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Non-Viral Ocular Gene Therapy: Assessment and Future Directions

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Abstract

The purpose of this review is to give the general reader a brief overview of the current state of the field of non-viral ocular gene therapy. For multiple reasons the eye is an excellent organ for gene therapy application and while non-viral gene therapy modalities have been around for quite some time; they have only been applied to the eye in the last few years. This review will cover the exciting current trends in non-viral gene therapy and their application to the eye in addition to a brief summary of the status of ocular gene therapy in general.

Keywords

gene therapy; retina; nanoparticle; non-viral; minicircle; integrase

2. Introduction

As a gene therapy target, the eye is a wonderful choice. First, it is easily accessible and various ocular tissues can be targeted by altering the mode of delivery. Second, the eye is reasonably immune privileged due to the blood-ocular barrier, and for the most part drug delivery to the eye is not associated with systemic drug exposure. This protection thus significantly limits extra-ocular toxicity. Third, the existence of animal models for retinal diseases and the ease of assessing structural and functional rescue after treatment make the eye a useful model for both proof-of-principle and advanced preclinical studies.

Gene therapy in the eye can be divided into three categories. The first is the use of gene replacement therapy to rescue diseases associated with loss-of-function mutations, most commonly mutations in retinal specific genes. The second involves using knockdown technology to eradicate mutant alleles associated with gain-of-function mutations. In recent years, the genetic basis for a wide range of inherited retinal diseases has been identified and over 118 retinal disease loci have been mapped, for which 55 genes have been isolated[1]. Mutations in these genes have been linked to a wide spectrum of retinal and RPE disorders (http://www.sph.uth.tmc.edu/RetNet/). Scientists have generated animal models for several of these mutations and made them available to the vision scientific community to develop therapeutic strategies and design rational treatment. The third group of ocular gene therapy studies aims to design genetic treatments for neurodegenerative disorders (glaucoma, age-related macular degeneration) that do not have a monogenic cause.

In order to summarize studies on ocular gene therapy and provide commentary on the future of the field, this review will contain discussions of the following subjects: ocular gene delivery

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options, examples of studies on the listed modes of therapy, the current status of non-viral ocular gene therapy and promising ideas to overcome current barriers to clinical applications.

3. Methods of Ocular Drug Delivery

In spite of its small size, the eye has multiple distinct tissues that can easily be targeted by therapeutic agents. Easy accessibility allows for carefully designed delivery methods to target therapeutic agents to different parts of the eye. For instance surface instillation of drugs has been effective in targeting corneal epithelium[2, 3], but high flow through tear ducts results in high drug turnover compared with other drug delivery methods.

Injections into the anterior chamber of the eye (intracameral) can be used to target the corneal endothelium, the iris and the aqueous outflow pathway[4]. Targeting the trabecular meshwork and Schlemm's canal are of particular relevance to researchers studying aqueous outflow and glaucoma. The utility of this delivery method is problematic for some of the same reasons as surface instillation. Aqueous humor turnover is high so maintaining therapeutically efficacious vector concentrations is difficult. Subconjunctival injection is a delivery pathway that is less invasive than any of the intraorbital injections; the subconjunctival space can accommodate a large volume and repeated injections without significant adverse procedural consequences[5]. Unfortunately, due to the blood-ocular barrier, it is of limited utility for applications apart from transfection of extraocular cells. Furthermore, delivery via this pathway has been associated with systemic drug exposure[6], so non-ocular side effects may be a concern.

Intravitreal injection is a common delivery method in both human and animal models. It allows targeting of the optic nerve, lens, inner retina and sometimes the outer retina or the anterior chamber. Similar to intracameral delivery however, excess fluid is typically quickly cleared from the vitreous so repeated dosing may be necessary. In some cases however, the therapeutic agents can accumulate and therapeutically effective concentrations may persist[7, 8]. Finally, subretinal injection is the most precise mode of therapeutic delivery to the photoreceptors and the retinal pigment epithelium (RPE). In some cases uptake of the therapeutic agents is limited to the site of injection[9, 10] although recent work suggests that some drugs can be taken up uniformly throughout the retina[11]. Even though subretinal injection is a technically challenging procedure but more importantly it is clinically viable. In the end, the choice of delivery method will be predicated on the desired target tissue. While all methods have limitations they are almost all applicable in certain instances. Furthermore, good therapeutic design can go a long way toward overcoming the shortcomings associated with the various delivery methods.

4. Examples of Ocular Gene Therapy

4.1 Gene Replacement Therapy in the Eye

Although rescue of different inherited retinal degenerations is underway, examination of all of them here would be space prohibitive and likely redundant as several excellent reviews addressing this issue have been published[12–14]. Pre-clinical investigations of therapeutic intervention in the disease course caused by retinal mutations have included gene delivery using modified viral vectors, liposomes, genetically modified cells, nanoparticles, or by direct DNA transfer to the retina[15–17]. These studies have targeted many different genes including Rds[18, 19] (macular degeneration/retinitis pigmentosa), RS1[20–22] (x-linked retinoschisin), RPE65[23–27] and RPGRIP[28] (Leber's congenital amaurosis), Gnat2[29] (achromatopsia) Myosin VIIA[30] (Usher syndrome 1B), PPT1[31] (infantile neuronal ceroid lipofuscinosis), and Mertk[32] (retinitis pigmentosa). Furthermore, many of these attempts to rescue genetic defects have proven quite effective[18, 33–35].

As an example, one of the most successful gene replacement trials thus far was conducted in Briard dogs harboring a mutation in the RPE65 gene[25, 27, 36]. Adeno-associated virus (AAV) to express the RPE65 cDNA, which restored retinal function to mutant dogs after a single subretinal injection[9, 10, 26]. The authors reported modest functional recovery in the dogs, on average maximum rod ERG amplitudes in treated dogs were 16% of the wild-type compared to approximately half that in untreated eyes. Additionally, visual thresholds were significantly reduced in treated eyes indicating better photoreceptor sensitivity. Most exciting, subsequent studies indicate that this level of rescue persisted over the long term; dogs were tracked for three years post injection and the functional rescue was not lost. Since an increase in serum antibodies against AAV was detected and several animals showed signs of inflammation or retinal degeneration at the site of injection, a subsequent study in preparation for human clinical trials assessed the safety of AAV-RPE65 in the human-like monkey retina[37]. The research group found no significant ocular or systemic toxicity up to 3 months after injection in monkeys. Currently, three clinical trials using AAV-RPE65 are ongoing as a next step in the development of treatment for RPE65-associated retinal diseases[38]. The results of these trials are eagerly awaited and will likely have significant impact on the direction of ocular gene therapy.

4.2 Gene Knockdown Therapies in the Eye

Many of the common disease-causing mutations in the retina are dominant, gain-of-function mutations. In these cases, gene replacement alone is not a viable treatment option. To that end, over the past few years experimentation with knockdown therapies has begun to appear. Researchers have confirmed the ability of small interfering RNAs contained in AAV-2 vectors to knockdown co-transduced reporter gene expression in retinal ganglion cells[39]. One of the looming problems in ocular gene therapy is that even if an ideal gene therapy vector is designed, the sheer number of mutated genes responsible for the different forms of inherited retinal degeneration means that designing treatments for each one will be time-consuming and likely cost-prohibitive. One of the exciting possibilities with RNAi involves non-mutation dependent knockdown, i.e. knockdown of all native and mutant proteins with concurrent supplementation of a slightly modified wild-type protein that resists the RNAi treatment[40]. In a proof-of-principle study designed to test the feasibility of this approach, researchers were able to specifically knockdown mouse rhodopsin expression (in cultured retinal explants) using short hairpin RNAs and concomitantly express (at ~90% of wild-type levels) a co-transfected mouse rhodopsin with silent mutations in the shRNA recognition sequence[40]. It remains to be seen whether this technology will be applicable in the eye *in vivo*, but it represents an exciting approach for future gain-of-function rescue studies.

4.3 Gene Enhancement Therapy in the Eye

The final type of gene therapy currently being studied in the eye is designed to treat diseases in which there is not a single genetic mutation responsible for the disease. Many neurodegenerative diseases such as glaucoma and age-related macular degeneration do not have a single causative genetic component. Recently significant effort has gone into evaluating the expression of neurotrophic or antiapoptotic factors in the eye or the suppression of angiogenic factors. As an example, AAV-mediated expression of brain-derived neurotrophic factor in retinal ganglion cells (RGCs) of rats was shown to increase cell survival in a laser induced ocular hypertension model of glaucoma[41]. Similar results were seen when ciliary neurotrophic factor (CNTF) was delivered via a self-inactivating lentiviral vector to the RGCs of mice who had undergone optic nerve transection[42] (although the putative benefits of CNTF need to be more fully explored[43]). Perhaps most encouragingly, PEDF (pigment epithelial derived factor) has been used pre-clinically to protect from ischemia reperfusion injury[44], delay the onset of retinitis pigmentosa[45], and inhibit choroidal neovascularization[46, 47] (CNV); and is now in clinical trials for the treatment of age-related macular degeneration[48]. In addition to

these examples which increased the expression of protective factors, RNAi has been used to inhibit detrimental factors such as vascular endothelial growth factor[49]; an angiogenic factor thought to be involved in retinal neovascularization associated with wet age related macular degeneration (AMD). In addition to pharmaceuticals targeting VEGF or its receptor, gene therapies utilizing siRNAs targeting VEGF are in development (reviewed in[16]).

5. Non-Viral Ocular Gene Therapy

In spite of the success of viral vectors in ocular gene therapy, there remains significant room for further improvements. Viral vectors have been able to alleviate hereditary retinal degeneration in mice[23, 35, 50], but they can be limited by cell tropism, size of the expression cassette to be transferred, and host immunity to repeated infections[12, 51, 52]. More importantly, concerns regarding the safety of using viral vectors in human patients have been raised and some trials have resulted in oncogenesis or even mortality[51–53]. Although the newer viral vectors such as non-integrating and self-inhibiting lentiviral vectors provide promising approaches to alleviate some of these concerns, there is a continued need for refinement and development of gene therapy vectors for the eye. As a result, recently significant research efforts have been directed towards the development of non-viral DNA delivery systems.

5.1 Naked DNA

Naked DNA is the most basic form of non-viral gene therapy and has been administered by almost all delivery methods[5, 11]. As it is typically not taken up into cells, naked DNA alone is not a viable ocular gene therapy modality. In several studies, significant uptake and expression of naked DNA was reported in the presence of electroporation or iontophoresis[54–57]. Both of these methods involve applying an electric current to cells to facilitate DNA uptake and movement. Electroporation is an excellent way to test DNA vectors in proof-of-principle studies undertaken in animal models, but side effects make it unlikely method to be a clinically viable delivery catalyst for clinical treatments. Iontophoresis has been used to successfully enhance retinal uptake of oligonucleotides without serious side effects[58] but there are conflicting reports on how effective this method would be in enhancing the uptake of complete genes or expression vectors[5, 57]. Further, the use of these methods in conjunction with unmodified DNA vectors is not associated with persistent transgene expression.

5.2 Liposomes

One of the most extensively studied types of non-viral vector packaging is cationic lipids. DNA encapsulating liposomes are delivered to the eye by any of the means already described above[5]. Surprisingly in addition to standard routes of delivery, two unexpected ones have been used to mediate liposome driven retinal gene expression. In the rat, topical instillation of cationic liposomes was capable of inducing β-galactosidase expression in retinal ganglion cells[59]. In the rhesus monkey, intravenous administration of cationic liposomes containing the β-galactosidase gene directed by the opsin promoter mediated gene expression in the retina without ectopic expression elsewhere[60]. This experiment took advantage of tissue targeting; by incorporating a monoclonal antibody against the insulin receptor into the liposome and a tissue specific promoter into the expression cassette, the vector easily passed through the blood-ocular barrier and expressed exclusively in retinal cells[60]. In spite of these promising results, liposomes also have limitations. Some retinal toxicity has been observed after liposome administration, and liposomes have been shown to aggregate significantly and form small vitreous bodies which can interfere with vision[61]. An exciting new type of liposome was recently developed in an attempt to overcome some of these limitations (transfection efficiency, toxicity, aggregation, and stability). Yamashita et al. have developed a protocol combining ultrasound treatment with novel PEG-ylated bubble liposomes which contain perfluoropropane gas[62]. Based on expression of transferred GFP, the liposomes are safe and efficient for

transfection of either cultured cells or rat conjunctiva. Unfortunately, in common with other liposome-style vectors, the group reported significant drop-off in gene expression after 4 days (although the use of the quickly-silenced CMV promoter likely contributed to this rapid down-regulation). In all studies examined and regardless of delivery method, liposome mediated gene expression was transient (never longer than four weeks) and would thus require repeated administration for the treatment of chronic diseases.

5.3 Compacted-DNA Nanoparticles

DNA nanoparticles are an additional technology that can be utilized to overcome many of the barriers to successful ocular gene therapy. Although the structural and energetic forces involved in DNA condensation have been studied by physical biochemists for the past 25 years, this area has experienced a recent resurgence of interest because of its application to gene therapy. Nanoparticle vectors have large payloads (tested up to 20 kb), generate no significant toxic responses, and offer efficient cell uptake due to their small sizes[11]. Various types of nanoparticles have been used for ocular gene therapy. Most DNA nanoparticles are between less than 10 and 400 nm in diameter. Nanospheres containing DNA surrounded by poly(lactic) acid and poly(glycolic) acid have been shown to effectively transduce RPE cells after intravitreal injection into the rat eyes[63]. While they exhibited no significant toxicity, these nanoparticles were only capable of transfecting between 10–35% of the exposed cells[63].

A second nanoparticle approach allows condensation of a single molecule of linear or plasmid DNA or RNA to a diameter between 8 and 20nm[11]. The plasmid DNA is bound by several molecules of a positively charged peptide, conferring properties that resemble the native DNA-histone complexes. Liu, et al. have described neutral DNA nanoparticles that allow for unimolecular packaging of DNA plasmids[64]. These compacted DNA polyethylene glycol/lysine nanoparticles have been shown to transduce genes 6,000-fold more efficiently than naked DNA, even in post-mitotic cells[64]. This is of particular importance with regard to potential nanoparticle-mediated gene delivery to photoreceptors and other retinal neurons, which are post-mitotic. In mouse lung, compacted DNA nanoparticles are several fold more effective than naked DNA[65]. These nanoparticles (<12nm in size) are currently undergoing evaluation in Phase II clinical trials for patients suffering from cystic fibrosis[66]. GLP toxicology studies showed no toxic endpoints and a transient histological finding of trace to grade 1 mononuclear cells around pulmonary veins only at the highest dose tested (100 μg of DNA)[65].

It has recently been shown that this technology can be used to drive high levels of gene expression throughout the eye[11]. After intravitreal injection of compacted-DNA nanoparticles containing GFP driven by the CMV promoter, expression of the GFP reporter gene is detected in retinal ganglion cells, cornea, lens, and trabecular meshwork. After subretinal injection in adult mice, expression was detected in RPE and photoreceptor cells. Excitingly, uptake was uniform throughout the retina; virtually 100% of RPE and photoreceptor cells expressed GFP after a single subretinal injection of the nanoparticles[11]. Furthermore, the expression level could be fine-tuned to mimic that of several different native proteins (rhodopsin, arrestin, peripherin/Rds) by altering the amount of the injected particles. No adverse toxic effects were observed with the particles; retinal structure and function were unaffected and healing after the injection procedure was unaltered from sham injected controls[11]. Currently, studies of the efficacy of this new technology in rescuing animal models of retinal diseases are ongoing. The effective and well-tolerated nature of these compacted-DNA nanoparticles suggests they are promising candidates for gene delivery to the retina and RPE cells.

The only impediment not yet overcome by any non-viral vector/vector delivery system used in the eye is the issue of transient expression. Since vector persistence remains a hurdle to clinically viable non-viral therapeutics, significant effort has gone into overcoming the problem

of vector silencing and valuable insight into this issue can be gained by examining approaches taken by researchers working with other (non-ocular) tissues.

6. Three Novel Ways to Overcome Transient Gene Expression

6.1 Genomic Integration

Michele Calos' group has done excellent work using the bacteriophage ΦC31 integrase system to promote persistent transgene expression in the eye as well as other tissues. This system involves delivery of a vector containing an *attB* recognition sequence and the ΦC31 integrase[55, 67]. The integrase mediates recombination between plasmids and genomic DNA that carry specific sequences known as *attP* or pseudo-*attP*. This sequence specificity makes the system superior to viral systems that rely on integration for persistence of expression; as the later is based on quasi-random integration. Some of these viral vectors indicate a preference for integration in actively transcribed genes significantly increasing the probability of insertional mutagenesis[68]. Extensive studies have shown that ΦC31 mediated recombination is sequence specific and that integration is typically confined to a few chromosomal hotspots[68]. Analysis of the primary insertion sites in the human genome has suggested very low risk of insertional mutagenesis. More importantly, genomic insertion is associated with persistent, therapeutically high levels of expression in multiple tissues including skeletal muscle, liver (expression up to 250 days), and rat retina (expression up to 4.5 months)[55, 67]. A parallel non-viral technology that utilizes the sleeping beauty transposon transposase system to mediate integration has given similar results without significantly increased toxicity when compared to the ΦC31 integrase system[69]. Thus far, the limitations of these technologies have been delivery and uptake into cells. In the rat retina, electroporation was used to increase uptake of the ΦC31 vector but with significant level of toxicity, including cataracts, inflammation, and in some cases small eye which make combination of this technology with an improved delivery method (such as liposomes or nanoparticles) of the utmost importance[55].

6.2 Minicircle DNA Technology

The second exciting technology involves vector modifications to overcome transgene silencing[70, 71]. Dr. Mark Kay's group and others have convincingly demonstrated that covalent linkage between bacterial DNA sequences and the expression cassette is associated with silencing[70, 72]. After injecting purified linear expression cassette into a mouse tail veins, researchers observed persistent transgene expression in the liver at much higher levels than after injection of either uncut plasmid or linearized plasmid[70]. This observation persists with multiple genes and multiple promoters; as an example, they report 28- to 40-fold higher serum levels of human α1 antitrypsin (hAAT) in animals injected with purified expression cassette when compared to those injected with circular DNA. Their subsequent work confirmed the hypothesis that covalent linkage between bacterial sequence and expression cassette is involved in silencing. When a plasmid containing HAAT or human Factor IX (FIX) designed to excise bacterial sequences *in vivo* was injected into mice, levels of transgene expression in serum were increased by 5- to 10-fold compared to normal circular DNA[73]. These levels persist for the duration of the experiment (up to eight months), significantly longer than that observed with traditional non-viral gene therapy mechanisms.

More recently, Dr. Kay's group has taken this work one step further and has developed DNA minicircle (MC) technology for use in gene therapy[74–76]. This technology is based on the core principle of including a site-specific intramolecular recombination site in the plasmid. Upon induction of the bacteria with L-arabinose, the plasmid recombines into two circular fragments via the ΦC31 integrase: one containing the expression cassette and one containing the prokaryotic sequence. The MC expression cassette can be easily and efficiently purified by standard methods. Dr. Kay's lab has elegantly demonstrated that the MC-DNA-vector is

capable of producing sustained expression of either hAAT or human factor IX at very high levels. Animals injected with MC vector exhibited serum hAAT levels 10- to 13-fold higher than those found in animals injected with purified expression cassette and 200- to 560-fold higher than those seen in animals injected with a traditional circular plasmid after hydrodynamic delivery to the mouse liver[74]. The increased expression level persisted for up to four months (duration of the experiment) and was not related to the promoter or enhancer sequence used.

6.3 Episomal Replicating Vectors

The final exciting new technology is based on the idea that the ideal vector for gene therapy should be based on chromosomal elements and behave as an independent functional unit after integration into the genome or when retained as an episome. Several elements have been shown to regulate mammalian gene expression and replication- e.g., enhancers, locus control regions, boundary elements, insulators and scaffold- or matrix-attachment regions (S/MARs). These elements have been used to design vectors that behave as artificial domains when integrating into the genome. Investigators have recently used some of these elements to develop replicating episomal vectors (REVs) for use as expression systems in mammalian cells. Such vectors are excellent alternative gene transfer vehicles and their main advantage is that they can persist in the recipient nucleus as independent units, without interfering with the host's genome[77]. Thus, REVs are intrinsically devoid of all the unpredictable consequences of integrating vectors. Investigators have developed a small circular vector named pEPI-1 show that it functions as a stable episome without coding for any protein of viral origin[78]. This vector contains a chromosomal scaffold/matrix attachment region (S/MAR) deriving from the 5′-region of the human interferon β-gene[79], as well as the origin of replication of the simian virus 40 genome (SV40 ori), the EGFP cDNA driven by the CMV immediate-early promoter and the gene conferring antibiotic resistance. By transfer of pEPI-1 into CHO cells, it was shown that this vector replicates episomally over many cell generations in the absence of large T antigen[78].

It is believed that the function of pEPI-1 as a stable episome relies on the ability of the S/MAR to recruit cellular factors, which mediate both its mitotic stability and its episomal replication. pEPI-1 is specifically associated through its S/MAR with the nuclear matrix and the chromosome scaffold *in vivo*[80], presumably via scaffold attachment factor-A (SAF-A)[81] and this interaction enables its co-segregation with the chromosomes upon mitosis. Moreover, the S/MAR in pEPI-1 likely interacts with other nuclear proteins mediating helix destabilization (a function of large T antigen in conventional SV40 ori-containing episomal vectors), allowing for the assembly of the replication machinery. Thus, in contrast to viral episomes which encode the factors required for their function, pEPI-1 exploits, through its S/MAR, factors provided by the host cell to ensure both functions required for its extrachromosomal maintenance: replication and segregation.

7. Future Prospects

We are generally optimistic about the future of non-viral ocular gene therapy. The eye is an excellent and accessible gene therapy target and significant research has laid excellent groundwork for future studies. The availability of animal models and the proliferation of vectors make gene-therapy mediated ocular treatments a viable option. The three main types of gene therapies described here are gene replacement for loss-of-function mutations, gene knockdown for gain-of-function mutations, and gene enhancement/knockdown for non-monogenic diseases. All of these approaches have historically been subject to the same limitations: 1) how to deliver the vector into the affected cells 2) how to achieve broad distribution throughout the tissue of interest 3) how to maintain persistent transgene expression and functional rescue and 4) how to avoid both local and systemic toxic responses. The use of newer viral vectors, particularly lentiviral vectors, and especially newly developed non-viral

vectors will enable researchers to overcome most of these obstacles. We are particularly excited about the prospects in ocular gene therapies which incorporate one of the technologies described above to circumvent the problem of transient gene expression. It has been shown that nanoparticles have been shown to distribute throughout the retina and are taken up efficiently[11]. By incorporating vector design strategies that have proven to be successful for non-viral therapy in other tissues, we anticipate the development of efficient, safe, and persistent gene therapy vectors that could be utilized to either replace or knockdown almost any gene of interest.

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