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Gene Therapy of *ABCA4*-Associated Diseases

Alberto Auricchio[1], Ivana Trapani[1], and Rando Allikmets[2]

[1]Telethon Institute of Genetics and Medicine, Naples, 80131 Italy

[2]Department of Ophthalmology, and Department of Pathology and Cell Biology, Columbia University, New York, New York 10032

Correspondence: rla22@cumc.columbia.edu

The ATP-binding cassette (ABC) transporter gene, *ABCA4 (ABCR)*, was characterized in 1997 as the causal gene for autosomal recessive Stargardt disease (STGD1). Shortly thereafter several other phenotypes were associated with mutations in *ABCA4*, which now have collectively emerged as the most frequent cause of retinal degeneration phenotypes of Mendelian inheritance. ABCA4 functions as an important transporter (or "flippase") of vitamin A derivatives in the visual cycle. Several ways to alleviate the effects of the defective ABCA4 protein, which cause accumulation of 11-*cis* and all-*trans*-retinal in photoreceptors and lipofuscin in the retinal pigment epithelium, have been proposed. Although *ABCA4* has proven to be a difficult research target, substantial progress through genetic, functional, and translational studies has allowed major advances in therapeutic applications for ABCA4-associated pathology, which should be available to patients in the (near) future. Here, we summarize the status of the gene therapy-based treatment options of *ABCA4*-associated diseases.

Somatic gene therapy has shown success in several animal models of (recessive) retinal degeneration (Campochiaro 2002; Allocca et al. 2006). Most well known have been reports that a recombinant adeno-associated virus (rAAV) carrying wild-type Rpe65 (rAAV-Rpe65) improved visual function in the Briard dog model of childhood blindness (Acland et al. 2001; Narfstrom et al. 2003; Le Meur et al. 2007). Similar successes have been reported for the $Rpe65^{-/-}$ and $Lrat^{-/-}$ mouse models with AAV and lentiviral vectors (Dejneka et al. 2004; Batten et al. 2005; Bemelmans et al. 2006; Yanez-Munoz et al. 2006).

Direct gene replacement represents an attractive therapeutic option also for treatment of all *ABCA4*-associated diseases, including Stargardt disease (STGD1) (Allikmets et al. 1997; Molday and Zhang 2010). Delivering a normally functioning gene to photoreceptors harboring mutant *ABCA4* via gene therapy should therefore be considered as a possible "cure" for *ABCA4*-associated diseases because: (1) All *ABCA4*-associated diseases are recessive, so that adding a functional gene could fully restore visual function and, (2) degeneration of the retinal cells in all *ABCA4*-associated diseases is relatively delayed, allowing a reasonable time window for therapeutic intervention.

Currently, two major approaches for the delivery of the genetic material to the eye are used: one based on viral vectors as delivery vehicles

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and the other including nonviral gene delivery methods. So far, the identification of an efficient vector for the delivery of the *ABCA4* gene to the retina has been hampered by the large size of the *ABCA4* coding sequence (6.8 kb) and by the expression of the ABCA4 gene/protein exclusively in photoreceptor (PR) cells, therefore requiring vectors with large cargo capacity and able to transduce PRs efficiently. Most delivery vehicles that accept large cargo capacity, such as lentiviruses, adenoviruses, and nonviral vectors, have been suggested not to able to efficiently transduce PRs, at least in the mouse models. At the same time, vectors with efficient PR transduction capability, for example those based on adeno-associated viruses (AAVs), have a packaging capacity limited to about 4.7 kb (Colella and Auricchio 2012; Lipinski et al. 2013) and therefore would not be able to package the large *ABCA4* coding sequence of ~7 kb. Recently, significant efforts have been directed toward both the development of AAV-based systems, which are able to deliver large genes and the identification of lentiviral and nonviral vectors with higher PR tropism. Here we will review the current status of *ABCA4* gene therapy development.

NONVIRAL GENE DELIVERY IN *ABCA4*-ASSOCIATED DISEASES

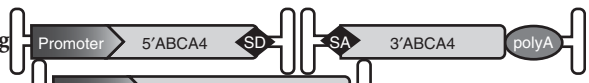
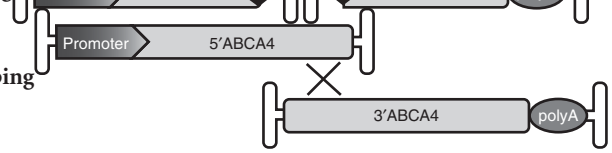
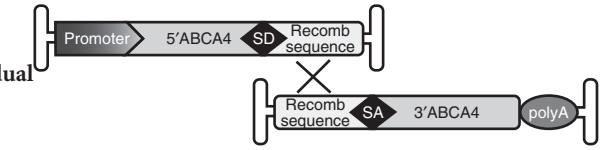
Nonviral vectors offer a number of advantages over viral-based strategies including: (1) reduced toxicity from the vector, (2) lack of an immune response against the vector and possibility to readminister the vector, (3) a large transgene capacity, and (4) simple and relatively inexpensive clinical grade production (Charbel Issa and MacLaren 2012). However, although viruses have evolved to optimize the delivery of the viral gene into the nucleus of the human host cell, "naked" DNA delivered by nonviral vectors needs to overcome several barriers to be expressed, such as: (1) extracellular degradation and immune response mediated by sensor of single stranded or double stranded DNA molecules, such as toll-like receptors, (2) cytoplasmic degradation, and (3) passage through the nuclear envelope during cell division, which is not possible in postmitotic cells, such as PRs. In addition, the presence of physical barriers in the eye, such as the vitreous, inner/outer limiting membranes, the inter-PR matrix, and the high concentrations of glycosaminoglycans present throughout the eye that sequester the DNA, further limit cellular access (Lipinski et al. 2013). As a consequence, many studies have provided evidence of the higher efficiency of viral versus nonviral retinal gene delivery (Andrieu-Soler et al. 2006). In particular, injection of naked DNA via subretinal injection between the PR and retinal pigment epithelium (RPE) (Liang et al. 2000) is highly inefficient (Charbel Issa and MacLaren 2012). Therefore, chemical or physical methods have been used to enhance the efficiency of nonviral vectors gene delivery to the outer retina. Chemical methods, such as liposomes, polymers, and compacted nanoparticles, are based on the conjugation of the DNA with synthetic or natural cationic compound that both protect DNA from nuclease-mediated degradation and allow passage through/into cell membranes via endocytosis and, in some instances, receptor-mediated uptake. Physical methods, such as electroporation or iontophoresis, typically use an electrical stimulus to temporarily permeabilize the membrane and allow the DNA to cross the cellular membranes. To date, however, evidence for effective gene delivery to outer retinal cells remains scarce and the success of the majority of the nonviral delivery technology has been limited to the RPE cell layer (Kachi et al. 2005; Johnson et al. 2008; Souied et al. 2008). However, recently, the polylysine-based compacted DNA nanoparticle (NP) CK30-NP has shown significant improvement in the efficacy of ocular gene transfer in comparison to the previous nonviral methods (Farjo et al. 2006). In the compacted nanoparticles, in particular, the DNA molecule is compacted by polyethylene glycol (PEG)-substituted 30-mer lysine peptides (CK30PEG) (Liu et al. 2003). Their minimal diameter (usually 8–20 nm) is such that allows to readily enter the nucleus (Liu et al. 2003) of dividing and nondividing cells, remaining episomal. Importantly, these particles have no theoretical DNA packaging limitations and have been successfully tested with plasmids up to 20 kb in length

(Fink et al. 2006). Notably, subretinal administration of CK30-NPs results in extensive PR transduction, even though with some unexplained patterns of transgene expression (Kumar-Singh 2008) in the absence of detectable immune responses or toxicity (Farjo et al. 2006; Ding et al. 2009). In addition, in contrast with other nonviral approaches, CK30-NPs drive long-term gene expression after subretinal delivery to the mouse eye (Conley and Naash 2010). Given the success of CK30-NPs to mediate phenotypic rescue in rodent models of retinitis pigmentosa (Cai et al. 2009, 2010) and their favorable safety and efficacy profile in a human clinical trial for cystic fibrosis (Konstan et al. 2004). Han et al. (2012) have recently tested CK30-NPs for *ABCA4* delivery in the *Abca4*$^{-/-}$ mouse model of Stargardt disease. This mouse model shows some of the clinical features associated with STGD1, such as accumulation of lipofuscin (A2E) in RPE (Weng et al. 1999; Mata et al. 2001), thickening of the RPE cells (Allocca et al. 2008; Radu et al. 2008; Conley et al. 2012), delayed recovery from light desensitization (Weng et al. 1999; Maiti et al. 2006; Allocca et al. 2008; Radu et al. 2008), and thinning of the outer nuclear (PR cell) layer in albino KO mice (Radu et al. 2008; Wu et al. 2010a). In this mouse model, after subretinal injection of the CK30-NPs carrying the human *ABCA4* gene, Han et al. (2012) detected *ABCA4* transgene expression for up to 8 months after injection and found both improved recovery of dark adaptation and reduced lipofuscin accumulation. These promising data have provided the first evidence of effective nonviral vector-mediated delivery of large genes, such as *ABCA4*, to the PR cell layer.

AAV GENE DELIVERY IN *ABCA4*-ASSOCIATED DISEASES

AAV-derived vectors are currently the most favored vehicles for therapeutic gene delivery to the retina, because they have low immunogenicity, favorable safety profile and support long-term transgene expression after a single administration (Colella and Auricchio 2012; Vandenberghe and Auricchio 2012). AAV is a small (25 nm), nonenveloped virus that packages a linear single-stranded DNA genome of ~4.7 kb. One of the strengths of the AAV vector platform is the availability of more than 100 different forms called AAV serotypes, which are isolated either as infectious or as molecular clones, and which differ in their external surface capsids proteins. Capsids can be easily exchanged among various AAVs to generate hybrid vectors that contain a genome with the same AAV inverted terminal repeats (ITRs) and the capsid from a different serotype (Auricchio 2003). AAVs obtained through this transcapsidation system are named AAV2/n, where the first number refers to the ITRs and the second to the capsid (Surace and Auricchio 2008). Different capsids confer different tropism and transduction characteristics. Initial AAV vectors were based on AAV serotype 2 (AAV2/2), the most prevalent serotype in humans. However, AAV2/2, although an excellent gene transfer vector for transducing RPE or retinal ganglion cells, is relatively inefficient at transducing other retinal cell types such as PR. Because the majority of mutations causing inherited retinal degeneration, including STGD1, occurs in genes expressed in PRs (Vandenberghe and Auricchio 2012), this concern prompted the search for AAV serotypes able to overcome this limitation. AAV2/5, 2/7, 2/8, and 2/9 vectors have all efficiently transduced PRs, in addition to RPE (Auricchio et al. 2001; Lotery et al. 2003; Allocca et al. 2007), with the AAV2/8 being the most efficient serotype in mice (Auricchio et al. 2001; Lotery et al. 2003; Allocca et al. 2007), pigs (Mussolino et al. 2011), dogs (Stieger et al. 2008), and nonhuman primates (Vandenberghe et al. 2011). The major limitation to the use of AAVs for gene replacement remains their packaging capacity, which is considered to be restricted to the size of the parental genome (4.7 kb), and thus hampers the treatment of certain forms of inherited retinal diseases caused by mutations in genes where cDNA exceeds 5 kb, such as *ABCA4*. Thus, different strategies to overcome AAV cargo limitations have been investigated. One is based on packaging of oversized genomes, that is, larger than 5 kb (Table 1) (Grieger and Samulski 2005; Allocca et al.

Table 1. Schematic representation of AAV-based strategies for large gene transduction

Approach	Mechanism for reconstitution
Oversized 	Reassembly of truncated genomes
Trans-splicing 	ITR-mediated concatemerization followed by splicing
Overlapping 	Homologous recombination
Hybrid dual 	Homologous recombination followed by splicing

SD, splicing donor signal; SA, splicing acceptor signal; polyA, poly-adenylation signal. Crosses show overlapping regions available for homologous recombination.

2008; Hirsch et al. 2010). Notably, oversized AAVs have been successfully used to express *ABCA4* in the PR of *Abca4*$^{-/-}$ mice resulting in significant and stable morphological and functional improvement of the *Abca4*$^{-/-}$ retina (Allocca et al. 2008). However, the mechanism underlying oversized AAV-mediated PR transduction remains elusive as the genomes contained in oversized AAV vectors appear highly heterogeneous in size and are predominantly shorter than expected (Dong et al. 2010; Hirsch et al. 2010; Lai et al. 2010; Wu et al. 2010b; Hirsch et al. 2013), which limits their use in clinical setting.

Alternatively, the ability of heterologous AAV genomes to form intermolecular concatemers in the nucleus of target cells (Duan et al. 1998) can be exploited to express large genes, which can be split into two halves, independently packaged in two different (dual) AAV vectors. Various dual AAVs strategies have been described (referred to as trans-splicing, overlapping, and hybrid dual-vector strategies, Table 1) and have been used to efficiently delivery large genes to different tissues. In the trans-splicing approach the 5′-half vector has a splice donor (SD) signal at the 3′ end, whereas the 3′-half vector carries a splice acceptor (SA) signal at the 5′end that allow trans-splicing of a single large mRNA molecule following head-to-tail concatemerization of the two AAVs (Yan et al. 2000). In the overlapping approach, the dual AAV genomes share overlapping sequences, thus the reconstitution of the large gene expression cassette relies on homologous recombination (Duan et al. 2001). The third dual AAV approach (hybrid dual) is a combination of the two previous approaches and it is based on the addition of a highly recombinogenic exogenous sequence to the trans-splicing vectors, to increase their recombination efficiency (Ghosh et al. 2008, 2011; Trapani et al. 2014). This recombinogenic sequence is placed downstream from the SD signal in the 5′-half vector and upstream of the SA signal in the 3′-half vector, so that, after recombination, it is spliced out from the mature RNA (Table 1). The recombinogenic sequences used so far to induce the recombination between dual AAV hybrid vectors derive from regions of either the alkaline phosphatase gene (AP) (Ghosh et al. 2008, 2011) or the F1 phage genome (AK) (Trapani et al. 2014). We

recently compared side-by-side the efficiency of the various oversized, dual AAV overlapping, trans-splicing, and hybrid strategies for AAV-mediated large gene transduction in the mouse and pig retina (Trapani et al. 2014), and found that dual AAV trans-splicing and hybrid AK vectors efficiently reconstitute the large *ABCA4* gene in mouse PRs. Notably, subretinal administration of dual AAV trans-splicing and hybrid AK vectors improved the phenotype of a mouse model of STGD1, providing evidence for the efficacy of these strategies for *ABCA4* gene therapy. Dozens of successful proofs of concept of the efficacy of AAV mediated gene therapy for recessive and dominant inherited retinal diseases have been generated in small and large animal models (Stieger et al. 2010) and have paved the way to the first clinical trials using AAV in patients with Leber congenital amaurosis, a severe form of childhood blindness (Bainbridge et al. 2008; Maguire et al. 2008, 2009; Cideciyan et al. 2009; Simonelli et al. 2010). The results from these initial trials suggest that retinal gene therapy with AAV is safe in humans, that vision can be improved in patients that have suffered from severe impairment of visual function, in some cases for decades, and that readministration of AAV to the subretinal space is feasible, effective, and safe. This, as well as the recent evidence of the efficacy of dual AAV-mediated *ABCA4* delivery in mice (Trapani et al. 2014), strongly supports further investigation using AAV for treatment of diseases caused by *ABCA4* mutation.

LENTIVIRAL GENE DELIVERY IN *ABCA4*-ASSOCIATED DISEASES

Lentiviral vectors offer several advantages for gene therapy applications in general, as well as in the specific case of *ABCA4*-associated diseases. First and foremost, lentiviruses are capable of delivering genes stably and permanently into the genome of transduced cells in vivo (Lois et al. 2002; Gouze et al. 2003; Kostic et al. 2003). Second, they can transduce nondividing cells, a crucial requirement for terminally differentiated cells such as photoreceptors (Kingsman 2003). Third, they can carry relatively large expression cassettes, which is essential in this case because the human *ABCA4* cDNA is almost 7 kb, which exceeds the capacity of the commonly used AAV vectors. However, two major problems associated with lentiviral-based gene therapy are, as suggested, the relatively low PR tropism of lentiviral vectors in mouse models and possible tumorigenic side effects resulting from uncontrolled, random integration of lentiviruses to the host genome.

The reported low efficiency of PR transduction with lentiviruses in (adult) rodents has been considered a major limiting factor for gene therapy of eye diseases with lentiviral vectors. Although the early experiments with rats were successful (Miyoshi et al. 1997; Takahashi et al. 1999), they were not confirmed subsequently, especially in adult rodent models (Kostic et al. 2003; Gruter et al. 2005). The best efficacy of PR transduction in mice (5%–15%) was achieved after subretinal injection of mice at postnatal 4 and 5 d (Kong et al. 2008). In adult mice, the efficacy rarely exceeded 5%, even after chemical modification of the subretinal space by neuraminidase X or chondroitinase ABC (Gruter et al. 2005). It had been noted, however, that the PR transduction by lentiviruses was more efficient in other animals, such as chicken and nonhuman primates (Lotery et al. 2002; Williams et al. 2006). Subsequently, we were able to show a more efficient PR transduction of the NHP retina by EIAV lentivectors using either GFP (Binley et al. 2013) or LacZ (data not shown) as reporters under the constitutive CMV promoter. Transduction of PRs, in addition to RPE, in the injected area of adult macaques suggests that lentiviral vectors can more efficiently transduce PRs in adult NHPs than in mice, thus raising hope for gene therapy in humans, especially for delivering large genes such as *ABCA4*. Additional studies with PR-specific promoters should unequivocally address the PR transduction efficiency issue for lentiviral vectors in NHPs.

However, Abca4$^{-/-}$ mice are still the only animal model for STGD1 disease, therefore all preclinical models have used this mouse strain. An efficient amelioration of a major disease marker, A2E accumulation, was successfully achieved in studies utilizing lentiviral vec-

tors in the Abca4$^{-/-}$ mouse model (Kong et al. 2008). After demonstrating safety and acceptable biodistribution of EIAV vectors carrying the human ABCA4 gene under the constitutive CMV promoter, phase I/II clinical trials for patients with STGD1 were started in 2012 by Oxford BioMedica (NCT01367444; see http://www.clinicaltrials.gov).

In addition to the suggested low PR transduction efficiency by lentiviruses in mice, two other concerns have slowed the development of the lentiviral platform. First, despite greatly improved biosafety, psychological concerns regarding using vectors derived from primate lentiviruses HIV-1, HIV-2, and simian immunodeficiency virus (SIV) have not been completely overcome, despite highly remote possibility of generating replication-competent lentivirus in clinical application. These concerns have been alleviated by using nonprimate lentiviral vectors, which cannot replicate in human cells (Maury 1998), such as those derived from equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), or feline immunodeficiency viruses (FIV) (Olsen 2001). Second, serious complications in early gene therapy clinical trials for X-linked SCID3 using retroviral vectors raised ethical and safety concerns about the use of retrovirus-based therapies in humans because of uncontrolled integration into genome (Kohn et al. 2003). These concerns are being addressed by creating either integration-deficient lentiviral vectors or vectors with targeted, clinically safe integration (Wanisch and Yanez-Munoz 2009).

CONCLUDING REMARKS

Several gene therapy applications are being used for *ABCA4*-associated diseases. Although currently only lentivirus-based therapy is in clinical trials, those based on AAV or nonviral vectors are close to follow. Another delivery vehicles not discussed above, but which are tried in preclinical studies are adenoviral (Ad) vectors. Although the Ad vectors, which are nonintegrating and transduce both dividing and nondividing cells, used to elicit immune responses that limited the duration of transgene expression, the current, helper-dependent Ad vectors, can substantially extend the duration of the expression of the transgene (Lamartina et al. 2007). Comparison of all methods will reveal which one is the most efficient and safe for patients; however, regardless of the final outcome, it is clear that efficient gene therapy will soon be available to patients affected with all *ABCA4*-associated diseases.

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