *FEBS Letters*, vol. 383, no. 3, pp. 185–190, 1996.

[122] T. B. Campbell, C. K. McDonald, and M. Hagen, "The effect of structure in a long target RNA on ribozyme cleavage efficiency," *Nucleic Acids Research*, vol. 25, no. 24, pp. 4985–4993, 1997.

[123] O. Matveeva, B. Felden, S. Audlin, R. F. Gesteland, and J. F. Atkins, "A rapid *in vitro* method for obtaining RNA accessibility patterns for complementary DNA probes: correlation with an intracellular pattern and known RNA structures," *Nucleic Acids Research*, vol. 25, no. 24, pp. 5010–5016, 1997.

[124] M. Scherr and J. J. Rossi, "Rapid determination and quantitation of the accessibility to native RNAs by antisense oligodeoxynucleotides in murine cell extracts," *Nucleic Acids Research*, vol. 26, no. 22, pp. 5079–5085, 1998.

[125] M. Scherr, J. J. Rossi, G. Sczakiel, and V. Patzel, "RNA accessibility prediction: a theoretical approach is consistent with experimental studies in cell extracts," *Nucleic Acids Research*, vol. 28, no. 13, pp. 2455–2461, 2000.

[126] N. K. Vaish, A. R. Kore, and F. Eckstein, "Recent developments in the hammerhead ribozyme field," *Nucleic Acids Research*, vol. 26, no. 23, pp. 5237–5242, 1998.

[127] A. D. Branch, "A good antisense molecule is hard to find," *Trends in Biochemical Sciences*, vol. 23, no. 2, pp. 45–50, 1998.

[128] H. E. Abdelmaksoud, E. H. Yau, M. Zuker, and J. M. Sullivan, "Development of lead hammerhead ribozyme candidates against human rod opsin mRNA for retinal degeneration therapy," *Experimental Eye Research*, vol. 88, no. 5, pp. 859–879, 2009.

[129] M. Homann, M. Tabler, S. Tzortzakaki, and G. Sczakiel, "Extension of helix II of an HlV-1-directed hammerhead ribozyme with long antisense flanks does not alter kinetic parameters *in vitro* but causes loss of the inhibitory potential in living cells," *Nucleic Acids Research*, vol. 22, no. 19, pp. 3951–3957, 1994.

[130] A. Khvorova, A. Lescoute, E. Westhof, and S. D. Jayasena, "Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity," *Nature Structural Biology*, vol. 10, no. 9, pp. 708–712, 2003.

[131] M. de la Peña, S. Gago, and R. Flores, "Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity," *EMBO Journal*, vol. 22, no. 20, pp. 5561–5570, 2003.

[132] D. M. Lilley, "Ribozymes—a snip too far?" *Nature Biotechnology*, vol. 10, pp. 672–673, 2003.

[133] J. C. Penedo, T. J. Wilson, S. D. Jayasena, A. Khvorova, and D. M. J. Lilley, "Folding of the natural hammerhead ribozyme is enhanced by interaction of auxiliary elements," *RNA*, vol. 10, no. 5, pp. 880–888, 2004.

[134] M. Martick and W. G. Scott, "Tertiary contacts distant from the active site prime a ribozyme for catalysis," *Cell*, vol. 126, no. 2, pp. 309–320, 2006.

[135] J. A. Nelson and O. C. Uhlenbeck, "When to believe what you see," *Molecular Cell*, vol. 23, no. 4, pp. 447–450, 2006.

[136] A. Lieber and M. Strauss, "Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library," *Molecular and Cellular Biology*, vol. 15, no. 1, pp. 540–551, 1995.

[137] J. J. Fritz, A. Lewin, W. Hauswirth, A. Agarwal, M. Grant, and L. Shaw, "Development of hammerhead ribozymes to modulate endogenous gene expression for functional studies," *Methods*, vol. 28, no. 2, pp. 276–285, 2002.

[138] T. P. Dryja, T. L. McGee, E. Reichel et al., "A point mutation of the rhodopsin gene in one form of retinitis pigmentosa," *Nature*, vol. 343, no. 6256, pp. 364–366, 1990.

[139] K. Gregory-Evans and S. S. Bhattacharya, "Genetic blindness: current concepts in the pathogenesis of human outer retinal dystrophies," *Trends in Genetics*, vol. 14, no. 3, pp. 103–108, 1998.

[140] S. P. Daiger, L. S. Sullivan, S. J. Bowne, and B. J. F. Rossiter, RetNet. Retinal information network, 2010, http://www.sph.uth.tmc.edu/retnet/.

[141] E. S. Lander, L. M. Linton, B. Birren et al., "Initial sequencing and analysis of the human genome," *Nature*, vol. 409, no. 6822, pp. 860–921, 2001.

[142] J. C. Venter, M. D. Adams, E. W. Myers et al., "The sequence of the human genome," *Science*, vol. 291, no. 5507, pp. 1304–1351, 2001.

[143] B. A. Sullenger and T. R. Cech, "Ribozyme-mediated repair of defective mRNA by targeted trans-splicing," *Nature*, vol. 371, no. 6498, pp. 619–622, 1994.

[144] B. A. Sullenger, "Revising messages traveling along the cellular information superhighway," *Chemistry and Biology*, vol. 2, no. 5, pp. 249–253, 1995.

[145] J. T. Jones, S. W. Lee, and B. A. Sullenger, "Tagging ribozyme reaction sites to follow trans-splicing in mammalian cells," *Nature Medicine*, vol. 2, no. 6, pp. 643–648, 1996.

[146] J. T. Jones and B. A. Sullenger, "Evaluating and enhancing ribozyme reaction efficiency in mammalian cells," *Nature Biotechnology*, vol. 15, no. 9, pp. 902–905, 1997.

[147] M. B. Long and B. A. Sullenger, "Evaluating group I intron catalytic efficiency in mammalian cells," *Molecular and Cellular Biology*, vol. 19, no. 10, pp. 6479–6487, 1999.

[148] M. B. Long, J. P. Jones, B. A. Sullenger, and J. Byun, "Ribozyme-mediated revision of RNA and DNA," *Journal of Clinical Investigation*, vol. 112, no. 3, pp. 312–318, 2003.

[149] U. Köhler, B. G. Ayre, H. M. Goodman, and J. Haseloff, "Trans-splicing ribozymes for targeted gene delivery," *Journal of Molecular Biology*, vol. 285, no. 5, pp. 1935–1950, 1999.

[150] P. P. Zarrinkar and B. A. Sullenger, "Optimizing the substrate specificity of a group I intron ribozyme," *Biochemistry*, vol. 38, no. 11, pp. 3426–3432, 1999.

[151] N. Lan, B. L. Rooney, S. W. Lee, R. P. Howrey, C. A. Smith, and B. A. Sullenger, "Enhancing RNA repair efficiency by combining transsplicing ribozymes that recognize different accessible sites on a target RNA," *Molecular Therapy*, vol. 2, no. 3, pp. 245–255, 2000.

[152] B. G. Ayre, U. Köhler, R. Turgeon, and J. Haseloff, "Optimization of trans-splicing ribozyme efficiency and specificity by *in vivo* genetic selection," *Nucleic Acids Research*, vol. 30, no. 24, article e141, 2002.

[153] J. Byun, N. Lan, M. Long, and B. A. Sullenger, "Efficient and specific repair of sickle β-globin RNA by trans-splicing ribozymes," *RNA*, vol. 9, no. 10, pp. 1254–1263, 2003.

[154] M. Hagen and T. R. Cech, "Self-splicing of the Tetrahymena intron from mRNA in mammalian cells," *EMBO Journal*, vol. 18, no. 22, pp. 6491–6500, 1999.

[155] A. S. Lewin, K. A. Drenser, W. W. Hauswirth et al., "Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa," *Nature Medicine*, vol. 4, no. 8, pp. 967–971, 1998.

[156] M. M. LaVail, D. Yasumura, M. T. Matthes et al., "Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 21, pp. 11488–11493, 2000.

[157] C. H. Sung, B. G. Schneider, N. Agarwal, D. S. Papermaster, and J. Nathans, "Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 19, pp. 8840–8844, 1991.

[158] C. H. Sung, C. M. Davenport, and J. Nathans, "Rhodopsin mutations responsible for autosomal dominant retinitis pigmentosa. Clustering of functional classes along the polypeptide chain," *The Journal of Biological Chemistry*, vol. 268, no. 35, pp. 26645–26649, 1993.

[159] C. H. Sung, C. Makino, D. Baylor, and J. Nathans, "A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment," *Journal of Neuroscience*, vol. 14, no. 10, pp. 5818–5833, 1994.

[160] J. Hwa, P. Garriga, X. Liu, and H. G. Khorana, "Structure and function in rhodopsin: packing of the helices in the transmembrane domain and folding to a tertiary structure in the intradiscal domain are coupled," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 20, pp. 10571–10576, 1997.

[161] L. Bosch, E. Ramon, L. J. el Valle, and P. Garriga, "Structural and functional role of helices I and II in rhodopsin. A novel interplay evidenced by mutations at Gly-51 and Gly-89 in the transmembrane domain," *The Journal of Biological Chemistry*, vol. 278, no. 22, pp. 20203–20209, 2003.

[162] J. A. Johnston, C. L. Ward, and R. R. Kopito, "Aggresomes: a cellular response to misfolded proteins," *Journal of Cell Biology*, vol. 143, no. 7, pp. 1883–1898, 1998.

[163] R. R. Kopito, "Aggresomes, inclusion bodies and protein aggregation," *Trends in Cell Biology*, vol. 10, no. 12, pp. 524–530, 2000.

[164] J. P. Chapple, C. Grayson, A. J. Hardcastle, R. S. Saliba, J. van der Spuy, and M. E. Cheetham, "Unfolding retinal dystrophies: a role for molecular chaperones?" *Trends in Molecular Medicine*, vol. 7, no. 9, pp. 414–421, 2001.

[165] R. S. Rajan, M. E. Illing, N. F. Bence, and R. R. Kopito, "Specificity in intracellular protein aggregation and inclusion body formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 23, pp. 13060–13065, 2001.

[166] M. E. Illing, R. S. Rajan, N. F. Bence, and R. R. Kopito, "A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system," *The Journal of Biological Chemistry*, vol. 277, no. 37, pp. 34150–34160, 2002.

[167] R. S. Saliba, P. M. G. Munro, P. J. Luthert, and M. E. Cheetham, "The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation," *Journal of Cell Science*, vol. 115, no. 14, pp. 2907–2918, 2002.

[168] H. F. Mendes, J. van der Spuy, J. P. Chapple, and M. E. Cheetham, "Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy," *Trends in Molecular Medicine*, vol. 11, no. 4, pp. 177–185, 2005.

[169] H. Mukai, T. Isagawa, E. Goyama et al., "Formation of morphologically similar globular aggregates from diverse aggregation-prone proteins in mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 31, pp. 10887–10892, 2005.

[170] E. Lai, T. Teodoro, and A. Volchuk, "Endoplasmic reticulum stress: signaling the unfolded protein response," *Physiology*, vol. 22, no. 3, pp. 193–201, 2007.

[171] D. Ron and P. Walter, "Signal integration in the endoplasmic reticulum unfolded protein response," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 7, pp. 519–529, 2007.

[172] T. Li, W. K. Snyder, J. E. Olsson, and T. P. Dryja, "Transgenic mice carrying the dominant rhodopsin mutation P347S: evidence for defective vectorial transport of rhodopsin to the outer segments," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 24, pp. 14176–14181, 1996.

[173] P. A. Sieving, J. E. Richards, F. Naarendorp, E. L. Bingham, K. Scorrt, and M. Alpern, "Dark-light: model for nightblindness from the human rhodopsin Gly-90 → Asp mutation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 3, pp. 880–884, 1995.

[174] P. Kurada, T. D. Tonini, M. A. Serikaku, J. P. Piccini, and J. E. O'Tousa, "Rhodopsin maturation antagonized by dominant rhodopsin mutants," *Visual Neuroscience*, vol. 15, no. 4, pp. 693–700, 1998.

[175] J. H. Wilson and T. G. Wensel, "The nature of dominant mutations of rhodopsin and implications for gene therapy," *Molecular Neurobiology*, vol. 28, no. 2, pp. 149–158, 2003.

[176] R. S. Rajan and R. R. Kopito, "Suppression of wild-type rhodopsin maturation by mutants linked to autosomal dominant retinitis pigmentosa," *The Journal of Biological Chemistry*, vol. 280, no. 2, pp. 1284–1291, 2005.

[177] M. Koizumi, Y. Hayase, S. Iwai, H. Kamiya, H. Inoue, and E. Ohtsuka, "Design of RNA enzymes distinguishing a single base mutation in RNA," *Nucleic Acids Research*, vol. 17, no. 17, pp. 7059–7071, 1989.

[178] G. Grassi, A. Forlino, and J. C. Marini, "Cleavage of collagen RNA transcripts by hammerhead ribozymes *in vitro* is mutation-specific and shows competitive binding effects," *Nucleic Acids Research*, vol. 25, no. 17, pp. 3451–3458, 1997.

[179] K. A. Drenser, A. M. Timmers, W. W. Hauswirth, and A. S. Lewin, "Ribozyme-targeted destruction of RNA associated with autosomal-dominant retinitis pigmentosa," *Investigative Ophthalmology and Visual Science*, vol. 39, no. 5, pp. 681–689, 1998.

[180] M. Zoumadakis, W. J. Neubert, and M. Tabler, "The influence of imperfectly paired helices I and III on the catalytic activity of hammerhead ribozymes," *Nucleic Acids Research*, vol. 22, no. 24, pp. 5271–5278, 1994.

[181] L. C. Shaw, A. Skold, F. Wong, R. Petters, W. Hauswirth, and A. S. Lewin, "An allele-specific hammerhead ribozyme gene therapy for a porcine model of autosomal dominant retinitis pigmentosa," *Molecular Vision*, vol. 7, pp. 6–13, 2001.

[182] J. M. Sullivan, K. M. Scott, H. F. Falls, J. E. Richards, and P. A. Sieving, "A novel rhodopsin mutation at the retinal binding site (Lys-296-Met) in adRP," *Investigative Ophthalmology & Visual Science*, vol. 34, article 1149a, 1993.

[183] S. Millington-Ward, B. O'Neill, G. Tuohy et al., "Strategems *in vitro* for gene therapies directed to dominant mutations," *Human Molecular Genetics*, vol. 6, no. 9, pp. 1415–1426, 1997.

[184] B. O'Neill, S. Millington-Ward, M. O'Reilly et al., "Ribozyme-based therapeutic approaches for autosomal dominant retinitis pigmentosa," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 10, pp. 2863–2869, 2000.

[185] J. M. Sullivan, K. M. Pietras, B. J. Shin, and J. N. Misasi, "Hammerhead ribozymes designed to cleave all human rod opsin mRNAs which cause autosomal dominant retinitis pigmentosa," *Molecular Vision*, vol. 8, pp. 102–113, 2002.

[186] S. M. Cashman, E. A. Binkley, and R. Kumar-Singh, "Towards mutation-independent silencing of genes involved in retinal degeneration by RNA interference," *Gene Therapy*, vol. 12, no. 15, pp. 1223–1228, 2005.

[187] M. S. Gorbatyuk, J. J. Pang, J. Thomas, W. W. Hauswirth, and A. S. Lewin, "Knockdown of wild-type mouse rhodopsin using an AAV vectored ribozyme as part of an RNA replacement approach," *Molecular Vision*, vol. 11, pp. 648–656, 2005.

[188] M. Gorbatyuk, V. Justilien, J. Liu, W. W. Hauswirth, and A. S. Lewin, "Preservation of photoreceptor morphology and function in P23H rats using an allele independent ribozyme," *Experimental Eye Research*, vol. 84, no. 1, pp. 44–52, 2007.

[189] M. O'Reilly, A. Palfi, N. Chadderton et al., "RNA interference-mediated suppression and replacement of human rhodopsin *in vivo*," *American Journal of Human Genetics*, vol. 81, no. 1, pp. 127–135, 2007.

[190] T. W. Kraft, D. M. Schneeweis, and J. L. Schnapf, "Visual transduction in human rod photoreceptors," *Journal of Physiology*, vol. 464, pp. 747–765, 1993.

[191] J. S. Penn and T. P. Williams, "Photostasis: regulation of daily photon-catch by rat retinas in response to various cyclic illuminances," *Experimental Eye Research*, vol. 43, no. 6, pp. 915–928, 1986.

[192] P. J. Rosenfeld, G. S. Cowley, T. L. McGee, M. A. Sandberg, E. L. Berson, and T. P. Dryja, "A null mutation in the rhodopsin gene causes rod photoreceptor dysfunction and autosomal recessive retinitis pigmentosa," *Nature Genetics*, vol. 1, no. 3, pp. 209–213, 1992.

[193] P. J. Rosenfeld, L. B. Hahn, M. A. Sandberg, T. P. Dryja, and E. L. Berson, "Low incidence of retinitis pigmentosa among heterozygous carriers of a specific rhodopsin splice site mutation," *Investigative Ophthalmology and Visual Science*, vol. 36, no. 11, pp. 2186–2192, 1995.

[194] R. R. McInnes and R. A. Bascom, "Retinal genetics: a nullifying effect for rhodopsin," *Nature Genetics*, vol. 1, no. 3, pp. 155–157, 1992.

[195] M. M. Humphries, D. Rancourt, G. J. Farrar et al., "Retinopathy induced in mice by targeted disruption of the rhodopsin gene," *Nature Genetics*, vol. 15, no. 2, pp. 216–219, 1997.

[196] J. Lem, N. V. Krasnoperova, P. D. Calvert et al., "Morphological, physiological, and biochemical changes in rhodopsin knockout mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 2, pp. 736–741, 1999.

[197] N. McNally, P. Kenna, M. M. Humphries et al., "Structural and functional rescue of murine rod photoreceptors by human rhodopsin transgene," *Human Molecular Genetics*, vol. 8, no. 7, pp. 1309–1312, 1999.

[198] J. M. Frederick, N. V. Krasnoperova, K. Hoffmann et al., "Mutant rhodopsin transgene expression on a null background," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 3, pp. 826–833, 2001.

[199] E. Tan, Q. Wang, A. B. Quiambao et al., "The relationship between opsin overexpression and photoreceptor degeneration," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 3, pp. 589–600, 2001.

[200] R. A. Montgomery and H. C. Dietz, "Inhibition of fibrillin 1 expression using U1 snRNA as a vehicle for the presentation of antisense targeting sequence," *Human Molecular Genetics*, vol. 6, no. 4, pp. 519–525, 1997.

[201] I. Ozaki, M. A. Zern, S. Liu, D. L. Wei, R. J. Pomerantz, and L. Duan, "Ribozyme-mediated specific gene replacement of the α1-antitrypsin gene in human hepatoma cells," *Journal of Hepatology*, vol. 31, no. 1, pp. 53–60, 1999.

[202] M. A. Zern, I. Ozaki, L. Duan, R. Pomerantz, S. L. Liu, and D. S. Strayer, "A novel SV40-based vector successfully transduces and expresses an alpha 1-antitrypsin ribozyme in a human hepatoma-derived cell line," *Gene Therapy*, vol. 6, no. 1, pp. 114–120, 1999.

[203] G. J. Farrar, P. F. Kenna, and P. Humphries, "On the genetics of retinitis pigmentosa and on mutation-independent approaches to therapeutic intervention," *EMBO Journal*, vol. 21, no. 5, pp. 857–864, 2002.

[204] M. Gorbatyuk, V. Justilien, J. Liu, W. W. Hauswirth, and A. S. Lewin, "Suppression of mouse rhodopsin expression *in vivo* by AAV mediated siRNA delivery," *Vision Research*, vol. 47, no. 9, pp. 1202–1208, 2007.

[205] A. S. Kiang, A. Palfi, M. Ader et al., "Toward a gene therapy for dominant disease: validation of an RNA interference-based mutation-independent approach," *Molecular Therapy*, vol. 12, no. 3, pp. 555–561, 2005.

[206] A. Palfi, M. Ader, A. S. Kiang et al., "RNAi-based suppression and replacement of rds-peripherin in retinal organotypic culture," *Human Mutation*, vol. 27, no. 3, pp. 260–268, 2006.

[207] K. Palczewski, T. Kumasaka, T. Hori et al., "Crystal structure of rhodopsin: a G protein-coupled receptor," *Science*, vol. 289, no. 5480, pp. 739–745, 2000.

[208] P. Shukla and J. M. Sullivan, "Normal and mutant rhodopsin activation measured with the early receptor current in a unicellular expression system," *Journal of General Physiology*, vol. 114, no. 5, pp. 609–636, 1999.

[209] J. M. Sullivan and M. F. Satchwell, "Development of stable cells lines expressing high levels of point mutants of human opsin for biochemical and biophysical studies," *Methods in Enzymology*, vol. 315, pp. 30–58, 2000.

[210] S. S. Karnik, T. P. Sakmar, H. B. Chen, and H. G. Khorana, "Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 22, pp. 8459–8463, 1988.

[211] J. M. Sullivan and R. T. Taggart, "Novel and enhanced approaches to determined local mRNA accessibility," *Investigative Ophthalmology & Visual Science*, vol. 48, article 4605, 2007.

[212] J. M. Sullivan and E. H. Yau, "Human-directed RNA drug discovery—are animal mRNA targets suitable representatives?" *Investigative Ophthalmology & Visual Science*, vol. 50, ARVO E-Abstract, 3016, 2009.

[213] M. C. Butler, T. Kolniak, and J. M. Sullivan, "Microscope-based high throughput cell protein quantitation for therapeutics development," *Investigative Ophthalmology & Visual Science*, vol. 48, ARVO E-Abstract, 4608, 2007.

[214] T. A. Kolniak and J. M. Sullivan, "Rapid, cell-based toxicity screen of potentially therapeutic post-transcriptional gene silencing agents," *Experimental Eye Research*, vol. 92, no. 5, pp. 328–337, 2011.

[215] M. C. Butler, T. A. Kolniak, and J. M. Sullivan, "A versatile instrument for high throughput and high content screening in gene-based drug discovery," *Investigative Ophthalmology & Visual Science*, vol. 49, ARVO E-Abstract, 5342, 2008.

[216] R. S. Yadava, E. M. Mahen, and M. J. Fedor, "Kinetic analysis of ribozyme-substrate complex formation in yeast," *RNA*, vol. 10, no. 5, pp. 863–879, 2004.

[217] M. C. Butler and J. M. Sullivan, "A novel real-time *in vivo* mouse retinal imaging system," *Investigative Ophthalmology & Visual Science*, vol. 51, ARVO E-Abstract, 3103, 2010.

[218] T. Kolniak, E. H. Yau, R. T. Taggart et al., "A novel fully humanized *RHO* adRP mouse model," *Investigative Ophthalmology & Visual Science*, vol. 51, ARVO E-Abstract, 5071, 2010.

[219] Y. Ding and C. E. Lawrence, "Statistical prediction of single-stranded regions in RNA secondary structure and application to predicting effective antisense target sites and beyond," *Nucleic Acids Research*, vol. 29, no. 5, pp. 1034–1046, 2001.

[220] Y. Ding and C. E. Lawrence, "A statistical sampling algorithm for RNA secondary structure prediction," *Nucleic Acids Research*, vol. 31, no. 24, pp. 7280–7301, 2003.

[221] Y. Ding, C. Y. Chan, and C. E. Lawrence, "Sfold web server for statistical folding and rational design of nucleic acids," *Nucleic Acids Research*, vol. 32, supplement, pp. W135–W141, 2004.

[222] D. H. Mathews, M. E. Burkard, S. M. Freier, J. R. Wyatt, and D. H. Turner, "Predicting oligonucleotide affinity to nucleic acid targets," *RNA*, vol. 5, no. 11, pp. 1458–1469, 1999.

[223] M. J. Lehmann, V. Patzel, and G. Sczakiel, "Theoretical design of antisense genes with statistically increased efficacy," *Nucleic Acids Research*, vol. 28, no. 13, pp. 2597–2604, 2000.

[224] R. A. Stull, L. A. Taylor, and F. C. Szoka, "Predicting antisense oligonucleotide inhibitory efficacy: a computational approach using histograms and thermodynamic indices," *Nucleic Acids Research*, vol. 20, no. 13, pp. 3501–3508, 1992.

[225] S. P. Ho, D. H. O. Britton, B. A. Stone et al., "Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries," *Nucleic Acids Research*, vol. 24, no. 10, pp. 1901–1907, 1996.

[226] S. P. Ho, Y. Bao, T. Lesher et al., "Mapping of RNA accessible sites for antisense experiments with oligonucleotide libraries," *Nature Biotechnology*, vol. 16, no. 1, pp. 59–63, 1998.

[227] M. Scherr, J. LeBon, D. Castanotto et al., "Detection of antisense and ribozyme accessible sites on native mRNAs: application to *NCOA3* mRNA," *Molecular Therapy*, vol. 4, no. 5, pp. 454–460, 2001.

[228] N. Milner, K. U. Mir, and E. M. Southern, "Selecting effective antisense reagents on combinatorial oligonucleotide arrays," *Nature Biotechnology*, vol. 15, no. 6, pp. 537–541, 1997.

[229] K. U. Mir and E. M. Southern, "Determining the influence of structure on hybridization using oligonucleotide arrays," *Nature Biotechnology*, vol. 17, no. 8, pp. 788–792, 1999.

[230] W. H. Pan, P. Xin, V. Bui, and G. A. Clawson, "Rapid identification of efficient target cleavage sites using a hammerhead ribozyme library in an iterative manner," *Molecular Therapy*, vol. 7, no. 1, pp. 129–139, 2003.

[231] Q. Yu, D. B. Pecchia, S. L. Kingsley, J. E. Heckman, and J. M. Burke, "Cleavage of highly structured viral RNA molecules by combinatorial libraries of hairpin ribozymes. The most effective ribozymes are not predicted by substrate selection rules," *The Journal of Biological Chemistry*, vol. 273, no. 36, pp. 23524–23533, 1998.

[232] W. H. Pan, H. F. Devlin, C. Kelley, H. C. Isom, and G. A. Clawson, "A selection system for identifying accessible sites in target RNAs," *RNA*, vol. 7, no. 4, pp. 610–621, 2001.

[233] J. Zu Putlitz, Q. Yu, J. M. Burke, and J. R. Wands, "Combinatorial screening and intracellular antiviral activity of hairpin ribozymes directed against hepatitis B virus," *Journal of Virology*, vol. 73, no. 7, pp. 5381–5387, 1999.

[234] H. T. Allawi, F. Dong, H. S. Ip, B. P. Neri, and V. I. Lyamichev, "Mapping of RNA accessible sites by extension of random oligonucleotide libraries with reverse transcriptase," *RNA*, vol. 7, no. 2, pp. 314–327, 2001.

[235] H. H. Chen, D. Castanotto, J. M. LeBon, J. J. Rossi, and A. D. Riggs, "In vivo, high-resolution analysis of yeast and mammalian RNA-protein interactions, RNA structure, RNA splicing and ribozyme cleavage by use of terminal transferase-dependent PCR," *Nucleic Acids Research*, vol. 28, no. 7, pp. 1656–1664, 2000.

[236] H. Y. Zhang, J. Mao, D. Zhou et al., "mRNA accessible site tagging (MAST): a novel high throughput method for selecting effective antisense oligonucleotides," *Nucleic Acids Research*, vol. 31, no. 14, article e72, 2003.

Research Article

Efficient Transduction of Feline Neural Progenitor Cells for Delivery of Glial Cell Line-Derived Neurotrophic Factor Using a Feline Immunodeficiency Virus-Based Lentiviral Construct

X. Joann You,[1] Ping Gu,[1, 2] Jinmei Wang,[1] Tianran Song,[1] Jing Yang,[1] Chee Gee Liew,[3] and Henry Klassen[1]

[1] *Department of Ophthalmology, School of Medicine, Gavin Herbert Eye Institute, University of California, Irvine, 101 The City Drive, Bldg. 55, 2nd Fl, Orange, CA 92868-4380, USA*

[2] *Department of Ophthalmology, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200011, China*

[3] *Department of Cell Biology and Neuroscience, Stem Cell Center, University of California, Riverside, Riverside, CA 92521, USA*

Correspondence should be addressed to
Henry Klassen, hklassen@uci.edu

Work has shown that stem cell transplantation can rescue or replace neurons in models of retinal degenerative disease. Neural progenitor cells (NPCs) modified to overexpress neurotrophic factors are one means of providing sustained delivery of therapeutic gene products in vivo. To develop a nonrodent animal model of this therapeutic strategy, we previously derived NPCs from the fetal cat brain (cNPCs). Here we use bicistronic feline lentiviral vectors to transduce cNPCs with glial cell-derived neurotrophic factor (GDNF) together with a GFP reporter gene. Transduction efficacy is assessed, together with transgene expression level and stability during induction of cellular differentiation, together with the influence of GDNF transduction on growth and gene expression profile. We show that GDNF overexpressing cNPCs expand in vitro, coexpress GFP, and secrete high levels of GDNF protein—before and after differentiation—all qualities advantageous for use as a cell-based approach in feline models of neural degenerative disease.

1. Introduction

The retina is susceptible to a variety of degenerative diseases, including age-related macular degeneration (AMD), retinitis pigmentosa (RP) and other inherited photoreceptor degenerations, photoreceptor loss following retinal detachment, ganglion cell loss in glaucoma and optic neuropathies, as well as the loss of retinal neurons associated with nondegenerative conditions such as diabetic retinopathy (DR), macular edema and ischemia, vascular occlusions, trauma, and inflammatory diseases. Any of these can lead to debilitating visual deficits. AMD is a particularly prevalent cause of blindness among elderly persons, affecting more than 30 million people globally. That number is expected to double over the next decade in association with demographic shifts towards an older population, particularly in developed countries [1]. Similar to the situation with many neurological diseases, little is available in the way of effective treatments for patients with AMD or other blinding disorders of the retina.

A large body of research has shown that the use of exogenous neurotrophic factors can reproducibly promote the survival of specific neurons in various parts of the central nervous system (CNS), including the retina [2, 3]. Frequently investigated neuroprotective neurotrophic factors have included glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF). Among these, GDNF has been associated with significant effects with respect to preventing cell death [4], including the protection of specific neuronal populations in the brain [5, 6], spinal cord [7],

and retina [8–11]. Receptors for GDNF are known to be expressed within the mature retina [8, 11, 12].

Stem and progenitor cell transplantation has also shown considerable promise in animal models of neural degeneration. Subretinal transplantation of neural progenitor cells (NPCs) has yielded intriguing evidence of cellular repopulation of damaged retinas, growth of neurites into the optic nerve head and retardation of ongoing retinal degeneration [13–17]. Both unmodified, as well as genetically modified, cortical human NPCs can survive for prolonged periods, migrate extensively, secrete growth factors, and rescue visual function following subretinal transplantation in the dystrophic Royal College of Surgeons rat [18], with sustained visual benefits following injection [19]. More recently, subretinal transplantation of human forebrain progenitor cells has been extended to nonhuman primates [20], although this model used nondystrophic hosts and therefore did not lend itself to evaluation of neuroprotective efficacy. When used for transplantation therapy, NPCs engineered to secrete GDNF contributed to reduced apoptotic death in vitro, enhanced survival in vivo, neuronal differentiation, and improved host cognitive function following traumatic brain injury as compared with nontransduced NPCs [21–24].

The visual system of the cat is quite sophisticated and one of the most extensively studied among higher mammals. There are many similarities to the human retina although that of the cat has a tapetum and is generally optimized for performance under scotopic conditions [25]. Like humans, the cat is a species with a robust intraretinal circulation [26]. The cat retina has also been the subject of decades of anatomical and physiological studies and has been used as an animal model of binocular visual function as well as studies involving drug treatment and research on retina detachment [27, 28]. In addition, the feline eye is large relative to that of rodents thereby allowing the application of surgical techniques similar to those typically used clinically. Finally, there exist feline models of retinal degeneration caused by spontaneous mutations in genes known to be involved in retinitis pigmentosa in humans [29, 30]. These animals provide excellent models for exploring the therapeutic potential of stem cell-based neuroprotective strategies in an animal with highly developed visual capabilities.

Previously, we showed that it is possible to derive NPCs from the developing cat brain and that these cells are capable of integration into the retina of dystrophic feline recipients [23]. To more fully exploit the potential of this model, it is useful to develop feline NPCs capable of sustained growth factor delivery to the host retina. Here we use a bicistronic feline lentiviral vector to generate genetically modified feline neural progenitor cells that exhibit sustained overexpression of GDNF before and after differentiation.

2. Materials and Methods

2.1. Isolation and Culture of Neural Progenitor Cells from Feline Brain.
Cat neural progenitor cells (cNPCs) were originally isolated from 47 day cat fetuses as previously described [23]. Briefly, forebrains were removed and finely minced with a surgical scalpel and the resulting tissue fragments digested for 20 minutes in 0.1% type I collagenase (Invitrogen, Carlsbad, CA). The supernatant containing dissociated cells was then passed through a 100 mm mesh strainer, centrifuged, and seeded in complete culture medium, designated here as standard medium (SM), consisting of advanced DMEM/F12, 1% N2 neural supplement, 2 mM L-glutamine, 50 mg/mL penicillin-streptomycin, and epidermal and basic fibroblast growth factors (recombinant human EGF and bFGF, Invitrogen), both at final concentrations of 20 ng/mL. After initial isolation, all medium was changed to an Ultraculture-based composition, identical to the above but in which DMEM/F12 was replaced with Ultraculture serum-free medium (Lonza, Basel, Switzerland). Therefore, in the present study standard proliferation medium was Ultraculture-based with growth factors and is designated (UM), whereas differentiation medium was Ultraculture-based as well, but did not contain added growth factors and did include 10% fetal bovine serum (UM-FBS). Culture medium was changed every 2 days and proliferating cells passaged at regular intervals of 4-5 days.

2.2. Lentivector Production and Titer Determination.
The lentiviral vector used in this study was an FIV-based bicistronic vector (GeneCopoeia, Germantown, Maryland) designated as lenti-GDNF-GFP, which carries a human GDNF gene driven by the cytomegalovirus (CMV) immediate-early promoter as well as an enhanced green fluorescent protein (GFP) reporter gene with an internal ribosome entry site (IRES). Lenti-GDNF-GFP vectors were prepared by transient transfection of 293T cells using a standard calcium phosphate precipitation protocol (Clontech, Mountain View, CA). Briefly, 293T cells cultured in 10 cm tissue culture dishes (BD Biosciences, San Jose, CA) were transfected with $2\,\mu$g of lentiviral transfer vector plasmid, along with $10\,\mu$g of the mixed envelope and packaging plasmids. The viral supernatants were harvested 48 and 72 hours posttransfection and concentrated by centrifugation of virus-containing supernatant through a Centricon Plus-70 filter (Millipore, Billerica, MA) following the manufacturer's instruction. Titers of the concentrated lentivector were estimated by transducing cNPC cells with a serial dilution of the virus and flow cytometric identification of GFP-positive cells.

2.3. Lentiviral Vector Transduction.
Cat neural progenitor cells were transduced with lenti-GDNF-GFP vectors at a MOI of 10 following the standard procedure. Briefly, cNPCs were seeded at a density that allowed them to grow to 90% confluency on the day of transduction. The cells were then transduced by 6–24 hours of exposure to virus-containing supernatant in the presence of 5–$8\,\mu$g/mL polybrene. Viral vector-containing medium was then replaced with fresh medium and cells were incubated at $37°$C in a CO_2 incubator.

2.4. FACS Analysis and Selection of Lenti-GDNF-GFP Positive cNPCs.
Cells were harvested using TrypLE Express (Invitrogen) and filtered through cell strainer caps ($35\,\mu$m mesh) to obtain a single cell suspension (approximately 10^6 cells per mL for analysis, 0.5–2×10^7 cells per mL for sorting).

The stained cells were analyzed and sorted on a fluorescence-activated cell sorter FACSAria (BD Biosciences) using FACS-Diva software (BD Biosciences). The fluorochromes were excited by the instrument's standard 488 nm and 633 nm lasers, and green fluorescence was detected using 490 LP and 510/20 filters. Prior to sorting, the nozzle, sheath, and sample lines were sterilized with 70% ethanol or 2% hydrogen peroxide for 15 minutes, followed by washes with sterile water. A 100 μm ceramic nozzle (BD Biosciences), sheath pressure of 20–25 pounds per square inch (PSI), and an acquisition rate of 1,000–3,000 events per second were used as conditions previously optimized for neuronal cell sorting.

2.5. Cell Growth Assessment. The growth properties of transduced and nontransduced cNPCs were assessed by culturing both types of cells under proliferation conditions in Ultraculture-based medium (UM). Cells of identical passage number (p17) were seeded in four T25 culture flasks at a density of 0.25 million cells/flask. One flask of each cell type were trypsinized and counted daily. Cell numbers were graphed at each time point to compare the growth properties of transduced versus nontransduced cells.

2.6. ELISA Analysis. Transduced and nontransduced cNPCs of identical passage number were seeded in T25 culture flasks (0.25 million/flask). Following attachment of cells (approx. 4 hours), the original media were replaced with 3 mL of fresh media. Subsequently, 3 mL of conditioned media were collected and replaced with fresh media at 24 hour intervals and conditioned samples were saved at $-80°C$ for ELISA analysis. ELISA was performed using a human GDNF DuoSet ELISA kit and protocol (R&D Systems, Minneapolis, MN). Wells of microtiter plates were coated (overnight, room temperature) with 2 μg/mL of GDNF capture antibody in 100 μL of coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and then blocked with 0.1% BSA in PBS for 1 hour at room temperature. Samples (100 μL) were loaded in triplicates and incubated for 2 hours at room temperature, followed by addition of 100 μL antibody detection antibody (0.1 μg/mL) for an additional 2 hours at room temperature. HRP-conjugated streptavidin (1 : 200) in blocking buffer was then added (20 minutes, room temperature) and the reaction visualized by the addition of 100 μL of substrate solution for 20 minutes. The reaction was stopped with 50 μL H_2SO_4 and absorbance at 450 nm was measured with reduction at 540 nm using an ELISA plate reader. Plates were washed five times with washing buffer (PBS, pH 7.4, containing 0.05% (v/v) Tween 20) after each step. As a reference for quantification, a standard curve was established by a serial dilution of recombinant GDNF protein (31.25 pg/mL–2.0 ng/mL).

2.7. Reverse Transcription and Quantitative PCR (qPCR) Analysis. Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen, Valencia, CA). DNaseI was used to eliminate the possibility of genomic DNA contamination. RNA concentration was measured at a wavelength of 260 nm (A260) for each sample, and the purity of isolated total RNA was determined by the A260/A280 ratio. Quantitative RT-PCR analyses were only performed on samples with A260/A280 ratios between 1.9 and 2.1. Two micrograms of total RNA in a 20 μL reaction were used for reverse transcription using an Omniscript cDNA Synthesis Kit (Qiagen, Valencia, CA). A primer set for each gene (Table 1) was designed using the cat genome browser (http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/) and the primers synthesized commercially (Invitrogen).

Quantitative PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR Detection System (Applied Biosystems, Foster, CA). Triplicate wells were used for each gene. A total volume of 20 μL per well containing 10 μL of 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA), 2 μL of cDNA and gene-specific primers were used. Cycling parameters for qPCR were as following: the initial denaturation was at 95°C for 10 minutes, followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C. To normalize template input, β-actin was used as an endogenous control and transcript level measured for each plate. The relative expression of the gene of interest was then evaluated using 7500 Fast System Sequence Detection Software, Version 1.4. The value obtained for Ct represents the number of PCR cycles at which an increase in fluorescence signal (and therefore cDNA) can be detected above background and the increase is exponential for the particular gene. Data were expressed as fold change relative to untreated controls after normalizing to β-actin. Error bars displayed the calculated maximum and minimum standard errors to the mean expression level of the triplicates.

2.8. Differentiation of Transduced cNPCs In Vitro. Transduced cNPCs were differentiated in UM without added EGF or bFGF and containing 10% FBS (UM-FBS). Cells (0.2 million) in UM were seeded in T25 culture flasks and allowed to attach, then culture medium was aspirated and replaced with either UM-FBS for differentiation or fresh UM for comparison. Conditioned media were collected and replaced with fresh media every 24 hours for 4 days and frozen for ELISA analysis. At the end of day 4, cells were trypsinized, counted, and ELISA analysis was performed on lysates as well as thawed media samples. For FACS analysis, transduced cNPCs were cultured in either UM-FBS or UM for 10 days prior to processing.

2.9. Immunocytochemistry. Transduced and nontransduced cNPCs were seeded in 4-well chamber slides (Nalge Nunc International, Rochester, NY) and allowed to grow for 3–5 days. Cells were re-fed every 2 days and fixed with freshly prepared 4% paraformaldehyde (Invitrogen) in 0.1 M phosphate-buffered saline (PBS) for 20 minutes at room temperature and washed with PBS. Cells were then incubated in antibody blocking buffer consisting of PBS containing 10% (v/v) normal goat serum (NGS) (Biosource, Camarillo, CA), 0.3% Triton X-100, 0.1% NaN3 (Sigma-Aldrich, Saint Louis, MO) for 1 hour at room temperature. Slides were incubated in primary antibodies (Table 2) overnight at 4°C. After washing the next morning, slides were incubated in

TABLE 1: Cat-specific primers for quantitative RT-PCR (GDNF = human).

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	GCCGTCTTCCCTTCCATC	CTTCTCCATGTCGTCCCAGT
Nestin	CTGGAGCAGGAGAAGGAGAG	GAAGCTGAGGGAAGCCTTG
Sox2	ACCAGCTCGCAGACCTACAT	TGGAGTGGGAGGAAGAGGTA
Vimentin	ATCCAGGAGCTACAGGCTCA	GGACCTGTCTCCGGTACTCA
Pax6	AGGAGGGGGAGAGAATACCA	CTTTCTCGGGCAAACACATC
Hes1	GCCAGCAGATATAATGGGAGA	GCATCCAAAATCAGTGTTTTCA
Hes5	CTCAGCCCCAAAGAGAAGAA	AGGTAGCTGACGGCCATCTC
Notch1	CAGTGTCTGCAGGGCTACAC	CTCGCACAGAAACTCGTTGA
Mash1	CATCTCCCCCAACTACTCCA	CCAACATCGCTGACAAGAAA
Ki-67	TCGTCTGAAGCCGGAGTTAT	TCTTCTTTTCCCGATGGTTG
DCX	GGCTGACCTGACTCGATCTC	GCTTTCATATTGGCGGATGT
β3-tubulin	CATTCTCGTGGACCTTGAGC	GCAGTCGCAATTCTCACATT
Map2	ACCTAAGCCATGTGACATCCA	CTCCAGGTACATGGTGAGCA
PKC-alpha	TTCACAAGAGGTGCCATGAA	CCATACAGCAATGACCCACA
GFAP	CGGTTTTTGAGGAAGATCCA	TTGGACCGATACCACTCCTC
Lhx2	GATCTGGCGGCCTACAAC	AGGACCCGTTTGGTGAGG
CD81	CCACAGACCACCAACCTTCT	CAGGCACTGGGACTCCTG
CD133	AGGAAGTGCTTTGCGGTCT	TGCCAGTTTCCGAGTCTTTT
NCAM (CD56)	AGAACAAGGCTGGAGAGCAG	TTTCGGGTAGAAGTCCTCCA
EGFR	AACTGTGAGGTGGTCCTTGG	CGCAGTCCGGTTTTATTTGT
NagoA	TTTGCAGTGTTGATGTGGGTA	TAACAGGAACGCTGAAGAGTGA
SDF1	ACAGATGTCCTTGCCGATTC	CCACTTCAATTTCGGGTCAA
CXCR4	TCTGTGGCAGACCTCCTCTT	TTTCAGCCAACAGCTTCCTT
Cyclin D2	CAAGATCACCAACACGGATG	ATATCCCGCACGTCTGTAGG
Pbx1	CTCCGATTACAGAGCCAAGC	GCTGACCATACGCTCGATCT
FABP7	TGGAGGCTTTCTGTGCTACC	TGCTTTGTGTCCTGATCACC
AQP4	TACACTGGTGCCAGCATGA	CACCAGCGAGGACAGCTC
Nucleostemin	CAGTGGTGTTCAGAGCCTCA	CCGAATGGCTTTGCTGTAA
Synapsin1	ACGACGTACCCTGTGGTTGT	CGTCATATTTGGCGTCAATG
Caspase 3	ATGGAGAACAGTGAAAACTCAGTGG	AATTATTATACATAAACCCATTTCAGG
Bax 4	CTGAGCAGATCATGAAGACAGG	GTCCAGTTCATCTCCGATGC
hGDNF*	TGGGCTATGAAACCAAGGAG	CAACATGCCTGCCCTACTTT

* Human GDNF gene.

fluorescent-conjugated secondary antibody (Alexa Fluor546 goat anti-mouse or goat anti-rabbit, 1 : 800 in PBS, BD) for 1 hour at room temperature. After washing, DAPI-containing Vectashield Hard Set Mounting Medium (Vector Laboratories, Burlingame, CA) was used to mount the slides for 20 minutes at room temperature. Negative controls for immunolabeling were performed in parallel using the same protocol but without primary antibody. Fluorescent staining was judged as positive only with reference to the negative controls. Immunoreactive cells were visualized and imaged using a fluorescent microscope (Eclipse E600, Nikon, Melville, NY).

3. Results

3.1. Transduction of Proliferating cNPCs by FIV-Based Vector.
Currently, there are relatively few molecular tools with enhanced specificity for feline cells. Recent development of

feline immunodeficiency virus- (FIV-) based vectors could present a means for improved delivery of transgenes into cells of this species. Here, we employed an FIV-based bicistronic vector for delivery of glial cell line-derived neurotrophic factor (GDNF) to cat neural progenitor cells (cNPCs). Forty eight hours after lenti-GDNF-GFP viral vector transduction, approximately 50% of cNPCs expressed the GFP reporter gene based on direct observation via fluorescence microscopy. To enrich for transgene-expressing cells, cNPCs were trypsinized at 72 hours postviral vector incubation and sorted by FACS based on GFP expression. The GFP-enriched population was subsequently cultured in Ultraculture-based proliferation medium (UM) for more than 60 days. High levels of GFP expression were sustained throughout this time period (Figure 1).

3.2. Expression of the GDNF Transgene Did Not Abrogate cNPC Proliferation.
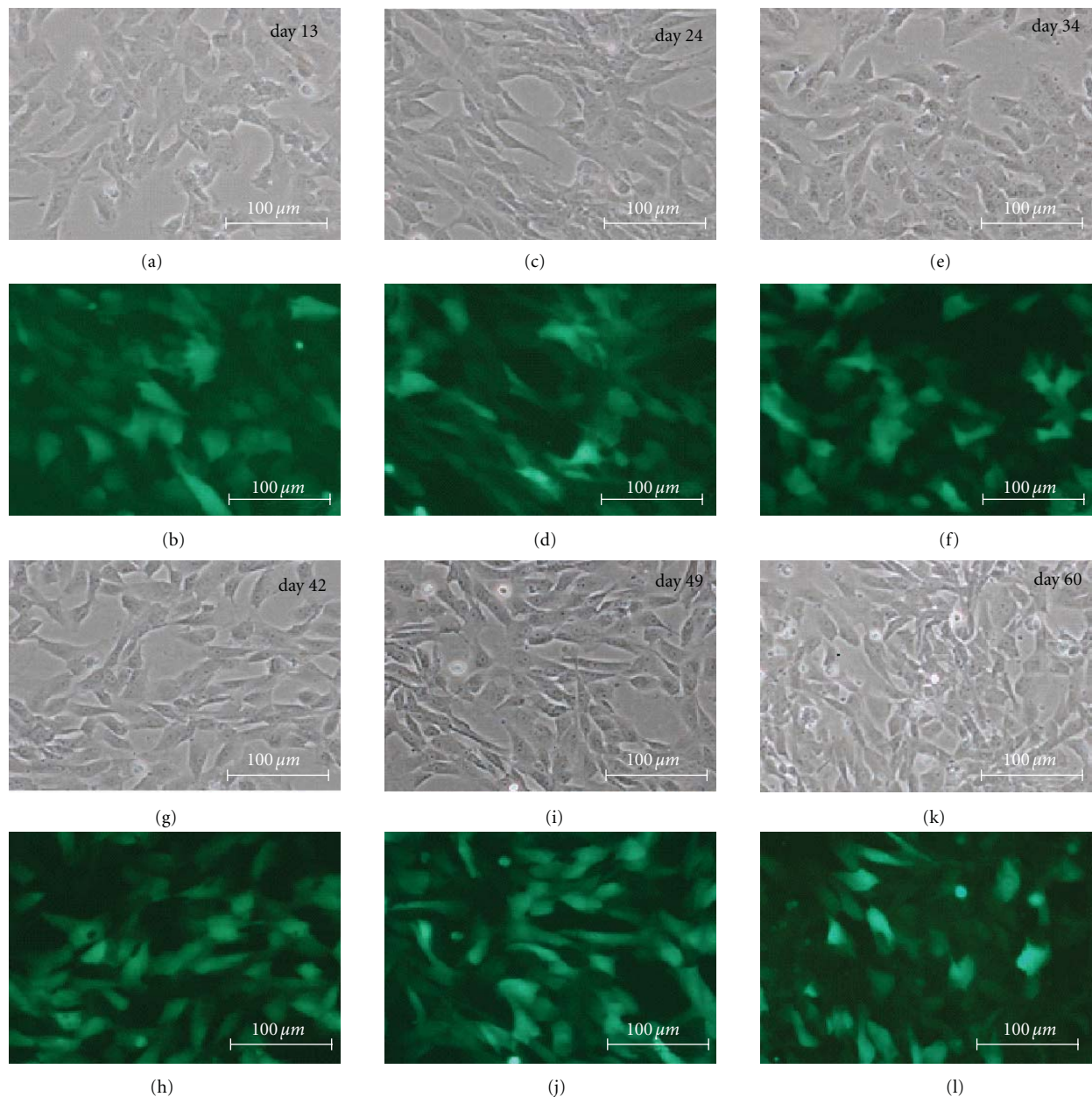GDNF is known to have a range of

FIGURE 1: GDNF-transduced cNPCs: morphology and reporter gene expression. Feline NPCs transduced using a bicistronic lenti-GDNF-GFP vector and cultured under proliferation conditions (UM) for 60 days (p9–p26). Cellular growth, morphology, and GFP expression were monitored over this time period. In this figure, paired phase contrast ((a), (c), (e), (g), (i), (k)) and fluorescence ((b), (d), (f), (h), (j), (l)) micrographs of the same field are presented for each of 6 sequential time points, as indicated. Transduced cNPCs exhibited consistent mophologies, continued growth, and sustained GFP expression throughout the period examined. Bars = 100 μm.

TABLE 2: Primary antibodies used for immunocytochemistry on cNPCs.

| Target	Antibody type	Reactivity in retina	Source	Dilution
Nestin	Mouse monoclonal	Progenitors, reactive glia	BD	1 : 200
Vimentin	Mouse monoclonal	Progenitors, reactive glia	Sigma	1 : 200
Ki-67	Mouse monoclonal	Proliferating cells	BD	1 : 200
GFAP	Mouse monoclonal	Astrocytes, reactive glia	Chemicon	1 : 200
β3-tubulin	Mouse monoclonal	Immature neurons	Chemicon	1 : 200
GDNF	Rabbit polyclonal	Growth factor	SCBT	1 : 200
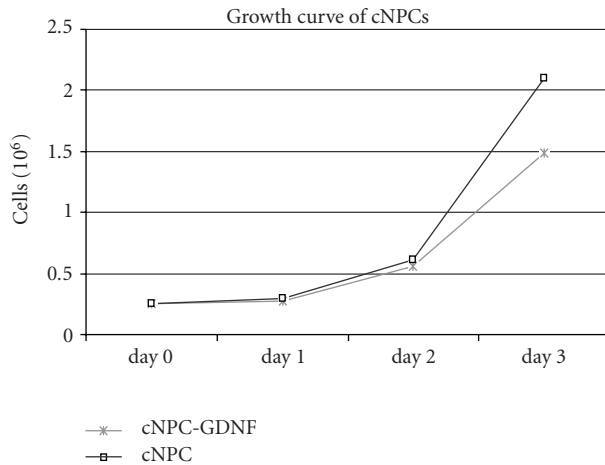
Figure 2: Growth properties of transduced versus nontransduced cNPCs. The growth of lenti-GDNF-GFP vector transduced cNPCs was compared to nontransduced cNPCs under proliferation conditions (UM). One flask of each type of cells was harvested and counted daily for 3 consecutive days. From this data it can be seen that the transduced cNPCs continued to proliferate despite overexpression of GDNF and that growth was similar to that of nontransduced cells out to day 2, after which the nontransduced cells exhibited relatively greater growth at the day 3 time point.
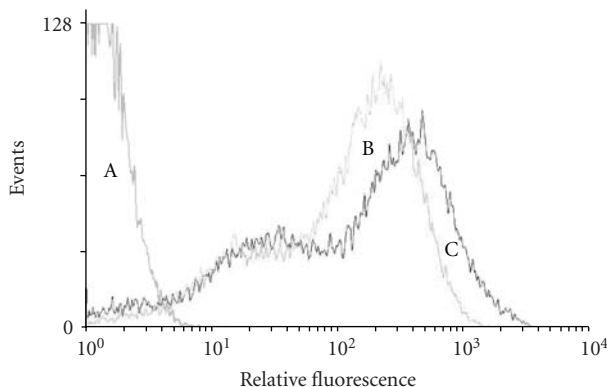

Figure 3: Flow cytometric analysis of GFP expression after induction of differentiation. Nontransduced cNPCs and lenti-GDNF-GFP vector transduced cNPCs cultured under proliferation conditions (UM) were compared to transduced cNPCs cultured for 10 days in Ultraculture-based medium without EGF or bFGF and containing 10% FBS in order to induce differentiation (UM-FBS). Curve A: nontransduced cNPCs as negative controls; curve B: lenti-GDNF-GFP transduced cNPCs and curve C: lenti-GDNF-GFP transduced cNPCs in UM-FBS. Induction of differentiation did not attenuate expression of the GFP reporter gene.

biological activities in the context of the nervous system and cultured neural cell populations. Because this activity might extend to neural progenitors, we examined the effect of GDNF transduction on cNPC behavior, specifically the ability to proliferate. Proliferation is an important consideration for large-scale expansion of modified donor cell populations for use in transplantation studies. Transduced cNPCs continued to proliferate in a logarithmic manner,

similar to but slightly slower than the nontransduced cNPCs (Figure 2). Conversely, the transduced cNPCs appeared to be somewhat more uniform, with less clumping and fewer floating cells, particularly when cells were cultured for more than 3 days in the same flask.

3.3. Transgene Expression Was Maintained under Differentiation Conditions. Neuronal differentiation has been implicated in gene silencing; therefore FACS analysis was performed to evaluate the effects of cell differentiation on GDNF transgene expression using the GFP reporter. Approximately 95% of transduced cNPCs expressed GFP, either when cultured in UM (proliferation conditions) or 10% FBS-containing UM (differentiation conditions). Among the cells expressing GFP, approximately 70% expressed GFP at high levels. There was no evidence of diminished GFP expression by the cells grown in the presence of FBS, thereby demonstrating maintained transgene expression was under differentiation conditions (Figure 3).

3.4. Transduced cNPCs Produced and Secreted Elevated Levels of GDNF. The levels of GDNF produced by transduced cNPCs, as present in conditioned culture medium and collected cell lysates, were analyzed by ELISA and compared to nontransduced controls. High levels of secreted GDNF were present in the culture medium of transduced cNPCs, measured on days 28, 33, and 38 posttransduction (Figure 4(a)). In addition, GDNF expression levels were considerably elevated in cell lysates extracted from transduced cultures on days 33 and 38 post-transduction (Figure 4(b)). Hence, transduced cNPCs continued to produce elevated levels of GDNF over a sustained period of time.

3.5. GDNF Expression Was Maintained under Differentiation Conditions. Having shown above that expression of the GFP reporter was sustained when transduced cNPCs were subjected to differentiation conditions, and that the transduced cells overexpress GDNF, we next verified that GDNF expression was sustained during cNPC differentiation (Figure 5). Transduced cNPCs were cultured in UM without added growth factors and containing 10% FBS to induce cell differentiation and media were collected for ELISA. The level of GDNF produced under differentiation conditions was not diminished relative to proliferation conditions.

3.6. Effect of GDNF Overexpression on Neural Differentiation. Neural progenitor cells have shown great promise as a source of neural cell types in transplantation studies. We therefore investigated whether genetically modified cNPCs retained their neural progenitor phenotype in the presence of high levels of GDNF expression, as assessed by a gene expression profile (Figure 6). qPCR analysis showed that transduced cNPC cells exhibited approximately 14,000-fold GDNF upregulation at the mRNA level compared to non-transduced controls. In transduced cells, expression levels of the progenitor cell markers nestin, vimentin, and sox2, as well as the neuronal marker β3-tubulin and the proliferation marker Ki-67 remained similar to that seen in nontransduced
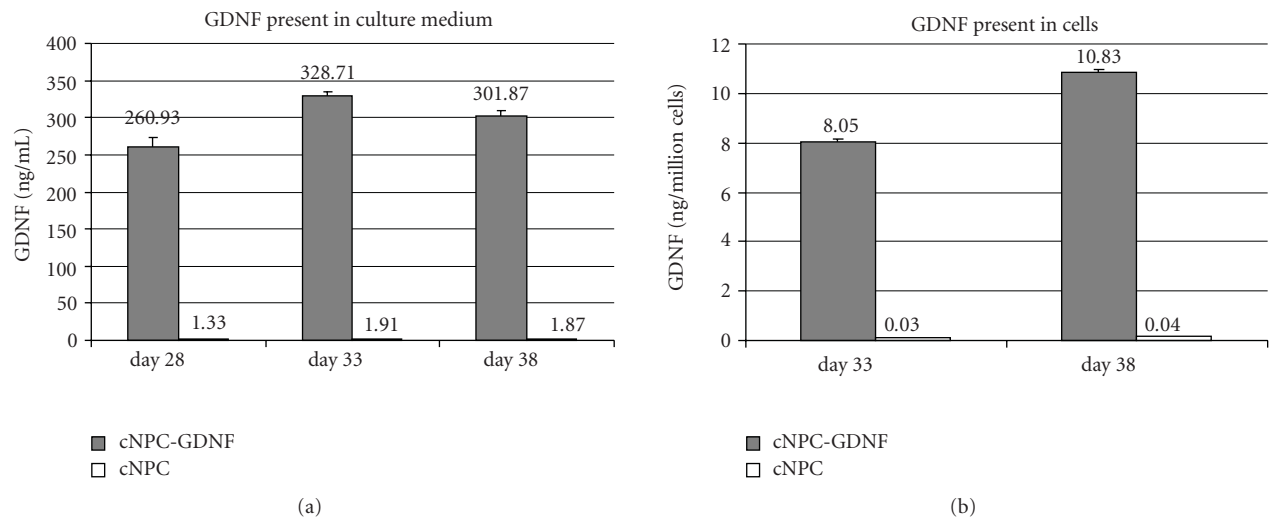
(a)

(b)

FIGURE 4: ELISA analysis of GDNF production by transduced cNPCs. (a) Lenti-GDNF-GFP vector transduced and nontransduced cells at passage 17 (cNPCp17) were seeded equally, under identical conditions, and allowed to grow for 15 days in UM, over which period the cells were passaged 3 times. At the time of each passage, culture media conditioned over the prior 48 hours was collected for ELISA assay. The conditioned media from transduced cNPCs was substantially enriched for GDNF compared to nontransduced cells. (b) Lenti-GDNF-GFP transduced and nontransduced cNPCp17 cells were trypsinized, lysed, and subjected to ELISA. GDNF was markedly elevated in lysates of transduced cells.
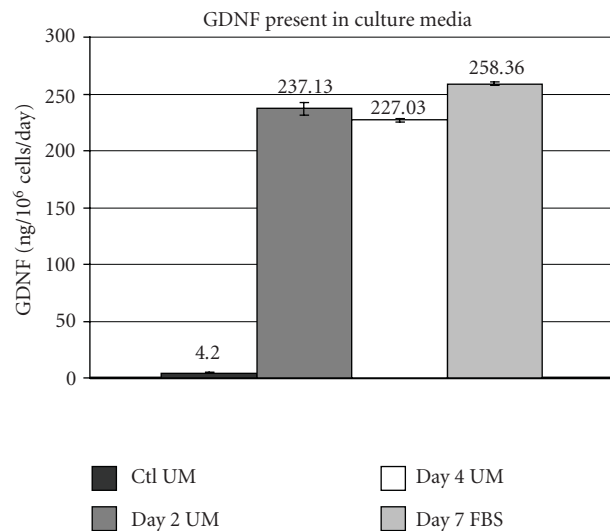

FIGURE 5: Effect of cell differentiation on transgene GDNF expression by ELISA. Lenti-GDNF-GFP vector transduced cNPCp24 cells were seeded equally in either UM (proliferation conditions) or Ultraculture-based medium without additional growth factors but containing 10% FBS (differentiation conditions, UM-FBS). Cultures were fed 24 hours prior to collecting GDNF conditioned media for ELISA assay at which time the cells were counted. ELISA data is presented as GDNF (ng) per million cells per day in order to further evaluate whether differentiation of transduced cNPCs had an influence on transgene expression. These data are consistent with sustained GDNF overexpression, confirming the flow cytometric data (Figure 3) that showed no evidence of diminished reporter gene expression in the UM-FBS treated population of transduced cNPCs.

cells. Transduced cells also exhibited increased transcript levels for stromal cell-derived factor-1 (SDF1, 4.2-fold), prominin (CD133, 2.9-fold), doublecortin (DCX, 2.4-fold), and Hes1 (1.45-fold), as well as lower transcript levels for CXCR4, FABP7 and NCAM.

3.7. Examination of Protein Expression Using Immunocytochemistry. Immunocytochemical analysis demonstrated that cNPCs produced low levels of GDNF protein at baseline (Figure 7(a)), but that expression of the protein was substantially elevated following transduction with Lenti-GDNF-GFP (Figure 7(b)). To investigate the effect of differentiation on GDNF protein overexpression, cNPCs were cultured in either serum-free UM or UM containing 10% FBS for 5 days. Following the induction of differentiation, the cells appeared larger in size and GDNF expression was sustained, although heterogeneity of expression levels across the population was evident (Figure 7(c)).

The expression of progenitor and lineage markers was also examined at the protein level, for both transduced and control cells, before and after induction of differentiation (Figure 8). The results verified the differentiating influence of the FBS-containing condition as follows. The neural progenitor cell marker nestin was only detected in cells grown in UM and was not seen in UM-FBS. Likewise, vimentin expression also decreased upon differentiation, although for this less-specific marker expression remained substantial. In contrast, β-tubulin III immunoreactivity was strikingly up-regulated in a subset of cells grown in UM-FBS, suggesting the induction of neuronal lineage. The proliferation marker Ki-67 was clearly downregulated in
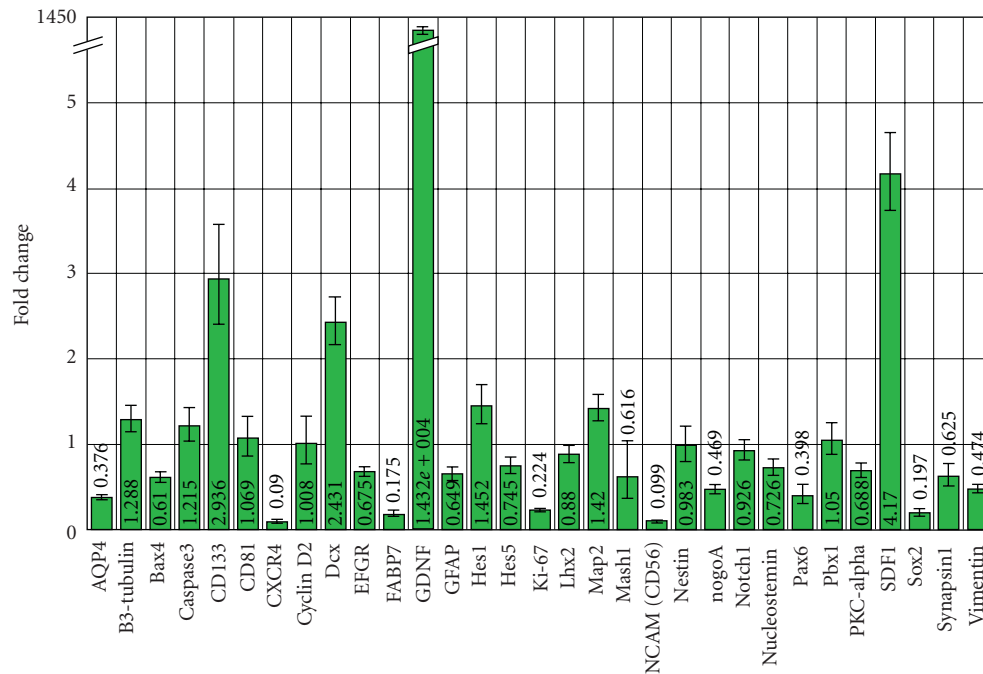
FIGURE 6: Expression profiles of cNPCs before and after transduction. The relative impact of GDNF overexpression on transcript expression levels was evaluated using qPCR analyses for a profile of 32 genes, which included β-actin as a housekeeping gene. Lenti-GDNF-GFP vector transduced cNPCp20 cells were compared to nontransduced cNPCp20 cells (with nontransduced cells set to 1.00). GDNF transcript level was over 14,000-fold higher in transduced versus nontransduced cells (note that Y-axis has break to accommodate value). The value for GDNF was vastly greater than any other changes in transcript level across the profile examined.
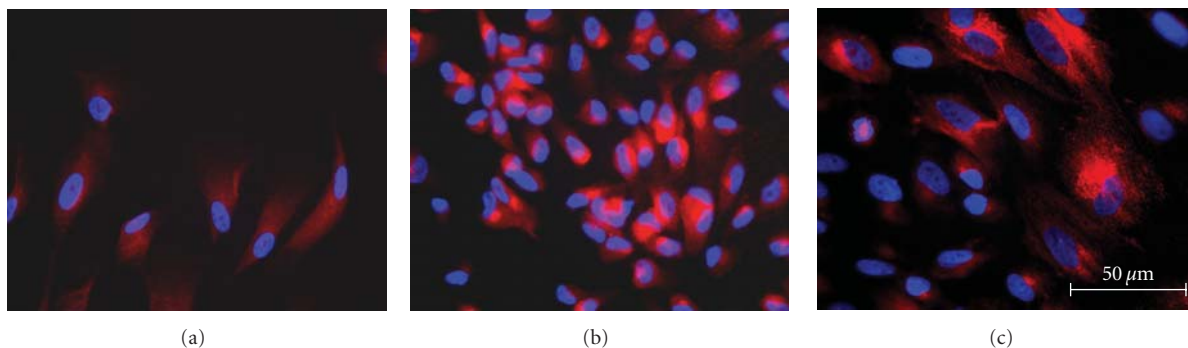

FIGURE 7: GDNF expression by cNPCs before and after transduction and differentiation. Immunocytochemistry (ICC) was performed on cNPCs using a rabbit anti-human GDNF antibody to evaluate expression of GDNF at the protein level, before and after transduction and before and after exposure to growth factor deprived/FBS-containing differentiation conditions (UM-FBS). (a) Nontransduced cNPCp20 cultured in UM (proliferation conditions) exhibit baseline cytoplasmic labeling for GDNF (red). (b) Lenti-GDNF-GFP vector transduced cNPCs cultured in UM show increased intensity of GDNF (red) labeling. (c) Transduced cNPCs cultured in UM-FBS (differentiation conditions) are larger in size and show persistent overexpression of GDNF (red), that is, heterogeneously distributed among the profiles. Nuclear labeling = DAPI (blue), scale bar = 50 μm.

UM-FBS cultured cNPCs, whereas the glial marker GFAP was not detected under proliferation conditions, but was strongly up-regulated by a subset of cells cultured in UM-FBS. Having confirmed the differentiating influence of the UM-FBS conditions, the same immunocytochemical analysis was repeated on cNPCs of identical age that had been transduced using the lenti-GDNF-GFP vector. The results were equivalent, suggesting that the differentiation of cNPCs

was not adversely influenced by transduction with GDNF (Figure 8).

4. Discussion

Among mammals, the highly developed visual system of the domestic cat has been studied in particular detail, owing in part to greater similarities with the human visual system
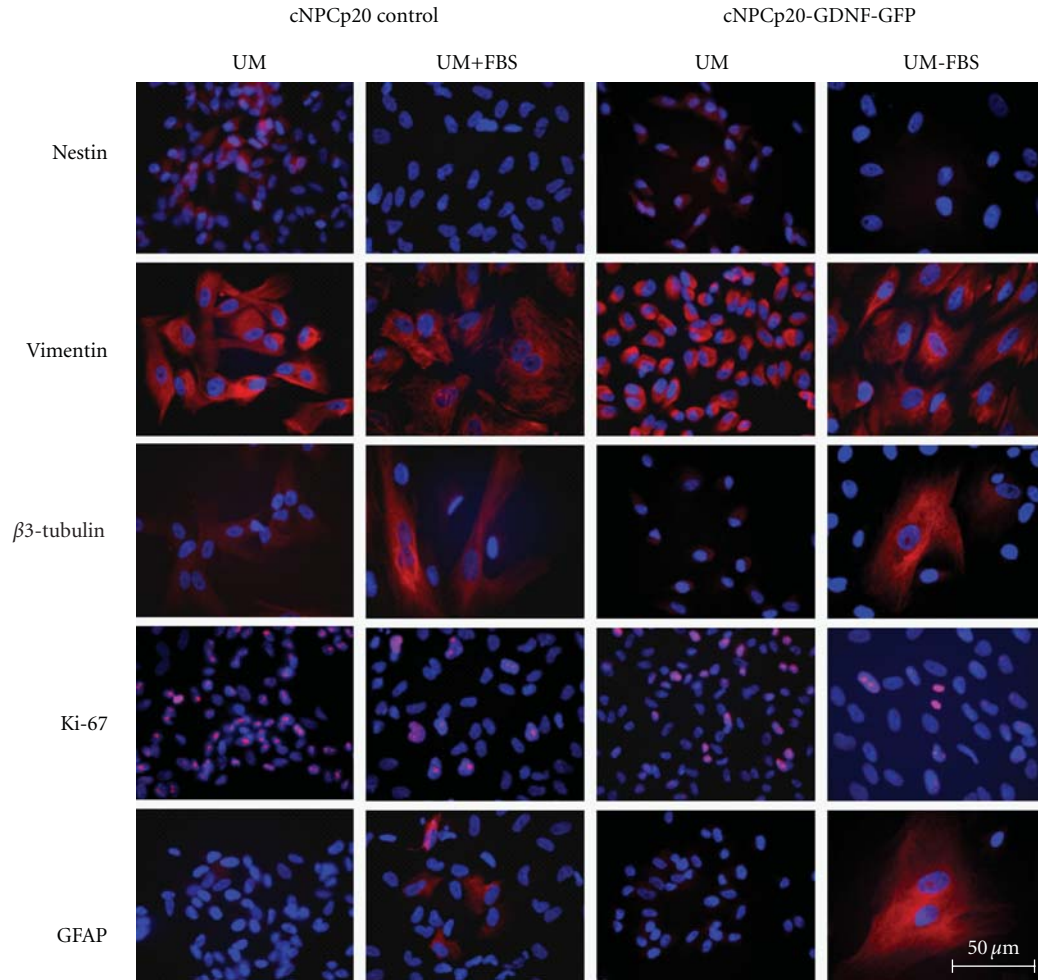
FIGURE 8: Expression of NPC and lineage markers before and after transduction and differentiation. The effects of passage number, induction of differentiation and GDNF transgene expression on the expression of 5 markers was evaluated using ICC. Nontransduced and lenti-GDNF-GFP vector transduced cNPCp20 were cultured in UM or UM-FBS, then immunolabeled with specific antibodies. The changes in expression patterns seen predominantly reflected exposure to differentiation conditions (alternating columns), with little that might be attributable to passage number or lenti-GDNF-GFP transduction. Scale bar = 50 μm.

as compared to laboratory rodents. This body of work, combined with the availability of naturally occurring retinal dystrophic mutants, would serve to recommend the cat as a powerful model for retinal regeneration research. A major limiting factor to regenerative research in this species is the paucity of available donor cells of the type suitable for such work, including stem, progenitor, or precursor cells of allogeneic origin. Furthermore, the use of these cells in transplantation studies would benefit from the inclusion of a reporter gene and, in some cases, additional transgenes of potential therapeutic value.

Here we demonstrate the feasibility of using feline lentiviral vectors to genetically modify cNPCs for sustained delivery of GDNF. These cells possess multiple desirable features for use in transplantation studies including ease of expansion in vitro, coexpression of a green fluorescence protein (GFP) reporter gene serving to both confirm GDNF expression as well as allowing easy tracking of donor cells after transplantation, and sustained transgene expression

following differentiation. In addition, they are allogeneic with respect to the targeted host species and therefore likely to be well tolerated without for the need of exogenous immune suppression [31].

The ability of a progenitor cell to sustain proliferation is important in order to avoid the necessity of repeated rederivation of the modified cell type. Importantly, the GDNF-GFP overexpressing cNPCs continued to exhibit log growth characteristics, indicating that neither the genetic modification process nor GDNF overexpression presents a major barrier to continued proliferation of these cells. Nevertheless, the growth of the GDNF-transduced cNPCs was less rapid than that of unmodified controls. This slower growth rate is also reflected in the lower number of cells that were Ki-67 positive following introduction of the transgene construct. Since we have recently shown that exogenous GDNF tends to promote, rather than hinder, the growth of murine RPCs [32], it seems unlikely that a feedback signaling mechanism involving the overexpressed cytokine would

explain the behavior seen here. Perhaps the particularly high levels of transgene expression maintained by the GDNF-GFP transduced cNPCs results in a metabolic load that slows growth relative to unmodified cells. Alternatively, genetic modification could introduce abnormalities to the host genome, for instance as a function of the sites of transgene integration.

Another consideration in terms of clinical application of transduced cells is the regulation of transgene expression. Sustained overexpression might result in undesired effects such as decreased sensitivity to the gene product, as might result from down-regulation of the corresponding growth factor receptor or, alternatively, toxic responses to high levels of the cytokine, either within the eye or systemically. Titrating the dose of transplanted cells should set an upper limit on GDNF delivery, since the progenitor cells tend to cease proliferation in vivo, however, a more sophisticated approach would be the use of inducible promoters which allow for the dynamic regulation of transgene expression levels.

Looking forward, the GDNF-GFP overexpressing cNPCs developed here are suitable for allogeneic transplantation to the vitreous cavity or subretinal space of cats with retinal disease. Of particular interest is the application of these cells to existing animals with photoreceptor dystrophy, such as the Swedish Abyssinian breed with the CEP290 mutation [29], with the goal of ameliorating visual loss through the sustained intraocular delivery of a neurotrophic factor. In vivo experiments in this nonrodent species would more realistically model the prospective treatment of analogous human conditions and could yield valuable information pertaining to the mechanisms of graft-mediated effects on host visual function.

Acknowledgments

References

[1] R. Klein, "Overview of progress in the epidemiology of age-related macular degeneration," *Ophthalmic Epidemiology*, vol. 14, no. 4, pp. 184–187, 2007.

[2] E. G. Faktorovich, R. H. Steinberg, D. Yasumura, M. T. Matthes, and M. M. LaVail, "Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor," *Nature*, vol. 347, no. 6288, pp. 83–86, 1990.

[3] M. M. LaVail, K. Unoki, D. Yasumura, M. T. Matthes, G. D. Yancopoulos, and R. H. Steinberg, "Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 23, pp. 11249–11253, 1992.

[4] A. Blesch and M. H. Tuszynski, "GDNF gene delivery to injured adult CNS motor neurons promotes axonal growth, expression of the trophic neuropeptide CGRP, and cellular protection," *Journal of Comparative Neurology*, vol. 436, no. 4, pp. 399–410, 2001.

[5] A. Björklund, C. Rosenblad, C. Winkler, and D. Kirik, "Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease," *Neurobiology of Disease*, vol. 4, no. 3-4, pp. 186–200, 1997.

[6] S. Behrstock, A. Ebert, J. McHugh et al., "Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates," *Gene Therapy*, vol. 13, no. 5, pp. 379–388, 2006.

[7] S. M. Klein, S. Behrstock, J. McHugh et al., "GDNF delivery using human neural progenitor cells in a rat model of ALS," *Human Gene Therapy*, vol. 16, no. 4, pp. 509–521, 2005.

[8] M. Frasson, S. Picaud, T. Léveillard et al., "Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 11, pp. 2724–2734, 1999.

[9] L. H. M. Sanftner, H. Abel, W. W. Hauswirth, and J. G. Flannery, "Glial cell line derived neurotrophic factor delays photoreceptor degeneration in a transgenic rat model of retinitis pigmentosa," *Molecular Therapy*, vol. 4, no. 6, pp. 622–629, 2001.

[10] C. Andrieu-Soler, A. Aubert-Pouëssel, M. Doat et al., "Intravitreous injection of PLGA microspheres encapsulating GDNF promotes the survival of photoreceptors in the rd1/rd1 mouse," *Molecular Vision*, vol. 11, pp. 1002–1011, 2005.

[11] S. M. Hauck, N. Kinkl, C. A. Deeg, M. Swiatek-De Lange, S. Schöffmann, and M. Ueffing, "GDNF family ligands trigger indirect neuroprotective signaling in retinal glial cells," *Molecular and Cellular Biology*, vol. 26, no. 7, pp. 2746–2757, 2006.

[12] A. Kretz, A. M. Jacob, S. Tausch, G. Straten, and S. Isenmann, "Regulation of GDNF and its receptor components GFR-α1, -α2 and Ret during development and in the mature retino-collicular pathway," *Brain Research*, vol. 1090, no. 1, pp. 1–14, 2006.

[13] E. Y. Snyder, C. Yoon, J. D. Flax, and J. D. Macklis, "Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 21, pp. 11663–11668, 1997.

[14] I. Ahmad, C. M. Dooley, W. B. Thoreson, J. A. Rogers, and S. Afiat, "In vitro analysis of a mammalian retinal progenitor that gives rise to neurons and glia," *Brain Research*, vol. 831, no. 1-2, pp. 1–10, 1999.

[15] D. M. Chacko, J. A. Rogers, J. E. Turner, and I. Ahmad, "Survival and differentiation of cultured retinal progenitors transplanted in the subretinal space of the rat," *Biochemical and Biophysical Research Communications*, vol. 268, no. 3, pp. 842–846, 2000.

[16] M. J. Young, J. Ray, S. J. O. Whiteley, H. Klassen, and F. H. Gage, "Neuronal differentiation and morphological integration of hippocampal progenitor cells transplanted to

the retina of immature and mature dystrophic rats," *Molecular and Cellular Neurosciences*, vol. 16, no. 3, pp. 197–205, 2000.

[17] A. Otani, M. I. Dorrell, K. Kinder et al., "Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells," *Journal of Clinical Investigation*, vol. 114, no. 6, pp. 765–774, 2004.

[18] D. M. Gamm, S. Wang, B. Lu et al., "Protection of visual functions by human neural progenitors in a rat model of retinal disease," *PLoS ONE*, vol. 2, no. 3, article e338, 2007.

[19] S. Wang, S. Girman, B. Lu et al., "Long-term vision rescue by human neural progenitors in a rat model of photoreceptor degeneration," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 7, pp. 3201–3206, 2008.

[20] P. J. Francis, S. Wang, Y. Zhang et al., "Subretinal transplantation of forebrain progenitor cells in nonhuman primates: survival and intact retinal function," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 7, pp. 3425–3431, 2009.

[21] B. Blits, B. M. Kitay, A. Farahvar, C. V. Caperton, W. D. Dietrich, and M. B. Bunge, "Lentiviral vector-mediated transduction of neural progenitor cells before implantation into injured spinal cord and brain to detect their migration, deliver neurotrophic factors and repair tissue," *Restorative Neurology and Neuroscience*, vol. 23, no. 5-6, pp. 313–324, 2005.

[22] A. Bakshi, S. Shimizu, C. A. Keck et al., "Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following traumatic brain injury," *European Journal of Neuroscience*, vol. 23, no. 8, pp. 2119–2134, 2006.

[23] H. Klassen, P. H. Schwartz, B. Ziaeian et al., "Neural precursors isolated from the developing cat brain show retinal integration following transplantation to the retina of the dystrophic cat," *Veterinary Ophthalmology*, vol. 10, no. 4, pp. 245–253, 2007.

[24] M. Suzuki, J. McHugh, C. Tork et al., "GDNF secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS," *PLoS ONE*, vol. 2, no. 1, article e689, 2007.

[25] R. H. Steinberg, M. Reid, and P. L. Lacy, "The distribution of rods and cones in the retina of the cat (Felis domesticus)," *Journal of Comparative Neurology*, vol. 148, no. 2, pp. 229–248, 1973.

[26] W. E. Morgan III and F. J. Macri, "Vascular responses of the posterior segment of the cat eye," *Archives of Ophthalmology*, vol. 79, no. 6, pp. 779–784, 1968.

[27] T. Nikara, P. O. Bishop, and J. D. Pettigrew, "Analysis of retinal correspondence by studying receptive fields of binocular single units in cat striate cortex," *Experimental Brain Research*, vol. 6, no. 4, pp. 353–372, 1968.

[28] K. A. Linberg, G. P. Lewis, C. Shaaw, T. S. Rex, and S. K. Fisher, "Distribution of S- and M-cones in normal and experimentally detached cat retina," *Journal of Comparative Neurology*, vol. 430, no. 3, pp. 343–356, 2001.

[29] M. Menotti-Raymond, V. A. David, A. A. Schäffer et al., "Mutation in CEP290 discovered for cat model of human retinal degeneration," *Journal of Heredity*, vol. 98, no. 3, pp. 211–220, 2007.

[30] M. Menotti-Raymond, K. H. Deckman, V. David, J. Myrkalo, S. J. O'Brien, and K. Narfström, "Mutation discovered in a feline model of human congenital retinal blinding disease," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 6, pp. 2852–2859, 2010.

[31] J. Hori, T. F. Ng, M. Shatos, H. Klassen, J. W. Streilein, and M. J. Young, "Neural progenitor cells lack immunogenicity and resist destruction as allografts," *Stem Cells*, vol. 21, no. 4, pp. 405–416, 2003.

[32] J. Wang, J. Yang, and H. Klassen, "GDNF augments proliferation and reduces apoptosis of murine retinal progenitor cells," in *Proceedings of the Association for Research in Vision and Ophthalmology Annual Meeting*, 2010, abstract 2650.

Review Article

Leber's Hereditary Optic Neuropathy-Gene Therapy: From Benchtop to Bedside

Rajeshwari D. Koilkonda[1, 2] and John Guy[1, 2]

[1] *Bascom Palmer Eye Institute, Miller School of Medicine, University of Miami, Miami, FL 33136, USA*
[2] *McKnight Vision Research Center, Miller School of Medicine, University of Miami, Miami, FL 33136, USA*

Correspondence should be addressed to John Guy, jguy@med.miami.edu

Leber's hereditary optic neuropathy (LHON) is a maternally transmitted disorder caused by point mutations in mitochondrial DNA (mtDNA). Most cases are due to mutations in genes encoding subunits of the NADH-ubiquinone oxidoreductase that is Complex I of the electron transport chain (ETC). These mutations are located at nucleotide positions 3460, 11778, or 14484 in the mitochondrial genome. The disease is characterized by apoplectic, bilateral, and severe visual loss. While the mutated mtDNA impairs generation of ATP by all mitochondria, there is only a selective loss of retinal ganglion cells and degeneration of optic nerve axons. Thus, blindness is typically permanent. Half of the men and 10% of females who harbor the pathogenic mtDNA mutation actually develop the phenotype. This incomplete penetrance and gender bias is not fully understood. Additional mitochondrial and/or nuclear genetic factors may modulate the phenotypic expression of LHON. In a population-based study, the mtDNA background of haplogroup J was associated with an inverse relationship of low-ATP generation and increased production of reactive oxygen species (ROS). Effective therapy for LHON has been elusive. In this paper, we describe the findings of pertinent published studies and discuss the controversies of potential strategies to ameliorate the disease.

1. Introduction

Leber's hereditary optic neuropathy (LHON) refers to a rare, neurodegenerative maternally inherited, mitochondrial genetic disease. The clinical features of LHON were first described by the German ophthalmologist Theodor Leber, in 1871 [1]. LHON is characterized by sudden painless loss of central vision. In the acute stages, the optic nerve head is swollen but later becomes atrophic [2]. Generally, visual loss is sequential with involvement of the second eye occurring weeks to months after the first [3, 4]. The mode of inheritance of LHON was thought to be X-linked, until the first report by Erickson in 1972, that described a non-Mendelian pattern of inheritance involving mitochondria [5]. In 1988, Wallace and his group reported the first mitochondrial DNA point mutation associated with LHON. It was the G to A transition at nucleotide 11778 in the *ND4* gene (NADH-ubiquinone oxidoreductase, subunit 4) of Complex I of the ETC that resulted in a substitution of histidine for arginine at amino acid position 340.

The disease shows variable penetrance with a male preponderance of 86% [6], and LHON is transmitted solely through females. The expression of LHON is heterogeneous. In some pedigrees, cardiac and neurological abnormalities have been documented along with the characteristic optic atrophy [7–13], loss of the retinal nerve fiber layer, and ganglion cells [14]. Fibers of the papillomacular bundle are highly sensitive to the degenerative process [15].

2. Epidemiology

There are few epidemiological studies of LHON. One of the largest was a population-based study done in the North-East of England. It reported a minimum point prevalence of visual failure to be 1 in 31,000. The minimum point prevalence was 1 in 8500 carriers of mitochondrial DNA mutations to be at risk of visual failure [16]. A similar prevalence was reported by two recent studies, one in the Netherlands of 1 in 39,000 and the other in Finland of 1

in 50,000, respectively, [17]. Analysis of Australian pedigrees showed approximately 0.42–2% of LHON-induced visual loss with a variability of prevalence among men (range: 7–58%) and women (range: 0–15%) [18]. Another study on a Finnish population reported the incidence based on the type of mutation. The prevalence of families with the 11778 mutation was 39% among men and 14% in women. In families harboring the 3460 mutation, the disease expressed in 32% of men and 15% of women [17].

3. Clinical Manifestations of LHON

Patients with LHON typically present with acute or subacute, sudden, painless, central vision loss leading to central scotoma and dyschromatopsia [14]. Ophthalmoscopic examination reveals peripapillary telangiectasia, microangiopathy, swelling of the optic nerve head, and vascular tortuosity (Figure 1) [3]. This later progresses to optic atrophy. Visual dysfunction usually starts at 18 to 30 years of age. However, it may range from 3 to 80 years, with a mean age of onset being 25 to 26 years in men and 27 to 29 years in women [19]. In most cases, visual deterioration is rapid and extreme, with Snellen visual acuities of 20/200 or even worse in each eye. In a few cases, visual loss is slow and insidious over a period of 2 years, with a mean progression time of 3.7 months.

The probability of spontaneous recovery among LHON patients varies depending on the causative mutation. The highest recovery rate occurs in patients with the 14484/ND6 mutation (37% in a period of 16 months). Patients possessing the 11778/ND1 mutation have the poorest recovery rate (4%) [6, 20, 21].

LHON can also be associated with minor neurological abnormalities defined as Leber's "plus." A study by Nikoskelainen et al. reported that 59% of their LHON patients harboring any of the three primary mtDNA mutations had neurological abnormalities [10]. Clinical manifestations included postural tremor, motor disorder, Parkinsonism with dystonia, peripheral neuropathy, multiple sclerosis-like syndrome, cerebellar ataxia, anarthria, dystonia, spasticity, or mild encephalopathy [10, 22–25].

Previous reports on the influence of environmental factors such as cigarette smoking and alcohol consumption on LHON pathology had somewhat contrasting results [26, 27]. A recent large study by Kirkman and his group showed no statistically significant association of smoking to LHON. They reported that carriers with heavy smoking habits were greatly susceptible to developing the disease, but the effects of alcohol were not statistically significant [28]. A single case report demonstrated the influence of malnutrition along with tobacco abuse as risk factors for LHON [29].

4. Genetics

Three-point mutations in the mtDNA respiratory chain Complex I subunit genes: m.3460G>A/ND1 [30, 31], m.11778G>A/ND4 [6], and m.14484 T>C/ND6 [32] are associated with LHON worldwide. They constitute approximately 95% of the LHON pedigrees belonging to patients of

northern European descent [33]. These three mtDNA mutations are considered to be the primary pathogenic mutations, as they alter evolutionarily conserved amino acids, and they are absent in the control individuals [30–32, 34]. In a small number of LHON cases, secondary mtDNA mutations that do not change evolutionarily conserved amino acids may be causative. In addition, a synergistic mechanism has been proposed whereby the secondary mutations along with the primary mutations increase the severity of LHON [35]. Since secondary mutations are also detected in unaffected control individuals, they may simply represent polymorphisms of the mitochondrial genome. The human mitochondrial genome database (MITOMAP:www.mitomap.org) lists most of the variants of mtDNA. A comprehensive list of all the mtDNA mutations associated with LHON is shown in Table 1.

Since all individuals with the pathogenic mtDNA mutations do not develop visual loss, the incomplete penetrance of LHON may be due to other genetic (nuclear or mitochondrial) or epigenetic factors. The mtDNA haplogroup is another major genetic determinant for LHON [36]. The mtDNA haplogroups include the nonsynonymous variants in Complex I and III subunit genes. European haplogroup J is preferably associated with the 11778/ND4 (32%) and 14484/ND6 (70–75%) pathogenic mutations. This haplotype increases the risk of visual loss [37–39]. The haplogroup J is further classified based on the amino acid changes in the cytochrome b gene [40]. There is an association of haplogroup J1 with the 14484 mutation and J1c and J2b with the 11778 mutation, thereby indicating the influence of specific combinations of amino acid changes influencing the mitochondrial respiratory chain Complexes I and III [40]. In addition, the 14484 LHON mutation showed low penetrance when present in the haplogroup H mtDNA background [41]. However, the distribution of the 3460 mutation was random among the haplotypes [37–39]. *In vitro* studies on cybrids with mitochondria that carry the 11778 on the haplogroup J background put onto the neutral nuclear background of osteosarcoma cells had lower oxygen consumption and delayed mitosis as compared to a nonhaplo J genotype [42]. However, another study reported no detectable differences in respiratory function between cybrids belonging to European haplogroups X, H, T, or J [43]. Recently investigation of the effect of mtDNA haplogroups on the assembly of oxidative phosphorylation (OXPHOS) complexes showed a differentially delayed assembly rate of respiratory chain Complexes I, III, and IV amongst mutants belonging to different mtDNA haplogroups. This indicates that specific mtDNA polymorphisms may modify the pathogenic potential of LHON mutations by affecting the overall assembly kinetics of OXPHOS complexes [44]. The influence of exposure to n-hexane neurotoxic metabolite 2,5-hexanedione (2,5-HD) on cell viability and mitochondrial function of different cell models (cybrids and fibroblasts) carrying the LHON mutations on different mtDNA haplogroups was studied. Cell death induced by 2,5-HD was greatly increased in LHON cells carrying the 11778/ND4 or the 14484/ND6 mutation on the haplogroup J background. On the other hand, the 11778/ND4 mutation in association with haplogroups U and H significantly improved cell survival [45]. Hence,

(a)

(b)

(c)

(d)

(e)

(f)
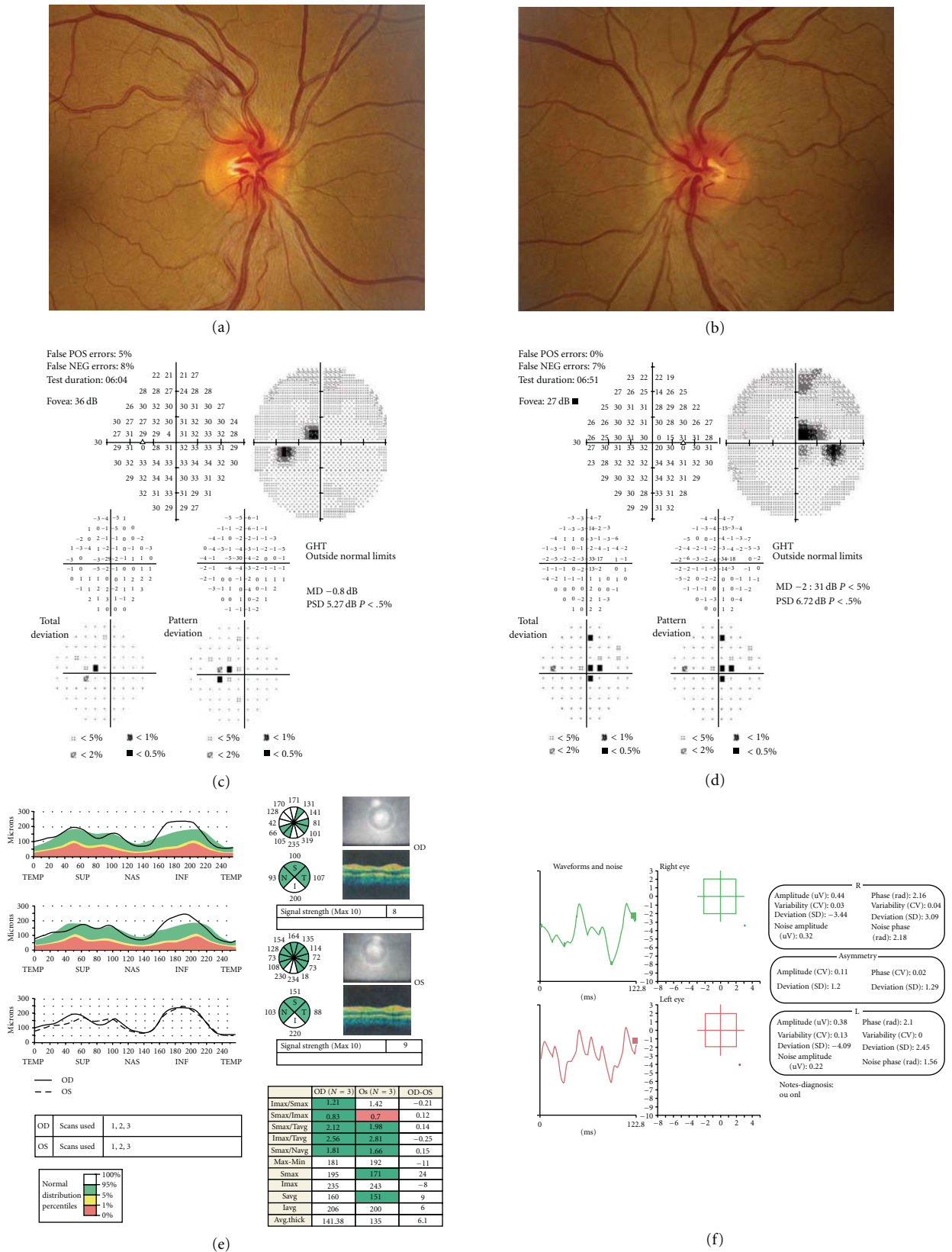
FIGURE 1: Fundus photographs of a patient with acute LHON revealed swelling of the right (a) and left (b) optic nerve heads. The arrow indicates the characteristic peripapillary telangiectasia. Automated visual fields showed central scotomas in the left (c) and right (d) eyes. OCT confirmed and quantitated the swelling of the retinal nerve fiber layer (e). Pattern electroretinograms illustrated a decline in ganglion cell function occurred during the acute stages of LHON and before structural evidence of RGC loss (f).

TABLE 1: Comprehensive list of genes/mutations involved in LHON.

Genes	Gen Bank ID	Nucleotide position	AA change	Phenotype	Hom/Het	Reference
MT-ND1	ACT53094.1	m.3316G>A	A4T	LHON/NIDDM	Hom	Matsumoto et al., 1999 [122]
		m.3376G>A	E24K	LHON/MELAS	Hom/Het	Blakely et al., 2005 [123]
		m.3394T>C	Y30H	LHON/NIDDM	Hom	Brown et al., 1992 [124]
		m.3460G>A	A52T	LHON	Hom/Het	Huoponen et al., 1991 [30]
		m.3496G>T	A64S	LHON	Hom	Matsumoto et al., 1999 [122]
		m.3497C>T	A64V	LHON	Hom	Matsumoto et al., 1999 [122]
		m.3635G>A	S110N	LHON	Hom	Brown et al., 2001 [125]
		m.3700G>A	A112T	LHON	Hom	Fauser et al., 2002 [126]
		m.3733G>A	E143K	LHON	Hom/Het	Valentino et al., 2004 [127]
		m.4025C>T	T240M	LHON	Hom	Huoponen et al., 1993 [128]
		m.4136A>G	Y277C	LHON	Hom	Howell et al., 1991 [129]
		m.4160T>C	L286P	LHON	Hom	Howell et al., 1991 [129]
		m.4171C>A	L289M	LHON	Hom/Het	Kim et al., 2002 [130]
		m.4216T>C	Y304H	LHON/Insulin resistance	Hom	Johns and Berman, 1991 [131]
MT-CO1	ACT53096.1	m.6261G>A	A120T	LHON/Prostrate Cancer	Hom	Abu-Amero and Bosley et al., 2006 [132]
		m.7444 G>A	Ter-K	LHON/SNH/DEAF	Hom	Brown et al., 1992 [133]
		m.7623 C>T	T13I	LHON	Hom	Abu-Amero and Bosley et al., 2006 [132]
MT-CO2	ACT53097.1	m.7868C>T	L95F	LHON	Hom	Yang et al., 2009 [134]
MT-ND2	ACT53095.1	m.4640C>A	I57M	LHON	Hom	Brown et al., 2001 [125]
		m.4917A>G	N150D	LHON/AMD/Insulin resistance/NRTI-PN	Hom	Johns and Berman, 1991 [131]
		m.5244G>A	G259S	LHON	Het	Brown et al., 1992 [135]
MT-ND3	ACT53101.1	m.10237T>C	I60T	LHON	Hom	Horvath et al., 2002 [136]
MT-ND4	ACT53103.1	m.11253T>C	I165T	LHON	Hom	Kjer 1959 [137]
		m.11696G>A	V312I	LHON + Spastic Dystonia	Het	De Vries et al., 1996 [138]
		m.11778G>A	R340H	LHON	Hom/Het	Wallace et al., 1988 [6]
		m.11874C>A	T372N	LHON	Hom	Abu-Amero and Bosley et al., 2006 [132]
MT-ND4L	ACT53102.1	m.10543A>G	H25R	LHON	Het	Abu-Amero and Bosley et al., 2006 [132]
		m.10591T>G	F41C	LHON	Het	Abu-Amero and Bosley et al., 2006 [132]
		m.10663T>C	V65A	LHON	Hom	Brown et al., 2002 [139]
		m.10680G>A	A71T	LHON	Hom	Yang et al., 2009 [134]
MT-ND5	ACT53104.1	m.12782T>G	I149S	LHON	Het	Abu-Amero and Bosley et al., 2006 [132]
		m.12811T>C	Y159H	LHON	Hom	Huoponen et al., 1993 [128]
		m.12848C>T	A171V	LHON	Het	Mayorov et al., 2005 [140]
		m.13045A>C	M237L	LHON/MELAS/LS	Het	Liolitsa et al., 2003 [141]
		m.13051G>A	G239S	LHON	Hom	Howell et al., 2003 [142]
		m.13379A>C	H348P	LHON	Hom	Abu-Amero and Bosley et al., 2006 [132]
		m.13528A>G	T398A	LHON-Like	Hom	Batandier et al., 2000 [143]
		m.13637A>G	Q434R	LHON	Hom	Huoponen et al., 1993 [128]
		m.13708G>A	A458T	LHON/MS risk	Hom	Johns and Berman, 1991 [131]
		m.13730G>A	G465E	LHON	Het	Howell et al., 1993 [144]

TABLE 1: Continued.

Genes	Gen Bank ID	Nucleotide position	AA change	Phenotype	Hom/Het	Reference
MT-ND6	ACT53105.1	m.14568C>T	G36S	LHON	Hom	Besch et al., 1999 [145]
		m.14279G>A	S132L	LHON	Hom	Zhadanov et al., 2005 [146]
		m.14459G>A	A72V	LHON + Spastic Dystonia	Hom/Het	Jun et al., 1994 [147]
		m.14482C>G	M64I	LHON	Hom/Het	Howell et al., 1998 [148]
		m.14484T>C	M64V	LHON	Hom/Het	Brown, et al., 1992 [124]
		m.14495A>G	L60S	LHON	Het	Chinnery et al., 2001 [149]
		m.14498C>T	Y59C	LHON	Hom/Het	Wissinger et al., 1997 [150]
		m.14596A>T	I26M	LHON	Hom	De Vries et al., 1996 [138]
		m.14325T>C	N117D	LHON	Hom	Howell et al., 2003 [142]
		m.14729G>A	S132L	LHON	Hom	Zhadanov et al., 2005 [146]
MT-CYB	ACT53106.1	m.14831G>A	A29T	LHON	Hom	Fauser et al., 2002 [126]
		m.14841A>G	N32S	LHON	Het	Yang et al., 2009 [134]
		m.15257G>A	D171N	LHON	Hom	Johns and Berman, 1991 [131]
		m.15674T>C	S310P	LHON	Hom	Abu-Amero Bosley et al., 2006 [132]
		m.15773G>A	V343M	LHON	Hom	La Morgia et al., 2008 [151]
		m.15812G>A	V356M	LHON	Hom	John et al., 1991 [152]
MT-CO3	ACT53100.1	m.9438G>A	G78S	LHON	Hom	Johns and Neufeld 1993 [153]
		m.9738G>T	A178S	LHON	Hom	Johns and Neufeld 1993 [153]
		m.9804G>A	A200T	LHON	Het	Johns and Neufeld 1993 [153]
MT-ATP6	ACT53099.1	m.8836A>G	M104V	LHON	Hom	Abu-Amero Bosley et al., 2006 [132]
		m.9016A>G	I164V	LHON	Het	Povalko et al., 2005 [154]
		m.9101 T>C	I192T	LHON	Hom	Puomila et al., 2007[17]
		m.9139G>A	A205T	LHON	Hom	La Morgia et al., 2008 [151]

the mtDNA haplotypes might act in association with the pathogenic mtDNA mutations to somehow modulate the phenotypic expression of LHON.

5. Heteroplasmy and Incomplete Penetrance

LHON exhibits incomplete penetrance with a male predominance. Approximately half of the men and 10% of females harboring one of the three pathogenic mtDNA mutations develop visual loss. This suggests that additional genetic factors and/or environmental factors modulate the phenotypic expression of LHON. The male preponderance in the disease manifestation could be also due to other anatomical, hormonal, or physiological factors [46].

Generally, cells contain 100–10,000 mitochondria, and each organelle harbors 2–10 mtDNA molecules. The copy number of mtDNA is therefore very high and shows heterogeneous distribution in different tissues based on the energy requirements [47]. Homoplasmy is defined as all the mitochondria of the cell possessing either wild-type or mutant mtDNA. When there is a mixture of wild-type and mutant mtDNA, it is called heteroplasmy. In the majority of LHON patients and family members, the pathogenic mtDNA mutation is homoplasmic. Still, 14% of the LHON

members have the mutation in heteroplasmic condition [48]. Clinically, there are no differences among the affected homoplasmic individuals from heteroplasmic patients [35].

Jacobi et al. reported variable prevalence of heteroplasmy based on the type of mutation possessed in 167 genealogically unrelated LHON families. Individuals with 11778/ND4, 3460/ND1, and 14484/ND6 mutations showed levels of heteroplasmy equal to 5.6%, 40% and 36.4%, respectively, [49]. However, a study analysing four large Thai LHON pedigrees showed a prevalence of 37% of heteroplasmic 11778/ND4 mtDNA [50, 51]. In heteroplasmic families, the level of heteroplasmy can vary extensively between generations and also between offspring in the same family due to a genetic bottle neck effect of mitochondrial distribution occurring in the early stages of oocyte formation [48, 52–55].

In addition, there have been controversial reports on the distribution of mutant mtDNA in different tissues. Yen et al. compared the mutant mtDNA from the leukocytes and hair follicles in an LHON proband carrying the 11778/ND4 mutation and observed mtDNA heteroplasmy in the hair follicle cells, but not in blood cells. This finding indicated the tissue variability in distribution of the wild-type to mutant mtDNA [56]. However, another report demonstrated comparable levels of mtDNA heteroplasmy in the blood, hair,

and urinary tract epithelia of LHON patients carrying the 11778/ND4 mutation [57].

In some heteroplasmic LHON families, an increase in the proportion of the mutant mtDNA in successive generations has been observed [48, 54]. This finding suggests a positive selection pressure. However, Puomila et al. quantified the level of heteroplasmy of the mtDNA mutations 11778/ND4 and 3460/ND1 in blood samples over a period of 4–12 years from nine members of four heteroplasmic LHON families. No major shift in heteroplasmy was demonstrated, thus no selection of either mtDNA genotypes. They proposed that the segregation of the wild-type mtDNAs and those carrying LHON mutations is a stochastic process governed by random genetic drift. In this respect, LHON mutations seem to behave like neutral polymorphisms [58]. These observations indicate that the role of selection is questionable.

The risk of visual failure in LHON increases as the threshold of heteroplasmy of primary pathogenic mtDNA mutations is increased to approximately 75%–80% [59]. The effect of heteroplasmy on phenotypic expression does not appear to be related to gender [60]. Howell et al. demonstrated in autopsied specimens of a woman with the 11778/ND4 mutation that the mutant mtDNA level was higher in the optic nerves (95%) and retina (100%) compared to circulating blood leukocytes (33%) [54]. This finding suggests that the susceptibility of certain tissues is due to their higher threshold of mutant to wild-type mtDNA. However, in a family of LHON, one of the two brothers with 98% mutant mtDNA lost vision, while his brother who had 100% mutant mtDNA was asymptomatic. Still, their ocular levels of mutated mtDNA were not evaluated.

The proposed risk for disease expression in homoplasmic families is only 30–50% in males and 5%–15% in females [35]. Chinnery et al. studied 17 independent LHON pedigrees to determine the risk of transmission of LHON in heteroplasmic families. He reported that mothers with 80% or less mutant mtDNA (measured in blood leuckocytes) were less likely to have clinically affected sons than mothers with 100% mutant mtDNA [61].

6. Mechanisms of Cell Death in LHON

Despite the presence of the mtDNA mutation in all retinal cells, it is predominantly RGCs of the papillomacular bundle region of the retina and their axons in the optic nerve that undergo degeneration in LHON. Why the disease spares other cell types such as the photoreceptors and the retinal pigment epithelium is unclear but may in part be due to the unique energy demands of RGCs, with their long axons and transition from unmyelinated to myelinated fibers in the retrobulbar nerve. Mitochondrial dysfunction from energy depletion has been proposed to disrupt axonal transport [61, 62]. Axonal transport is driven by the motor proteins kinesin and dyenin, both of which require large amounts of ATP for this function [61]. Therefore, proteins synthesized in the RGC cytoplasm as well as the mitochondria themselves that do not move down the axon towards the brain may contribute to the visual loss and degeneration of LHON.

As most LHON mutations involve the NADH-ubiquinone oxidoreductase, a decrease in Complex I activity resulting in apoptotic cell death is paramount [63]. LHON cybrids grown in galactose media, as the sole carbon source, force the cells to rely on oxidative phosphorylation rather than glycolysis to generate ATP. Under such restrictive conditions, LHON cells with mutated mitochondrial DNA undergo apoptotic cell death in a calcium [Ca(2+)-]dependent [64] and caspase-independent pathway [65, 66]. In addition, cytochrome c along with the apoptosis inducing factor (AIF) and endonuclease G (Endo G) are released from the mitochondria into the cytosol. Control cells with normal mitochondrial DNA remained unaffected by this restrictive media [65]. Cells harboring the 3460 and 14484 mtDNA mutations in the same nuclear background were comparitively more sensitive to apoptotic death than those harboring the 11778 mtDNA mutation. Battisti et al. treated the peripheral blood lymphocytes of LHON patients and controls with 2-deoxy-D-ribose and found a higher apoptotic rate in cells of LHON patients in comparison to controls, thus indicating mitochondrial involvement in this susceptibility [67].

In addition, mechanisms relating to increased oxidative stress have been proposed in LHON pathophysiology. Studies on the osteosarcoma-derived cybrids made from the mitochondria of LHON patients, carrying the 11778/ND4, 3460/ND1, or the 14484/ND6 mtDNA mutations, showed an excitotoxic mechanism of impaired glutamate transport. Defective activity of the excitatory amino acid transporter 1 (EAAT1) led to oxidative stress and increased mitochondrial ROS within RGCs. This in turn contributed to the apoptotic pathway of cell death of RGCs, loss of axons, and optic nerve atrophy [68].

In a study of oxidative stress of a cell line previously thought to be of RGC lineage (RGC-5), it was found that endogenous levels of superoxide anion were significantly lower than that found in neurons of the rat brain. Increases in ROS caused by mtDNA mutations that trigger the apoptotic cascade in ganglion cells of the retina may be better tolerated by neurons of the brain [69]. That mitochondrial DNA mutations result in Fas-induced apoptosis were demonstrated in osteosarcoma-derived cybrid cells carrying the 11778/ND4 or 3460/ND1 mutations. Control cells with the same mitochondrial halogroup J, but without the pathogenic G11778A mutation, were not sensitive compared to other controls. This finding indicates the pathogenicity of the LHON mutations [70]. Figure 2 shows potential pathways implicated in the optic nerve degeneration of LHON, as deduced from cellular and animal models.

7. Current Therapies

Management of LHON has been supportive, primarily by the use of low-vision aids. Current therapies are inadequate, but they deserve mention. The mainstay of treatment includes pharmaceutical compounds that are believed to restore electron flow or increase antioxidant defenses. One of these agents is idebenone, a short chain derivative of coenzyme
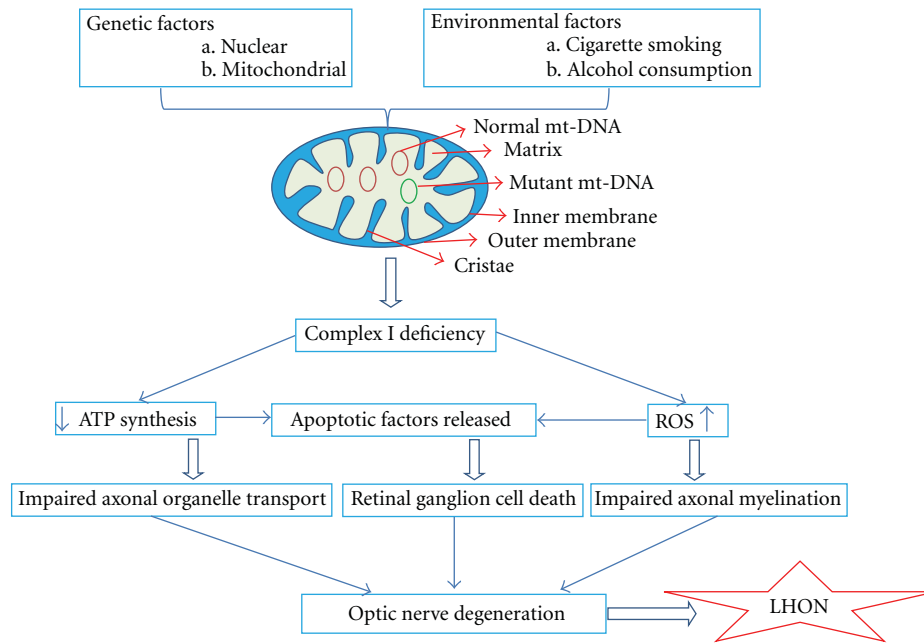
FIGURE 2: A schematic diagram illustrates the interaction of Complex I dysfunction, decreased ATP production, increased ROS, and apoptosis that culminate in the optic nerve degeneration of LHON and LHON cellular and animal models. ATP—adenosine triphosphate; ROS—reactive oxygen species.

Q_{10} (CoQ$_{10}$) [71–73]. Mashima et al. used idebenone combined with vitamin B2 and vitamin C, to "stimulate ATP formation" in LHON patients. Treatment for at least one year hastened the recovery process. Visual improvement that was defined as being greater or equal to 0.3 logMAR occurred within 17.6 months of treatment relative to 34.4 months without it [73]. In another report of idebenone and vitamin B12 therapy, a North African LHON patient harboring a homoplasmic 14484/ND4 mtDNA mutation recovered vision. Serum lactate levels normalized over a period of 3.5 months [72]. In contrast, two other patients who were treated with idebenone and multivitamins failed to improve [74]. The effectiveness of idebenone therapy for LHON is currently the subject of a controlled double-masked randomized study in Europe and Canada. While the results of a recent press release were favorable, details have not yet been published.

The clinical phase of the patient at which time therapy is initiated might determine treatment outcome. As a prophylactic measure to prevent vision loss, a topically applied agent, brimonidine purite 0.15% (Alphagan), with potentially antiapoptotic properties was administered to the as yet unaffected eyes of LHON patients. Unfortunately, this therapy proved unsuccessful in preventing them from undergoing visual loss. Thus, the study was terminated after enrollment of only 8 patients [75]. The search for an effective treatment continues.

8. Animal Models

The genetics of LHON have steadily accumulated for more than two decades. However, the pathogenic mechanisms leading to the apoplectic visual failure with subsequent retinal ganglion cell and optic nerve degeneration that could lead to the development of an effective treatment strategy are poorly understood. This is in large part due to the lack of bona fide animal models for LHON. The deficiency of animal models is also a general problem for most mitochondrial diseases, where the complete deletion of any subunit of the respiratory chain often results in a lethal phenotype [76]. Still, using different approaches, a few animal models resembling LHON have been generated in recent years. The first animal model for LHON was made by Zhang and his group by administering rotenone, an irreversible Complex I inhibitor, to mice. Histologic analysis showed thinning of the RGC layer, by 43%, one day after the rotenone injections [77].

Next, Qi et al. used a genetic approach to knockdown Complex I activity. They designed ribozymes to degrade the mRNA encoding a critical nuclear-encoded subunit gene of Complex I (NDUFA1). It markedly reduced Complex I activity in murine cells. Using the AAV vector as a vehicle to deliver the ribozymes into the mouse vitreous cavity, the authors found loss of RGCs and axons that resembled the histopathology of LHON [78]. This model system also implicated oxidative stress in the pathogenesis of the degenerative process.

As further evidence for involvement of ROS in optic nerve degeneration, intraocular injections of AAV-expressing hammerhead ribozymes designed to degrade mitochondrial superoxide dismutase (*SOD2*) mRNA induced further loss of axons and myelin in the optic nerve and ganglion cells of the retina, the very hallmarks of LHON histopathology [79]. RGC and axonal loss were ameliorated by intraocular

injections of an AAV overexpressing *SOD2* into eyes that also had received the NDUFA1 ribozymes [80]. Later, Qi and associates proposed augmenting mitochondrial antioxidative defensive mechanisms to rescue cybrid cells with the G11778A mutation in mtDNA from galactose-induced apoptotic cell death by infecting them with AAV-*SOD2*. The control cells were treated with AAV-GFP (green fluorescent protein). Within 2 and 3 days of growth in galactose media, LHON cell survival increased by 25% and 89%, respectively, [81]. The ROS superoxide anion has recently been shown to mediate apoptosis in RGCs [82]. Dismutation of the superoxide anion by SOD suppressed RGC apoptosis. Taken together, these findings suggest that antioxidant genes may offer a therapeutic strategy directed at the pathophysiologic mechanisms of LHON.

Still, it was unclear whether such findings in those mouse models are truly representative of events in LHON patients. The ribozyme and rotenone animal models illustrate the pathogenic effects of severe loss of Complex I activity in the vertebrae visual system. However, the activity of the NADH-ubiquinone oxidoreductase is reduced only slightly in cells with the G11778A mtDNA. To generate a more representative animal model of LHON, Qi and associates constructed a mutant ND4 subunit gene that was designed to express the arginine-to-histidine substitution at amino acid 340 characteristic of the mutant human LHON ND4 protein [83]. Delivery of this construct with the AAV vector injected into the mouse vitreous cavity resulted in optic nerve head swelling. Several months later, the optic nerve became atrophic and ganglion cells of the retina were lost. Both optic nerve head swelling and visual loss are characteristics of acute LHON. They are followed by optic atrophy. Thus, the phenotype of the murine model and the human disease appear comparable.

Ultrastructural analysis of mutant ND4-injected eyes revealed disruption of mitochondrial cytoarchitecture, elevated reactive oxygen species that culminated in apoptosis of RGCs [83]. Mouse eyes injected with AAV containing the normal human ND4 showed no evidence of pathology whatsoever. Since the mutant and human ND4 constructs differed only in the arginine to histidine transition at amino acid 340 (mutant ND4), these studies affirm that the pathogenicity of this mutation is the cause of LHON. Ellouze et al. who later introduced the mutant human ND4 gene into rat eyes found that it caused RGC degeneration and a decline in visual performance [84]. An important difference between human LHON and rodent models is worth mentioning. Disease in rodents occurred even in the presence of endogenous mouse (or rat) ND4. In human LHON, wild-type ND4 is typically absent. That being the case, rescue of the LHON rodent model by the addition of more wild-type ND4 may not be possible.

9. Genetic Therapy and Future Directions

Curative treatments for mitochondrial disorders are currently lacking. However, extensive exciting research advances are being made. One of the most promising emerging technologies is "allotopic expression," wherein a nuclear version of the mitochondrial gene is constructed by partially recoding the mtDNA gene in the nuclear genetic code. It was through allotopic expression of the mutant human ND4 subunit gene that an LHON-like phenotype was induced in rodent models as discussed in the previous section. Changing the ATA codon to ATG is necessary to achieve allotopic expression, since the ATA encodes for methionine in mitochondria, but isoleucine in the nucleus. In addition, the TGA codon that specifies tryptophan in mitochondria is a stop codon in the nucleus. Therefore, this codon must also be corrected for the full-length ND4 protein to be translated on cytoplasmic ribosomes. Protein import into mitochondria is then directed by the addition of a mitochondrial targeting sequence (MTS) to the amino terminus [85, 86]. Protein expression can then be monitored with an epitope tag appended to the carboxy terminus. Guy et al. were the first to use this approach with a human ND4 gene to rescue the defects of oxidative phosphorylation in G11778A LHON cells [86]. They constructed a synthetic ND4 subunit from overlapping 80 mer oligonucleotides. After packaging in an adenoassociated viral vector, it was used to transduce cells harboring 100% G11778A-mutated mtDNA. One of their constructs successfully increased ATP synthesis by threefold in LHON cell lines relative to controls treated with GFP or transduced with the same ND4 gene that had a different MTS and epitope tag that was not imported into the mitochondria [86].

Last year, our group demonstrated that allotopic delivery of the normal human ND4 subunit gene into the vitreous cavity of the murine eye is safe. There was no difference in total RGC counts, measured as Thy1.2 positive cells, between these experimental eyes and controls injected with AAV-GFP. Moreover, the pattern and flash electroretinogram amplitudes after the injections remained unchanged from their baseline values before the injections. This important finding indicates that injection of the allotopic human ND4 did not compromise murine RGC function [87]. Using immunoprecipitation of the 45 subunit Complex I, we demonstrated that the FLAG-tagged human ND4 incorporated into the holoenzyme of infected murine retinal and optic nerve tissues. To validate the technique, we submitted nine bands pulled down by Complex I immunoprecipitation of murine mitochondria isolated from the optic nerve, brain, spinal cord, or retina for identification by mass spectroscopy. They were positively identified as subunits of the NADH-ubiquinone oxidoreductase (Figure 3). No other respiratory Complexes (II–V) were detected. Therefore, the FLAG-tagged human ND4 detected by this assay proves that it effectively integrated into the murine Complex I.

Prior evidence of cross complementation in dissimilar mammalian species was previously shown by Tsukihara and coworkers [88], where a bovine allotopic COX1 had integrated into the human cytochrome oxidase enzyme. They used blue-native electrophoresis to pull down the assembled COX holoenzyme. Using this technique, Figueroa-Martínez and coworkers [89] were unable to find integration of their construct that used a short COX6 MTS to direct import of ND6 tagged with hemagglutinin (HA), the only construct

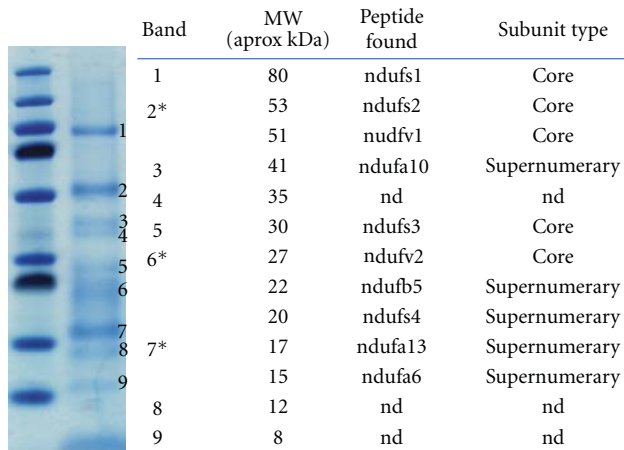| Band	MW (aprox kDa)	Peptide found	Subunit type
1	80	ndufs1	Core
2*	53	ndufs2	Core
	51	nudfv1	Core
3	41	ndufa10	Supernumerary
4	35	nd	nd
5	30	ndufs3	Core
6*	27	ndufv2	Core
	22	ndufb5	Supernumerary
	20	ndufs4	Supernumerary
7*	17	ndufa13	Supernumerary
	15	ndufa6	Supernumerary
8	12	nd	nd
9	8	nd	nd

FIGURE 3: Electrophoresis of mitochondrial proteins isolated from the mouse brain following Complex I immunoprecipitation and counterstained by Coomassie blue are shown in the second lane. The first lane has the molecular weight standards. The table indicates the identity of bands submitted for mass spectroscopy to be subunits of Complex I. (*)—subunits identified within the same excised band. (nd)—not determined.

tested. Brookes and coworkers [90] used mass spectroscopy to identify more than 30 bands that were separated by 2D blue-native PAGE. Of these bands, a single 33 kilodalton (kDA) subunit of Complex I was identified [90]. In addition, the authors pulled down many proteins that were not respiratory complexes. The authors further go on to show that inhibition of mitochondrial protein synthesis of hydrophobic mtDNA-encoded proteins with chloramphenicol did not alter the assembly of respiratory complexes, as judged by blue-native electrophoresis. Therefore, unambiguous data showing that hydrophobic Complex I subunits are isolated by blue-native electrophoresis has yet to be demonstrated.

Using electron microscopy as further evidence of allotopic import, ND4 labeled by immunogold decorated the interior of the organelle and it colocalized with MnSOD [83, 87]. The latter is a nuclear-encoded mitochondrial protein that too is imported into the organelle along with 85% of all mitochondrial proteins that are encoded by the nucleus. Had the ND4FLAG immunogold been stuck in the membrane pores as suggested by Oca-Cossio [91] it would have decorated the exterior of the organelle as shown by Gilkerson and colleagues [92] for the membrane protein porin. Thus, the allotopic ND4 protein did not get stuck in the mitochondrial import channels or induce cell death as suggested by Oca-Cossio and coworkers [87, 91]. Injections of wild-type human ND4 also had a small, but quantifiable, biological effect that was manifested by shortening of the pattern electroretinogram (PERG) latency. The implications of this finding are unclear. With the mouse and human ND4 being approximately 80% homologous and amino acid 340 being highly conserved, along with the fact that the latency of the mouse PERG is almost double that of humans, it is tempting to speculate that human ND4 integration into the mouse Complex I made the mouse RGC responses faster

than that observed in control eyes. This may reflect an evolutionary adaptation of the human respiratory chain for the relatively fast RGC responses required in man.

Controversies in allotopic expression based entirely on studies of cultured cells continue to be debated in the recent literature. However, they have shifted somewhat from the views of Oca-Cossio [91] that showed no colocalization of allotopic proteins (except ATP8) with a bona fide mitochondrial marker to those now showing colocalization suggestive of import, but lacking integration into functional respiratory complexes [89]. In 2002, Guy et al. showed that LHON cells expressing the same allotopic human ND4, but fused to a different mitochondrial targeting sequence (aldehyde dehydrogenase) or epitope tag (GFP) did import into the mitochondria [86]. Consistent with this finding, the latter construct did not rescue LHON cells from glucose-free galactose media-induced cell death or improve their ATP synthesis.

In support of a paramount role for the MTS in directing mitochondrial trafficking, Superkova and coworkers [93] showed that the allotopic import of a mutant COX2 was dependent on the mitochondrial targeting sequence, but not the mitochondrial targeting 3′UTR. Still, the studies of Bonnet et al. [94] clearly demonstrated the benefits of the COX10 3′UTR when used in conjunction with the CIS acting elements of the COX10 MTS. Relative to controls, the COX10-ND4 or COX10-ND4 3′UTR constructs each increased G11778A LHON cell survival in galactose media and improved their ATP synthesis. Their findings support the earlier studies of Guy and coworkers' successful allotopic ND4 import into mitochondria. Clearly, the testing of constructs for allotopic expression that include the mitochondrial targeting sequence, protein, epitope tag, or 3′UTR is largely a trial-and-error endeavor [95]. Since cell culture studies can sometimes be misleading [96], confirmation in appropriate animal models is vital to demonstrating the safety and effectiveness of allotopic ND4 expression before it can be applied to LHON patients.

Using their MTS and 3′UTR model system, Ellouze and colleagues introduced the mutant human ND4 subunit gene harboring the G11778A mutation into rat eyes, by in vivo electroporation. This led to loss of vision and degeneration of almost half the RGCs, as previously described by Qi and coworkers [83]. By introducing a normal copy of the human ND4 gene, visual and RGC loss were averted [84]. Thus, the data accumulated to date provide overwhelming evidence that allotopic expression of a mutant ND4 causes RGC degeneration and a wild-type version does not. More importantly, they show that the wild-type ND4 can rescue an LHON animal model. Clearly, allotopic delivery of a normal ND4 is a promising approach in the quest for an effective remedy for LHON caused by mutated G11778A mtDNA.

For this to occur, an effective and safe delivery system is necessary for ND4 gene therapy. The single-stranded (ss) AAV2 used in allotopic mouse experiments has been proven safe in several phase I human ocular gene therapy trials [97]. Thus, the AAV vector has a proven track record in human clinical trials [98–101]. There has been extensive research on these viral vectors with much advancement, particularly in

the areas of transduction efficiency, stability, tropism, and most importantly safety. The retinal layer exclusively affected in LHON can be targeted by optimizing the vector serotype (AAV2), and by choosing the route of vector administration (intravitreal injection).

Newer generation vectors include the self-complementary (sc) AAV that contains both positive and negative complementary strands. Since second strand synthesis is believed to be the rate-limiting step for expression of single-stranded vectors, it is not surprising that scAAV vectors increase the speed and efficiency of transgene expression [102–104]. Other AAVs with mutations in the capsid proteins also increase the efficiency of transgene expression [105, 106]. They were designed to reduce cellular degradation of AAV, thus increasing cellular levels of AAV virions. By taking advantage of scAAV to deliver the allotopic ND4 into the mouse eye, our group doubled RGC expression relative to the single-stranded AAV that is the current standard vehicle for gene delivery. With scAAV, FLAG-tagged ND4 was seen in almost all murine RGCs (90%) [107]. Such newer generation vectors may be highly advantageous for LHON gene therapy. They can be used at lower doses, thus minimizing immunologic responses against the viral capsid that could prevent expression of ND4 and also with maximal efficiency [108]. This could have important implications for treatment of LHON patients, where prior injection into the first eye, should it generate an immune response, may limit expression with later injections of AAV-ND4 into the second eye. LHON is a bilateral disease, thus both eyes need treatment.

Great care must be taken in extrapolating the results achieved in rodents to the human disorder, particularly under pathological conditions. As an example, immuno-precipitation of Complex I following intravitreal injections of the normal allotopic human ND4 revealed a greater distribution of the FLAG-tagged ND4 in the murine optic nerve than that observed in the retina [87]. In contrast, the mutant ND4 had greater incorporation into the murine retina than in the optic nerve [83]. These findings suggest that cellular events associated with the optic disc edema may impede movement of the ND4 integrated into the holoenzyme from the retina to the nerve. The studies of Oca-Cossio suggest that if ND4 is not correctly processed into mitochondria, it may be harmful [91]. In addition, if LHON is primarily an axonopathy, then the allotopic ND4 may not get to the target tissue (axonal mitochondria in the optic nerve) for rescue in acute LHON patients who typically have optic nerve head swelling. On the other hand, if LHON is primarily a disorder of RGCs, then it will rescue. Further studies in lower vertebrates are needed to delineate the best window for intervention.

Many other experimental techniques have been proposed to address disorders caused by mutated mtDNA. They include mitochondrial gene replacement in embryonic stem cells [109], protoFection [110], importing genes from other species, changing the ratio of heteroplasmy with specific restriction endonucleases [111], or selecting for respiratory function or regeneration (in muscle) [112, 113]. None of these techniques are directly applicable to the treatment of LHON that is caused predominantly by 100% mutated mtDNA. An approach worth mentioning here is that of transkingdom allotopic expression coined "xenotopic expression." This technique was pioneered by Ojaimi and coworkers, who experimentally restored defects in Complex V of the electron transport chain [114]. Using a similar approach, Seo et al. used the NDI1 gene of Saccharomyces cerevisiae to rescue the respiratory deficiency of Complex I deficient Chinese hamster CCL16-B2 cell lines [115]. The NDI1 gene is a single subunit NADH-ubiquinone oxidoreductase that appears to perform the function of the 45 subunit mammalian Complex I. NDI1 is encoded in the nuclear genome, expressed on cytoplasmic ribosomes, and successfully transported into the mitochondrial inner membrane with an N-terminus mitochondrial targeting sequence.

The Yagi laboratory has applied their NDI1 technology to alleviating the consequences of a human cell line carrying a homoplasmic frame shift mutation in the ND4 gene [116]. Recently, they utilized an AAV expressing NDI1 to rescue the Complex I deficiency induced by rotenone in the mouse visual system [117]. Xenotopic technology has the advantage whereby a single construct, NDI1, can treat all LHON cases caused by mutated ND4, ND1, or ND6 Complex I subunits. Allotopic expression of human Complex I subunits requires three separate constructs, one for each of the three mutated subunit genes. Still, it is unclear whether introduction of a gene from an entirely different species is acceptable for human therapy. Moreover, it has not been demonstrated how NDI1 interacts with Complexes II–V of the human respiratory chain. A recent publication showing the extension of the fly lifespan with the NDI1 gene suggests that the mechanism of benefit was not achieved by improving oxidative phosphorylation, but rather by decreased production of ROS [118]. Still, who is an appropriate candidate for allotopic or xenotopic gene therapy?

10. Candidates for Genetic Therapies

The stage of disease may dictate the outcome of gene therapy. Lam and coworkers found that optical coherence tomography (OCT) measurements of the retinal nerve fiber layer (RNFL) averaged $72\,\mu$m for as long as 3 years after visual loss [119]. After this time, RNFL thickness dropped to $42\,\mu$m. With loss of more than half of their RGCs, these late-stage patients may not have a sufficient population of remaining cells for meaningful rescue of vision. Still, the scAAV that expressed in almost all RGCs of the mouse has the potential to restore function even in those remaining axons described in autopsied LHON eyes as exhibiting accumulation of mitochondria and dissolution of cristae [15]. If so, visual function may improve even with long-standing optic atrophy. That this may be possible is suggested by the Leber's congenital amaurosis (LCA) clinical trials where partial return of visual function occurred even in eyes with severe and long-standing photoreceptor loss [97, 120, 121].

The rapidity of gene expression is clinically relevant in treating LHON patients who present with bilateral simultaneous onset of acute visual loss. Still, oxidative injury and apoptosis may already be irreversible at this time. Considering the window period of 2–3 months between the involvement of the first and the second eye, treatment may even be employed before loss of vision. Thus, rescue prior to visual loss in the second eye may be possible during this window period, particularly if introduction of the normal ND4 subunit gene in those eyes with acute optic disc edema after visual loss proves ineffective.

The studies of Elouze et al. suggest that gene therapy prevents visual loss in lower vertebrates [84]. Whether successful rescue in symptomatic patients will support intervention in asymptomatic carriers may be dependent on tests capable of predicting conversion to the phenotype. Using the PERG as a sensitive measure of ganglion cell function, Lam et al. found that the PERG amplitude was substantially reduced in some asymptomatic G11778A carriers [119]. They plan to follow those patients for several years to see if they develop LHON.

In summary, due to the research of many groups all over the world who are working in the field, the molecular and biochemical basis of this disease has been unraveling. Efficient gene delivery techniques tested in vertebrae animal models that mimic the optic nerve degeneration of LHON provide a renewed hope for an effective and long-lasting remedy for this disorder in the coming years.

Acknowledgments

References

[1] T. Leber, "Ueber hereditaere und congenital angelegte sehnervenleiden," *Graefes Arch Ophthalmol*, vol. 17, pp. 249–291, 1871.

[2] N. J. Newman, "Hereditary optic neuropathies: from the mitochondria to the optic nerve," *American Journal of Ophthalmology*, vol. 140, no. 3, pp. 517.e1–517.e9, 2005.

[3] E. Nikoskelainen, W. F. Hoyt, and K. Nummelin, "Ophthalmoscopic findings in Leber's hereditary optic neuropathy. II. The fundus findings in the affected family members," *Archives of Ophthalmology*, vol. 101, no. 7, pp. 1059–1068, 1983.

[4] D. C. Wallace, "A new manifestation of Leber's disease and a new explanation for the agency responsible for its unusual pattern of inheritance," *Brain*, vol. 93, no. 1, pp. 121–132, 1970.

[5] R. P. Erickson, "Leber's optic atrophy, a possible example of maternal inheritance," *American Journal of Human Genetics*, vol. 24, no. 3, pp. 348–349, 1972.

[6] D. C. Wallace, G. Singh, M. T. Lott et al., "Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy," *Science*, vol. 242, no. 4884, pp. 1427–1430, 1988.

[7] P. Riordan-Eva, M. D. Sanders, G. G. Govan, M. G. Sweeney, J. Da Costa, and A. E. Harding, "The clinical features of Leber's hereditary optic neuropathy defined by the presence of a pathogenic mitochondrial DNA mutation," *Brain*, vol. 118, no. 2, pp. 319–337, 1995.

[8] R. M. Chalmers and A. E. Harding, "A case-control study of Leber's hereditary optic neuropathy," *Brain*, vol. 119, no. 5, pp. 1481–1486, 1996.

[9] F. M. Meire, R. Van Coster, P. Cochaux, B. Obermaier-Kusser, C. Candaele, and J. J. Martin, "Neurological disorders in members of families with Leber's hereditary optic neuropathy (LHON) caused by different mitochondrial mutations," *Ophthalmic Genetics*, vol. 16, no. 3, pp. 119–126, 1995.

[10] E. K. Nikoskelainen, R. J. Marttila, K. Huoponen et al., "Leber's 'plus': neurological abnormalities in patients with Leber's hereditary optic neuropathy," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 59, no. 2, pp. 160–164, 1995.

[11] S. P. C. Bower, I. Hawley, and D. A. Mackey, "Cardiac arrhythmia and Leber's hereditory optic neuropathy," *Lancet*, vol. 339, no. 8806, pp. 1427–1428, 1992.

[12] Y. Mashima, K. Kigasawa, H. Hasegawa, M. Tani, and Y. Oguchi, "High incidence of pre-excitation syndrome in Japanese families with Leber's hereditary optic neuropathy," *Clinical Genetics*, vol. 50, no. 6, pp. 535–537, 1996.

[13] E. K. Nikoskelainen, M. L. Savontaus, K. Huoponen, K. Antila, and J. Hartiala, "Pre-excitation syndrome in Leber's hereditary optic neuropathy," *Lancet*, vol. 344, no. 8926, pp. 857–858, 1994.

[14] V. Carelli, F. N. Ross-Cisneros, and A. A. Sadun, "Mitochondrial dysfunction as a cause of optic neuropathies," *Progress in Retinal and Eye Research*, vol. 23, no. 1, pp. 53–89, 2004.

[15] A. A. Sadun, P. H. Win, F. N. Ross-Cisneros et al., "Leber's hereditary optic neuropathy differentially affects smaller axons in the optic nerve," *Transactions of the American Ophthalmological Society*, vol. 98, pp. 223–235, 2000.

[16] P. Y. W. Man, P. G. Griffiths, D. T. Brown, N. Howell, D. M. Turnbull, and P. F. Chinnery, "The epidemiology of Leber hereditary optic neuropathy in the North East of England," *American Journal of Human Genetics*, vol. 72, no. 2, pp. 333–339, 2003.

[17] A. Puomila, P. Hämäläinen, S. Kivioja et al., "Epidemiology and penetrance of Leber hereditary optic neuropathy in Finland," *European Journal of Human Genetics*, vol. 15, no. 10, pp. 1079–1089, 2007.

[18] D. A. Mackey and R. G. Buttery, "Leber hereditary optic neuropathy in Australia," *Australian and New Zealand Journal of Ophthalmology*, vol. 20, no. 3, pp. 177–184, 1992.

[19] W. M. Carroll and F. L. Mastaglia, "Leber's optic neuropathy: a clinical and visual evoked potential study of affected and asymptomatic members of a six generation family," *Brain*, vol. 102, no. 3, pp. 559–580, 1979.

[20] N. J. Newman, "Leber's hereditary optic neuropathy: new genetic considerations," *Archives of Neurology*, vol. 50, no. 5, pp. 540–548, 1993.

[21] D. R. Johns, K. L. Heber, N. R. Miller, and K. H. Smith, "Leber's hereditary optic neuropathy: clinical manifestations of the 14484 mutation," *Archives of Ophthalmology*, vol. 111, no. 4, pp. 495–498, 1993.

[22] J. Palace, "Multiple sclerosis associated with Leber's Hereditary Optic Neuropathy," *Journal of the Neurological Sciences*, vol. 286, no. 1-2, pp. 24–27, 2009.

[23] T. Murakami, S. Mita, M. Tokunaga et al., "Hereditary cerebellar ataxia with Leber's hereditary optic neuropathy mitochondrial DNA 11778 mutation," *Journal of the Neurological Sciences*, vol. 142, no. 1-2, pp. 111–113, 1996.

[24] A. Gropman, T. J. Chen, C. L. Perng et al., "Variable clinical manifestation of homoplasmic G14459A mitochondrial DNA mutation," *American Journal of Medical Genetics*, vol. 124, no. 4, pp. 377–382, 2004.

[25] K. K. Abu-Amero, T. M. Bosley, S. Bohlega, and D. McLean, "Complex I respiratory defect in LHON plus dystonia with no mitochondrial DNA mutation," *British Journal of Ophthalmology*, vol. 89, no. 10, pp. 1380–1381, 2005.

[26] J. B. Kerrison, N. R. Miller, F. C. Hsu et al., "A case-control study of tobacco and alcohol consumption in leber hereditary optic neuropathy," *American Journal of Ophthalmology*, vol. 130, no. 6, pp. 803–812, 2000.

[27] N. J. Newman, "Leber hereditary optic neuropathy: bad habits, bad vision," *Brain*, vol. 132, no. 9, pp. 2306–2308, 2009.

[28] M. A. Kirkman, P. Yu-Wai-Man, A. Korsten et al., "Gene-environment interactions in Leber hereditary optic neuropathy," *Brain*, vol. 132, no. 9, pp. 2317–2326, 2009.

[29] U. Lachmund and D. S. Mojon, "Leber's hereditary optic neuropathy in malnutrition: a case report," *Klinische Monatsblatter fur Augenheilkunde*, vol. 223, no. 5, pp. 393–396, 2006.

[30] K. Huoponen, J. Vilkki, P. Aula, E. K. Nikoskelainen, and M. L. Savontaus, "A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy," *American Journal of Human Genetics*, vol. 48, no. 6, pp. 1147–1153, 1991.

[31] N. Howell, L. A. Bindoff, D. A. McCullough et al., "Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees," *American Journal of Human Genetics*, vol. 49, no. 5, pp. 939–950, 1991.

[32] D. R. Johns, M. J. Neufeld, and R. D. Park, "An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy," *Biochemical and Biophysical Research Communications*, vol. 187, no. 3, pp. 1551–1557, 1992.

[33] D. A. Mackey, R. J. Oostra, T. Rosenberg et al., "Primary pathogenic mtDNA mutations in multigeneration pedigrees with Leber hereditary optic neuropathy," *American Journal of Human Genetics*, vol. 59, no. 2, pp. 481–485, 1996.

[34] D. Mackey and N. Howell, "A variant of Leber hereditary optic neuropathy characterized by recovery of vision and by an unusual mitochondrial genetic etiology," *American Journal of Human Genetics*, vol. 51, no. 6, pp. 1218–1228, 1992.

[35] K. Huoponen, "Leber hereditary optic neuropathy: clinical and molecular genetic findings," *Neurogenetics*, vol. 3, no. 3, pp. 119–125, 2001.

[36] X. Bu and J. I. Rotter, "X chromosome-linked and mitochondrial gene control of Leber hereditary optic neuropathy: evidence from segregation analysis for dependence on X chromosome inactivation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 18, pp. 8198–8202, 1991.

[37] M. D. Brown, F. Sun, and D. C. Wallace, "Clustering of Caucasian Leber hereditary optic neuropathy patients containing the 11778 or 14484 mutations on an mtDNA lineage," *American Journal of Human Genetics*, vol. 60, no. 2, pp. 381–387, 1997.

[38] A. Torroni, M. Petrozzi, L. D'Urbano et al., "Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484," *American Journal of Human Genetics*, vol. 60, no. 5, pp. 1107–1121, 1997.

[39] P. Y. Man, N. Howell, D. A. Mackey et al., "Mitochondrial DNA haplogroup distribution within Leber hereditary optic neuropathy pedigrees," *Journal of Medical Genetics*, vol. 41, no. 4, article e41, 2004.

[40] V. Carelli, A. Achilli, M. L. Valentino et al., "Haplogroup effects and recombination of mitochondrial DNA: novel clues from the analysis of leber hereditary optic neuropathy pedigrees," *American Journal of Human Genetics*, vol. 78, no. 4, pp. 564–574, 2006.

[41] N. Howell, C. Herrnstadt, C. Shults, and D. A. Mackey, "Low penetrance of the 14484 LHON mutation when it arises in a non-haplogroup J mtDNA background," *American Journal of Medical Genetics*, vol. 119, no. 2, pp. 147–151, 2003.

[42] L. Vergani, A. Martinuzzi, V. Carelli et al., "MtDNA mutations associated with Leber's hereditary optic neuropathy: studies on cytoplasmic hybrid (cybrid) cells," *Biochemical and Biophysical Research Communications*, vol. 210, no. 3, pp. 880–888, 1995.

[43] V. Carelli, L. Vergani, B. Bernazzi et al., "Respiratory function in cybrid cell lines carrying European mtDNA haplogroups: implications for Leber's hereditary optic neuropathy," *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1588, no. 1, pp. 7–14, 2002.

[44] R. Pello, M. A. Martín, V. Carelli et al., "Mitochondrial DNA background modulates the assembly kinetics of OXPHOS complexes in a cellular model of mitochondrial disease," *Human Molecular Genetics*, vol. 17, no. 24, pp. 4001–4011, 2008.

[45] A. M. Ghelli, A. M. Porcelli, C. Zanna et al., "The background of mitochondrial DNA haplogroup J increases the sensitivity of Leber's hereditary optic neuropathy cells to 2,5-hexanedione toxicity," *PLoS One*, vol. 4, no. 11, Article ID e7922, 2009.

[46] P. Yu-Wai-Man, P. G. Griffiths, G. Hudson, and P. F. Chinnery, "Inherited mitochondrial optic neuropathies," *Journal of Medical Genetics*, vol. 46, no. 3, pp. 145–158, 2009.

[47] B. Lemire, "Mitochondrial genetics," *WormBook*, pp. 1–10, 2005.

[48] K. H. Smith, D. R. Johns, K. L. Heher, and N. R. Miller, "Heteroplasmy in Leber's hereditary optic neuropathy," *Archives of Ophthalmology*, vol. 111, no. 11, pp. 1486–1490, 1993.

[49] F. K. Jacobi, B. Leo-Kottler, K. Mittelviefhaus et al., "Segregation patterns and heteroplasmy prevalence in Leber's hereditary optic neuropathy," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 6, pp. 1208–1214, 2001.

[50] N. Phasukkijwatana, W. L. Chuenkongkaew, R. Suphavilai, K. Luangtrakool, B. Kunhapan, and P. Lertrit, "Transmission of heteroplasmic G11778A in extensive pedigrees of Thai Leber hereditary optic neuropathy," *Journal of Human Genetics*, vol. 51, no. 12, pp. 1110–1117, 2006.

[51] N. Phasukkijwatana, W. L. Chuenkongkaew, R. Suphavilai et al., "The unique characteristics of Thai Leber hereditary optic neuropathy: analysis of 30 G11778A pedigrees," *Journal of Human Genetics*, vol. 51, no. 4, pp. 298–304, 2006.

[52] M. T. Lott, A. S. Voljavec, and D. C. Wallace, "Variable genotype of Leber's hereditary optic neuropathy patients," *American Journal of Ophthalmology*, vol. 109, no. 6, pp. 625–631, 1990.

[53] P. A. Bolhuis, E. M. Bleeker-Wagemakers, N. J. Ponne et al., "Rapid shift in genotype of human mitochondrial DNA in a family with Leber's hereditary optic neuropathy," *Biochemical*

and Biophysical Research Communications, vol. 170, no. 3, pp. 994–997, 1990.

[54] N. Howell, M. Xu, S. Halvorson, I. Bodis-Wollner, and J. Sherman, "A heteroplasmic LHON family: tissue distribution and transmission of the 11778 mutation," American Journal of Human Genetics, vol. 55, no. 1, pp. 203–206, 1994.

[55] R. N. Lightowlers, P. F. Chinnery, D. M. Turnbull, and N. Howell, "Mammalian mitochondrial genetics: heredity, heteroplasmy and disease," Trends in Genetics, vol. 13, no. 11, pp. 450–455, 1997.

[56] M. Y. Yen, T. C. Yen, C. Y. Pang, J. H. Liu, and Y. H. Wei, "Mitochondrial DNA mutation in Leber's hereditary optic neuropathy," Investigative Ophthalmology and Visual Science, vol. 33, no. 8, pp. 2561–2566, 1992.

[57] P. Barboni, V. Mantovani, P. Montagna et al., "Mitochondrial DNA analysis in Leber's hereditary optic neuropathy," Ophthalmic Paediatrics and Genetics, vol. 13, no. 4, pp. 219–226, 1992.

[58] A. Puomila, T. Viitanen, M. L. Savontaus, E. Nikoskelainen, and K. Huoponen, "Segregation of the ND4/11778 and the ND1/3460 mutations in four heteroplasmic LHON families," Journal of the Neurological Sciences, vol. 205, no. 1, pp. 41–45, 2002.

[59] M. Y. Yen, AN. G. Wang, and Y. H. Wei, "Leber's hereditary optic neuropathy: a multifactorial disease," Progress in Retinal and Eye Research, vol. 25, no. 4, pp. 381–396, 2006.

[60] W. L. Chuenkongkaew, R. Suphavilai, L. Vaeusorn, N. Phasukkijwatana, P. Lertrit, and B. Suktitipat, "Proportion of 11778 mutant mitochondrial DNA and clinical expression in a Thai population with Leber hereditary optic neuropathy," Journal of Neuro-Ophthalmology, vol. 25, no. 3, pp. 173–175, 2005.

[61] P. F. Chinnery, R. M. Andrews, D. M. Turnbull, and N. Howell, "Leber hereditary optic neuropathy: does heteroplasmy influence the inheritance and expression of the G11778A mitochondrial DNA mutation?" American Journal of Medical Genetics, vol. 98, no. 3, pp. 235–243, 2001.

[62] S. T. Brady, R. J. Lasek, and R. D. Allen, "Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms," Cell Motility, vol. 5, no. 2, pp. 81–101, 1985.

[63] V. Carelli, F. N. Ross-Cisneros, and A. A. Sadun, "Optic nerve degeneration and mitochondrial dysfunction: genetic and acquired optic neuropathies," Neurochemistry International, vol. 40, no. 6, pp. 573–584, 2002.

[64] A. Wong and G. Cortopassi, "mtDNA mutations confer cellular sensitivity to oxidant stress that is partially rescued by calcium depletion and cyclosporin A," Biochemical and Biophysical Research Communications, vol. 239, no. 1, pp. 139–145, 1997.

[65] C. Zanna, A. Ghelli, A. M. Porcelli, A. Martinuzzi, V. Carelli, and M. Rugolo, "Caspase-independent death of Leber's hereditary optic neuropathy cybrids is driven by energetic failure and mediated by AIF and Endonuclease G," Apoptosis, vol. 10, no. 5, pp. 997–1007, 2005.

[66] C. Zanna, A. Ghelli, A. M. Porcelli, V. Carelli, A. Martinuzzi, and M. Rugolo, "Apoptotic cell death of cybrid cells bearing Leber's hereditary optic neuropathy mutations is caspase independent," Annals of the New York Academy of Sciences, vol. 1010, pp. 213–217, 2003.

[67] C. Battisti, P. Formichi, E. Cardaioli et al., "Cell response to oxidative stress incluced apoptosis in patients with Leber's hereditary optic neuropathy," Journal of Neurology, Neurosurgery and Psychiatry, vol. 75, no. 12, pp. 1731–1736, 2004.

[68] S. Beretta, L. Mattavelli, G. Sala et al., "Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines," Brain, vol. 127, no. 10, pp. 2183–2192, 2004.

[69] L. A. Levin, "Mechanisms of retinal ganglion specific-cell death in Leber hereditary optic neuropathy," Transactions of the American Ophthalmological Society, vol. 105, pp. 379–391, 2007.

[70] S. R. Danielson, A. Wong, V. Carelli, A. Martinuzzi, A. H. V. Schapira, and G. A. Cortopassi, "Cells bearing mutations causing Leber's hereditary optic neuropathy are sensitized to Fas-induced apoptosis," Journal of Biological Chemistry, vol. 277, no. 8, pp. 5810–5815, 2002.

[71] P. Cortelli, P. Montagna, G. Pierangeli et al., "Clinical and brain bioenergetics improvement with idebenone in a patient with Leber's hereditary optic neuropathy: a clinical and 31P-MRS study," Journal of the Neurological Sciences, vol. 148, no. 1, pp. 25–31, 1997.

[72] V. Carelli, P. Barboni, A. Zacchini et al., "Leber's hereditary optic neuropathy (LHON) with 14484/ND6 mutation in a North African patient," Journal of the Neurological Sciences, vol. 160, no. 2, pp. 183–188, 1998.

[73] Y. Mashima, K. Kigasawa, M. Wakakura, and Y. Oguchi, "Do idebenone and vitamin therapy shorten the time to achieve visual recovery in Leber hereditary optic neuropathy?" Journal of Neuro-Ophthalmology, vol. 20, no. 3, pp. 166–170, 2000.

[74] N. Barnils García, E. Mesa, S. Muñoz, A. Ferrer-Artola, and J. Arruga, "Response to idebenone and multivitamin therapy in Leber's hereditary optic neuropathy," Archivos de la Sociedad Espanola de Oftalmologia, vol. 82, no. 6, pp. 377–380, 2007.

[75] N. J. Newman, V. Biousse, R. David et al., "Prophylaxis for second eye involvement in leber hereditary optic neuropathy: an open-labeled, nonrandomized multicenter trial of topical brimonidine purite," American Journal of Ophthalmology, vol. 140, no. 3, pp. 407.e1–407.e11, 2005.

[76] G. Manfredi and M. F. Beal, "Poison and antidote: a novel model to study pathogenesis and therapy of LHON," Annals of Neurology, vol. 56, no. 2, pp. 171–172, 2004.

[77] X. Zhang, D. Jones, and F. Gonzalez-Lima, "Mouse model of optic neuropathy caused by mitochondrial complex I dysfunction," Neuroscience Letters, vol. 326, no. 2, pp. 97–100, 2002.

[78] X. Qi, A. S. Lewin, W. W. Hauswirth, and J. Guy, "Suppression of complex I gene expression induces optic neuropathy," Annals of Neurology, vol. 53, no. 2, pp. 198–205, 2003.

[79] X. Qi, A. S. Lewin, W. W. Hauswirth, and J. Guy, "Optic neuropathy induced by reductions in mitochondrial superoxide dismutase," Investigative Ophthalmology and Visual Science, vol. 44, no. 3, pp. 1088–1096, 2003.

[80] X. Qi, A. S. Lewin, L. Sun, W. W. Hauswirth, and J. Guy, "SOD2 gene transfer protects against optic neuropathy induced by deficiency of complex I," Annals of Neurology, vol. 56, no. 2, pp. 182–191, 2004.

[81] X. Qi, L. Sun, W. W. Hauswirth, A. S. Lewin, and J. Guy, "Use of mitochondrial antioxidant defenses for rescue of cells with a leber hereditary optic neuropathy-causing mutation," Archives of Ophthalmology, vol. 125, no. 2, pp. 268–272, 2007.

[82] A. Kanamori, M.-M. Catrinescu, N. Kanamori, K. A. Mears, R. Beaubien, and L. A. Levin, "Superoxide is an associated signal for apoptosis in axonal injury," Brain, vol. 133, no. 9, pp. 2612–2625, 2010.

[83] X. Qi, L. Sun, A. S. Lewin, W. W. Hauswirth, and J. Guy, "The mutant human ND4 subunit of complex I induces optic neuropathy in the mouse," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 1, pp. 1–10, 2007.

[84] S. Ellouze, S. Augustin, A. Bouaita et al., "Optimized allotopic expression of the human mitochondrial ND4 prevents blindness in a rat model of mitochondrial dysfunction," *American Journal of Human Genetics*, vol. 83, no. 3, pp. 373–387, 2008.

[85] N. G. Larsson, "Leber hereditary optic neuropathy: a nuclear solution of a mitochondrial problem," *Annals of Neurology*, vol. 52, no. 5, pp. 529–530, 2002.

[86] J. Guy, X. Qi, F. Pallotti et al., "Rescue of a mitochondrial deficiency causing Leber Hereditary Optic Neuropathy," *Annals of Neurology*, vol. 52, no. 5, pp. 534–542, 2002.

[87] J. Guy, X. Qi, R. D. Koilkonda et al., "Efficiency and safety of AAV-mediated gene delivery of the human ND4 complex I subunit in the mouse visual system," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 9, pp. 4205–4214, 2009.

[88] T. Tsukihara, K. Shimokata, Y. Katayama et al., "The low-spin heme of cytochrome c oxidase as the driving element of the proton-pumping process," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 26, pp. 15304–15309, 2003.

[89] F. Figueroa-Martínez, M. Vázquez-Acevedo, P. Cortés-Hernández et al., "What limits the allotopic expression of nucleus-encoded mitochondrial genes? The case of the chimeric Cox3 and Atp6 genes," *Mitochondrion*. In press.

[90] P. S. Brookes, A. Pinner, A. Ramachandran et al., "High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes," *Proteomics*, vol. 2, no. 8, pp. 969–977, 2002.

[91] J. Oca-Cossio, L. Kenyon, H. Hao, and C. T. Moraes, "Limitations of allotopic expression of mitochondrial genes in mammalian cells," *Genetics*, vol. 165, no. 2, pp. 707–720, 2003.

[92] R. W. Gilkerson, J. M. L. Selker, and R. A. Capaldi, "The cristal membrane of mitochondria is the principal site of oxidative phosphorylation," *FEBS Letters*, vol. 546, no. 2-3, pp. 355–358, 2003.

[93] L. Supekova, F. Supek, J. E. Greer, and P. G. Schultz, "A single mutation in the first transmembrane domain of yeast COX2 enables its allotopic expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 11, pp. 5047–5052, 2010.

[94] C. Bonnet, V. Kaltimbacher, S. Ellouze et al., "Allotopic mRNA localization to the mitochondrial surface rescues respiratory chain defects in fibroblasts harboring mitochondrial DNA mutations affecting complex I or V subunits," *Rejuvenation Research*, vol. 10, no. 2, pp. 127–143, 2007.

[95] I. N. Shokolenko, M. F. Alexeyev, S. P. Ledoux, and G. L. Wilson, "The approaches for manipulating mitochondrial proteome," *Environmental and Molecular Mutagenesis*, vol. 51, no. 5, pp. 451–461, 2010.

[96] E. Perales-Clemente, P. Fernandez-Silva, R. Acin-Perez, A. Perez-Martos, and J. A. Enriquez, "Allotopic expression of mitochondrial-encoded genes in mammals: achieved goal, undemonstrated mechanism or impossible task?" *Nucleic Acids Research*. In press.

[97] W. W. Hauswirth, T. S. Aleman, S. Kaushal et al., "Treatment of Leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene

vector: short-term results of a phase I trial," *Human Gene Therapy*, vol. 19, no. 10, pp. 979–990, 2008.

[98] B. B. Seo, E. Nakamaru-Ogiso, T. R. Flotte, A. Matsuno-Yagi, and T. Yagi, "In vivo complementation of complex I by the yeast Ndi1 enzyme: possible application for treatment of Parkinson disease," *Journal of Biological Chemistry*, vol. 281, no. 20, pp. 14250–14255, 2006.

[99] L. Dudus, V. Anand, G. M. Acland et al., "Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV," *Vision Research*, vol. 39, no. 15, pp. 2545–2553, 1999.

[100] J. Guy, X. Qi, N. Muzyczka, and W. W. Hauswirth, "Reporter expression persists 1 year after adeno-associated virus-mediated gene transfer to the optic nerve," *Archives of Ophthalmology*, vol. 117, no. 7, pp. 929–937, 1999.

[101] G. M. Acland, G. D. Aguirre, J. Ray et al., "Gene therapy restores vision in a canine model of childhood blindness," *Nature Genetics*, vol. 28, no. 1, pp. 92–95, 2001.

[102] D. M. McCarty, "Self-complementary AAV vectors; advances and applications," *Molecular Therapy*, vol. 16, no. 10, pp. 1648–1656, 2008.

[103] D. M. McCarty, P. E. Monahan, and R. J. Samulski, "Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis," *Gene Therapy*, vol. 8, no. 16, pp. 1248–1254, 2001.

[104] D. M. McCarty, H. Fu, P. E. Monahan, C. E. Toulson, P. Naik, and R. J. Samulski, "Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo," *Gene Therapy*, vol. 10, no. 26, pp. 2112–2118, 2003.

[105] LI. Zhong, B. Li, G. Jayandharan et al., "Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression," *Virology*, vol. 381, no. 2, pp. 194–202, 2008.

[106] LI. Zhong, B. Li, C. S. Mah et al., "Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 22, pp. 7827–7832, 2008.

[107] R. D. Koilkonda, T.-H. Chou, V. Porciatti, W. W. Hauswirth, and J. Guy, "Induction of rapid and highly efficient expression of the human ND4 complex I subunit in the mouse visual system by self-complementary adeno-associated virus," *Archives of Ophthalmology*, vol. 128, no. 7, pp. 876–883, 2010.

[108] A. K. Zaiss and D. A. Muruve, "Immunity to adeno-associated virus vectors in animals and humans: a continued challenge," *Gene Therapy*, vol. 15, no. 11, pp. 808–816, 2008.

[109] M. Tachibana, M. Sparman, H. Sritanaudomchai et al., "Mitochondrial gene replacement in primate offspring and embryonic stem cells," *Nature*, vol. 461, no. 7262, pp. 367–372, 2009.

[110] P. M. Keeney, C. K. Quigley, L. D. Dunham et al., "Mitochondrial gene therapy augments mitochondrial physiology in a Parkinson's disease cell model," *Human Gene Therapy*, vol. 20, no. 8, pp. 897–907, 2009.

[111] S. R. Bacman, S. L. Williams, D. Hernandez, and C. T. Moraes, "Modulating mtDNA heteroplasmy by mitochondria-targeted restriction endonucleases in a 'differential multiple cleavage-site' model," *Gene Therapy*, vol. 14, no. 18, pp. 1309–1318, 2007.

[112] S. DiMauro, M. Hirano, and E. A. Schon, "Approaches to the treatment of mitochondrial diseases," *Muscle and Nerve*, vol. 34, no. 3, pp. 265–283, 2006.

[113] M. P. Bayona-Bafaluy, B. Blits, B. J. Battersby, E. A. Shoubridge, and C. T. Moraes, "Rapid directional shift of mitochondrial DNA heteroplasmy in animal tissues by a mitochondrially targeted restriction endonuclease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 40, pp. 14392–14397, 2005.

[114] J. Ojaimi, J. Pan, S. Santra, W. J. Snell, and E. A. Schon, "An algal nucleus-encoded subunit of mitochondrial ATP synthase rescues a defect in the analogous human mitochondrial-encoded subunit," *Molecular Biology of the Cell*, vol. 13, no. 11, pp. 3836–3844, 2002.

[115] B. B. Seo, T. Kitajima-Ihara, E. K. L. Chan, I. E. Scheffler, A. Matsuno-Yagi, and T. Yagi, "Molecular remedy of complex I defects: rotenone-insensitive internal NADH-quinone oxidoreductase of Saccharomyces cerevisiae mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 16, pp. 9167–9171, 1998.

[116] Y. Bai, P. Hájek, A. Chomyn et al., "Lack of complex I activity in human cells carrying a mutation in MtDNA-encoded ND4 subunit is corrected by the Saccharomyces cerevisiae NADH-quinone oxidoreductase (NDI1) gene," *Journal of Biological Chemistry*, vol. 276, no. 42, pp. 38808–38813, 2001.

[117] M. Marella, B. B. Seo, B. B. Thomas, A. Matsuno-Yagi, and T. Yagi, "Successful amelioration of mitochondrial optic neuropathy using the yeast NDI1 gene in a rat animal model," *PLoS One*, vol. 5, no. 7, Article ID e11472, 2010.

[118] S. Bahadorani, J. Cho, T. Lo et al., "Neuronal expression of a single-subunit yeast NADH-ubiquinone oxidoreductase (Ndi1) extends Drosophila lifespan," *Aging cell*, vol. 9, no. 2, pp. 191–202, 2010.

[119] B. L. Lam, W. J. Feuer, F. Abukhalil, V. Porciatti, W. W. Hauswirth, and J. Guy, "Leber hereditary optic neuropathy gene therapy clinical trial recruitment: year 1," *Archives of Ophthalmology*, vol. 128, no. 9, pp. 1129–1135, 2010.

[120] J. W. B. Bainbridge, "Prospects for gene therapy of inherited retinal disease," *Eye*, vol. 23, no. 10, pp. 1898–1903, 2009.

[121] A. M. Maguire, F. Simonelli, E. A. Pierce et al., "Safety and efficacy of gene transfer for Leber's congenital amaurosis," *New England Journal of Medicine*, vol. 358, no. 21, pp. 2240–2248, 2008.

[122] M. Matsumoto, S. Hayasaka, C. Kadoi et al., "Secondary mutations of mitochondrial DNA in Japanese patients with Leber's hereditary optic neuropathy," *Ophthalmic Genetics*, vol. 20, no. 3, pp. 153–160, 1999.

[123] E. L. Blakely, R. de Silva, A. King et al., "LHON/MELAS overlap syndrome associated with a mitochondrial MTND1 gene mutation," *European Journal of Human Genetics*, vol. 13, no. 5, pp. 623–627, 2005.

[124] M. D. Brown, A. S. Voljavec, M. T. Lott, A. Torroni, C. C. Yang, and D. C. Wallace, "Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy," *Genetics*, vol. 130, no. 1, pp. 163–173, 1992.

[125] M. D. Brown, S. Zhadanov, J. C. Allen et al., "Novel mtDNA mutations and oxidative phosphorylation dysfunction in Russian LHON families," *Human Genetics*, vol. 109, no. 1, pp. 33–39, 2001.

[126] S. Fauser, J. Luberichs, D. Besch, and B. Leo-Kottler, "Sequence analysis of the complete mitochondrial genome in patients with Leber's hereditary optic neuropathy lacking the three most common pathogenic DNA mutations," *Biochemical and Biophysical Research Communications*, vol. 295, no. 2, pp. 342–347, 2002.

[127] M. L. Valentino, P. Barboni, A. Ghelli et al., "The ND1 gene of complex I is a mutational hot spot for Leber's hereditary optic neuropathy," *Annals of Neurology*, vol. 56, no. 5, pp. 631–641, 2004.

[128] K. Huoponen, T. Lamminen, V. Juvonen, P. Aula, E. Nikoskelainen, and M. L. Savontaus, "The spectrum of mitochondrial DNA mutations in families with Leber hereditary optic neuroretinopathy," *Human Genetics*, vol. 92, no. 4, pp. 379–384, 1993.

[129] N. Howell, I. Kubacka, M. Xu, and D. A. McCullough, "Leber hereditary optic neuropathy: involvement of the mitochondrial ND1 gene and evidence for an intragenic suppressor mutation," *American Journal of Human Genetics*, vol. 48, no. 5, pp. 935–942, 1991.

[130] J. Y. Kim, J. M. Hwang, and S. S. Park, "Mitochondrial DNA C4171A/ND1 is a novel primary causative mutation of Leber's hereditary optic neuropathy with a good prognosis," *Annals of Neurology*, vol. 51, no. 5, pp. 630–634, 2002.

[131] D. R. Johns and J. Berman, "Alternative, simultaneous complex I mitochondrial DNA mutations in Leber's hereditary optic neuropathy," *Biochemical and Biophysical Research Communications*, vol. 174, no. 3, pp. 1324–1330, 1991.

[132] K. K. Abu-Amero and T. M. Bosley, "Mitochondrial abnormalities in patients with LHON-like optic neuropathies," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 10, pp. 4211–4220, 2006.

[133] M. D. Brown, C.-C. Yang, I. Trounce, A. Torroni, M. T. Lott, and D. C. Wallace, "A mitochondrial DNA variant, identified in Leber hereditary optic neuropathy patients, which extends the amino acid sequence of cytochrome c oxidase subunit I," *American Journal of Human Genetics*, vol. 51, no. 2, pp. 378–385, 1992.

[134] J. Yang, Y. Zhu, YI. Tong et al., "The novel G10680A mutation is associated with complete penetrance of the LHON/T14484C family," *Mitochondrion*, vol. 9, no. 4, pp. 273–278, 2009.

[135] M. D. Brown, A. S. Voljavec, M. T. Lott, I. MacDonald, and D. C. Wallace, "Leber's hereditary optic neuropathy: a model for mitochondrial neurodegenerative diseases," *FASEB Journal*, vol. 6, no. 10, pp. 2791–2799, 1992.

[136] J. Horvath, R. Horvath, V. Karcagi, S. Komoly, and D. R. Johns, "Sequence analysis of Hungarian LHON patients not carrying the common primary mutations," *Journal of Inherited Metabolic Disease*, vol. 25, no. 4, pp. 323–324, 2002.

[137] P. Kjer, "Infantile optic atrophy with dominant mode of inheritance: a clinical and genetic study of 19 Danish families," *Acta Ophthalmologica. Supplement*, vol. 164, supplement 54, pp. 1–147, 1959.

[138] D. D. De Vries, L. N. Went, G. W. Bruyn et al., "Genetic and biochemical impairment of mitochondrial complex I activity in a family with Leber hereditary optic neuropathy and hereditary spastic dystonia," *American Journal of Human Genetics*, vol. 58, no. 4, pp. 703–711, 1996.

[139] M. D. Brown, A. Torroni, C. L. Reckord, and D. C. Wallace, "Phylogenetic analysis of Leber's hereditary optic neuropathy mitochondrial DNA's indicates multiple independent occurrences of the common mutations," *Human Mutation*, vol. 6, no. 4, pp. 311–325, 1995.

[140] V. Mayorov, V. Biousse, N. J. Newman, and M. D. Brown, "The role of the ND5 gene in LHON: characterization of a new, heteroplasmic LHON mutation," *Annals of Neurology*, vol. 58, no. 5, pp. 807–811, 2005.

[141] D. Liolitsa, S. Rahman, S. Benton, L. J. Carr, and M. G. Hanna, "Is the mitochondrial complex I ND5 gene a hot-spot for MELAS causing mutations?" *Annals of Neurology*, vol. 53, no. 1, pp. 128–132, 2003.

[142] N. Howell, R. J. Oostra, P. A. Bolhuis et al., "Sequence analysis of the mitochondrial genomes from Dutch pedigrees with leber hereditary optic neuropathy," *American Journal of Human Genetics*, vol. 72, no. 6, pp. 1460–1469, 2003.

[143] C. Batandier, A. Picard, N. Tessier, and J. Lunardi, "Identification of a novel T398A mutation in the ND5 subunit of the mitochondrial complex I and of three novel mtDNA polymorphisms in two patients presenting ocular symptoms," *Human Mutation*, vol. 16, no. 6, p. 532, 2000.

[144] N. Howell, S. Halvorson, J. Burns, D. A. McCullough, and J. Poulton, "When does bilateral optic atrophy become Leber hereditary optic neuropathy?" *American Journal of Human Genetics*, vol. 53, no. 4, pp. 959–963, 1993.

[145] D. Besch, B. Leo-Kottler, E. Zrenner, and B. Wissinger, "Leber's hereditary optic neuropathy: clinical and molecular genetic findings in a patient with a new mutation in the ND6 gene," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 237, no. 9, pp. 745–752, 1999.

[146] S. I. Zhadanov, V. V. Atamanov, N. I. Zhadanov, O. V. Oleinikov, L. P. Osipova, and T. G. Schurr, "A novel mtDNA ND6 gene mutation associated with LHON in a Caucasian family," *Biochemical and Biophysical Research Communications*, vol. 332, no. 4, pp. 1115–1121, 2005.

[147] A. S. Jun, M. D. Brown, and D. C. Wallace, "A mitochondrial DNA mutation at nucleotide pair 14459 of the NADH dehydrogenase subunit 6 gene associated with maternally inherited Leber hereditary optic neuropathy and dystonia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 13, pp. 6206–6210, 1994.

[148] N. Howell, C. Bogolin, R. Jamieson, D. R. Marenda, and D. A. Mackey, "mtDNA mutations that cause optic neuropathy: how do we know?" *American Journal of Human Genetics*, vol. 62, no. 1, pp. 196–202, 1998.

[149] P. F. Chinnery, D. T. Brown, R. M. Andrews et al., "The mitochondrial ND6 gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy," *Brain*, vol. 124, no. 1, pp. 209–218, 2001.

[150] B. Wissinger, D. Besch, B. Baumann et al., "Mutation analysis of the ND6 gene in patients with lebers hereditary optic neuropathy," *Biochemical and Biophysical Research Communications*, vol. 234, no. 2, pp. 511–515, 1997.

[151] C. La. Morgia, A. Achilli, L. Iommarini et al., "Rare mtDNA variants in Leber hereditary optic neuropathy families with recurrence of myoclonus," *Neurology*, vol. 70, no. 10, pp. 762–770, 2008.

[152] D. R. Johns and M. J. Neufeld, "Cytochrome b mutations in Leber hereditary optic neuropathy," *Biochemical and Biophysical Research Communications*, vol. 181, no. 3, pp. 1358–1364, 1991.

[153] D. R. Johns and M. J. Neufeld, "Cytochrome c oxidase mutations in Leber hereditary optic neuropathy," *Biochemical and Biophysical Research Communications*, vol. 196, no. 2, pp. 810–815, 1993.

[154] N. Povalko, E. Zakharova, G. Rudenskaia et al., "A new sequence variant in mitochondrial DNA associated with high penetrance of Russian Leber hereditary optic neuropathy," *Mitochondrion*, vol. 5, no. 3, pp. 194–199, 2005.

Review Article

Conditional Gene Targeting: Dissecting the Cellular Mechanisms of Retinal Degenerations

Yun-Zheng Le[1, 2, 3, 4]

[1] *Departments of Medicine, University of Oklahoma Health Sciences Center, 941 S. L. Young Boulevard, BSEB 302G, Oklahoma City, OK 73104, USA*
[2] *Departments of Cell Biology, University of Oklahoma Health Sciences Center, 941 S. L. Young Boulevard, BSEB 302G, Oklahoma City, OK 73104, USA*
[3] *Harold Hamm Oklahoma Diabetes Center, University of Oklahoma Health Sciences Center, 941 S. L. Young Boulevard, BSEB 302G, Oklahoma City, OK 73104, USA*
[4] *Dean A. McGee Eye Institute, Oklahoma City, OK 73104, USA*

Correspondence should be addressed to Yun-Zheng Le, yun-le@ouhsc.edu

Retinal neuron degeneration and survival are often regulated by the same trophic factors that are required for embryonic development and are usually expressed in multiple cell-types. Therefore, the conditional gene targeting approach is necessary to investigate the cell-specific function of widely expressed and developmentally regulated genes in retinal degeneration. The discussion in this review will be focused on the use of Cre/*lox*-based conditional gene targeting approach in mechanistic studies for retinal degeneration. In addition to the basic experimental designs, this article addresses various factors influencing the outcomes of conditional gene targeting studies, limitations of current technologies, availability of Cre-drive lines for various retinal cells, and issues related to the generation of Cre-expressing mice. Finally, this review will update the current status on the use of Cre/*lox*-based gene targeting approach in mechanistic studies for retinal degeneration, which includes rod photoreceptor survival under photo-oxidative stress and protein trafficking in photoreceptors.

1. Introduction

The use of gene targeting with homologous recombination in murine embryonic stem (ES) cells has led to many mechanistic insights about human diseases. However, global gene disruption has two major limitations that may prevent the identification of gene function in a target tissue or in adults. First, disruption of essential genes often causes embryonic or early postnatal lethality [1]. Second, disruption of a ubiquitously expressed gene may not yield mechanistic insights regarding the function of a protein of interest in a particular cell type [2, 3]. In these scenarios, temporal or/and spatial gene disruption is far more advantageous. The seminal work on the utilization of bacteriophage P1 site-specific recombination system in mammals by Dr. Brian Sauer and his coworkers [4, 5] established a firm foundation

for the Cre/*lox*-based gene targeting, which is the most widely used conditional gene targeting approach to date.

Cre recombinase is a 38 kDa protein and belongs to the integrase family of recombinases [6]. Biochemically Cre catalyzes site-specific DNA recombination, both intra- and intermolecularly, between the 34 base pair *loxP* sites [7]. Cre carries a eukaryotic nuclear targeting sequence [8] and is efficient in performing site-specific DNA recombination in mammals [9]. Therefore, Cre/*lox* system has become the primary choice for the site-specific DNA recombination-based manipulation of the mouse genome. Efficient Cre-mediated excision of DNA between directly repeated *loxP* sites has been widely used in gene activation and deletion of small or large segment of chromosomal DNA [9–11]. Cre-mediated recombination also permits the translocation of large DNA fragments on chromosomes [12] and integration

(knock-in) or replacement of a gene or DNA segment [13–15]. Conditional gene knockout is by far the most widely used application of Cre-mediated site-specific recombination [16]. The use of this strategy in retinal degeneration studies will be the focus of this paper. In addition to the general strategy of Cre/*lox* gene targeting, this review will address various factors influencing the outcomes of conditional gene targeting studies, limitations of current technologies, availability of Cre-drive lines for various retinal cells, and issues related to the generation of Cre-drive lines. Finally, this paper will update the current status on the use of Cre/*lox*-based gene targeting approach in mechanistic studies for retinal degeneration, including the two most advanced areas, rod photoreceptor survival under photo-oxidative stress and protein trafficking in photoreceptors.

2. Strategy in Experimental Design

2.1. Basic Scheme of Experimental Design. Cre/*lox* conditional gene targeting requires a mouse that has been pre-engineered with a *loxP*-flanked gene (or gene segment), generated with homologous recombination in murine ES cells (Figure 1). As the *loxP* sites are placed in introns, this engineered mouse is phenotypically wild type. A conditional gene knockout mouse is generated by breeding this mouse with a mouse that expresses Cre under the control of a tissue-specific promoter for two generations (Figure 1). In the conditional gene knockout mouse, the *loxP*-flanked gene is removed in a tissue-specific fashion. Only cells/tissues that express Cre carry the deleted gene, and thus they are phenotypically mutants (Figure 1). In this way, one can analyze the gene function in Cre-expressing tissues without affecting the gene expression in nontargeted tissues.

2.2. Considerations in Experimental Design. One concern regarding the use of conditional gene targeting *in vivo* is that the Cre-mediated excisive recombination is usually not 100 percent. Therefore, the effect of gene disruption may not be observed. It is important to understand that there is a fundamental difference between Cre-mediated gene disruption and conventional gene knockdown. As only four Cre molecules are required for a productive Cre-mediated recombination [7], Cre-mediated gene disruption occurs usually in an all-or-none fashion in a particular cell. A most likely scenario for a 20 percent efficiency of Cre-mediated recombination is that approximately 20 percent of targeted cells have 100 percent gene knockout. This is completely different from 20 percent gene knockdown in all cells. This characteristic has made Cre/*lox*-based gene targeting a useful approach in gene function analysis, even though it is rare that transgenic Cre mice express the recombinase in all targeted cells/tissues. Since most gene function studies are targeting the effect of gene inside the cells, a fraction of targeted cells with gene deletion could produce stable phenotypic changes in animals [44, 45]. However, in a scenario that no phenotypic change is observed in animals that have a small portion of targeted cells carrying Cre-mediated gene disruption, the interpretation of data needs to be cautious.

Another misconception in designing conditional gene targeting studies is that a complete Cre-mediated excision is more desirable. This is not always true, particularly, in a situation that Cre may have toxic effect to the cells or phenotypic changes are too strong to be characterized. In a previous study, we intentionally used a rod-expressing Cre line with a lower efficiency of Cre-mediated recombination to avoid unnecessary complication derived from potential Cre toxicity in rods [44], as observed by others [21]. In a scenario that conditional gene targeting results in a massive or/and rapid phenotypic change that hampers the understanding of the biology and diseases, a lower level of Cre expression in targeted tissues/cells may produce a genetic mosaic that attenuates the development of pathological changes in animal models [46].

3. Cre-Drive Lines

3.1. Available Cre-Drive Lines. Although Cre can be exogenously delivered to a targeted tissue, it is usually expressed under the control of tissue/cell specific promoters. A critical factor for a successful conditional gene inactivation study is the availability of a suitable Cre-expressing drive line. Table 1 includes a list of published Cre-expressing drive lines for various retinal cells. Since most retinal degeneration studies are related to the photoreceptors and RPE, all published rod-, cone-, and RPE-expressing Cre mouse lines are listed in Table 1. Retinal Müller glia is the major supporting cell and plays a critically role in maintaining structural and functional integrity in the retina under stress conditions. As most Cre-drive lines for Müller glia were usually developed for brain and Cre expression occurred outside ocular tissues in these mice, Table 1 only lists a few that either have been characterized more thoroughly or have been shown to be successful in conditional gene targeting in the retina [3, 47, 48]. Degeneration of retinal ganglion cells (GCs) is becoming a focused research area for their role in glaucoma and for the relevance to the safety of treating AMD patient with anti-VEGF strategies [49]. A number of characterized GC-expressing Cre-drive lines are thus listed in Table 1. While inner nuclear layer (INL) neurons are not often investigated for retinal degeneration, they are retinal neurons. The Cre-drive lines for INL neurons can be used for studies related to retinal neurobiology and are listed in Table 1. Finally, Cre-drive lines that are expressed in almost all retinal neurons are also listed in Table 1. It is worth noting that some of the listed Cre-expressing mouse lines were originally designed to trace cell lineage and had strong developmental Cre expression. These Cre lines may not be suitable for retinal degeneration studies. Although some promoters employed for Cre expression are useful in circumventing embryonic lethality, due to their ubiquitous expression they cannot be utilized to study a tissue/cell type-specific gene function.

3.2. Redundancy of Cre-Drive Lines. For most retinal cell-types, Table 1 lists more than one Cre-drive line. It is important to know that these seemly redundant Cre-drive lines are necessary. As most published Cre-drive lines derived from
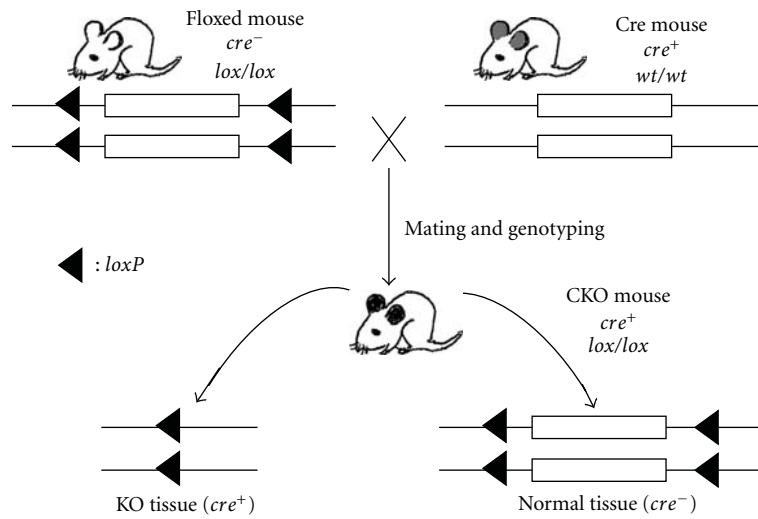
FIGURE 1: Schematic diagram of generating a conditional knockout (CKO) mouse from breeding a tissue-specific Cre mouse (top right) with a mouse carrying homozygous floxed gene (top left). A CKO mouse carrying a homozygous floxed gene and *cre* (either heterozygous or homozygous) is obtained by genotyping the F2 offspring. Tissue-specific Cre expression is shown as grey-eared (top right). Tissue-specific gene KO is diagrammed as black-eared (bottom).

the same or similar promoters are not identical, it is ideal to have several usable Cre-drive lines for a particular cell-type due to the following considerations. First, a range of Cre expression levels provide choice to achieve a suitable degree of gene inactivation for a particular study. Second, variable ecotopic expression patterns between the Cre-expressing lines may produce unintended phenotypes that may be beneficial [24]. Third, transgenic *cre* is localized on one of the 20 chromosomes in mice. There is a 5 percent of possibility that *cre* may be residing on the same chromosome where a *loxP*-flanked gene is localized. Having more than one Cre-drive line for a targeted tissue/cell-type is likely to provide a choice for the successful generation of a conditional gene knockout mouse. Therefore, publishing a Cre-drive line for a particular cell-type with already established drive lines should be encouraged. Since there have not been many side-by-side studies comparing different Cre-drive lines as performed by Ivanova et al. recently [31], it is not possible to give an accurate account of the differences among Cre-drive lines that target a particular cell-type. This review only provides a roadmap about the available resources. To select the most desirable Cre-drive line, end users should perform side-by-side comparison, if necessary.

3.3. Types of Cre-Drive Lines. While the traditional transgenic approaches have proved to be useful for generating Cre-drive lines, the inherent problems associated with this approach [50] may cause variability in mutant phenotypes among animals. This variability sometimes may result in unintended expression pattern that may or may not be useful for other studies [24]. The use of knock-in or bacterial artificial chromosome based transgenic approaches is likely to produce Cre-drive lines with the expression patterns that more closely resemble the characteristics of the promoters. In addition, the variability in Cre expression among animals

can be reduced using these transgenic approaches. For these reasons, the Cre-drive lines referenced in Table 1 also provide information on how these Cre-expressing mice were generated. It is important to keep in mind that a Cre-drive line generated with a knock-in approach may affect the expression of the native gene and careful phenotyping of Cre-expressing mice are necessary.

Table 1 also includes information about whether Cre-expressing lines are generated using an inducible promoter system such as tetracycline- or tamoxifen-inducible systems [51, 52]. While inducible tissue-/cell-specific gene knockout approach is more advantageous, there are inherent problems associated with these systems, such as leakiness [53, 54]. Efficient delivery of inducing agents to the targeted retinal cells at the peak of promoter activity is the key to the success of inducible Cre expression. Although inducing gene expression in a tetracycline-inducible system with doxycycline for a short period of time may not be harmful to the retina [55], one should always keep in mind that tamoxifen may be toxic to the retina [56]. One distinctive advantage of using inducible systems is their ability to turn off/down the expression of Cre, which may be toxic to the targeted cells [19, 21].

3.4. Cre Toxicity. Cre is a DNA recombinase and may cause unintended chromosomal rearrangement at cryptic sites [57, 58]. Proper control of Cre expression is required for Cre-drive lines and a careful phenotypic analysis of Cre-drive lines is a prerequisite for conditional gene targeting. However, the Cre toxicity may not be the only contributing factor that caused retinal denegation in Cre-expressing rod-specific Cre mice [19, 21]. As expression of human rhodorpsin-GFP fusion, a nontoxic protein, also caused progressive rod photoreceptor degeneration [59], it is likely that a high level of expression of an exogenous protein may be toxic to the

TABLE 1: Published potentially useful Cre-drive lines in designing studies related to retinal degeneration.

Major targeted cells	Minor/other expression	Promoter	References
Photoreceptors			
M- and S-cone	Not reported	*hRgp*	[17]
M-cone	Not reported	*mMo*	[18]
S-cone	Not reported	*mSo*	[18]
Rod	Rod bipolar	*mRho*	[19]
Rod	Not reported	*Irbp*	[20]
Rod	Not reported	*hRho*	[21, 22]
RPE			
*RPE	Optic nerve	*hVmd2*	[23]
*RPE	Müller cells/optic nerve/INL	*hVmd2*	[24]
RPE	Pigmented cells	*Dct*	[25]
RPE	Neural retina	*Trp1*	[26]
RPE	Lens/neural retina	Modified *αA-crystallin*	[27]
Müller glia			
#Müller cells	GC and ONL	*Pdgfra*	[28]
*Müller cells	INL	*hVmd2*	[24, 29]
!#Müller cells	Brain	*Glast*	[30]
Müller cells	INL/Brain	*Thy1*	[31, 32]
Müller cells	Brain	*Foxg1*	[31]
Ganglion cells			
GC	Brain	*Grik4*	[31]
Melanopsin-expressing GC	Not reported	*Opn4*	[33]
$*GC*	Amacrine and horizontal cells	*Math5*	[34]
*GC/n*eural retina	Brain	*Thy1.2*	[35]
GC/Amacrine cells	Brain	*Chat*-(BAC transgenic)	[31, 36]
Inner nuclear layer neurons			
$Amacrine cells	Not reported	*Chat*-(knockin-Jackson Lab)	[31]
Bipolar cells	photoreceptor/Brain	*Pcp2*	[37]
#Rod bipolar cells	Brain	*Pcp2*	[38]
$Amacrine and horizontal cells	Not reported	*Ptf1a*	[39]
Neural retina			
#All retinal neurons	Not reported	*Chx10*	[40]
!All retinal neurons	Brain	*PrP*	[41]
Neural retina	Brian/multiple tissues	*Six3*	[42]
#All retinal neurons	Not reported	*Dkk3*	[43]

*Expression with a tetracycline-inducible approach. !Expression with a tamoxifen-inducible approach. #Expression with BAC transgenic approach. $expression with knock-in approach. Abbreviations: *Chat*: choline acetyl transferase *Dct*: dopachrome tautomerase *Dkk3*: Dickkopf family protein 3 *Foxg1*: Forkhead box G1, *Glast*: glutamate/aspartate transporter, *Grik4*: glutamate receptor, ionotropic kainate 4 precursor, *hRgp*: human red/green pigment, *Math5*: murine atonal homolog 5, *mRho*: mouse rhodopsin, *mMo*: mouse M-opsin, *mSo*: Mouse S-opsin, *Opn4*: melanopsin, *Pcp2*: purkinje cell protein 2, *Pdgfra*: platelet-derived growth factor receptor-α, *PrP*: Prion protein, *Ptf1a*: pancreas specific transcription factor 1a, *Six3*: six/sine oculis subclass of homeobox gene, *Thy1.2*: Thymus cell antigen 1.2, and *Trp1*: tyrosinase-related protein.

host protein transcription/translation/maturation system in rods.

3.5. New Cre-Drive Lines. For the past decade or so, many laboratories have contributed considerable effort in establishing various Cre-drive lines. While Cre-expressing mice have been used successfully in conditional gene targeting, there are not sufficient Cre-drive lines, even for the most advanced field, photoreceptor biology. Due to a high level of Cre expression causes rod degeneration, it would be ideal to have at least one inducible Cre-drive line for rods. As there are at least fifty types of retinal neurons, the current list (Table 1) is far from completion. However, for most retinal cell-types, a major shortcoming of most currently available Cre-drive lines is a lack of temporal or spatial specificities and desired efficiencies. Significant improvement in this area is needed. At present, a major challenge for Cre/*lox*-based conditional gene targeting is the difficulties to obtain Cre-drive lines with desired tissue-specificities. A lack of "ideal" promoters is the major reason. Therefore, it is worthwhile

to invest some effort on studying the expression pattern of potential promoters that drive Cre expression before making a mouse.

4. Dissecting Cellular Mechanisms of Retinal Degeneration

4.1. Photoreceptor Survival under Photo-Oxidative Stress. A major focus in retinal denegation is to reveal the mechanisms of photoreceptor survival. As many of the survival factors are essential for development, global disruption of these essential genes often causes embryonic lethality. Using Cre/*lox*-based conditional gene targeting approach, Haruta et al. demonstrated that Rac1, a component of NADPH oxidase that produces reactive oxygen species, was required for the rod photoreceptor protection from photo-oxidative stress [60]. To determine photoreceptor survival mechanisms under photo-oxidative stress, Ueki et al. used rod-specific gp130 knockout mice and showed that preconditioning of mice with a sublethal photo-oxidative stress activated an autonomous protective mechanism in rods through gp130, an IL6 cytokine receptor, and, its downstream target STAT3 [61]. To determine further whether Müller cells, major retinal supporting cells often played a role in photoreceptor protection by releasing survival factors, were involved in this process, they demonstrated that gp130 activation in Müller cells had no additional effect for rod survival under photo-oxidative stress [47]. While this study demonstrates the neuroprotective role of gp130-STAT3 pathway in the rod photoreceptors under the chronic photo-oxidative stress, another series of studies showed that the PI-3 kinase/AKT pathway could protect rod photoreceptors under the acute photo-oxidative stress. Using a conditional gene knockout approach, Rajala et al. showed that insulin receptor, a PI-3 kinase upstream regulator, had a protective effect to rod photoreceptors under the acute photo-oxidative stress [62]. In another study using a conventional gene targeting approach, disruption of AKT2, a PI-3 kinase downstream target, accelerated the acute photo-oxidative stress-induced rod photoreceptor degeneration [63]. Finally, Zheng et al. demonstrated that BCL-xl, a downstream target of AKT, was a rod survival factor under acute photo-oxidative stress [44]. These studies clearly mapped the significance of PI-3 kinase/AKT pathway in stress-induced rod photoreceptor survival *in vivo*.

4.2. Protein Trafficking and Photoreceptor Degeneration. Kinesin-II is a molecular motor localized to the inner segment, connecting cilium, and axoneme of mammalian photoreceptors. The involvement of kinesin-II in protein trafficking through the mammalian photoreceptor cilium was initially probed with Cre/*lox*-based conditional gene targeting. Loss of kinesin-II in rods caused significant accumulations of opsin, arrestin, and membrane proteins within the photoreceptor inner segment, which ultimately led to the death of photoreceptors, a phenotype that is commonly observed in retinitis pigmentosa [20]. Further experiments also suggested that ectopic accumulation of opsin was a primary result of rod-specific kinesin-II deletion [21]. Using a conditional gene targeting approach, Avasthi et al. recently demonstrated that heterotrimeric kinesin-II acted as a molecular motor for proper trafficking of membrane proteins within the cone photoreceptors [64]. These conditional gene targeting studies established an unequivocal role of kinesin-II as a molecular motor that facilitates protein membrane trafficking in the photoreceptors.

4.3. Conditional Gene Targeting in the RPE. RPE is the gatekeeper of the retina and plays a pivotal role in the maintenance of retinal neurons. Abnormal RPE function is associated with both the wet and dry-forms of age-related macular denegation (AMD) (for review see [65, 66]). Although the pathogenic mechanisms for dry-AMD is unclear, clinical evidence suggests that photoreceptor degeneration is a consequence of impaired RPE functions [67, 68]. RPE-specific gene targeting will be a powerful approach for functional analysis of the RPE-expressed genes in the pathogenesis of dry-AMD. Whereas the use of conditional gene targeting in the PRE is still at its infancy, investigating the role of vascular endothelial growth factor (VEGF or VEGF-A), a potent angiogenic factor whose polymorphisms are associated with AMD [69, 70], in choroidal vascular development has yield some information related to the relationship between the RPE-derived VEGF and choroidal vasculature [2, 71]. As abnormal choroidal vasculature is clearly associated with both the dry- and wet-AMD [72–75], the genetic systems established in these studies may have some utility for AMD research. While the conditional gene targeting approach has yet to reach its full potential in AMD research, Lewin et al. recently demonstrated that disruption of mitochondrial manganese superoxide dismutase (SOD) in the RPE produced a geographic atrophy-like phenotypes in mice [76]. Here again, tissue/celltype-specific disruption of widely expressed genes, such as VEGF and SOD, circumvents the interference of nontargeting tissues/cells and is likely a direction for generating animal models used for mechanistic, diagnostic, and therapeutic investigations in the years to come.

5. Concluding Remarks

Remarkable progress has been made since the publication of the first study on the retinal denegation using a conditional gene targeting approach a decade ago [20]. It is also important to realize that, except in protein trafficking and photoreceptor survival, progress in other areas of retinal biology is not keeping the pace. At present, cellular mechanisms of many trophic factors and their signaling pathways in the retina remains unclear. Although the RPE and Müller cells are two major retinal supporting cell-types, the post-developmental functions of RPE and retinal Müller cell-derived trophic factors and their signaling mechanisms have remained largely uninvestigated. Substantial effort is necessary to establish a framework for cellular mechanisms of inherited retinal degeneration, AMD, and diabetes-induced retinal neuron degeneration. Many of these investigations

will require the use of conditional gene targeting approach. With the improved Cre-drive lines and effort in investigating cell-specific function of trophic factors and their signaling, significant progress in our understanding of retinal degeneration will be achieved in the near future. Ultimately, these findings will help to design therapeutic approaches for the treatment of the retinal degenerative diseases.

References

[1] N. Ferrara, K. Carver-Moore, H. Chen et al., "Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene," *Nature*, vol. 380, no. 6573, pp. 439–442, 1996.

[2] Y. Z. Le, Y. Bai, M. Zhu, and L. Zheng, "Temporal requirement of RPE-derived VEGF in the development of choroidal vasculature," *Journal of Neurochemistry*, vol. 112, no. 6, pp. 1584–1592, 2010.

[3] Y. Bai, J. X. Ma, J. Guo et al., "Müller cell-derived VEGF is a significant contributor to retinal neovascularization," *Journal of Pathology*, vol. 219, no. 4, pp. 446–454, 2009.

[4] S. Fukushige and B. Sauer, "Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 17, pp. 7905–7909, 1992.

[5] B. Sauer and N. Henderson, "Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 14, pp. 5166–5170, 1988.

[6] S. E. Nunes-Düby, H. J. Kwon, R. S. Tirumalai, T. Ellenberger, and A. Landy, "Similarities and differences among 105 members of the Int family of site-specific recombinases," *Nucleic Acids Research*, vol. 26, no. 2, pp. 391–406, 1998.

[7] F. Guo, D. N. Gopaul, and G. D. van Duyne, "Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse," *Nature*, vol. 389, no. 6646, pp. 40–46, 1997.

[8] Y. Le, S. Gagneten, D. Tombaccini, B. Bethke, and B. Sauer, "Nuclear targeting determinants of the phage P1 Cre DNA recombinase," *Nucleic Acids Research*, vol. 27, no. 24, pp. 4703–4709, 1999.

[9] S. Gagneten, Y. Le, J. Miller, and B. Sauer, "Brief expression of a GFPcre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions," *Nucleic Acids Research*, vol. 25, no. 16, pp. 3326–3331, 1997.

[10] M. Lakso, B. Sauer, B. Mosinger Jr. et al., "Targeted oncogene activation by site-specific recombination in transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 14, pp. 6232–6236, 1992.

[11] L. Madsen, N. Labrecque, J. Engberg et al., "Mice lacking all conventional MHC class II genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 18, pp. 10338–10343, 1999.

[12] A. J. H. Smith, M. A. de Sousa, B. Kwabi-Addo, A. Heppell-Parton, H. Impey, and P. Rabbitts, "A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination," *Nature Genetics*, vol. 9, no. 4, pp. 376–385, 1995.

[13] Y. Le, S. Gagneten, T. Larson et al., "Far-upstream elements are dispensable for tissue-specific proenkephalin expression using a Cre-mediated knock-in strategy," *Journal of Neurochemistry*, vol. 84, no. 4, pp. 689–697, 2003.

[14] S. Soukharev, J. L. Miller, and B. Sauer, "Segmental genomic replacement in embryonic stem cells by double lox targeting," *Nucleic Acids Research*, vol. 27, no. 18, article e21, 1999.

[15] B. Bethke and B. Sauer, "Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants," *Nucleic Acids Research*, vol. 25, no. 14, pp. 2828–2834, 1997.

[16] H. Gu, J. D. Marth, P. C. Orban, H. Mossmann, and K. Rajewsky, "Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting," *Science*, vol. 265, no. 5168, pp. 103–106, 1994.

[17] Y. Z. Le, J. D. Ash, M. R. Al-Ubaidi, Y. Chen, J. X. Ma, and R. E. Anderson, "Targeted expression of Cre recombinase to cone photoreceptors in transgenic mice," *Molecular Vision*, vol. 10, pp. 1011–1018, 2004.

[18] M. Akimoto, E. Filippova, P. J. Gage, X. Zhu, C. M. Craft, and A. Swaroop, "Transgenic mice expressing Cre-recombinase specifically in M- or S-cone photoreceptors," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 1, pp. 42–47, 2004.

[19] Y. Z. Le, L. Zheng, W. Zheng et al., "Mouse opsin promoter-directed Cre recombinase expression in transgenic mice," *Molecular Vision*, vol. 12, pp. 389–398, 2006.

[20] J. R. Marszalek, X. Liu, E. A. Roberts et al., "Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors," *Cell*, vol. 102, no. 2, pp. 175–187, 2000.

[21] D. Jimeno, L. Feiner, C. Lillo et al., "Analysis of kinesin-2 function in photoreceptor cells using synchronous Cre-loxP knockout of Kif3a with RHO-Cre," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 11, pp. 5039–5046, 2006.

[22] S. Li, D. Chen, Y. Sauvé, J. McCandless, Y. J. Chen, and C. K. Chen, "Rhodopsin-iCre transgenic mouse line for Cre-mediated rod-specific gene targeting," *Genesis*, vol. 41, no. 2, pp. 73–80, 2005.

[23] Y. Z. Le, W. Zheng, P. C. Rao et al., "Inducible expression of Cre recombinase in the retinal pigmented epithelium," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 3, pp. 1248–1253, 2008.

[24] M. Zhu, Y. Ueki, J. D. Ash, L. Zheng, and Y. Z. Le, "Unexpected transcriptional activity of human VMD2 promoter," *Advances in Experimental Medicine and Biology*, vol. 664, pp. 211–216, 2010.

[25] L. Guyonneau, A. Rossier, C. Richard, E. Hummler, and F. Beermann, "Expression of Cre recombinase in pigment cells," *Pigment Cell Research*, vol. 15, no. 4, pp. 305–309, 2002.

[26] M. Mori, D. Metzger, J. M. Garnier, P. Chambon, and M. Mark, "Site-specific somatic mutagenesis in the retinal pigment epithelium," *Investigative Ophthalmology and Visual Science*, vol. 43, no. 5, pp. 1384–1388, 2002.

[27] H. Zhao, Y. Yang, C. M. Rizo, P. A. Overbeek, and M. L. Robinson, "Insertion of a Pax6 consensus binding site into the αA-crystallin promoter acts as a lens epithelial cell enhancer in transgenic mice," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 6, pp. 1930–1939, 2004.

[28] K. Roesch, A. P. Jadhav, J. M. Trimarchi et al., "The transcriptome of retinal Müller glial cells," *Journal of Comparative Neurology*, vol. 509, no. 2, pp. 225–238, 2008.

[29] Y. Ueki, J. D. Ash, M. Zhu, L. Zheng, and Y. Z. Le, "Expression of Cre recombinase in retinal Muller cells," *Vision Research*, vol. 49, no. 6, pp. 615–621, 2009.

[30] M. Slezak, C. Göritz, A. Niemiec et al., "Transgenic mice for conditional gene manipulation in astroglial cells," *GLIA*, vol. 55, no. 15, pp. 1565–1576, 2007.

[31] E. Ivanova, G. S. Hwang, and Z. H. Pan, "Characterization of transgenic mouse lines expressing Cre recombinase in the retina," *Neuroscience*, vol. 165, no. 1, pp. 233–243, 2010.

[32] I. Dewachter, D. Reversé, N. Caluwaerts et al., "Neuronal deficiency of presenilin 1 inhibits amyloid plaque formation and corrects hippocampal long-term potentiation but not a cognitive defect of amyloid precursor protein [V7171] transgenic mice," *Journal of Neuroscience*, vol. 22, no. 9, pp. 3445–3453, 2002.

[33] M. Hatori, H. Le, C. Vollmers et al., "Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses," *PLoS ONE*, vol. 3, no. 6, article e2451, 2008.

[34] Z. Yang, K. Ding, L. Pan, M. Deng, and L. Gan, "Math5 determines the competence state of retinal ganglion cell progenitors," *Developmental Biology*, vol. 264, no. 1, pp. 240–254, 2003.

[35] K. D. Campsall, C. J. Mazerolle, Y. de Repentigny, R. Kothary, and V. A. Wallace, "Characterization of transgene expression and Cre recombinase activity in a panel of Thy-1 promoter-Cre transgenic mice," *Developmental Dynamics*, vol. 224, no. 2, pp. 135–143, 2002.

[36] S. Gong, M. Doughty, C. R. Harbaugh et al., "Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs," *Journal of Neuroscience*, vol. 27, no. 37, pp. 9817–9823, 2007.

[37] H. Saito, H. Tsumura, S. Otake, A. Nishida, T. Furukawa, and N. Suzuki, "L7/Pcp-2-specific expression of Cre recombinase using knock-in approach," *Biochemical and Biophysical Research Communications*, vol. 331, no. 4, pp. 1216–1221, 2005.

[38] X. M. Zhang, B. Y. Chen, A. H. L. Ng et al., "Transgenic mice expressing Cre-recombinase specifically in retinal rod bipolar neurons," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 10, pp. 3515–3520, 2005.

[39] H. Nakhai, S. Sel, J. Favor et al., "Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina," *Development*, vol. 134, no. 6, pp. 1151–1160, 2007.

[40] S. Rowan and C. L. Cepko, "Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter," *Developmental Biology*, vol. 271, no. 2, pp. 388–402, 2004.

[41] P. Weber, D. Metzger, and P. Chambon, "Temporally controlled targeted somatic mutagenesis in the mouse brain," *European Journal of Neuroscience*, vol. 14, no. 11, pp. 1777–1783, 2001.

[42] Y. Furuta, O. Lagutin, B. L. M. Hogan, and G. C. Oliver, "Retina- and ventral forebrain-specific Cre recombinase activity in transgenic mice," *Genesis*, vol. 26, no. 2, pp. 130–132, 2000.

[43] S. Sato, T. Inoue, K. Terada et al., "Dkk3-Cre BAC transgenic mouse line: a tool for highly efficient gene deletion in retinal progenitor cells," *Genesis*, vol. 45, no. 8, pp. 502–507, 2007.

[44] L. Zheng, R. E. Anderson, M. P. Agbaga, E. B. Rucker 3rd, and Y. Z. Le, "Loss of BCL-X in rod photoreceptors: increased susceptibility to bright light stress," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 12, pp. 5583–5589, 2006.

[45] Y. Ueki, J. Wang, S. Chollangi, and J. D. Ash, "STAT3 activation in photoreceptors by leukemia inhibitory factor is associated with protection from light damage," *Journal of Neurochemistry*, vol. 105, no. 3, pp. 784–796, 2008.

[46] U. A. K. Betz, C. A. J. Vosshenrich, K. Rajewsky, and W. Muller, "Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination," *Current Biology*, vol. 6, no. 10, pp. 1307–1316, 1996.

[47] Y. Ueki, S. Chollangi, Y. Z. Le, and J. D. Ash, "gp130 activation in Müller cells is not essential for photoreceptor protection from light damage," *Advances in Experimental Medicine and Biology*, vol. 664, pp. 655–661, 2010.

[48] J. Wang, E. Xu, M. H. Elliott, M. Zhu, and Y.-Z. Le, "Müller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage," *Diabetes*, vol. 59, no. 9, pp. 2297–2305, 2010.

[49] K. Nishijima, Y. S. Ng, L. Zhong et al., "Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury," *American Journal of Pathology*, vol. 171, no. 1, pp. 53–67, 2007.

[50] R. Jaenisch, "Transgenic animals," *Science*, vol. 240, no. 4858, pp. 1468–1474, 1988.

[51] M. Gossen, S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard, "Transcriptional activation by tetracyclines in mammalian cells," *Science*, vol. 268, no. 5218, pp. 1766–1769, 1995.

[52] R. Feil, J. Brocard, B. Mascrez, M. LeMeur, D. Metzger, and P. Chambon, "Ligand-activated site-specific recombination in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 20, pp. 10887–10890, 1996.

[53] Y. Le, J. L. Miller, and B. Sauer, "GFPcre fusion vectors with enhanced expression," *Analytical Biochemistry*, vol. 270, no. 2, pp. 334–336, 1999.

[54] S. Feil, N. Valtcheva, and R. Feil, "Inducible Cre mice," *Methods in Molecular Biology*, vol. 530, pp. 343–363, 2009.

[55] M. A. Chang, J. W. Horner, B. R. Conklin, R. A. DePinho, D. Bok, and D. J. Zack, "Tetracycline-inducible system for photoreceptor-specific gene expression," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 13, pp. 4281–4287, 2000.

[56] E. W. Gerner, "Ocular toxicity of tamoxifen," *Annals of Ophthalmology*, vol. 21, no. 11, pp. 420–423, 1989.

[57] A. Loonstra, M. Vooijs, H. B. Beverloo et al., "Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells," *Proceedings of the National Academy of*

Sciences of the United States of America, vol. 98, no. 16, pp. 9209–9214, 2001.

[58] E. E. Schmidt, D. S. Taylor, J. R. Prigge, S. Barnett, and M. R. Capecchi, "Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13702–13707, 2000.

[59] F. Chan, A. Bradley, T. G. Wensel, and J. H. Wilson, "Knock-in human rhodopsin-GFP fusions as mouse models for human disease and targets for gene therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 24, pp. 9109–9114, 2004.

[60] M. Haruta, R. A. Bush, S. Kjellstrom et al., "Depleting Rac1 in mouse rod photoreceptors protects them from photo-oxidative stress without affecting their structure or function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 23, pp. 9397–9402, 2009.

[61] Y. Ueki, Y. Z. Le, S. Chollangi, W. Muller, and J. D. Ash, "Preconditioning-induced protection of photoreceptors requires activation of the signal-transducing receptor gp130 in photoreceptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 50, pp. 21389–21394, 2009.

[62] A. Rajala, M. Tanito, Y. Z. Le, C. R. Kahn, and R. V. S. Rajala, "Loss of neuroprotective survival signal in mice lacking insulin receptor gene in rod photoreceptor cells," *Journal of Biological Chemistry*, vol. 283, no. 28, pp. 19781–19792, 2008.

[63] G. Li, R. E. Anderson, H. Tomita et al., "Nonredundant role of Akt2 for neuroprotection of rod photoreceptor cells from light-induced cell death," *Journal of Neuroscience*, vol. 27, no. 1, pp. 203–211, 2007.

[64] P. Avasthi, C. B. Watt, D. S. Williams et al., "Trafficking of membrane proteins to cone but not rod outer segments is dependent on heterotrimeric kinesin-II," *Journal of Neuroscience*, vol. 29, no. 45, pp. 14287–14298, 2009.

[65] J. S. Sunness, "The natural history of geographic atrophy, the advanced atrophic form of age-related macular degeneration," *Molecular Vision*, vol. 5, article 25, 1999.

[66] M. A. Zarbin, "Current concepts in the pathogenesis of age-related macular degeneration," *Archives of Ophthalmology*, vol. 122, no. 4, pp. 598–614, 2004.

[67] J. P. Sarks, S. H. Sarks, and M. C. Killingsworth, "Evolution of geographic atrophy of the retinal pigment epithelium," *Eye*, vol. 2, no. 5, pp. 552–577, 1988.

[68] S. H. Sarks, "Ageing and degeneration in the macular region: a clinico pathological study," *British Journal of Ophthalmology*, vol. 60, no. 5, pp. 324–341, 1976.

[69] A. J. Churchill, J. G. Carter, H. C. Lovell et al., "VEGF polymorphisms are associated with neovascular age-related macular degeneration," *Human Molecular Genetics*, vol. 15, no. 19, pp. 2955–2961, 2006.

[70] S. S. Boekhoorn, A. Isaacs, A. G. Uitterlinden et al., "Polymorphisms in the vascular endothelial growth factor gene and risk of age-related macular degeneration. The Rotterdam Study," *Ophthalmology*, vol. 115, no. 11, pp. 1899–1903, 2008.

[71] A. G. Marneros, J. Fan, Y. Yokoyama et al., "Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function," *American Journal of Pathology*, vol. 167, no. 5, pp. 1451–1459, 2005.

[72] J. E. Grunwald, S. M. Hariprasad, J. DuPont et al., "Foveolar choroidal blood flow in age-related macular degeneration," *Investigative Ophthalmology and Visual Science*, vol. 39, no. 2, pp. 385–390, 1998.

[73] D. S. McLeod, R. Grebe, I. Bhutto, C. Merges, T. Baba, and G. A. Lutty, "Relationship between RPE and choriocapillaris in age-related macular degeneration," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 10, pp. 4982–4991, 2009.

[74] X. Yi, N. Ogata, M. Komada et al., "Vascular endothelial growth factor expression in choroidal neovascularization in rats," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 235, no. 5, pp. 313–319, 1997.

[75] T. Ishibashi, Y. Hata, H. Yoshikawa, K. Nakagawa, K. Sueishi, and H. Inomata, "Expression of vascular endothelial growth factor in experimental choroidal neovascularization," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 235, no. 3, pp. 159–167, 1997.

[76] A. Lewin, S. Gibson, J. Liu, W. W. Hauswirth, and Y. Z. Le, "Generation of a Mouse Model of Early AMD by Induction of Oxidative Stress," ARVO Abstract, 6414, 2010.

Research Article

Mouse Model Resources for Vision Research

Jungyeon Won, Lan Ying Shi, Wanda Hicks, Jieping Wang, Ronald Hurd, Jürgen K. Naggert, Bo Chang, and Patsy M. Nishina

The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA

Correspondence should be addressed to Patsy M. Nishina, patsy.nishina@jax.org

The need for mouse models, with their well-developed genetics and similarity to human physiology and anatomy, is clear and their central role in furthering our understanding of human disease is readily apparent in the literature. Mice carrying mutations that alter developmental pathways or cellular function provide model systems for analyzing defects in comparable human disorders and for testing therapeutic strategies. Mutant mice also provide reproducible, experimental systems for elucidating pathways of normal development and function. Two programs, the Eye Mutant Resource and the Translational Vision Research Models, focused on providing such models to the vision research community are described herein. Over 100 mutant lines from the Eye Mutant Resource and 60 mutant lines from the Translational Vision Research Models have been developed. The ocular diseases of the mutant lines include a wide range of phenotypes, including cataracts, retinal dysplasia and degeneration, and abnormal blood vessel formation. The mutations in disease genes have been mapped and in some cases identified by direct sequencing. Here, we report 3 novel alleles of Crx^{tvrm65}, $Rp1^{tvrm64}$, and $Rpe65^{tvrm148}$ as successful examples of the TVRM program, that closely resemble previously reported knockout models.

1. Introduction

The Eye Mutant Resource (EMR) and the Translational Vision Research Models (TVRMs) programs currently housed at The Jackson Laboratory are tailored to provide genetically defined models of vision-associated diseases to the Research Community. The EMR screens for spontaneous mutations in the large production and repository colonies, while the TVRM program screens for chemically induced mutations in third-generation (G3) offspring of mutagenized mice. Both programs are motivated by the need for well-characterized models for studying the function of particular molecules in the eye, for examining disease pathology, and for providing a resource to test therapeutic regimens.

In the early phases of the EMR program, the tools for examining mice for ocular abnormalities were adapted for the small size of the mouse eye [1, 2]. These tools included indirect ophthalmoscopy, slit lamp biomicroscopy, fundus photography, and electroretinography (ERG). Initially, mice from various stocks and inbred strains were screened to identify spontaneous ocular mutants using the first two

methodologies. Currently, ERG screening is done as well to identify and characterize new retinal mutants. As secondary screens, fluorescein angiography is used to detect vascular changes [2], and noninvasive tonometry [3] is used to assess changes in intraocular pressure. Screening has also been expanded to include genetically engineered strains from the Jackson Laboratory's Genetic Resource Sciences (GRS) repository that are systematically examined as they are removed from the shelf or are retired from breeding. Also, in addition to the initial phenotypic characterization, the EMR strives to identify the mutations underlying the disorders.

Systematic chemical mutagenesis screens have been successfully carried out in several model organisms, including *Drosophila* [4], *C. elegans* [5], and zebrafish [6, 7]. The zebrafish screens have provided valuable eye models, especially those pertaining to eye development [8]. In addition to our efforts, other mutagenesis screens for eye phenotypes in mice have been reported in which a number of mutants have been described [9–11]. Although different methods for mutagenizing mice are available, the alkylating agent, N-ethyl-N-nitrosourea (ENU), is the mutagen most commonly

used [12]. ENU mainly induces point mutations resulting in a range of consequences including total or partial loss-of-function, dominant-negative, or gain-of-function alleles [13–16]. Its effectiveness as a mutagen is dependent on dosage, frequency of administration, and mouse strain. Effectiveness, in terms of identifying mutants, depends upon the type of screen (e.g., dominant versus recessive) and the reproducibility of the phenotypic assay utilized. Mutant recovery has ranged from a rate of 1/175 [17], to ~1/666 in Specific Locus Tests (SLTs) [12], and to an average of 1/1470 based on recessive screening in a defined chromosomal region [18]. The mutation rates for individual loci can vary by almost tenfold [12, 17, 18].

The majority of large-scale mutagenesis screens have been dominant screens. This is probably due to the relative ease of creating mutagenized mice for dominant screens compared to recessive ones. Screening for dominants on a genome-wide basis can be done in one generation (G_1), while recessives generally require three. The Neuherburg Cataract Mutant Collection of ~170 dominant mutants was assembled through screening over 500,000 first-generation mice exposed to various mutagens [19]. The GSF-Munich [14] and MRC-Harwell [13, 20] programs were established using a phenotype-based approach to screen thousands of mice for dominant mutations affecting a variety of biological processes. A major drawback to dominant screens, however, is that not all mutations have dominant effects. A dominant screen will, therefore, miss many of the induced mutations. Estimates suggest that the frequency of diseases caused by recessive mutations is 4–10-fold higher than for dominant ones. In fact, of 218 eye mutants surveyed in the Mouse Genome Informatics Database, 80% were recessive mutations and only 20% were dominant or semidominant. Therefore, the TVRM program screened a G3 population of mutagenized mice for recessive mutations.

Screening for spontaneous and chemically induced mutants provides an important source of models to study the effects of single-gene mutations found in human patients. Additionally, new mutations within the same gene provide allelic series in which splice variants or domain-specific effects can be queried. Finally, mutations in novel genes that lead to retinal disorders can be discovered using a forward genetic approach.

2. Materials and Methods

2.1. Origins of Mice and Husbandry. The ages at which the visual system is affected by disease can vary considerably. For the EMR program, an initial screen of JAX Mice & Services (JMSs) production colonies and mice removed from the GRS Repository is routinely performed at ~2 months of age and if necessary, additional screening is done at an older age, usually at 6 months of age. Also, as with other neuronal diseases, diseases of the visual system are not reversible, so ocular diseases can be captured in retired breeders. Therefore, when available, retired breeders that are older than 1 year of age are screened. C57BL/6J (B6) G3 ENU mutagenized mice were screened by the TVRM program. For
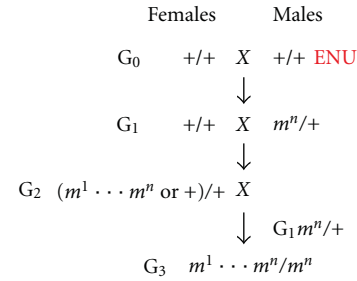
<center>Females   Males</center>

$$G_0 \quad +/+ \quad X \quad +/+ \text{ ENU}$$
$$\downarrow$$
$$G_1 \quad +/+ \quad X \quad m^n/+$$
$$\downarrow$$
$$G_2 \quad (m^1 \cdots m^n \text{ or } +)/+ \quad X$$
$$\downarrow \quad G_1 m^n/+$$
$$G_3 \quad m^1 \cdots m^n/m^n$$

FIGURE 1: Schematic representation of the mating scheme of dominant (G_1) or recessive (G_3) screens. Male mice were mutagenized (3 weekly doses, 80 mg/kg) and mated to WT females after 4 weeks. If any female was pregnant within 5 weeks, the mating was discarded. If, however, male mice impregnated a female after that, the resulting G_1 males were crossed to their respective female counterparts, and the G_2 progeny were backcrossed to the G_1 fathers to generate G_3 offspring.

the chemically induced mutations, ENU was administered to male B6 mice in three weekly injections of 80 mg/kg. G3 offspring were generated using a three-generation backcross mating scheme (Figure 1). G3 mice were screened at 24 weeks of age in order to enhance our ability to identify late onset diseases.

To determine if the disease phenotype was inheritable, mutant mice were outcrossed to wild-type (WT) mice to generate F1 progeny with subsequent intercrossing of the resultant F1 mice to generate F2 progeny. Both F1 and F2 mice were examined by indirect ophthalmoscopy or ERG. If F1 mice were affected, the pedigree was designated as segregating a dominant mutation. If F1 mice were not affected but ~25% of F2 mice were affected, the pedigree was designated as segregating a recessive mutation. Once the observed ocular phenotype was determined to be genetically heritable, mutants were bred and maintained in the Research Animal Facility at JAX. Mice were provided with NIH 6% fat chow diet and acidified water, with 12:12 hour dark:light cycle in pressurized individual ventilation caging which are monitored regularly to maintain a pathogen-free environment. Procedures utilizing mice were approved by the Institutional Animal Care and Use Committee.

2.2. Clinical Evaluation and Electroretinography. Mice, dark adapted for a minimum of 1 hour, were treated with atropine prior to examination by indirect ophthalmoscopy with a 60 or 78 diopter aspheric lens. Fundus photographs were taken with a Kowa small animal fundus camera using a Volk superfield lens held 2 inches from the eye as previously described [2].

For electroretinographic evaluation of mutants, following a 2-hour dark adaptation, mice were anesthetized with an intraperitoneal injection of xylazine (80 mg/kg) and ketamine (16 mg/kg) in normal saline. Additional anesthetic was given if akinesia was inadequate. The equipment and protocol used here were those previously described [21]. Briefly, dark-adapted, rod-mediated ERGs were recorded

with the responses to short-wavelength flashes over 4.0-log unit to the maximum intensity by the photopic stimulator. Cone-mediated ERGs were recorded with white flashes after 10 min of complete light adaptation. The signals were sampled at 0.8 msec intervals and averaged.

2.3. Genetic Mapping. Genomic DNA was isolated from tail tips using a PBND (PCR buffer with nonionic detergents) preparation, which was adapted from a protocol from Perkin Elmer Cetus [22]. Tail tips were digested in PBND buffer + Proteinase K overnight at 55°C. Samples were heated to 95°C for 10 minutes, and 1 μL of the DNA preparation was used in a 12 μL PCR reaction. Amplicons were visualized with ethidium bromide after electrophoretic separation on a 4% agarose gel.

For mapping purposes, phenotypically affected mice, presumed to be homozygous for the mutations, were mated with DBA/2J mice. The resulting F1 offspring were intercrossed to generate F2 offspring if recessive and backcrossed (BC) to WT parental if dominant. Resulting progeny were phenotyped by indirect ophthalmoscopy. DNA isolated from tail tips from a minimum of 10 affected and 10 unaffected mice was pooled and subjected to a genome-wide scan using 48–80 simple sequence length polymorphic markers distributed throughout the genome. Samples used in the DNA pools were tested individually to confirm the map location [23].

2.4. Preparation of RNA Samples and Subsequent Analysis. Total RNA was isolated from whole eyes and brains of affected mutants and B6 mice using TRIzol Reagent (Life Technologies) per manufacturer's protocol. Total RNA was treated with RNase-free DNaseI (Ambion) and quantity was determined using a NanoDrop spectrophotometer (Thermo Scientific). RNA quality was evaluated with an Agilent Technologies 2100 Bioanalyzer. cDNA was generated using the Retroscript kit (Ambion).

Primers to sequence the coding region of the candidate genes were designed from exon sequences obtained from the Ensembl Database. RT-PCR was done using eye cDNA in a 24 μL PCR reaction containing 1xPCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 250 μM of each dATP, dCTP, dGTP, dTTP, 0.2 μM of each forward and reverse primer, 1.5 mM MgCl2, and 0.6 U Taq polymerase. The following PCR program was used: 94°C for 1 minute 30 sec followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 45 sec, and a final extension of 72°C for 2 minutes. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. DNA fragments were sequenced on an Applied Biosystems 3730XL (using a 50 cm array and POP7 polymer).

2.5. Histological Analysis. Mice were asphyxiated by carbon dioxide inhalation, and enucleated eyes were fixed overnight in cold methanol/acetic acid solution (3:1, v/v). The paraffin-embedded eyes were cut into 6 μm sections, stained by hematoxylin and eosin (H and E), and examined by light microscopy.

3. Results and Discussion

3.1. Status of the EMR Program. Since its inception in the 1980s, the EMR program has identified and/or imported more than 100 mouse models with ocular abnormalities for research. Table 1 lists some of the retinal degeneration mouse models of human disease developed and/or currently maintained in the EMR that are available to the Research Community. Other models are described on the EMR web page (http://eyemutant.jax.org/).

3.2. Status of the TVRM Program. The TVRM program was built upon the success of the Neuromutagenesis Facility (NMF) at The Jackson Laboratory, and 15 of the 60 mutant lines (Tables 2 and 3) in which a disease phenotype has been subsequently fixed as a coisogenic inbred strain by the TVRM program were first identified in screens conducted by the NMF. The remaining 45 TVRM lines were established by screening ~14,000 G3 mice for anterior and posterior segment abnormalities by indirect ophthalmoscopy and/or slit lamp biomicroscopy. Six of the 60 mutations (10%) are inherited in a dominant or codominant manner, and the remaining are recessive mutations. Forty six of the mutants have retinal phenotypes ranging between pan-retinal spots or patches, pigmentation defects, and/or attenuation of blood vessels with or without morphological changes that were detectable by light microscopy. Six of the mutant lines have reduced or absent ERG responses for either rod and/or cone cells without photoreceptor loss. Five mutant lines presented with vitreal fibroplasia and three with cataracts. Forty six of the mutations (23 reported in Table 3) have been localized to a chromosome, and the molecular basis has been identified for 23 of them (Table 2). Fourteen lines are still in the process of being mapped (data not shown). Nineteen of the 23 mutations in Table 2 were novel alleles in genes in which mutations had previously been reported. Some of these mutants are described below. It should be noted that the current bias for reoccurrences of mutations, herein referred to as remutations, versus identification of novel genes in Table 2 is probably due to the fact that once a mutation is mapped, candidate genes previously associated with an eye disease can be quickly sequenced. Regions containing no obvious candidate genes need to be narrowed further and/or all genes within the region may need to be sequenced to identify the disease-causing mutation.

Interestingly, new phenotypes were observed in 8 of the remutations that have been examined (see; [51–55], personal communication PMN). For example, outer segments (OSs) were either formed abnormally or did not initiate in retinas from homozygous $Rpgrip1^{nmf247}$ mice [51]. This was in contrast to the $Rpgrip1^{tmlTili}$ targeted null mutant, hereafter, $Rpgrip1^{-/-}$ in which OS discs were formed and stacked vertically rather than horizontally [56]. Targeted alleles of $Lama1$ were reported to be embryonic lethal [57, 58]. The ENU-induced allele, $Lama1^{nmf223}$, provides a viable, hypomorphic allele in which abnormalities in the adult animal could be examined. Clinically, vitreal fibroplasia and abnormal retinal vasculature were observed. Histologically,

Table 1: Mouse retinal mutants maintained in the Eye Mutant Resource (EMR) at The Jackson Laboratory.

Model	Mode	Gene	Chr.	Clinical phenotype
rd1	AR	Pde6b	5	Early onset, severe retinal degeneration [24]
pcd	AR	Agtpbp1	13	Slower retinal degeneration associated with Purkinje cell degeneration [25]
nr	AR	UN	8	Progressive retinal degeneration with hyperactive ataxic behavior (nervous) [25]
Rd2	AD	Prph2	17	Slow progressive retinal degeneration [26]
rd3	AR	Rd3	1	Retinal degeneration, beginning at 3 weeks of age [27]
Rd4	AD	Gnb1	4	Autosomal dominant retinal degeneration [28]
Tub	AR	Tub	7	Retinal degeneration, hearing loss, and late-developing obesity, also known as rd5 [29]
mnd	AR	Cln8	8	Early onset retinal degeneration with a late-onset progressive motor neuron degeneration [30]
rd6	AR	Mfrp	9	Small, white retinal spots and progressive photoreceptor degeneration [31]
rd7	AR	Nr2e3	9	Retinal spots and progressive photoreceptor degeneration [32]
rd8	AR	Crb1	1	Focal photoreceptor degeneration [33]
Rd9	XD	UN	X	Progressive retinal white spotting and degeneration [33]
rd10	AR	Pde6b	5	Early onset, mild retinal degeneration [34]
rd11	AR	Lpcat1	13	Retinal degeneration with white retinal vessels at 4 weeks of age [35]
rd12	AR	Rpe65	3	Poor ERG response and late onset retinal degeneration [36]
rd14	AR	UN	18	Slow retinal degeneration with white retinal spots [37]
rd15	AR	UN	7	Retinal degeneration with retinal outer plexiform dystrophy [38]
rd16	AR	Cep290	10	Early onset retinal degeneration [39]
rd17	AR	Gnat1	9	Poor rod ERG response and slow retinal degeneration [40]
cpfl1	AR	Pde6c	19	Cone photoreceptor function loss-1 [41]
Cpfl2	AD	UN	3	Cone photoreceptor function loss-2 with white retinal spots [42]
cpfl3	AR	Gnat2	3	Cone photoreceptor function loss-3 [43]
Cpfl4	AD	UN	17	Cone photoreceptor function loss-4 [44]
cpfl5	AR	Cnga3	1	Cone photoreceptor function loss-5 [45]
cpfl6	AR	Hcn1	13	Cone photoreceptor function loss-6 [46]
cpfl7	AR	UN	19	Cone photoreceptor function loss-7 [47]
nob2	XR	Cacna1f	X	Anatomical and functional abnormalities (no b-wave-2) in the outer retina [48]
nob3	AR	Grm6	11	Retinal functional abnormalities (no b-wave 3) [49]
arrd2	AR	Mdm1	10	Age-related retinal degeneration-2 [50]

AR: autosomal recessive, AD: autosomal dominant, XR: X-linked recessive, UN: unknown.

persistent hyaloid vessels and fibrous tissue were found in the vitreal space, and the inner limiting membrane was disrupted [52]. In an allelic series of mutations within the rhodopsin gene, light-induced retinal degeneration was observed. Heterozygous Rho^{Tvrm1} and Rho^{Tvrm4} mice raised in standard vivarium lighting did not exhibit any morphological changes until exposed to bright light [54]. Previously Rho alleles showed spontaneous and pan-retinal degeneration, even when mice were reared from birth in darkness [59].

3.3. New Alleles of Crx^{tvrm65}, $Rp1^{tvrm64}$, and $Rpe65^{tvrm148}$

3.3.1. Crx^{tvrm65}. tvrm65 segregates as a recessive mutation that is characterized by a pan-retinal, grainy fundus appearance that eventually progresses with age to patches of depigmentation within the central retina (data not shown). The mutation was mapped to chromosome (Chr.) 7 between flanking markers D7Mit75 and D7Mit190. A single nucleotide polymorphic (SNP) marker (SNP ID: RS13479126) served to narrow the interval. Crx, a reasonable

biological candidate gene, contained within the minimal interval, was examined for a mutation.

CRX is an evolutionary conserved protein. Mice and humans share a 97% sequence similarity. To date, two Crx transcripts have been reported. The long isoform (Genbank nm_001113330) has 25 additional amino acids (aa) in its N terminus when compared to the shorter isoform (Genbank nm_007770). A T>A nonsense mutation identified in Crx^{tvrm65} is located in the last exon and is expected to affect both isoforms. The tvrm65 mutation is predicted to cause an early termination at Leu277 (TTG) of the 323 aa from the longer isoform or at Leu253 of a 299 aa product from the shorter isoform (Figure 2(a)).

Phenotypically, Crx^{tvrm65} mutants resemble the null mouse model in which the single homeodomain containing region [60] of Crx was targeted. Homozygous Crx^{tm1Clc} mice do not develop OS and photoreceptors degenerate. Crx^{tvrm65} mutants show a rapid photoreceptor degeneration (Figure 2(b)). At postnatal day (P) 14 and P21, OSs were absent and inner segments (ISs) were rarely observed (Figure 2(b)). By P21, photoreceptor cell bodies were reduced to ~60% of

TABLE 2: Mouse mutants from the Translational Vision Research Models (TVRMs) program in which the molecular basis for the disease phenotype has been identified.

Model	Mode	Gene	Chr.	Clinical phenotype
tvrm64	AR	Rp1	1	Juvenile onset retinal degeneration
nmf12	AR	Mertk	2	Late onset slow degeneration
tvrm148	AR	Rpe65	3	Late onset retinal degeneration
nmf192	AR	Nphp4	4	Early rapid retinal degeneration
nmf364	AR	Pde6b*	5	Early rapid retinal degeneration
nmf449	AR	Pde6b*	5	Early rapid retinal degeneration
Tvrm1	AD	Rho	6	Light inducible retinal degeneration [51]
Tvrm4	AD	Rho	6	Light inducible retinal degeneration [51]
Tvrm144	AD	Rho	6	Light inducible retinal degeneration
tvrm65	AR	Crx	7	Early rapid retinal degeneration
tvrm27	AR	Trpm1	7	No B-wave
tvrm89	AR	Myo6	9	Attenuated ERG
tvrm84	AR	Grm1	10	Attenuated ERG
nmf246	AR	Uchl3	14	Juvenile onset retinal degeneration
nmf247	AR	Rpgrip1	14	Early rapid retinal degeneration [52]
nmf5a	AR	Pfnd5	15	Early rapid retinal degeneration
nmf240	AR	Clcn2	16	Early rapid retinal degeneration [53]
nmf223	AR	Lama1	17	Vitreal fibroplasia, vascular abnormalities [54]
tvrm124	AR	Tulp1*	17	Early rapid retinal degeneration
nmf282	AR	Pde6a	18	Early rapid retinal degeneration [55]
nmf363	AR	Pde6a	18	Early rapid retinal degeneration [55]
tvrm58	AR	Pde6a*	18	Early rapid retinal degeneration
tvrm32	AR	Hps1*	18	Pigmentation defect

*Established by complementation testing.

TABLE 3: Mouse mutants from the Translational Vision Research Models (TVRMs) program in which the molecular basis of the disease phenotype has not yet been identified.

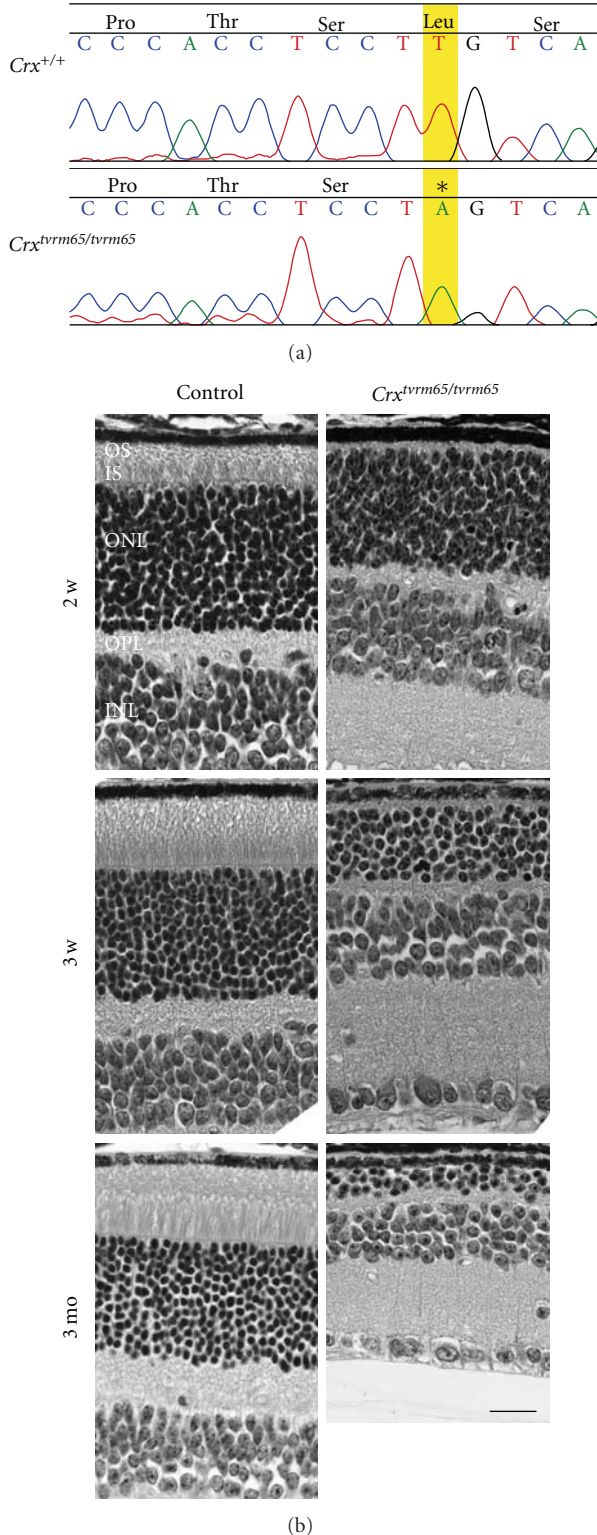| Model	Mode	Chr.	Clinical phenotype
tvrm9	AR	1	Retinal spots
tvrm113	AR	4	Retinal spots, grainy fundus appearance
Tvrm6	AD	7	Retinal spots
tvrm116	AR	12	Retinal spots, late onset
tvrm111	AR	14	Retinal spots
nmf289	AR	16	Retinal spots
tvrm5	AR	18	Retinal spots in central retina
tvrm10	AR	19	Retinal spots, coloboma, and vascular defects
tvrm77	AR	6	Central patches
tvrm119	AR	18	Retinal patches
tvrm127	AR	18	Retinal patches
tvrm102	AR	6	Grainy retina
tvrm101	AR	10	Grainy retina
nmf67	AR	7	Fine web-like fundus appearance
Tvrm122	AD	3	Shiny flecks
tvrm64a	AR	12	None, identified through histology, lamination defect
tvrm111b	AR	8	Abnormal ERG
tvrm87	AR	4	Vitreal fibroplasia
tvrm114	AR	4	Vitreal fibroplasia, cataracts
tvrm53	AR	7	Vitreal fibroplasia
tvrm85	AR	18	Vitreal fibroplasia
Tvrm49	AD	15	Cataracts
tvrm129	AR	13	Cataracts

(a)

(b)

Figure 2: The mouse model Crx^{tvrm65}. (a) The mutation in homozygous Crx^{tvrm65} causes a premature termination at aa residue Leu277. The mutated nucleotide is highlighted (b). Histology of control and Crx^{tvrm65} mutant retina at P14, P21, and 3 months of age. OSs were absent at all ages in homozygous Crx^{tvrm65}, and progressive thinning of IS, ONL, and OPL was observed. OSs: outer segments, ISs: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer. Scale bar = 20 µm.

controls. The outer plexiform layer (OPL) was also thinner, approximately 40% of controls. By 3 months of age, the OSs and ISs were absent and only 2~3 layers of outer nuclear layer (ONL) were remained. The photoreceptor degeneration observed in the Crx^{tvrm65} mutants occurs more rapid than reported for the null allele [60]. This may, in part, be due to the difference in genetic background of the two alleles as Crx^{tvrm65} was generated on a B6 background, whereas the previous null allele was described on a segregating B6 and 129Sb genetic background.

3.3.2. $Rp1^{tvrm64}$. *tvrm64* segregates as a recessive mutation that is characterized by a grainy fundus appearance and attenuated retinal vessels (data not shown). The mutation mapped to Chr.1 between the centromere and *D1Mit427*, an interval in which *Rp1* resides. *Rp1* encodes a large protein of 2095 aa in mouse and 2156 aa in humans. RP1 localizes in the connecting cilia and appears to play a structural and/or functional role in molecular transport through the connecting cilia [61, 62]. Mouse RP1 shares 72% similarity with human RP1. Structurally, it has two ubiquitin homolog (UBQ) domains in its amino terminus. *Rp1* was tested for a mutation, as the phenotype of homozygous *Tvrm64* mutants was similar to that of mice carrying either of two targeted *Rp1* alleles, involving homologous recombination in which exons 2 and 3 were targeted ($Rp1^{tm1Jn2}$) [61] or a truncation after codon 662, $Rp1^{tm1Eap}$, analogous to the R667ter mutation in humans [62]. Direct sequencing of homozygous $Rp1^{tvrm64}$ retinal cDNA revealed an A>T transversion at nucleotide 1769 (Genbank nm_011283), creating a nonsense mutation in which Arg522 (AGA) is changed to a termination codon (TGA; Figure 3(a)). The mutation is localized adjacent to the two UBQ domains in RP1.

The OS length of $Rp1^{tvrm64}$ mutant retina was approximately 50% shorter than WT controls at 1 month of age (Figure 3(b)). The difference in IS length between mutant and controls, however, was barely discernable at 1 month of age but was obviously shorter in $Rp1^{tvrm64}$ mutants at 3 months of age. The photoreceptor degeneration was progressive with little difference in cell body number in the ONL at 1 month of age but by 3 months, cell nuclei were reduced to ~50% in mutants in comparison to controls. In contrast, the photoreceptor morphology of $Rp1^{tm1Jn2}$ mice [61] appeared normal by light microscopy at P30 with comparable length of OS in mutant and controls. Also, $Rp1^{tm1Eap}$ mice [62] at P30 showed shorter OS lengths and a 1–2-layer reduction in ONL. Therefore, the disease progression in $Rp1^{tvrm64}$ at similar age appears to be more severe than observed in $Rp1^{tm1Jn2}$ mice but less severe than $Rp1^{tm1Eap}$ mice.

This difference between the models was also discernable functionally. At 1 month of age, dark-adapted ERGs of $Rp1^{tvrm64}$ mice were comparable to WT (Figures 3(c) and 3(d)). In $Rp1^{tm1Eap}$, these responses were significantly reduced at 4~5 weeks of age [62].

3.3.3. $Rpe65^{tvrm148}$. The recessive *tvrm148* mutation is characterized by late onset retinal spotting and by patches
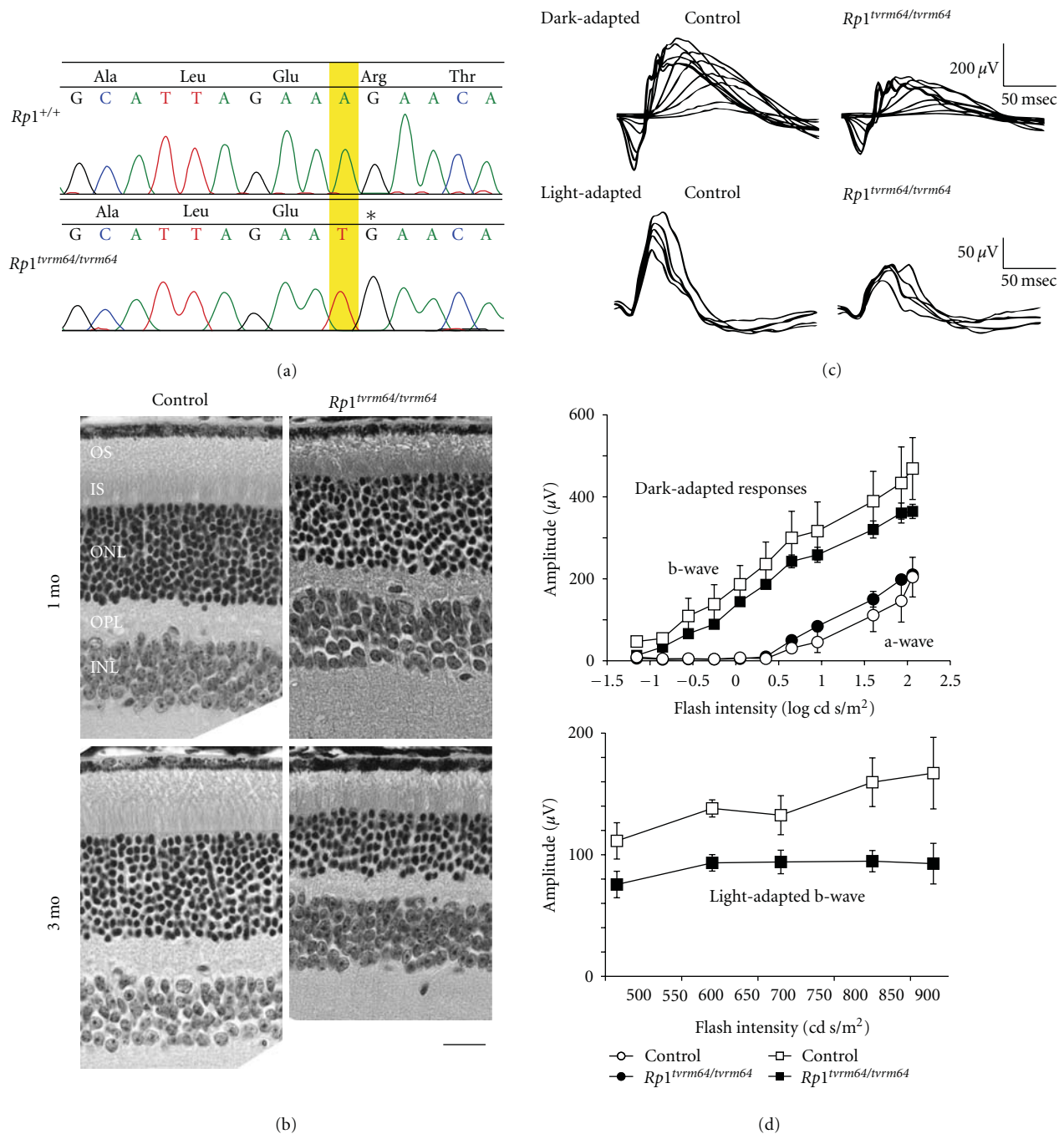
(a)

(c)

(b)

(d)

FIGURE 3: The mouse model $Rp1^{tvrm64}$. (a) Direct sequencing of control and $Rp1^{tvrm64}$ homozygous mutant identified an A to T mutation, predicting early termination at Arg522. The position of the mutation is highlighted and an asterisk indicates the termination. (b) The retinal morphology of control and $Rp1^{tvrm64}$ mice was examined at 1 and 3 months of age (mo). OSs: outer segments, ISs: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer. Scale bar = 20 μm. (c) Electroretinogram of dark-adapted (scotopic) and light-adapted (photopic) control at 9 weeks of age and $Rp1^{tvrm64}$ at 4 weeks of age. (d) The amplitude of dark-adapted a and b-wave and light-adapted b-wave ($\pm$SEM, $n = 3$) of 4 weeks old $Rp1^{tvrm64}$ mice and age matched controls.

of depigmentation that is readily discernable by indirect ophthalmoscopy at 5 months of age (data not shown). The mutation mapped to Chr. 3 between markers, *D3Mit147* and *D3Mit19*. Rpe65 was screened by direct sequencing for a mutation as it fell within the minimal interval identified, and the disease phenotype was similar to that reported

for the $Rpe65^{tm1Tmr}$ targeted knockout animal (herein referred to as $Rpe65^{-/-}$) [63] and $Rpe65^{rd12}$ [64] alleles. A T>C point mutation was found by direct sequencing of retinal cDNA from $Rpe65^{tvrm148}$ mice and is expected to generate a mutant protein with an F229S point mutation (Figure 4(a)). F229 is evolutionarily conserved from
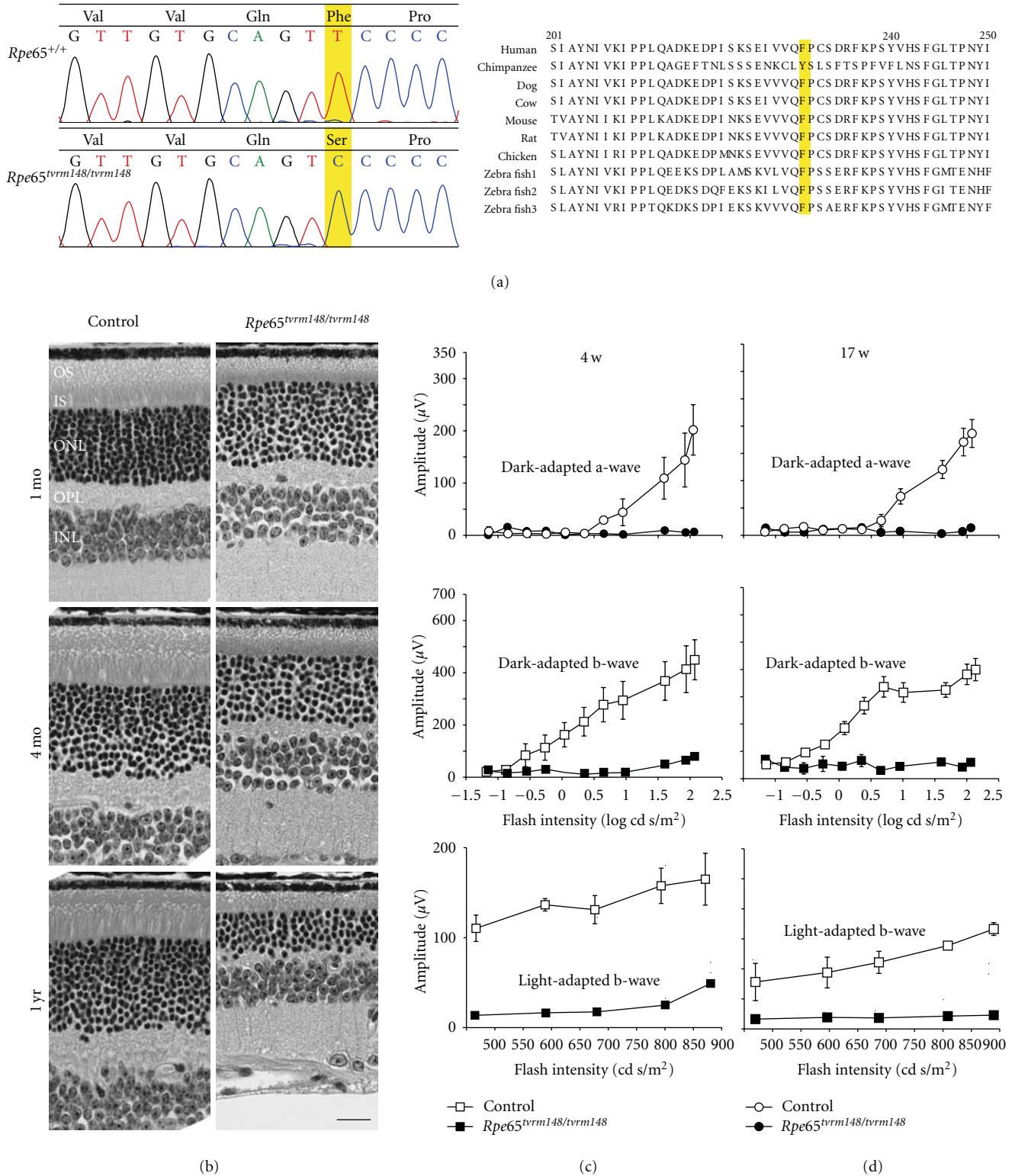
(a)

(b)

(c)

(d)

FIGURE 4: The $Rpe65^{tvrm148}$ mouse model. (a) Mutation analysis by direct sequencing revealed that the homozygous $Rpe65^{tvrm148}$ mouse harbored a missense mutation at aa residue 229, causing an amino acid change from Phe to Ser. The highlighted nucleotide indicates the mutation in the $Rpe65^{tvrm148}$ mouse (left). RPE65 protein is an evolutionarily conserved protein, and F229 is a nearly invariant residue from human to zebra fish (right). (b) Retinal morphology at 1 and 4 months and 1 year of age was analyzed by light microscopy. ONL thinning was progressive, and IS/OS was shorter than controls at all ages examined. OSs: outer segments, ISs: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer. Scale bar = 20 μm. (c, d) Physiological retinal function was analyzed by ERG at 4 weeks (c) and 17 weeks of age (d). The plotted amplitude was obtained at 9 weeks from control and $Rpe65^{tvrm148}$ mice (c) or at 17 weeks from control and from homozygous $Rpe65^{tvrm148}$ mice. $N = 3$.

humans to zebra fish but interestingly not in chimpanzee (Figure 4(a)).

The $Rpe65^{tmlTmr}$ mutant [63] had a nonfunctional rod ERG response due to the lack of 11-*cis*-retinal production in the RPE and showed disorganized rod outer segments. Another targeted allele mimicking a human R91W mutation was found in Leber Congenital Amaurosis (*LCA2*) patients ($Rpe65^{tmlLreb}$) [65], and a spontaneous model $Rpe65^{rd12}$ [64] showed a similar disease progression to that observed in $Rpe65^{tvrm148}$ mutants. Photoreceptors degenerated progressively in homozygous $Rpe65^{tvrm148}$ mouse from 1 month to 1 year of age, the latest time point examined (Figure 4(b)). At 1 month of age, OS and IS lengths were approximately 50% shorter than controls with no obvious thinning of the ONL. The photoreceptor nuclei were reduced in thickness by ~20% at 4 months and ~60% by 1 year of age.

Like the three previously reported mouse models, $Rpe65^{tvrm148}$ exhibited severely impaired rod ERGs and relatively spared cone ERGs. Rod responses were absent by 4 weeks of age. However, cone b-wave ERGs were comparable to controls at 4 weeks of age but by 17 weeks, the amplitudes were reduced compared to controls (Figures 4(c)–4(d)).

4. Conclusions

4.1. The Utility of Spontaneous and Chemically Induced Mutations. Spontaneous or chemically induced mutations in mice provide a rich source of animal models. These mutations offer some advantages for the study of human genetic diseases and basic gene function over mutations obtained by homologous recombination. First, these mutations are generally identified because they cause a clinically relevant phenotype. By starting with a known phenotype, information about the physiological function of the mutant gene and its biomedical relevance is immediate. Second, the forward genetic approach has the potential for discovery of new genes involved in ocular development and function that were previously unappreciated. Further, spontaneous and chemically induced mutations may better model naturally occurring human genetic conditions. They produce a full and unbiased array of mutation types—single base pair changes or deletions, and in the case of spontaneous mutations, retroviral insertions, repeat sequence expansions, and chromosomal rearrangements. These mutations can create alternatively spliced transcripts or nonsense or missense reading frames. They can abolish all protein function (null), partially diminish function (hypomorphic), or change function (dominant negative or gain-of-function). Moreover, allelic series—collections of mutant alleles of the same gene—can provide domain specific information about protein function and information on alternatively spliced variants. Biomedically relevant phenotypes associated with some human genetic disorders may be revealed by the different alleles that are not replicated by knockout alleles. For example, whereas the null alleles of *Lama1* [57, 58] were embryonic lethal, the hypomorphic ENU *nmf223* allele allowed for the examination of ocular phenotypes in adult mice [52]. In another example, the *rd10* allele of *Pde6b* [66]

identified by the EMR program has a later onset and slower rate of degeneration than the original *rd1* allele, thus allowing for the opportunity to test therapeutic strategies [67]. Finally, two phosphodiesterase 6a mutations first described by the TVRM program cause missense mutations that lead to different biochemical outcomes and rates of photoreceptor degeneration, suggesting a difference in the importance of the particular mutant residues to the function of the protein [55].

It should also be noted that spontaneous mutations occur on a wide variety of strain backgrounds, and chemical mutagenesis can be carried out in different genetic backgrounds. The observation of altered mutant phenotypes in different genetic backgrounds can provide a means for identifying interacting genes and molecular pathways of pathophysiology. For example, $Nr2e3^{rd7}$ was observable clinically only in the B6 genetic background [68], and a number of genetic backgrounds act to ameliorate the disease [69]. $Crb1^{rd8}$ is observable clinically in the C3H/HeJ background but not in the B6 background [70], and the null mutation is phenotypically different on a segregating 129X1/SvJ and B6 background [71]. Finally, a wide variety of disease phenotypes are observed in *rd3* [27] and $Gnb1^{rd4}$ [28] in different strain backgrounds, indicating interactions with genetic background modifiers. The variation in genetic background enables discovery of modifiers and gene interactions and could be essential to the discovery of important mutant phenotypes and potential targets for therapeutic intervention.

4.2. The Future of the EMR and TVRM Programs. In the future, the EMR will continue to screen for spontaneous mutations in the large production colonies at The Jackson Laboratory. The mutants identified in the TVRM program will be incorporated into the EMR distribution colonies as the molecular bases of the mutations are identified. Finally, sensitized chemical mutagenesis screens are planned that will uncover pathways important in retinal development, maintenance, and function.

Acknowledgments

References

[1] B. Chang, N. Hawes, M. Davisson, and J. Heckenlively, "Mouse models of RP," in *Retinal Degenerations: Biology, Diagnostics, and Therapeutics*, J. Tombran-Tink and C. Barnstable, Eds., pp. 149–164, The Human Press, 2007.

[2] N. L. Hawes, R. S. Smith, B. Chang, M. Davisson, J. R. Heckenlively, and S. W. John, "Mouse fundus photography and angiography: a catalogue of normal and mutant phenotypes," *Molecular Vision*, vol. 5, p. 22, 1999.

[3] W.-H. Wang, J. C. Millar, I.-H. Pang, M. B. Wax, and A. F. Clark, "Noninvasive measurement of rodent intraocular pressure with a rebound tonometer," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 12, pp. 4617–4621, 2005.

[4] C. Nusslein-Volhard and E. Wieschaus, "Mutations affecting segment number and polarity in Drosophila," *Nature*, vol. 287, no. 5785, pp. 795–801, 1980.

[5] P. E. Kuwabara, "Worming your way through the genome," *Trends in Genetics*, vol. 13, no. 11, pp. 455–460, 1997.

[6] W. Driever, L. Solnica-Krezel, A. F. Schier et al., "A genetic screen for mutations affecting embryogenesis in zebrafish," *Development*, vol. 123, pp. 37–46, 1996.

[7] P. Haffter, M. Granato, M. Brand et al., "The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*," *Development*, vol. 123, pp. 1–36, 1996.

[8] J. J. Malicki, Z. Pujic, C. Thisse, B. Thisse, and X. Wei, "Forward and reverse genetic approaches to the analysis of eye development in zebrafish," *Vision Research*, vol. 42, no. 4, pp. 527–533, 2002.

[9] P. N. Baird, R. H. Guymer, D. Chiu et al., "Generating mouse models of retinal disease using ENU mutagenesis," *Vision Research*, vol. 42, no. 4, pp. 479–485, 2002.

[10] C. Thaung, K. Arnold, I. J. Jackson, and P. J. Coffey, "Presence of visual head tracking differentiates normal sighted from retinal degenerate mice," *Neuroscience Letters*, vol. 325, no. 1, pp. 21–24, 2002.

[11] M. M. Jablonski, X. Wang, L. Lu et al., "The Tennessee Mouse Genome Consortium: identification of ocular mutants," *Visual Neuroscience*, vol. 22, no. 5, pp. 595–604, 2005.

[12] S. Hitotsumachi, D. A. Carpenter, and W. L. Russel, "Dose-repetition increases the mutagenic effectiveness of N-ethyl-N-nitrosourea in mouse spermatogonia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 19, pp. 6619–6621, 1985.

[13] M. J. Justice, B. Zheng, R. P. Woychik, and A. Bradley, "Using targeted large deletions and high-efficiency N-ethyl-N-nitrosourea mutagenesis for functional analyses of the mammalian genome," *Methods*, vol. 13, no. 4, pp. 423–436, 1997.

[14] S. D. M. Brown and P. M. Nolan, "Mouse mutagenesis—systematic studies of mammalian gene function," *Human Molecular Genetics*, vol. 7, no. 10, pp. 1627–1633, 1998.

[15] M. Hrabe de Angelis and R. Balling, "Large scale ENU screens in the mouse: genetics meets genomics," *Mutation Research*, vol. 400, no. 1-2, pp. 25–32, 1998.

[16] R. P. Woychik, M. L. Klebig, M. J. Justice, T. R. Magnuson, and E. D. Avrer, "Functional genomics in the post-genome era," *Mutation Research*, vol. 400, no. 1-2, pp. 3–14, 1998.

[17] A. Shedlovsky, J. D. McDonald, D. Symula, and W. F. Dove, "Mouse models of human phenylketonuria," *Genetics*, vol. 134, no. 4, pp. 1205–1210, 1993.

[18] E. M. Rinchik and D. A. Carpenter, "N-ethyl-N-nitrosourea mutagenesis of a 6- to 11-cM subregion of the Fah- Hbb interval of mouse chromosome 7: completed testing of 4557 gametes and deletion mapping and complementation analysis of 31 mutations," *Genetics*, vol. 152, no. 1, pp. 373–383, 1999.

[19] J. Favor and A. Neuhäuser-Klaus, "Saturation mutagenesis for dominant eye morphological defects in the mouse Mus musculus," *Mammalian Genome*, vol. 11, no. 7, pp. 520–525, 2000.

[20] S. D. Brown and J. Peters, "Combining mutagenesis and genomics in the mouse–closing the phenotype gap," *Trends in Genetics*, vol. 12, no. 11, pp. 433–435, 1996.

[21] N. L. Hawes, B. Chang, G. S. Hageman et al., "Retinal degeneration 6 (*rd6*): a new mouse model for human retinitis punctata albescens," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 10, pp. 3149–3157, 2000.

[22] R. Higuchi, "Rapid, efficient DNA extraction for PCR from cells or blood. Perkin Elmer Cetus," *Amplifications*, vol. 2, pp. 1–3, 1989.

[23] B. A. Taylor, A. Navin, and S. J. Phillips, "PCR-amplification of simple sequence repeat variants from pooled DNA samples for rapidly mapping new mutations of the mouse," *Genomics*, vol. 21, no. 3, pp. 626–632, 1994.

[24] C. E. Keeler, "The inheritance of a retinal abnormality in white mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 10, pp. 329–333, 1924.

[25] R. J. Mullen and M. M. LaVail, "Two new types of retinal degeneration in cerebellar mutant mice," *Nature*, vol. 258, no. 5535, pp. 528–530, 1975.

[26] R. van Nie, D. Ivanyi, and P. Demant, "A new H-2-linked mutation, rds, causing retinal degeneration in the mouse," *Tissue Antigens*, vol. 12, no. 2, pp. 106–108, 1978.

[27] B. Chang, J. R. Heckenlively, N. L. Hawes, and T. H. Roderick, "New mouse primary retinal degeneration (*rd-3*)," *Genomics*, vol. 16, no. 1, pp. 45–49, 1993.

[28] T. H. Roderick, B. Chang, N. L. Hawes, and J. R. Heckenlively, "A new dominant retinal degeneration (*Rd4*) associated with a chromosomal inversion in the mouse," *Genomics*, vol. 42, no. 3, pp. 393–396, 1997.

[29] J. R. Heckenlively, B. Change, L. C. Erway et al., "Mouse model for usher syndrome: linkage mapping suggests homology to usher type I reported at human chromosome 11p15," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 24, pp. 11100–11104, 1995.

[30] B. Chang, R. T. Bronson, N. L. Hawes et al., "Retinal degeneration in motor neuron degeneration: a mouse model of ceroid lipofuscinosis," *Investigative Ophthalmology and Visual Science*, vol. 35, no. 3, pp. 1071–1076, 1994.

[31] N. L. Hawes, B. Chang, G. S. Hageman et al., "Retinal degeneration 6 (rd6): a new mouse model for human retinitis punctata albescens," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 10, pp. 3149–3157, 2000.

[32] B. Chang, J. R. Heckenlively, N. L. Hawes, and M. T. Davisson, "A new mouse model of retinal dysplasia and degeneration (*rd7*)," *Investigative Ophthalmology & Visual Science*, vol. 39, p. S880, 1998.

[33] B. Chang, N. L. Hawes, R. E. Hurd, M. T. Davisson, S. Nusinowitz, and J. R. Heckenlively, "Retinal degeneration mutants in the mouse," *Vision Research*, vol. 42, no. 4, pp. 517–525, 2002.

[34] B. Chang, N. L. Hawes, M. T. Pardue et al., "Two mouse retinal degenerations caused by missense mutations in the β-subunit of rod cGMP phosphodiesterase gene," *Vision Research*, vol. 47, no. 5, pp. 624–633, 2007.

[35] N. L. Hawes, B. Chang, R. E. Hurd, S. Nusinowitz, J. R. Heckenlively, and M. T. Davisson, "A new mouse model of retinal degeneration (*rd11*)," *Investigative Ophthalmology & Visual Science*, vol. 43, E-Abstract 3669, 2002.

[36] B. Chang, N. L. Hawes, R. E. Hurd, M. T. Davisson, S. Nusinowitz, and J. R. Heckenlively, "A point mutation in the *Rpe65* gene causes retinal degeneration (*rd12*) in mice," *Investigative Ophthalmology & Visual Science*, vol. 43, E-Abstract 3670, 2002.

[37] J. Zhang, N. L. Hawes, J. Wang et al., "A new mouse model of retinal degeneration (*rd14*)," *Investigative Ophthalmology & Visual Science*, vol. 46, E-Abstract 3170, 2005.

[38] N. L. Hawes, R. E. Hurd, J. Wang et al., "A new mouse model of retinal degeneration (*rd15*) with retinal outer plexiform dystrophy," *Investigative Ophthalmology & Visual Science*, vol. 46, E-Abstract 3175, 2005.

[39] B. Chang, H. Khanna, N. Hawes et al., "In-frame deletion in a novel centrosomal/ciliary protein CEP290/NPHP6 perturbs its interaction with RPGR and results in early-onset retinal degeneration in the *rd16* mouse," *Human Molecular Genetics*, vol. 15, no. 11, pp. 1847–1857, 2006.

[40] B. Chang, N. L. Hawes, R. E. Hurd, J. Wang, M. T. Davisson, and S. Nusinowitz, "A new mouse model of retinal degeneration (*rd17*)," *Investigative Ophthalmology & Visual Science*, vol. 48, E-Abstract 4513, 2007.

[41] B. Chang, N. L. Hawes, R. E. Hurd, M. T. Davisson, S. Nusinowitz, and J. R. Heckenlively, "A new mouse model of cone photoreceptor function loss (cpfl1)," *Investigative Ophthalmology & Visual Science*, vol. 42, p. S527, 2001.

[42] N. L. Hawes, R. E. Hurd, N. Haider et al., "A new mouse model of cone photoreceptor function loss (Cpfl2)," *Investigative Ophthalmology & Visual Science*, vol. 44, E-Abstract 4531, 2003.

[43] B. Chang, M. S. Dacey, N. L. Hawes et al., "Cone photoreceptor function loss-3, a novel mouse model of achromatopsia due to a mutation in *Gnat2*," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 11, pp. 5017–5021, 2006.

[44] N. L. Hawes, R. E. Hurd, J. Wang et al., "A new mouse model of cone photoreceptor function loss (*Cpfl4*)," *Investigative Ophthalmology & Visual Science*, vol. 45, E-Abstract 3590, 2004.

[45] N. L. Hawes, X. Wang, R. E. Hurd et al., "A point mutation in the Cnga3 gene causes cone photoreceptor function loss (cpfl5) in mice," *Investigative Ophthalmology & Visual Science*, vol. 47, E-Abstract 4579, 2006.

[46] B. Chang, N. L. Hawes, R. E. Hurd et al., "A new mouse model of cone photoreceptor function loss (cpfl6) associated with the targeted *Hcn1* gene mutation," *Investigative Ophthalmology & Visual Science*, vol. 47, E-Abstract 2294, 2006.

[47] N. L. Hawes, B. S. Harris, R. E. Hurd et al., "A new mouse model of cone photoreceptor fuction loss (*cpfl7*)," *Investigative Ophthalmology & Visual Science*, vol. 48, E-Abstract 1350, 2007.

[48] B. Chang, J. R. Heckenlively, P. R. Bayley et al., "The *nob2* mouse, a null mutation in *Cacna1f*: anatomical and functional abnormalities in the outer retina and their consequences on ganglion cell visual responses," *Visual Neuroscience*, vol. 23, no. 1, pp. 11–24, 2006.

[49] D. M. Maddox, K. A. Vessey, G. L. Yarbrough et al., "Allelic variance between GRM6 mutants, *Grm6^{nob3}* and *Grm6^{nob4}* results in differences in retinal ganglion cell visual responses," *Journal of Physiology*, vol. 586, no. 18, pp. 4409–4424, 2008.

[50] B. Chang, M. N. A. Mandal, V. R. M. Chavali et al., "Age-related retinal degeneration (*arrd2*) in a novel mouse model due to a nonsense mutation in the Mdm1 gene," *Human Molecular Genetics*, vol. 17, no. 24, pp. 3929–3941, 2008.

[51] J. Won, E. Gifford, R. S. Smith et al., "RPGRIP1 is essential for normal rod photoreceptor outer segment elaboration and morphogenesis," *Human Molecular Genetics*, vol. 18, no. 22, pp. 4329–4339, 2009.

[52] M. M. Edwards, E. Mammadova-Bach, F. Alpy et al., "Mutations in *Lama1* disrupt retinal vascular development and inner limiting membrane formation," *Journal of Biological Chemistry*, vol. 285, no. 10, pp. 7697–7711, 2010.

[53] M. M. Edwards, C. Mar de Evsikova, G. B. Collin et al., "Photoreceptor degeneration, azoospermia, leukoencephalopathy, and abnormal RPE cell function in mice expressing an early stop mutation in CLCN2," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 6, pp. 3264–3272, 2010.

[54] E. Budzynski, A. K. Gross, S. D. McAlear et al., "Mutations of the opsin gene (Y102H and I307N) lead to light-induced degeneration of photoreceptors and constitutive activation of phototransduction in mice," *Journal of Biological Chemistry*, vol. 285, no. 19, pp. 14521–14533, 2010.

[55] K. Sakamoto, M. Mccluskey, T. G. Wensel, J. K. Naggert, and P. M. Nishina, "New mouse models for recessive retinitis pigmentosa caused by mutations in the *Pde6a* gene," *Human Molecular Genetics*, vol. 18, no. 1, pp. 178–192, 2009.

[56] Y. Zhao, D.-H. Hong, B. Pawlyk et al., "The retinitis pigmentosa GTPase regulator (RPGR)-interacting protein: subserving RPGR function and participating in disk morphogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 3965–3970, 2003.

[57] F. Alpy, I. Jivkov, L. Sorokin et al., "Generation of a conditionally null allele of the laminin α1 gene," *Genesis*, vol. 43, no. 2, pp. 59–70, 2005.

[58] S. Schéele, M. Falk, A. Franzén et al., "Laminin α1 globular domains 4-5 induce fetal development but are not vital for embryonic basement membrane assembly," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 5, pp. 1502–1506, 2005.

[59] M. I. Naash, H. Ripps, S. Li, Y. Goto, and N. S. Peachey, "Polygenic disease and retinitis pigmentosa: albinism exacerbates photoreceptor degeneration induced by the expression of a mutant opsin in transgenic mice," *Journal of Neuroscience*, vol. 16, no. 24, pp. 7853–7858, 1996.

[60] T. Furukawa, E. M. Morrow, T. Li, F. C. Davis, and C. L. Cepko, "Retinopathy and attenuated circadian entrainment in Crx-deficient mice," *Nature Genetics*, vol. 23, no. 4, pp. 466–470, 1999.

[61] J. Gao, K. Cheon, S. Nusinowitz et al., "Progressive photoreceptor degeneration, outer segment dysplasia, and rhodopsin mislocalization in mice with targeted disruption of the retinitis pigmentosa-1 (*Rp1*) gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 8, pp. 5698–5703, 2002.

[62] Q. Liu, A. Lyubarsky, J. H. Skalet, E. N. Pugh Jr., and E. A. Pierce, "RP1 is required for the correct stacking of outer segment discs," *Investigative Ophthalmology and Visual Science*, vol. 44, no. 10, pp. 4171–4183, 2003.

[63] T. M. Redmond, S. Yu, E. Lee et al., "Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle," *Nature Genetics*, vol. 20, no. 4, pp. 344–351, 1998.

[64] J.-J. Pang, B. Chang, N. L. Hawes et al., "Retinal degeneration 12 (*rd12*): a new, spontaneously arising mouse model for human Leber congenital amaurosis (LCA)," *Molecular Vision*, vol. 11, pp. 152–162, 2005.

[65] M. Samardzija, J. von lintig, N. Tanimoto et al., "R91W mutation in *Rpe65* leads to milder early-onset retinal dystrophy

due to the generation of low levels of 11-cis-retinal," *Human Molecular Genetics*, vol. 17, no. 2, pp. 281–292, 2008.

[66] B. Chang, N. L. Hawes, M. T. Pardue et al., "Two mouse retinal degenerations caused by missense mutations in the beta-subunit of rod cGMP phosphodiesterase gene," *Vision Research*, vol. 47, no. 5, pp. 624–633, 2007.

[67] J.-J. Pang, S. L. Boye, A. Kumar et al., "AAV-mediated gene therapy for retinal degeneration in the rd10 mouse containing a recessive PDEβ mutation," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 10, pp. 4278–4283, 2008.

[68] N. B. Akhmedov, N. I. Piriev, B. Chang et al., "A deletion in a photoreceptor-specific nuclear receptor mRNA causes retinal degeneration in the *rd7* mouse," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 10, pp. 5551–5556, 2000.

[69] N. B. Haider, W. Zhang, R. Hurd et al., "Mapping of genetic modifiers of $Nr2e^{3rd7/rd7}$ that suppress retinal degeneration and restore blue cone cells to normal quantity," *Mammalian Genome*, vol. 19, no. 3, pp. 145–154, 2008.

[70] A. K. Mehalow, S. Kameya, R. S. Smith et al., "CRB1 is essential for external limiting membrane integrity and photoreceptor morphogenesis in the mammalian retina," *Human Molecular Genetics*, vol. 12, no. 17, pp. 2179–2189, 2003.

[71] S. A. van de Pavert, A. Kantardzhieva, A. Malysheva et al., "Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure," *Journal of Cell Science*, vol. 117, no. 18, pp. 4169–4177, 2004.

Review Article

The Domestic Cat as a Large Animal Model for Characterization of Disease and Therapeutic Intervention in Hereditary Retinal Blindness

Kristina Narfström,[1, 2] Koren Holland Deckman,[3] and Marilyn Menotti-Raymond[4]

[1] *Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, Mason Eye Institute,*
  University of Missouri-Columbia, MO 65211, USA
[2] *Department of Ophthalmology, Mason Eye Institute, University of Missouri-Columbia, MO 65212-0001, USA*
[3] *Department of Chemistry, Gettysburg College, Gettysburg, PA 17325, USA*
[4] *Laboratory of Genomic Diversity, National Cancer Institute-Frederick, Frederick, MD 21702-1201, USA*

Correspondence should be addressed to Kristina Narfström, narfstromk@missouri.edu

Large mammals, including canids and felids, are affected by spontaneously occurring hereditary retinal diseases with similarities to those of humans. The large mammal models may be used for thorough clinical characterization of disease processes, understanding the effects of specific mutations, elucidation of disease mechanisms, and for development of therapeutic intervention. Two well-characterized feline models are addressed in this paper. The first model is the autosomal recessive, slowly progressive, late-onset, rod-cone degenerative disease caused by a mutation in the *CEP290* gene. The second model addressed in this paper is the autosomal dominant early onset rod cone dysplasia, putatively caused by the mutation found in the *CRX* gene. Therapeutic trials have been performed mainly in the former type including stem cell therapy, retinal transplantation, and development of ocular prosthetics. Domestic cats, having large human-like eyes with comparable spontaneous retinal diseases, are also considered useful for gene replacement therapy, thus functioning as effective model systems for further research.

1. Introduction

The value of appropriate animal models to advance our understanding and treatment of human retinal disease processes that cause severe visual impairment or blindness cannot be overemphasized. Animal models have led to the identification of disease genes, and elucidation of the molecular genetic and cellular mechanisms underlying retinal pathology. Moreover, they provide the basis for testing the efficacy of therapeutic approaches, including the use of drugs and gene replacement [1], novel genetic approaches (siRNA) [2], stem cell therapy [3], surgical intervention, such as retinal transplantation [4], and the use of ocular or retinal prosthetics [5]. Additionally, animal models can lead to the identification of novel genes underlying human

retinal pathology. Though 157 genes have been identified as causative of nonsyndromic human retinitis pigmentosa (RP; http://www.sph.uth.tmc.edu/retnet/home.htm), over 50% of the genetic causality of RP still remains uncharacterized [6].

Though the mouse has been the classic animal model of retinal disease, the advent of comprehensive genetic maps of many mammals has led to the identification of a number of non-rodent animal models of human hereditary retinal disease. Many large animal models offer a complement to existing rodent models. The size of the rodent eye is restrictive for visualization using regular clinical ophthalmic instrumentation and also in conjunction with therapeutic intervention. Even for detailed morphological studies the small size of the mouse eye may be a problem. As a case in point Pazour et al. previously reported that in their research

examining the trafficking of ciliary protein in photoreceptor cells, the physical limitations of the mouse retina led them to resort to the use of a bovid eye [7].

2. Dogs and Cats as Large Animal Models of Spontaneous Retinal Disease

Dog and cat populations offer a wealth of potential as large animal models of human retinal disease. Small effective population sizes, the use of popular sires and inbreeding have contributed to the "load" of inherited diseases, especially in dog breeds [8]. Hereditary and primary photoreceptor diseases, or progressive retinal atrophies (PRA) have been described in more than 100 dog breeds [9], many of which are likely to be caused by the same mutation which is observed across related breeds. This phenomenon has been observed in a number of gene-defining phenotypes in the dog [10–12]. Thirteen genes have been mapped and characterized as causative of canine PRA, including *ADAM9* [13], *CCDC66* [14], *CNGB3* [15], *PDE6α* [16], *PDE6β* [17], *PRCD* [18], *RD3* [19], *RHO* [20], *RPE65* [21], *RPHP4* [22], *RPGR* [23], *RPGRIP1* [24], and *VMD2* [25].

Cats have been considered to be affected less frequently by hereditary disease. However, the informative website Online Mendelian Inheritance in Animals (http://omia.angis.org.au/) catalogues 288 distinctive pathologies with an inherited component in the cat, with cited references. Only in recent years have specific mutations been elucidated for hereditary retinal diseases in cats [26, 27], clinically similar to the PRA complex in dogs [28]. Domestic dogs and cats of today experience a level of medical surveillance second only to human kind, thus increasing the likelihood, that individuals with rare or unique mutations are identified.

3. Sequencing of the Cat Genome

Report of two partial sequences (1.9X, 3X) of the cat genome [29, 30] has been invaluable in the initial mapping and characterization of feline hereditary diseases [26, 27, 31]. A full genome sequence (14X) of the cat has currently been completed (Wes Warren, Washington University, personal communication). The identification of single-nucleotide polymorphisms (SNP) in cat breeds, an integral part of the 14X full genome sequence project and the 3X sequencing of the cat genome [30] is currently being utilized in development of a domestic cat SNP chip. With the availability of these genomic resources, the mapping and characterization of feline monogenic disorders will largely be dependent on obtaining an appropriate sample set. Genome-wide association (GWA) studies in dog breeds are proving extremely successful in identifying genes associated with breed-defining phenotypes and monogenic disorders [13, 32, 33]. Often this is accomplished with surprisingly small sample sizes. The mapping of the canine cone-rod dystrophy 3 gene (*ADAMS9*) in the Glen of Imaal Terrier breed was recently accomplished with as few as 22 unaffected and 19 affected individuals [13] while Awano et al. reported identification of the gene causative of canine degenerative myelopathy

(*SOD1*) in the Pembroke Welsh corgi with 38 affected and 17 control individuals. Extended linkage disequilibrium (LD) in dog breeds [19, 34, 35] contributes to the success of GWA mapping in dogs and is an important factor underlying successful mapping with small sample sizes in the dog. Preliminary studies suggest that extended blocks of LD are also observed in cat breeds, though the length of LD appears to be abbreviated to that which is observed in dog breeds [29].

4. The Abyssinian Retinal Degeneration Cat Model (*rdAc*)

The female Abyssinian cat (Cinnamon), subject of the feline whole genome sequencing efforts, is a member of a pedigree developed for genetic mapping of the gene defect for the *rdAc (retinal degeneration in Abyssinian cats)* model, first described in 1982 [36]. The autosomal recessive (AR) trait, *rdAc,* has become an important model of human RP [37, 38]. At birth, affected cats have normal vision, but, by 1.5–2 years of age they develop early changes that can be observed by ophthalmoscopy [39] (Figures 1(a)–1(c)). By 7 months of age, affected cats demonstrate significantly reduced retinal function by electroretinography (ERG; Figure 1(d)). ERG a-wave amplitudes are then reduced more than 50% as compared to normal individuals, with a parallel reduction in retinal oxygen tension [40]. Rod photoreceptor outer segments exhibit the first morphological changes in individuals 5–8 months of age, observed as a disorganization and disruption of rod outer segment lamellar discs and the appearance of vacuoles near the connecting cilium [41]. Progression of the disease results in further degeneration of the rods (Figures 2(a) and 2(b)), followed also by disruption of the cone photoreceptors. By 3–5 years of age, the clinical end stage of the disease has been reached with generalized photoreceptor degeneration, and subsequently retinal atrophy leads to blindness [42].

The molecular genetic basis of *rdAc* was recently established in the *CEP290* gene. A single-nucleotide polymorphism in an intron of the felid *CEP290* gene generates a novel strong canonical splice-donor site resulting in a 4-bp insertion, a frame shift, and the introduction of a premature stop codon (Figures 3(a) and 3(b)). The putative truncated CEP290 peptide would lack the more 3′ KIDV and VI domains. The protein is an important component of the intraflagellar transport (IFT) system whereby specialized proteins critical for phototransduction are transferred from their site of synthesis in the inner segment of photoreceptors through the connecting cilium to the outer segment [7]. As the rod photoreceptor discs are in a constant state of regeneration, a fully functional IFT system is critical for the maintenance of the photoreceptors [7]. In the *rd16* mouse model, the phototransduction proteins opsin and rhodopsin are found concentrated in the inner segment, which led Chang et al. [44] to propose a ciliary trafficking role for the CEP290 protein. Mutations in *CEP290* have been reported in RP, Leber's congenital amaurosis (LCA), as well as the syndromic retinopathies, Joubert, Meckel-Gruber, and Bardet-Biedl [45–48].
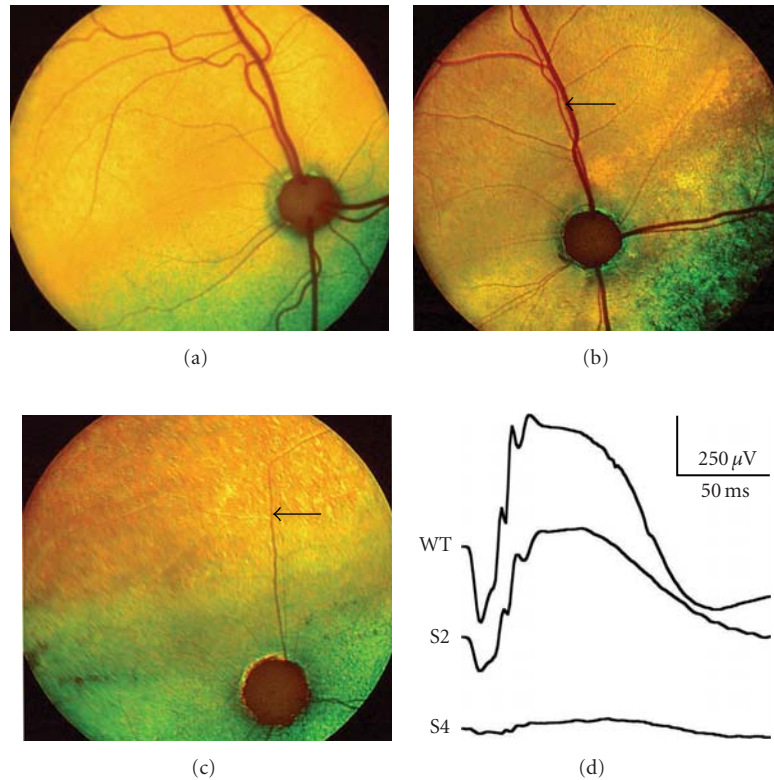
FIGURE 1: Fundus appearance and electroretinograms (ERGs) of *rdAc* individuals with the *CEP290* mutation. Fundus photographs demonstrate (a) a 1-year-old unaffected Abyssinian cat (wildtype, WT), (b) a 2-year-old affected Abyssinian cat with an early disease stage (S2) [39], and (c) a 6-year-old Abyssinian with an advanced disease stage (S4) [39]. Arrows in (b) and (c) indicate retinal vasculature that is attenuated, more so in the advanced stage (c) than in early stage of disease (b). For the same three cats, the waveforms of the dark-adapted full-field flash ERG recordings are shown, using 4 cd.s/m^2 of white light stimulation for each of the recordings. Amplitude and implicit time calibrations are indicated in the figure, vertically and horizontally, respectively. Reproduced with permission from [43].
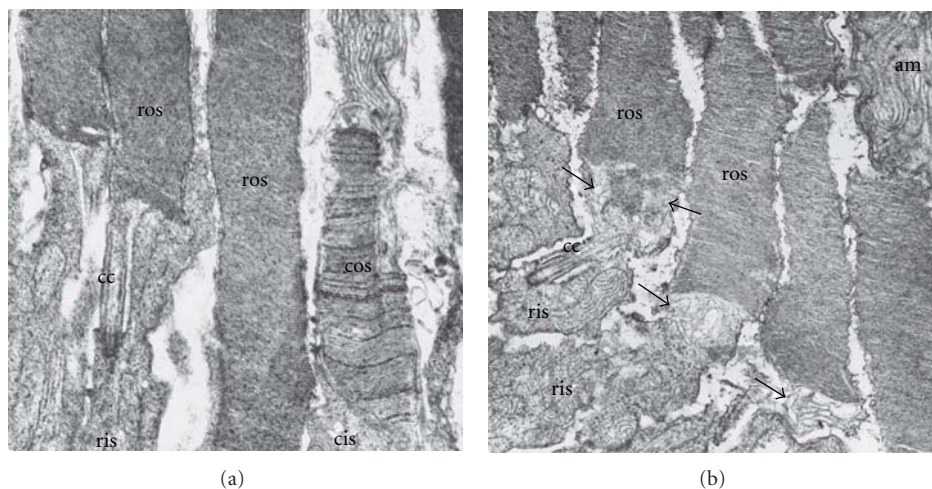

FIGURE 2: Electron micrographs of outer retina showing photoreceptor outer and inner segments of normal (a) Abyssinian cat and young affected (b) *rdAc* cat. Note abnormalities at the base of the rod outer segments near the connecting cilium in (b); membranes are not formed as in the normal (a), instead there is vacuolization and degeneration (arrows) of membranes in the affected retina. Am: apical microvilli of the retinal pigment epithelium, ros: rod outer segments, ris: rod inner segments, cos: cone outer segments, cis: cone inner segments, cc: connecting cilium of the photoreceptor. Original magnification: ×19152. Reproduced with permission from [27].
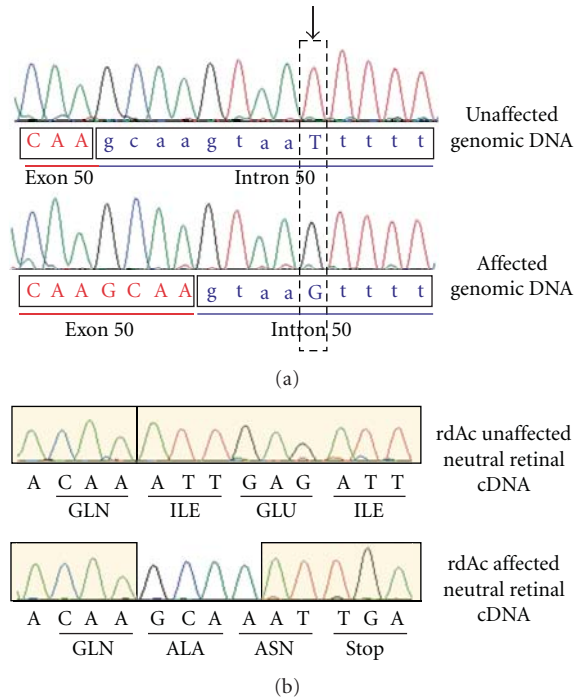
FIGURE 3: (a) Electropherograms of genomic DNA of *CEP290* sequenced in unaffected and *rdAc* affected individuals of exon 50/intron 50 junction. Arrow indicates position of SNP in intron 50, which uncovers a canonical GT splice donor site, resulting in alternative splicing in affected individuals. Exon 50 (red letters) and intron 50 (blue letters) nucleotides were identified by cDNA sequence analysis. GenBank Accession No. for feline *CEP290*: EF028068. (b) Electropherograms of cDNA for *CEP290* 3′ region of exon 50 generated from neural retinal tissue in affected and unaffected individuals. Alternative splicing in affected individuals results in a frame shift and introduction of a premature STOP codon. Reproduced with permission from [27].

Recently, it has been shown that *rdAc* cats exhibit some degree of phenotypic variation, with end-stage blindness reached in individuals anywhere from three to seven years [49]. Interestingly, it appears that the slow progression of disease may be one of the factors leading to the cat's exceptional ability to adapt to decreasing retinal function [49]. The condition thus evaded detection by both owners and veterinarians in a highly popular cat breed, the Siamese, which demonstrates a high frequency (~33%) for the *rdAc* disease allele [49]. Breeding practices have caused the *CEP290* mutation to spread to multiple cat breeds [49], and to exhibit a worldwide distribution [49].

5. The Rod Cone Dysplasia Cat Model (*Rdy*)

A second feline model of human retinal dystrophy, the *Rdy* cat, was first described in a single Abyssinian cat, from which a pedigree was developed and extensively studied on a phenotypic level [50–53]. The disease is an early onset primary photoreceptor disorder with an autosomal dominant (AD) mode of inheritance in which affected individuals exhibit

abnormal photoreceptor development at 22 days of age. The disease leads rapidly to blindness usually within the first few months of life. Further characterization of the dystrophy has demonstrated that the photoreceptors never develop normally, and the disease has therefore been designated as a rod-cone dysplasia with early onset degeneration of both cones and rods.

The molecular genetic basis for *Rdy* was recently elucidated [26]. A single-base deletion in the *CRX* gene introduces a frameshift and a stop codon immediately downstream, truncating a region previously demonstrated as critical for gene function [26, 54] (Figure 4). The *CRX* gene product is critical in transcriptional activation of a number of genes involved in photoreceptor development and maintenance [55, 56]. In humans, mutations in *CRX* are associated with human AD cone-rod dystrophy (CoRD), and both AD and AR Leber's congenital amaurosis (LCA) [54, 57–59]. The *Rdy* cat is the first large animal model for *CRX*-linked spontaneous retinal disease. A large screening of cat breeds has failed to detect any other domestic feline breeds with the disease allele [26].

The *Rdy* model provides one of the very few large animal models of an autosomal dominant retinal disease. These disorders are challenging from a therapeutic standpoint. Causality of the disease can arise from haploinsufficiency of product, or in some circumstances from gain of function or competition from a truncated or aberrant protein product [60]. The presence of both mutant and wildtype RNA in *Rdy* individuals, initially suggestive that a truncated CRX product might be generated [26] has been supported by recent findings (K. Holland Deckman, unpublished). The truncated peptide would retain the CRX motifs involved in nuclear localization and DNA-binding, but lack the region critical for transcriptional activation of photoreceptor specific genes [61]. This truncated product could thus compete with the wildtype CRX product and other transcription factors for promoter binding regions of target genes, which is currently under investigation.

6. Other Cat Models under Investigation

In the late 1960s, a new feline breed, the Bengal, which has gained huge popularity, was developed through hybridization of domestic cats and the Asian leopard cat [62]. Recently, a novel, early onset autosomal recessive disorder was described in this breed [63]. The disease is under investigation but appears to be an early onset primary photoreceptor disorder, leading to blindness within the first year of age. Genetic mapping and further characterization of the disorder are in progress. A second feline retinal disease model has been described in the Persian cat breed [64]. The rod cone dysplasia demonstrates an autosomal recessive mode of inheritance [64]. Affected individuals showed clinical signs of disease 2-3 weeks after birth and clinical blindness at 16 weeks of age. Photoreceptors in affected individuals never reach full maturity. The molecular genetic defect for both of these disorders has not as yet been elucidated. It appears that the Bengal cat retinal disease should become
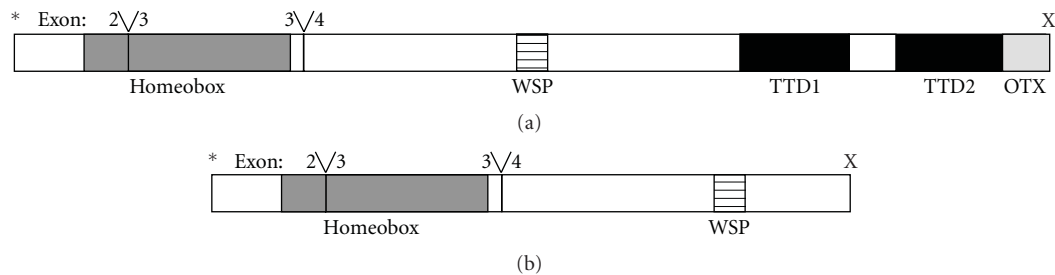
(a)

(b)

FIGURE 4: CRX protein structure in *Felis catus*. Wildtype feline CRX protein (a) compared to the putative truncated CRX protein (b). The exon splice junctions are noted as "Y". The start codon and stop codons are labeled as (*) and (X), respectively. The protein domains are highlighted as shaded boxes and defined as the homeobox, the WSP domain, the transcriptional transactivation domains 1 and 2 (TTD1 and TTD2), and the OTX tail. Domains are drawn to scale. Reproduced with permission from [26].

an important animal model for the research community in regards to the study of various treatment modalities. The disease starts out from a mainly normal appearing retina but due to the fast progression of the disorder, retinal atrophy ensues comparatively early thus functioning as an effective model system for retinal research.

7. Therapeutic Intervention

The retinas of large animal models more closely approximate that of humans, and are thus more easily amenable for visualization and imaging [65] of the disease process, for surgical intervention, and for clinical evaluation of therapeutic effects. Dogs and cats also offer the potential of long-term followup studies in conjunction with treatment trials.

The rod and cone photoreceptors (the latter; short and middle wavelength sensitive cones) of both species are distributed in the retina in a mosaic pattern comparable to that of the human retina. Neither cats nor dogs have a macula. However, in cats, in the same region as the human macula, there sre a cone-rich region called the area centralis where the concentration of cones in comparison to that of rods is higher than at any other location. Along with the holangiotic configuration of the retinal vasculature, the cat retina becomes structurally similar to the human counterpart. Further, cats in particular, have historically been important models in neuroanatomy and neurophysiology, especially with respect to visual function.

Successful therapeutic intervention is the ultimate goal of research using animal models for human retinal disease processes. In recent years, groundbreaking research has been performed by independent groups in regards to gene therapy using dogs with spontaneous hereditary retinal disease. Proof of principle that the technology works was achieved by an *in vivo* study by Acland et al. [66], using AAV2/2 as a safe and effective vector. The well-characterized *rdAc* and *Rdy* feline models of spontaneous hereditary retinal disease, now with known mutations, are excellent candidates for gene therapy-based approaches, especially for the late onset type of retinal degeneration (Jean Bennett, personal communication, 2007). Gene therapy approaches targeting the *Rdy* model, which has been recently elucidated on the molecular genetic level, are currently under investigation.

Novel therapeutic interventions have recently been developed to target aberrant RNA species that survive nonsense mediated decay. Short interfering RNA (shRNA), short double stranded RNA molecules, can be designed to degrade specific target mRNAs [67], while ribozymes, which are small catalytic RNAs, are designed to cleave complementary RNA sequences [2]. RNA interference-mediated suppression and replacement aims to remove both wildtype and aberrant RNA copies of a targeted gene while replacing wildtype expression with a copy of the gene.

Other methods of treatment include retinal transplantation of viable cells or tissue. Experimentation in this regard includes the replacement of dying visual cells with healthy neuroblastic progenitor cells and retinal pigment epithelial (RPE) cells as sheets of normal tissue [68]. It has been demonstrated that retinal transplants in rats can morphologically reconstruct a damaged retina and restore retinal sensitivity [69]. Affected cats with the *CEP290* defect (*rdAc*) have been used in trials with transplantation of sheets of allogeneic fetal retinal tissue [70]. Surgeries have been successful as to graft survival in the retina, although cellular connectivity has not been shown and ERG testing has not demonstrated improvement in retinal function. So far the cat model in regards to transplantation of large sheets of normal tissue has shown a comparatively high risk for complications. The tight structures of the cat eye presents difficulties to manipulate the globe in the orbit in comparison to other large animal models (such as dog, pig, and rabbits) and the high frequency of hemorrhage from the deep venous plexus region of the domestic cat renders this surgery difficult even for experienced surgeons [71].

Transplantation of stem and neural progenitor cells appears to offer considerable promise. Subretinal transplantation of neural progenitor cells in rats has shown evidence of cellular repopulation of damaged retinas and retardation of ongoing retinal degeneration [72, 73]. Neural progenitor cells can also be engineered to secrete specific growth factors such as glial cell line-derived neurotrophic factor (GDNF). When used for transplantation studies such cells contributed to enhanced cellular survival, neuronal differentiation, and improved host cognitive function following brain injury, in comparison to transplantation of nontransduced neuronal progenitor cells [74]. Recent studies, using *rdAc* animals

have shown promising results when retinal progenitor cells from transgenic fluorescent red cats were transplanted to cats affected by the *CEP290* mutation (*rdAc*) by subretinal injections of progenitor cell suspensions. No adverse reactions were observed in the transplanted cat eyes. There was further development and migration of transplanted cells in the outer and inner retina, and development of donor progenitor cells specifically into Müller-like cells observed by immunohistochemistry [63]. Further studies are in progress.

Another modality under development using the feline species is intraocular implantation of retinal prosthesis [75]. Either epiretinal or subretinal implantation can be utilized in the degenerate retina. The electrodes in the prosthesis may emit electrical currents and stimulate residual retinal cells, such as second- and third- order neurons, for example, bipolar and ganglion cells. Signals to the visual cortex are transmitted to produce a visual sensation. It appears that the cat eye, with the visual processes already thoroughly investigated, would be an optimal animal model for further development of research in regards to retinal prosthesis.

8. Future Directions

Through discoveries of causative mutations and their detrimental effects on retinal cell function, new insights into retinal degenerative disease mechanisms have been gained. It is now possible to aim therapies at correcting disease mutations in the eye directly or indirectly. The cat species, with disease entities that are comparable to those of humans, and with large human-like eyes, is amenable to treatment using similar surgical techniques and instrumentation as those used for humans. We now have an effective model system that can be used for cell replacement therapy, retinal transplantation using tissue from healthy retinas or retinal progenitor cells, artificial retinal prosthesis, or combinations of one or more of the above. There definitely is some hope of further advancement in the field of spontaneously occurring hereditary retinal blinding disease using the cat as a valuable large animal model.

Acknowledgments

References

[1] A. J. Smith, J. W. Bainbridge, and R. R. Ali, "Prospects for retinal gene replacement therapy," *Trends in Genetics*, vol. 25, no. 4, pp. 156–165, 2009.

[2] M. Gorbatyuk, V. Justilien, J. Liu, W. W. Hauswirth, and A. S. Lewin, "Preservation of photoreceptor morphology and function in P23H rats using an allele independent ribozyme," *Experimental Eye Research*, vol. 84, no. 1, pp. 44–52, 2007.

[3] O. Comyn, E. Lee, and R. E. MacLaren, "Induced pluripotent stem cell therapies for retinal disease," *Current Opinion in Neurology*, vol. 23, no. 1, pp. 4–9, 2010.

[4] R. B. Aramant and M. J. Seiler, "Progress in retinal sheet transplantation," *Progress in Retinal and Eye Research*, vol. 23, no. 5, pp. 475–494, 2004.

[5] J. Dowling, "Current and future prospects for optoelectronic retinal prostheses," *Eye*, vol. 23, no. 10, pp. 1999–2005, 2009.

[6] E. Pomares, G. Marfany, M. J. Brión, A. Carracedo, and R. Gonzàlez-Duarte, "Novel high-throughput SNP genotyping cosegregation analysis for genetic diagnosis of autosomal recessive retinitis pigmentosa and Leber congenital amaurosis," *Human Mutation*, vol. 28, no. 5, pp. 511–516, 2007.

[7] G. J. Pazour, S. A. Baker, J. A. Deane et al., "The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance," *Journal of Cell Biology*, vol. 157, no. 1, pp. 103–113, 2002.

[8] L. Asher, G. Diesel, J. F. Summers, P. D. McGreevy, and L. M. Collins, "Inherited defects in pedigree dogs. Part 1: disorders related to breed standards," *Veterinary Journal*, vol. 182, no. 3, pp. 402–411, 2009.

[9] S. M. Petersen-Jones, "A review of research to elucidate the causes of the generalized progressive retinal atrophies," *Veterinary Journal*, vol. 155, no. 1, pp. 5–18, 1998.

[10] E. K. Karlsson, I. Baranowska, C. M. Wade et al., "Efficient mapping of mendelian traits in dogs through genome-wide association," *Nature Genetics*, vol. 39, no. 11, pp. 1321–1328, 2007.

[11] H. G. Parker, B. M. VonHoldt, P. Quignon et al., "An expressed *fgf4* retrogene is associated with breed-defining chondrodysplasia in domestic dogs," *Science*, vol. 325, no. 5943, pp. 995–998, 2009.

[12] E. Cadieu, M. W. Neff, P. Quignon et al., "Coat variation in the domestic dog is governed by variants in three genes," *Science*, vol. 326, no. 5949, pp. 150–153, 2009.

[13] O. Goldstein, J. G. Mezey, A. R. Boyko et al., "An *ADAM9* mutation in canine cone-rod dystrophy 3 establishes homology with human cone-rod dystrophy 9," *Molecular Vision*, vol. 16, pp. 1549–1569, 2010.

[14] G. Dekomien, C. Vollrath, E. Petrasch-Parwez et al., "Progressive retinal atrophy in Schapendoes dogs: mutation of the newly identified CCDC66 gene," *Neurogenetics*, vol. 11, pp. 163–174, 2009.

[15] D. J. Sidjanin, J. K. Lowe, J. L. McElwee et al., "Canine CNGB3 mutations establish cone degeneration as orthologous to the human achromatopsia locus ACHM3," *Human Molecular Genetics*, vol. 11, no. 16, pp. 1823–1833, 2002.

[16] S. M. Petersen-Jones, D. D. Entz, and D. R. Sargan, "cGMP phosphodiesterase-α mutation causes progressive retinal atrophy in the Cardigan Welsh corgi dog," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 8, pp. 1637–1644, 1999.

[17] M. L. Suber, S. J. Pittler, N. Qin et al., "Irish setter dogs affected with rod/cone dysplasia contain a nonsense mutation in the rod cGMP phosphodiesterase β-subunit gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 9, pp. 3968–3972, 1993.

[18] B. Zangerl, O. Goldstein, A. R. Philp et al., "Identical mutation in a novel retinal gene causes progressive rod-cone degeneration in dogs and retinitis pigmentosa in humans," *Genomics*, vol. 88, no. 5, pp. 551–563, 2006.

[19] A. V. Kukekova, O. Goldstein, J. L. Johnson et al., "Canine RD3 mutation establishes rod-cone dysplasia type 2 (rcd2) as ortholog of human and murine rd3," *Mammalian Genome*, vol. 20, no. 2, pp. 109–123, 2009.

[20] J. W. Kijas, A. V. Cideciyan, T. S. Aleman et al., "Naturally occurring rhodopsin mutation in the dog causes retinal dysfunction and degeneration mimicking human dominant retinitis pigmentosa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 9, pp. 6328–6333, 2002.

[21] G. D. Aguirre, V. Baldwin, S. Pearce-Kelling, K. Narfström, K. Ray, and G. M. Acland, "Congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect," *Molecular Vision*, vol. 4, pp. 23–29, 1998.

[22] A. C. Wiik, C. Wade, T. Biagi et al., "A deletion in nephronophthisis 4 (*NPHP4*) is associated with recessive cone-rod dystrophy in standard wire-haired dachshund," *Genome Research*, vol. 18, no. 9, pp. 1415–1421, 2008.

[23] Q. Zhang, G. M. Acland, W. X. Wu et al., "Different *RPGR* exon ORF15 mutations in Canids provide insights into photoreceptor cell degeneration," *Human Molecular Genetics*, vol. 11, no. 9, pp. 993–1003, 2002.

[24] C. S. Mellersh, M. E. G. Boursnell, L. Pettitt et al., "Canine RPGRIP1 mutation establishes cone-rod dystrophy in miniature longhaired dachshunds as a homologue of human Leber congenital amaurosis," *Genomics*, vol. 88, no. 3, pp. 293–301, 2006.

[25] K. E. Guziewicz, B. Zangerl, S. J. Lindauer et al., "Bestrophin gene mutations cause canine multifocal retinopathy: a novel animal model for best disease," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 5, pp. 1959–1967, 2007.

[26] M. Menotti-Raymond, K. H. Deckman, V. David, J. Myrkalo, S. J. O'Brien, and K. Narfström, "Mutation discovered in a feline model of human congenital retinal blinding disease," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 6, pp. 2852–2859, 2010.

[27] M. Menotti-Raymond, V. A. David, A. A. Schäffer et al., "Mutation in *CEP290* discovered for cat model of human retinal degeneration," *Journal of Heredity*, vol. 98, no. 3, pp. 211–220, 2007.

[28] K. Narfström and S. Petersen-Jones, "Diseases of the canine ocular fundus," in *Veterinary Ophthalmology*, K. N. Gelatt, Ed., vol. 2, pp. 944–1025, Blackwell, Ames, Iowa, USA, 2007.

[29] J. U. Pontius, J. C. Mullikin, D. R. Smith et al., "Initial sequence and comparative analysis of the cat genome," *Genome Research*, vol. 17, no. 11, pp. 1675–1689, 2007.

[30] J. C. Mullikin, N. F. Hansen, L. Shen et al., "Light whole genome sequence for SNP discovery across domestic cat breeds," *BMC Genomics*, vol. 11, no. 1, article 406, 2010.

[31] J. C. Fyfe, M. Menotti-Raymond, V. A. David et al., "An ∼140-kb deletion associated with feline spinal muscular atrophy implies an essential *LIX1* function for motor neuron survival," *Genome Research*, vol. 16, no. 9, pp. 1084–1090, 2006.

[32] K. M. Meurs, E. Mauceli, S. Lahmers, G. M. Acland, S. N. White, and K. Lindblad-Toh, "Genome-wide association identifies a deletion in the 3′ untranslated region of Striatin in a canine model of arrhythmogenic right ventricular cardiomyopathy," *Human Genetics*, vol. 128, pp. 315–324, 2010.

[33] T. Awano, G. S. Johnson, C. M. Wade et al., "Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles namyotrophic lateral sclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2794–2799, 2009.

[34] M. M. Gray, J. M. Granka, C. D. Bustamante et al., "Linkage disequilibrium and demographic history of wild and domestic canids," *Genetics*, vol. 181, no. 4, pp. 1493–1505, 2009.

[35] N. B. Sutter, M. A. Eberle, H. G. Parker et al., "Extensive and breed-specific linkage disequilibrium in *Canis familiaris*," *Genome Research*, vol. 14, no. 12, pp. 2388–2396, 2004.

[36] K. Narfström, "Hereditary progressive retinal atrophy in the Abyssinian cat," *Journal of Heredity*, vol. 74, no. 4, pp. 273–276, 1983.

[37] K. Narfström, G. B. Arden, and S. E. G. Nilsson, "Retinal sensitivity in hereditary retinal degeneration in Abyssinian cats: electrophysiological similarities between man and cat," *British Journal of Ophthalmology*, vol. 73, no. 7, pp. 516–521, 1989.

[38] S. G. Jacobson, C. M. Kemp, K. Narfström, and S. E. G. Nilsson, "Rhodopsin levels and rod-mediated function in Abyssinian cats with hereditary retinal degeneration," *Experimental Eye Research*, vol. 49, no. 5, pp. 843–852, 1989.

[39] K. Narfström, "Progressive retinal atrophy in the Abyssinian cat. Clinical characteristics," *Investigative Ophthalmology and Visual Science*, vol. 26, no. 2, pp. 193–200, 1985.

[40] J. J. Kang Derwent, L. Padnick-Silver, M. McRipley, E. Giuliano, R. A. Linsenmeier, and K. Narfström, "The electroretinogram components in Abyssinian cats with hereditary retinal degeneration," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 8, pp. 3673–3682, 2006.

[41] K. Narfström and S. E. Nilsson, "Morphological findings during retinal development and maturation in hereditary rod-cone degeneration in Abyssinian cats," *Experimental Eye Research*, vol. 49, no. 4, pp. 611–628, 1989.

[42] K. Narfström and S. E. Nilsson, "Progressive retinal atrophy in the Abyssinian cat. Electron microscopy," *Investigative Ophthalmology and Visual Science*, vol. 27, no. 11, pp. 1569–1576, 1986.

[43] S. Thompson, R. E. H. Whiting, R. H. Kardon, E. M. Stone, and K. Narfström, "Effects of hereditary retinal degeneration due to a CEP290 mutation on the feline pupillary light reflex," *Veterinary Ophthalmology*, vol. 13, no. 3, pp. 151–157, 2010.

[44] B. Chang, H. Khanna, N. Hawes et al., "In-frame deletion in a novel centrosomal/ciliary protein *CEP290/NPHP6* perturbs its interaction with RPGR and results in early-onset retinal degeneration in the rd16 mouse," *Human Molecular Genetics*, vol. 15, no. 11, pp. 1847–1857, 2006.

[45] C. C. Leitch, N. A. Zaghloul, E. E. Davis et al., "Hypomorphic mutations in syndromic encephalocele genes are associated with Bardet-Biedl syndrome," *Nature Genetics*, vol. 40, no. 4, pp. 443–448, 2008.

[46] V. Frank, A. I. den Hollander, N. O. Brüchle et al., "Mutations of the *CEP290* gene encoding a centrosomal protein cause meckel-gruber syndrome," *Human Mutation*, vol. 29, no. 1, pp. 45–52, 2008.

[47] J. A. Sayer, E. A. Otto, J. F. O'Toole et al., "The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4," *Nature Genetics*, vol. 38, no. 6, pp. 674–681, 2006.

[48] E. M. Valente, J. L. Silhavy, F. Brancati et al., "Mutations in *CEP290*, which encodes a centrosomal protein, cause pleiotropic forms of Joubert syndrome," *Nature Genetics*, vol. 38, no. 6, pp. 623–625, 2006.

[49] K. Narfström, V. David, O. Jarret et al., "Retinal degeneration in the Abyssinian and Somali cat (*rdAc*): correlation between genotype and phenotype and *rdAc* allele frequency in two continents," *Veterinary Ophthalmology*, vol. 12, no. 5, pp. 285–291, 2009.

[50] K. C. Barnett and R. Curtis, "Autosomal dominant progressive retinal atrophy in Abyssinian cats," *Journal of Heredity*, vol. 76, no. 3, pp. 168–170, 1985.

[51] R. Curtis, K. C. Barnett, and A. Leon, "An early-onset retinal dystrophy with dominant inheritance in the Abyssinian cat. Clinical and pathological findings," *Investigative Ophthalmology and Visual Science*, vol. 28, no. 1, pp. 131–139, 1987.

[52] A. Leon and R. Curtis, "Autosomal dominant rod-cone dyksplasia in the Rdy cat 1. Light and electron microscopic findings," *Experimental Eye Research*, vol. 51, no. 4, pp. 361–381, 1990.

[53] A. Leon, A. A. Hussain, and R. Curtis, "Autosomal dominant rod-cone dysplasia in the Rdy cat," *Experimental Eye Research*, vol. 53, no. 4, pp. 489–502, 1991.

[54] S. Chen, Q. L. Wang, Z. Nie et al., "Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes," *Neuron*, vol. 19, no. 5, pp. 1017–1030, 1997.

[55] T. H. C. Hsiau, C. Diaconu, C. A. Myers, J. Lee, C. L. Cepko, and J. C. Corbo, "The Cis-regulatory logic of the mammalian photoreceptor transcriptional network," *PLoS One*, vol. 2, no. 7, article e643, 2007.

[56] A. K. Hennig, G. H. Peng, and S. Chen, "Regulation of photoreceptor gene expression by Crx-associated transcription factor network," *Brain Research*, vol. 1192, no. C, pp. 114–133, 2008.

[57] C. L. Freund, C. Y. Gregory-Evans, T. Furukawa et al., "Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (*CRX*) essential for maintenance of the photoreceptor," *Cell*, vol. 91, no. 4, pp. 543–553, 1997.

[58] T. Furukawa, E. M. Morrow, and C. L. Cepko, "*Crx*, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation," *Cell*, vol. 91, no. 4, pp. 531–541, 1997.

[59] A. Swaroop, Q. L. Wang, W. Wu et al., "Leber congenital amaurosis caused by a homozygous mutation (R90W) in the homeodomain of the retinal transcription factor *CRX*: direct evidence for the involvement of *CRX* in the development of photoreceptor function," *Human Molecular Genetics*, vol. 8, no. 2, pp. 299–305, 1999.

[60] T. Rio Frio, N. M. Wade, A. Ransijn, E. L. Berson, J. S. Beckmann, and C. Rivolta, "Premature termination codons in PRPF31 cause retinitis pigmentosa via haploinsufficiency due to nonsense-mediated mRNA decay," *Journal of Clinical Investigation*, vol. 118, no. 4, pp. 1519–1531, 2008.

[61] S. Chen, Q. L. Wang, S. Xu et al., "Functional analysis of cone-rod homeobox (*CRX*) mutations associated with retinal dystrophy," *Human Molecular Genetics*, vol. 11, no. 8, pp. 873–884, 2002.

[62] B. Fogle, *The New Encyclopedia of the Cat*, DK Publishing, New York, NY, USA, 2001.

[63] K. Narfström, "Development of Müller-like cells after subretinal transplantation of feline red fluorescent retinal progenitors in Abyssinian cat hereditary retinal degeneration," in *The Association for Research in Vision and Ophthalmology Annual Meeting Abstracts*, Fort Lauderdale, Fla, USA, May 2010.

[64] H. Rah, D. J. Maggs, T. N. Blankenship, K. Narfström, and L. A. Lyons, "Early-onset, autosomal recessive, progressive retinal atrophy in Persian cats," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 5, pp. 1742–1747, 2005.

[65] M. W. Seeliger, S. C. Beck, N. Pereyra-Muñoz et al., "In vivo confocal imaging of the retina in animal models using scanning laser ophthalmoscopy," *Vision Research*, vol. 45, no. 28, pp. 3512–3519, 2005.

[66] G. M. Acland, G. D. Aguirre, J. Ray et al., "Gene therapy restores vision in a canine model of childhood blindness," *Nature Genetics*, vol. 28, no. 1, pp. 92–95, 2001.

[67] M. Gorbatyuk, V. Justilien, J. Liu, W. W. Hauswirth, and A. S. Lewin, "Suppression of mouse rhodopsin expression in vivo by AAV mediated siRNA delivery," *Vision Research*, vol. 47, no. 9, pp. 1202–1208, 2007.

[68] M. J. Seiler, B. Rao, R. B. Aramant et al., "Three-dimensional optical coherence tomography imaging of retinal sheet implants in live rats," *Journal of Neuroscience Methods*, vol. 188, no. 2, pp. 250–257, 2010.

[69] M. J. Seiler, R. B. Aramant, B. B. Thomas, Q. Peng, S. R. Sadda, and H. S. Keirstead, "Visual restoration and transplant connectivity in degenerate rats implanted with retinal progenitor sheets," *European Journal of Neuroscience*, vol. 31, no. 3, pp. 508–520, 2010.

[70] M. J. Seiler, R. B. Aramant, M. W. Seeliger, R. Bragadottir, M. Mahoney, and K. Narfström, "Functional and structural assessment of retinal sheet allograft transplantation in feline hereditary retinal degeneration," *Veterinary Ophthalmology*, vol. 12, no. 3, pp. 158–169, 2009.

[71] R. Bragadóttir and K. Narfström, "Lens sparing pars plana vitrectomy and retinal transplantation in cats," *Veterinary Ophthalmology*, vol. 6, no. 2, pp. 135–139, 2003.

[72] M. J. Young, J. Ray, S. J. O. Whiteley, H. Klassen, and F. H. Gage, "Neuronal differentiation and morphological integration of hippocampal progenitor cells transplanted to the retina of immature and mature dystrophic rats," *Molecular and Cellular Neurosciences*, vol. 16, no. 3, pp. 197–205, 2000.

[73] D. M. Gamm, S. Wang, B. Lu et al., "Protection of visual functions by human neural progenitors in a rat model of retinal disease," *PLoS One*, vol. 2, no. 3, article e338, 2007.

[74] A. Bakshi, S. Shimizu, C. A. Keck et al., "Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following traumatic brain injury," *European Journal of Neuroscience*, vol. 23, no. 8, pp. 2119–2134, 2006.

[75] K. Narfström, "Subretinal implantation: a step forward to restoring dying photoreceptors," *Expert Reviews in Ophthalmology*, vol. 2, pp. 521–524, 2007.