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Ocular gene therapies in clinical practice: viral vectors and nonviral alternatives

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Ocular gene therapy has entered into clinical practice. Although viral vectors are currently the best option to replace and/or correct genes, the optimal method to deliver these treatments to the retinal pigment epithelial (RPE) cells and/or photoreceptor cells remains to be improved to increase transduction efficacy and reduce iatrogenic risks. Beyond viral-mediated gene replacement therapies, nonviral gene delivery approaches offer the promise of sustained fine-tuned expression of secreted therapeutic proteins that can be adapted to the evolving stage of the disease course and can address more common nongenetic retinal diseases, such as age-related macular degeneration (AMD). Here, we review current gene therapy strategies for ocular diseases, with a focus on clinical stage products.

Viral gene therapy

Viral vectors for inherited retinal disorders (IRDs)

In December 2017, ~20 years after the first publication of gene therapy proof-of-concept results by Jean Bennett's group [1], the US Food and Drug Administration (FDA) approved the first gene therapy product for a retinal disease, voretigene neparvovec (Luxturna) developed by Spark Therapeutics [2]. This serotype 2 adeno-associated virus (AAV2)-based vector encodes RPE65 to treat patients with Leber congenital amaurosis type 2 (LCA2), an IRD caused by mutations in the *RPE65* gene. When administered in the subretinal space following vitrectomy, AAV2-RPE65 vectors proved to be safe and effective, with patients demonstrating clinically significant improvements in their ability to navigate in dim lighting conditions and in light sensitivity [3–5]. Importantly, the initially reported effects persisted over a 3-year follow-up period in most of the studies [6–8]. Immunogenicity of the AAV capsid was shown not to be an obstacle to effective treatment of the contralateral eye several months and/or years after the first injection with the same vector [9]. This allowed the bilateral treatment of patients in the pivotal Phase III clinical trial [10]. This first success has given hope to thousands of patients who have no other treatment option, and paved the way for the development of other AAV vectors targeting different retinal gene mutations. To date, more than 30 ocular gene replacement clinical trials are underway with new AAV vectors (Table 1 in Ref. [11]).

AAV vectors are currently the viral vector of choice for retinal gene transfer. Compared with other viral vectors, AAV exhibits an improved safety profile because of its low integration frequency and low immunogenicity and nonpathogenicity of its parental form [12]. Importantly, AAV recombinant genomes persist as episomal concatemers in transduced cells, leading to long-lasting expression of the transgene in nondividing retinal cells. When administered subretinally, AAV vectors efficiently transduced RPE and photoreceptor cells [13,14], while initially developed adenovirus (Ad)-based vectors failed to transduce photoreceptors possibly because the large size of Ad particles prevented their diffusion through the adult retina [15]. Several AAV serotypes were evaluated to provide specificity in cell transduction; following subretinal delivery, AAV1, AAV4, and AAV6 are the most specific to efficiently transduce RPE, and AAV5 and AAV8 are the most efficient to transduce photoreceptors [16].

More recently, lentiviral vectors (LV) have also been developed, particularly for the transduction of large genes with a transgene-

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carrying capacity of up to 8 kb [16,17]. Although first results with HIV-1-derived LV reported limited ability to transduce photoreceptors [18], improved transduction of rods and cones was later described with equine infectious anemia virus (EIAV)-derived LV pseudotyped with VSV-G envelope protein in adult macaque [19]. These results led to two Phase I/II clinical trials (StarGen™ and UshStat®, respectively) to deliver *ABCA4* and *MYO7A* genes to patients with advanced Stargardt disease and Usher syndrome type IB (USHIB), respectively. To date, no significant changes in BCVA have been reported in either the 23 patients enrolled in the StarGen™ study, or in the nine adult patients enrolled in UshStat®, motivating enrollment of younger patients with less-advanced disease [20].

Viral vectors for noninherited retinal disorders

Wet AMD

Beyond gene replacement therapies for monogenic IRDs, gene augmentation therapy approaches offer the promise of long-term sustained expression of therapeutic secreted proteins to address more frequent nongenetic retinal disease, such as AMD (Table 1). Antivascular endothelial growth factor (VEGF) therapies (aflibercept, ranibizumab, and bevacizumab) proved to be efficient for treatment of the neovascular (exudative or wet) form of the disease (wet AMD), reducing vascular leakage and macular edema. Despite impressive results during clinical development, 'real-world' outcomes of anti-VEGF-A therapy are generally worse than those obtained in clinical trials, because of undertreatment [21]. The need for repeated intravitreal (IVT) injections (typically ranging from every 4–8 weeks) to maintain adequate intraocular concentrations poses a burden for both caregiver and patient. In addition, frequent IVT injections increase the risk of injection-related adverse events, including endophthalmitis, hemorrhage, retinal detachment, and transient intraocular hypertension [22]. To overcome these limitations, viral gene therapy approaches are being explored for the continuous expression of antiangiogenic proteins with a single administration. Despite extensive encouraging preclinical proof-of-concept results, the first clinical trials with AAV vectors encoding the natural anti-VEGF-A inhibitor, soluble FMS-like tyrosine kinase-1 (rAAV.sFLT-1 from Adverum Biotechnologies; ClinicalTrials.gov: NCT01494805) or a fusion protein of the sFLT-1 domain 2 with the Fc domain of IgG1 (AAV2-sFLT01 from Sanofi; NCT01024998), were disappointing (recently reviewed in [23]). Although the IVT injection procedure was safe, and the anti-VEGF protein was detected in aqueous humor at the highest dose (above 2E10 vector genome), no statistically significant difference in best-corrected visual acuity (BCVA) improvement could be demonstrated [24]. It was hypothesized that expression levels were too low. Similar disappointing results were obtained after subretinal delivery of an EIAV-derived lentiviral vector encoding two antiangiogenic proteins, endostatin and angiostatin (OXB-201 or RetinoStat® from Oxford Biomedica; NCT01301443). Despite an apparent reduction in fluorescein angiographic leakage and visual acuity stabilization, no significant change in mean lesion size was observed [25].

More encouraging, however, are Regenxbio's interim results from a Phase I, open-label dose-escalation trial evaluating the safety and early efficacy of the subretinal injection of a novel AAV8 vector (RGX-314) encoding a soluble anti-VEGF monoclonal antibody fragment (NCT03066258). Dose-dependent protein expression levels were observed at 1 month, with sustained expression demonstrated at 6 months in patients treated at a dose of 6E10 vector genome (vg) per eye. Over 6 months, most patients treated at that dose had required minimal or no anti-VEGF injections (50% of patients), with maintenance of central retinal thickness

TABLE 1

Gene therapy and RNA-based clinical trials for non-inherited retinal diseases.

Vector	Disease	Drug	Route	Development status	Company	Clnical trial no.
AAV	Wet AMD	AVA 001 (AA V.sFLt1)	Subretinal	Phase I/II (completed)	Avalanche Biotechnologies	NCT01494805
		RGX-314(AA V8-AntiVEGF)	Subretinal	Phase I (recruiting)	Regenxbio	NCT03066258
		AAV2-sFLT01	IVT	Phase I (completed)	Genzyme (Sanofi)	NCT01024998
		ADVM-022(AA V2.7m8-Aflibercept)	IVT	Phase I (recruiting)	Adverum Biotechnologies	NCT03748784
		HMR59(AA VCAGsCD59)	IVT	Phase I (recruiting)	Hemera Biosciences	NCT03585556
	Dry AMD	HMR59(AA VCAGsCD59)	IVT	Phase I (active)	Hemera Biosciences	NTC03144999
LV	Wet AMD	RetinoStat(EIA V-endostatin-angiostatin)	Subretinal	Phase I completed	Oxford Biomedica	NCT01678872 NCT01301443
siRNA	Wet AMD	PF-655(siRNA RTP801 gene)	IVT	Phase II (completed)	Quark/Pfizer	NCT00725686 NCT00713518
	DME	PF-655(siRNA RTP801 gene)	IVT	Phase II (completed)	Quark/Pfizer	NCT00701181 NCT01445899
	NAION	QPI-1007(siRNA caspase-2)	IVT	Phase II/III (recruiting)	Quark	NCT02341560
	Glaucoma	QPI-1007(siRNA caspase-2)	IVT	Phase II (completed)	Quark	NCT01965106
Plasmid DNA	NIU	EYS606 (hTNFR-Is/hIgG1)	Electrotransfection of ciliary muscle	Phase I/II (recruiting)	Eyevensys	NCT03308045

and BCVA assessments versus baseline showing maintenance or improvements in visual acuity. Results from a fourth cohort (1.6E11 vg/eye) have yet to be reported. It remains to be seen whether durability can extend beyond 1 year.

Similar promising results were recently reported by Adverum, which is now pursuing the development of a new candidate, ADVM-022 (AAV2.7m8-aflibercept) to be administered as a single IVT injection. In the nonhuman primate laser induced-choroidal neovascularization (CNV) model, a single IVT injection of ADVM-022 allowed for sustained intraocular expression of aflibercept for up to 16 months and long-term efficacy in preventing the development of Grade IV lesions [27]. The company initiated a Phase I trial in November 2018 to assess the safety and tolerability of a single IVT administration of ADVM-022 (up to 6E12 vg/eye) in anti-VEGF patient responders (NCT03748784).

Dry AMD

Viral gene therapy is also being explored for treatment of dry AMD (Table 1), the most common form of AMD that affects ~85–90% of all patients with AMD and for which no treatment exists. Dry AMD is associated with loss of RPE, formation of deposits beneath the macula, called drusen, and loss of choriocapillaris vessels. The disease usually progresses with slow deterioration of the macula, sometimes transitioning from dry to wet AMD. In most severe cases of dry AMD, the disease evolves to geographic atrophy (GA) with the loss of the RPE cells and consequent degeneration of the overlying retina, leading to vision loss. Recent evidence implicates a significant role for complement activation in the pathogenesis of AMD, ending in the incorporation of the membrane attack complex (MAC) in biological membranes and subsequent cell lysis (reviewed in [28]). Elevated levels of MAC have been documented on the RPE as well as choroidal blood vessels of patients with AMD, suggestive of a possible role for MAC in the transition of dry to wet AMD. CD59 is a naturally occurring membrane-bound inhibitor of MAC formation [29]. CD59 functions by binding the C5b678 terminal complement protein complex and preventing the incorporation of the multiple C9 molecules required to complete the formation of a pore in the cell membrane [30]. Hemera Biosciences is developing AAVCAGsCD59 (also named HMR59), an AAV2 vector driving the expression of a soluble form of CD59 (sCD59), to inhibit MAC formation. A Phase I dose-escalating trial is evaluating the safety of AAVCAGsCD59 after a single intravitreal injection in 17 patients with advanced dry AMD and GA (NCT03144999). Interestingly, the same vector is also being tested in patients with newly diagnosed wet AMD who have never received treatment in the affected eye (NCT03585556). All patients will be treated with a single IVT injection of HMR59 administered 7 days after a first IVT injection of anti-VEGF. Patients will be followed monthly and treated with anti-VEGF injection as needed over 12 months. The number of anti-VEGF injections over the period is considered to be the primary outcome.

Future challenges for viral gene therapy approaches

Although successful for LCA2, future AAV retinal gene therapies will face several challenges. Among them, surgical considerations remain an important issue, possibly affecting both safety and efficacy. Subretinal injection of the viral vector suspension requires highly invasive surgery performed by trained surgeons through pars plana vitrectomy, sometimes under intraoperative optical coherence tomography (OCT) to ensure the subretinal location of the vector (Fig. 1). The injection induces subretinal bleb formation, resulting in transient detachment of RPE from the photoreceptors, which could aggravate the ongoing degenerative process. Complications reported to date in the various LCA2 clinical trials include macular holes, unresolved retinal detachment requiring surgical repair, choroidal effusions, hypotonia, and retinal tears [3,4,7,31]. In addition, the retinal zone transduced is restricted to the bleb, limiting the benefit of the treatment to that area. For these reasons, the ability of AAV vectors to transduce the entire retina through less invasive intravitreal injections is being explored (Fig. 1). Unfortunately, conventional AAV serotypes or newly engineered AAV variants (AAV2.7m8 or AAV8BP2) developed so far tend not to effectively transduce photoreceptors and RPE when injected intravitreally in nonhuman primates unless high potentially toxic doses are used [32]. The presence of physical barriers, such as the inner limiting membrane, was presumed to prevent access of the virus to the outer retina. To date, IVT delivery of AAV was used in a limited number of clinical trials, particularly for Leber hereditary optic neuropathy (LHON), for which photoreceptors are not the primary target (NCT03293524), or in trials evaluating optogenetic approaches to turn light-naive retinal cells into photosensitive cells (NCT02556736; NCT03326336).

AAV vectors also have a limited cargo capacity to ~4.7 kb of DNA, preventing the effective packaging of large transgenes into AAV particles [33]. Strategies to overcome limited AAV capacity are being developed by splitting large transgenes into multiple separate AAV vectors [34]. Orphan drug designation was granted by the European Medical Agency (EMA) for a first dual AAV8 vector developed to deliver the large myosin 7A (*MYO7A*) gene into patients with USHIB [35]. When administered in a mouse model of USHIB, intermolecular recombination between the two AAV vector genomes allowed for full-length *MYO7A* cDNA reconstitution and some therapeutic efficacy. However, the efficiency of recombination event is, by definition, limited in cell biology, and protein expression levels were lower than with single AAV vector, raising questions about the translation of this strategy to humans [36,37].

Long-term expression and efficacy in patients remain open questions. Although trials with Luxturna reported long-term encouraging efficacy data, other studies with similar AAV2-RPE65 vectors indicated progressing retinal degeneration despite gene augmentation [38,39]. In addition, although long-term transgene expression is required for gene replacement, one could question the safety of prolonged transgene expression of therapeutic proteins, such as anti-VEGF or anticomplement proteins. In wet AMD, CNV is part of a primarily degenerative disease for which a lifetime suppression of VEGF might aggravate the ongoing choroidal vascular atrophy [40].

Finally, AAV gene therapies raise major economic challenges. Given that manufacturing processes of recombinant AAV vectors still have too low yield, costs for production are exorbitant [41]. Thus, there is an urgent need for more efficient manufacturing methods [42]. When on the market, the price of such a therapy creates an economic burden for healthcare systems and raises questions about reimbursement and patient access to treatments [43]. Luxturna, for example, is marketed in the USA at a price of US

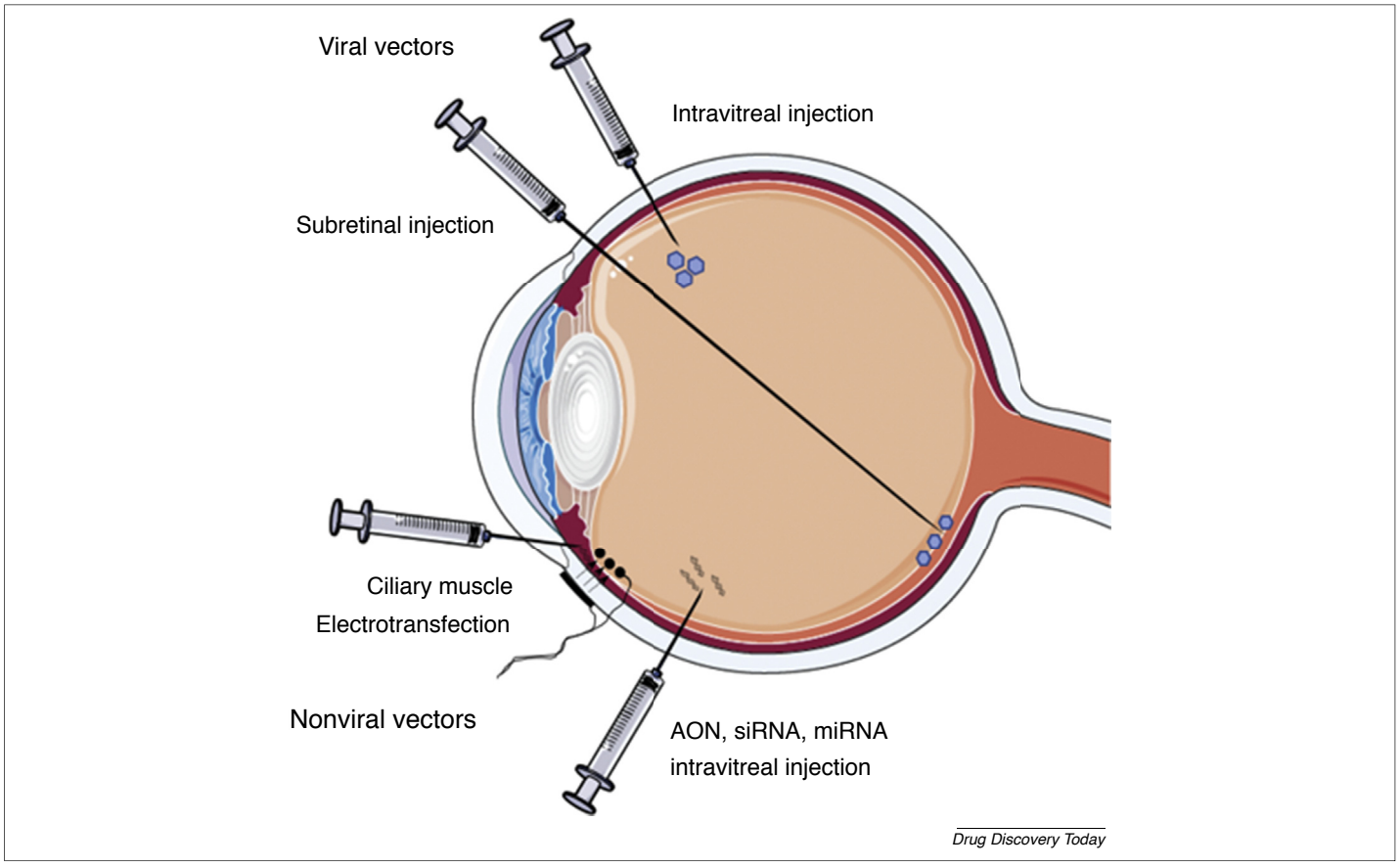

FIGURE 1

Ocular gene therapy and RNA-based approaches in clinical trials. Current routes of administration used to deliver viral and nonviral vectors under clinical investigations are shown. In most trials, viral vectors are delivered by subretinal injection through three port-pars plana vitrectomy and, to a lesser extent, by direct intravitreal injection. Naked plasmid DNA is injected and electrotransfered into the ciliary muscle to express proteins secreted directly into the eye. Antisense oligonucleotides (AON), small-interfering RNA (siRNA), or miRNA are intravitreally injected.

$425 000 per eye. With 106 new drug applications for gene therapies submitted to FDA in 2017 (up 34% from 2016), new payment systems will have to be developed to ensure timely patient access and affordability, while continuing to reward the innovation.

Nonviral gene therapy

For the reasons discussed earlier, alternatives to viral vectors for ocular gene delivery are being considered. Compared with viral vectors, no-viral vectors (naked plasmid DNA, oligonucleotides, and RNA) have a better safety profile (less immunogenic and lower risk of insertional mutagenesis), a larger cargo capacity, the potential for repeated administration, and are more easily produced on a large scale. However, the ability of naked DNA molecules to transduce cells is usually not effective unless the DNA is complexed with other chemical molecules or physical methods are applied to force its cellular entry and nuclear import.

Chemical methods

Development of DNA nanoformulations is an active research topic that has been extensively reviewed recently and will not be covered in detail here. Among these chemical delivery methods, lipid-based delivery systems [44,45], polymers [46], physical particles [47], or functionalized cell-penetrating peptides have been the most studied. Although transduction of retinal cells and some therapeutic benefits have been demonstrated in animal models, DNA nanoparticles (NPs) have not yet been evaluated in ocular gene therapy clinical trials, mainly because of low and/or short-term transduction [48,49]. The most advanced program in this field is the DNA NP developed by Copernicus Therapeutics, comprising a single plasmid DNA compacted with a 10-kDa polyethylene glycol (PEG)-substituted 30-mer lysine peptide (CK30PEG), which uses the nucleolin-dependent endocytic process to quickly reach the nucleus [50]. When delivered in the subretinal space, these compacted DNA NPs target the photoreceptors and RPE cells without significant toxicity, with persistence up to 2 years in mice [51,52]. CK30PEG NPs also mediate histological and functional improvement in several mouse models of IRD, including the $Rds^{+/-}$, the $Rho^{-/-}$ and P23H models of RP, the $Rpe65^{-/-}$ LCA model, and the $Abca4^{-/-}$ model of Stargardt disease (reviewed in [53]). Importantly, NPs were shown to transduce the retina and RPE even when delivered by intravitreal injection in nonhuman primates, offering a great advantage over AAV vectors, which up to now have failed to treat the retina outside of the subretinal injection bleb [54]. With almost no cargo limitation, these DNA NPS could become an alternative for ocular gene replacement therapy, especially for genes too large for AAV vectors, provided that long-term expression is proven in large animals.

Physical methods

As mentioned earlier, gene transfer with naked plasmid DNA is ineffective because of limited cellular uptake, poor nuclear import, and rapid degradation by nucleases, resulting in low and transient gene expression. Physical methods have been developed to improve DNA cell entry into ocular cells, such as iontophoresis [55,56], bioballistic [57], electrotransfection [58], magnetofection [59], sonoporation [60], and optoporation [61]. Among them, plasmid DNA electrotransfection for ocular gene delivery is the most promising, with a first clinical trial underway offering new avenues for treatment of sight-threatening diseases (Table 1).

Electrotransfection, also known as electroporation or electropermeabilization, involves applying a local and short external electric field to the cell to transiently modify the permeability of the cell membrane, facilitate the penetration of naked plasmid DNA, and promote its intracellular trafficking through electrophoresis [62]. Demonstration of highly efficient plasmid DNA transfer using electro transfer in vivo was first reported in skeletal muscle fibers using square-wave electric pulses, enabling sustained transgene expression for at least 9 months, with levels up to 100-fold higher than those obtained by simple injection of plasmid DNA (Mir 1998; Mir 1999; Aihara 1998). Subsequently, in vivo electroporation was successfully applied to a variety of tissues, leading to 90 clinical trials (reviewed in [63,64]). In the eye, plasmid DNA electroporation was applied to transduce retinal cells. Electrotransfection following subretinal injection of naked plasmids resulted in efficient transduction of the neuroretina when applied to newborn rodents [65], and of RPE and, to a lower extent, photoreceptors when performed in adult animals [66,67]. When subretinal injection and electroporation were applied to the Royal College of Surgeons (RCS) rat model, increased photoreceptor survival was achieved using brain-derived neurotrophic factor (BDNF) gene transfection in RPE. Similarly, injection of a soluble VEGF receptor-1 (sFlt-1)-encoding plasmid into the suprachoroidal space followed by electrotransfection allowed the transduction of choroidal and RPE cells, and potentially photoreceptors, providing a significant inhibition of laser induced-CNV [68]. Electroporation after intravitreal injection of DNA resulted in transfection of adult rat retinal ganglion cells [69]. Finally, delivering DNA expression plasmids to the corneal endothelium was demonstrated after plasmid injection into the anterior chamber and electroporation [70]. Although successful in preclinical settings, these approaches require invasive surgery to place microelectrodes to produce a localized electric field in proximity to targeted cells, making translation to humans challenging.

More promising is the electrotransfection of the ocular ciliary muscle to serve as a biofactory for high and long-lasting intraocular expression and secretion of therapeutic proteins (Fig. 1) [71]. In the eye, the ciliary muscle is a smooth muscle that allows lens accommodation. Located at the crossroads between the anterior and posterior ocular segments, this muscle is easily accessible and amenable to gene electrotransfer for treatment of both anterior and posterior segment diseases. Using a contact external plaque electrode positioned on the sclera overlying the ciliary muscle and a needle cathode electrode inserted in the ciliary muscle with previously published electrical parameters for skeletal muscle electroporation, Behar-Cohen and co-workers demonstrated safe, specific, and efficient electrotransfection of the ciliary muscle after trans-scleral injection of reporter gene-encoding plasmids in adult rats [71,72] and rabbits [73]. In the absence of electrical pulses, almost no reporter gene expression was achieved, confirming previous observations made in skeletal muscle. Using a plasmid encoding a secreted form of luciferase (*Gaussia* luciferase) under control of the CMV promoter, transgene expression was observed in the vitreous and, to a lower extent, in aqueous humor for at least 5 months, with essentially no systemic exposure [73]. Protein expression for up to 9 months was observed with a plasmid encoding a monomeric form of the human soluble TNF-alpha receptor type I (hTNFR-Is) [74]. Intraocular protein levels also correlated with the plasmid dose and the surface of transfected muscle, allowing this system to be adapted as a treatment regimen to meet medical needs. This nonviral method was used to deliver plasmids encoding different therapeutic proteins, including hTNFR-Is proteins [71–73], glial-derived neurotrophic factor [75], soluble VEGF receptor-1 (sFlt-1) [76], or transferrin [77]. For each protein tested, therapeutic concentrations were achieved, allowing preservation of the retina in disease animal models of uveitis, retinitis pigmentosa, macular edema, or retinal degeneration. For example, rat sFlt-1variant-encoding plasmids significantly diminished vascular leakage and neovascularization in a rat model of laser-induced CNV, with inhibition maintained for up to 6 months after plasmid electrotransfection in the ciliary muscle. Treatment effect correlated with downregulation of VEGF mRNA levels in the RPE and the choroid, demonstrating that the secreted sFlt-1 proteins reached the back of the eye [76].

Based on these promising results, Eyevensys (Paris, France) is moving this technology forward into clinical development (Table 1). A major first step was the development of a proprietary medical device comprising a pulse generator to deliver electrical pulses in a controlled manner and a disposable ocular device to ensure a guided precise plasmid injection into the ciliary muscle once positioned on the surface of the eye (Fig. 2). This was achieved by modeling the human eye according to published eye anatomic measures [78] and MIR measurements of the ciliary muscle characteristics, which showed that the thickness of the sclera, the distance between the cornea and the ciliary muscle, and the length of the ciliary muscle were stable among different patient group [73]. The accurate targeting of the posterior longitudinal ciliary muscle fibers was validated in nonhuman primates as well as in human cadaveric eyes (Eyevensys, unpublished data, 2016). Electrical parameters were also refined to suit this new design.

Non-infectious uveitis (NIU) was chosen as the first indication to clinically validate this technology platform. NIU is a leading cause of visual impairment and manifests as the presence of inflammatory cells in the eye. TNF-α is a major player in the induction and maintenance of intraocular inflammation and in retinal cell death [79]. Systemic adalimumab (Humira®), a fully humanized anti-TNF monoclonal antibody, was recently approved for the treatment of NIU [80]. Although adalimumab has shown efficacy for treating posterior segment ocular diseases, systemic anti-TNF therapy exposes patients to unnecessary potentially adverse effects, such as increased risks of malignancies [81]. Sustained intraocular delivery of anti-TNF proteins through intraciliary muscle plasmid electrotransfection might be a safer alternative. Electroporation of pVAX2.hTNFR-Is/mIgG1, a plasmid encoding the soluble human TNF-α p55 receptor linked to the

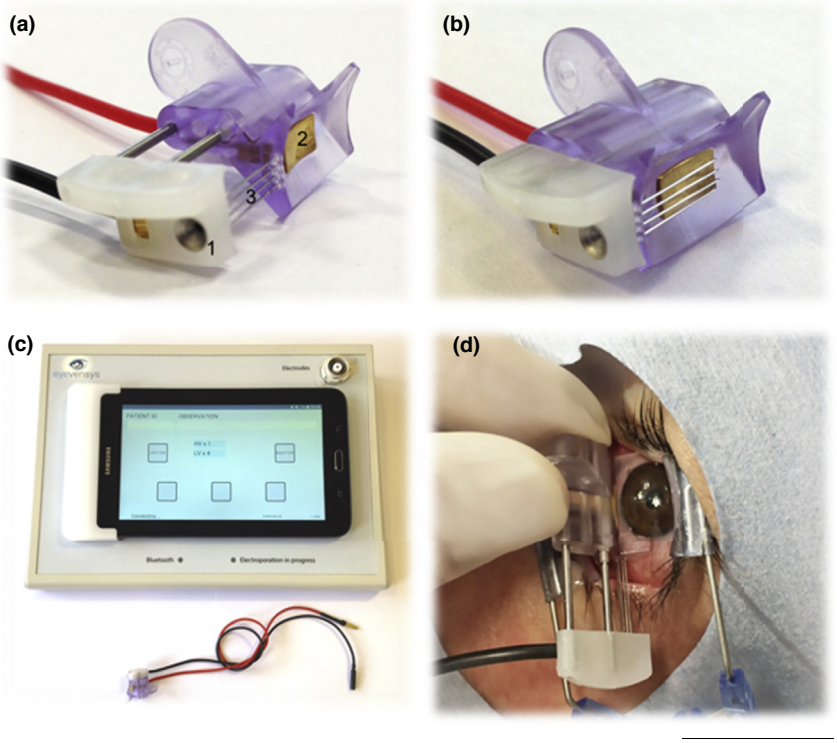

FIGURE 2

The Eyevensys electrotransfection system for plasmid DNA electrotransfection into the ciliary muscle. The Eyevensys proprietary electrotransfection system comprises a disposable ocular device **(a,b)** and an electrical pulse generator **(c)**. When positioned on top of the sclera **(d)**, the ocular device allows for plasmid injection into the posterior longitudinal ciliary muscle fibers by inserting an injection needle containing a plasmid solution through the needle guide (i) into the ciliary muscle between the plate surface electrode (ii), and the comb needle electrode (iii). A short and safe pulse sequence is then delivered to allow plasmid entry into muscle cells.

murine IgG1 Fc domain, reduced intraocular proinflammatory mediators and reduced the severity of disease in rat models of uveitis [71,72,82]. Touchard *et al.* recently reported similar results with a medical grade plasmid, pEYS606, devoid of antibiotic selection gene and encoding a fully humanized hTNFR-Is fusion protein (hTNFR-Is/hIgG1) [83]. In single-dose and repeat-dose toxicity studies conducted with pEYS606 electrotransfection, neither changes in electroretinography or intraocular pressure, nor retinal histological findings were reported (Eyevensys, unpublished data, 2017–2018). A clinical Phase I/II dose-escalating trial evaluating the safety, tolerability, and clinical activity of pEYS606 in patients with posterior, intermediate, and panuveitis is currently underway in the UK and France (NCT03308045). Electrotransfer of plasmids in the ciliary muscle is proposed as an alternative to repeated intravitreal injection of therapeutic proteins for the treatment of retinal disorders without limitations in proteins size (Table 2). To fine-tune the levels and duration of subsequent transgene expression to the specific needs of various retinal disorders, the pulse protocol used for plasmid delivery can be adapted to each therapeutic application.

Future challenges for nonviral gene therapy approaches

Better understanding of mechanisms involved in nonviral gene delivery is required to identify outstanding factors that might limit the efficiency of this approach. Unlike the traditional theory stating that electroporation creates transient pores that permit plasmid DNA to enter the cell, recent results suggest that endocytosis is involved in uptake and intracellular transport of electrotransfected plasmid DNA [84,85]. Approaches that would improve endocytosis of gene delivery vectors could enhance electrotransfection efficiency. Additionally, delay of endosomal escape might offer more protection of naked plasmid DNA against endonuclease degradation in the cytosol [85].

Continuous improvement in plasmid engineering is also key to overcome limitations to long-term expression associated with nonviral gene delivery. Adding a scaffold/matrix attachment region (S/MAR), not only maintains plasmid as replicating episomes, but also prevents epigenetic silencing by anchoring the transgene sequence to regulatory sequences within heterochromatin, maintaining the vector in a transcriptionally active state and for a prolonged period in RPE and photoreceptors [52,86]. Lowering CpG motifs and minimizing all nonessential sequences from the plasmid backbone was also shown to lower gene silencing (i.e., minicircle DNA, Nanoplasmid^{TM}). Future generations of plasmid DNA will allow for the combination of several transgenes in one vector; for example, an ideal drug candidate for macular edema might combine antiangiogenic, antifibrotic, anti-inflammatory, and neuroprotective genes.

In addition to DNA vectors, antisense oligonucleotides (AON), small-interfering RNAs (siRNAs) or miRNA show promise as ocular therapies; these are not be reviewed in detail here because of space constraints (Table 1). Of note, the first antisense oligonucleotide

TABLE 2

Gene therapies as an alternative to repeated intravitreal injection of recombinant proteins.

Approach/route of administration	Advantages	Current limitations
Recombinant proteins: IVT injection	Local, simple administration	Peak-and-trough protein levels; limited efficacy; potential safety issues with repeated injection; burden to patient
Viral gene therapy: subretinal or IVT injection	High transfection capacity; high level of protein; long-term expression; clinically validated (RPE65-Luxturna)	Invasive subretinal injection procedure; potential safety issues; low transduction of RPE and photoreceptors with IVT injection; no turn-off strategy; cargo limitation (AAV~4.7 kb; LV ~8 kb); repeated administration not possible; high manufacturing costs
Plasmid electrotransfection: ciliary muscle	Minimally invasive; safety of procedure and vector shown in animals; sustained expression (possibly 1–2 years); repeated administration possible; no cargo limitation (combination possible); turn-off strategy possible after photocoagulation; low manufacturing cost	Temporary expression (might require repeat administrations); still early stage (clinical efficacy under evaluation in humans)

approved for marketing by the FDA was for treatment of cytomegalovirus retinitis: fomivirsen (Vitravene). Since then, ProQR Therapeutic is moving forward with QR-110 (sepofarsen), an antisense oligonucleotide to restore correct splicing in patients with LCA10 with point mutation in the ciliopathy gene encoding centrosomal protein 290 (*CEP290*). Interim results from a Phase I/II study reported no serious adverse events following 3-monthly injections of AONs (NCT03140969). Visual acuity even improved at 3 months with 26-letter differences between treated and untreated eyes, motivating the company to plan for Phase II/III pivotal trials in 2019 [87]. Similar encouraging safety results were reported with siRNAs targeting the *RT801* gene or caspase-2 (QPI-1007) in AMD [88] or nonarteritic anterior ischemic optic neuropathy (NAION) and glaucoma, respectively (Table 1). With an ever-greater understanding of pathologies, the number of candidates will continue to increase.

Although the regulatory pathway for the development of non-viral vector is complicated by the combination of a gene therapy product and a medical device (i.e., a pulse generator and/or electrodes, or liposomes), the increasing number of such drug–device combination products will enable establishment of guidelines that will facilitate their development and evaluation by regulatory agencies [89].

Concluding remarks

Gene therapies will soon expand the therapeutic arsenal for retinal diseases. At the same time, novel targets are being identified. In multifactorial non-monogenic diseases, numerous pathways involving multiple soluble regulating proteins are involved. It is unlikely that one single therapeutic protein will be sufficient to control chronic diseases over the long term. Flexibility of the gene delivery system, and the possibility of combining therapies, together with biomarkers for more personalized medicine, might be the best option.

Conflict of interest statement

T.B. is employee of Eyevensys; F.B.C. is the founder and shareholder of Eyevensys.

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