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Gene therapy for retinal disease

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Abstract

Gene therapy strategies for the treatment of inherited retinal diseases have made major advances in recent years. This review focuses on adeno-associated viral (AAV) vector approaches to treat retinal degeneration and thus prevent or delay the onset of blindness. Data from human clinical trials of gene therapy for retinal disease show encouraging signs of safety and efficacy from AAV vectors. Recent progress in enhancing cell-specific targeting and transduction efficiency of the various retinal layers plus the use of AAV-delivered growth factors to augment the therapeutic effect and limit cell death suggest even greater success in future human trials is possible.

INTRODUCTION

Inherited retinal diseases (IRDs) are heterogeneous disorders that cause degeneration of the retina, a multi-layered structure composed of various cell types (Figure 1). Light must pass through layers of neuronal cells (primarily ganglion and bipolar cells) to reach the light-sensitive rod and cone photoreceptors at the back of the retina. The outer segments of these photoreceptors contain pigments that absorb various wavelengths of light and via a G-protein cascade convert the light into electrochemical signals that are transferred back through the bipolar and ganglion cells, through the optic nerve to the brain. There are three types of cone photoreceptor, defined by one of three cone opsins expressed. Opsins form the photopigments that sense different wavelengths of light (short, S; medium, M and long, L). Rod photoreceptors have just one photopigment (rhodopsin) and are more sensitive to low levels of light. This leads to cone photoreceptors providing us with colour vision and visual acuity in daylight (photopic vision) whereas the rod photoreceptors allow us to see in dim light conditions (scotopic vision). Supporting the photoreceptors at the back of the retina is a single layer of cells known as the retinal pigment epithelium (RPE) and normal function of these cells is essential for photoreceptor survival. Depending on the genetic cause of a particular IRD, cell death may be confined to one cell type within the retina, such as in the cone dystrophies (1) or result in loss of more than one cell type, for example as in retinitis pigmentosa (RP), which begins with a primary loss of rod photoreceptor cells but over time the cone photoreceptors also degenerate as their survival is dependent upon rod survival (2, 3).

Mutations in genes specific to the inner retinal layer can result in IRD but for the majority of cases the cause is mutation in genes expressed in the photoreceptor or RPE cells (4, 5). Mutations in over 200 genes have been identified that cause IRDs (<http://www.sph.uth.tmc.edu/RetNet/>) with a range of inheritance patterns exhibited. The most common form of IRD is retinitis pigmentosa, for which at least 30 genes have been

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associated (5). Around 5% of retinal disease cases are the result of early onset retinal dystrophy, Leber congenital amaurosis (LCA), which may show a more severe phenotype than RP (6). Cone and cone-rod dystrophies that primarily affect the cone photoreceptors are more rare as are macular disorders in which retinal degeneration is confined to the central retina (7). Due to the importance of RPE function in the survival of photoreceptors, mutations in genes specific to the RPE cells can lead to photoreceptor death prior to RPE degeneration (8). It is therefore essential to understand the etiology and subsequent pathophysiology of any particular IRD before attempting to intervene with gene therapy.

As a gene therapy target, the retina has many features that may facilitate therapeutic interventions. Surgical access is relatively easy and the route of administration can be altered to enable targeting of either the inner retina (by intravitreal injection) or the outer retina and RPE (by subretinal injection). The blood-retina barrier separates the subretinal space from the blood supply, enhancing its prospects for effective gene therapy by protecting it from immune-mediated damage. Any therapeutic effect can then be safely evaluated by non-invasive methods such as electroretinography (ERG) and optical coherence tomography (OCT).

There are numerous mouse models of the common forms of IRD available, both naturally occurring (9) and transgenic (10), for studying the pathophysiology of IRDs. In the case of disorders such as autosomal recessive RP in which retinal degeneration is progressive over time, gene replacement therapy may be attempted at an early stage of disease before there is complete loss of photoreceptors (11). It may also be appropriate to deliver anti-apoptotic factors that restrict further cell loss (12). Alternatively, an optogenetic approach may be considered in the terminal stage of disease when only the inner neuronal layers of the retina remain. This strategy attempts to provide a light-inducible pigment such as Channelrhodopsin-2 to residual cells in order to make them intrinsically photosensitive (13). It is apparent that the physiology and progression of a particular IRD will have an impact on determining the most effective gene therapy approach. As the retina degenerates, transduction of the remaining retina may be less effective due to the structural changes (14). In a mouse model of moderate RP, *Rpe65*^{R91W/R91W}, the loss of rod followed by cone photoreceptors is not as rapid as in other models (such as the *rd10* mouse model), thus allowing for investigation regarding how late into the disease progression cone rescue can still occur (15). The results suggest that for RP cases resulting from missense mutations, it may be possible to treat with gene therapy even in the later stages of disease and still rescue cone function. Given that cone photoreceptors are responsible for visual acuity and daylight vision, the ability to rescue and prevent their loss could have a critical impact on a patient's life. Knowing when to treat and being able to target specific cell types of the retina will increase the safety and efficacy of the gene therapy treatment.

CELL-SPECIFIC TARGETING WITHIN THE RETINA: SEROTYPE TROPISM

The majority of IRDs are caused by mutations in genes specifically expressed in the photoreceptor or RPE cells, therefore the ideal gene therapy would provide efficient transduction with restricted transgene expression to these cell types. Adeno-associated virus (AAV) is currently the favoured vector for retinal gene therapy but lentiviral (LV) vectors are also being pursued. Whilst LV vectors have been shown to be efficient in targeting murine RPE cells (16), transduction of photoreceptors has been less successful (17) except during development when both human immunodeficiency 1 virus (HIV1) and equine infectious anaemia virus (EIAV) derived LV vectors were able to target photoreceptors soon after birth before a physical barrier is formed between the RPE and photoreceptor cells (18). This suggested that the lack of the photoreceptor transduction seen following subretinal injection may be due to the size of the LV vectors, resulting in their inability to diffuse from

the subretinal space, through the outer limiting membrane (OLM) and inter-photoreceptor matrix to the photoreceptor cells. However, in degenerate retina where the OLM structure is disrupted, two different HIV1-derived LV pseudotypes still show relatively poor photoreceptor transduction (19). In contrast to this, recombinant AAV (rAAV) viral vectors have shown consistent success in being able to transduce all layers of the retina, particularly the RPE and photoreceptors (20). Intravitreal injections allow for targeting of the ganglion cells whereas subretinal injections of rAAV transduce the RPE, photoreceptors and inner neuronal layers, depending on the serotype. Over 100 AAV serotypes have been identified and pseudotyping the variants has been a useful method of manipulating the viral vector target specificity in addition to enhancing retinal transduction efficiency. AAV vectors have been pseudotyped to contain the genome of one AAV variant packaged in the capsid of another (21). For the retina, the genome of AAV2 is commonly used and the ability of pseudotyped AAV vectors to transduce the various retinal cell types has been extensively assessed following both intravitreal and subretinal delivery. Intravitreal injections of rAAV2/2 and rAAV2/8 in C57BL/6 mice led to transduction of retinal ganglion cells (22). Subretinal injections of rAAV2/1 and rAAV2/6 were shown to transduce only RPE cells (23) as was rAAV2/4 (24) whilst rAAV2/2, rAAV2/5, rAAV2/7, rAAV2/8 and rAAV2/9 all show RPE and photoreceptor transduction in mice (25). Expression onset of the transgene varies between the serotypes with rAAV2/2 showing later onset of photoreceptor expression than rAAV2/1, rAAV2/5, rAAV2/8 and rAAV2/9, for which GFP was evident 3-5 days post-injection. It was noted that rAAV2/8 and rAAV2/9 were particularly successful in transducing cone photoreceptors (75% compared to 35% shown by rAAV2/5, (25)). Photoreceptor transduction by rAAV2/5 was more efficient than with rAAV2/2 (26). Whilst these studies are helpful as an indication of ideal AAV vector choice for *in vivo* testing of mouse models, it is important to remember that serotype tropism may differ between species. In nonhuman primates, rAAV2/2 showed good transduction of rod photoreceptors and RPE (27) whilst rAAV2/5 was efficient in transducing rod photoreceptors but not cones (28). rAAV2/5 was later shown successfully to target primate cones when used in combination with a cone-specific enhancer and promoter (29). It is thought that receptors on the various cell surfaces are responsible for the serotype tropisms observed (30). In liver transduction studies, the ability of the host cells to uncoat different serotypes appeared to vary, effecting transduction and subsequent expression (31). It may be that a similar phenomenon also occurs in cells of the retina.

Recently the development of tyrosine mutant serotypes has greatly enhanced transduction efficiencies of the rAAV vectors. Entry of AAV into a host cell triggers ubiquitin-mediated degradation by the proteasome, a process which affects the transduction efficiency of rAAV vectors (32). The conversion of tyrosine residues to phenylalanine on the AAV capsid is thought to prevent ubiquitination of the virus within the host cell and subsequent degradation, thus enhancing transduction efficiency (33). Targeting the inner retinal layer, such as bipolar cells, has previously proven to be particularly difficult but the rAAV2/8 Y733F vector revealed reporter gene expression in bipolar cells of *rd10* mice (34). Studies of these mutant capsid forms have revealed a significant improvement in transduction of all retinal layers, intravitreal injections of rAAV2/2 Y444F even revealed consistent transduction of the photoreceptor layer (35). Similarly, rAAV2/2 Y444F revealed ganglion cell transduction following subretinal delivery, suggesting AAV vectors can diffuse through all the layers of the retina but require particular conditions to survive and transduce the various cell types. Of the rAAV2/8 mutants, Y733F showed quicker onset of transgene expression compared to rAAV2/8 following subretinal injection, with EGFP expression seen at 10 days post-injection. The single tyrosine mutants created appeared not to affect the cellular tropism of the serotype but did enhance the transduction potential. However, when multiple mutations were created in a single capsid, vectors exhibited different *in vivo* transduction properties with the sextuple mutant rAAV2/2 vector (Y252, 272, 444, 500, 704,

730F) exhibiting transduction of all the retinal layers, including the ganglion cells, following subretinal injection (36). Clearly these tyrosine mutant capsid variants have the potential to greatly enhance retinal gene therapy and studies of their success in animal models of IRD will be discussed in another section of this review.

PROMOTER CHOICE IN RETINAL GENE THERAPY

The use of ubiquitous promoters has helped reveal transduction patterns of AAV vectors (as described above) but as human gene therapy trials are becoming more frequent, the development of vectors that specifically target the cell of interest will increase safety and efficacy of the treatment. In retinal gene therapy animal studies, the commonly used ubiquitous promoters have been immediate-early cytomegalovirus (CMV) enhancer-promoter (37) and the CAG promoter, which combines the CMV enhancer with the chicken α -actin (CBA) promoter (38). Despite the good expression levels generated from these promoters, the ideal scenario in a clinical application of retinal gene therapy will be to deliver therapeutic gene expression using a promoter specific to the targeted cell type. For RPE-specific expression, promoters from two genes involved in retinal disease have been tested. *RPE65* mutations cause both RP and LCA and a fragment of the human *RPE65* promoter (−1556 to +23bp) was used to induce RPE-specific expression of *RPE65* in the RPE cells of *RPE65*-deficient Briard dogs, although this was found to be only 10% as strong as the CMV promoter and ineffective in older animals (39). A similar fragment of the human *REP65* promoter was used to drive *RPE65* expression in a human LCA clinical trial (40). A second potential RPE-specific promoter is that of *VMD2* (41), a gene that encodes bestrophin, mutations in which cause the macular dystrophy known as Best disease (42). This promoter appeared to drive only weak expression compared to CBA, cone arrestin and mouse opsin promoters in *in vitro* transductions of ARPE19 (RPE-derived) and 661W (cone photoreceptor-derived) cell lines (43). Concise reports of activity following rAAV administration to the retina are unavailable but a 2005 review (44) reported that use of a −585 to +38bp *VMD2*-promoter fragment in rAAV1 was able to limit GFP expression to the RPE but for a few GFP-positive photoreceptor cells. More recently, the *RPGR* promoter region has been defined and characterised (45) and may prove useful in future RPE-targeting.

Photoreceptor targeting in the murine retina has been successfully achieved using the rhodopsin and rhodopsin kinase promoters (25). The combination of serotype and cell-specific promoter led to significantly higher photoreceptor cell expression in subretinally injected mice, though the onset of expression was reduced compared to the ubiquitous CMV and CBA promoter (3-4 weeks compared to 5-12 days). The human rhodopsin kinase (*hGRK1*) promoter is ideal for rAAV photoreceptor targeting due to its small size (292bp) and effective targeting and activity in mice (46), Figure 2, and nonhuman primate retina (47). However, in a canine model, GFP expression is limited to rods except with high doses, at which levels cone toxicity is observed (48). Numerous gene therapy studies using mouse models have been reported in which photoreceptor-specific expression was achieved using the *hGRK1* promoter with effective rescue of phenotype (11, 46, 49-52). For some IRDs such as the cone and cone-rod dystrophies, cone-specific photoreceptor expression may be desired. Various combinations of the L/M opsin promoter were tested using rAAV2/5 in a canine model and the largest version at 2.1kb (named PR2.1) was found to be effective in specifically transducing L/M cone photoreceptors (53). An issue with the PR2.1 promoter is not only its large size, which limits remaining space within the transgene, but also that it will only target L and M cone photoreceptors, excluding S cones from treatment. For this reason the cone arrestin (*CAR*) promoter may be a better option for cone-specific expression (54), as used in the *CNGB3* treatment of the *Cngb3* knockout mouse model of achromatopsia (55).

If significant degeneration of photoreceptors occurs before gene therapy intervention then targeting of the bipolar cells for optogenetic strategies may be the desired treatment approach. For this purpose a 200bp fragment of the murine *Grm6* enhancer, 445bp *Cabp5* promoter and 164bp *Chx10* enhancer have been identified as potential fragments to drive bipolar-specific gene expression (56). When the *Grm6* enhancer was fused to a 203bp fragment of the SV40 promoter, reporter gene expression was restricted to bipolar cells following subretinal injection in the *rd10* mouse model of RP (34). The ganglion cell layer is another optogenetic target for when the outer retina layers are completely lost, so far a fragment of the human connexin 36 promoter (2.8kb) has revealed selective transduction of foveal bipolar ganglion cells following intravitreal delivery of rAAV2/2 to macaque eyes (57).

Whilst use of a cell-specific promoter is desirable, often the size can restrict the vector capacity, limiting the therapeutic genes that can be delivered, as in the case of PR2.1. Expression levels may also vary between species with further testing required before using other cell-specific promoters in clinical trials. Clearly the correct combination of tyrosine mutants, capsid serotype and promoter have the potential to significantly increase safety and efficacy of retinal gene therapy treatments but such combinations require further optimisation and testing in larger animal models. Improvements in *ex vivo* retina survival (58) will also allow for tropism testing of various serotype and promoter combinations in human tissue using explants from routine retinectomy procedures (Figure 2). Another issue to consider with cell-specific promoters is that their activity may be affected in degenerating retina as expression patterns from the targeted cell types may change during the pathophysiology of disease. This may explain, for instance, the poor results in older Briard dogs (39) and in the LCA clinical trial using the *hRPE65* promoter (40), as discussed later in this review.

SELF-COMPLEMENTARY AAV VECTOR USE IN RETINAL GENE THERAPY STUDIES

The onset of expression following AAV infection is delayed by numerous factors but the rate-limiting step is the synthesis of the complementary strand to the ssDNA AAV genome (59, 60). Self-complementary AAV (scAAV) vectors have been developed that can bypass this critical step. These vectors package a single-stranded transgene that contains both a forward and reverse copy of the therapeutic gene separated by an altered ITR. Following entry into the target cell and capsid uncoating, the single-stranded genome folds to form a dsDNA hairpin, a formation likely to be triggered by the ITR in the centre of the genome (61). Several studies investigating scAAV transduction of the retina have been conducted in various animal models. Compared to the standard rAAV2/2 equivalent vector, scAAV initiated more rapid onset of reporter gene expression following subretinal delivery to mouse retina in both RPE and photoreceptor cells (62). Comparisons of transduction from scAAV2/2, scAAV2/5 and scAAV2/8 compared to rAAV2/8 found consistently faster onset of reporter gene expression and higher levels of stable gene expression from the self-complementary vectors in adult murine retina (63).

By design this form of vector offers both an enhancement to retinal gene therapy but also a downside: a reduction in packaging capacity, with two copies of the coding sequence needing to fit within ~5kb of DNA. If inclusion of a larger cell-specific promoter is also required then scAAV vectors may not be the ideal choice for the gene therapy treatment of many IRDs. scAAV vectors have been used in retinal gene therapy studies of retinal disease in animal models and these will be considered below.

AAV TREATMENT OF AUTOSOMAL RECESSIVE MODELS OF RETINAL DISEASE

The majority of animal models of IRD exhibit null mutations, relevant to the study of autosomal recessive IRDs. Many studies have shown the feasibility of gene replacement therapy in such animal models. Peripherin2 is a rod photoreceptor-specific structural protein mutations in which lead to autosomal recessive RP or macular dystrophy (64). The *rd*s (retinal degeneration slow) mouse is homozygous for a null mutation in the *rd*s/*peripherin-2* gene, completely lacking functional peripherin2 (65). Gene replacement therapy was attempted with rAAV2/2 carrying *Prph2* driven by a rhodopsin promoter sequence (66). Peripherin2 was detected in rod cells and appeared to initiate morphological changes that led to an improvement in retinal function for up to 14 weeks post-treatment, beyond which improvements were not sustained. When treatment was given to younger animals there was a greater initial restoration of structure but again the effect dropped over time (67). Other models of autosomal recessive RP have shown more long-term success in gene replacement animal studies. *Rd10* mice show rapid degeneration of rod photoreceptors due to lack of

Pde6 (68), a protein integral to rod outer segments that is essential in the phototransduction cascade (69). Subretinal delivery of rAAV2/8Y733F-*hPDE6* restored retinal function and preserved the retinal structure for up to 6 months (70). RCS rat retinal degeneration is due to lack of *Mertk* (71), an RPE receptor tyrosine kinase required for phagocytosing shed photoreceptor outer segments, mutations in which cause RP in humans (72). Initial rescue of the degeneration exhibited in the RCS rats was demonstrated using a recombinant adenovirus vector (73), a rAAV2/2 vector (74) and more recently rAAV2/8Y733F with expression of *hMERTK* driven by the RPE-specific promoter *hVMD2* promoter. This latter study revealed rescue of the RPE function and slowing of photoreceptor degeneration for at least 8 months following treatment (75). A less well-studied model of autosomal recessive RP is the *rd6* mouse, degeneration in which is caused by a null mutation in *Mfrp* (76). MFRP (membrane-type frizzled-related protein) is expressed in the RPE and human mutations are rare (77-80). Following a subretinal injection of rAAV2/8Y733F-*mMfrp* (using a ubiquitous CBA promoter) into *rd6* mice, *Mfrp* was found to localise to the RPE apical membrane with subsequent evidence of rod and cone photoreceptor rescue (81).

Other models of autosomal recessive IRD for which gene replacement therapy has shown promising recovery of function include various models of LCA. AIPL1 (Aryl hydrocarbon receptor interacting protein like-1) mutations in humans cause a severe form of childhood blindness and account for approximately 7% of LCA cases worldwide (82). AIPL1 is necessary for cone photoreceptor survival (83) and several studies of gene replacement therapy have shown promising results using rAAV2/2, rAAV2/5 and rAAV2/8 with both ubiquitous and photoreceptor-specific promoters driving expression (11, 49). The *Aip11*^{-/-} mouse model shows a rapid degeneration and severe phenotype and subretinal delivery of a scAAV2/8Y733F vector in this model successfully transduced *mAip11* during the active phase of degeneration, showing preservation of photoreceptors following treatment and indicating a greater phenotypic effect was induced compared to the rAAV vector (84).

The *GC1KO* mouse is a cone degeneration model that lacks the gene *Gucy2d*(GC), which encodes the cone-specific guanylate cyclase-1 protein. This protein is essential for photoreceptor sensitivity and functioning (85) and mutations are generally associated with LCA (86) but RP (87), cone (88) and cone-rod dystrophy (89) cases have also been reported. Photoreceptor function has been restored in the *GC1KO* mouse following photoreceptor-specific expression using a rAAV2/5 vector driving *mGC1* expression from the *hGRK1* promoter (50). Restoration of cone function was later shown to last for up to one year post-treatment using a rAAV2/8Y733F vector to improve transduction and expression (90). In the *RPGRIP1*^{-/-} mouse model of LCA, there is a lack of RPGRIP1 (retinitis pigmentosa

GTPase regulator interacting protein 1), a protein that localises to the photoreceptor cilium and interacts with RPGR (retinitis pigmentosa GTPase regulator, mutations in which cause X-linked RP (91) and aids in photoreceptor disc morphogenesis (92). Mutations in *RPGRIP1* are a common cause of LCA (93) and studies in the *RPGRIP1*^{-/-} mouse have indicated prolonged photoreceptor survival and morphology occur following subretinal delivery of rAAV2/8 expressing *hRPGRIP1* under the control of the *hGRK1* promoter (51). In the *rd12* mouse model of LCA, rescue of cone photoreceptors and halting of degeneration was shown in older mice following delivery of scAAV2/5-*hRPE65*, suggesting that improvements in morphology and function of remaining cones can be made even in the later stages of disease (94).

Achromatopsia, also known as rod monochromacy, is a rare autosomal recessive disorder that affects all three cone classes due to mutations in genes encoding proteins essential for function of the cone photoreceptors (95). Gene replacement studies in several models of achromatopsia have indicated successful long-term restoration of function. GNAT2 is a cone-specific G-protein necessary for the phototransduction cascade and encodes the subunit of cone transducin, mutations in which cause achromatopsia (96). Treatment of the *Gnat^{cpfl3}* mouse model with PR2.1 driven expression of *Gnat2* from a rAAV2/5 vector indicated cone-targeted gene therapy (97). A naturally occurring mouse model, *cpfl5*, lacks *Cnga3* (cone photoreceptor cGMP-gated cation channel α -subunit), mutations in which also result in achromatopsia in humans (98). Gene replacement therapy with rAAV2/5 Y719F mutant capsid carrying *Cnga3* driven by the murine blue cone promoter in the knockout mouse model (99) and rAAV2/5-*hCNGA3* treatment in the *cpfl5* mouse model of achromatopsia (100) have shown positive effects of cone-mediated light response restoration following treatment. Similarly, the *Cngb3*^{-/-} mouse model has also been used for gene therapy studies of achromatopsia. *CNGB3* encodes the β -subunit of the cone photoreceptor cGMP-gated cation channel and use of the cone-specific promoter *hCAR* to drive expression of *hCNGB3* via a rAAV2/8 vector increased cone density and survival over several months following treatment (55). Positive effects on retinal function were noted even when delaying treatment until the mice were 6 months of age. In two canine models of *CNGB3* mutations, treatment with rAAV2/5-*hCNGB3* driven by the PR2.1 promoter revealed effective treatment was not mutation dependent but was promoter and age dependent, with younger animals treated with the cone-specific promoter vector achieving the best results (101). This is an important indication of optimal therapy design in human trials.

It is apparent that various models of autosomal recessive IRD are available and that gene replacement studies using these models are showing consistent success in recovery of function and improved retina morphology. These positive effects are continuing over a relatively long time course in mice (6-12 months) and dogs (33 months) and include successes when gene therapy intervention is provided during the later stages of disease. Combinations of serotypes and promoters to allow specific tropism and expression are adding to these positive results. The data are very encouraging for future prospects of human retinal gene therapy.

One issue in the reliance of rAAV vectors for gene-replacement studies of autosomal recessive IRDs is that its maximum packaging size is limited to around 5kb (102). To achieve greater packaging (up to 6kb) and for successful transduction with rAAV carrying such oversized genomes requires additional treatment with proteasome inhibitors (103, 104). However, many of the genes implicated in retinal degenerations exceed even this size, including *ABCA4*, mutations in which are the most common cause of autosomal recessive IRD. *ABCA4* (ATPase binding cassette, sub-family A, member 4) is localised to the photoreceptor outer segments and acts as a membrane transporter important for the recycling of the light-sensitive chromophore, a process necessary for phototransduction. A lack of

ABCA4 function leads to build up of a toxic product, A2E, in the RPE cells. This disrupts correct RPE function and leads to both RPE and photoreceptor death (105). Mutations in *ABCA4* can cause Stargardt's disease (106), RP (107), cone-rod dystrophy (108) and bull's-eye maculopathy (109). Retinal gene therapy for ABCA4 would therefore have a broad spectrum of patients that would benefit from such a treatment. Until now, lentiviral vectors have been favoured for large gene delivery due to their greater packaging size and LV vectors were able to successfully deliver ABCA4 to the *Abca4* knockout mouse model (110). However, just 5-20% of photoreceptors were transduced and these were limited to within the site of injection. AAV effective transduction of the retina is widespread and more extensively studied, leading to recent approaches that split a large AAV2-based transgene into two halves, packaging each half into a separate AAV capsid. Upon transduction, both viruses need to infect the same host cell, within which the two single-stranded genomes of the separate transgenes recombine, forming the complete transcript. This can either be achieved using an overlapping vector approach (111-118) or a trans-splicing approach (119-125). The overlapping dual vector approach has been successful in the recombination of a split ABCA4 transgene *in vitro* and *in vivo*, with transductions leading to generation of the full length protein (115). Studies for muscular dystrophy have shown the dual vector approach can lead to sufficient co-transduction with subsequent homologous recombination of the overlapping transgenes to elicit a functional improvement in a mouse model of the disorder (116, 118). This evidence of functional improvement in a model of disease suggests there is potential for such positive effects to be achieved in the treatment of IRD. Co-transduction efficiency will clearly be extremely important for this dual vector approach (126) with correct serotype selection necessary to achieve optimal co-transduction (122). This dual rAAV vector approach offers promise that effective gene therapy for IRDs will not be limited to smaller genes. In addition to this approach, helper-dependent adenoviral vectors (which have a larger cloning capacity than AAV) successfully transduce mouse retina with long-term strong levels of reporter gene expression (127). Nanoparticles also offer potential for gene therapy treatment of large genes, with *ABCA4* delivery to knockout mice showing ABCA4 production 8 months after treatment (128).

AAV TREATMENT OF AUTOSOMAL DOMINANT MODELS OF RETINAL DISEASE

The majority of animal models available for study of IRD carry null mutations yet there are numerous IRDs that do not show a complete lack of protein. Dominant mutations often yield mutated versions of a protein, if these impart a dominant-negative effect then provision of the wild-type gene and subsequent protein should resolve the phenotype as in the gene replacement strategies above. Dominant-negative mutations tend to eliminate one or more functions of the encoded protein, for example, the common rhodopsin mutation P23H (129) is thought to preserve an ability of the opsin to oligomerise that interferes with normal function (130). However, if a toxic gain-of-function effect is generated then the gene therapy approach will need to silence the mutant mRNA and provide a correct copy. Gain-of-function mutations can alter the protein to generate a novel activity, for example, the rhodopsin mutation K296E that leads to RP (131) results in the photopigment constitutively activating rod transducin and the phototransduction pathway (132). Clearly it is important to determine the etiology and molecular pathology of autosomal dominant retinal diseases in order to determine the appropriate gene therapy approach. For rhodopsin alone it is apparent that different mutations can trigger different responses. The main function of rod photoreceptors is to produce rhodopsin for initiating phototransduction and around 85% of total protein in the outer segment is rhodopsin, which also makes it important for outer segment structure (133). Outer segment proteins are synthesised in the inner segment of the photoreceptor and transported unidirectionally to the outer segments and mutations that

cause autosomal dominant RP affect the stability, folding and trafficking stages of this process (134-136). Due to the high production rate of rhodopsin, build up of mutants tend to have a toxic effect and therefore silencing of the mutant form is desired before supplying the wild-type form. AAV-delivered ribozymes have been used to destroy targeted mutant mRNA in an autosomal dominant RP rat model carrying the P23H rhodopsin mutation (137, 138). The vector was injected at different ages, including a later stage of degeneration when 40% of photoreceptor cells were missing. In all age groups photoreceptor cell loss was delayed following injection of the AAV-ribozyme vector. AAV delivery of RNA interference (RNAi) has also been used to suppress rhodopsin whilst co-delivery of a codon-modified rhodopsin replacement gene resistant to suppression allows for wild type expression (139, 140). Whereas these approaches utilise codon-modified gene replacements, complete codon optimisation of the transgene would not only be helpful in resistance to silencing but it may also enhance expression levels and therefore increase the chance and degree of physiological outcome (141). *In vitro* studies in HEK293 cells have shown that codon optimised genes can produce a nine-fold increase in protein level compared to the WT coding sequence (142). Whilst no retinal codon optimised comparisons have currently been reported, a codon optimised microdystrophin transgene showed significantly improved expression and physiological outcome in *mdx* mice following rAAV2/8 delivery (143).

Gain-of-function mutations in *PRPH2* also result in autosomal dominant RP, macular and cone-rod dystrophies in humans (144). Use of microRNA (miRNA)-based hairpins to silence *Prph2* in the murine retina showed fast and effective gene silencing via rAAV2/8-mediated subretinal delivery (145).

For the treatment of dominant-negative mutations, injections of rAAV2/5-*RHO* vector without use of a silencing mechanism into the *RHO*^{P23H} and *RHO*^{+/+} mouse models revealed expression of the wild type rhodopsin slowed the rate of degeneration in the P23H mutant mice. In the wild type mice, expression from the vector resulted in damaging effects, suggesting that whilst expression of normal rhodopsin can suppress the P23H mutant effects, over production of rhodopsin can also be damaging (146). In this scenario use of a photoreceptor-specific promoter may be favoured over a strong ubiquitous promoter as expression is more likely to be regulated, limiting the chance of over-expression.

Whilst more complex than gene-replacement strategies, the co-suppression of a mutant gene and provision of the wild type replacement is showing promise via AAV delivery, with many autosomal dominant IRDs candidates for therapy.

HUMAN AAV CLINICAL TRIALS FOR THE TREATMENT OF RETINAL DISEASE

In 2008, data from three independent trials revealed evidence of improved visual function following rAAV2/2 subretinal delivery of *hRPE65* to LCA patients (reviewed in (147)). RPE65 (retinal pigment epithelium-specific protein 65kDa) is expressed in the RPE and is essential in the visual pathway for its role in regenerating the active chromophore required for light absorption, loss of function of RPE65 therefore leads to loss of vision (148). Whilst all three clinical trials were consistent in their use of rAAV2/2, the transgene structure differed in each: in one, a 1,400bp fragment of the *hRPE65* promoter was used (40). The other two studies used the ubiquitous CAG promoter in combination with a modified Kozac consensus upstream of the start codon (149) or with a hybrid intron in the 5' UTR region upstream of the therapeutic gene (150). The use of the less active *hRPE65* promoter is suggested to be why less impressive vision changes were observed from that study compared to the others. Despite this, both studies using the CAG promoter did reveal varied evidence of improved visual function. In one study, improvements in visual acuity were seen

together with improved pupil responses (149), whereas the other study reported a shift in fixation towards an extrafoveal region exposed to vector (151) – both objective assessments. Interpretation of mean vision outcomes in both these studies were however impaired to some extent by surgical complications such as macular hole formation. The other study with the *hRPE65* promoter suggested anecdotal visual improvements in one of three patients, but the observed microperimetry gains did not match the area exposed to vector and should be interpreted with caution when retesting an amblyopic eye (40). Similarly whilst the maze navigation test showed apparent improvements for the treated eye, it also showed a similar improvement in the control eye, which raises questions about the reliability of the baseline measurements of this test. It was interesting to note in the preclinical testing of this vector that visual improvements were only seen in younger Briard dogs injected up to 11 months of age, but not at 30 months (39). Hence the lack of efficacy at later stages of the disease process in dogs and humans may be because the *hRPE65* promoter is less active in RPE cells undergoing stress responses, where translation of cell-specific transcription factors would be inhibited (147). Nevertheless although evidence of vector efficacy is doubtful, this study did show safe detachment of the retina without surgical complications such as macular hole formation. A follow on study using the *hRPE65* promoter but at a higher dose is planned, however, many would argue that CAG/CBA is now the promoter of choice for clinical trials targeting the RPE, in view of the evidence of efficacy from a number of separate independent studies (147).

One concern over AAV gene therapy is its ability to initiate an immune response, neutralising antibodies to rAAV2/2 capsid temporarily increased in one patient (149) with a marginal increase observed in another patient (150) but neither were significant. The findings from all three studies suggest very good safety and low immune response from the AAV gene therapy. Given that 30% of adults have neutralising antibodies to AAV2 with others having antigen-specific memory CD8+ T cells (152) use of other AAV serotypes may minimise further the chance of immune response (153). Treatment of the second eye in three patients has now been reported with no adverse effects after 6 months (154). These LCA trials have shown great encouragement for future trials and with recent developments in promoter and serotype choices, gene therapy treatment of IRDs has a promising future. There are several AAV gene therapy trials currently approved and ongoing, including further studies to assess safety and efficacy of *RPE65* AAV2/2 subretinal delivery to LCA patients (NCT00481546, NCT00516477, NCT00643747, NCT01208389, NCT00749957, NCT00999609), *MERTK* AAV2/2 subretinal delivery to RP patients (NCT01482195), AAV2/2 *REPI* subretinal injection in choroideraemia patients (NCT01461213), and AAV2/2 subretinal delivery of *FLT1* (also known as vascular endothelial growth factor, VEGF) in patients with age-related macular degeneration (NCT01024998, NCT01494805). Other clinical trials ongoing include a Phase I/II study of the lentiviral-based vector UshStat (Oxford BioMedica) to deliver the large gene *MYO7A* to Usher 1B patients (NCT01505062). Usher syndrome is the most common form of deaf-blindness and exhibits an RP phenotype of varying severity in addition to deafness (155). Other clinical trials currently underway offering therapy for IRDs include a non-gene therapy based treatment using the synthetic retinoid QLT091001 (NCT01543906, NCT01521793, NCT01014052). Many IRDs including LCA and RP will have a reduced availability of the chromophore 11-*cis*-retinal as a consequence of the molecular changes that occur within the retinal cells due to the causative disease mutation. These trials are attempting to determine if oral administration of a synthetic retinoid replacement can aid the symptoms of the IRDs.

NEUROPROTECTION OF THE RETINA

AAV delivery of growth and anti-apoptotic factors can be used to prevent cell loss when retinal degeneration is advanced and the causative mutation unknown and also to enhance

the effects of a co-delivered therapeutic gene. Apoptosis of photoreceptor cells is common to most forms of IRD therefore preventing cell death could significantly slow degeneration and allow a greater window for gene therapy intervention. Minimising rod photoreceptor death would also prolong cone survival and AAV delivery of various neurotrophic factors have been shown to preserve the photoreceptors. rAAV2/2 subretinal delivery of glial cell line derived neurotrophic factor (GDNF) to a rat model of autosomal dominant RP led to increased rod photoreceptor survival (156). Another study of fibroblast growth factor 2 (FGF-2) had a similar preservation effect of photoreceptors though improvements in ERG (as a measure of rod function) were minimal (157). Other studies have confirmed the ability of GDNF to slow photoreceptor cell death but similar testing of ciliary neurotrophic factor (CNTF) has shown an adverse affect on retinal function (12). In *rd*s mice, photoreceptor degeneration was shown to be delayed following subretinal and intravitreal delivery of AAV-*CNTF* but too much CNTF appeared to have a detrimental effect on photoreceptor function in a dose-dependent manner (158, 159). In *rd*s gene replacement therapy with rAAV2/2-*Prph2*, the beneficial effects of the gene therapy were minimised by co-delivery of CNTF (160). These data suggest that low, regulated levels of CNTF may prove to be beneficial but perhaps require the control of a weaker or inducible promoter to allow for consistent sub-toxic but therapeutic levels of expression. Ectopic expression of neurotrophic factors have also been shown to improve survival and integration following transplantation of photoreceptors (161). AAV delivered insulin-like growth factor 1 (IGF-1) enhanced cell integration, whilst FGF-2 had no apparent effect and CNTF exhibited a detrimental effect on cell integration.

rAAV2/2 delivery of X-linked inhibitor of apoptosis (XIAP) has been shown to enhance survival of cultured human RPE cells following induced oxidative cells death (162). XIAP therapy has also been shown to aid survival of transplanted rod precursors in a degenerating host retina (163) and to prolong the effects of rAAV2/5-*PDE6* gene therapy (164).

These data suggest that anti-apoptotic factors and growth factors have the potential to prevent further cell loss within the retina and also to enhance the effects of gene therapy. However, caution needs to be taken as the long term effects and dose levels need to be better understood.

SUMMARY

Gene therapy of retinal diseases is an advancing field. Identification of serotype tropism and enhancement of transduction efficiencies of the various retinal cell layers is allowing for targeted AAV treatment in various forms: growth factors and anti-apoptotic factors to prevent cell loss and also to enhance gene therapy in addition to gene-replacement and suppression-replacement therapies. All are showing success in animal models of retinal disease. Clinical trials of AAV-based gene therapy have shown that treatment is safe in humans and well tolerated, with improvements in vision only likely to improve further with advancements in understanding of serotype and promoter selection.

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Figure 1. Basic vertebrate retinal structure.

Light must pass through the ganglion cell layer (GCL, consisting of ganglion cells and displaced amacrine cells) through the inner nuclear layer (INL) of bipolar, Müller and horizontal cells and then beyond the outer nuclear layer (ONL) to the photoreceptor outer segments that lie at the back of the retina prior to the supporting retinal pigment epithelium (RPE).

Figure 2. C57BL/6 retina following subretinal injection of rAAV2/5 *hGRK1* promoter-GFP vector

a) nuclei staining and GFP expression; b) GFP only revealing photoreceptor-specific expression. Human retinal explants transduced *ex-vivo* with rAAV2/5 CAG promoter-GFP: c) nuclei staining and GFP expression; d) GFP only revealing mostly photoreceptor expression with some INL expression. INL = inner nuclear layer, ONL = outer nuclear layer.