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A review of therapeutic prospects of non-viral gene therapy in the retinal pigment epithelium

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Abstract

Ocular gene therapy has been extensively explored in recent years as a therapeutic avenue to target diseases of the cornea, retina and retinal pigment epithelium (RPE). Adeno-associated virus (AAV)-mediated gene therapy has shown promise in several RPE clinical trials but AAVs have limited payload capacity and potential immunogenicity. Traditionally however, non-viral alternatives have been plagued by low transfection efficiency, short-term expression and low expression levels. Recently, these drawbacks have begun to be overcome by the use of specialty carriers such as polylysine, liposomes, or polyethyleneimines, and by inclusion of suitable DNA elements to enhance gene expression and longevity. Recent advancements in the field have yielded non-viral vectors that have favorable safety profiles, lack immunogenicity, exhibit long-term elevated gene expression, and show efficient transfection in the retina and RPE, making them poised to transition to clinical applications. Here we discuss the advancements in nanotechnology and vector engineering that have improved the prospects for clinical application of non-viral gene therapy in the RPE.

Keywords

RPE65; non-viral gene therapy; retinal pigment epithelium; DNA nanoparticle

1. Introduction

The retinal pigment epithelium (RPE), a monolayer of cuboidal epithelial cells lining the back of the retina, performs multiple critical functions required for the maintenance of the neural retina and proper vision including nourishing ENREF 1 and protecting the photoreceptor cells, absorbing excess light entering the retina, secreting growth factors, and recycling retinoids as part of the visual cycle [1]. In addition, tight junctions between RPE cells form the blood retina barrier and help the eye maintain its immune privilege [2]. Many blinding retinal disorders including Leber's congenital amaurosis (LCA) and retinitis pigmentosa (RP) occur due to mutations in RPE-specific genes, such as RPE65, bestrophin, and lecithin retinol acyltransferase (LRAT) (<http://www.retina-international.org/sci-news/databases/mutation-database/>). Choroidal neovascularization, age-related macular

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degeneration (AMD), and various other retinal diseases are also associated with defects in RPE-related structures, gene products, and metabolism [1]. Most often, in these cases blindness arises due to photoreceptor loss or malfunction secondary to RPE degeneration or functional defect.

Currently there are no curative therapies for RPE-associated diseases although many strategies are being explored. These include oral/intravitreal administration of retinoids, delivery of agents designed to alleviate ER stress, and cell transplantation. For example, treatment with 9-cis retinal, a precursor for the visual chromophore has mediated improvements in rod function in *rpe65*^{-/-} mice [3] and in *Irat*^{-/-} mice [4], and the pharmacological agent TUDCA (tauroursodexychoic acid) reduced ER stress and apoptosis resulting in significantly increased cone survival in *Irat*^{-/-} mice [5]. Cell transplantation using human embryonic stem cells or retinal progenitor cells to replace dying photoreceptors and RPE is a rapidly expanding field, and recent results demonstrating successful formation of an optic-cup in 3D-embryonic stem cell culture [6, 7] suggest that cell therapy has a highly promising future for ocular diseases. Due to limitations on cell integration and survival after transplantations much work remains to be done before this type of therapy finds clinical application for transplantation of retinal neurons [8-10], however, progress in the RPE is more advanced and several initial clinical trials are underway or completed testing cell transplantation therapies for RPE-associated diseases [11] (Clinicaltrials.gov NCT00401713, NCT01691261, NCT01469832, NCT01344993).

While these approaches have a bright future, particularly cell transplantation, by far the most well-developed field of research for the treatment of RPE-based diseases is gene therapy. Adeno-associated virus (AAV)-based gene delivery has been particularly fruitful. Many clinical trials testing AAV-based therapies for LCA associated with RPE65 and LRAT deficiency are underway based on extensive preliminary research using animal models [12-14] ENREF 25. In spite of these successes, there remain concerns about the safety of viral vectors, and production of neutralizing antibodies upon re-treatment [15], and although initial reports from the clinical trials were promising, concerns about damage from subretinal injection and limited rescue have prompted additional basic science research [16, 17]. From a drug development standpoint, AAV vectors are limited by their small payload capacity (<5kb of genetic cargo) [18] which cannot accommodate large genes or extensive promoter/regulatory elements such as the RPE gene *LRAT* [19] as well as photoreceptor genes such as *ABCA4* [20] (Stargardt's macular degeneration) and *USH2A* [21] (Usher syndrome type 2). Thus, development of alternative gene delivery strategies to complement AAVs for the treatment of RPE-associated diseases is a current research focus. Here we explore options for non-viral gene delivery to the RPE, discuss vector-based factors that contribute to the success of these strategies, and consider the prospects of these vectors for future clinical viability.

2. Non-viral gene therapy strategies

Successful non-viral gene delivery must overcome two traditional barriers; limited uptake into the cell and nucleus, and limited stability or transient gene expression once in the nucleus. Viral vectors have the advantage in cellular entry because they bind to the cellular receptors and co-receptors which help internalize and traffic them to the nucleus [22]. Such targeting and maintenance of plasmid vectors *in vivo* requires compaction with efficient gene carriers and modifications of the vector DNA. First we address options for gene carriers that have been tested in animal models of blindness including liposomes, organic polymers, nanoparticles, and plasmids.

2.1 Lipid cations (liposomes)

Liposomes are composed of lipid cations with hydrophobic head groups and hydrophilic tails and can incorporate negatively charged DNA or other drugs [23] (Fig. 1A). They can fuse with the plasma membrane for uptake making them exciting tools for gene and drug delivery [23-26] ENREF 6. Once inside the cell, the liposomes are processed via the endocytic pathway and the DNA is then released from the endosome/carrier into the cytoplasm (Fig. 1B) [27, 28]. Liposome-mediated gene delivery is most successful in mitotic cells where DNA entry into the nucleus can take place during cell division. DNA has also been shown to enter the nucleus in the absence of mitosis but at a low frequency. In the past decade these lipid cations have been extensively studied and modified to reduce cytotoxicity and increase gene expression levels. Some of these liposomes are now being explored in clinical trials for periodontal disease and various types of cancer [29-32], and further research is focusing on improvements in shape, size, cytotoxicity, transfection efficiency, and biocompatibility, which may yield an efficacious method of gene delivery in the future.

Topical and intravitreal delivery of liposomes to the eye has been tried [33, 34], however these delivery methods have yielded very low transfection efficiency for the retina and RPE. The inner limiting membrane and blood retina barrier severely limit passage of most gene delivery constructs including AAV after intravitreal delivery [35]. Subretinal delivery of liposomes has yielded better results with varying degrees of expression and longevity [36, 37], but liposome-mediated gene delivery is not currently a highly active area for RPE therapeutic research.

2.2 Polymers

Biocompatibility and safety concerns have driven the development of many novel biodegradable polymers such as polyethyleneimine (PEI), polyamidoamine (PAA), chitosan, polyesters, and polylysine for use as gene delivery tools in the past two decades. In many cases these polymers are complexed with polyethylene glycol. Some of the more widely used polymers are discussed below:

2.2.1 Polyethyleneimine—PEI is a cationic polymer with a high charge density that can incorporate DNA into polyplexes and has been used to deliver DNA *in vitro* and *in vivo* [38-40]. DNA degradation in the lysosomes and low transfection efficiency associated with liposomes led to research in PEI as an alternative. With its proton sponge-like characteristics enabling it to escape lysosomal degradation, PEI-compacted nanoparticles are considered more efficient for gene transfer. Although higher molecular weight PEI (~70kD) has been shown to hamper cellular growth [41], modifying the PEI backbone by adding histidine-lysine (KH) short peptides reduced cytotoxicity and increased transfection efficiency [42]. In addition, smaller PEI (25kD) can be used and showed no cytotoxicity when used to deliver oligonucleotides to cultured primary rat Müller glial cells [43]. One significant advantage of PEI-based complexes is that they can provide sustained release [44].

PEI-based gene delivery has been tested in the eye. In common with other delivery vectors, intravitreal delivery was not very successful [45, 46], however, results from topical treatment (for example for corneal conditions) has been more positive ENREF 25[47]. Its ability to transfect RPE cells has been mostly studied *in vitro* [48, 49], and the ability of these polymers to generate panretinal transfection and rescue of RPE disease models has not been established.

2.2.2 Chitosan—Chitosan is a linear polysaccharide, a polymer of β -(1-4) D glucosamine and N-acetyl-glucosamine [50]. It is commercially produced by de-acetylation of chitin, a

naturally occurring compound, and is biodegradable, and non-toxic. Combined with the ability to form complexes with DNA [51], these characteristics make chitosan an attractive polymer for gene delivery [52]. Like other non-viral delivery strategies, chitosan was initially considered an inefficient gene delivery tool. To alleviate this, various modifications to the chitosan-DNA compaction process have been studied. Coating with anionic polymers [53], polyethylene glycol (PEG) [54], and other aromatic compounds [55] has been shown to increase efficacy *in vivo*. Toxicity studies with chitosan based delivery have been controversial; some groups have claimed that chitosan nanoparticles are safe based on cell viability assays [56], while others have shown persistent inflammation following chitosan mediated gene delivery [57].

Chitosan-based gene delivery has been tested in several animal models of genetic diseases. It has been shown to transfect the cornea [58], retina [59], lungs [60] and other cells and tissues as well, although therapeutic rescue after ocular delivery has yet to be demonstrated. Chitosan particles have been tested for RPE transfection *in vitro*, and studies demonstrated efficient transfection, biocompatibility, and cell viability after treatment [61, 62] portending positive outcomes in future *in vivo* studies if toxicity issues can be resolved.

2.2.3 Polylysine—Positively charged polylysine peptides (CK30) can be used to compact negatively charged DNA or RNA into nearly charge neutral nanoparticles [63], but these particles tend to aggregate and become unstable [64]. However, conjugation of the CK30 to PEG (CK30PEG) overcomes these limitations and has been shown to facilitate improved gene expression [64]. These particles (Fig. 2A) are quite small, with a diameter of <25 nm [65] with the smallest ones having minor diameters in the range of 8-11 nm [66]. Particle size and homogeneity are some of the advantages of these CK30PEG compacted DNA nanoparticles compared to other polymeric compactations such as PEI and chitosan. Critically, and in contrast to AAVs, CK30PEG nanoparticles have a large capacity, tested up to 20 kbp in the lung [67] and 14 kbp in the eye [68], making them an excellent choice for delivery of large genes. The primary route of cell entry for these nanoparticles in some cells has been shown to be by binding to lipid raft-associated cell surface nucleolins [69, 70] (Fig. 2B), although it is not clear if this pathway governs RPE uptake. The nucleolin-NP complex traffics rapidly to the nucleus, although the small size of these nanoparticles also makes them able to enter the nucleus alone via the nuclear pore [63]. These transport features make CK30PEG nanoparticles efficient at transfecting both mitotic and post-mitotic cells; a benefit for RPE therapy. In addition to their positive uptake and trafficking characteristic, these nanoparticles have been shown to be non-toxic and non-immunogenic in mice [71, 72], rats [73] and humans [74].

CK30PEG nanoparticles have been used *in vivo* to drive gene expression and mediate therapeutic rescue in the lung epithelium and nasal mucosa [63, 65], brain [73, 75] ENREF 71, retina [68, 76, 77], and retinal pigment epithelium [66, 77]. Retinal expression was several fold higher than that achieved with naked DNA [68, 76, 78] and on the same scale as that observed with AAVs [79]. In adult mice at two days post-treatment, we demonstrated that a single subretinal injection of CK30PEG nanoparticles yielded reporter gene expression in 55% percent of RPE cells [66]. Importantly, we showed that this expression and therapeutic rescue was sustained in the RPE for 2 years or more when the reporter gene plasmid contained a scaffold/matrix attachment region (S/MAR-further discussed below) [80]. We have also demonstrated that subretinal delivery of these particles is safe and non-toxic after delivery to both normal and diseased eyes [72, 78, 81]. We observe no inflammatory reaction except a transient response to subretinal injection which is observed equally in saline (vehicle) injected eyes [72], and no sign of ectopic expression in the brain [79]. Importantly, this positive safety profile is preserved even after repeat dosing [81].

Combined, these features make CK30PEG nanoparticles exciting tools for RPE-based gene therapy.

2.3 Uncompacted DNA

Plasmid DNA can be delivered without any sort of additional packaging, however limited uptake into most cells and inefficient gene expression levels generally make this an unattractive option for gene therapy. However, the unique phagocytic capacity of the RPE makes this tissue more amenable to naked DNA transfection than other tissues. The apical RPE microvilli phagocytose and degrade a large quantity of outer segments each day as part of normal photoreceptor turnover [1]. Consistent with this cellular function, it has been observed that most materials including DNA, nanoparticles, and drugs delivered into the subretinal space are readily internalized into the RPE [66, 82]. We have previously shown that naked DNA effectively makes it to the cytoplasm in the RPE within 6-12 hours of subretinal delivery [66]. However, trafficking of naked DNA into the nucleus is an inefficient process ultimately resulting in low gene expression levels. Some evidence suggests that vector modifications can increase the efficiency of naked DNA-based gene therapy [80], but in general, delivery of DNA without compaction or another physical method (such as electroporation) is unlikely to be a clinically viable gene transfer method, even in the RPE.

3. Vector engineering: Changing the paradigm for non-viral vectors

Bacterial plasmid DNA [83] has been the building block for most non-viral gene delivery constructs. Although plasmids are often used during the construction of various viral vectors, the final packaged viruses have DNA content significantly different from plasmid DNA. Although plasmids have been highly useful for non-viral gene transfer (especially when coupled with efficient compaction), the episomal nature of the plasmid and the presence of bacterial elements required for propagation have contributed to chronic problems with gene silencing and transient expression. To help overcome these barriers, recent work has focused on incorporating additional DNA elements. This field of vector engineering has identified and evaluated the effect of enhancers, insulators, S/MARs, inverted terminal repeats (ITRs), and CpG depletion on transgene expression levels and longevity with variable effects. Delivery tools such as CK30PEG nanoparticles are especially suited to delivering these highly engineered vectors, as their large capacity enables them to compact larger, more complex vectors containing additional DNA elements.

3.1 Enhancers

Enhancers are DNA sequences that sequester transcription factors to help upregulate the rate of formation/binding of the pre-initiation complex to the core promoter [84] and the ability of enhancers to augment gene transcription in gene therapy vectors has been demonstrated by several groups [85-87]. Although these are usually considered cis-acting elements, they may or may not be located in the same gene or even on the same chromosome as the gene which they regulate [88]. The SV40 72 base pair enhancer has been extensively used in plasmid DNA to increase levels of expression [89]. The location of the enhancer element and its distance from the promoter within a plasmid vector is very important for expression augmentation. Several combinations of promoter-enhancer have been studied to maximize gene expression [85, 90]; however, the efficacy varies between tissue types.

In ocular tissues, one of the primary enhancers to be tested comes from the human interphotoreceptor retinoid binding protein (hIRBP) promoter. In an *in vitro* study, addition of the IRBP enhancer into equine infectious anemia virus (EIAV) vectors carrying the phosphodiesterase (PDE) or rhodopsin promoters led to increases in luciferase expression

levels in a retinoblastoma cell line [91]. However, this increase was promoter specific; the enhancer increased expression 15-fold with the PDE promoter but only 2-4 fold with the rhodopsin promoter [91]. Another enhancer that has been tested in ocular cells is from the intron of the GDEP (gene differentially expressed in prostate cancer aka PCAN1) gene. When the GDEP enhancer was paired with the GDEP promoter, reporter gene expression in Y79 retinoblastoma cells increased by 8 fold compared to cells transfected with vectors lacking the enhancer [92]. The advantages of additional retinal cis-regulatory elements have been discussed at length [93], but the true efficacy of these and all DNA elements can only be assessed following *in vivo* experiments using the specific construct of interest as results for individual elements are not generalizable from vector to vector or tissue to tissue.

3.2 Insulators

Chromatin insulators are DNA elements with two distinct functions, enhancer blocking and barrier formation [94]. Enhancer blocking insulators prevent enhancer mediated transcription of genes, and barrier type insulators prevent the spreading of heterochromatin into the expression cassette. The physical presence of the insulators close to the promoter or the gene is important for their function. Due to their ability to reduce genetic interaction between vectors and the host genome [95] (heterochromatin spreading), one often mentioned cause of gene silencing, insulators can be an ideal tool for gene transfer studies. Insulators have also been claimed to reduce vector related genotoxicity in viral vectors [96, 97], and although the dynamics of epigenetic regulation have not been well studied in basic gene therapy studies, it has been well documented that insulators are beneficial for promoting persistence of expression [98, 99]. The most well-characterized vertebrate insulator is derived from the chicken β -globin gene cluster (cHS4). It acts as both an enhancer blocker and as a barrier unit [100] and has been shown to protect the genome against position effects [101], of particular interest to help maintain transferred genes in the cell in a non-native location. In addition to cHS4, several human insulator elements such as the gamma satellite sequence, and a segment upstream of the ankyrin-1 gene are currently being explored for their ability to serve as insulators [102].

Due to their ability to prevent silencing from adjacent regions of DNA, insulators have been thought to be particularly beneficial for integrating vectors. When the cHS4 insulator element was tested in a transformed RPE cell line (ARPE19) in combination with an integrating vector system utilizing sleeping beauty transposons, it was shown to increase the efficiency of transposition and yield increased gene expression [98]. Interestingly, the cHS4 element did not have any effect on persistence of gene expression [98]. It is also not clear what the benefits of the insulator region may be for non-integrating vectors, so further studies are warranted.

3.3 CpG depletion

In DNA sequences, cytosine/guanine pairs are known as CpG groups, and cytosine methylation at the CpG site can result in decreased gene expression or gene silencing [103], although silencing also occurs by other mechanisms [104]. Bacterial backbones are hubs of CpG sites and strong promoters such as CMV and CBA (chicken-beta-actin) also contain numerous CpG sites. This has the potential to have a large impact on the efficiency of expression of transferred genes as in mammals 70%-80% of all CpGs are methylated [105]. Apart from silencing concerns associated with methylated CpGs, non-methylated CpGs present a separate set of difficulties. The bacterial genome contains unmethylated CpGs which are detected by toll-like receptor-9 [106] as part of the immune system's response to bacterial genes [107]. This response has been shown to block long term expression of AAV2 vectors in some cases [108], and other studies have also shown that recognition of these unmethylated sites cause inflammatory immune responses including activation of

macrophages, monocytes, natural killer cells, and dendritic cells [107, 109, 110] ENREF 152.

To combat these multiple silencing mechanisms, various groups have generated and tested CpG depleted plasmids [104, 111]. This depletion resulted not only in increased gene transcription levels but also reduced the amount of toxicity associated with the use of plasmid vectors in murine lung and liver cells [111]. Conversely, introducing CpG motifs led to reduced transcription levels *in vivo* in xenopus oocytes [103] ENREF 101. In one case, a partial loss in transcriptional activity was observed for the CpG depleted plasmids, but the authors suggested that the sustainability of expression made up for the reduced levels [111]. CpG depleted vectors have not yet been evaluated in the retina or RPE, but present an exciting avenue for further exploration.

3.4 Minicircles

Minicircles are circular DNA vectors that are devoid of bacterial backbone. They are produced from larger, more traditional plasmids, by a special site specific recombination in *E. coli* mediated by phage lambda integrase [112]. This recombination produces circular DNA containing the mammalian expression cassette but no antibiotic resistance gene or the bacterial origin of replication. In mouse hepatocytes, these stable minicircles have provided 10-12 fold higher levels of expression which persisted for 7 weeks compared to the unrecombined parent plasmid for which elevated expression only lasted for a day [113]. Moreover, these minicircles not only increased the transgene expression levels but also reduced toxicity associated with the bacterial sequences [114, 115]. This strategy eliminates the inconvenience of CpG depletion which may have an impact on levels of expression. After many years of optimization by Dr. Mark Kay's group, the minicircle vectors are now easy to produce and are also available commercially which eases production for many labs and research institutions not equipped to generate them in-house. Although the initial minicircles contained only a basic expression cassette (promoter, gene, polyA), recently minicircles containing S/MARs have also been generated and tested, demonstrating that more complex expression cassettes can be incorporated into this approach [116].

3.5 Linearization

Linearized vectors consisting only of the expression cassette serve a similar purpose to minicircle vectors, in that they are devoid of potentially detrimental bacterial backbone elements. While they have been historically used for building transgenic animal models they are also useful for gene therapy applications. PCR-generated linear fragments have been shown to yield higher expression levels than plasmid counterparts following electroporation in cultured cells as well as in mouse liver cells [117]. However, the *in vivo* utility of this approach is not clear. Linear vectors have been shown to integrate into the genome and drive sustained gene expression after hydrodynamic delivery to the liver [118, 119] ENREF 123. However, others have shown that linear vectors are often targets of exonuclease activity leading to their subsequent degradation [120]. This limitation can be overcome by using phosphorothioate modified-primers [120] or by ligation of hairpin nucleotides at the 5' and 3' end of the DNA [121]. We have shown that linear vectors can generate elevated gene expression in the RPE after subretinal delivery [81]. At one day and three days post-injection we showed that linear vectors carrying an eGFP reporter construct and an RPE-specific promoter drove gene expression at comparable levels to circular plasmid vectors (when delivered as either naked DNA or CK30PEG compacted nanoparticles), and did not show any signs of toxicity [81]. However, we have not yet studied the longevity of this expression.

3.6 Curved DNA sequences

Curved DNAs are bent DNA segment of repeats of nonaltering oligo (A) • oligo (T) (where • indicates intervening nucleotides), called A•T tracts, that form a left hand curved sequence. They are commonly found in proximity to origins of replication, matrix attachment regions, and recombination loci as well as in other regions [122]. These sequences act as acceptors for the histone core and allow nucleosome sliding for transcription augmentation by allowing transcription factors to access the promoter and TATA box [122]. Artificially created curved DNA sequences have been shown to activate promoter sequences in plasmid DNA in culture and *in vivo* in liver cells [122, 123]. These synthetic curved DNA sequences were shown to activate and increase activity for the HSV *tk* promoter (herpes simplex virus thymidine kinase) by 70 fold in a transient transfection assay, and similar results were observed with other promoters [123]. Several TATA box-containing-promoters possess endogenous curved DNA segments upstream, including the ubiquitously expressed β -actin gene [124], suggesting that the incorporation of synthetic curved sequences into gene therapy vectors may be beneficial for a wide range of promoters. To date these elements have not been tested for retinal gene therapy, but are an exciting additional tool for future vector engineering approaches.

3.7 S/MARs

S/MARs are AT-rich matrix associated regions with chromatin related functions and are one of the better characterized DNA elements useful for gene therapy. *In vivo*, S/MARs are thought to organize the nucleosomes into chromatin loops and play a critical role in nuclear architecture and function [125]. S/MARs have affinity for the nuclear matrix and play a role in stress induced DNA duplex destabilization [126]. These functions are thought to be mediated by interactions between S/MARs and several nuclear factors such as p300 [127], SAF-A [128] and SATB1 [129]. An S/MAR sequence obtained from the 5' region of the beta-interferon gene has been extensively studied for its ability to augment gene transcription and promote the stability of virally and non-virally delivered DNA [130]. This 2 kb S/MAR DNA sequence consists of base unpairing regions with a well-defined nucleation center (core-unpairing element) and a series of additional evenly spaced unpairing elements [131]. Base unpairing regions are specific regions of recognition for DNA binding proteins and unpairing elements are specific regions where DNA duplexes are destabilized [125]. The features of S/MARs most beneficial for gene therapy are discussed below.

3.7.1 Transcription augmentation—Incorporation of S/MARs into plasmid DNA vectors led to increased expression levels in CHO cells likely due to modulation of super helical stress during transcription *in vitro* [130, 132] ENREF 156. However, this may also be attributed to targeting of vectors to the active transcription hub via S/MAR interactions with the nuclear matrix and nuclear matrix targeting elements SAF-A and p300 [127, 128]. Interestingly, augmentation of transcription was only seen when the S/MAR was directly downstream of the expression cassette and upstream of the polyadenylation site [133]. In addition, S/MARs have been shown to augment transcription by shielding against repression effects, a function similar to that of the *chs4* insulator but mediated by a different mechanism [134]. There is some evidence in *Drosophila* that S/MARs coexist with enhancers [135], and may play an enhancing role. S/MAR-mediated enhancement is thought to be mediated by binding to several factors such as nuclear factor μ negative regulator, SAF-A, demethylases etc. [136, 137] ENREF 143.

3.7.2 Episomal maintenance/DNA stability—One of the most favorable features of S/MARs is that they play a role in maintaining the episomal status of vectors *in vivo*. It has been shown that S/MARs help plasmid DNA replicate episomally over several generations

in cell culture and *in vivo* in liver cells [133, 138]. Stehle et al., showed that active transcription upstream of the S/MAR is required and probably sufficient for episomal maintenance and replication in CHO cells [133]. Episomal maintenance of gene therapy vectors is highly desirable over random integration for toxicity and safety-related reasons, but loss of episomal vectors over time leads to reduced expression. Thus, the ability of S/MARs to promote episomal maintenance is of substantial benefit.

We have recently conducted extensive testing with S/MAR-containing vectors in the RPE and retina [68, 80]. We generated S/MAR-containing plasmids carrying an RPE-specific promoter (VMD2) and eGFP-reporter vectors, and delivered them to the subretinal space as either CK30PEG nanoparticles or naked DNA. Both nanoparticles and naked DNA drove elevated gene expression for the life of the animal (up to two and a half years, longest timepoint tested) without a significant drop in expression levels [80]. More importantly, when a therapeutic gene (RPE65) was included instead of eGFP, this vector mediated significant structural and functional improvements in the *rpe65*^{-/-} mouse, a model of LCA. Our initial therapeutic studies on the *rpe65*^{-/-} model showed improvement at 6 months post-injection, and our more recent studies have shown that therapeutic improvements also persist long term (data showing improvement at post-injection 15 months have been submitted for publication). These results highlight the utility of S/MARs in non-viral therapy for RPE-based diseases, and others have shown similar beneficial effects from the inclusion of S/MARs in liver and hematopoietic stem cells [139, 140].

4. Route of delivery to the RPE

The most efficient method to deliver genes to the RPE is by subretinal injection (Fig. 3A-B), and this has been the method of choice for the ongoing RPE65 gene therapy trials [141-143]. Unfortunately, this is also the most invasive method and it comes with significant risk of complications such as inflammation, damage to the lens and retinal detachment. Intravitreal delivery (Fig. 3A) is currently the delivery route of choice for drug administration but thus far intravitreal delivery of non-viral (liposomes [144], PEI [145], CK30PEG nanoparticles [77]) and viral vectors (AAVs [35]) does not result in efficient RPE expression. In an effort to eliminate subretinal injection, significant ongoing research is being devoted to increasing retina and RPE based gene expression after intravitreal or subconjunctival (Fig. 3A) delivery.

For local delivery of nucleic acids, physical methods such as electroporation [146, 147], ultrasound [148] and gene guns [149, 150] ENREF 50 have also been explored to increase efficiency of uptake without subretinal injection (or in concert with it). Electroporation increases the permeability of the plasma membrane by an externally applied electrical field to allow nucleic acids to enter the cell. Though this is a routine process for transforming bacterial cells, yeast cells and human cells *in vitro* [151], its use in live animals requires a great deal of caution. In the eye, recent advancements in electrotransfer have allowed transfection of the retina and the RPE by supra-choroidal electrotransfer without detaching the retina [152]. If this approach is mechanically feasible in larger animals such as non-human primates and humans without causing retinal tear or injuries to the eye, this may be a significant upgrade compared to subretinal injections for ocular gene transfer.

While intravitreal or suprachoroidal delivery is less invasive than subretinal delivery, the ideal form of administration would be topical instillation. While topical instillation of drugs is effective for treatment of corneal and anterior chamber disorders, little has been done on topical delivery for retinal gene expression. Liposomes applied topically to the eye in albino rabbits of dry eye model have yielded retinal gene expression [34, 144] but as yet at levels far too low to be biologically relevant. Topical delivery would likely require different

compaction/packaging technology, one such approach has been the use of poly (ethylene-oxide)-poly (propylene-oxide)-poly (ethylene-oxide) (PEO-PPO-PEO) polymeric micelles for gene delivery [153]. Following topical instillation of PEO-PPO-PEO-compacted CMV-*lacZ* vector in rabbit and mouse eyes, transgene expression was detected in cornea, anterior chamber and RPE; albeit at low levels. Similarly, topical application of herpes-simplex viral vectors led to transfection of approximately 20-25% of the cornea and RPE cells [154]. With no risks of corneal and retinal damage, topical delivery is an ideal treatment modality, and with more research to improve delivery efficiency, this approach may become clinically relevant for retinal and RPE-based diseases. For now, however, subretinal injection remains the most viable option.

5. Conclusions

The phagocytic nature of the RPE makes it an excellent target for non-viral gene therapy, and with the advancement of DNA compaction technology and vector engineering strategies, efficient transfection and prolonged gene expression are achievable goals. Epigenetics and tissue-specific differences play a major role in the efficacy of the DNA elements and delivery approaches discussed in this review, and only properly controlled *in vivo* experiment can determine which modifications and compaction techniques will yield best results for the target of interest. Although non-viral gene therapy has been gaining popularity as a gene delivery approach over the past decade, many of these modifications that we have describe are still being tested *in vitro*. Our results on RPE-targeting with compacted CK30PEG nanoparticles and S/MAR containing vectors are highly encouraging, but the variety of options available means that vector composition, compaction strategies, and delivery routes can be precisely tailored for a given application. We look forward to future studies focused on preclinical investigation to determine the efficacy of these approaches in non-human primates and higher mammals, thus promoting the clinical relevance of non-viral gene therapy targeting the RPE.

Developing effective therapies for diseases associated with the RPE and the retina is critical. Many disease genes of the RPE and retina are too large to fit into AAV vectors, including some associated with, Stargardt's macular dystrophy, retinitis pigmentosa, Usher syndrome, LCA, etc. As ocular gene and cell therapy continues to evolve, it has become more evident that a single type of therapy will not likely be successful in all cases and the development of multiple effective strategies is both prudent and necessary. Thus, we suggest that investigation and development of clinically-relevant non-viral treatment options is a valuable pursuit, particularly since the current success of RPE-directed clinical trials has demonstrated that genetic targeting of the RPE is a viable treatment strategy.

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Figure 1. Liposome-mediated transfection and endocytosis. **A.** Cationic lipids can form micelles that fuse with DNA molecules to form liposomes. **B.** These liposomes can be internalized by endocytosis, after which the DNA can be released into the cytosol or degraded in lysosomes.

Figure 2.

A. Formulation of compacted DNA nanoparticles [63]. CK30PEG is prepared by mixing HPLC purified CK30 with PEG-maleimide (10kD). Plasmid DNA is added while vortexing the solution of CK30PEG under specific conditions. The lysine counterion present at the time of compaction influences particle shape. Acetate (Ac) yields rod shaped particles. Shown is TEM of representative CK30PEG nanoparticles, scale bar 200nm. **C.** CK30PEG nanoparticles are taken into epithelial cells [70] by a nucleolin-mediated pathway. Nanoparticles bind to nucleolin in lipid rafts and are internalized via raft mediated endocytosis. Nanoparticles traffic to the nucleus through the microtubules in association with the glucocorticoid receptor (GCR) and dynein complexes. In the nucleus, they accumulate in the nucleolus.

Figure 3.

A. Common routes of intraocular delivery. **B.** Example of retinal detachment/retinal bleb after subretinal injection. Shown is a retinal section from an eye at one hour after subretinal injection of fluorescent microbeads.