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Clinical applications of retinal gene therapy

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ABSTRACT

Many currently incurable forms of blindness affecting the retina have a genetic etiology and several others, such as those resulting from retinal vascular disturbances, respond to repeated, potentially indefinite administration of molecular based treatments. The recent clinical advances in retinal gene therapy have shown that viral vectors can deliver genes safely to the retina and the promising initial results from a number of clinical trials suggest that certain diseases may potentially be treatable. Gene therapy provides a means of expressing proteins within directly transduced cells with far greater efficacy than might be achieved by traditional systemic pharmacological approaches. Recent developments have demonstrated how vector gene expression may be regulated and further improvements to vector design have limited side effects and improved safety profiles. These recent steps have been most significant in bringing gene therapy into the mainstream of ophthalmology. Nevertheless translating retinal gene therapy from animal research into clinical trials is still a lengthy process, including complexities in human retinal diseases that have been difficult to model in the laboratory. The focus of this review is to summarize the genetic background of the most common retinal diseases, highlight current concepts of gene delivery technology, and relate those technologies to pre-clinical and clinical gene therapy studies.
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1. Introduction

Of all diseases that cause blindness, arguably those with genetic etiology, such as retinitis pigmentosa, are the most devastating, because they affect both eyes and may often lead to total blindness. Frequently, the diagnosis is made early in affected patients providing possible opportunities for treatment, where a therapeutic window exists in which correction of the gene defect before the onset of significant cellular pathology, may serve to prevent cell death and thereby preserve vision. In addition to examining the potential therapeutic applications of gene therapy in the context of various monogenic and complex retinal diseases, this review deals with the challenges in molecular biology and vector technology associated with translation from bench to clinic.

2. The eye as a target for gene therapy

The eye is a highly specialized organ which has evolved to transduce light stimuli into electrical signals and to relay those signals to the visual cortex. Light sensation and image formation is mediated through the activation of photoreceptor cells located in the outermost layer of the neurosensory retina, where incident light focused by the cornea and lens results in the activation of a signalling cascade and the propagation of an electrical impulse. This photoactivation is initiated by the isomerisation of light-sensitive 11-cis retinal within the photoreceptor outer segments, triggering the opening of cyclic-GMP gated ion channels. The continuation of the visual cycle is dependent on the constant replenishment of 11-cis retinal, a function performed by the retinal

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pigment epithelium (RPE), an underlying monolayer of cells in close contact with the photoreceptors. In addition to the isomerisation of photobleached retinoids, the RPE contributes directly to photoreceptor survival through the phagocytosis of shed outer-segment discs. Indeed, the maintenance of correct photoreceptor function is critically dependent on the RPE, and death of RPE cells invariably results in photoreceptor dysfunction and their secondary degeneration. Maintenance of the complex photoreceptor–RPE interaction requires the precise regulation of highly specialized intracellular protein interactions and the harmonic function of several hundred genes, almost any one of which can lead to retinal dysfunction or cell death when mutated. Despite its complexity the eye has many traits which make it an attractive organ for gene therapy: it is relatively immune privileged, has a small compartment size, is easily visualized and examined, and readily accessible with minimal risk to patients undergoing surgery. The photoreceptors and the RPE are the primary targets for gene therapy treatments of retinal disease due to their key role in the visual cycle and susceptibility to a large number of single gene defects. The retina has a highly organized laminar structure which can be exploited for accurate spatial delivery of therapeutic material to the photoreceptors and RPE. Delivery is usually achieved through injection of a fluid suspension containing the therapeutic particles into the anatomically constrained subretinal space, which is conveniently interposed between the two tissue layers. Injection into the subretinal space results in a temporary detachment of the retina, but otherwise causes minimal damage.

3. Retinal pigment epithelium expressed gene targets

The RPE consists of a monolayer of hexagonally arranged cuboidal or low columnar epithelial cells interposed between the choroid and the neural retina, where the layer's basal aspect forms part of Bruch's membrane, and the apical aspect is highly invaginated and closely associated with the photoreceptors. The RPE forms a selectively permeable barrier between the choroid and the neurosensory retina and is involved in many aspects of photoreceptor maintenance, including phagocytosis of rod and cone outer segment discs and cycling of retinoids. The phagocytotic nature of the RPE greatly facilitates gene delivery, with exogenous genetic material efficiently internalized possibly through interactions with RGD binding domains. Virtually all gene delivery vectors so far described transduce the RPE with a high degree of efficacy, making it an ideal target for gene delivery.

3.1. Leber's congenital amaurosis

Leber's congenital amaurosis (LCA), or early-onset retinal dystrophy, is a severe form of retinitis pigmentosa (RP) first described by Theodore Leber in the 19th century (Leber, 1869) and defined by visual impairment within the first three years of life. Patients suffer severe vision loss with best corrected visual acuity (BCVA) generally ranging from 20/200 to little or no perception of light. The electroretinogram (ERG) is frequently absent and pupillary responses are diminished (Chung and Traboulsi, 2009). Whilst more than 400 mutations have been identified across 14 LCA associated genes, in 30% of cases the mutation remains unknown. Three RPE expressed genes are implicated in LCA, accounting collectively for up to 17% of LCA (den Hollander et al., 2008).

3.1.1. LRAT and RPE65

Lecithin retinol acetyltransferase (LRAT) is an essential component of vitamin A metabolism, catalyzing the esterification of all-trans retinol, received to the RPE via shed outer segment discs, into all-trans retinyl ester. LRAT is closely associated with the function of retinal pigment epithelium-specific protein 65 kDa (RPE65), an enzyme involved in trans- to cis-retinoid isomerisation, converting all-trans retinyl ester to 11-cis retinol. *LRAT* and *RPE65* account for 0.5% and 7–16% of LCA, respectively, and mutations affecting either result in disruption of the phototransduction cascade and accumulation of all-trans retinol (Gu et al., 1997). While the fundus of LCA patients typically appears normal at birth, RPE and photoreceptor degeneration occurs within the first decade. In the case of RPE65 deficiencies, for instance, cell death likely occurs due to deposition of all-trans retinol, resulting in cellular toxicity presenting as chorioretinal atrophy, pigment migration and rarely macular aplasia (den Hollander et al., 2008).

3.1.2. MERTK

c-mer proto-oncogene tyrosine kinase (*MERTK*) encodes a tyrosine kinase receptor localized to the apical aspect of the RPE, consisting of an intracellular kinase domain, transmembrane region and an extracellular signalling domain. MERTK is activated through binding of Gas6 and PS ligands associated with the outer segment disc membrane which trigger RPE cytoskeletal remodelling and outer segment phagocytosis (den Hollander et al., 2008; Hall et al., 2005, 2001). Mutations of *MERTK* occur in 0.6% of LCA patients causing a severe retardation of the RPE's ability to phagocytose shed discs (Gu et al., 1997). Accumulation of outer segment debris in the subretinal space leads to rapid loss of vision and subsequently photoreceptor degeneration, often presenting as childhood-onset rod–cone dystrophy and macular atrophy, and in some cases presenting as classic LCA (Mackay et al., 2010). A gene therapy trial has recently started at the King Khaled Eye Hospital in Saudi Arabia to treat patients with RP caused by MERTK mutations (NCT01482195).

Although an autosomal dominant form of LCA caused by a 12 bp deletion in the *CRX*-homeobox gene has been identified (Sohocki et al., 1998), all other forms so far reported, including those arising from mutations in *RPE65*, *LRAT* or *MERTK*, follow an autosomal recessive inheritance pattern. The disease phenotype is somewhat heterogeneous within RPE65 cohorts, particularly in the first two decades where residual cone function is variable, and remaining vision is broadly correlated to anatomical preservation, with central and peripheral islands of retina surviving until later stages of disease (Jacobson et al., 2009; Lorenz et al., 2008). By the third decade of life phenotypic heterogeneity is greatly reduced indicating that mutations typically result in loss-of-function, regardless of mutation class (Thompson et al., 2000). While this is also likely the case concerning *LRAT* and *MERTK* mutations, their relatively low incidence means that a detailed genotype–phenotype correlation has not been carried out (den Hollander et al., 2008). Recessive mutations resulting in loss-of-function are ideal candidates for straightforward gene replacement therapy, where expression of wild-type protein is likely to significantly ameliorate the disease phenotype (see Section 10.1). However, identifying the areas with the best preserved retinal structure, in particular areas with a relatively intact outer nuclear layer, is critical to successful gene delivery in RPE65 patients, where targeted gene replacement in these areas would preserve vision to the greatest extent.

RPE mutations primarily cause vision loss through dysfunction of phagocytosis or the visual cycle and are diagnosed early, typically in the first year of life when lack of fixation associated with nystagmus, or oculodigital reflex (eye poking/pressing/rubbing) become apparent. Except in cases of *RPE65* mutations where a loss of fundus autofluorescence may be noted, the fundus of young patients with RPE gene defects show no specific features that distinguish them from retinal degenerations caused by photoreceptor gene defects (Weleber et al., 1993). Early diagnosis, the preservation of photoreceptors until late stages of disease, and the

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amenability of the RPE to gene delivery makes treatment of LCA arising from RPE mutations a good prospect for treatment with gene therapy. Indeed, several gene therapy trials are currently underway targeting RPE65 mutations resulting in LCA.

4. Photoreceptor expressed gene targets

Photoreceptors are located at the outermost aspect of the neural retina and are the primary light sensitive cells of the retina. Photoreceptors are highly specialized neurons, each consisting of an outer and inner segment separated by connecting cilium, a cell body situated in the outer nuclear layer, and an axonal synaptic terminal (cone pedicle or rod spherule) extending to the outer plexiform layer, through which signalling to second order neurons (bipolar cells) is conducted. A significant proportion of retinal disease arises from genetic defects which affect the photoreceptors directly; likely attributable in part to their complex structure and high energy demands, such that disruptions to gene function may have a significant impact on intracellular function and survival. Gene delivery to photoreceptors is comparatively more difficult than to the RPE, possibly due to their non-phagocytotic nature and the constant movement of outer segment discs away from the nucleus. The densely-packed lipid rich discs may also provide a physical barrier between the point of delivery (subretinal space) and the cell nucleus located in the outer nuclear layer, particularly for enveloped vectors such as lentivirus. However, various vector technological enhancements have optimized photoreceptor gene delivery (see Sections 7–9).

4.1. Achromatopsia

Achromatopsia, or rod-monocromatism, is an autosomal recessive condition affecting an estimated 1 in 30,000 people which results in severe cone dysfunction (Kohl et al., 2002). Clinically, achromatopsia can either be complete, with total dysfunction of all three cone classes, or rarely, incomplete, where one or more cone-classes may be partially functional. Complete achromats typically have visual acuity of 20/200 or worse, suffer from severe photophobia (day-blindness) due to reliance on rod-based vision, have pendular nystagmus, and are unable to determine colour (Kohl et al., 2010; Pang et al., 2010b). Genetic mutations affecting one of four genes can be identified in approximately 80% of patients with complete achromatopsia. In achromats degeneration is typically absent or relatively mild, making them ideal candidates for retinal gene therapy. However, the fact that these patients generally live reasonably normal lives with rod mediated vision raises the threshold for intervention.

4.1.1. CNGB3 and CNGA3

Mutations affecting the cone-specific cyclic nucleotide gated (*CNG*) channel beta (*B3*) and alpha (*A3*) subunits account for approximately 50% and 25% of complete achromatopsia, respectively (Kohl et al., 2000; Wissinger et al., 2001). Like all CNG channels, those in cones are heterotetrameric complexes, likely consisting of three CNGA3 (principal) subunits and one CNGB3 (modulatory) subunit (Gerstner et al., 2000). In photoreceptors CNG channels control outer segment membrane hyperpolarisation, where activation by incident light results in channel closure preventing the steady influx of calcium ions (dark current) (Kaupp and Seifert, 2002). Mutations in *CNGA3* are predominantly missense resulting in amino acid substitutions, the most common of which (R277C and R283W) affect function of the voltage-sensing S4 domain, or the cyclic-GMP (cGMP) binding domain and associated linker (F547L and R436W) (Kaupp and Seifert, 2002). In contrast, *CNGB3* mutations are typically null, resulting in no protein or production of functionless truncations (Pang et al., 2010b). Although functional characterisation of mutant CNGB3 or A3 protein has not been performed, it can be inferred from clinical observations that these mutations cause severe impairment to channel function, resulting in cone dysfunction. More recently it has been observed that cone degeneration is a feature of many types of achromatopsia in humans, which merges the boundary with cone dystrophy. To date, both mouse and dog models have shown successful restoration of cone-mediated vision following gene therapy for CNGA3 and CNGB3 mutations (Carvalho et al., 2011a; Komaromy et al., 2010; Pang et al., 2012; Michalakis et al., 2010).

4.1.2. GNAT2 and PDE6C

Mutations in *GNAT2*, the cone-specific alpha subunit of transducin, account for less than 2% of complete achromatopsia, with mutations in *PDE6C*, the cGMP-specific cone phosphodiesterase, accounting for a further 2% (Kohl et al., 2010). As the alpha subunit of transducin, *GNAT2* binds to and activates cGMP phosphodiesterase, catalysing the breakdown of cGMP – Mutations in GNAT2 consequently result in high levels of intracellular cGMP. *PDE6C* has only recently been associated with complete achromatopsia, functioning as it does to convert second messenger cGMP to 5′-GMP during light exposure (Chang et al., 2009; Thiadens et al., 2009). Mutations in either *GNAT2* or *PDE6C* result in an increase in intracellular cGMP concentration which inhibits the closure of CNG channels and prevents hyperpolarisation in response to light. AAV-medicated gene therapy has been used successfully to restore cone function to the GNAT2 knockout mouse (Alexander et al., 2007), but PDE6C is a recent discovery and animal models showing restoration of function following gene therapy have yet to be described.

The clinical manifestations of complete achromatopsia relates to a presumed inability of cone photoreceptors efficiently to activate CNG channels. Whilst the resulting phenotype results in severe visual impairment, it is due predominantly photoreceptor dysfunction, rather than degeneration, which raises the possibility of visual improvement, even in late stages of the disease. Affected individuals are usually diagnosed in childhood, presenting with nystagmus, eccentric fixation and increased light sensitivity. Patients present a fundus of normal appearance, and though some may develop subtle foveal RPE changes, the disease is typically stable for many of years, with visual acuity remaining constant. The combination of early diagnosis and the non-progressive disease nature provide a large treatment window in which gene therapy might be applied. However, these same factors, and the reliance of patients on rod-based vision, increase the threshold for intervention – of particular consideration is the potential damage caused to rod photoreceptors during surgical detachment of the retina. As a recessive condition complete achromatopsia is likely to be best treated through direct gene replacement, particularly in cases of *CNGB3* null mutations where there is an absence of protein production. In instances of missense mutations, as are often observed in *CNGA3*, truncated or functionless protein is frequently incorporated into inactive CNG channels that remain cytosolic (Matveev et al., 2010). Consequently, incorporation of wild-type protein during channel formation is likely to be competitive, and may necessitate a high level of protein expression from the therapeutic transgene to ensure a significant proportion of channels produced are functional. Nevertheless despite these theoretical challenges in humans, the pre-clinical data to date in both mouse and dog models is compelling (see Section 11.1).

4.2. Retinitis pigmentosa

Retinitis pigmentosa (RP) is a genetically and phenotypically heterogeneous disorder – with mutations collectively affecting

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between 1 in 4000 and 1 in 2500 individuals worldwide – and is characterised by progressive loss of photoreceptors (Haim, 2002; Pagon and Daiger, 2005). Generally, in typical rod–cone dystrophies, rod loss occurs early (first two decades) but many years may pass until central vision drops as a result of cone degeneration. It is still not entirely clear why cones degenerate in cases of RP in which the gene defect is manifest only in rods (e.g. RHO, NRL, PDE6β) but is believed to occur through loss of a rod-related sustaining factor (Leveillard and Sahel, 2010). Other more general ocular pathologies can also affect cone mediated vision, such as cystoid macular edema, cataracts, epiretinal membranes and uveitis. It is therefore important to screen patients with RP for these other confounding conditions as many are treatable using standard non-genetic approaches. RP can be inherited as an autosomal dominant (30–40% of cases), autosomal recessive (50–60%) or X-linked (5–15%) trait. Generally, X-linked cases are caused by mutations in RPGR and are at the severe end of the spectrum (Fishman, 1978; Hartong et al., 2006). Approximately 50% of dominant RP and 30% of recessive non-syndromic RP have no known genetic cause, although more genetic causes are almost certainly going to be identified as next generation sequencing techniques become more routinely used.

4.2.1. RHO

Rhodopsin (RHO) mutations have a high prevalence in dominant RP and outcomes are heterogeneous, ranging from mild to severe phenotypes and even congenital stationary night blindness (Hartong et al., 2006). There are over 120 described rhodopsin mutations categorized in 7 classes (I–VI, and unknown) based on the behaviour of the mutant protein (Mendes et al., 2005). Rhodopsin is the rod specific visual pigment in humans responsible for scotopic (dim light) vision, localised to the outer segment disc membrane where it binds 11-cis retinal. The rhodopsin molecule may also be important for the maintenance of outer segment disc structure, where outer segments form incorrectly in the absence of rhodopsin (Humphries et al., 1997). Mutations of rhodopsin are implicated in 25–30% of autosomal dominant RP, but only 1% of autosomal recessive RP, reflecting the tendency for rhodopsin mutants to abnormal gain-of-function, which may involve damage incurred by abnormal light activation. The most common of the gain-of-function mutations is the P23H substitution (class-II), which results in accumulation of unfolded rhodopsin protein in the endoplasmic reticulum and either activation of the unfolded-protein response (UPR) and cell apoptosis, or opsin aggregation, leading to cytotoxicity (Mendes et al., 2005; Rutkowski and Kaufman, 2004). Class-I mutations result in protein which is inappropriately localised, leading to disruption of vesicular transport, synaptic transmission or undue metabolic burden through aggregation. Other classes of mutations lead to abnormalities in arrestin binding, endocytosis, or constitutive activation; for a truly excellent review of rhodopsin mutation classes see Mendes et al. (2005). Direct dominant negative effects of rhodopsin mutations have also been observed, where the aberrant protein inhibits opsin production from the wild-type allele (Kurada et al., 1998).

In principle, the majority of RP (rod–cone) is highly amenable to gene therapy, where the disease is typically diagnosed early and central cone-mediate vision is preserved until late stages of disease, giving a wide window of intervention; this is particularly the case for recessive forms of RP. However, in practice the window for preservation of rod function is narrow, with significant rod degeneration usually evident by the time patients present to the clinic. Also troublesome is the secondary loss of cones, which occurs for reasons that are not well understood, indicating that ideally rods would need to be rescued early in the disease course. Additionally, the genetic heterogeneity makes development of gene specific therapies impractical for all but the most prevalent alleles, such as rhodopsin.

Gene therapy for the treatment of autosomal dominant RP arising from rhodopsin mutations faces several challenges. First, transgene replacement would be in competition with any mutant gene expression, and so an additional molecular strategy to knockdown or silence mutant mRNA transcripts may be necessary. This is particularly the case in patients with dominant-negative mutations, where aberrant protein directly interferes with correct expression of the therapeutic gene (e.g. mutation in TIMP3) (Knupp et al., 2002). Second, the mutation class may dictate the mechanism of rod photoreceptor degeneration, and treatment approaches may not be applicable to more than one class of mutant. For example, photoreceptor neuroprotection has been shown to work best for disease arising from class-I rhodopsin mutations (Liang et al., 2001; McGee Sanftner et al., 2001; Okoye et al., 2003). Third, treatments designed to inhibit the aberrant function of mutant rhodopsin, e.g. through binding of stabilizing molecules, typically also target wild-type rhodopsin to some extent (Noorwez et al., 2003). This is similarly the case for gene silencing; however, a novel approach has been proposed of simply over-expressing normal rhodopsin cDNA using a highly efficient vector which then presumably puts it in competition with the mutant protein – a form of competitive knockdown (Mao et al., 2011). Consequently, dominant RP arising from rhodopsin mutations may prove complex to treat via gene therapy. A number of therapeutic strategies for treatment of dominant and dominant-negative retinal disease, such as in rhodopsin, are discussed in Section 10.2, including neuroprotection, inhibition of apoptosis and gene silencing.

4.3. Leber's congenital amaurosis

Mutation of photoreceptor expressed genes account for a significant proportion of LCA (~53%), with the most frequently mutated recently identified as *CRB1* (10%), *GUCY2D* (12%) and *CEP290* (15%) (den Hollander et al., 2001; den Hollander et al., 2006; Perrault et al., 2005).

4.3.1. CRB1

CRB1, a mammalian homolog of the Drosophila Crumbs gene (*crb*) (den Hollander et al., 2001; den Hollander et al., 1999), encodes a transmembrane protein localized to the photoreceptor inter segment that is critical for the establishment of apico-basal cell polarity during photoreceptor morphogenesis (Bujakowska et al., 2012). In vertebrates, CRB1 is also expressed in the sub-apical region of Müller glia cells, where expression is thought to control the number and size of apical villi (van de Pavert et al., 2007). Whilst the role of CRB1 in the adult retina remains unclear, mutations cause developmental anomalies including unusual retinal thickening, an absence of lamination and possibly formation of photoreceptor rosettes (Jacobson et al., 2003). Over 150 mutations of the *CRB1* gene have been described, with disease in all patients following a recessive inheritance pattern (Bujakowska et al., 2012). The CRB1 gene at 4.2 kb coding sequence for the cDNA is at the very upper end of what might be packaged into an AAV vector (discussed fully in Section 7), but there are no reports yet of successful rescue of the retinal degeneration in animal models.

4.3.2. GUCY2D

Retina-specific guanylate cyclase (*GUCY2D*), also known as *RETGC-1*, encodes an enzyme localized to the nuclei and inner segments of both rod and cone photoreceptors which catalyses the hydrolysis of GTP to cGMP. Consequently, *GUCY2D* expression is critical for the visual cycle, facilitating the opening of membrane bound CNG channels and reestablishment of the dark current (Perrault et al., 1996). GUCY2D protein consists of a large extracellular domain separated by a single transmembrane region from

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an intracellular catalytic domain containing two Calcium switches (Krishnan et al., 1998). Mutations in the catalytic domain (e.g. F514S, R976L, M1009L, H1019P and Q1036Z) typically result in the severe retardation or ablation of basal cyclase activity, while those occurring in the extracellular domain do not (Duda et al., 1999; Rozet et al., 2001). Over 125 mutations have been identified in *GUCY2D*, and whilst some mutations (e.g. R838H) result in dominant cone–rod dystrophy, LCA arising from *GUCY2D* disruption is recessively inherited (Xiao et al., 2011). The murine model of human GUCY2D disease (GC1 mouse) has been treated successfully in the laboratory using subretinal AAV5 and AAV8 capsid vectors (Boye et al., 2010; Mihelec et al., 2011).

4.3.3. CEP290

The centrosome, comprised of two functionally and structurally asymmetric centriole subunits, is the primary microtubule organizing centre (MTOC) in eukaryotic cells, and plays a critical role in mitotic spindle orientation, genome stability and determination of cellular polarity (Bornens, 2012). In 2003, a protein with a mass of 290 kDa (CEP290) was identified as one of the most abundant components of the centrosome (Andersen et al., 2003). In the retina, CEP290 has been observed to localize primarily to the basal body of the photoreceptor cilium, a structure which connects the photoreceptor inner and outer segments. CEP290 is thought to play a critical role in intraflagellar transport of proteins between cellular compartments, and has been associated through protein immunoprecipitation experiments with retinitis pigmentosa GTPase regulator (RPGR) mutations, which are implicated in 15% of retinitis pigmentosa cases (Chang et al., 2006b; Hosch et al., 2011; Khanna et al., 2005). Though the pathophysiology of LCA associated with *CEP290* mutations is poorly understood, it is likely that photoreceptor dysfunction arises as a result of improper transport across the cilium, as evidenced by the inappropriate distribution of rhodopsin and arrestin in *rd16* mice, which have an in-frame deletion of *CEP290* (Chang et al., 2006b; den Hollander et al., 2008). To date, successful gene therapy in animal models of CEP290 deficiency has not been achieved, most likely due to the challenge of expressing a large gene (7.5 kb) in photoreceptors.

The prospect for treating LCA arising from gene defects within photoreceptors depends greatly on the gene involved. Despite mutations affecting CRB1 accounting for approximately 10% of LCA, it is an unlikely candidate for gene therapy, as it is developmentally expressed and mutations may lead to severe abnormalities of retinal morphology, such as thickening of the outer nuclear layer, which would probably not be reversible, even with successful gene delivery. In such instances, where irreversible morphological abnormalities are present, alternative approaches such as neuroprotection may be more appropriate to preserve any remaining function (see Section 10.2).

Mutations affecting *GUCY2D* or *CEP290* would likely be more amenable, where vision loss and subsequent degeneration arise from dysfunction, rather than morphological disruption. *GUCY2D* and *CEP290* LCA are recessively inherited, and providing the re-expressed protein is able to localise to the correct cellular compartment, the inner segment membrane in the case of GUCY2D, or the cilium basal body in the case of CEP290, both conditions might be significantly improved by direct gene replacement. As mentioned above however, the large size of the CEP290 gene may limit progress using current AAV vectors.

4.4. Usher syndrome

Usher syndrome, arguably first described in 1858 by Albrecht von Graefe, but named after Charles Usher, is a syndromic condition affecting the development of sensorineural cells characterised by a duel sensory impairment of the visual and audiovestibular systems (Usher, 1914; von Graefe, 1858). Classically, Usher syndrome has been divided in to three clinically distinct types (I, II and III) based on severity of hearing loss, the presence or absence of vestibular dysfunction and the age at which visual impairment manifests (Yan and Liu, 2010). Usher syndrome is a genetically heterogeneous disease, with 11 associated loci and nine genes, mutations of which collectively affect approximately 1 in 25,000 people (Moller et al., 1989; Spandau and Rohrschneider, 2002). Herein, this review will focus on Usher syndrome 1B due to its prevalence (39–55% of Usher type I), its clinical severity, and the possibility for treatment using gene therapy (Ouyang et al., 2005; Roux et al., 2006).

4.4.1. MYO7A

Usher 1B is caused by mutations of the *MYO7A* gene, which encodes myosin (VIIA). In eukaryotes, transport of proteins along cilia, such as those connecting photoreceptor inner and outer segments, relies on myosin proteins. Myosins consist of a substrate binding domain, an actin binding domain, and a mechanochemical motor, which utilizes ATP-hydrolysis to drive the myosin along actin filaments, thus transporting the bound substrate between cellular compartments. Myosin VIIA is thought to play a role in the transport of opsin to the outer segment discs; absence of myosin VIIA results in an abnormal accumulation of opsin in the photoreceptor cilium and retards disc membrane morphogenesis (Liu et al., 1999). Myosin VIIA likely carries out this function in conjunction with kinesin-2, deficit of which results in the total absence of opsin transport and rapid photoreceptor degeneration (Jimeno et al., 2006; Marszalek et al., 2000; Williams, 2008).

Usher 1B is, in many respects, a promising candidate for gene therapy, with disease following a recessive inheritance pattern and arising from an absence of myosin VIIA function. Additionally, whilst deafness and photoreceptor dysfunction are present at birth, photoreceptor degeneration does not generally begin until adolescence, giving a therapeutic window in which to intervene. However, the transgene required to express myosin VIIA protein is too large (6.7 kb) to be delivered using conventional adeno-associated virus (AAV) vectors, and photoreceptors may not be as efficiently targeted by other viral vectors or non-viral means. Nevertheless a commercial clinical trial led by Oxford Biomedica using an equine lentiviral vector to deliver MYO7A has started (NCT01505062). Further aspects of lentiviral-mediated gene delivery technology are discussed in Section 8.1.

4.5. Stargardt's disease

Stargardt's disease, also known as fundus flavimaculatus, was first described in the early twentieth century by Karl Stargardt and is the most common form of juvenile macular degeneration, affecting an estimated 1 in 10,000 individuals worldwide (Blacharski, 1988; Stargart, 1909). However, the carrier frequency of *ABCA4* mutant variants is thought to be as high as 2 in 100, and hence *ABCA4*-retinopathies, such as those presenting as classic rod–cone dystrophies, may be under-reported (Jaakson et al., 2003).

4.5.1. ABCA4

Stargardt's arises from mutations affecting the photoreceptor-specific ABC transporter gene (*ABCA4*), which encodes an ATP-binding cassette localized to the membrane of photoreceptor outer segment discs that plays a role in retinoid cycling. During phototransduction, isomerisation of 11-cis retinal following light activation leads to accumulation of all-trans retinal in the outer segment lumen. Due to its hydrophobicity, the majority of all-trans

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retinal spontaneously translocates across the disc membrane, where it is converted to all-trans retinol by all-trans retinol-dehydrogenase 8 (RDH8) (Maeda et al., 2007). However, approximately 24% of all-trans retinal reversibly complexes with phosphatidylethanolamine (PE) to form N-retinylidene-PE (Mata et al., 2000), a hydrophilic compound which is unable to cross the disc membrane independently. ABCA4 is thought to catalyse the translocation of N-retinylidene-PE across the membrane using energy from ATP hydrolysis (Tsybovsky et al., 2010). In the absence of functional ABCA4 protein, N-retinylidene-PE accumulates in the lumen, where it can irreversibly bind to a second all-trans retinal molecule to form di-retinoid-pyridinium-PE (A2PE). Following disc shedding and RPE phagocytosis, A2PE is eventually released into the acidic environment of the phagosome, where it is hydrolysed to A2E. A2E is unable to be metabolised further and accumulates in the RPE where it forms a major toxic component of lipofuscin. Lipofuscin build-up severely retards RPE phagocytotic and lysosomal functions and creates reactive oxygen species (ROS) leading to RPE degeneration and subsequently loss of the overlying photoreceptors. A2E deposition is particularly rapid in the macular region where retinoid turnover is high, leading to a range of phenotypically heterogeneous diseases, including "bulls-eye" maculopathy, autosomal recessive retinitis pigmentosa, and cone–rod dystrophy (Burke and Tsang, 2011).

Though Stargardt's disease is monogenic, and is often diagnosed prior to complete loss of central vision, treatment with gene therapy remains problematic. This is primarily due to the size of the transgene necessary to express functional ABCA4 protein, which like that of *MYO7A* is too large to be package efficiently in AAV, but is within the approximate 9 kb packaging capacity of lentiviral vectors. A commercial clinical trial led by Oxford Biomedica using EIAV to deliver ABCA4 is running in parallel with the Usher trial mentioned above (NCT01367444).

5. Disorders affecting multiple tissues

5.1. Choroideremia

Choroideremia, first described in 1871 by Mauthner, is a condition leading to degeneration of the choroid, RPE and neural retina that affects an estimated 1 in 50,000 individuals (MacDonald et al., 2010; Mauthner, 1871). The disease follows an X-linked inheritance pattern, with affected males suffering progressive chorioretinal degeneration characterized by night blindness and loss of peripheral visual field. Central vision is usually preserved, but deteriorates later in life. Carrier females are generally asymptomatic, though often have a slowly progressing degeneration which does not lead to visual impairment. Occasionally, as a consequence of skewed X-inactivation or the presence of a balanced X-autosome translocation, females can be severely affected and develop a full choroideremia phenotype (Lorda-Sanchez et al., 2000; MacDonald et al., 2010). Choroideremia is monogenic and arises from mutations (almost always null) affecting the *CHM* gene.

5.1.1. CHM/CHML

CHM encodes Rab-escort protein-1 (REP1), which is expressed ubiquitously and plays a role in intracellular trafficking. Transport of vesicles within eukaryotic cells is mediated through association of membrane-bound GTP-binding proteins (Rab), which act to guide vesicles (endosomes, melanosomes etc) to the appropriate cellular compartment. There are currently 60 human Rab proteins, each trafficking between specific compartments, and Rab binding to the correct vesicular/compartment membrane requires that the protein is geranyl-geranylated through the transfer of isoprene lipids to one or both c-terminal cysteine residues (Preising and Ayuso, 2004; Seabra et al., 2002; Sergeev et al., 2009). Transfer of geranyl–geranyl moieties to a Rab protein is catalysed by geranyl–geranyl transferase (RabGGTase) and requires that the Rab is presented in a complex with REP-1 or REP-2 (Farnsworth et al., 1994). REP-2 is an alternative rab-escort protein that is encoded by the *CHML* gene, which is likely a retro gene of *CHM* and has 75% sequence identify. In the absence of functional REP-1 protein, geranyl-geranylation of Rab proteins should be assured by the homologous function of REP-2. However, for reasons that are incompletely understood, REP-2 expression does not compensate for the absence of REP-1 in ocular tissues (Sergeev et al., 2009).

Most reported mutations affecting CHM are null, with very few missense, generally resulting in a total absence of protein production. Disease is often diagnosed in the first two decades of life and progresses slowly, with patients only losing central vision later (fifth decade) due to preservation of the fovea area until the very late stages (MacDonald et al., 2010). Thus, choroideremia appears to be a promising candidate for gene therapy, likely through direct gene replacement (re-expression of *CHM*). However, the relative gulf in knowledge regarding the mechanisms of disease pathogenesis, and the lack of functional compensation by REP-2 gives rise to certain concerns. In particular, it has been suggested that photoreceptor and RPE degeneration follow secondary to atrophy of the choroid (Ghosh and McCulloch, 1980; McCulloch, 1950). The choroid is a tissue not easily accessible to gene delivery, and while re-expression of *CHM* in the RPE has been demonstrated in an animal model of choroideremia, it is uncertain if rescue of prenylation defects in the RPE alone is sufficient to ameliorate the disease phenotype (Tolmachova et al., 2012). The current ongoing clinical trial using AAV mediated gene replacement of REP1 – designed to target both RPE and photoreceptors – should soon answer many of these questions (NCT01461213).

5.2. Age related macular degeneration

Age related macular degeneration (AMD), defined by presence of drusen and altered pigmentation in the macular region, is the leading cause of visual impairment in the developed world, affecting approximately 10% of individuals over 65 years of age, rising to 12% in the over 80 age group (Klein et al., 2010; Leveziel et al., 2011). In particular, neovascular (wet) AMD (NV-AMD), caused by abnormalities of angiogenesis which lead to choroidal neovascularisation and retinal angiomatosis proliferation, accounts for over 60% of late AMD cases, and 90% of AMD related blindness (Lotery et al., 2007). Current treatments focus on the prevention of choroidal neovascularisation in NV-AMD through the delivery of anti-angiogenic factors, particularly bevacizumab (Avastin) or Ranibizumab (Leucentis). These compounds both function as inhibitors of vascular endothelial growth factor A (VEGF-A), expression of which is strongly linked to the growth and increased permeability of new blood vessels (Folk and Stone, 2010).

Whilst AMD is a complex disorder with numerous environmental risk factors, including smoking and high HDL cholesterol levels, several major genetic factors have been implicated, including complement factor H (CFH), complement C3, complement factor I (CFI), Age-related maculopathy susceptibility 2 (ARMS2) and ApoE (For review, see Leveziel et al., 2011). However, no individual gene accounts for a significant proportion of AMD, and each might be considered risk factors, rather than the defining underlying cause of disease pathogenesis. Due to the complex nature of AMD and the absence of a standout candidate gene for replacement, it might seem an unlikely candidate for gene therapy. However, the success of VEGF-antagonists bevacizumab and ranibizumab in the treatment of AMD has demonstrated that long-term expression of anti-angiogenic molecules may prove an effective

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alternative treatment route, and would be applicable even after development of neovascularisation. Genetic therapies for the treatment of AMD, including delivery of sFlt-1, PEDF and soluble statins are discussed in detail in Sections 10.3 And 11.4.

6. Gene delivery

The success of any gene therapy for the treatment of retinal disease is dependent upon the efficiency with which the therapeutic transgene can be delivered to the appropriate cell type. There are presently two major approaches for the delivery of genetic material: Virally vectored gene delivery and non-viral gene delivery.

7. Viral

A number of different viral vectors have been shown to have tropism for specific cell types of the eye in animal models and tissue culture, including AAV (Reichel et al., 1998), adenovirus (Campochiaro et al., 2006), herpes simplex virus (Spencer et al., 2000) and lentivirus (Miyoshi et al., 1997). The most commonly used of these vectors both for pre-clinical and clinical gene transfer is adeno-associated virus (AAV).

7.1. Adeno-associated virus

Adeno-associated virus (AAV) is non-pathogenic dependovirus (subfamily: *Parvovirinae*) that is unable to replicate in the absence of an active synergistic virus infection, typically of adenovirus or herpes simplex virus (Berns, 1996). AAV virions are approximately 25 nm in diameter, non-enveloped and package a single stranded DNA (ssDNA) genome (Baltimore class II) with a maximum length of 5.1 kb (Arsuaga et al., 2002; Berns, 1996; Srivastava et al., 1983). The wild-type AAV genome has two open reading frames (ORF) each containing a single gene. ORF-1 contains *rep,* which encodes four proteins (Rep40, Rep52, Rep68 and Rep78) required for virus replication, and ORF-2 contains *cap*, which encodes the three structural proteins (VP1, VP2 and VP3) that comprise the icosahedral capsid. The coding sequence is flanked by two inverted terminal repeats (ITRs), palendromic sequences 145 bp in length which form hairpin-loop secondary structures at the strand termini (Fig. 1). ITRs are critical components, facilitating genome packaging, which occurs from the 3′ terminal ITR. Additionally, ITRs prevent recognition of the ssDNA genome by host cell immunity and direct sustained eukaryotic transcription.

There are presently at least 11 naturally occurring AAV serotypes (AAV1 − 11), though over one hundred non-redundant species of proviral sequence have been recovered, with sequence

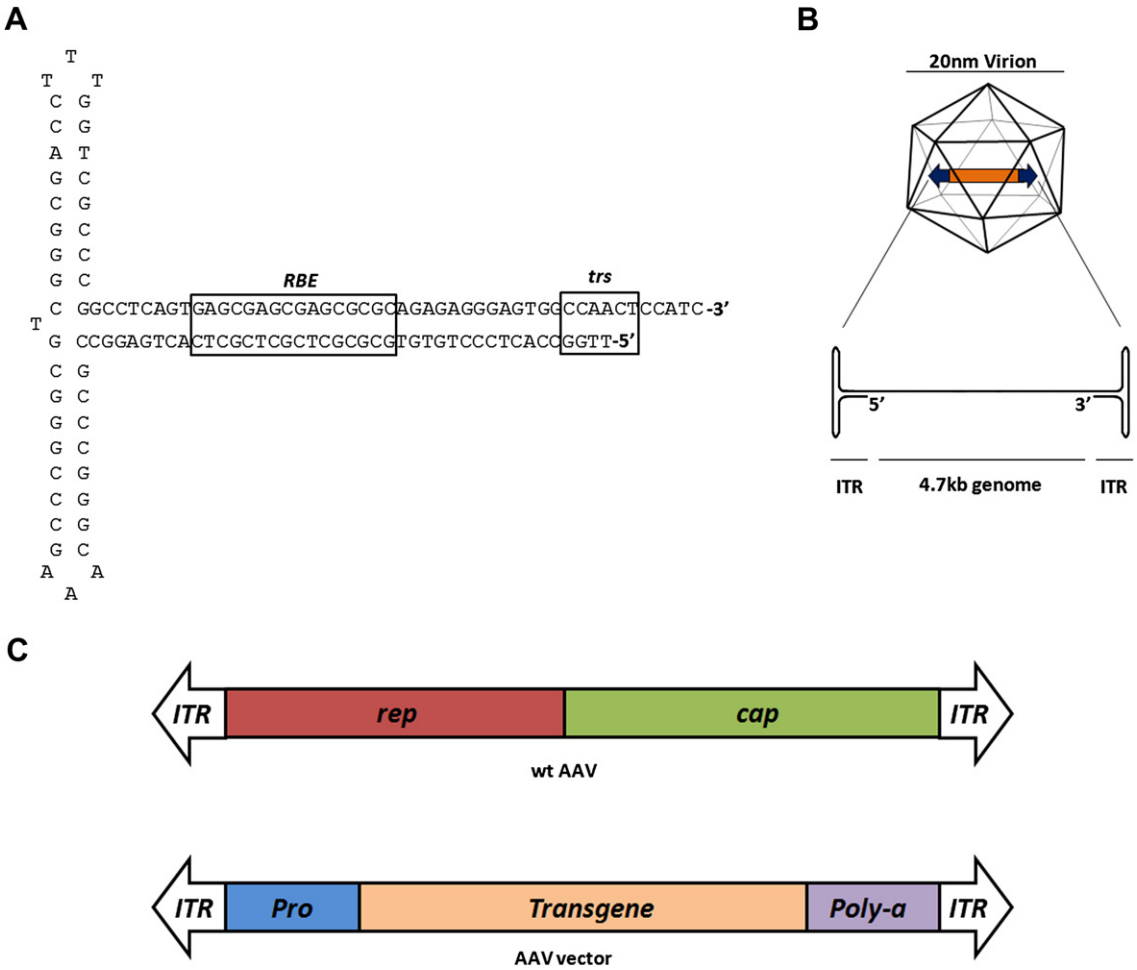

Fig. 1. Schematic representation of an AAV vector and inverted terminal repeats (ITR). (A) Hairpin structure of AAV2 ITR. RBE = rep binding element; trs = terminal resolution site. (B) 20 nm icosahedral capsid of the AAV virion containing a single-stranded DNA AAV genome. (C) Removal of the viral Rep and Cap genes allows the insertion of an expression cassette with a maximum size of 4.7 kb; note the 146 bp inverted terminal repeats (TR) which form *t*-shaped hairpin loops at both the 5′ and 3′ termini which facilitates genome packaging, second strand synthesis and genome replication. The transgene cassette minimally contains a promoter, a transgene and poly-a tail: regulatory elements which are required for gene expression.

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identity of *cap* ranging from 45% to 99% (Gao et al., 2005; Mori et al., 2004). The polymorphic nature of *cap* gives rise to capsid proteins with affinities for different cell surface receptors. The primary receptor mediating cell binding of AAV serotype 2 (AAV2) is heparan sulphate (Summerford and Samulski, 1998), with $\alpha_v\beta5$ integrin (Summerford et al., 1999), fibroblast growth factor-1 (Qing et al., 1999), hepatocyte growth factor (Kashiwakura et al., 2005) and laminin all implicated as co-receptors (Akache et al., 2006). By contrast, AAV4 and AAV5 bind preferentially to α2,3 sialic acid or platelet-derived growth factor (Di Pasquale et al., 2003; Kaludov et al., 2002), while AAV3, AAV8 and AAV9 bind laminin (Akache et al., 2006). Differential binding significantly affects the natural tropism of each AAV serotype, an aspect which is exploited when using AAV derived vectors (Wang et al., 2011).

7.1.1. Vector construction and gene packaging

AAV vector construction requires modification of a wild-type virus genome, usually that of AAV2. ORF-1 and ORF-2, containing the *rep* and *cap* genes, are deleted, and replaced with a transgene cassette which minimally consists of a promoter, therapeutic cDNA and poly-adenylation (poly-a) signal. The ITRs are retained, and are the only viral sequence required for cis-packaging of a recombinant vector genome (McLaughlin et al., 1988). The necessary inclusion of ITRs reduces the total coding capacity of AAV vectors to approximately 4.7 kb, and limits the size of the therapeutic gene that can be delivered. However, if one were to assume inclusion of the cytomegalovirus (CMV) promoter (~700 bp) and bovine growth hormone poly-a (~200 bp), genes for the treatment of achromatopsia (≤2.6 kb) or certain forms of LCA could still be packaged comfortably. For a complete summary of target genes implicated in retinal disease, see Table 1.

The delivery of larger genes using AAV remains problematic. Efforts to artificially expand AAV packaging capacity have focused on intermolecular re-arrangement through trans-splicing, where the therapeutic transgene is split across two separately package AAV genomes, one containing a splice donor site and the other a splice acceptor (Duan et al., 2003; Lai et al., 2005b; Yan et al., 2000). Once released into the nucleoplasm, trans-splicing vectors perform homologous recombination or non-homologous end-joining of the ITRs to form a concatomeric genome containing both a splice donor and splice acceptor site. Transcription of the concatameric ssDNA genome consequently results in production of a spliced full-length transcript (Fig. 2). While an elegant solution, trans-splicing is inefficient due to dependence on recombination and vector design is more complex.

In 2008 a landmark publication demonstrated expression of ABCA4 protein in the mouse retina from a single 8.9 kb transgene cassette packaged into AAV (Allocca et al., 2008). Recent studies have confirmed that larger genomes are unable to be packaged into a single virion. The proposed mechanism of expression is instead that terminally truncated plus and minus strands are packaged up to a length of 5.1 kb into separate virions (Lai et al., 2010). Following release into the nucleoplasm, direct annealing of truncated genomes of opposite polarity occurs in the region of overlap, followed by single-strand synthesis in both directions primed by the free 3′ termini (Hirsch et al., 2010; Wu et al., 2010) (Fig. 2). Though the optimum length of the overlapping region required for spontaneous reannealing has not been elucidated, if the mechanism is reproducible, it would increase significantly the number of visual disorders that could be treated using AAV. This would be of particular relevance for the treatment of Stargardt's disease and Usher 1B, caused by defects of the ABCA4 (6.8 kb) and MYO7A (6.6 kb) genes, respectively. However, even with the increased coding capacity, genes linked to Usher syndrome 1D (CDH23, 10 kb) and 2C (GRP98, 18.9 kb), would remain unpackageable as the gene size would span across more than two AAV genomes.

7.1.2. Gene expression from AAV vectors

Although recombinant AAV vectors have many advantages, one potential problem remains the delay in the onset of transgene expression, often requiring up to six weeks for peak levels of protein, which could limit applications in acute retinal disease (Bennett et al., 1997). There are two major rate-limiting steps affecting the speed and efficiency of transgene expression after administration of rAAV vectors. The first is the breakdown of AAV virions in the endosome following internalization.

Following cell-surface attachment, AAV virions are internalized by clathrin-mediated endocytosis, or occasionally by caleolae, in the case of AAV5, in a process requiring dynamin (Duan et al., 1999). Transport to the nucleus and escape of the virion from the endosome is a poorly understood process, but requires acidification of the endosome and likely a conformational change in capsid structure (Bartlett et al., 2000; Sonntag et al., 2006) (Fig. 3). Although not an uncommon property in viruses, AAV requires endosome maturation to reach the highly acidic end-stage prior to externalization of VP1 and VP2 domains thought to be required for escape (Sonntag et al., 2006). However, prior to release the AAV capsid frequently undergoes phosphorylation of exposed surface-tyrosine resides, which marks the virion for ubiqutination and proteosome-mediated degradation, and significantly reduces the number of viral genomes that are successfully delivered to the nucleus for expression (Zhong et al., 2008).

The second rate-limiting step is the time taken for synthesis of the complementary strand from the single-stranded vector DNA by the host replication machinery (Ferrari et al., 1996).

7.1.3. Capsid-mutant AAV

An elegant solution to avoid degradation of AAV virions is the use of capsid serotypes containing targeted mutations, specifically, replacement of exposed surface tyrosine (Y) residues (Petrs-Silva et al., 2009). Tyrosine-to-Phenylalanine (F) substitutions are most common, where phenylalanine is an aromatic amino acid with similar hydrophobic properties to tyrosine, allowing replacement without significant impact on capsid protein structure. Advantageously, phenylalanine lacks the para-hydroxyl group which enables tyrosine to be phosphorylated, thus preventing ubiquitination and subsequent proteolysis of the capsid. AAV vectors with capsid mutant serotypes have proven to significantly increase the levels of transduction following subretinal and intravitreal administration in comparison to non-mutant serotypes (Petrs-Silva et al., 2009). Most strikingly capsid mutant vectors have demonstrated efficient transduction of photoreceptors following intravitreal delivery, a property not previously observed, and which has implications for the treatment of stationary diseases such as achromatopsia, as there is less chance of surgical complications compromising residual vision if the fovea can be targeted from the vitreous side without needing to detach the retina.

7.1.4. Self-complementary AAV

Self-complementary AAV vectors (scAAV) have been proposed to reduce the time until gene expression by eliminating the requirement for second strand synthesis. The genome of scAAV vectors are designed such that two copies of the transgene cassette are included, one in sense orientation and one reverse complement, separated by a short linker. Following release of the ssDNA genome into the nucleoplasm the complementary plus and minus sequences anneal forming a simple hairpin loop structure. The resultant double stranded template allows the transgene to be

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Table 1
Genetic etiology, cellular role and distribution for some of the commonest retinal diseases, including typical heritability patterns. Size of the minimal functional cDNA sequence for each gene is given in addition to an indication as to their ability to be packaged efficiently in standard adeno associated vectors (serotype rAAV2/2) or HIV1 based lentivirus vector.

Condition	Type	Gene	Role	Cell type affected	Normal heritability	cDNA size	Package in AAV[a]	Package in lentivirus[b]
Retinitis Pigmentosa (RP)	AR-RP, AD-RP	Rhodopsin (Rho)	Phototransduction cascade	Photoreceptor (Rod)	Autosomal dominant, Autosomal recessive	1.0 kb	Yes	Yes
	AR-RP	Phosphodiesterase 6A, cGMP-specific, rod alpha (PDE6A)	Phototransduction cascade	Photoreceptor (Rod)	Autosomal Recessive	2.6 kb	Yes	Yes
	AR-RP	Phosphodiesterase 6B, cGMP-specific, rod beta (PDE6B)	Phototransduction cascade	Photoreceptor (Rod)	Autosomal Recessive	2.6 kb	Yes	Yes
	X-RP	Retinitis pigmentosa GTPase regulator (RPGR)	Intracelluar trafficking	Photoreceptor (All)	X-linked	3.0 kb	Yes	Yes
	AR-RP	Cyclic nucleotide gated channel alpha 1 subunit (CNGA1)	Phototransduction cascade	Photoreceptor (Rod)	Autosomal Recessive	2.3 kb	Yes	Yes
	AR-RP	Cyclic nucleotide gated channel beta 1 subunit (CNGB1)	Phototransduction cascade	Photoreceptor (Rod)	Autosomal Recessive	3.8 kb	Yes	Yes
Leber's Congenital Amaurosis (LCA)		mer tyrosine kinase proto-oncogene (MERTK)	Outer segment phagocytosis	RPE	Autosomal recessive	3.0 kb	Yes	Yes
	LCA1	Guanylate cyclase 2D	Phototransduction cascade	Photoreceptor (All)	Autosomal recessive	3.3 kb	Yes	Yes
	LCA2	Vitamin A -trans-cis isomerase (RPE65)	Vitamin A metabolism	RPE	Autosomal recessive	1.6 kb	Yes	Yes
	LCA3	Spermatogenesis associated 7 (SPATA7)	Unknown	Unknown	Autosomal recessive	1.8 kb	Yes	Yes
	LCA4	Aryl hydrocarbon receptor interacting protein-like 1 (AIPL1)	PDE chaperone	Photoreceptor (All)	Autosomal recessive	1.2 kb	Yes	Yes
	LCA5	Lebercillin (LCA5)	Intracelluar trafficking	Photoreceptor (All)	Autosomal recessive	2.1 kb	Yes	Yes
	LCA6	Retinitis pigmentosa GTPase regulator interacting 1 (RPGRIP1)	Intracelluar trafficking	Photoreceptor (All)	Autosomal recessive	3.8 kb	Yes	Yes
	LCA7	Cone–Rod Homeobox (CRX)	Transcription factor	Photoreceptor (All)	Autosomal recessive	0.9 kb	Yes	Yes
	LCA8	Crumbs homolog (CRB1)	Embryological development	Photoreceptor (All)	Autosomal recessive	4.2 kb	No	Yes
	LCA9	Unknown (locus q1.36)	Unknown	Unknown	Unknown	N/A	N/A	N/A
	LCA10	Centrosomal protein 290 kDa (CEP290)	Intracelluar trafficking	Photoreceptor (All)	Autosomal recessive	7.5 kb	No	No
	LCA11	Inosine 5′-monophosphate dehydrogenase 1 (IMPDH1)	Guanine synthesis	Photoreceptor (All)	Autosomal recessive	1.8 kb	Yes	Yes
	LCA12	Retinal degeneration 3 (RD3)	Unknown	Photoreceptor (All)	Autosomal recessive	0.6 kb	Yes	Yes

(*continued on next page*)

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Table 1 (*continued*)

Condition	Type	Gene	Role	Cell type affected	Normal heritability	cDNA size	Package in AAV[a]	Package in lentivirus[b]
	LCA13	Retinal dehydrogenase 12 (RDH12)	Vitamin A metabolism	Photoreceptor (All)	Autosomal recessive	1.0 kb	Yes	Yes
	LCA14	Lecithin retinol acyltransferase (LRAT)	Vitamin A metabolism	RPE	Autosomal recessive	0.7 kb	Yes	Yes
	LCA15	Tubby like protein (TULP1)	Intracelluar trafficking	Photoreceptor (All)	Autosomal recessive	1.7 kb	Yes	Yes
Stargardt's Disease	STGD1	ATP-binding cassette, sub-family A, member 4 (ABCA4)	Retinoid Flipase	Photoreceptor (All)	Autosomal recessive	6.8 kb	No	No
Achromatopsia	ACHM2	Cyclic nucleotide gated channel alpha 3 subunit (CNGA3)	Gated ion channel	Photoreceptor (Cone)	Autosomal recessive	2.1 kb	Yes	Yes
	ACHM3	Cyclic nucleotide gated channel beta 3 subunit (CNGB3)	Gated ion channel	Photoreceptor (Cone)	Autosomal recessive	2.4 kb	Yes	Yes
	ACHM4	Guanine nucleotide alpha transducing polypeptide 2 (GNAT2)	Phototransduction cascade	Photoreceptor (Cone)	Autosomal recessive	1.1 kb	Yes	Yes
	ACHM5	Phosphodiesterase 6C, cGMP-specific, cone alpha prime (PDE6C)	Phototransduction cascade	Photoreceptor (Cone)	Autosomal recessive	2.6 kb	Yes	Yes
Usher's Syndrome	USH1B	Myosin 7A (MYO7A)	Intracellular (cillia) trafficking	Neurosensory retina	Autosomal recessive, severe syndromic	6.7 kb	No	No
	USH1C	Harmonin (USH1C)	Cellular scaffold	Neurosensory retina	Autosomal recessive, severe syndromic	2.7 kb	Yes	Yes
	USH1D	Cadherin 23 (CDH23)	Cell-to-cell adhesion	Neurosensory retina	Autosomal recessive, severe non-syndromic	10 kb	No	No
	USH1F	protocadherin-related 15 (PCDH15)	Cell-to-cell adhesion	Neurosensory retina	Autosomal recessive, severe syndromic	5.9 kb	No	No
	USH1G	Usher syndrome 1G (USH1G)	Cellular scaffold	Neurosensory retina	Autosomal recessive, severe syndromic	1.4 kb	Yes	Yes
	USH2A	Usherin (USH2A)	Cell-to-cell adhesion	Neurosensory retina	Autosomal recessive, mild syndromic	15.6 kb	No	No
	USH2C	G-coupled receptor 98 (GPR98)	cell signalling	Neurosensory retina	Autosomal recessive, mild syndromic	18.9 kb	No	No
	USH3A	Clarin 1 (CLRN1	cell signalling	Neurosensory retina	Autosomal recessive, mild syndromic	0.7 kb	Yes	Yes
Choroidaremia	CHM	Rab-escort protein 1 (CHM)	Intracellular trafficking	Choroid, RPE Photoreceptor (All)	X-linked	2.0 kb	Yes	Yes

[a] AAV vector packaging (max = 5.1 kb) in this instance assumes inclusion of viral inverted terminal repeats (ITRs; ~300 bp), a cytomegalovirus (CMV) promoter (~700 bp) and bovine growth hormone (bGH) poly adenylation signal (200 bp) in the transgene cassette.

[b] Lentiviral expression cassette (max 9.0 kb) assumes inclusion of minimal regulatory elements: 5′ long terminal repeat (LTR), packaging signal (ψ), Rev-response element (RRE), poly-purine tracts (cPPT), woodchuck post-translational regulatory element (WPRE), 3′ LTR, splicing and regulatory sequences, introns and a murine phosphoglycerate kinase (PGK) promoter (~3200 bp total). AR-RP = autosomal recessive retinitis pigmentosa; AD-RP = autosomal dominant retinitis pigmentosa; X-RP = X-linked retinitis pigmentosa; LCA = Leber's congenital amaurosis.

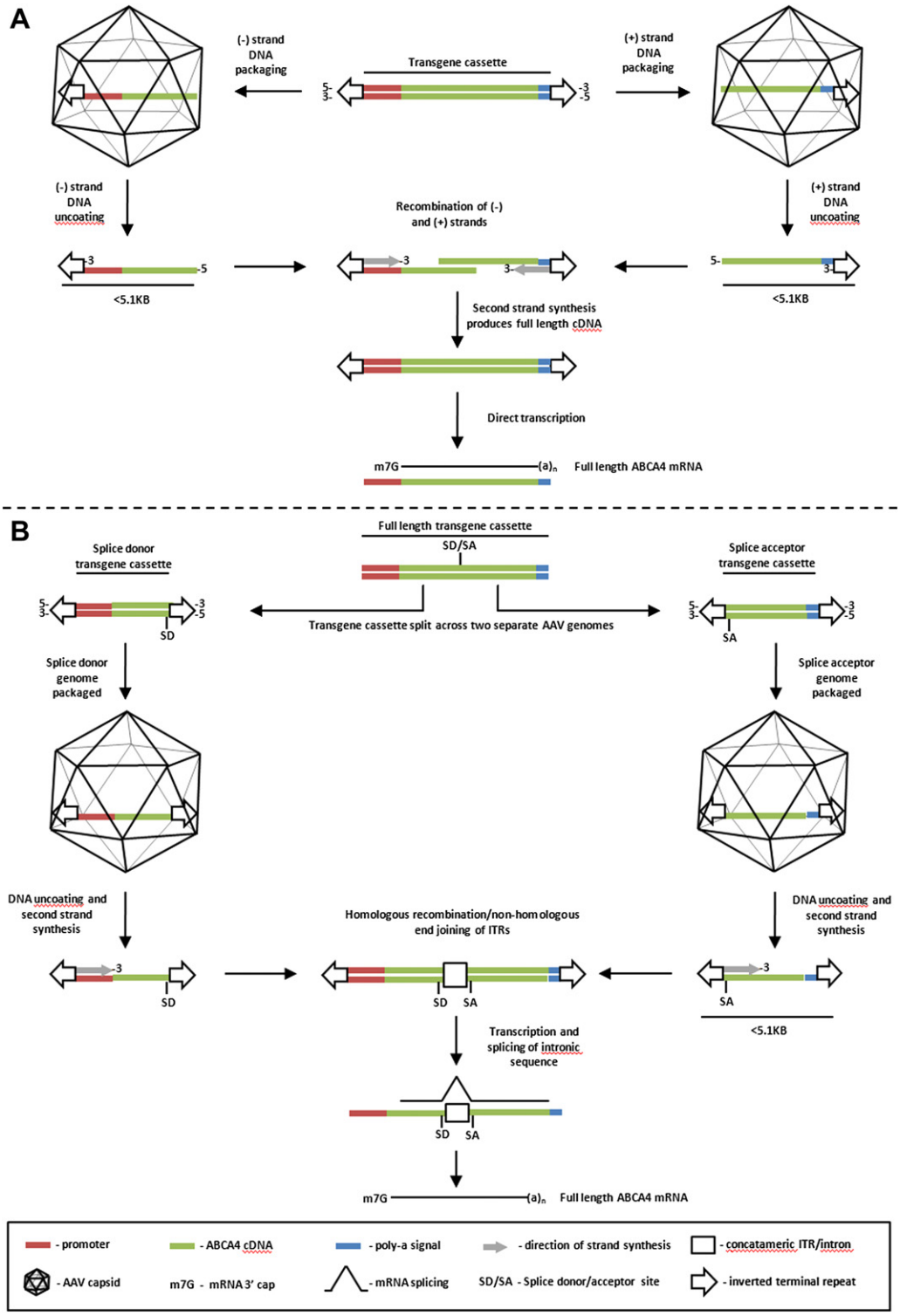

Fig. 2. Diagrammatic representation of two techniques for the packaging of large transgene cassettes into AAV vectors, using ABCA4 cDNA (6.8 kb) as an example. A) Recombination approach – the transgene cassette is packaged as randomly truncated single DNA strands of up to 5.1 kb. Strands are of opposite polarity and packaged with equal efficiency in to separate virions from the 3′ ITR. Following uncoating of virions containing complementary strands within the nucleoplasm overlapping regions anneal. Strands are extended from the 3′ terminus by host DNA repair machinery and ligated forming a double stranded duplex which can be directly transcribed. B) Trans-splicing approach – the transgene cassette is fragmented into a splice donor genome (containing SD site) and a splice acceptor genome (containing SA site), each flanked by 3′ and 5′ ITRs and being no more than 5.1 kb in length. The splice donor and acceptor genomes are packaged untruncated from the 3′ ITR in to separate virions. Upon release into the nucleoplasm both genomes are extended from the 3′ terminus to form separate DNA duplexes. The ITRs of two separate genomes form a concatameric structure through homologous recombination of non-homologous end joining. Transcription results in the concatameric ITRs being lost through a traditional splicosome mediated reaction. Both technique rely on the entry of two virions of opposite design/orientation into the same cell, and are greatly inefficient.

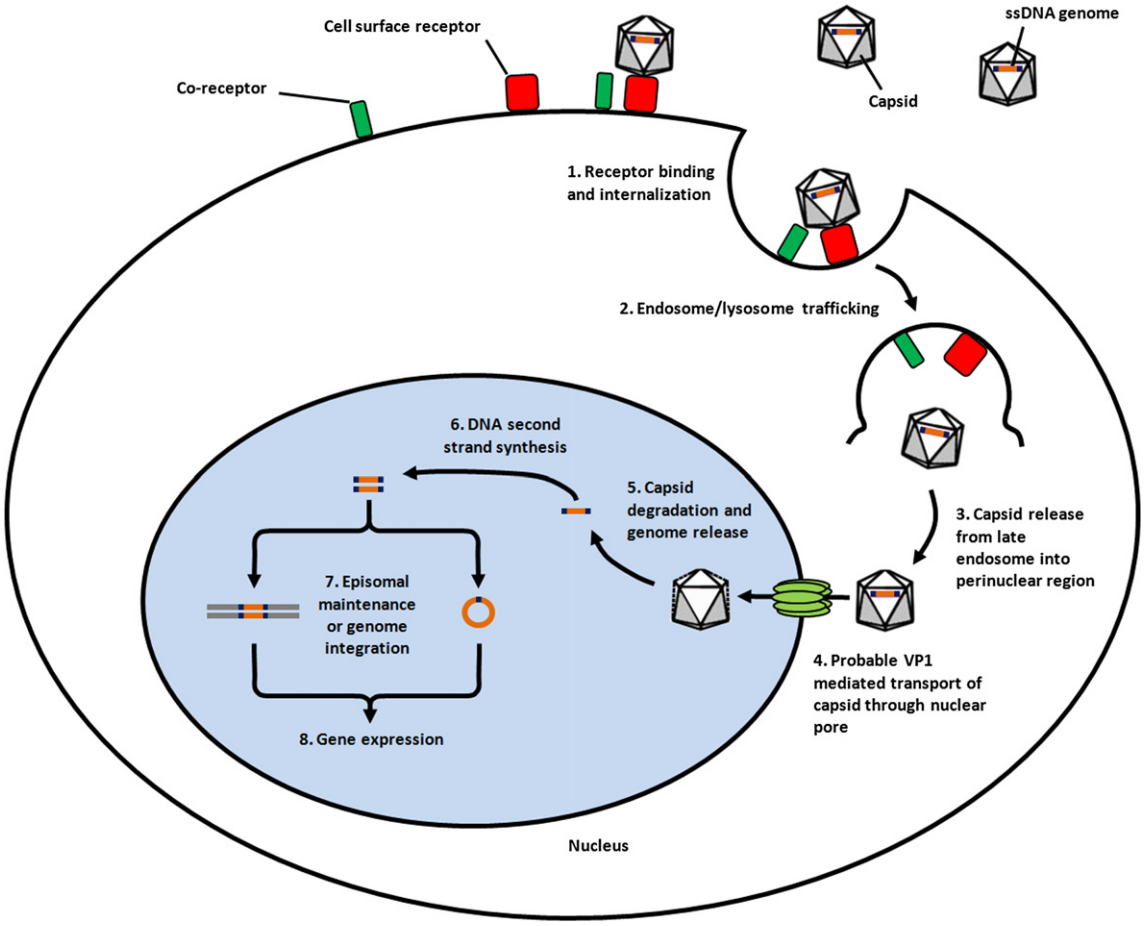

Fig. 3. Gene expression from an AAV vector requires the completion of multiple steps. 1) Binding of the AAV virion to specific cell surface receptors, which include integrins, FGFR-2 and heperan sulphate; endocytosis likely requires secondary binding of co-receptors. 2) Trafficking of internalized endosome/lysozyome through the cytoplasm and 3) release of the AAV virion in to the perinuclear region. 4) Nuclear internalization through a pore complex. This process is incompletely understood, but is likely to be dependent on nuclear localisation signals found within the N-terminus tail of the VP1 capsid protein, which is exposed during capsid escape from the late endosome. 5) Degradation of the capsid and release of the single stranded DNA genome. 6) Synthesis of double stranded DNA which is either maintained 7) Episomally or integrated into the host genome. 8) Expression of viral genome or transgene.

transcribed directly without need for recruitment of the host DNA replicatory machinery. Whilst the need to include sense and antisense copies of the transgene cassette reduces the coding capacity considerably, scAAV vectors have been shown to reduce the time from infection to peak transduction in the liver, brain cells and the eye (Yokoi et al., 2007). In diseased RPE cells of the RPE65 deficient mouse, scAAV REPE65 transgene expression has been detected as early as four days after subretinal injection (Pang et al., 2010a). Whilst this may be relevant for mouse models with a rapid retinal degeneration, it is not entirely clear which human retinal diseases would benefit from scAAV, as the most effective applications of AAV gene therapy will most likely be for chronic degenerative conditions active throughout the lifetime of the patient. However, capsid mutant serotypes and self-complementary genomes have recently been used in conjunction to restore vision in a mouse model of early onset LCA, and were found to be more efficacious than single stranded vectors (Ku et al., 2011).

7.2. Spatial regulation of gene expression

The accurate spatial control of transgene expression is integral to the success of any gene therapy intervention, and is determined by vector design, but also the route by which the vector is subsequently administered.

7.2.1. Recombinant AAV serotypes

The cellular tropism of AAV is conferred predominantly by the capsid, which harbours receptor binding domains with affinity for specific cell-surface residues (summarized above), such as the heparin binding domain located on the externalized loop-domain of AAV2 VP3 protein (Wu et al., 2000). It is possible to utilize this differential tropism through production of recombinant AAV (rAAV) vectors, formed by the process of 'pseudotyping', where an AAV2 based expression cassette is packaged into a capsid originating from a different serotype. Using recombinant vectors gene delivery to RPE has been effectively shown with several pseudotypes, particularly rAAV2/1 (AAV2-based genome in AAV1 capsid), rAAV2/2 and rAAV2/4 following delivery into the subretinal space (Ali et al., 1996; Le Meur et al., 2007; Lebherz et al., 2008). rAAV2/2 also exhibits photoreceptor transduction, but to a lesser degree than either rAAV2/5 or rAAV2/8 when delivered subretinally (Bennett et al., 1999; Flannery et al., 1997; Mussolino et al., 2011; Vandenberghe et al., 2011), but uniquely demonstrates transduction of ganglion cells and Müller glia following intravitreal administration (Ali et al., 1998; Yin et al., 2011). It is not known if any rAAV can transduce the choroid effectively, although various serotypes are known to transduce vascular endothelial cells and muscle (Kilian et al., 2008; Zincarelli et al., 2008).

Consequently, selection of capsid serotype depends predominantly on the tissue type to which the therapeutic gene is to be delivered. For this reason, rAAV2/2 has been used extensively in the current ongoing LCA trials, where *RPE65* re-expression is desirable in the RPE. In contrast, expression of a photoreceptor specific gene, such as GUCY2D for the treatment of LCA type 1, might be best achieved with a rAAV2/8 vector, which efficiently targets all photoreceptor types. One minor confounding factor affecting vector targeting is the method of virus preparation, particularly the presence of surfactants or osmotic diuretic agents (e.g. mannitol) which have been demonstrated to affect tropism (Ayuso et al., 2010; Mancuso et al., 2009) (Table 2).

7.2.2. Promoter usage

The use of alternative capsid serotypes gives spatial control at the tissue level; however for many clinical applications it is likely that tropism may need to be restricted further to specific cell types. This may be of particular importance where ectopic transgene expression might be detrimental. In the retina, promoters driving expression of genes required for opsin function are particularly useful for targeting photoreceptors, with *rhodopsin kinase* promoters used extensively to target both rod and cone photoreceptors while eliminating off-target RPE expression (Boye et al., 2010; Flannery et al., 1997; Sun et al., 2010; Zou et al., 2011). Cone-specific targeting can be achieved through use of the promoter driving expression of *arrestin-3*, a negative modulator of signal transduction that is active in red-, green- and blue-sensitive cone classes, and is a promising candidate for regulating therapeutic gene expression in complete achromatopsia (Carvalho et al., 2011b; Sakuma et al., 1996). Specific cone classes might also be targeted through the use of opsin promoters, either medium/long wavelength (Mancuso et al., 2009) or short wavelength (Michalakis et al., 2010). The former might be applied for the treatment of red–green colour blindness, or potentially blue-cone monochromatism, though the promoter would likely require extensive transcriptional regulatory elements (locus control region) to direct expression.

Whilst allowing greater control, cell specific promoters often give lower levels of expression than ubiquitous promoters, especially in stressed cells, as would be observed in clinical presentations of retinal disease (MacLaren, 2009). In the RPE, the cytomegalovirus (CMV) promoter is estimated to express ten-fold as much protein compared with the RPE65 promoter, which is down regulated as a response to cellular stress (Rolling et al., 2006). For packaging into AAV, the CMV promoter is ideal because of its small size (~700 bp); however, it can undergo methylation silencing in the long term, which limits its use *in vivo* (Al-Dosari et al., 2006). This has not been observed in the CBA promoter, with sustained transgene expression seen over a number of years in RPE65 deficient dogs (Bennicelli et al., 2008). However, lower levels of gene expression from cell-specific promoters can be addressed, for instance, through introduction of a synthetic intron in the expression cassette, which whilst reducing the effective transgene capacity, has been shown to increase gene expression in neuronal cells up to six hundred fold (Choi et al., 1991).

Table 2
Relative ability of well characterized AAV serotypes to transduce different ocular cells following administration A) subretinally, or B) intravitreally in mice. Transduction of the choroid, inner retinal neurons and Müller glia are not included due to a lack of available information regarding tropsism. – = no transduction; * = limited transduction; ** = moderate transduction; *** = high transduction.

Genome	Capsid	Notation	RPE	Photoreceptors	Ganglion cells
A) Subretinal administration					
AAV2	AAV1	rAAV2/1	+++	–	–
AAV2	AAV2	rAAV2/2	++	+	–
AAV2	AAV4	rAAV2/4	++	–	–
AAV2	AAV5	rAAV2/5	+	++	–
AAV2	AAV8	rAAV2/8	+	+++	–
AAV2	AAV9	rAAV2/9	+	+++	–
B) Intravitreal administration					
AAV2	AAV1	rAAV2/1	–	–	–
AAV2	AAV2	rAAV2/2	–	–	++
AAV2	AAV4	rAAV2/4	–	–	–
AAV2	AAV5	rAAV2/5	–	–	–
AAV2	AAV8	rAAV2/8	–	–	–
AAV2	AAV9	rAAV2/9	–	–	–

7.2.3. MicroRNA

An alternative to the use of cell specific promoters for restricting transgene expression is the exploitation of microRNAs, naturally occurring non-coding RNAs which bind to defined sequences within the 3′UTRs of mammalian mRNAs, resulting in translational suppression or silencing (Huntzinger and Izaurralde, 2011). The number of miRNAs coded for in the human genome has been estimated to be between 800 and 1000 (Bentwich et al., 2005). MicroRNAs are specific to the transcript against which they are raised and their expression profiles are consequently cell specific (Landgraf et al., 2007). Inclusion of miR-124 or miR-204 microRNA binding sites in the transgene cassette has been shown efficiently to silence expression occurring ectopic to RPE or photoreceptors, respectively, when administered by subretinal injection of rAAV2/5 (Karali et al., 2011). Conceptually, the small size (20–25 nt) of microRNA-binding sites makes their inclusion in AAV vectors an attractive method of spatially restricting transgene expression. However, suppression is rarely absolute, resulting in variable off-target expressivity, and their inclusion in tandem arrays often negates the advantage of their small size, especially when compared to promoters such as *rhodopsin kinase*, which is highly specific for photoreceptors in addition to being only ~200 bp in length.

Spatial control of transgene expression is therefore a complex balance between cellular specificity and efficiency of expression, and depends greatly on the therapeutic set up. Recent studies have shown that the best results are achieved when serotype selection and promoter choice are optimal, as demonstrated by the superior expression of RPE65 when using the human RPE65 promoter packaged in serotype rAAV2/4 (RPE specific tropism) than rAAV2/2 (RPE-photoreceptor tropism) (Le Meur et al., 2007).

7.3. Temporal regulation of gene expression

Temporal regulation, the ability to switch expression of a therapeutic gene 'on' or 'off', is highly desirable, allowing maintenance of appropriate dosing, or cessation of treatment if toxicity or harmful side effects are observed. In vivo gene regulation exploits inducible promoters which are activated or suppressed in the presence of a pharmacological agent, typically an antibiotic (tetracycline/doxycycline) or antimycotic (rapamycin) (Gossen and Bujard, 1992; Serguera et al., 1999; Ye et al., 1999), though other agonists exist (e.g. mifepristone or ecdysone) (No et al., 1996; Rivera et al., 1996).

7.3.1. Tetracycline-inducible system

The tetracycline system has its origin in the Tn10 *E. coli* tetracycline (Tet) resistance operon, which contains a Tet repressor protein (TetR) and a Tet responsive element (TetO). In Tn10 and similar *E. coli*, the absence of tetracycline allows TetR binding to TetO, which is located in the control region of the tetracycline resistance gene, suppressing expression. TetR has a high affinity for tetracycline and is bound in the presence of sub-lethal antibiotic levels, releasing it from TetO which activates expression of the resistance gene (Wissmann et al., 1986). TetR can be modified through fusion to the VP16 Herpes simplex virus transactivator,

allowing the production of systems which are either 'Tet-off' or 'Tet-on'. In Tet-off systems, the TetR-VP16 fusion protein (tTA) binds TetO in the absence of tetracycline, or its derivative doxycycline, activating expression. In Tet-on systems, the fusion protein is modified through amino acid substitutions to the TetR domain (rtTA), so that it binds to TetO only when conjugated to a molecule of tetracycline or doxycycline. Consequently, gene expression in Tet-on systems is activated by the presence of the appropriate antibiotic, whilst in Tet-off systems, gene expression is suppressed by their presence (Stieger et al., 2009).

7.3.2. Rapamycin-inducable system

In 2002, Auricchio et al developed a novel recombinant transcriptional activator (TF1Nc) sensitive to rapamycin (Auricchio et al., 2002). In the presence of rapamycin, TF1Nc binds to ZFHD1 domains upstream of a minimal IL-2 promoter, resulting in activation and gene expression. In this respect, the rapamycin system in analogous to that of Tet-on in that the presence of the substrate results in gene expression.

For gene therapy applications the therapeutic transgene is put under the control of the inducible promoter. Inducible gene expression technologies are particularly attractive for the treatment of neovascular AMD, where expression of a VEGF antagonist could be placed under the control of a Tet-on system and expression activated through periodic courses of doxycycline. A Tet-on or analogous rapamycin system would be generally preferable to Tet-off, as expression can be switched on through peripheral administration (oral) of the agonist, and so in the event of an adverse reaction or toxic side effect, gene expression can be stopped simply through drug discontinuation. Tetracycline and doxycycline both have well established safety profiles and are well tolerated, so repeated administration is unlikely to be problematic (Folliot et al., 2003; Stieger et al., 2006). In contrast, rapamycin has potent immunosuppressive activity, inhibiting cell cycle through blocking of the mTOR pathway, and so repetitive or continuous use might be contraindicated. However, the use of a non-immunosuppressive rapamycin analogue might be an alternative, though lower levels of gene expression and differences in tissue uptake have been described (Lebherz et al., 2005).

8. Alternative viral vectors

8.1. Lentivirus

Wild-type lentiviruses (family: *Retroviridae*) are large (~120 nm) integrating pathogenic viruses with a complex genome and virion structure. Mature virions consist of a conical or rod shape capsid packaging multiple copies of a single stranded positive sense RNA genome which undergoes reverse transcription (Baltimore class VI), surrounded by a matrix and host-derived lipid envelope.

8.1.1. Genome and packaging

Vectors are typically derived from primate lentiviruses, such as human immunodeficiency virus type 1 (HIV-1). Lentivector genomes consist minimally of a 5′ long terminal repeat (LTR), packaging signal (ψ), *Rev* response element (RRE), promoter, transgene and 3′ LTR. A poly-a signal is not included in the transgene cassette as this would lead to truncation of the RNA genome during replication. Instead, mRNA expressed from the transgene is poly-adenylated by a native signal located in the 3′ LTR, which is not active during replication or vector production (Freed and Martin, 1996). The deletion of the majority of viral sequence gives lentivectors a significant advantage of AAV vectors in terms of coding capacity. However, the future refinement of large-gene packaging technologies in AAV may allow the delivery of similar size transgenes in both AAV and lentiviral vectors (e.g. Allocca et al., 2008).

8.1.2. Integration (versus AAV)

Lentivectors are able to transduce dividing and non-dividing cells, leading to sustained expression from the integrated provirus. However, integration is a property that is less critical for ocular gene transfer, where target cells are post mitotic and consequently the therapeutic gene is not subject to dilution through cell division. Indeed, the tendency for lentiviruses to integrate could be considered detrimental in non-dividing cells, where integration increases unnecessarily the risk of cell mutagenesis.

Wild-type AAV viruses are also integrating, though do so preferentially at the AAVS1 locus (c19q13.3), rather than randomly (Kotin et al., 1992). Deletion of AAV *Rep* during vector construction significantly reduces levels of integration, with greater than 99.5% of vector DNA remaining non-integrated (episomal) following transduction with rAAV (Schnepp et al., 2003). In the absence of *Rep* AAV integrates randomly, though typically at existing DNA breaks, and transduction with rAAV has not been linked to increased incidence of mutation in human cells (Miller et al., 2004). However, there is some evidence to suggest that *Rep* protein may be present in rAAV preparations, either as a result of contamination with wild-type AAV particles (Wu et al., 1998) or from carryover of *Rep* protein bound directly to the capsid during preparation (Kube et al., 1997). Nevertheless, integration of rAAV vectors remains a rare event and consequently the risk of insertional mutagenesis is very low.

Conversely, other members of the *Retroviridae* family have already been implicated in causing insertional mutagenesis (Hacein-Bey-Abina et al., 2008; Howe et al., 2008). Whilst lentiviruses do not preferentially target CpG islands or 5′ flanking regions, integration of the provirus is predominantly the default mechanism of genome maintenance, and so the risk of mutagenesis persists (Mitchell et al., 2004). However, the discovery of retroviral episomes lead to the advent of integration deficient lentiviruses (IDLVs) which lack protein mediated integration through mutation of the integrase (Gianni et al., 1975). IDLVs have been demonstrated to efficiently target the RPE, though there have been too few studies to determine the rate of residual integration, and whether it is lower than with rAAV (Fabes et al., 2006; Wanisch and Yanez-Munoz, 2009; Yanez-Munoz et al., 2006).

8.1.3. Immunogenicity

The eye is to a certain extent immune privileged. A well documented example is that of anterior chamber associated immune deviation (ACAID), a form of immune tolerance where within the anterior chamber antigen presenting cells (APCs) act to sequester antigens, rather than present them to the central immune system, thus down regulating the Th1 humoral response (Kosiewicz et al., 1998). In spite of such mechanisms, however, lentivectors are still commonly immunoreactive, where the virus membrane is derived from human cell lines during virus packaging, and thus easily recognized as foreign. This is particularly acute in response to subretinal administration, which results in induction of strong humoral Th2 responses including induction of IgG1 and IgG2b antibodies, in addition to IL-4, IL-10 and TGF-β, possibly mediated through the RPE acting as APCs (Bennett, 2003). AAV by contrast has only weak immunogenicity, though does provoke IgG1 and IgG2b antibodies following intraocular injection (Bennett et al., 2000; Dudus et al., 1999).

8.1.4. Tropism

Lentiviruses have the ability to be pseudotyped, a process where the native surface glycoproteins (gp41 and gp120), are replaced

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with the transmembrane receptor(s) of another enveloped virus. The most common pseudotype utilizes the glycoproteins of vesicular-stomatitis virus (VSVg), a zoonotic virus (family *Rhabdoviridae*) with broad cellular tropism. In the eye VSVg pseudotyped lentivectors have been shown to transduce RPE effectively (Balaggan et al., 2006a; Bemelmans et al., 2006). In contrast to AAV, there is little evidence so far of any lentiviral pseudotype that consistently targets photoreceptors. In spite of concerns involving insertional mutagenesis and immunogenicity, lentiviruses have been used in several pre-clinical animal studies for retinal disease, particularly for the treatment of NV-AMD and RPE65 LCA.

8.2. Adenovirus

Adenoviruses (Family: *Adenoviridae*) are large, non-enveloped viruses with complex icosahedral capsid packaging a linear double stranded DNA (Baltimore class I) of up to 45 kb. For an excellent review of Adenoviral structure and genome organization see (Berk, 2006). Adenoviral vectors used for ocular gene transfer are typically E1 or E1/E3 deleted, making them replication deficient and freeing up to 36 kb of coding space for insertion of the transgene cassette.

8.2.1. Tropism

Subretinal injection of adenovirus type 5 (Ad5) results in high level RPE transduction, and to a lesser extent rod and cone photoreceptors. Gene transfer into photoreceptors is dependent on age, with transduction levels shown to be greater when administered in younger mice. Interestingly, photoreceptor transduction with Ad5 was also more successful in degenerate retinas (Bennett et al., 1994; Li et al., 1994). Recombinant adenoviruses can be produced, also through pseudotyping, by substitution of capsid fibre receptors which mediate cellular binding. Ad5/F35 (Ad5 capsid with fibres of Ad35), Ad37 and Ad5ΔRGD (Ad5 capsid with deletion in the penton base) have all been shown to target photoreceptors with greater efficiency than Ad5 (Sweigard et al., 2010).

8.2.2. Immunogenicity

Adenoviruses are *highly* immunogenic and can result in severe reactions, particularly where an individual has prior adenoviral exposure, as typified by the onset of fatal systemic inflammatory response syndrome (SIRS) in an ornithine transcarbamylase deficient patient following intra-arterial adenoviral vector administration (Raper et al., 2003). However, due to ocular immune deviation the humoral responses to adenoviral administration to the eye are predominantly Th2 derived, resulting in high levels of IgG2b and IgG3, compared to systemic responses which are predominantly Th1 based (Bennett, 2003). Subretinal adenovirus administration also results in activation of RPE, retinal microglia or choroidal dendritic cells as antigen presenting cells, and infiltration of macrophages.

Adenoviruses have significant advantages in coding capacity compared to either AAV or lentivirus vectors, and are able to transduce photoreceptors efficiently. Consequently, adenovirus could be utilized for the delivery of large genes such as CDH23 (10 kb) or GRP98 (18.9 kb). However, transgene expression can be transient as a result of immunogenicity (Hoffman et al., 1997; Reichel et al., 1998).

9. Non-viral

Therapeutic gene delivery with viral vectors presents issues with regard to both packaging capacity, which can be limiting as in the case of AAV, and immunogenicity, which can lead to adverse patient outcomes. Non-viral delivery of exogenous nucleotides, normally DNA, is an alternative approach which, in theory at least, allows the delivery of large nucleotide fragments in a form unlikely to trigger an immunogenic reaction.

9.1. Challenges of non-viral gene delivery

To be expressed, exogenous nucleotides have to negotiate a daunting journey from the point of delivery to the nucleus, avoiding along the way extracellular degradation and immune response, cytoplasmic enzymatic breakdown, and the crossing of the cell membrane and nuclear envelope, which are not permeable outside of cell division. In general the efficiency of non-viral gene transfer to post-mitotic cells is substantially lower than virally mediated delivery. This is particularly true for delivery to the eye, where physical barriers, such as the vitreous, inner/outer limiting membranes and the inter-photoreceptor matrix significantly restrict cellular access. Additionally, high concentrations of glycosaminoglycans present throughout the eye, particularly in the vitreous, sequester DNA leading to aggregation and impeding cellular uptake further. Non-viral transfer is typically facilitated by either chemical or physical means. To be clinically applicable, any approach must be minimally invasive, with administration not resulting in significant damage to retinal structure of disruption of cellular function (Charbel Issa and Maclaren, 2012).

9.1.1. Physically-mediated delivery

Physical means of mediating nucleotide delivery typically focus on the use of an electrical stimulus to temporarily permeablize the cell membrane, facilitating DNA transport across the lipid leaflet. Electroporation and ionoporation are most common, where the former utilizes short pulses of high intensity electric field to destabilize the plasma membrane, and the later employs higher current to facilitate increased tissue penetration of charged molecules (Bejjani et al., 2007). Whilst seemingly efficacious in newborn animals, electroporation is not an effective method of delivering genetic material to the neurosensory retina in adults. Electroporation results in limited RPE targeting, though far less efficiently than any viral means (Johnson et al., 2008; Kachi et al., 2005). Ionoporation has the potential to allow control of nucleotide movement in the direction of current, and may facilitate transscleral gene delivery (Andrieu-Soler et al., 2006; Souied et al., 2008). However, ionoporation, like electroporation, has so far only been effective in the retina of newborn mice, and so presently does not represent a clinically viable option for the delivery of therapeutic genes to the adult neurosensory retina or RPE.

9.1.2. Chemically-mediated delivery

Chemical mediation involves the conjugation of DNA to either a nanoparticle or a cationic polymer to facilitate membrane transport. Cationic polymers, such as polyethyleneimine (PEI), are commonly used for plasmid transfection of cells *in vitro*, though appears to have limited applications *in vivo* due to high levels of toxicity, especially when injected subretinally (authors own observations). Nanoparticles are a significantly more promising prospect for ocular gene delivery. Nanoparticles are formed of a DNA strand compacted with polyethylene glycol (PEG)-substituted 30-mer lysine peptides (CK30PEG) which facilitates membrane transport (Charbel Issa and Maclaren, 2012; Liu et al., 2003). DNA nanoparticles encoding *peripherin-2*, mutations of which cause autosomal dominant macular dystrophy or retinitis pigmentosa, partially rescue the disease phenotype of the *rds* mouse model when delivered subretinally (Cai et al., 2010, 2009).

Whilst the majority of non-viral delivery technology is not suitable for clinical gene delivery due to either inefficiency or

toxicity, gene transfer with DNA conjugated nanoparticles remains promising, and has been the only non-viral delivery strategy to be applied successfully to a model of retinal disease to date.

10. Choice of therapeutic approach

10.1. Autosomal recessive and X-linked disease

As discussed in previous sections, treatment of autosomal recessive and X-linked conditions is typically approached through gene replacement therapy. In most cases recessive and X-linked mutations (with the exception of RPGR ORF15) cause an absence of protein, or production of functionally null protein, and consequently the expression of wild-type protein is likely to significantly ameliorate the disease phenotype. Recessive genotypes where gene replacement would be appropriate include, but is certainly not limited to, CNGA3$^{-/-}$ and CNGB3$^{-/-}$ (achromatopsia), GUCY2D$^{-/-}$, CEP290$^{-/-}$ and RPE65$^{-/-}$ (LCA), MYO7A$^{-/-}$ (Usher 1B) and ABCA4$^{-/-}$ (Stargardt's). X-linked disease includes CHM$^{x/-}$ (Choroideremia), and RP2$^{x/-}$ (X-linked RP). However, some recessive disorders will remain at least in part untreatable if they lead to profound developmental abnormalities, e.g. NR2E3 (no rod development), or CRB1 (no formation of the outer limiting membrane), whilst RPGR X-linked mutations may be less responsive to gene replacement if the ORF15 variants have a gain-of-function (Hong et al., 2004).

10.2. Dominant and dominant negative disease

Dominant mutations pose certain challenges to treatment with gene therapy, not least in the case of dominant negative mutations, where gain-of-function results in interference of wild-type protein processing. One example is rhodopsin S137F mutations where aberrant protein impacts on the post-translational processing of opsin produced by the wild-type allele (Kurada et al., 1998; Rajan and Kopito, 2005; Saliba et al., 2002). Several therapeutic strategies exist for treatment of dominant disease, including neuroprotection, inhibition of apoptosis and silencing of the dominant gene.

10.2.1. Neuroprotection

Neuroprotection focuses not on the correction of a specific deficit in gene function, but on prolonging the lifespan of cells in spite of their genetic abnormality, and is the simplest of the three approaches. This is typically accomplished through the expression of naturally occurring low molecular weight 'growth factors', which when present at high concentration elicit a neuroprotective effect. A distinct advantage of this approach is that such factors are highly diffusible, and so the therapeutic gene can be delivered to any tissue, typically the RPE or ganglion cell layer for ease, from where the factor is secreted resulting in a paracrine effect. Several neurotrophic factors have been demonstrated to have efficacy in the treatment dominant RP arising from rhodopsin mutations, including ciliary neurotrophic factor (CNTF) (Liang et al., 2001), brain-derived neurotrophic factor (BDNF) (Okoye et al., 2003) and glial-cell derived neurotrophic factor (GDNF) (McGee Sanftner et al., 2001). The neuroprotective approaches utilized in these studies were dependent on mutation class (all class-I), which implies that neuroprotection is a strategy that is unlikely to be successful in many dominant conditions depending on the nature of the disease mechanism. However, neuroprotective approaches have also been used successfully in models of recessive retinal disease both *in vitro* and *in vivo*, and present one of the only approaches for the treatment of inherited retinal disease which do not have identified genes (Buch et al., 2006; Lipinski et al., 2011; Schlichtenbrede et al., 2003). One attractive candidate for the treatment of non-specific retinal degeneration is erythropoietin (EPO), a cytokine requires for red-blood cell production (erythropoiesis), which has been shown to have neuroprotective properties in several models of retinal disease (Grimm et al., 2005, 2006; Rex et al., 2004; Sullivan et al., 2011).

10.2.2. Inhibition of apoptosis

Inhibition of apoptosis follows a similar approach as neuroprotection, in that gene defects are not corrected, but the effect they have on the cell are instead counteracted. The functional mechanism of anti-apoptotic proteins require that they are expressed intracellularly and so, in contrast to neuroprotection, the therapeutic gene must be delivered directly to the mutation harbouring cell.

X-linked inhibitor of apoptosis (XIAP) has shown considerable promise in attenuating the degeneration of photoreceptors in models of ischaemic injury and in models of retinitis pigmentosa (Chen et al., 1996; Leonard et al., 2007; Renwick et al., 2006; Shan et al., 2011; Zadro-Lamoureux et al., 2009). Similarly, delivery of BiP/Grp78, a molecular chaperone, has proved effective in suppression of apoptosis in missense mutations where activation of the unfolded protein response (UPR) is triggered. UPR activation occurs when the accumulation of incorrectly folded protein is detected by a transmembrane kinase (IRE1) present in the endoplasmic reticulum (ER) (Shamu, 1997). Subsequently, translational attenuation is triggered by phosphorylation of the α-subunit of elF2 resulting in the non-specific down regulation of translation, and ultimately to apoptosis. Expression of Bip/Grp78 delivered subretinally in rAAV resulted in photoreceptor preservation in animals with dominant P23H rhodopsin mutations (Gorbatyuk et al., 2010). Whilst anti-apoptotic factors are promising, there are concerns relating to the potential for oncogenesis, where control of apoptosis is deregulated in many cancers and malignancies.

10.2.3. Gene silencing

Rather than protecting the cell from death through expression of external factors, it may be feasible to directly alter expression of the dominant allele through the delivery of regulatory nucleotide sequences to permanently silence aberrant gene expression. The principle of gene silencing to reduce levels of mutant mRNA in the eye has previously been demonstrated through expression of ribozymes. Ribozymes are catalytic RNA molecules with developed tertiary structures which allow them to bind and directly cleave complementary mRNA sequences, and have been shown to significantly reduce expression of mutant P23H rhodopsin protein when expressed from rAAV vectors (Drenser et al., 1998; Gorbatyuk et al., 2007; Hauswirth et al., 2000; LaVail et al., 2000; Lewin et al., 1998). The specificity of ribozymes for cleaving mRNA produced by the mutant allele only is both their strength and weakness. This specificity prevents down regulation of mRNA from the wild-type allele, but does require that a new ribozyme is engineered for every dominant point mutation, of which over 120 are described for rhodopsin alone, making widespread application of ribozymes for the treatment of retinal disease more challenging. Specifically, it is likely that each ribozyme would have to undergo a separate clinical trial – a lengthy process even if granted orphan drug status, or similar.

An alternative to the use of ribozymes is RNA interference (RNAi). RNAi typically involves the direct delivery or expression of short single stranded RNA molecules, which have sequence homology to the mRNA strand that they are designed to silence, and are able to form hairpin loops. The resulting short hairpin RNA (shRNA) molecules are processed by DICER, a protein which cleaves the stem loop leaving a short double stranded RNA molecule (dsRNA) of approximately 21 nt in length. The dsRNA molecule is

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subsequently transferred to an argonaute protein, the RNA duplex is unwound and positive sense strand is degraded; the negative sense RNA strand is retained bound to argonaute, forming the RNA-induced silencing complex (RISC). The negative sense strand directs RISC binding to specific mRNA transcripts through sequence homology, and once bound, RISC catalyses the cleavage of the bound mRNA, triggering mRNA degradation. RNAi effectively prevents protein production by silencing at the transcriptional level, and has been used to silence expression of the dominant P23H rhodopsin allele *in vivo*. Additionally, RNAi has been used to silence expression of aberrant peripherin protein *in vitro* (Chadderton et al., 2009; O'Reilly et al., 2007; Palfi et al., 2006; Tam et al., 2008) and *in vivo* (Georgiadis et al., 2010). In contrast to ribozymes, which are engineered to have their own catalytic activity, RNAi relies on the catalytic activity of host enzymes, and is therefore considerably easier to implement, requiring only the design of an RNA molecule complementary to the target sequence. Consequently, RNAi represents an encouraging option for the treatment of dominant and dominant negative mutations involved in retinal disease, where elimination or reduction of mutant protein production would likely increase survival of the host cell. However, RNAi techniques often result in targeting of both wild type and mutant mRNA, especially where sequence differences between alleles are small, and so may result in the down regulation of any functional wild-type protein production.

10.3. Complex disease

In complex disease, where no genes show involvement in a significant proportion of disease, one approach is the expression of compounds which treat the most damaging symptoms of disease. In NV-AMD this approach has been demonstrated with the repeated (monthly) injection of either bevacizumab (Avastin) or Ranibizumab (Lucentis) to prevent angiogenesis. Long term expression via gene therapy of anti-angiogenic compounds represents an attractive treatment option for NV-AMD, leading to an improvement in patient care (less invasive), and a reduction of cost, particularly in relation to repetitive injections of Lucentis. Several candidate molecules have been explored, the most promising of which are summarised below.

10.3.1. PEDF and sFlt-1

Pigment epithelium-derived factor (PEDF) is a serine protease inhibitor (member of the SERPIN family) which has been demonstrated to increase survival of cultured neurons and protect photoreceptors in models of light damage and oxidative stress (Bilak et al., 1999; Cao et al., 1999, 2001; Steele et al., 1993). PEDF is naturally produced by foetal and adult RPE and secreted apically into the interphotoreceptor matrix (IPM) where it likely acts as a VEGF antagonist and promotes photoreceptor survival (Campochiaro, 2011; Dawson et al., 1999; Maminishkis et al., 2006; Tombran-Tink et al., 1995). The anti-angiogenic activity of PEDF likely arises through binding of glycosaminoglycans in the IPM, such as heparin sulphate, chondroitin sulphate and dermatin sulphate, which are known to be distributed throughout the retina and choroid (Clark et al., 2011). sFlt-1, the soluble extracellular domain of the VEGF receptor-1 (VEGFR-1), is a naturally occurring alternative splice variant of the full length membrane bound VEGFR-1 (He et al., 1999; Kendall et al., 1996). As a natural receptor of VEGF, sFlt-1 binds with high affinity and may act to sequester VEGF.

10.3.2. Angiostatin and endostatin

Endostatin is a c-terminal fragment of collagen type XVIII that exhibits anti-tumorigenic properties through inhibition of angiogenesis (O'Reilly et al., 1994a; O'Reilly et al., 1994b). Angiostatin is derived from cleavage of plasminogen and pro-angiogenic plasmin, and is similarly anti-angiogenic (Campochiaro, 2011; O'Reilly et al., 1997). The mechanism by which angiostatin and endostatin inhibit formation of new vessels is poorly understood. However, these molecules have been shown to limit the development of CNV in the mouse laser model (Balaggan et al., 2006b) when delivered by EIAV lentivirus and a commercial clinical trial led by Oxford Biomedica to test the effects of this vector in patients has recently started (NCT01301443).

Due to their potent anti-angiogenic properties, PEDF, sFlt-1 or soluble statins are promising candidates to prevent neovascularisation in NV-AMD. However, our knowledge of angiogenesis homeostasis and subsequent deregulation is incomplete. In particular, toxicity leading to rapid retinal degeneration has been observed following administration of sFlt-1, which cautions against excessive suppression of endogenous VEGF through over expression of antagonists (Nishijima et al., 2007; Saint-Geniez et al., 2008). The application of inducible promoters, particularly the Tet-on system, is attractive for the long term treatment of NV-AMD, where toxic effects resulting from constitutive expression might be negated by temporally controlling transgene expression through peripheral drug delivery, e.g. short monthly courses of doxycycline to induce expression of an anti-angiogenic factor.

11. Animal models of retinal disease and pre-clinical studies

There are now many natural and experimentally-engineered animal models that enable the assessment of genetic and environmental factors on visual defects. However, species differences must be accounted for in vector design and this is becoming increasingly important as research moves into the clinical phase. Of particular note is that non-primate mammals, specifically laboratory rodents, do not possess a macula. Caution must therefore be applied when extrapolating the results from mice. However, many excellent animal models exist and have played a pivotal role in the development of clinically applicable gene therapy treatments for retinal disease.

11.1. Achromatopsia

Achromatopsia has several well established naturally occurring or transgenic animal models on which to evaluate therapeutics, including *Gnat2*, *Cnga3* and *Cngb3* deficient mouse strains, all of which have absent ERG responses. Recent studies have demonstrated that re-expression of murine *Cnga3* protein using a rAAV vector leads to restoration of a recordable light-adapted ERG which was maintained for at least 2 months (Michalakis et al., 2010; Pang et al., 2009, 2010b). *Gnat2*$^{-/-}$ (*cpfl3*) mice have a recessive mutation affecting exon 6 of the cone α-transducin subunit, which results in progressive loss of light-adapted ERG response over 9 months and secondary loss of rod function (Chang et al., 2006a). Re-expression of murine Gnat2 using a minimal human red–green opsin promoter delivered by rAAV2/5 vector leads to restoration of visual acuity and correction of cone-mediated ERG phenotype (Alexander et al., 2007). The effect of rescue was significantly more pronounced in the Gnat2 model of achromatopsia, with ERG responses reaching almost wild-type levels, in contrast to treatment of the Cnga3 model which resulted in only 30% ERG recovery. This may indicate that some forms of achromatopsia in the mouse may be more amenable to gene replacements than others.

For the modelling of CNGB3 achromatopsia, which accounts for approximately half of all human disease, there is both a murine and a canine model. The former occurs as a result of targeted deletion within the CNGB3 gene resulting in progressive loss of ERG

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response and cone photoreceptor degeneration. The canine model is due to a naturally occurring recessive mutation in the Alaskan Malamute breed which results in day-blindness and an absence of retinal cone function. The mouse model has been effectively treated through expression of human CNGB3 protein, resulting in a restoration of ERG to 90% of wild-type levels and preservation of cone photoreceptors (Carvalho et al., 2011b). However, expression of human CNGB3 protein in the canine model failed to restore ERG responses significantly, likely reflecting the relatively small area of retina transduced compared to the mouse model (Komaromy et al., 2010). In humans one might expect more promising results from CNGB3 gene therapy than in the canine model, as the main target in primates would likely be the fovea, and so transduction of this cone-rich area could restore central vision and greatly improve vision despite a small area of transduction.

Whilst these studies give great promise for the future clinical treatment of achromatopsia, one issue remains that the largely stationary nature of achromatopsia in humans, and patients' reliance on rod mediated vision, increases the threshold for intervention substantially in comparison to rapidly progressive degenerative diseases. In particular, surgical detachment of the retina, even if largely confined to the macula, would have detrimental short and potentially long term affects on remaining rod function. One solution to this problem would be the use of capsid mutant AAV vectors to target cone photoreceptor by intravitreal injection.

11.2. Choroideremia

In mice knockout of the REP gene typically causes embryonic lethality in males due to abnormally development of extra-embryonic membranes such as the yolk-sac and placenta. This occurs in all affected males and in females where the mutated allele is inherited maternally, likely due to preferential X-inactivation the paternal allele in such tissues (van den Hurk et al., 1997). Female carriers inheriting the null X-allele paternally are viable and develop retinal degeneration (Tolmachova et al., 2010). An increase in prenylation activity has been observed in the RPE of $CHM^{null/WT}$ females following subretinal administration of CHM/REP1 cDNA using a lentiviral vector, indicating that expression of CHM may serve to reduce the presence of unprenylated Rabs and slow degeneration (Tolmachova et al., 2012).

11.3. Stargardt's disease

ABCA4 knockout mice exhibit delayed dark adaptation, increased all-trans retinal levels following light exposure, and accumulation of A2E in the RPE contributing to increased fundus autofluorescence (Charbel Issa et al., 2012; Weng et al., 1999). However, $ABCA4^{-/-}$ mice do not exhibit a significant ERG phenotype, evidence of photoreceptor degeneration or atrophy is absent, and fleck like deposits are not observed on examination of the fundus. Consequently, the ABCA4 knockout mouse is a poor model of the human disease phenotype. Nevertheless, though A2E accumulation in the mouse appears not to result in RPE cell death or photoreceptor degeneration as is observed in humans, any therapy would invariably aim to reduce A2E levels, and one could use those levels as an outcome measure. Expression of human ABCA4 protein has been claimed to result in lowered levels of A2E following expression from a lentiviral vector (Kong et al., 2008). However, the outcome of study remains troubling due to the low levels of reported photoreceptor transduction ($\sim$5%) and the significant effect of a mock virus (not containing ABCA4 transgene), indicating a sham effect related to surgical intervention rather than to transgene expression. ABCA4 expression using rAAV vectors have shown to result in a reduction in the number of lipofuscin granules accumulated in the RPE, where A2E is a major component of such inclusions (Allocca et al., 2008). The lack of appropriate animal model in conjunction with the phenotypic heterogeneity of the disease and difficultly of delivering an ABCA4 transgene to the photoreceptors makes Stargardt's a difficult prospect for treatment with gene therapy at the present. In spite of this a commercial company has developed a lentivirus vector which was recently given orphan drug status to deliver hABCA4 for the treatment of Stargardt's, although it would need to target ABCA4 to photoreceptors and without a significant inflammatory response in order to be clinically effective.

11.4. Age related macular degeneration (AMD)

As a complex genetic disease there are no reliable transgenic models of NV-AMD. However, the most damaging acute complication, choroidal neovascularisation (CNV), can be modelled in animals through the delivery of a laser insult which disrupts Bruch's membrane and overlying RPE, triggering angiogenesis from the choriocapillaris into the subretinal space (Francois et al., 1975). Laser induced CNV has been used in rodents and has been optimized in several species of primate including *Macaca mulatta* (Rhesus macaque), *Macaca fascicularis* (Cynomolgus monkey) and *Chlorocebus sabaeus* (African green monkey) (Ryan, 1979; Shen et al., 2004; Tolentino et al., 2000; Zhang et al., 2008). Primate models of laser induced CNV are generally preferred due to similarities in eye size and structure, and have been used to show the efficacy of the current anti-VEGF treatments (Goody et al., 2011; Husain et al., 2005; Kim et al., 2006; Krzystolik et al., 2002; Lichtlen et al., 2010).

Alternatively, ischemic insults can also be used to induce retinal neovascularization, and have been used to demonstrate that intravitreal administration of rAAV2/2 encoding sFlt-1 reduces the number of neovascular endothelial cells compared to the untreated contra-lateral eye (Bainbridge et al., 2002). Similarly, sFlt-1 has been used to treat laser-induced CNV following subretinal administration in rAAV2/8 in mice (Igarashi et al., 2010). Long term suppression of neovascularisation following subretinal sFlt-1 administration has been shown up to 17 months post rAAV-mediated delivery in primates, implicating the potential of soluble Flt-1 as a viable long-term treatment for NV-AMD (Lai et al., 2005a) – a commercial dose-escalation trial of sFlt-1 is currently underway (Genzyme, NCT01024998). PEDF over-expression has also been shown significantly to reduce CNV in both laser and ischaemia models either when constitutively expressed or delivered by AAV vector (Mori et al., 2002; Park et al., 2011; Renno et al., 2002).

Angiostatin and endostatin have both been shown to reduce vessel permeability and inhibit CNV formation, and currently a lentivirus vector expressing both has been commercialized for use in NV-AMD (Balaggan et al., 2006b; Berns et al., 2001; Lai et al., 2001) (Oxford Biomedica, NCT01301443).

11.5. Leber's congenital amaurosis

As LCA is a genetically heterogeneous disease there exist numerous models, including various rodent models in addition to avian models. One such is the GUCY2D chicken, which has a large naturally occurring deletion in exons 4–7 of the gene encoding retGC1, likely leading to the production of functionless unstable protein (Laura et al., 1996; Semple-Rowland et al., 1998). These animals have no functional ERG recording and have erratic reflexive visual responses from birth. It has been shown using lentiviral vectored retGC1 transgene that retinal morphology can be preserved, and ERG and optokinetic nystagmus responses elicited

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(Williams et al., 2006). However, due to the early onset of photoreceptor degeneration in this model, treatment must be delivered prior to hatching (*in ovo*) through injection into the neural tube. Consequently it is difficult to draw conclusions about the likely success of any translational treatment, which would certainly be delivered in a later stage of disease.

Another extensively studied model is the Royal College of Surgeon's rat, which has a recessive MERTK mutation resulting in accumulation of shed outer segment discs in the subretinal space and degeneration of the overlying photoreceptors (D'Cruz et al., 2000). While in this respect the RCS rat faithfully represents the human disease phenotype, the mechanism of disease pathology makes evaluation of the effectiveness of interventions difficult to interpret. In particular, the RCS rat is particularly susceptible to removal of debris through surgical intervention, and the observed improvements are as much likely due to the 'flushing out' of the subretinal space by the injection of vector suspension, than to any vector-related expression of MERTK. This highlights the need for accurate controls in evaluation of prospective treatments, in particular, the use of the contra-lateral eye treated with a 'sham' injection. However, it is likely that re-expression of MERTK in the RPE leads partial correction of the disease phenotype, with an increase in the number of phagosomes, clearance of subretinal debris and preservation of retinal morphology observed following viral delivery (Smith et al., 2003; Vollrath et al., 2001).

Probably the most widely studied models of retinal disease are those with mutations affecting RPE65. The Rpe65/Rd12 mouse model has RPE dysfunction resulting in degeneration of rod photoreceptors with preservation of cones, and as such accurately reflects the human disease phenotype (Pang et al., 2005; Redmond et al., 1998). Additionally, a subset of Briard dogs has a natural 4 bp deletion in the RPE65 gene which results in the introduction of a premature stop codon. Dogs have RPE inclusions and rod photoreceptor with abnormal morphology (shortened inner segments) which progressively degenerate, leading to reduction in ERG amplitude with age (Aguirre et al., 1998). The Briard dog model represents an excellent model for human RPE65-LCA as it has both a similar phenotype in addition to having eyes of similar size and structure, despite the absence of a macula. Acland et al. (2001) first demonstrated subretinal administration of RPE65 cDNA could significantly improve dark-adapted ERG and blue flicker response. Additionally, performance of visually guided tasks improved significantly to wild-type levels, reflecting that visual evoked cortical potentials (VEPs) from treated eyes, which were significantly higher than untreated eyes, were being transduced into useful visual input (Acland et al., 2001). Human and canine RPE65 expression when delivered by AAV vectors has since been shown to be consistent and long-lived, with ERG response stable for over three years (Acland et al., 2005; Bennicelli et al., 2008). However, this study demonstrated that there is an effective treatment window after which gene replacement therapy is ineffective, where dogs treated after 30 months of age did not respond. Presumably this reflects permanent morphological changes of the overlying photoreceptors, and is likely to have an analogous stage in human disease. Interestingly, use of a rAAV2/4 vector expressing the transgene from the native RPE65 promoter resulted in more rapid expression than rAAV2/2 containing a ubiquitous promoter, highlighting that optimization of both transgene cassette and delivery method is likely to result in the best clinical outcome (Le Meur et al., 2007; Rolling et al., 2006). Following demonstration of therapeutic efficacy in Briard dogs, safety studies were conducted in both Briards and non-human primates which demonstrated that there was no systemic toxicity in response to either the vector or transgene, though moderate inflammation and localized retinal thinning at high doses was observed (Jacobson et al., 2006a, 2006b). Additionally, of slight concern was a serotype independent presence of rAAV sequences in the optic nerve, brain, lymph nodes and peripheral blood mononuclear cells following intraocular administration (Jacobson et al., 2006b; Provost et al., 2005; Stieger et al., 2008). These findings reinforce the importance of proper spatial control of vector expression in any vector for clinical use where ectopic expression might be harmful. The intraocular immunoreactivity levels were also shown to be slightly increased in a small number of the dogs, but this was not specific for RPE65. Additionally they did not detect extra-ocular expression of RPE65, using RT-PCR screening of bone marrow, brain and other tissues.

These animal studies demonstrated the clinical potential of RPE65 gene transfer using AAV as a viral vector and led the way for RPE65-LCA to be the first ocular disease treated by gene therapy.

12. Conclusions and future outlook

The use of AAV derived vectors has led to significant advances in retinal gene transfer in both pre-clinical and clinical research. These advances make it increasingly conceivable that gene therapy may become a commonly used tool for the treatment of retinal disease, although it is likely to take many years before gene therapy becomes a part of mainstream medicine. A greater understanding of the mutations and mechanisms that cause visual defects is essential to the future development of effective treatments and consideration must be taken as to the possible side effects of any gene therapy treatment strategies that are developed. In cases where degeneration occurs too early or the progression is too severe it is unlikely to be feasible to restore photoreceptor function through direct gene replacement, inhibition of apoptosis or the expression of neuroprotective compounds. Therefore, it is essentially that research continues in to alternative therapies, such as photoreceptor transplantation and stem cell treatments, retinal prosthesis, or the sensitization of remaining retinal cells to light.

Conflict of interest

The authors have no conflict or commercial interest to disclose.

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