

Chapter 2

Gene-Based Medicines Targeting Genetic Defects Directly and Molecular Pathologies Common to Multiple Forms of Disease

Leber congenital amaurosis (LCA) was an attractive initial target for gene-based therapies targeting retinopathies for several reasons. Firstly, the disease is transmitted in an autosomal recessive mode, and hence gene replacement therapy, as opposed to a requirement to selectively target dominant acting mutations either at the DNA or transcript levels, would be required. Secondly, while, in many forms of disease congenital blindness is evident, photoreceptors remain viable, though non-functional. Since some forms of LCA are caused by defects in enzymes expressed in the pigmented epithelium, which re-cycle all trans retinal into the chromophore 11-cis retinal, it was envisaged that restoration of gene function within the RPE could well result in an immediate improvement of vision, in other words, a rapid and effective readout of treatment efficacy. A major success in gene therapy was reported by Acland et al. [1]. These workers targeted the Briard dog, a naturally occurring model of LCA. These animals have a four base pair deletion in the RPE65 gene and animals homozygous for this deletion are congenitally blind. Acland et al., injected sub-retinally into these animals an adeno-associated virus (AAV2/5) expressing a functional RPE65 gene driven by a chicken β -actin promoter. 150–200 μ l of AAV (at a titre of 2.3×10^{11} infectious particles per ml), was injected sub-retinally into the eyes of dogs. Post-treatment, animals were shown to regain retinal function, assessed by a substantial increase in rod derived electroretinographic (ERG) waveforms. These encouraging results have now lead to the initiation of clinical trials in human subjects, a total of nine such trials for LCA having so far been registered (Clinicaltrials.gov) in the US, the UK, France and Israel, all of these studies employing AAV vectors for gene delivery.

Several gene therapy trials for retinitis pigmentosa have also been registered targeting diseases induced by recessive mutations within the Mer thymidine kinase (MERTK) and MyosinVIIA genes. In regard to MERTK, the protein encoded by this gene is a receptor tyrosine kinase, homozygous mutations within which were initially shown by D'Cruz et al. [2] to cause a degenerative retinopathy in the Royal College of Surgeons (RCS) rat. Subsequently, mutations within this gene were found in some patients with autosomal recessive RP by Andreas Gal and

colleagues [3]. Successful gene replacement therapy in the RCS rat, using an AAV virus expressing the MERTK gene, was reported by Smith and colleagues in 2003 [4] and in 2005, Tschernutter et al. reported long-term preservation of the function of the retina in the RCS rat using a lentiviral vector [5]. A human clinical trial involving gene replacement using an AAV2 vector is currently in phase 1 in Saudi Arabia (Clinicaltrials.gov NCT01482195).

Mutations within the myosin VIIA gene were shown initially by Gibson et al. [6] to underlie the autosomal recessive disease manifested in the *Shaker 1* mouse, these animals displaying symptoms of vestibular (balance) dysfunction. In parallel, Weil et al. [7] discovered mutations within the same gene in the human syndrome, Usher syndrome type 1B. This autosomal recessive condition involves progressive auditory impairment together with retinitis pigmentosa. Hashimoto et al. [8] reported that lentiviral vectors could accommodate cDNAs large enough to encode Myosin XIIa and demonstrated rescue of the retinas of MYO7A null mice. A phase I/II clinical trial has been registered (Clinicaltrials.gov NCT01505062) in the UK in which a medication, *UshStat*, will be used. The latter is a product of the company, Oxford Biomedica, and is a lentiviral vector expressing the Myosin VII gene. The same company has also registered a clinical trial of their product, StarGen, for treatment of Stargardt macular dystrophy. This is a rare autosomal recessive retinopathy involving degeneration of the cone-rich central portion of the retina, the macula. A major feature of this disease is a progressive buildup within the retinal pigmented epithelium of lipofuscin, a major component of which is N-retinylidene-N-retinyl ethanolamine (A2E), which is regarded as a major pathological feature [9]. Stargardt disease is caused by mutations within the ABCA4 gene, which were first identified by Rando Allikmets and colleagues [10], the gene encoding a transporter located in the outer segments of rod and cone photoreceptors, which assists in the transport of vitamin A intermediates from the photoreceptors into the retinal pigmented epithelium. Kong and colleagues subsequently showed that a lentiviral vector expressing the ABCA4 gene from a constitutive CMV or Rhodopsin promoter, subretinally introduced into the retinas of ABCA4 mice significantly reduced the accumulation of A2E and suggested that lentiviral gene therapy would likely represent an efficient method of treating this disease [11]. The StarGen product is a lentiviral vector expressing the ABCA4 gene.

A gene therapy trial has also been registered in the UK (Clinicaltrials.gov NCT01461213) in which an AAV vector will be used in phase I/II trials for treatment of choroideremia. The latter is a rare X-linked recessive condition in which the choroid underlying the retina degenerates and is caused by mutations within the RAB Escort protein 1 (REP-1). In this case, the medication is an AAV2 vector expressing the REP-1 gene.

Over the last 10 years, a growing number of proof of efficacy studies have been reported in which gene replacement strategies have been used in rodent and other animal models of retinopathy and many of these undoubtedly represent the forerunners of human gene therapy trials. The majority of these have involved the use of AAV vectors. For example, Pawlyk et al. [12] reported successful gene replacement therapy in a mouse model of LCA caused by null mutations within the

RPGRIP gene, demonstrating correct localization of the protein to the photoreceptor cilia, and preservation of function of photoreceptors by electroretinography. Additional studies validating gene therapy for forms of LCA caused by mutations in other genes were reported in 2010 and 2011 (see below).

Successful gene replacement therapy was also reported in 2005 by Min et al. [13], in a mouse model of retinoschisis. This is a rare X-linked retinopathy characterized by loss of central vision and with the appearance of small cysts within the retina. The retina can also split peripherally, between the inner and outer nuclear layers (hence the name of the disease). The disease is caused in males by a null mutation within the retinoschisin gene (*Rs1*), an AAV vector expressing *RS1* cDNA having been shown by these workers to restore retinoschisin expression and to preserve retinal function and morphology. Additional studies on gene replacement therapy for retinoschisis were reported by Kjellstrom and colleagues [14]. These workers injected an AAV2/2 vector subretinally into *Rs1h* knockout mice at post-natal day 14 and examined retinal structure and function at 14 months, where improved outer and inner retinal structure were observed together with improved ERG amplitudes. These studies were undertaken using the subretinal route of AAV delivery, which induces a detachment of the retina in the region of the inoculations, which subsequently reattaches. Park and colleagues [15] reasoned that an intravitreal route of viral administration might be more feasible in a human scenario in that the procedure does not detach the retina, which is innately fragile in retinoschisis. They were able to demonstrate in retinoschisin knockout mice efficient transduction of the retina following intravitreal injection of the virus.

Further evidence of the efficacy of AAV-mediated gene replacement therapy for retinal degenerations was provided by Alexander et al. [16] in a study targeting achromatopsia. This is an exceedingly rare autosomal recessive retinopathy affecting no more than 1 in 30,000 people in which there is loss of color vision. Defects within four different genes have been identified in different individuals or families. These encode three of the subunits of cyclic nucleotide gated channels, *CNGA3*, *CNGB3* (the α and β subunits of cone photoreceptor cGMP-gated channel respectively), *GNAT2* (guanine nucleotide binding protein 2), in addition to the α subunit of cone cGMP phosphodiesterase (*PDE6C*). Mutations within these genes are encountered with widely differing frequencies, the most common being *CNGB3* in about half of all cases of the disease. These workers demonstrated rescue of cone-ERG responses and visual acuity in a murine model of this disease. In a later study, Komaromy and colleagues [17] used an AAV5 vector expressing the *CNGB3* gene in a naturally occurring dog model of achromatopsia and using a red opsin promoter they were able to show a therapeutic effect for up to 3 years post inoculation. Quoting the authors: ‘Our results hold promise for future clinical trials of cone-directed gene therapy in achromatopsia and other cone-specific disorders’. More recently in 2011, Carvalho and colleagues used an AAV2/8 vector expressing a human cone-arrestin-driven human *CNGB3* gene subretinally inoculated in *CNGB3*^{−/−} mice and were able to demonstrate highly significant restoration of cone function—‘This study represents achievement of the most substantial restoration of visual function reported to date in an animal model

of achromatopsia using a human gene construct, which has the potential to be utilized in clinical trials' [18]. In an additional study of this disease, Michalakis and colleagues [19] reported that cone vision could be restored in *CNGA3*^{-/-} mice using an AAV vector and in this study were able to demonstrate activation of the output neurons of the retina, ganglion cells.

Efficacy of AAV-mediated gene replacement was also demonstrated in 2008 in the naturally occurring rd10 mouse model of autosomal recessive RP. These animals are homozygous for null mutations within the gene encoding the β -subunit of rod photoreceptor cGMP phosphodiesterase, a central enzymatic component of the visual transduction cycle. Pang and colleagues injected an AAV5 vector expressing the β PDE gene subretinally into the animals at P14 and examined animals after a further period of 3 weeks, both scotopic and photopic ERG waveforms being notably preserved, as well as good preservation of the outer nuclear layer of the retina and of photoreceptor outer segments [20]. This work was extended in 2011 with a demonstration that preservation of vision was achievable for at least 6 months post AAV8 injection [21].

While gene therapy trials are well established for LCA involving null mutations within the RPE65 gene, mutations within 16 genes, as outlined above, have been implicated in this condition. One of these is the gene encoding aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1). Mutations within this gene not only cause LCA, but have been encountered in some forms of RP and cone-rod dystrophy. Tan and colleagues [22], reported the use of AAV2/2 and AAV2/8 vectors as gene replacement vehicles in several murine models of LCA bearing mutations within this gene and were able to demonstrate significant rescue of the photoreceptor degeneration. In a subsequent study, Sun and colleagues [23] were also able to demonstrate significant and long-term retinal rescue in mice expressing mutated AIPL1 genes using an AAV vector. Pawlyk and colleagues [24] also investigated gene replacement therapy for LCA in a murine model of disease caused by null mutations within the RPGRIP1 gene, this time using an AAV8 vector expressing a human gene replacement driven by a rhodopsin kinase promoter. Significant therapeutic benefit was obtained, the authors commenting: 'This study demonstrates the efficacy of human gene replacement therapy and validates a gene therapy design for future clinical trials in patients afflicted with this condition'. Another gene involved in LCA (LCA1) encodes guanylate cyclase 1 (Gucy2d), mutations within this gene being estimated to cause up to 20 % of cases of LCA. Boye and colleagues have shown that subretinal inoculation of an AAV5 vector expressing the murine GC1 gene driven either by a rhodopsin kinase or a ubiquitous chicken β -actin promoter resulted in extensive restoration of cone function in treated animals. Quoting the authors: 'These results lay the ground work for the development of an AAV-based gene therapy vector for the treatment of LCA1' [25]. A further study involving AAV-mediated gene replacement of guanylate cyclase 1 in a mouse LCA1 model was reported by Mihelec and colleagues [26]. They subretinally inoculated an AAV2.8 vector expressing a human rhodopsin kinase promoter-driven human GUCY2D gene. The authors concluded: '...we observed a dose-dependent restoration of rod and cone function

and an improvement in visual behavior of transduced area up to 6 months post injection. To date, this is the most effective rescue of the Gucy2e^{-/-} mouse model of LCA and we propose that a vector, similar to the one used in this study, could be suitable for use in a clinical trial of gene therapy for LCA1’.

Gene therapies have also been validated in murine models of a number of hereditary syndromes that incorporate RP. AAV-mediated gene replacement therapy has been validated in a murine model of Usher syndrome type 2D, with a targeted disruption of the DFNB31 gene, encoding the PZD scaffold protein, Whirlin [27]. In that study, Zou and colleagues [27] subretinally inoculated an AAV2/5 vector into DFNB31^{-/-} mice expressing a rhodopsin kinase promoter driven replacement gene. They comment: ‘The combined hRK [kinase promoter] and AAV gene delivery system could be an effective gene therapy approach to treat retinal degeneration in USH2D patients’. A syndromic form of RP involving microphthalmos, foveoschisis and drusen deposition at the optic disk, is caused by mutations within the membrane-tuppe frizzled-related protein (MFRP). In another example of AAV-mediated retinal gene therapy, Dinculescu and colleagues have recently shown in a mouse model of retinal degeneration with mutations in this gene (the rd6 mouse) that an AAV8 vector expressing the murine gene driven by a chicken β -actin promoter subretinally injected at day 14 rescues rod and cone photoreceptors as assayed at 2 months of age [28]. Another well known, though rare syndrome incorporating RP is Bardet-Biedl syndrome. This (usually recessive) condition may feature hypogonadism, mental retardation, polydactyly, obesity and kidney disease and displays extensive genetic heterogeneity, to date, mutations in 15 different genes having been implicated in the disease, which, when mutated result in ciliary dysfunction. One of the consequences of this is that the primary photoreactive pigment of rod cells, rhodopsin, cannot be transported from the inner to the outer segments of the photoreceptors and photoreceptor cell death occurs as a result. The Bbs4 null mouse is a naturally occurring model of Bardet-Biedle syndrome. Simons and colleagues [29] have recently shown that subretinal inoculation of an AAV5 vector expressing the mouse Bbs4 gene results in correct localization of rhodopsin and preservation of rod cells. The authors conclude: ‘...our treatment prevents photoreceptor death, improves electrophysiological function of the retina and preserves visually evoked behavioral responses. These data from a mammalian model suggest that BBS-associated retinal degeneration can be treated’.

It is notable that all human clinical trials so far registered, and all animal studies outlined above, have involved treatment of autosomal recessive or X-linked recessive forms of disease. Here, both versions of the gene in question are non-functional, and therefore a strategy of gene replacement can be employed. However, while essentially all forms of LCA are recessive, up to 30 % of cases of RP display autosomal dominant modes of transmission. Here, the gene product is either ‘haplo-insufficient’, perhaps a rather cumbersome term, simply meaning that one allele is nonfunctional and that expression from the remaining functional gene is insufficient to maintain photoreceptor viability, or alternatively, one gene homolog is functionally normal, while the mutated version exerts a ‘dominant negative’

effect in causing photoreceptor cell mortality. While the distinction between these two basic mechanisms of photoreceptor cell death remains to be fully established for all genes or mutations, many dominant forms of RP fall into the latter category, including, for example, those caused by mutations within the rhodopsin, RDS-peripherin, and IMPDH1 genes. A substantial number of studies in animal models of autosomal dominant retinal disease have been reported in which therapeutic efficacy has been obtained using methods that target disease-causing genes directly. Strategies have included RNAi-mediated down regulation of both normal and mutant alleles at the same time, which in some cases has been shown to be of therapeutic benefit because lack of expression of both alleles results in only a mild phenotype. Alternatively both normal and mutant alleles can be suppressed, followed by introduction of a functional gene that has been modified such as to escape RNAi-mediated suppression. Another strategy has involved overexpression of the normal allele such as to reduce, in relative terms, the amount of mutant gene product.

The concept of a gene-based mutation-independent approach to therapy for autosomal dominant disease was published initially by Millington-Ward and colleagues [30] and reviewed by Farrar et al. [31]. According to this concept, a gene encoding shRNA targeting transcripts derived from a dominant-acting gene is introduced into an AAV vector together with a replacement gene which has been structurally modified at third base degenerate codon sites such as to encode a normal transcript that can not be bound by the shRNA. In this way, a single shRNA molecule can be used to down regulate both normal and mutant transcripts, while at the same time a functional gene encoding transcripts resistant to the shRNA is introduced. This way a single gene therapy vector can, in principle, be used in targeting multiple mutations within a given gene. This is especially relevant, where multiple mutations have been identified within a given gene, such as is the case for rhodopsin, where up to 100 different mutations have so far been encountered in patients with autosomal dominant RP. Studies of this approach in a murine dominant RP model with a mutation in the rhodopsin gene were reported by O'Reilly et al. [32], Chadderton et al. [33], Palfi et al. [34] and Millington-Ward et al. [35]. In an alternative approach, Mao et al. [36] demonstrated that subretinal inoculation of an AAV vector expressing a normal murine rhodopsin gene into mice expressing a dominant acting mutation within the rhodopsin gene (P23H) induced significant protection of the photoreceptors by decreasing, in relative terms the amount of mutant rhodopsin present in rod cells. In an RP10 model of autosomal dominant RP, simultaneous ablation both of normal and dominantly mutated transcripts has been shown to have therapeutic benefit. This form of disease is caused by dominant acting mutations within the inosine monophosphate dehydrogenase gene (IMPDH1). In humans the disease is early in onset, producing significant visual handicap within the second decade of life, current evidence indicating that disease pathology is induced by aggregation of mutant IMPDH1 protein [37]. On the other hand, mice with a targeted disruption of the IMPDH1 gene display a much slower retinal degeneration and even at 1 year of age (equivalent to late middle age in humans) retain much of their outer nuclear layer

and retinal function [37]. Tam and colleagues [38] have shown that subretinal inoculation into mice of an AAV virus expressing shRNA directed towards a mutant human IMPDH1 together with the equivalent endogenous murine transcript, substantially alleviates disease progression.

Given the North Face of genetic heterogeneity that must be surmounted in developing gene-based medicines targeting individual genes, there is clearly a strong rationale in looking for gene-based products that are not required to target primary genetic defects, but rather, molecular pathologies common to more than one, and hopefully many individual retinal conditions. Significant progress is being made in this area and has included studies of the efficacies of retinal protection afforded by expression within the retina of rod-derived cone viability factor (RDCVF), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), the chaperone, Grp78/BiP, lens epithelium-derived growth factor, C1q, X-linked inhibitors of apoptosis (XIAP) and of genes encoding artificial light sensors, including channelrhodopsin and halorhodopsin in output neurons of the retina (ganglion cells) or indeed in surviving, but nonfunctional cone cells, this approach having been referred to as optogenetic therapy.

It is of note that the primary degenerative feature of retinitis pigmentosa is the progressive death of rod photoreceptors, resulting in the initial symptom of the disease—nyctalopia. However, cone photoreceptors persist for longer periods although they can remain viable for substantial periods of time after their functional activity has been lost. In addition, the output ganglion cells of the retina persist long after photoreceptors have degenerated. Optogenetics refers to the concept of genetically modifying ganglion cells or surviving nonfunctional cones by introducing into them genes that encode membrane channels that can be directly activated by light. Two such channels have been extensively explored, channelrhodopsin-2 from *Chlamydomonas reinhardtii*, a flagellated green algae, and halorhodopsin, originally identified in the halophylic microorganism, *Natronomonas pharaonis*, originally isolated from a salt lake in Africa. When inserted into the membranes of neurons, light activation of channelrhodopsin-2 results in direct cell depolarization. On the other hand, light activation of halorhodopsin results in cell hyperpolarization. Bi et al. [39] incorporated a gene encoding channelrhodopsin-2 and green fluorescent protein into an AAV2 vector driven by a hybrid chicken β -actin-CMV promoter. Intravitreal injection of this virus into rd1—/— mice, in which photoreceptor cells were absent, resulted in transfection of retinal ganglion cells and extensive expression within the inner plexiform layer of the retina. It is of note that stable transfection of the retina was achievable for at least 12 months. Dissociated retinal ganglion cells expressing channelrhodopsin-2 could be identified by virtue of green fluorescence, patch clamp recordings from individual cells indicating light-evoked responses were generated and in addition, visual evoked responses were recordable in such animals. However, the authors pointed out that AAV-mediated transfection of ganglion cells targets both ON- and OFF-ganglion cells at the same time and how this would impact on visual perception remained to be established. Restoration of

visual response by channelrhodopsin in the RCS rat has also been reported by Tomita and colleagues [40, 41]. Busskamp and colleagues [42] have more recently incorporated a halorhodopsin gene driven either by a red cone opsin or cone arrestin-3 promoter into an AAV vector, with the objective of transfecting viable, though nonfunctional cone photoreceptors in slow and fast mouse models of retinal degeneration (*CNGA3*^{-/-}*Rho*^{-/-} and *rd1* respectively). The advantage of halorhodopsin is that in response to light-activation, transfected neurons are hyperpolarized, as is the case in natural light activation of cone photoreceptors. Visual evoked potentials could be detected in treated animals and in both dark light box and optomotor tests, treated animals performed better. Moreover, these authors have identified patients with RP whom, by OCT analysis have no photoreceptor outer segments but who retain cone cell bodies and potentially these individuals could be subjects for clinical trials.

Extensive studies have been undertaken into the efficacy of expression neurotrophic factors in protecting photoreceptors. Faktorovich and colleagues, in an early pioneering study reported in 1990, that intravitreal inoculation of basic fibroblast growth factor (bFGF) directly into the vitreous humor of RCS rats, a model of autosomal recessive RP homozygous for null mutations within the *MERTK* gene, afforded extensive protection to photoreceptor cells [43]. LaVail and colleagues demonstrated in 1992 that direct intravitreal inoculation of a variety of neurotrophic factors into the eyes of wild type rats, protected the retinas from the effects of light damage [44]. Effective agents included bFGF, BDNF, CNTF, interleukin 1 β , and acidic fibroblast growth factor. Subsequently, La Vail and colleagues also reported results of direct intravitreal inoculation of a variety of neurotrophic factors into mouse and rat retinopathy models and concluded that retinal degeneration can be slowed down by CNTF and leukemia inhibitory factor and in some cases by BDNF [45]. Given the proinflammatory role of IL-1 β , Whiteley and colleagues [46], also demonstrated that doses of IL-1 β much lower than had previously been used, still afforded retinal protection while minimizing inflammatory damage in the RCS rat model. The authors conclude, 'Since the survival-promoting effects of IL-1 β are comparable to those of bFGF, even when given at much lower dosages, this cytokine may play an important role in the phenomenon of injury-related PRC [photoreceptor cell] rescue and represents another potentially useful survival factor for retinal degenerative diseases' [46]. Cayouette and colleagues, subsequently, demonstrated that intravitreal injection of an adenoviral vector expressing CNTF significantly protected the retinas of *rd*^{-/-} mice, with a naturally occurring null mutation of the gene encoding the β subunit of cGMP phosphodiesterase [47]. They also found that intravitreal inoculation of CNTF produced a protective effect on the retina, but less so than expression from the AV genome. Liang and colleagues [48] were also able to demonstrate a protective effect of this polypeptide in *Rho*^{-/-} mice. These authors incorporated a CNTF gene along with a GFP gene into an AAV vector, which was subretinally inoculated into the rhodopsin knockout mice. In such animals, all rod photoreceptors die over a period of approximately 3 months, and in these experiments, expression of CNTF produced a protective effect over the entire 3-month period.

An additional study was reported by these authors in 2001 in which an AAV vector expressing CNTF was intravitreally as well as subretinally inoculated into the eyes of *rds*^{-/-} mice and into the eyes of P23H and S334ter rhodopsin transgenic rats [49]. They found significant structural protection of the photoreceptors in all animals examined, although in treated rats, ERG amplitudes were lower than controls. The authors suggest: ‘the need for greater understanding of the mechanism of action of CNTF before human application can be considered’. Further studies of the potentially beneficial effects of CNTF therapy were reported in 2002 by Bok and colleagues [50]. These authors introduced AAV vectors expressing a CNTF gene driven either by CMV or chicken β -actin promoters into the retinas of mice expressing a dominant mutation within the *rds*-peripherin gene. They observed long-term pan-retinal protection of the retinas of these animals, which was more readily achievable when the virus was subretinally inoculated. However, as noted previously in other models, both scotopic a and b wave ERG amplitudes were reduced in treated animals. The authors conclude: ‘...proper doses of CNTF administration should be determined before human clinical trials are considered for the amelioration of inherited retinal degenerations with CNTF’. Schlichtenbrede and colleagues [51], using *rds*^{-/-} mice, while observing improved photoreceptor structure as a result of intravitreal injection of AAV expressing CNTF, also saw a marked deterioration in retinal function assessed by ERG, and even found such deterioration in wild type mice similarly injected. They conclude: ‘Our results demonstrate that intraocular CNTF gene delivery may have a deleterious effect on the retina and caution against its application in clinical trials’. Adamus and colleagues [52] studied the effects of AAV-mediated expression of CNTF in a mouse model of cancer-associated retinopathy. In humans, this is a rare syndrome and when it occurs it is most frequently associated with lung carcinoma. The disease is associated with the presence of autoantibodies against the retina expressed protein, recoverin, and can be modeled in mice by inoculating anti-recoverin antibodies. These researchers subretinally inoculated an AAV vector expressing CNTF into mice that were then inoculated with anti-recoverin antibody to induce a retinal degeneration. The authors found a significant protective effect in retinas expressing CNTF and conclude: ‘CNTF may be a useful treatment for human antibody-mediated retinal degeneration’. In an additional study reported in 2004, Huang and colleagues examined the effect of direct inoculation into the eye of recombinant CNTF as well as AV-mediated expression of the cytokine in the RCS rat [53]. They found that photoreceptor cell death was potentially delayed and that treated animals showed improved retinal function assessed by ERG. They conclude: ‘this study indicates that adenoviral CNTF effectively rescues degenerating photoreceptors in RCS rats’. In 2006, Buch and colleagues reported deleterious effects of AAV-mediated expression of CNTF were dose dependent, but administering levels of CNTF that did not affect photoreceptor function, did not protect photoreceptors from degeneration [54]. In the same study, however, they reported that AAV-mediated expression of GDNF afforded protection to photoreceptors in two rodent models of RP. In humans, a phase I, and two phase II clinical studies have been undertaken using Encapsulated Cell Technology (Neurotech Inc) in which a

small 6 mm long implant containing human cells genetically engineered to secrete CNTF is surgically implanted in a removable way into the vitreous of the eye. In a recent study, cone cell structure and function were reported in detail on three patients having received such implants, one patient with autosomal dominant RP with a rhodopsin mutation, one with Usher syndrome type 2 and one with simplex RP [55]. The authors conclude: ‘In this study presenting the first images of cone photoreceptors in human eyes treated with CNTF, the results suggest that CNTF may slow cone photoreceptor loss in eyes with retinal degeneration. They also provide evidence to support the pursuit of additional, larger prospective, masked clinical trials of CNTF using AOSLO images as an outcome measure of disease progression and treatment response’.

Many studies of the protective effects of GDNF on photoreceptors have been reported. Frasson and colleagues [56] investigated the effects of direct intraocular injection of GDNF in the *rd*–/– mouse, a naturally occurring model of retinal degeneration homozygous for null mutations within the RDS-peripherin gene. While at 22 days of age rod-derived ERG responses were unrecordable in untreated animals, a proportion of treated animals displayed improved retinal function and all animals treated had significantly greater numbers of rod cells. Given that mutations within the RDS-peripherin gene are found in human RP, these authors concluded, ‘GDNF represents a candidate neurotrophic factor for palliating some forms of hereditary human blindness’. McGee and colleagues [57] investigated the protective effects of AAV-mediated expression of GDNF in photoreceptor cells of a rat model of RP expressing a dominant S334-ter mutation within the rhodopsin gene. While a significant protection of the retina was observed, the effects diminished after 60 days as the photoreceptors expressing the cytokine degenerated. Wu and colleagues [58] examined the long-term safety of delivering a gene expressing GDNF incorporated into an AAV vector by the intravitreal route in wild type Sprague–Dawley rats. Accumulation of GDNF was immunologically confirmed for a period of up to 1 year, and there appeared to be no negative effects on retinal function or histology. In 2006, Hauck and colleagues showed that receptors for GDNF, GFRalpha, and RET are on Muller glial cells but not on photoreceptors in pig retinas [59]. They showed that GDNF induces ERK phosphorylation and that such signaling results in the induction of FGF-2. Thus, the protective effects of GDNF were shown to be mediated by Muller cells. In 2007, Dong and colleagues reported a study in which degeneration of the retinas of mice was induced by oxidative damage following treatment with paraquat, FeSO₄, and hyperoxia [60]. These were transgenic animals incorporating a GDNF gene that was inducible within the retina by doxycycline. They observed a significant positive effect on retinal function as assessed by ERG and on retinal morphology. These authors concluded that their data ‘suggest that gene transfer of GDNF should be considered as a component of therapy for retinal degenerations in which oxidative damage plays a role’, therefore suggesting that this neurotrophic factor may prove to be of value in treatment of age-related macular degeneration in addition to hereditary retinopathies such as RP. Further indication of the benefits of GDNF expression within the retina was provided by Gregory-Evans and

colleagues [61]. They used mouse embryonic stem cells generated to express elevated levels of GDNF and up to 200,000 of such cells were intra-vitreally inoculated into the retinas of rats expressing a dominant-acting mutation (S334ter) within the rhodopsin gene—a model of autosomal dominant RP at day 21 post-birth. Stem cells expressing GDNF were still detectable in the retinas of these animals at day 90, photoreceptor cell counts being significantly higher in treated animals. Further studies on the mechanisms of neuroprotection induced by GDNF were reported in 2011 by Del Rio and colleagues, in a study of transcripts induced in retinal tissues by GDNF [62]. One of these encoded the cytokine, osteopontin (OPN). OPN was further shown to display a protective effect on photoreceptors in culture and on retinal explants from *Pde6brd1* mice, the latter an autosomal recessive retinopathy, the authors concluding that these findings ‘suggest that RMG [retina muller glia]-derived OPN is a novel candidate protein that transmits part of the GDNF-induced neuroprotective activity of RMG to PR [photoreceptor] cells’. In a recent (2011) study, Dalkara and colleagues [63] targeted expression of GDNF to retina Muller glial cells, reasoning that these cells are natural secretors of neurotrophins, that they span essentially the entire retina and that they persist even in extensively degenerating retinas. The AAV used in these experiments was a capsid variant that was able to selectively transduce Muller cells [64]. Expression of GDNF from Muller cells leads to long-term protection of the retinas of transgenic rats expressing a dominant-acting mutation (Ser334-ter) in the rhodopsin gene, a model of RP.

It has been established for some time that introduction of wild type murine photoreceptors into the eyes of *rd*—/— mice protected cone photoreceptors from degeneration and that the protective effect appeared to be the result of a diffusible factor which was probably a protein [65–68]. In order to identify the gene or genes involved, Leveillard and colleagues screened up to 200,000 cDNAs derived from a normal mouse retina in an in vitro cone cell survival assay and identified a clone, encoding a protein which was termed RdCVF [69]. The RdCVF gene (*Nxn1*) encodes a thiol-oxidoreductase enzyme and is believed to protect the retina from oxidative stress [70]. Supporting the protective role of this protein, *Nxn1*—/— mice display a gradual degeneration in cone function and injection of the purified protein into the retinas of transgenic rats expressing the common P23H mutation within the rhodopsin gene resulted in significant protection of cone photoreceptors [71]. These data suggest that retinal protection could be achieved in genetically diverse forms of retinopathy by introduction into the retina of an AAV vector expressing the *NXNL1* gene to compensate for the loss of this secreted protein as rod cells die off.

A number of approaches to retinal protection have been explored which involve the direct targeting of apoptotic processes, approaches involving both inhibition of apoptosis, and enhancement of its efficiency, having been explored. In regard to the former, the rationale was to preserve photoreceptors by suppressing the mechanism inducing their death. In regard to the latter, the reasoning was that if apoptotic mechanisms were enhanced, dying photoreceptors may be engulfed more efficiently by the RPE or by resident retinal macrophages, thus reducing the

number of cells dying by necrotic processes, where the contents of cells would be liberated, thus exacerbating inflammatory processes which may be to the detriment of remaining retinal neurons. Apoptosis, or programmed cell death, involves the condensation of dying cells into apoptotic bodies, which are readily phagocytosed, and many studies have indicated that it is a common mechanism of cell death in degenerative retinopathies [72–74]. A number of studies in which the ability of anti-apoptotic members of the Bcl family, Bcl-2 and the related protein Bcl-XL, to slow down the death of photoreceptors in animal models of degenerative retinopathy have been reported [75–78]. Bcl-2 exerts its protective effect by inhibiting the export of cytochrome C from photoreceptor mitochondria [79, 80]. However, somewhat mixed results have been obtained from these experiments. Chen et al. [76] generated transgenic mice in which relatively high levels of Bcl-2 protein were expressed using an opsin promoter to drive the gene. They crossed these animals with mice expressing a truncated rhodopsin gene (S334ter) in which retinal degeneration is normally rapid, all photoreceptors degenerating in such animals by 3 weeks of age. They also crossed Bcl-2 expressing mice onto an *rd*–/– background, *rd*–/– mice normally losing their photoreceptors, again within about 3 weeks of birth. The effects of elevated expression of Bcl-2 on light-induced photoreceptor degeneration, where, after 14 days of constant light exposure, most photoreceptors are normally dead, were also tested. In all three scenarios, significant protection of photoreceptors was achieved. The authors concluded, ‘These results strongly support the idea that retinal degeneration may be treated by modulating the entry of PRs [photoreceptors] into the apoptotic cell death pathway’. Tsang and colleagues [77] undertook similar experiments in which mice with a homozygous mutation within the gamma subunit of retinal cGMP phosphodiesterase, normally developing a very rapid retinal degeneration where photoreceptors are essentially all dead within 4 weeks of birth, were crossed with a strain of mouse expressing an opsin-driven Bcl-2 gene. The results of these experiments clearly indicated a protective effect induced by expression of Bcl-2, but this was only of limited duration. Joseph and Li [75], however, were unable to demonstrate any protective effect of expression of Bcl-2, or Bcl-XL in the retinas of *rd*–/– mice and in mice expressing a dominant mutation (K296E) within the rhodopsin gene, nor were they able to show any protective effect of these genes against light damage. Nir and colleagues [78] investigated the possible protective effects of expression of Bcl2 in the retinas of *rds*–/– mice. These animals were homozygous for recessive null mutations within the *rds*-peripherin gene, mutations within which are known to cause both recessive and dominant forms of RP as well as forms of macular degeneration. At 3 months of age, the retinas of *rds*–/– mice normally have about three rows of photoreceptor nuclei in their outer nuclear layer. When crossed onto mice expressing a Bcl-2 gene driven by a mouse rhodopsin promoter, up to six rows of photoreceptor nuclei were observed, the authors concluding that ‘Bcl-2 expression may be an effective therapeutic strategy in humans with mutations in the RDS or other genes that affect the integrity of photoreceptor outer segments’. The reasons for such discrepancies in these studies are unclear, although *rds*–/– mice display a relatively slow degeneration in

comparison to the other models of retinal degeneration that were employed, which may partially account for the differences in protective effects observed. To date, no clinical trial involving expression of Bcl-2 or Bcl-XL has been registered.

Given the commonality of apoptosis as a mechanism of photoreceptor cell death in degenerating retinas, research has also focused on elevating the expression of the most extensively characterized natural apoptotic inhibitor, XIAP, or X-linked inhibitor of apoptosis. This protein, as its name implies, is encoded by a gene on chromosome X, binding through its baculovirus-inhibitor of apoptosis repeat (BIR) domains, caspases 3, 7 and 9, thus inhibiting the process of apoptosis [81]. Many studies have been reported in which the protective effects of XIAP expression have been assessed in animal models of retinal degeneration, glaucoma and retinal detachment and on RPE cells in relation to oxidative stress [82–86]. In addition, XIAP expression has recently been shown to enhance the viability of transplanted photoreceptor precursor cells in mice [87]. In a study reported by Leonard and colleagues [85], the potentially protective effects of XIAP expression were assessed in two well characterized rat models of autosomal dominant RP, expressing either P23H or S334ter mutations of the rhodopsin gene. In this series of experiments, a full length XIAP gene was incorporated into the genome of an AAV5 vector under the control of a chicken β -actin promoter. The virus was introduced into the retinas of these animals by sub-retinal inoculation. Animals were injected between post-natal days 14 and 17 and were functionally and structurally assessed 28 weeks after inoculation. The ONL of treated animals was significantly thicker than controls in both animal models. Functional assessment of the retinas of these animals was also undertaken by electroretinography. Little or no improvement in ERG waveforms was observed in S334ter animals, although both ERG a- and b-wave amplitudes were significantly improved in animals with the P23H transgene. The authors conclude that their results ‘clearly demonstrate the potential of XIAP gene therapy for the treatment of inherited retinal degenerations. It is important to note [however] that the XIAP treatment does not represent an all-encompassing gene therapy strategy for retinal degeneration’. In our opinion, XIAP therapy, based on these data, could be potentially effectively used in combination with therapy targeting specifically-mutated genes.

Interestingly, XIAP therapy has also recently been used by Yao and colleagues to enhance the viability of transplanted photoreceptor precursors in a murine model [87]. MacLaren and colleagues initially demonstrated in 2006, that transplantation of photoreceptor precursors from donor mice at the time of peak of rod photoreceptor genesis (post natal day 1) resulted in the development of transplanted precursors into apparently mature photoreceptors that were able to migrate into the outer nuclear layer of transplanted retinas [88]. Yao and colleagues pre-transfected immature photoreceptor cells with a XIAP-expressing AAV vector and were able to show that transplanted cells showed increased survival over those that were not expressing XIAP, the authors concluding that ‘preventing programmed cell death through XIAP therapy may be an important component of future therapeutic retinal cell transplantation strategies’. It is of interest also to note that expression of XIAP has also been shown by Shan and colleagues

to protect retinal RPE cells from apoptosis induced by exposure of such cells to peroxide [89]. The authors suggest that expression of XIAP may, therefore, be beneficial in protecting the RPE and photoreceptors from oxidative stress in age-related macular degeneration.

In terms addressing the concept of improving the efficiency of the apoptosis in degenerating retinas to reduce the likelihood of exacerbating retinal degeneration owing to cell disruption by necrotic processes, we have recently shown that cone photoreceptor survival in mice homozygous for a targeted mutation of the rhodopsin gene, a model of autosomal recessive RP, is significantly enhanced by the presence of C1q, a primary component of the classical complement pathway [90]. The rationale for addressing this hypothesis was that C1q is known to bind to cells in late-stages of apoptosis, which results in deposition onto such cells of complement components C3 and C4, facilitating their phagocytosis. In the absence of C1q, the efficiency of apoptotic clearance of dying photoreceptors may be reduced, favoring necrosis and cell lysis and the spilling of cell contents into the retina, which may negatively impact on photoreceptor survival. These results suggest that optimum cone cell survival and hence daytime vision, will be achieved by maintaining, or possibly enhancing levels of C1q within the retina. It is also of interest to note that Galvan and colleagues have recently shown that C1q enhances the expression within the retina of the MERTK which is active in facilitating engulfment of shed outer segment photoreceptor disk membranes into the RPE, providing an additional possible explanation for the protective effects of C1q on photoreceptor survival [91].

In conclusion, gene therapies are now in human clinical trial for some forms of degenerative retinopathy, while an impressive portfolio of gene-based medicines targeting primary genetic lesions, in addition to those targeting disease pathologies common to multiple forms of disease have now been convincingly validated in a growing number of animal disease models, setting the scene for translation into the clinical setting.

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