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Authors
Amador, Cynthia
Shah, Ruchi
Ghiam, Sean
et al.

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Gene Therapy in the Anterior Eye Segment

Cynthia Amador[#1], **Ruchi Shah**[#1], **Sean Ghiam**[2], **Andrei A. Kramerov**[1], **Alexander V. Ljubimov**[1,3,*]

[1]Eye Program, Board of Governors Regenerative Medicine Institute and Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, USA;

[2]Sackler School of Medicine, New York State/American Program of Tel Aviv University, Tel Aviv, Israel;

[3]Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA

[#] These authors contributed equally to this work.

Abstract

This review provides comprehensive information about the advances in gene therapy in the anterior segment of the eye, including cornea, conjunctiva, lacrimal gland, and trabecular meshwork. We discuss gene delivery systems, including viral and non-viral vectors as well as gene editing techniques, mainly CRISPR-Cas9, and epigenetic treatments, including antisense and siRNA therapeutics. We also provide a detailed analysis of various anterior segment diseases where gene therapy has been tested with corresponding outcomes. Disease conditions include corneal and conjunctival fibrosis and scarring, corneal epithelial wound healing, corneal graft survival, corneal neovascularization, genetic corneal dystrophies, herpetic keratitis, glaucoma, dry eye disease, and other ocular surface diseases. Although most of the analyzed results on the use and validity of gene therapy at the ocular surface have been obtained *in vitro* or using animal models, we also discuss the available human studies. Gene therapy approaches are currently considered very promising as emerging future treatments of various diseases, and this field is rapidly expanding.

Keywords

Gene therapy; cornea; corneal dystrophy; corneal wound healing; keratitis; corneal neovascularization; glaucoma; corneal dystrophy; dry eye; graft survival; non-viral vector; nanoconstruct; drug delivery; adenovirus; adeno-associated virus; retrovirus; lentivirus; antisense; siRNA; CRISPR-Cas9

[*]Address correspondence to this author at the Eye Program, Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, AHSP-A8319, Los Angeles, CA, USA; Tel: 3233367949; ljubimov@cshs.org.

CONFLICT OF INTEREST

AVL is an officer and stockholder of Arrogene Nanotechnology, Inc., 8560 West Sunset Boulevard, Suite 424, Los Angeles, CA 90069, USA.

1. INTRODUCTION

Gene therapy is the technique of transfer of genetic material to remove, replace, repair, or introduce a gene in order to treat diseases [1]. Although this concept has existed for nearly 50 years, gene therapy has gained momentum only recently as a promising treatment option for many human diseases [2, 3]. After encountering several hurdles, including serious side effects and safety and efficiency issues in early clinical studies, today more than 2,500 clinical studies are underway for a wide variety of diseases, with six gene therapies already approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) [4].

The anterior segment of the eye, particularly the cornea, is an attractive target for gene therapy due to its accessibility and immune privilege. Current pharmacological approaches for corneal diseases only provide a short-term benefit, often require repeated treatment, and have at times proved to be ineffective, requiring further surgical intervention. The conventional approach also does not usually target the root cause of the disease. Therefore, corneal and other ocular surface disorders may greatly benefit from gene therapy approaches [5–9]. In this communication, we present a broad overview of gene therapy vectors and discuss what is known about its anterior segment applications for disease treatment (summarized in Table 1).

2. GENE DELIVERY SYSTEMS

2.1. Viral Vectors

Viral vectors have shown great promise for transgene delivery to target cells in the anterior segment of the eye [6, 7, 9]. Viral vectors are commonly used as vehicles for therapeutic genes due to the high efficacy of transduction. Even though using viral vectors has positive results, there are also many limitations and possible contingencies. A significant concern is the pre-existing immunity against viral vehicles (*e.g.*, for adenovirus) that may result in low rates of transduction and decrease the expression of the therapeutic gene within cells. In addition, viral capsids and remnant viral proteins have the ability to cause inflammation in their target [10].

2.1.1. Adenovirus—Adenovirus (Ad) is a double-stranded DNA virus with more than 50 serotypes. This class of viruses has a genomic length of approximately 20–40 thousand base pairs [11]. Ad has become an efficient vehicle for ocular gene therapy since it is utilized to treat short-term alterations in gene expression [12]. Delivery of genes coding for interleukins and other cytokines, *sFLT-1, c-Met,* and other therapeutic genes *via* Ad has provided treatment of various conditions such as corneal neovascularization, limbal graft rejection, and wound healing in different human and animal models [13–15]. Unfortunately, Ad vectors may still retain the ability to trigger adaptive and innate immunity generating adverse effects such as cytotoxic T-cell response [16]. Modern generations of Ad have been modified by excising their entire genome and preserving only inverted terminal repeats, and packaging genes with the hopes of decreasing immune response and making room to carry larger therapeutic material [9, 17]. The issue that arises from mass deletion of the viral genome is that infected cells will once again have significant immune responses due

to innate immune receptors that will identify viruses and trigger an immune reaction based on conserved molecular motifs [10]. Nonetheless, recombinant Ad (rAd) vectors are being successfully used as vehicles for ocular gene therapy.

2.1.2. Adeno-associated Virus—Adenoviruses and adeno-associated viruses (AAV) are by far the most used viral vehicles for gene therapy. Unlike Ad, AAV vectors elicit little or no immune response [16]. However, due to its prevalence in the environment, up to 90% of the population is naturally exposed to AAV, resulting in pre-existing immunity from neutralizing antibodies that can decrease transduction efficiency. Anti-AAV antibodies in the sera generate a varying range of seropositivity and immune response to different AAV serotypes, as detailed in several reviews [18–21]. AAV vectors can transduce both dividing and non-dividing cells. They do not integrate into the host cell genome but exist in the cells as episomal DNA, due to which they are diluted over time as the cell undergoes division [22]. In the eye, AAV serotype 5 (AAV5) is often used as a vehicle because it is known to deliver therapeutic genes that inhibit ocular disease, like corneal vascularization, while only producing an insignificant number of adverse effects [23]. AAV serotypes 6, 8, and 9 have been successfully transduced into both mouse and human corneal stromal cells. Compared to each other, AAV9 showed 1.1–1.4-fold higher efficiency compared to AAV8 and was 3.5–5.5-fold more effective as a delivery system compared to AAV6 [24]. Regarding the anterior segment of the eye, rAd and rAAV both have been reported to transduce human and rabbit corneas effectively. rAAV serotypes were significantly and statistically better viral vectors compared to Ad, although the level of gene expression judged by GFP levels was markedly higher with Ad [25]. All the rAAV serotypes tested produced distinct but different GFP staining in the corneal epithelium upon topical administration. Transduction of rAAV1 and rAAV8 produced higher staining intensities as compared to rAAV2, rAAV5 and rAAV7 [25]. The subconjunctival injection also produced serotype-dependent patterns, with AAV6 restricted to the corneal endothelium, whereas AAV8 efficiently transduced the stroma [26].

Recombinant AAV can deliver a wide range of genetic material to manipulate protein expression within cells and alleviate some ocular diseases. Currently, two AAV-based gene therapies have been approved by the FDA for human use, and many are in clinical trials [27]. As corneal angiogenesis is one of the most common forms of ocular diseases, extensive work is dedicated to disrupting the processes related to VEGF-A and PIGF1 expression *via* AAV vehicles [28]. Using AAV as a vector will potentially alleviate the problem of repeated intravenous injections to induce a systemic blockade of VEGF-A that is normally expressed in the human retina [29]. Anti-angiogenic microRNAs (miR) have also been applied *via* rAAV multi-target biological therapy to reduce the amount of corneal neovascularization [30]. In order to prevent significant immunogenicity of the viral carrier, delivery of human leukocyte antigen G (HLA-G) was used due to its natural induction of immune tolerance mechanisms, which would ensure reduced immune infiltration. As a result, T-cell infiltration was reduced in alkali-burned rabbit corneas, with decreased corneal neovascularization and fibrosis [31]. A common issue related to corneal transplantation and eye disease is corneal scarring [32, 33], which could be successfully improved using rAAV5 as a carrier for the anti-fibrotic *Smad7* gene [34]. Furthermore, *transforming growth factor-β1 (TGF-β1)* gene

delivered *via* rAAV has been used in rat models of high-risk penetrating keratoplasty (PKP), with prolongation of corneal allograft survival [35].

The most recent application of rAAV vectors is through synthetic contact lenses made of hydrogels of hydroxyethyl methacrylate (HEMA) with aminopropyl methacrylamide (APMA), which provide controlled release of vectors containing therapeutic genes [36]. The functionality of the hydrogel was assessed by transgene delivery of reporter *β-galactosidase* gene activity and red fluorescent protein to human mesenchymal stem cells with no apparent toxicity. Hydrogels carrying rAAV with the highest functional group (H2: 80 mM APMA) yielded the most transgene expression within the first hour and maintained transduction for several days. Contact lenses dispensing AAV-loaded hydrogels onto the corneal surface were found to be functional and represent a novel potential method of non-invasive ocular gene therapy.

2.1.3. Retrovirus and Lentivirus—Retroviruses have been used extensively in gene therapy due to their moderately high efficiency and long-term transgene expression. They generally have an 80–130 nm capsid and a single-stranded RNA genome of 3–9 kb in size. An important characteristic of a retrovirus is its integration into the host genome with a possibility of insertional mutagenesis and spread through cell division [12]. Lentiviruses share the same characteristics as retroviruses, except that lentiviruses do not usually cause insertional mutagenesis and can infect non-dividing cells like Ad or AAV [9]. Integration of retroviruses and lentiviruses into the host cell genome upon infection allows for stable gene and protein expression, making them versatile vehicles for gene delivery [37, 38].

Clinically pertinent equine infectious anemia virus (EIAV)-derived lentivirus has shown success in transgene delivery of *endostatin* and *angiostatin* genes to inhibit corneal neovascularization [39]. Delivery of *interleukin-10* gene *via* lentiviral vectors significantly increased survival of corneal grafts [15]. Furthermore, subepithelial fibrosis in rat corneal stroma was greatly reduced by Smad7 gene expression. Lentiviral delivery of therapeutic genes mediated the reduction of TGFβ/Smad signaling caused by decreased phosphorylation of Smad2, and Smad7 additionally downregulated the expression of pro-fibrotic TGF-β2 [40].

Integrating lentiviruses have 7% transduction efficacy after 3 days of application and 14.1% after 5 days in mouse and rabbit corneal epithelial cells. This makes them a less attractive vehicle for human use. In addition, the efficacy of transduction is dose-dependent based on the number of lentiviral particles, with doses 5×10^3 cfu/mL and 10^4 cfu/mL having the highest efficacy [41].

Most viral vectors encounter the issue of immune cell infiltration as a defense mechanism against foreign pathogens. Additionally, it is not uncommon to find insertional mutagenesis because retroviruses and, to a lesser extent, lentiviruses are notorious for randomized integration, even if their genomes have undergone modifications to prevent this effect [9, 23]. For this reason, retroviral and lentiviral vehicles still require additional investigations and perfecting before they become applicable to humans.

2.2. Non-viral Delivery Vectors and Methods

Non-viral vectors are also used to deliver therapeutic genes to cells in the anterior segment of the eye. In contrast to viral vectors, non-viral vectors have been shown to be more biologically safe as there is less immunogenicity and pathogenicity observed [42]. Additionally, an important advantage of non-viral vectors is their usually low cost, and they may be readily manufactured. However, there may be a lower yield of transfection by non-viral vehicles [43]. Various classes of non-viral vectors that have been used in gene therapy for the anterior segment of the eye are discussed below.

2.2.1. Naked DNA—Naked DNA is used without proteins, lipids, or other structures that would protect it. Naked DNA itself may be applied without a vector or vehicle for gene therapy, but its structural instability may prevent its proper uptake by the cells. For this reason, naked or plasmid DNA expression is up to 50% more effective when encapsulated into a vehicle or into a vector, in which gene delivery into the cells will be most productive [44]. Plasmid DNA, due to its bacterial origin, contains unmethylated CpG dinucleotides and can elicit an innate immune response by activating TLR receptors [16]. This issue can be resolved by suppressing the inflammatory response by methylating or eliminating the unmethylated CpG sequences [45]. Several approaches to do so have been tested and include site-directed mutagenesis, removal of non-essential regions within the plasmid backbone, generation of synthetic fragments without CpG sequences, or use of specific inhibitors of the CpG signaling pathway [46–48].

Although applying therapeutic DNA *via* recombinant viral vectors would yield higher protein expression levels within cells, using non-viral vehicles may have the benefit of the absence of immunogenicity.

2.2.2. Nanoparticles and Nanopolymers

2.2.2.1. Metal: Introducing nucleic acids or plasmid DNA to treat corneal diseases by coating metal nanoparticles (NP) is a reliable non-viral gene therapy method. Heavy metals and heavy metal compounds such as silver, gold, cerium dioxide, and titanium dioxide have shown no toxicity to rabbit corneal cells. Zinc oxide as a nano carrier could stimulate the production of reactive oxidative species and overexpression of apoptotic biomarkers Bax and Bcl-2 [49]. Metal NPs may be introduced with force by gene gun, but transfection of these gene carrier particles will also occur when introduced topically, in solution, to corneal and conjunctival cells. Topical administration of polyethyleneimine-conjugated gold NPs containing the *BMP7* gene could inhibit corneal fibrosis *in vivo* [50]. Gold NPs may be one of the most commonly used types for drug and gene delivery as they are ultra-stable, do not accumulate and aggregate in the body, and can be made into compounds with molecular weights ranging from 800 to 6000 g/mole [51].

2.2.2.2. Magnetic: There are few data reporting the effects of magnetic nanoparticles as a method of treatment, but it still remains a potential vehicle for gene therapy. Magnetic NPs are investigated due to the unique physical properties that allow them to have biological interactions with cells when they are exposed to an external magnetic field [52]. Superparamagnetic iron oxide NPs (SPIONP) have been introduced into bovine corneal

endothelial cells (CEC) and were found to move CECs into injured areas and alter metabolic activity as the concentrations of magnetic particles were increased. There was a significant difference between the metabolic activity of CEC (100×10^6 SPIONP/cell) with and without a magnetic field applied [53]. In a recent study, instead of directly delivering magnetic NPs into corneal or ocular cells, superpara-magnetic NPs were loaded into human corneal endothelial cells and injected into the anterior chamber of the rabbit cornea after Descemet's membrane stripping. This method reduced the risk of toxicity in the eye and showed that magnetic cell delivery outperformed nonmagnetic cell delivery by gravity alone in retention and efficacy [54]. By loading magnetic NPs into human CEC and guiding them with a magnetic field, drug and therapeutic gene delivery were shown to increase up to 2.4-fold without compromising the transfected cell viability or identity [55]. Such results suggest that magnetic NPs may be good alternative candidates for gene therapy of anterior eye disease, although more studies should be done.

2.2.2.3. Micellar and Liposomal Nanoparticles: Micelles are self-assembling amphipathic molecules that expose their polar surface to the external environment, and their non-polar portions are enclosed inside. This assembly is a possible carrier of genetic material at its core. Micelles allow for easy entry into tissue with little to no immune reaction. Some examples of micelles include naturally occurring chitosan and hyaluronic acid-based polymers, in addition to poly(alkylcyanoacrylate), poly(ε-caprolactone), and poly(butylcyanoacrylate). The advantage of using micellar NPs is that their structures are biologically compatible and biodegradable, which allows for facilitated transfection of genetic material into recipient cells. The use of such NPs in the cornea remains limited. Topical application of polymeric micelles allowed the correct expression of the reporter gene with specific promotors in the epithelial and stromal corneal compartments [56]. An aqueous mixed nanomicellar formulation (MNF) of dexamethasone was shown to increase the solubility of the drug with no cytotoxicity in isolated rabbit corneal epithelial cells [57]. Such a formulation could also be potentially used to deliver gene constructs to diseased corneal cells.

2.2.2.4. Cationic Nanoparticles: Cationic NPs are potential gene delivery vehicles due to their biodegradable properties and enhanced biological compatibility. Some biologically compatible NPs may contain the osmoregulatory protein albumin that is also an effective gene and drug delivery vehicle or is lipid-coated with cationic cores. Albumin NPs loaded with bevacizumab, an antiangiogenic antibody used to treat multiple cancers and specific retinal diseases, significantly reduced corneal neovascularization in an animal model compared to free bevacizumab [58]. Coating cationic NPs such as DNA-carrying chitosan with a lipid can increase cellular uptake of such liponanoparticle 5-fold compared to a chitosan NP. The endocytosis of these cationic particles is greatly facilitated by an external lipid layer [59].

2.2.2.5. Nanopolymers: The advantage of using nanopolymers is that their structures offer biological compatibility and degradability, allowing for an easy cellular uptake, as well as for the attachment of multiple and interchangeable functional moieties [60, 61]. As the corneal surface is exposed to the external environment, it is susceptible to epithelial

debridement. Topically administered polymeric micelles containing anti-apoptotic genes of the bcl-2 family were shown to reduce cell death and heal epithelial debridement injuries [62]. Similar delivery vehicles were made to contain *β-galactosidase* gene under the control of corneal epithelium- and stroma-specific promotors of keratin 12 and keratocan genes, respectively. Such topically applied micelles were able to induce specific gene expression in the intended corneal parts suggesting their validity for gene therapy [56].

2.2.2.6. Dendrimers: Dendrimers are advantageous nano vehicles for gene therapy due to their structural design to favor biodegradability and drug release mechanisms. Dendrimers have three main structural components, the central core, the highly branched interior (that resembles dendrites) in which genetic material may be intercalated, and the exterior surface with customizable functional groups [63]. Polyamidoamine dendrimer hydrogels designed to deliver anti-glaucoma drugs proved to be more effective than common PBS-formulated eye drops [64]. Dendrimer hydrogels have a significantly increased uptake in human corneal epithelial cells and bovine corneal epithelium, stroma, and endothelium, by up to 4.6--fold *vs.* brimonidine and timolol maleate [64]. Dendrimer-dexamethasone gel containing the *D-Cy5* gene may also be injected subconjunctivally to reduce corneal inflammation caused by immune cell infiltration and inflammatory cytokine expression [65]. Dendrimers have been found non--toxic *in vitro* in corneal and conjunctival cells post 6-hour incubation with 0.2–20 μM nanocarriers [66]. Dendrimers have the potential to deliver the necessary material to treat a wide range of ocular diseases without adverse side effects.

2.2.3. Microinjection—Microinjections are administered through a bore glass needle that is about 0.2 μm or less in diameter. Due to the minuscule size and precision of microinjections, they are widely used as a physical method to deliver DNA directly into the cell nucleus or in surrounding structures that will enhance gene expression [67]. Application to cells is very accurate but can become tedious when multiple cells are being injected individually. Reports have shown successful delivery of reporter genes coding for GFP, interleukin-18 that plays a role in anti-angiogenesis, Flt intraceptors to inhibit injury-induced angiogenesis, and metalloproteinase-14 to reduce corneal scarring [68–70]. Microinjections have shown great promise as a reliable method of transgene delivery and a possible treatment for medical conditions affecting the anterior segment of the eye. Microinjections continue to be multi-functional tools with potential to deliver gene therapy to the anterior segment of the eye.

2.2.4. Electroporation—Electroporation utilizes high-intensity electric impulses to form pores within the cell membrane to facilitate plasmid gene transfer. Electro gene therapy is difficult to conduct on most organs due to their internal location, but as the anterior segment of the eye is easily accessible, it has become a potential method for treating eye diseases. Studies have shown that increasing electrical intensity for gene transport into the cell resulted in enhanced gene expression [71]. The optimal electrical field strength for this method of gene transfer is 200 V/cm since no corneal damage, edema, or inflammation was observed. Gene uptake in the cornea is increased by 1000-fold compared to injection of DNA alone, and expression will occur within the first 6 hours after the application [72]. The main concern when using electroporation at extreme intensities is that the

created local and transient permeability becomes irreversible due to the generated heat [73]. Nonetheless, transfection of therapeutic genes, such as *Kringle 5 plasminogen*, into rat corneal cells *via* electroporation led to robust gene expression and effective inhibition of corneal neovascularization [74].

2.2.5. Sonoporation—Similar to electroporation, sonoporation physically creates transient and localized pores within cell membranes *via* ultrasound waves, thus facilitating the transfer of DNA to the nucleus. As compared to naked DNA, sonoporation can increase the amount of therapeutic gene expression up to 15--fold, but electroporation still yields a higher efficacy of protein expression [75]. Both physical modalities show promise in gene delivery as neither of them has been reported to cause significant immunogenicity or pathogenicity. But they are limited as the size of the molecule delivered may affect the amount of transfection [76]. Research *in vivo* on non-human tissue suggested that it is possible to use sonoporation as a vehicle for gene therapy, but there is not enough data on how it may work *in vivo* on human tissue.

2.2.6. Iontophoresis—Iontophoresis is a method used to deliver ionized molecules through the cell membrane *via* transient and localized pores that are created by low currents [77]. A direct electrical current may be applied to the cornea at 0.5–5.0 mA/cm^2, and for the conjunctiva, it may be applied at 0.5–20 mA/cm^2 for 30 min. Gene or drug delivery across the cell membrane *via* iontophoresis has been reported to increase by 2.3- and 2.5-fold for the cornea and 4.0- and 3.4--fold for the conjunctiva, respectively; the transfer returned to baseline levels in rabbit cornea and conjunctiva once the current was no longer applied [78]. Introducing genes and drugs into the cornea utilizing current is carried out by loading the electrode with, for example, riboflavin solution and connecting it to a current generator aiming at enhancing corneal cross-linking in keratoconus patients after epithelial debridement [79]. Three different protocols have been assessed to treat corneal thinning disease keratoconus, one of which is iontophoresis. Compared to conventional and accelerated methods, iontophoresis has the benefit of non-invasively improving riboflavin penetration throughout the cornea. However, even with successful results with iontophoresis in reducing or avoiding epithelial debridement in keratoconus treatment, the conventional procedure still remains most effective, being quick, safe, and delicate enough for thin corneas [80]. Nonetheless, iontophoresis may become standard procedure for treating anterior ocular disease due to having a short procedural duration of 10 minutes and being more comfortable for patients. However, long-term studies still need to be done to further assess the efficacy and risks that come from using this method for corneal cross-linking [81].

2.2.7. Gene Gun—A biolistic particle delivery system, also known as a gene gun (ballistic gene transfer), is an instrument that allows for the transfer of DNA, RNA, or proteins into tissues and cells by coating them with inert, heavy metal (such as gold or silver), making micro-projectiles and delivering them with force. The benefit of using a gene gun in the corneal epithelium is that the application yields high levels of therapeutic gene expression without causing measurable adverse effects [82]. Opioid growth factor and its receptor (OGFr) negatively regulate ocular surface epithelial proliferation and wound healing. To block this negative regulation in rat cornea, the transfer of OGFr plasmid

antisense DNA (intended to inhibit OGFr) to the injured corneas *in vivo* was used *via* gene gun delivery and resulted in the acceleration of wound healing [83, 84]. The effects of gene gun delivered gold-coated vector DNA for *IL-4,* and *CTLA-4* genes were also explored *vs.* the use of gene gun alone in the model of corneal grafting. The procedure itself caused increased migration of F4/80+ macrophages into the stroma, impeding graft survival. The increase could be a response to eye irritation due to the application. However, treating recipient tissue with vector DNA using a gene gun improved graft survival [85]. The data show that this method should be used with caution for plasmid DNA delivery, as there are factors limiting its efficacy, including the amount of genetic material supplied, the force used during application, shallow or deep penetration, the number of cells penetrated, and temperature. With greater force applied, there is more damage or inflammation observed in the affected cells. The Gene gun method should be further investigated in the cornea [86].

2.2.8. Laser—The conventional excimer laser is used to correct eye conditions such as astigmatism, myopia, and hyperopia. Photorefractive keratectomy surgeries utilizing this laser have the purpose of reshaping the cornea, but a common adverse effect is a corneal haze [87]. More recently, the femtosecond laser was introduced that has the advantage of ultrafast and ultrashort pulses that favor proper healing of the cornea [88]. Due to its high precision and safety, the femtosecond laser has been used to create minuscule stromal pockets of 110 μm depth to introduce genes into *ex vivo* pig cells to assess the efficacy of gene expression [89]. Once the pocket has been created, therapeutic genes may be introduced at the site *via* injection. As viral gene transfer has been assessed with this method [89], the next steps could include introducing non-viral vehicles and potentially trying this method on human cells or *ex vivo* human organ cultures. The femtosecond laser has also been used to activate carbon NPs that carry genetic material in the *ex vivo* human corneal endothelium [90]. As a result, macromolecules can be applied to the human cornea with a high delivery rate.

2.2.9. Chemical—Both synthetic and natural chemicals, including lipids and polymers, have been widely tested as vehicles for therapeutic gene delivery in the *in vivo* models of ocular gene therapy. Cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane and dioleoylphosphatidyl-ethanolaminehave have been used in transfection mixtures to deliver genes to human epithelial cells in culture and to corneal rabbit epithelium *in vivo* as eye drops [91]. Furthermore, a concoction of 5 different non-viral lipid vectors was applied to human epithelial cells, which resulted in up to a 17% increase in transgene delivery with nearly no cytotoxicity [92]. What makes these cationic lipids efficient as a carrier is their affinity to negatively charged DNA.

2.2.10. Antisense Oligonucleotides—The use of short single-stranded DNA that interacts with mRNA to block the translation of a specific protein is widely used in gene therapy. These antisense oligonucleotides (AONs) are complementary to their targets and, once bound, mark mRNA for degradation to prevent protein synthesis. The advantage of AONs compared to genetic knockdowns or knockouts is that they are gene-specific and do not cause changes in the expression of other genes [93]. There are several versions of AONs, with the Morpholino type being preferable due to its stability, superior target specificity, and

resistance to nuclease attack [94]. What makes the antisense use special is that it can be non-invasive, as AONs can be applied topically through eye drops. In the eye, a clinical phase was first conducted in 2014 to access the efficacy of AON (aganirsen) targeting insulin substrate-1 receptor expression to block corneal neovascularization and found it effective [95]. The aganirsen topical eye drops successfully inhibited corneal neovascularization in keratitis patients reducing the need for corneal transplantation. Similarly, GS-101 AON, a potent anti-angiogenic compound, was found most optimal when applied at a dose of 86 μg per day, inhibiting and causing regress of corneal neovascularization [96]. Introducing AONs *via* non-viral vehicles may render it less immunogenic. However, it was reported that AONs targeting TNF-α and used against herpetic keratitis might still interfere with the antiviral response and therefore lead to recurrence of herpetic stromal keratitis [97, 98]. AONs may be a potential tool to correct molecular genetic disorders, such as Fuchs' endothelial corneal dystrophy (FECD), by targeting the sense RNA transcript of *TCF4* triplet repeat expansion, which will reverse the toxic nuclear RNA foci formation as well as splicing defects [99]. In addition, AONs are practical tools of ocular gene therapy due to the small amount needed for the application. They can also be readily synthesized and modified for stability, for instance, to produce Morpholino variants that have shown efficacy for corneal wound healing [100]. The efficacy of AONs may be dependent on the stage of the ocular disease. Generally, patients who are at the early stages of FECD would respond better to AON gene therapy since they have not yet experienced significant endothelial cell loss due to *TCF4* repeat expansion-mediated toxicity [101].

The functionality of AON can be maximized when used with other methods for facilitating drug delivery, such as nanoconstructs or iontophoresis. When used together, AON transfer is increased, the delivered AONs can persist for 24 hours and may result in lower expression of target genes for as many as 7 days as reported by Gibson *et al.* 2017, although they may act even longer [102]. It should be noted that in the cornea, free AONs can effectively, although slowly, transduce the endothelium. Still, the epithelium requires additional tools for delivery, including nanoconstructs or transfection enhancers [100, 103].

2.2.11. siRNA—Small interfering RNA (siRNAs) are a class of double-stranded, non-coding RNA molecules that are about 20–25 base pairs, whose function is to regulate the gene expression of mRNA by targeting them for degradation, ultimately leading to silencing of that target gene [104]. siRNAs have been tested in various ocular diseases such as retinal disorders, glaucoma, Meesmann's epithelial corneal dystrophy (MECD), wound healing, and neovascularization [104–107]. Most of these studies have been conducted on rabbit, mouse, and rat tissues, although some were tested in human diseases, and some siRNAs are in the clinical trials [108]. siRNAs can be delivered as naked constructs, inside NPs, with cell-penetrating peptides or as parts of plasmids as short hairpin RNA (shRNA) [108–112]. Although they are widely used primarily to knock down gene expression, there are some concerns about their off-target effects, stability, and short duration of their action [108]. However, recent advances in their design would make these shortcomings less significant [104].

2.3. Next Generation of Genome Editing System: CRISPR-Cas Delivery *via* Viral and Non-Viral Vehicles

The CRISPR-Cas9 genome editing system is a fairly novel tool for gene therapy application in the anterior segment of the eye. Like most drugs and therapeutic material, the CRISPR-Cas9 system cannot be delivered systemically due to the blood-ocular barrier [113]. Fortunately, all of the aforementioned vehicles for drug delivery are feasible methods to deliver CRISPR-Cas9. The biggest concern with using these vehicles is their carrying capacity; they are often too small to carry the full genome editing system. In AAV vectors, the maximum capacity for genetic material is approximately 5 kilobases [114], 36 kilobases in Ad vectors [115], and up to 10 kilobases in lentiviral vectors [116]; all of which include inverted terminal repeats. A dual-AAV design has been used to correct this issue by delivering the Cas9 nuclease and the sgRNA cassettes in two separate viral vehicles yielding high expression of the genome editing system [117]. Further engineering must be investigated to optimize the space within these current vehicles. Ad has been used to deliver CRISPR-Cas9 treatment for myocilin (MYOC)-associated glaucoma. The editing components were found to be successfully delivered into primary human trabecular meshwork cells and in MYOC mouse models for primary open-angle glaucoma. Transduction efficiency of up to 70% using Ad5-cas9 or Ad5-crMYOC was reported alongside a significant reduction in IOP. Reports showed Cas9 expression in the corneal endothelium and parts of the iris and ciliary body [118]. Although not many studies have been conducted using AAV, Ad, and lentivirus to deliver CRISPR-Cas9 materials to the anterior segment of the eye, a few have been done on the posterior ocular delivery *via* viruses. By using AAV and lentivirus, researchers have targeted retinal pigment epithelial (RPE) cells [119] and successfully transduced the editing system due to the larger carrying capacity compared to Ad vectors [116, 120].

CRISPR-Cas9 gene-editing system delivered by electroporation was used in mouse retinas to test the role of cis-regulatory module B108, an enhancer of a transcription factor Blimp1 that controls the proper ratio of rods and bipolar cells. The targeted regions of the genome could be successfully deleted *in vivo* [121]. In a rat model of autosomal dominant retinitis pigmentosa, a subretinal injection of plasmid bearing guide RNA/Cas9 combined with electroporation was able to disrupt the mutant Rho(S334) allele of the rhodopsin gene, which prevented retinal degeneration and improved vision [122]. Recently, CRISPR-RNP was packaged into glutathione (GSH)-cleavable covalently cross-linked polymer or a nanocapsule and delivered to the retina, showing functional results [123]. The advantages of using this nanocapsule for delivery are its biodegradability and lack of toxicity. Another NP delivery of CRISPR-Cas9 *via* silica–metal–organic framework (SMOF) hybrid nanoparticle was reported to have 7% expression of the edited gene in the retinal area [124].

Genome editing *via* CRISPR-Cas9 is a rising topic of interest in gene therapy. Since there are not many studies that demonstrate the delivery of the CRISPR-Cas9 system to the anterior segment of the eye, more research must be done. The successes reported from using viral and non-viral methods for delivery of this gene-editing system to the posterior eye are a starting point for further investigations related to the anterior segment pathologies.

3. ROUTES OF ADMINISTRATION FOR DRUG DELIVERY

There are four main routes of drug administration to treat diseases of the anterior segment of the eye, namely topical, subconjunctival, intracameral, and systemic, as illustrated in (Fig. 1). The most important factors of ophthalmic drug administration are the duration of drug release, target location and ocular barriers, and patient compliance [125]. Based on these factors, each mode of administration has its advantages and limitations.

3.1. Topical

Topical delivery is the most common form of ocular drug delivery for anterior segment diseases. It is simple, non-invasive, self-administrable, and usually formulated as eye drops. Several factors such as blinking, solution drainage, and the tear film reduce the bioavailability of topically applied drugs. Due to constant tear turnover, topical drugs get washed out of the eye and require repeated application, which is affected due to poor patient compliance [126]. Recent studies have focused on the use of nanocarriers to improve bioavailability of topically applied drugs. Nanocarriers have a better ability to adhere to the ocular surface, sustained delivery, and improved delivery of poorly soluble drugs [127]. Corneal and conjunctival barriers can also create impediments to effective topical treatment due to the various layers of the cornea and the presence of conjunctival blood capillaries [128]. It is also important to note that whereas this route is preferred for treating several corneal and conjunctival disorders, many successful studies utilizing topical administration are performed in mice that have thinner cornea (~150 μm thick) as compared to humans (~550 μm thick). Hence, to reach the deeper layers of the cornea, other routes of drug administration, such as intrastromal delivery or penetration enhancers, may be required.

3.2. Sub-conjunctival

Sub-conjunctival implants or injections are administered under the conjunctiva of the eyeball (epibulbar) or the conjunctiva lining the eyelid (subpalpebral). Sub-conjunctival delivery bypasses the corneal barrier and also circumvents the washout and precorneal drainage limitations associated with topical drug use. Injected drugs may be designed as sustained release formulations that provide effective treatment for longer duration or high concentration with a single injection [129].

3.3. Intracameral

Intracameral injections are administered directly into the anterior chamber. This method delivers high concentration of the drug and circumvents the corneal side effects seen with topical administration. It is often used to deliver an anesthetic or to inject antibiotics to prevent endophthalmitis that may occur during cataract surgery [125].

3.4. Systemic

Systemic drug delivery is achieved by intravenous, intraperitoneal, or oral administration. It is most often used for treating posterior segment diseases but has also been used to treat conditions affecting the eyelids and sclera, as well as in the treatment of herpetic stromal keratitis. However, the blood-aqueous barrier limits the effective delivery of a drug by this

method to the anterior segment [130]. Moreover, systemic delivery is also associated with side effects and toxicity related to high drug concentration [129].

4. APPLICATIONS OF GENE THERAPY IN DISEASES

Diseases of the anterior segment of the eye, particularly corneal opacity, are the fourth leading cause of visual impairment and blindness, as reported by the World Health Organization [131, 132]. These diseases encompass a wide variety of pathological conditions, including various types of trauma, infections, and hereditary diseases, among others, that cause abnormal wound healing, inflammatory reactions, corneal cloudiness, scarring, and graft rejection. Due to its relative accessibility, the anterior segment is uniquely suited for gene delivery to treat these diseases. We describe here the applications and advances of gene therapy for the treatment of various such conditions.

4.1. Corneal and Conjunctival Fibrosis and Scarring

The wound healing response to corneal injury often results in stromal scar formation and fibrosis characterized by the emergence of myofibroblasts and the abnormal deposition of extracellular matrix (ECM) proteins. TGF-β plays a key role in the transdifferentiation of stromal fibroblasts to myofibroblasts and the pathogenesis of ocular fibrosis [133]. The expression of α-smooth muscle actin (α-SMA) due to TGF-β activity is the hallmark of myofibroblast phenotype along with the deposition of various ECM proteins such as collagens types I, III, IV, and fibronectin. The excessive synthesis of ECM proteins alters the properties of the existing ECM, resulting in scarring and haze [134]. Therefore, TGF-β has become the primary target of gene therapy for the prevention and treatment of fibrotic ocular surface disease.

For anti-fibrotic gene therapy, Mohan *et al.* [23, 135] have extensively studied decorin, a small leucine-rich proteoglycan [136] involved in ECM organization. Decorin forms a complex with TGF-β, thus decreasing its bioavailability, blocking its binding to its receptors, and resulting in diminished fibrotic activity [137–139]. Transfection of *decorin* gene cloned into the mammalian expression vector in human corneal fibroblast cultures resulted in a downregulation of expression of α-SMA, collagens types I, III, IV, and fibronectin that was induced by TGF-β1, thus resulting in a significant decrease in TGF-β induced transdifferentiation of corneal fibroblasts to myofibroblasts and a reduction in fibrosis [135]. Subsequent *in vivo* studies by the same group showed therapeutic potential of AAV-5 based *decorin* gene therapy with inhibition of fibrosis in rabbit corneal stroma after PRK, without significant keratocyte apoptosis or immune cells infiltration [23]. Similarly, non-viral gene therapy using polyethylenimine (PEI) NP was also employed to deliver decorin gene safely and efficiently in equine corneal cells and reduce fibrosis *in vitro* [140].

Although TGF-β is central to the process of fibrosis, it is also important for the normal wound healing process. Therefore, more recent therapeutic approaches were directed towards the downstream targets of TGF-β signaling. PEI NP--mediated delivery of soluble *TGF-β receptor 2* gene (*sTGFBRII*) fused to the Fc portion of human IgG showed attenuation of TGF-β1 induced transformation of cultured human corneal fibroblasts to myofibroblasts with no significant cell death. This effect could possibly be due to

sequestering adsorption of TGF-β or by acting as a dominant-negative receptor [141]. Other targets of TGF-β signaling include cytokines such as hepatocyte growth factor (HGF) and bone morphogenic protein 7 (BMP7) that inhibit TGF-β activity in tissue fibrosis [142, 143], as well as inhibitory Smad7 protein, a negative regulator of TGF-β signaling [144]. In a mouse model of corneal alkali burn, burned corneas treated with Ad-BMP7 showed decreased scarring after 20 days as compared to the control. The authors further demonstrated that the effect was due to the activation of Smad1/5/8 signaling and partial suppression of the phospho-Smad2 signal, thus counteracting TGF-β effects [145]. Similarly, localized *BMP7* gene delivery by PEI-conjugated gold NPs inhibited fibrosis by counterbalancing TGF-β1 mediated pro-fibrotic Smad signaling in rabbit corneas in a PRK model of fibrosis [50]. The Mohan group also used combination therapy with BMP+HGF to treat corneal fibrosis and restore transparency in rabbits after alkali injury by two independent pathways. BMP7 acted by antagonizing TGF-β and inhibited the activation of new myofibroblasts, while HGF promoted apoptosis of established myofibroblasts. This combination therapy caused minimal ocular toxicity, but this was a short-term study of 3 weeks, and long-term effects remain to be seen [146]. Wang *et al.* 2013 also showed that lentivirus-mediated overexpression of the Smad7 gene mitigated the activation of TGF-β signaling in rat corneas by decreasing the phosphorylation of Smad2 and the expression of TGF-β2 after PRK surgery [40]. Similarly, in an *in vivo* rabbit model of corneal fibrosis induced by PRK (Fig. 2), AAV-5 mediated Smad7 gene therapy was safe and effective in inhibiting corneal scarring [34].

Apart from the cornea, TGF-β has also been targeted for another ocular surface scarring. In a model of aggressive scarring after glaucoma filtration surgery, TGF-β2 AON treatment significantly reduced conjunctival scarring after a single administration and was more potent than AON to TGF-β1, most likely because TGF-β2 is the predominant TGF-β isoform associated with conjunctival scarring [147, 148]. Similarly, reduction in subconjunctival scarring post glaucoma filtration surgery in a rabbit model was also achieved by inhibiting SPARC (secreted protein, acidic and rich in cysteine) that is associated with excessive accumulation of collagen, using positive-charge tuned gelatin hydrogel-based delivery [149].

Other approaches to reduce corneal scarring include the overexpression of peroxisome proliferator-activated receptor-γ (PPAR-γ) *via* Ad gene transfer to suppress TGF-β1 induced profibrotic generation of myofibroblasts. Ad-PPAR-γ treatment resulted in the suppression of profibrotic matrix metalloproteinase MMP-9 in macrophages as well as of collagen type I in the presence of TGF-β1. Moreover, it also stimulated the *in vitro* proliferation of corneal epithelial cells but not of fibroblasts, possibly by the inhibition of TGF-β1. In addition to the *in vitro* effects, PPAR-γ overexpression in a mouse model of corneal alkali burns also promoted epithelial healing post-injury *via* the mRNA reduction of profibrotic MMP and rapid restoration of the basement membrane [150]. Galiacy *et al.* (2011) prevented abnormal collagen deposition by overexpressing MMP-14 *via* a single injection of AAV vector in a mouse model of corneal full-thickness incision [70]. This treatment also caused a marked reduction in corneal opacity as well as decreased expression α-SMA and collagen type III, but with toxicity at higher doses [70]. Keratocyte proliferation was targeted by topical administration of retroviral gene therapy to express dominant-negative cyclin G1 in excimer laser-induced corneal haze after phototherapeutic

keratectomy (PTK) in rabbits. The use of eye drops of mutant cyclin G1 retroviral vector inhibited keratocyte proliferation and significantly reduced abnormal deposition of ECM proteins. The treatment had no negative effects on epithelial proliferation, possibly due to the protection of the limbal area from vector exposure and the poor *in vivo* transduction efficiency of retroviral agents in epithelial cells [151]. AAV-mediated expression of the immunomodulatory and anti-inflammatory molecule, human leukocyte antigen-G (HLA-G) in rabbit corneas also inhibited α-SMA expression indicating a reduction in myofibroblast activation and fibrosis. However, it is unclear if HLA-G has a direct link to myofibroblast generation and the effect seems more likely to be linked to its anti-inflammatory actions [31].

4.2. Corneal Epithelial Wound Healing

Wound healing of the corneal epithelium is a clinically relevant phenomenon and has been a subject of numerous studies. It is a complex process involving cell adhesion, migration, proliferation, differentiation, stratification, and ECM remodeling mediated by various growth factors, cytokines, and cell signaling events [152]. Our group has extensively used both viral and non-viral gene therapy for the delivery of target genes in the human diabetic corneas characterized by slow epithelial wound healing. These genes with altered expression in diabetic corneas [153, 154] coded for HGF receptor, c-Met, and proteinases, cathepsin F and MMP-10. HGF/c-Met signaling plays an important role in wound healing-related processes of cell migration, proliferation, and apoptosis in various organ systems, including the cornea [155–159]. Gene microarray analysis showed that HGF was upregulated in diabetic corneas with downregulation of c-Met [154]. The effect of c-Met on epithelial wound healing was tested using *c-Met* gene transduction with rAd vector in human diabetic organ-cultured corneas. Overexpression of c-Met normalized the expression of diabetic markers, laminin, nidogen-1, and integrin α3β1, and stimulated wound healing (Fig. 3) by restoring HGF signaling and activation of p38 MAPK pathway [13]. The diabetic corneas also have increased expression of proteinases, cathepsin F and MMP10 [153, 154]. Ad-mediated overexpression of these genes in non-diabetic corneas resulted in diabetes-like changes [160]. Conversely, rAd-driven shRNA silencing of *cathepsin F* and *MMP-10* genes improved diabetic epithelial wound healing *via* the activation of EGFR and Akt signaling and also restored the expression of diabetic markers integrin α3β1 and nidogen-1, as well as diabetes-suppressed putative stem cell markers, ΔNp63α, keratin 17, and ABCG2 [109, 161]. Combined therapy with c-Met overexpression was even more efficient [109]. rAd gene therapy with c-Met and shRNA to cathepsin F and MMP-10 for wound healing was also successful if only the limbus harboring epithelial stem cells was transduced [110]. This Ad-mediated gene therapy was unexpectedly toxic for limbal epithelial stem cell (LESC)-enriched primary limbal epithelial cell (LEC) cultures and resulted in impaired wound healing [161]. Therefore, a non-viral approach was tested with nanobioconjugates (NBC) constructed on natural polymalic acid (PMLA) scaffold to deliver AON to target cathepsin F, MMP-10, and c-Met inhibiting miR-409 in human diabetic LEC as well as human diabetic organ-cultured corneas. The application of NBC (Fig. 4) safely and efficiently ameliorated diabetes-impaired wound healing and normalized the LESC and diabetic marker expression [100]. This may be a promising new approach for normalizing wound healing in diabetic corneas.

Another growth factor that modulates corneal epithelial wound healing is the OGF that acts as a negative regulator of epithelial proliferation [162]. Transfection of OGFr antisense using gene gun into rat corneas after central corneal abrasion resulted in fewer epithelial defects and accelerated wound healing, whereas the sense OGFr construct delayed healing [83, 84]. Delivery of anti-apoptotic gene *bcl-xL* has also been used to reduce cell death triggered by corneal epithelial debridement [62].

MiRs can also be utilized for achieving gene silencing and hence can be used to modulate target genes [152]. Saghizadeh *et al.* demonstrated that antisense (antagomir) to diabetes-elevated miR-146a enhanced corneal wound healing *via* the activation of EGFR and p38 signaling and normalized the expression of diabetic and stem cell markers [163, 164]. MiR-155-5p also promoted the repair of corneal injury and reduced corneal epithelial permeability in rats by decreasing the expression of myosin light chain kinase and phosphorylation of myosin light chain [165]. However, the use of miRs for gene therapy should be thoroughly validated for due to the known effect of most miRs on multiple targets.

4.3. Corneal Graft Survival

The cornea is the most commonly transplanted tissue in humans worldwide. Although avascular corneas have the immune privilege [166], a substantial number of corneal grafts undergo allograft rejection after PKP, varying between 5 and 40% [167]. There are significant side effects with the prolonged use of systemic immunosuppression to reduce graft rejection [168]. A gene modification approach was thus used in various models by introducing immunoregulatory molecules, anti-angiogenic factors, or by inhibiting apoptotic pathways in donor and recipient corneas to prevent graft rejection [169].

4.3.1. Immunomodulatory Factors—Overexpression of immunomodulatory growth factors and cytokines has been extensively studied to prevent corneal graft rejection. Oral *et al.* (1997) showed that it was possible to infect *ex vivo* human corneas with recombinant Ad to transduce the gene encoding the immunomodulatory CTLA-4 Ig protein to modify the alloresponse directed against the graft [170]. They further demonstrated significantly prolonged corneal graft survival after a single administration of this viral vector [171]. Zhou *et al.* (2010) targeted TGF-β1, a potent immunosuppressive cytokine in the corneal endothelium using rAAV prior to transplantation to prolong graft survival in a high-risk rat model [172]. Corneal endothelial cells are the most important targets for graft survival gene modification due to the irreversible damage caused to these cells during rejection because of their negligible replication capacity in humans. Ad-mediated local delivery of anti-inflammatory *IL-10* gene into donor corneal endothelium immediately before transplantation could prolong corneal allograft survival in sheep [173]. In contrast, local *ex vivo* liposome-mediated viral *IL-10* gene transfer or *ex vivo* Ad-IL-10 gene transfer had no effect on graft survival, but the systemic expression of IL-10 using rAd extended graft survival in rats [174]. Lentivirus carrying *IL-10* transgene also prolonged ovine orthotopic corneal allograft survival, but not as efficiently as Ad vector on account of the relatively low transgene levels [175]. More recently, Ad and lentiviral vectors were used to transduce limbal graft tissue *ex vivo* with biologically active IL-10, leading to delayed rejection in rats [15]. In addition to *IL-10* gene transfer, blocking of pro-inflammatory IL-12

by local overexpression of the IL-12 p40 inhibitory subunit also prolonged sheep corneal graft survival [176]. On the other hand, no prolongation of allogeneic graft survival was obtained in rat corneas by Ad-mediated IL-12 p40 transfer [177]. Non-viral Entranster™, an NP vector, was employed to deliver *CD25* siRNA that resulted in prolonged graft survival in rat corneas *via* the upregulation of IL-10 expression [107]. Increased graft survival and inhibition of neovascularization (Fig. 5) were observed after stromal (before grafting) or anterior chamber (after grafting) injection of a plasmid bearing *IL-1 receptor antagonist* gene [178]. Inhibiting T cell activation by lentiviral gene therapy was also used to suppress graft rejection. Lentivirus mediated overexpression of programmed cell death-ligand 1 (PD-L1) in organ-cultured allogeneic rat corneas decreased $CD3^+CD8^+CD161^-$ and $CD3^+CD8^+CD161^+$ T cells upon transplantation of modified corneas and reduced inflammatory cytokines IFN-γ and IL-6 resulting in significantly better graft survival [179].

4.3.2. Pro- and Anti-apoptotic Factors—Corneal endothelial damage caused during graft rejection may be a result of apoptotic cell death [180]. Overexpression of the anti-apoptotic baculoviral p35 protein in corneal epithelial sheets reduced the priming of T cells in draining lymph nodes after transplantation [181]. Anti-apoptotic genes coding for bcl-xL, bcl-2, survivin, and p35 were also tested in cultured corneal endothelial cells, with the best anti-apoptotic effect obtained with bcl-xL. The respective gene was then introduced in the endothelium of donor corneas using lentivirus resulting in improved survival of allografts in mice [182]. Endothelial cell loss during storage is also a cause for donor cornea rejection for transplantation. Therefore, Fuchsluger *et al.* applied lentivirus-mediated gene transfer of *bcl-xL* and *p35* to successfully prolong the survival of endothelial cells during preservation/storage, which could prove beneficial for future corneal transplantations [183, 184].

4.4. Corneal Neovascularization

Pathological corneal neovascularization (CNV) causes a loss of corneal transparency and visual acuity and is a major risk factor for graft rejection after corneal transplantation and a postoperative complication. Angiogenic privilege, the balance between a low level of angiogenic and a high level of antiangiogenic factors, has been the key to maintaining corneal avascularity [76]. Significant progress has been made in the understanding of the mechanisms involved in angiogenic privilege, which has led to gene therapy approaches using transgenic expression of antiangiogenic factors or inactivation of proangiogenic factors *via* gene silencing [185]. The angiogenic VEGF pathway has been targeted by gene transfer of VEGF receptors, Flt-1 and Flk-1. Gene transfer of soluble Flt-1 using rAAV in cauterized rats [186] and using non-viral micellar nano vector in mice [187] successfully inhibited CNV. Recombinant Ad-mediated delivery of soluble Flk-1 in cauterized rats had a similar effect [188]. Recombinant Ad-driven VEGF AON [189] and poly(lactic co-glycolic acid) NP-mediated delivery of VEGF-A shRNA [190] were both able to downregulate VEGF and reduce corneal neovascular response. Subconjunctival delivery of lipoplexes carrying the gene encoding GA-binding protein (GABP), a transcription factor that regulates the expression of angiogenic factors VEGF and roundabout4 (Robo4), delayed CNV in a mouse model of corneal injury, but the effect was relatively short-lived [191]. In addition to these, endostatin and angiostatin, two antiangiogenic factors that act through the inhibition of VEGF and FGF-2, have been employed for CNV gene therapy. AAV-mediated *endostatin*

and *angiostatin* subconjunctival gene delivery (Fig. 6) inhibited CNV in mouse and rat corneal injury models [192, 193]. Equine infectious anemia virus (EIAV)-based lentiviral vector harboring both *endostatin* and *angiostatin* genes suppressed CNV and decreased immune cell infiltration and corneal opacification in a rabbit model of corneal rejection [39]. Recombinant retroviral vectors encoding multiple murine antiangiogenic genes (soluble *Flk-1, Tie2, endostatin*) inhibited the proliferation and migration of human umbilical vein endothelial cells (HUVEC) *in vitro* and reduced CNV in mice [194].

The immunomodulatory and anti-inflammatory molecule, human leukocyte antigen-G (HLA-G) has also been delivered *via* an AAV vector to reduce injury-induced CNV and immune cell infiltration in rabbits [31]. Although gene therapy for CNV has been extensively investigated, only aganirsen AON that inhibits insulin receptor substrate-1 expression has been successfully tested clinically and safely administered as eye drops resulting in the inhibition of keratitis-induced CNV, reducing the need for corneal transplantation [95, 96].

Other therapeutic approaches include overexpression of miR-204 (downregulated in neovascularized mouse corneas) that attenuated CNV in injured mouse cornea *via* multiple pathways (Angpt1/Tie2/PI3K/Akt) [30]. Synthetic amphiphile INTeraction-18 (SAINT-18) plasmid carrying anti-angiogenic *pigment epithelium-derived factor* gene inhibited CNV in an experimental model of rat corneal angiogenesis [195]. More recently, downregulation of proangiogenic MMP-9 was achieved using lipid NPs to deliver shRNA for treating CNV [196]. Similarly, a cholesterol-modified siRNA delivery system targeting stromal cell-derived factor 1 (SDF-1) inhibited neovascularization and HUVEC proliferation by inhibiting Akt signaling [197]. Overall, gene therapy to inhibit pathological corneal neovascularization by acting on a number of promising targets may produce newly approved drugs in the near future.

4.5. Genetic Corneal Dystrophies

Corneal dystrophies are heritable progressive disorders that can alter the corneal shape, decrease corneal transparency, and ultimately cause vision loss in both eyes. There are 29 disorders characterized as corneal dystrophies, with 22 disorders associated with genetic abnormalities, which may possibly be amenable to treatment by gene therapy [27]. Existing treatments for severe corneal dystrophies that are routinely used today are keratoplasty and corneal transplantation. However, there are drawbacks of these procedures, such as graft rejection, procedural complications, and a limited supply of healthy donor corneas outside of the United States. Additionally, there is a wide range of phenotypes and severity of clinical symptoms associated with corneal dystrophies. Therefore, other current treatments, such as ointments and eye drops, do not address the individualized concerns and are largely symptomatic [198]. By identifying the underlying genetic defect, gene therapy can emerge as a better and more individualized intervention in treating corneal dystrophies.

Most corneal dystrophies exhibit a monogenic or Mendelian pattern of inheritance, which makes them ideal candidates for gene therapy. Gene therapy interventions that are able to knock out mutant genes or block translation of mutant proteins may be more effective in autosomal dominant corneal dystrophies, such as Meesmann epithelial corneal dystrophy

(MECD), lattice corneal dystrophy type 1 (LCD1), granular corneal dystrophy (GCD), and FECD. Allele-specific siRNAs and the CRISPR-Cas9 system already emerged as potential treatment options for the prevention of the MECD and LCD corneal pathology.

In a study using corneal limbal biopsy from patients with MECD, siRNA treatment completely blocked endogenous mutant keratin 12 alleles in limbal epithelial cell cultures with no effect on wild-type allele expression. This approach, in conjunction with a suitable siRNA delivery vector, may have the potential to treat individuals with MECD [106]. A later study by the same group using the CRISPR-Cas9 system showed *in vivo* gene editing of a heterozygous disease--causing SNP that results in a novel protospacer adjacent motif to target keratin 12 mutation [199]. Recently, this group has designed a corneal bioluminescence K12-luciferase multitarget knock-in mouse that will support the *in vivo* real--time study of siRNA delivery of mutant K12 allele expression for the prevention of MECD pathology [200].

In an *ex vivo* model of limbal biopsy from patients with LCD1, a siRNA specific to the *TGF-β-induced (TGFBI)* gene allele was shown to efficiently suppress the mutant allele, indicating this promising gene silencing approach in other TFBI-associated corneal dystrophies as well [201]. A similar approach using the CRISPR-Cas9 system also showed interference of the *TGFBI* mutant alleles in corneal dystrophy models. However, this approach lacked the ability to distinguish between wild-type and mutant alleles, thus yielding cleavage of the wild-type copy as well [202]. GCD is another corneal dystrophy that involves the mutant *TGFBI* gene; however, it is manifested as multiple discrete and irregular shaped granular opacities deposited in the corneal stroma. The CRISPR-Cas9 system was also utilized to correct the *TFGBI* mutation in GCD patient-derived primary corneal keratocytes *in vitro* with the use of a homology-directed repair [203]. However, a limitation of this study was modest correction efficiency ranging from 20.6% in heterozygous cells to 41.3% in homozygous cells. Although the CRISPR-Cas9 gene-editing system may eventually get into clinic to treat hereditary corneal dystrophies [204, 205], more work needs to be done to increase its efficacy.

FECD is the most common posterior corneal dystrophy and is characterized by guttae (extracellular matrix outgrowths of Descemet's membrane), endothelial cell degeneration, and stromal edema [7]. Most cases of FECD (70%) are caused by a *TCF4* trinucleotide repeat expansion leading to widespread changes in mRNA splicing. However, rare FECD cases are associated with mutations in other genes, including *COL8A2, SLC4A11, ZEB1,* and *LOXHD1* [206]. Emerging data point to the validity of gene therapy in FECD by targeting the *TCF4* gene. In a FECD subject-derived corneal endothelial cell model, the potency of AON therapy in ameliorating TCF4 repeat expansion-mediated toxicity was shown [101]. Additionally, the CRISPR-Cas9 system showed promise for FECD treatment. Recently, Rong *et al.* (2020) delivered dCas9 and *TCF4* repeat-targeting single guide RNA to patient-derived corneal endothelial cells by lipofection or lentiviral transduction. This treatment resulted in a significant, 10-fold reduction in the percentage of cells with toxic nuclear RNA foci and the number of foci per 100 cells [206]. With effective delivery to corneal endothelial cells, this method may become a viable treatment for FECD, reducing the necessity for corneal transplantation.

4.6. Herpetic Keratitis

Herpetic keratitis is a viral infection caused by the herpes simplex virus type 1 (HSV-1), commonly known as the cold sore virus, leading to corneal blindness and corneal graft rejection [98]. After infection, it develops indefinite latency in the trigeminal ganglion and then moves to other systems, including the cornea [207, 208]. Corticosteroids are commonly used to treat HSV-1 infection [209], but gene therapy approaches have also been tested in the cornea by targeting corneal inflammation and neovascularization, and more recently, by directly targeting the HSV-1 genome.

Several inflammatory mediators have been targeted to prevent HSV-1 mediated corneal inflammation. Topical administration of plasmid DNA with *IL-4, IL-10, IL-12,* and *IL-18* genes has shown a reduction in inflammatory lesion severity in various studies [68, 210–212]. Similarly, other mouse studies demonstrated that topical delivery of interferon (IFN)-α1, IFN-β, and IFN-γ through a plasmid DNA 12–24 hours before infection resulted in increased survival [213–217].

Some studies were aimed at developing a vaccine using gene therapy to introduce plasmid DNA encoding HSV-1 glycoproteins (gB, gC, gD, gE, and gI) to elicit humoral and cellular immunity with varying effects on herpetic keratitis [218, 219]. Inoue *et al.* used NP-mediated delivery of IL-21 and gD vaccine and inhibited herpetic keratitis in HSV-1 infected mice, with the combination showing better results than gD alone [220–222]. Although reducing inflammation and increasing survival is beneficial in treating herpetic keratitis, it does not actually cure the disease. For this reason, in recent years, the focus has shifted to targeting the HSV-1 genome for degradation using ribozymes [223], AONs [224], siRNA [225], aptamers [226], and homing endonucleases [227].

4.7. Glaucoma

Glaucoma is often associated with elevated intraocular pressure (IOP) caused by increased resistance to the drainage of aqueous humor through the conventional outflow system comprising the trabecular meshwork (TM) and the Schlemm's canal. It results in blindness in more than 60 million people worldwide. The TM located around the base of the cornea is the preferred pharmacological target for gene therapy in the treatment of glaucoma by reducing intraocular pressure to eventually retard visual field loss [228].

AAV vectors were able to induce long-term, safe delivery of transgenes to the TM of rats and monkeys [229]. Several AAV and Ad were also tested *in vitro* (in human immortalized TM cell lines) and *in vivo* by intracameral injection in mice, with varying results [230]. AAV was also efficiently used to deliver *MMP-3* (decreased in human glaucomatous aqueous humor) gene resulting in increased MMP-3 expression and decreased IOP [231]. In a rabbit model of glaucoma filtration surgery, Ad-mediated gene transfer of $p27^{KIP1}$, a member of the cyclin-dependent kinase inhibitor family, reduced IOP (Fig. 7) and prevented scarring [232]. Ad-mediated gene transfer of dominant-negative RhoA increased outflow facility in perfused human anterior segment cultures due to loosening of the cell-substrate and cell-cell attachments in the cells of the outflow pathway [233]. Numerous siRNA-based strategies have been studied for the treatment of glaucoma [234]. Silencing of RhoA using

siRNA in TM reduced IOP in mice [105]. Similarly, topical administration of siRNA to P2Y(2) purinergic receptor [235] and β2 adrenergic receptors [236] also reduced IOP in New Zealand rabbits, which could be used for glaucoma treatment. Recent *in vivo* data also showed that AAV2-mediated transfer of *exoenzyme C3 transferase* gene, which inactivates Rho by ADP ribosylation [237], *via* intracameral injection into mouse and monkey eyes, resulted in morphological changes in TM and IOP reduction [238]. These results suggest the importance of gene therapy as a promising future treatment of glaucoma.

4.8. Dry Eye Disease

Dry eye disease is caused by deficient tear production by the lacrimal gland or excessive evaporation. If left untreated, it may lead to corneal irritation and scarring [239]. Several autoimmune diseases like Sjögren's syndrome, rheumatoid arthritis, and lupus erythematosus are associated with dry eye disease [240]. Ad-mediated transfer of TNF-α inhibitor gene in an induced autoimmune dacryoadenitis rabbit model restored tear production to normal levels and reduced corneal surface defects [241]. This group also showed that AAV-mediated delivery of *IL-10* gene suppressed lacrimal gland immunopathology with decreased number of CD18+ cells and a smaller ratio of CD4/CD8 cells [242]. Lacrimal gland fluid movement was also restored by *aquaporin-1* gene therapy using an AAV vector administered to the submandibular glands in a mouse model of Sjögren's syndrome. The effect was due to BMP6 regulation [243]. MUC5AC, glycosylated mucin that acts as an ocular surface lubricant, is decreased in keratoconjunctivitis sicca, Sjögren's syndrome, and Stevens-Johnson syndrome [244–247]. Cationized gelatin-based NPs have been used to deliver the *MUC5AC* gene resulting in restoration of MUC5AC levels and reduction in inflammation with no vascularization or edema, and increased tear production in a mouse model of dry eye [248]. These promising data warrant further translation of dry eye gene therapy into human trials.

4.9. Mucopolysaccharidosis

Mucopolysaccharidosis (MPS) is an autosomal recessive disease affecting lysosomal storage resulting in corneal clouding [249]. In the eyes, the lack of lysosomal enzymes causes an accumulation of glycosaminoglycans (GAG). Based on the enzyme defect and disease severity, seven different forms of MPS have been recognized [250, 251]. Administration of Ad expressing human *β-glucuronidase* gene, which is mutated in MPS-VII, into the anterior chamber or intrastromal region of the affected mouse corneas effectively treated corneal clouding in MPS-VII [252]. A similar effect was also seen in a model of canine MPS VII [253]. Recombinant AAV-mediated transduction of the gene encoding *α-L-iduronidase* gene (IUDA) (mutation in this gene causes MPS-I) was able to restore IUDA function in the cornea with a potential to reverse MPS-associated corneal blindness [33]. Overall, gene therapy appears to have great potential in mitigating the ocular effect in various forms of MPS.

4.10. Aniridia

Aniridia is a congenital condition resulting in partial or complete iris hypoplasia/loss and most commonly affects both eyes. It may result from an ocular trauma or, more frequently, from a genetic disorder that may lead in the cornea to aniridia-associated keratopathy (AAK)

[254–256]. Most cases of aniridia present with haploinsufficiency truncating mutation in the *PAX6* gene, although about one-third of cases are associated with chromosomal rearrangements at the 11(13p) region [255, 256]. CRISPR-Cas9 system has been recently tested for gene therapy correction of *PAX6* mutations. A novel aniridia mouse model containing the small eye *PAX6* mutation (*Sey*) also found in patients was used for *in vitro* and *in vivo* germline correction of the *Sey* mutation *via* the CRISPR-Cas9 system. This correction was successful *in vitro* at about 35%, and *in vivo*, it resulted in the restoration of FLAG-tagged PAX6 protein expression and normalized eyes [257]. CRISPR-Cas9 technology has also been used to introduce a heterozygous nonsense mutation found in AAK patients' *PAX6* gene into cultured human telomerase-immortalized LECs. This resulted in a significant functional change negatively affecting cell proliferation, migration and detachment. The abnormal phenotype could be rescued by the introduction of recombinant PAX6 protein [258]. CRISPR-Cas9 technology has thus enabled the development of cellular and animal models for aniridia and the rescue of mutant phenotype as a proof-of-concept treatment to treat associated vision loss.

CONCLUSION

In the last decade, the gene therapy field has benefited from a variety of vectors and delivery methods, and important efficacy data have been obtained in animal models and humans. Currently, many of these newer approaches have been tested in the anterior eye segment as well and produced promising results. Although quite a few gene therapy drugs have been approved lately for human use, the major advances in this field pertaining to the anterior eye segment relate only to animal models or *in vitro* studies. The translation into the clinic is clearly lagging behind the developments for the posterior segment, including retinal diseases and age-related macular degeneration. In the anterior segment, topical AON aganirsen for corneal neovascularization has completed phase III clinical trial in Europe with positive results [95]. Another clinical trial in China for the use of CRISPR-Cas9 gene-editing therapy to treat refractory viral keratitis is currently recruiting patients. Although just a few, these developments give hope for the translation of gene therapy for the anterior segment conditions into the clinic in the near future.

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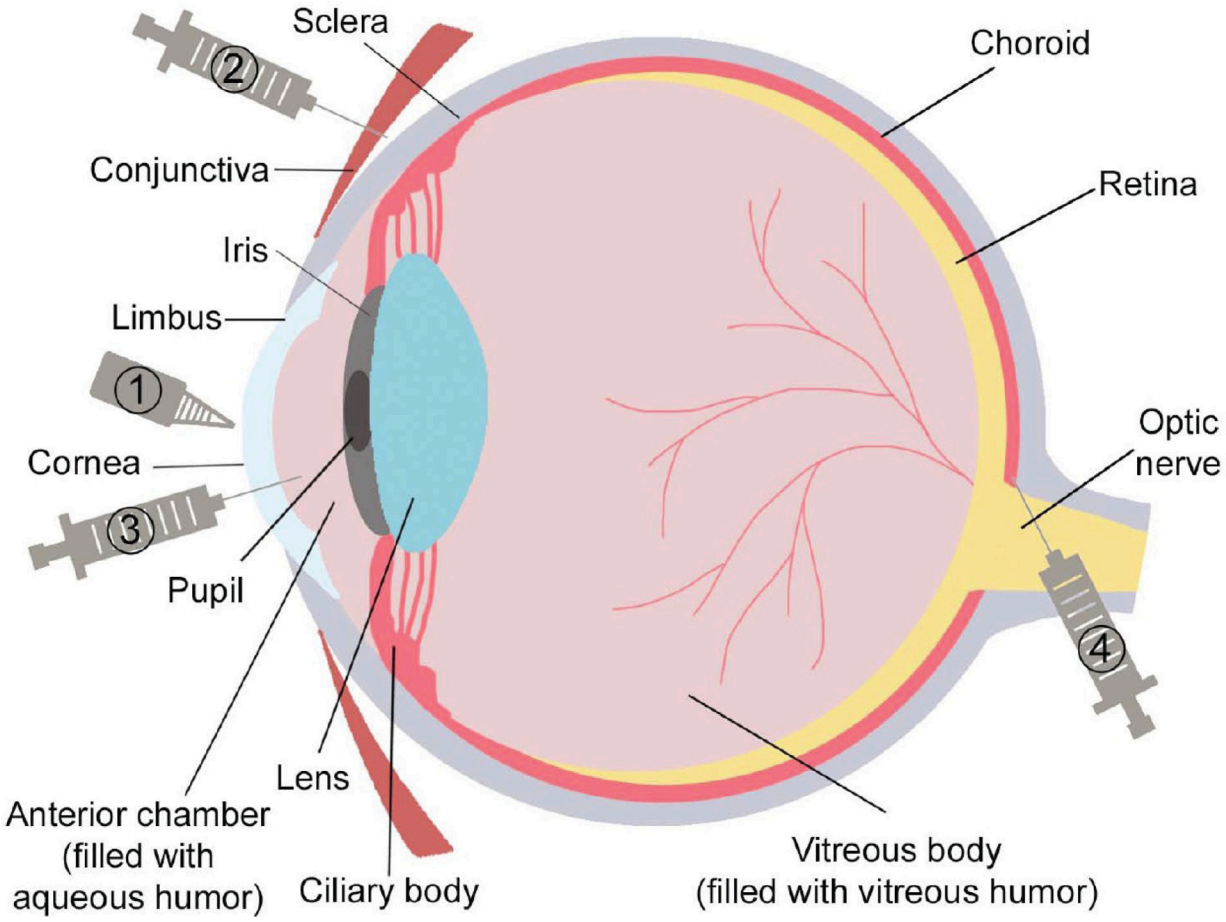

Fig. 1.

Routes of drug administration for the anterior segment of the eye. (1) Topical, (2) sub-conjunctival, (3) intracameral, and (4) systemic administration.

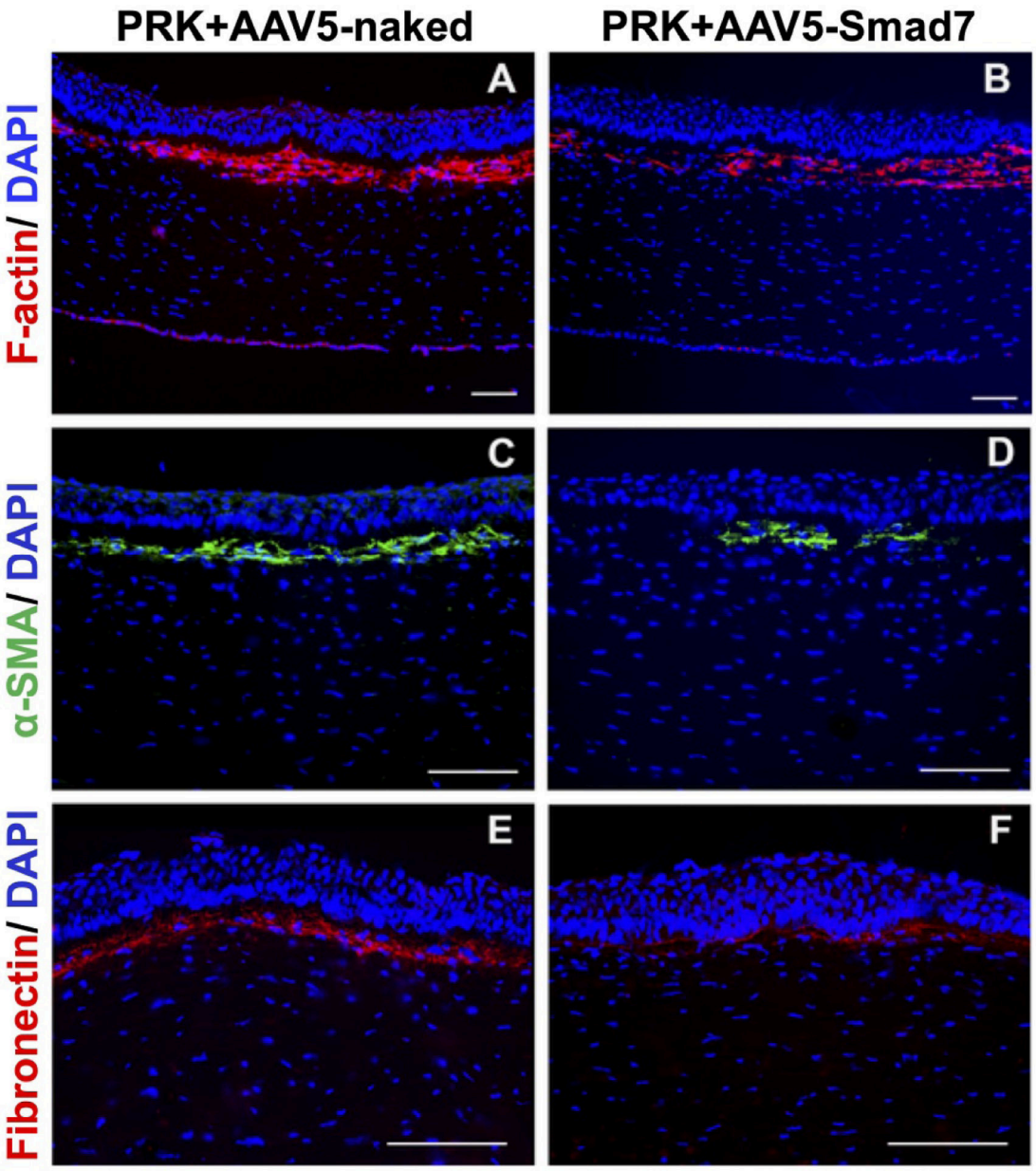

Fig. 2.

The images illustrate levels of profibrotic proteins in PRK-applied and AAV5-naked or AAV5-Smad7 treated eyes. Immunostaining for F-actin (A, B), α-SMA (C, D), and fibronectin (E, F), respectively. Scale Bar: 100 μm. (*Reproduced from: Gupta S, Rodier JT, Sharma A, Giuliano EA, Sinha PR, Hesemann NP, Ghosh A, Mohan RR. (2017) Targeted AAV5-Smad7 gene therapy inhibits corneal scarring in vivo. PLoS One 2017;12:e0172928).*

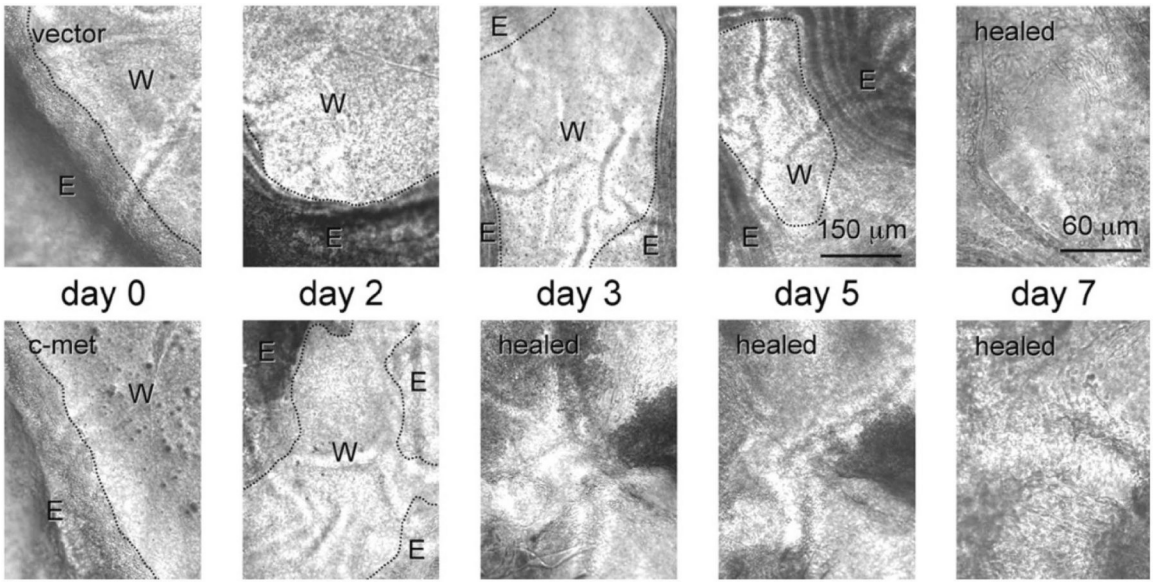

Fig. 3.

Dynamics of wound healing. A typical course of healing is presented for a c-met–transduced and vector-transduced cornea. rAV-vector–transduced diabetic cornea (top row) healed in 7 days, whereas rAV-met–transduced fellow cornea (bottom row) healed in 3 days. Pictures of live healing corneas are shown. Dashed line: shows the boundaries of the unhealed wound region. W, wound zone; E, migrating epithelium. *(Reproduced from: Saghizadeh M, Kramerov AA, Yu FS, Castro MG, Ljubimov AV. Normalization of wound healing and diabetic markers in organ cultured human diabetic corneas by adenoviral delivery of c-Met gene. Invest Ophthalmol Vis Sci. 2010;51:1970–1980).*

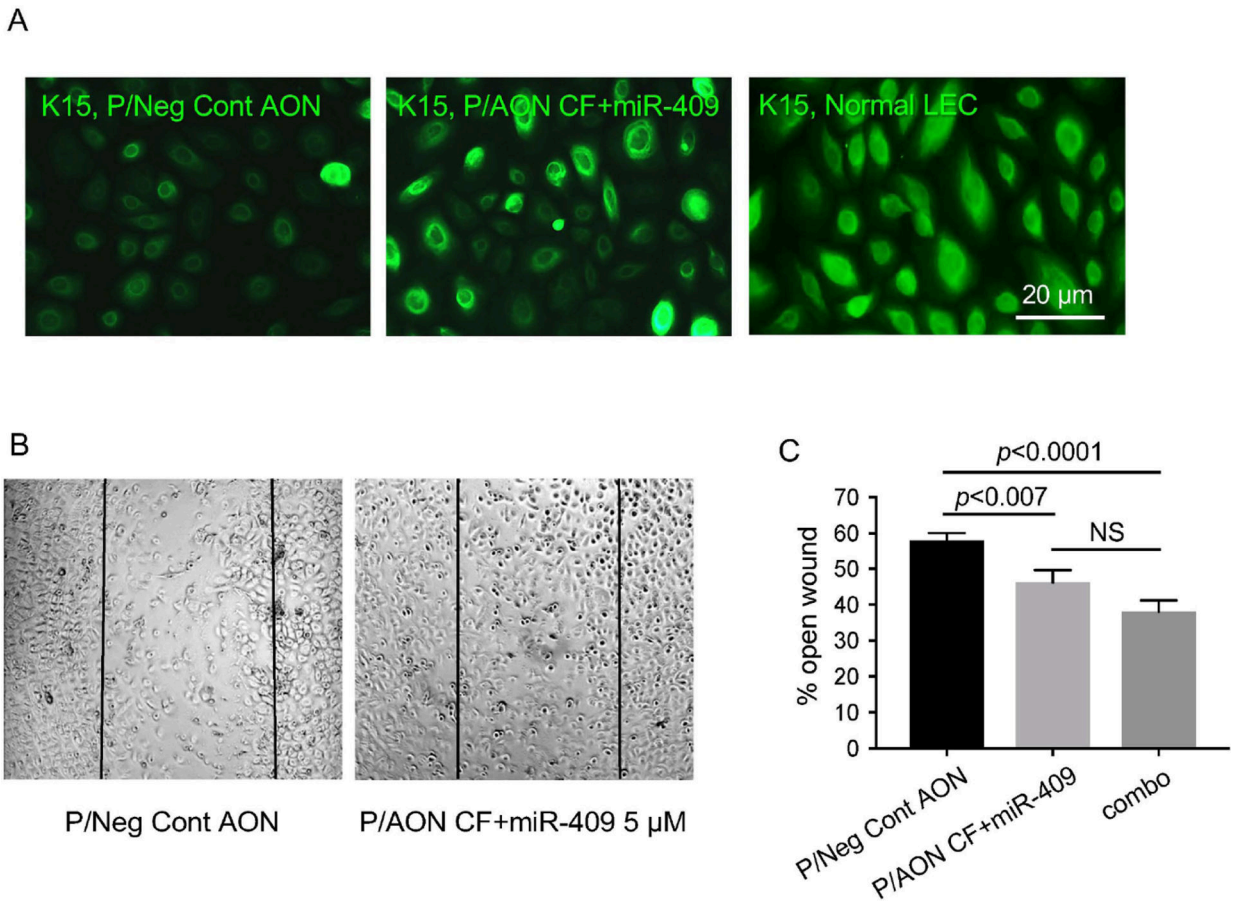

Fig. 4.

Normalizing effects of nanobioconjugates (NBCs) in diabetic limbal epithelial cell (LEC) cultures. A, By immunostaining, control LEC showed rare keratin (K)15-positive cells. After lead NBC treatment, the number of positive cells markedly increased and became similar to normal LEC. B, Lead NBC boosting c-Met expression by inhibiting miR-409 and blocking cathepsin F (CF) expression by the attached antisense oligonucleotides (AON) accelerated scratch wound healing in diabetic LEC (phase contrast). C, Lead NBC significantly reduced open scratch wound area compared to NBC with standard AON. The addition of a second NBC inhibiting MMP-10 (combo) further accelerated wound healing; however, the difference from lead NBC was not significant. The graph comprises data from four to five independent experiments using LECs from three independent cases. *(Reproduced with permission from: Kramerov AA, Shah R, Ding H, et al. Novel nanopolymer RNA therapeutics normalize human diabetic corneal wound healing and epithelial stem cells. Nanomedicine. 2021;32:102332).*

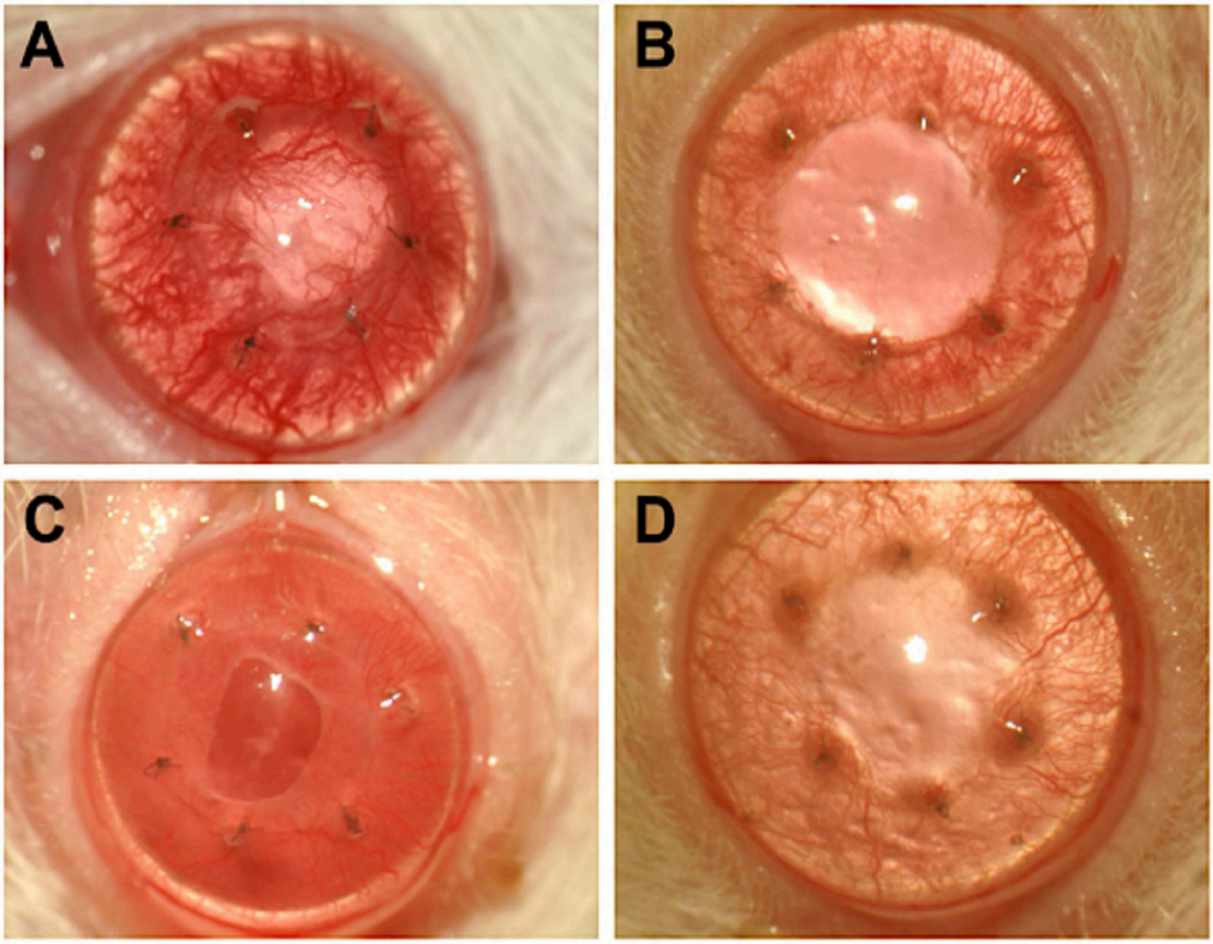

Fig. 5.

The appearance of the corneal graft 14 days after the operation. A, In group I (negative control; subconjunctival injection of saline after surgery), the graft showed oedema and new blood vessel growth into the centre of the graft. B, in the group II (IL-1ra plasmid gene injection into the corneal stroma before donor graft collection), the graft showed mild oedema, and fewer new blood vessels were observed than in controls. C-D, in the groups III (IL-1ra plasmid gene injection into the anterior chamber after graft-graft bed alignment suturing) and group IV (positive control; subconjunctival injection of IL-1ra protein after surgery), the graft was transparent, and no neovascularisation was found in the centre of the graft. *(Reproduced from: Yuan J, Liu Y, Huang W, Zhou S, Ling S, Chen J. The experimental treatment of corneal graft rejection with the interleukin-1 receptor antagonist (IL-1ra) gene. PLoS One. 2013;8:e60714).*

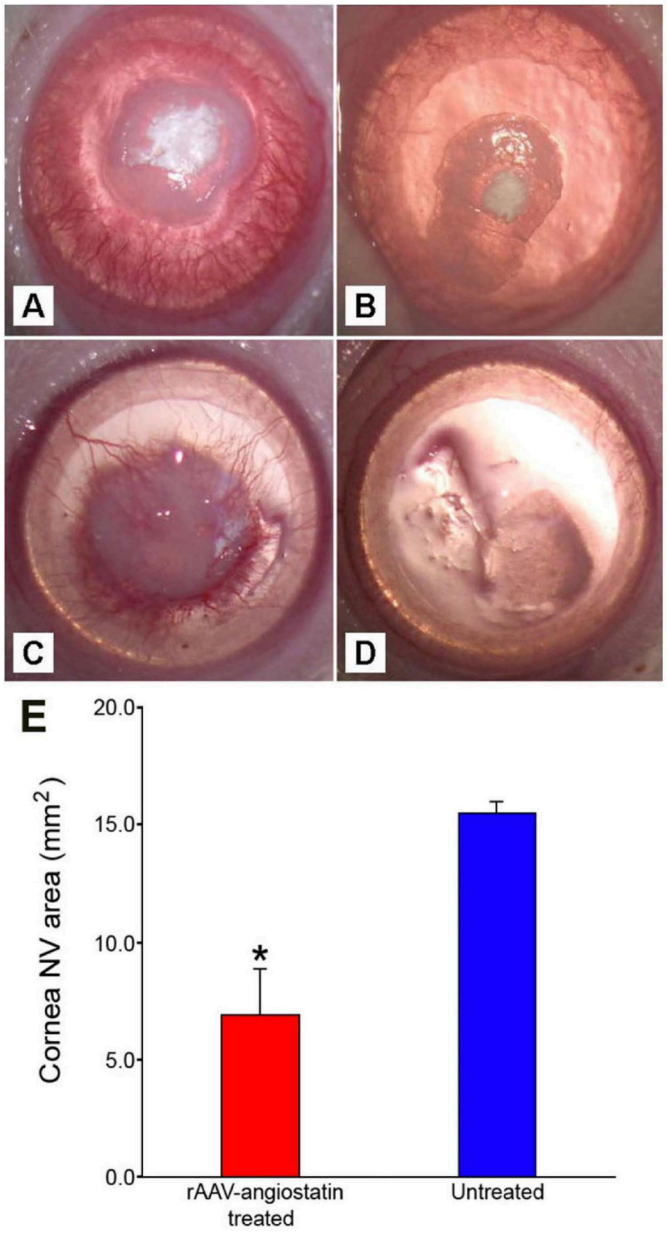

Fig. 6.

Effect of subconjunctival injection of rAAV-angiostatin on alkali burn-induced cornea neovascularization regression. One week before infliction of the alkali burn with 1 N NaOH in rats, the rats were assigned randomly either to a treatment group or control group. The treatment group received a subconjunctival injection of rAAV-angiostatin three weeks before the alkali burn, and the control group received blank rAAV viral vector treatment. Corneal NV was examined daily by a slit lamp for two weeks. Representative photographs of alkali burn-induced corneal NV at days 7 and 14are shown. A: Dense NV is growing toward the central cornea. Seven days after the alkali burn, there was severe corneal opacity and epithelial defects in the rAAV viral vector control eye. B: The rAAV-angiostatin injected eye seven days after the alkali burn is shown. Note the scarcity of corneal NV and the mild

to moderate corneal opacity. C: The rAAV viral vector control eye at day 14 follow-up is shown. Corneal NV and pannus maturation were evident. D: The rAAV-angiostatin injected eye 14 days after the alkali burn is shown. Not only the corneal NV was absent, but also the central corneal opacity was mild. E: The corneal NV area (mean±SD, n=6) measured at seven days after exposure to the alkali burn is shown. The rAAV-angiostatin treated group demonstrated a significantly smaller NV area compared with the rAAV viral vector control group (the asterisk means that $p<0.05$). *(Reproduced from: Cheng HC, Yeh SI, Tsao YP, Kuo PC. Subconjunctival injection of recombinant AAV-angiostatin ameliorates alkali burn-induced corneal angiogenesis. Mol Vis. 2007;13:2344–2352).*

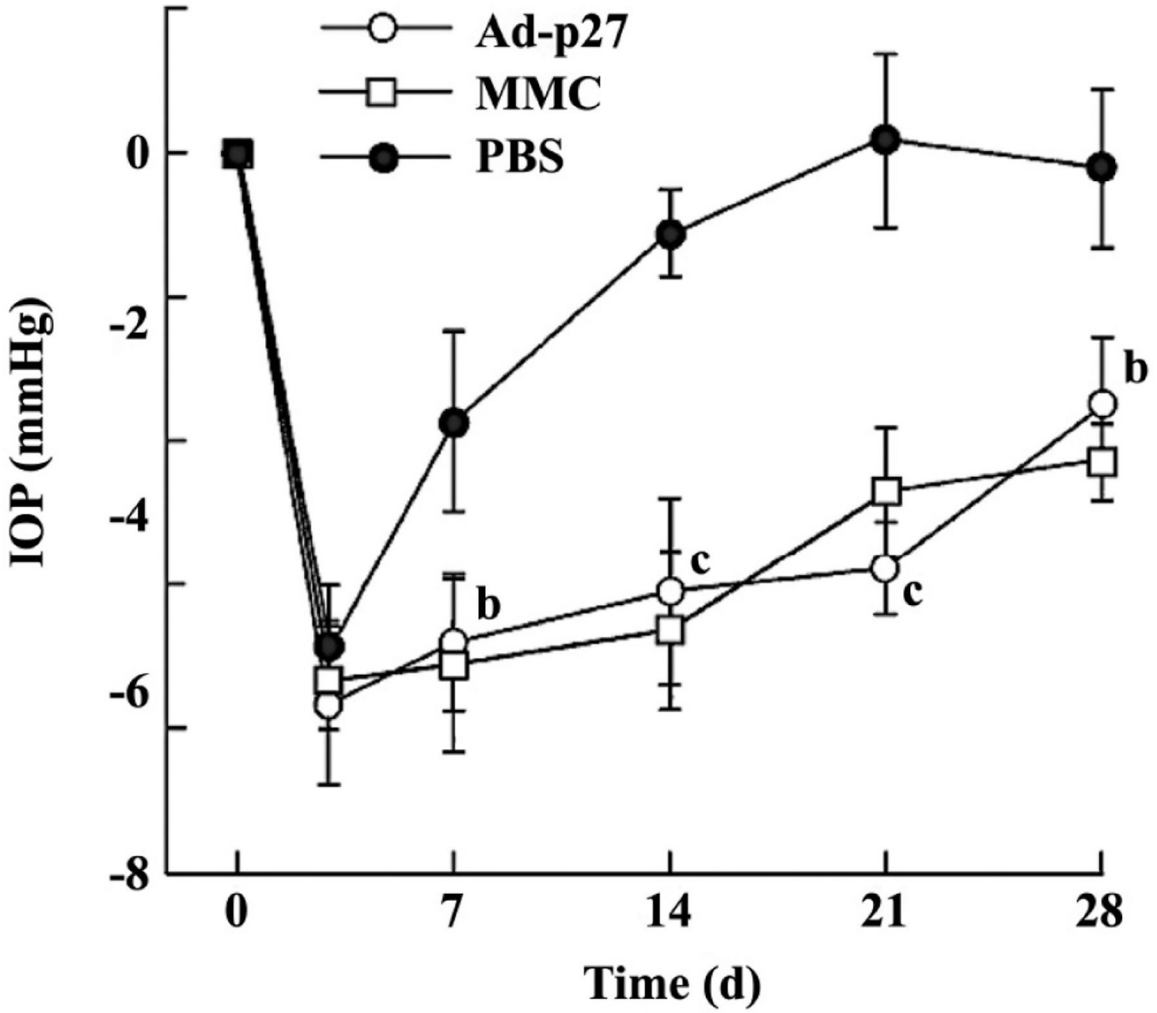

Fig. 7.

IOP of rabbits during a 28-d period treated with Ad-p27 *vs.* controls (PBS or mitomycin C, MMC) (n=6). Data are given as mean ± SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. *(Reproduced with permission from: Yang JG, Sun NX, Cui LJ, Wang XH, Feng ZH. Adenovirus-mediated delivery of p27(KIP1) to prevent wound healing after experimental glaucoma filtration surgery. Acta Pharmacol Sin. 2009;30:413–423).*

Table 1.

Gene therapy for the treatment of diseases of the anterior segment of the eye.

Corneal and Conjunctival Fibrosis					
Vector	**Gene**	**Organism**	**Route of Administration**	**Result**	**References**
Ad	PPAR-γ	Mouse	*In vitro* incubation with corneal cells; *in vivo,* the route is not specified, possibly topical	Suppression of pro-fibrotic factors and promotion of epithelial healing after corneal alkali burn	[150]
Ad	BMP-7	Mouse	*in vivo,* topical	Decrease in scarring of alkali burn corneas after 20 days of Ad-BMP-7	[145]
AAV-5	Decorin	Rabbit	*in vivo,* topical	Inhibition of fibrosis in corneal stroma after PRK	[23]
AAV-5	Smad7	Rabbit	*in vivo,* topical	Inhibition of corneal scarring after PRK	[34]
AAV (AAV8G9 vector)	HLA-G	Rabbit	*in vivo,* intrastromal injection	Inhibited α-SMA expression	[31]
Retrovirus	Cyclin G1	Rabbit	*in vivo,* topical	Inhibition of keratocyte proliferation, and reduction in abnormal deposition of ECM proteins after PTK; no effect on epithelial proliferation	[151]
Lentivirus	Smad7	Rat	*in vivo,* topical	Decrease in activation of TGF-β/Smad signaling after PRK	[40]
AON	TGF-β2	Mouse	*in vivo,* sub-conjunctival injection	Reduction in conjunctival scarring	[148]
PEI nanoparticle	Decorin	Horse	*in vitro*	Reduction in fibrosis in corneal cells	[140]
PEI nanoparticle	sTGFBRII	Human	*in vitro*	Attenuation of TGF-β1 induced transformation of corneal fibroblasts to myofibroblasts	[135]
PEI gold nanoparticle	BMP-7	Rabbit	*in vivo,* topical	Inhibition of fibrosis after PRK	[50]
PEI gold nanoparticle	BMP-7, HGF	Rabbit	*in vivo,* topical	Reduction in fibrosis, restoration of transparency after alkali injury	[146]
Positive charge tuned gelatin hydrogel siRNA	SPARC	Rabbit	*in vivo,* sub-conjunctival injection	Reduction in subconjunctival scarring post glaucoma filtration surgery	[149]
Corneal Epithelial Wound Healing					
Vector	**Gene**	**Organism**	**Route of Administration**	**Result**	**Reference**
Ad	c-Met	Human	*ex vivo,* corneal treatment in organ-culture	Normalization of diabetic marker expression and stimulation of epithelial wound healing in diabetic organ-cultured corneas	[13]
Ad-shRNA	Cathepsin F, MMP-10	Human	*ex vivo,* corneal treatment in organ culture	Restoration of putative stem cell and diabetic marker expression; stimulation of epithelial wound healing in diabetic organ-cultured corneas	[109, 161]
Ad-shRNA	c-Met and cathepsin F, MMP10	Human	*ex vivo,* limbal treatment in organ--cultured cornea	Restoration of putative stem cell and diabetic marker expression; stimulation of epithelial wound healing in diabetic organ-cultured corneas	[110]

Gold particle gene gun	OGFr	Rat	*in vivo*	Acceleration in corneal epithelial wound healing	[84]
Polymeric micelles	Bcl-xL	Mouse	*in vivo,* topical	Reduction in apoptosis triggered by corneal epithelial debridement in wounded corneas	[62]
Nanobioconjugates with AON	miR-409, Cathepsin F	Human	*ex vivo,* corneal treatment in organ-culture	Restoration of putative stem cell and diabetic marker expression; normalization of diabetic epithelial wound healing	[100]
Corneal Graft Survival					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**
Ad	CTLA-4	Human	*ex vivo* cornea organ culture, topical	Prolonged corneal graft survival	[170]
Ad	IL-10	Sheep	*ex vivo* cornea organ culture, topical	Reduced corneal graft rejection and prolonged graft survival	[173]
Ad	IL-12 p40	Sheep	*ex vivo* cornea organ culture, topical	Prolonged graft survival due to local intraocular production of p40 IL-12	[176]
Ad	IL-12p40	Rat	*ex vivo* cornea organ culture, topical	Increased graft survival due to increased IL-4 mRNA-expression	[177]
Corneal and Conjunctival Fibrosis					
Vector	**Gene**	**Organism**	**Route of Administration**	**Result**	**References**
Ad-liposome mediated	IL-10	Rat	*ex vivo* cornea organ culture, topical	Prolonged corneal graft survival *via* AdvIL-10	[174]
AAV-2	TGF-β1	Rat	*ex vivo* cornea organ culture, topical	Prolonged corneal allograft survival and inhibition of Ox-62 expression in grafts after high-risk PKP	[172]
Ad, Lentivirus	IL-10	Rat	*ex vivo* cornea organ culture, topical	Transfected limbal graft tissue, *ex vivo*, with IL-10, delayed graft rejection	[15]
Lentivirus	IL-10	Sheep	*ex vivo* cornea organ culture, topical	Prolonged corneal graft survival by 7 days in human compared to sheep	[175]
Lentivirus	PD-L1	Rat	Topical *ex vivo* transduction of organ-cultured corneas before transplantation	Significant increase in corneal graft survival *via* suppression of T-cell response and reduction of IFN-γ and IL-6	[179]
Lentivirus	p35	Mouse	*ex vivo* cornea organ culture, topical	Reduction of graft-mediated immune response due to $CD4^+$ T-cells decrease by p35	[181]
Lentivirus	p35, bcl-xL	Human	*ex vivo* cornea organ culture, topical	Increased survival and prolonged retention of EC morphology in cells expressing either p35 or Bcl-xL	[183]
Lentivirus	p35, bcl-xL	Human	*in vitro*, human endothelial cells/*ex vivo* cornea organ culture	Prevention of cell death by bcl-xL through apoptotic pathways. p35 was more protective *via* the intrinsic pathway	[184]
Lentivirus	bcl-xL, bcl-2, survivin, p35	Mouse	*ex vivo* cornea organ culture, topical	Bcl-xL had the highest yield in corneal graft survival in comparison to bcl-2, survivin, and p35 by reduction of apoptosis	[182]
Entranster™ NP-siRNA	CD25	Rat	*ex vivo* cornea organ culture, topical	Prolonged graft survival in rat corneas *via* the upregulation in IL-10 expression, reduced endothelial cell apoptosis, inflammatory cell infiltration and graft neovascularization	[107]

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Plasmid DNA	IL-1 receptor antagonist	Rat	*In vivo* subconjunctival or anterior chamber injection	Longer graft survival after gene therapy, higher corneal transparency, lower levels of TGF-β1, IL-1α, IL-1β and RANTES	[178]
Corneal Neovascularization (CNV)					
Vector	**Gene**	**Organism**	**Route of Administration**	**Result**	**Reference**
Ad	Flk-1	Rat	*in vivo,* anterior chamber injection	Inhibition of development of CNV in cauterized rat	[188]
Ad-AON	VEGF	Rat	*in vivo,* anterior chamber injection	Downregulation of VEGF production and inhibition of CNV	[189]
Ad	Vasohibin-1	Mouse	*in vivo,* subconjunctival injection	Reduction of CNV induced by alkali burn	[172]
AAV-2	Flt-1	Rat	*in vivo,* anterior chamber injection	Reduction in development of CNV in the stroma in cauterized rat	[186]
AAV	Endostatin	Mouse	*in vivo,* subconjunctival injection	Inhibition of CNV induced by silver nitrate cauterization	[193]
AAV	Angiostatin	Rat	*in vivo,* subconjunctival injection	Inhibition of CNV induced by alkali burn	[192]
AAV (AAV8G9 vector)	HLA-G	Rabbit	*in vivo,* intrastromal injection	Reduction in injury induced CNV and immune cell infiltration	[31]
AAV (AAVrh.8)	miR-204	Mouse	*in vivo,* intrastromal injection; sub-conjunctival injection	Attenuation of CNV in alkali burned cornea	[30]
Lentivirus	Endostatin, angiostatin	Rabbit	*ex vivo,* cornea	Suppression of CNV, decrease in immune cell infiltration and corneal opacification	[39]
Retrovirus	Flk-1, Tie2, endostatin	Mouse	*in vivo,* subconjunctival injection	Reduction in CNV	[194]
Polyplex micelle	Flt-1	Mouse	*in vivo,* subconjunctival injection	Inhibition of CNV	[187]
NP-shRNA	VEGF-A	Mouse	*in vivo,* intrastromal injection	Regression of CNV	[190]
Lipoplex	GA-binding protein	Mouse	*in vivo,* subconjunctival injection	Delay in CNV development, but short-lived effect	[191]
Lipid NP-shRNA	MMP-9	Human	*in vitro,* HCE-2 cells, HUVEC	Inhibition of cell migration, tube formation	[196]
AON aganirsen	Insulin receptor substrate-1 (IRS-1)	Human	*in vivo,* topical	Inhibition of keratitis induced CNV and decrease in a need for transplantation	[95, 96]
Corneal and Conjunctival Fibrosis					
Vector	**Gene**	**Organism**	**Route of Administration**	**Result**	**References**
Synthetic Amphiphile INTeraction-18	PEDF	Rat	*in vivo,* subconjunctival injection	Inhibition of CNV	[195]
Cholesterol-modified siRNA	SDF-1	Rat	*in vivo,* injection in Matrigel into the conjunctival sac	anti-angiogenic effect in corneal alkali burn model through downregulation of Akt signaling	[197]
Genetic Corneal Dystrophies					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**

siRNA lipofection	KRT12	Human	*in vitro*, corneal limbal epithelial cell cultures from limbal biopsy of MECD patients	Specific knockdown of mutant K12-Leu132Pro	[106]
Plasmid DNA (sgK12LP) CRISPR--Cas9	KRT12	Human HEK AD293 cells, Mouse	*in vitro, in vivo,* intrastromal injection	*in vitro* reduction of mutant *KRT12* mRNA expression *in vivo,* non-homologous end-joining repair resulting in frame-shifting deletions within the mutant *KRT12* allele	[199]
siRNA lipofection	TGFBI	Human	*ex vivo* limbal biopsy from LCD1 patients	siRNA specific to TGFBI suppressed the mutant allele	[201]
CRISPR-Cas9	TGFBI (R124H)	Human	*in vitro,* GCD2 patient-derived corneal keratocytes	CRISPR-Cas9-mediated homology-directed repair was effectively applied to correct the R124H mutation associated with GCD2 with no off-target effects.	[203]
AON	TCF4 repeats	Human	*in vitro,* FECD patient-derived corneal endothelial cell culture	Significant reduction in the incidence of toxic nuclear RNA foci, splicing protein (MBN-L1) recruitment to the foci, and downstream aberrant splicing	[101]
lipofection or lentivirus with CRISPR-dCas9	TCF4 repeats	Human	*in vitro,* FECD patient-derived corneal endothelial cell culture	10-fold reduction in the incidence of toxic nuclear RNA foci and the number of foci per cell	[206]
Antisense	Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)	Human, mouse	Long-term incubation (*ex vivo*), intracameral injection (*in vivo*)	Decreased expression of *MALAT1* gene as proof of principle for FECD treatment	[103]
Herpetic Keratitis					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**
Plasmid DNA	IL-4, IL-10	Mouse	*in vivo,* topical and systemic	Reduction of inflammatory lesion associated with corneal infection of HSV	[210]
Plasmid DNA	IL-12	Mouse	*in vivo,* cornea topical	Inhibition of corneal neovascularization and indirect inhibition of angiogenesis	[212]
Plasmid DNA	IL-18	Mouse	*in vivo,* cornea topical	Inhibition of VEGF in the cornea and reduced angiogenesis	[68]
Plasmid DNA	IFN-α1	Mouse	*in vivo,* cornea topical	Reduction in viral Ag expression in the eyes and immune cell infiltration into the cornea and iris	[213]
Plasmid DNA	IFN-α1	Mouse	*in vivo,* cornea topical	Decrease in the viral load and expression of HSV-1 immediate-early and early gene transcripts	[214]
Plasmid DNA	IFN-α1	Mouse	*in vivo,* cornea topical	Increased protection against ocular HSV-1 infection	[215]
Plasmid DNA	IFN-β	Mouse	*in vitro/ in vivo,* cornea topical	*In vitro,* reduction in the viral load of HSV-1 at an MOI. *In vivo,* increased survival and reduced the viral load of HSV-1 in the eyes	[217]
Plasmid DNA	gB1	Rabbit	*in vivo,* periocular injection	Reduced ocular symptoms, elicited neutralizing antibody response, and prevented the death of rabbits from encephalitis	[218]
Plasmid DNA	IL-2	Mouse	*in vivo,* topical	Complete inhibition of stromal keratitis, but no prevention/ reduction of HSV-1 epithelial lesions	[220]
Plasmid DNA	IL-2	Mouse	*in vivo,* hypodermal and subconjunctival injection, topical	Increased cell-mediated immunity against stromal herpetic keratitis	[221]

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Corneal and Conjunctival Fibrosis					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**
Plasmid DNA	IL-2	Mouse	*in vivo*, hypodermal/ subconjunctival injection	Inhibition of stromal herpetic keratitis	[222]
Naked DNA	gB, gC, gD, gE, and gI (5gP DNA)	Mouse	*in vivo*, intramuscular injection	Reduced ocular virus replication, blepharitis and latency	[219]
Glaucoma					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**
Ad	$p27^{KIP1}$	Rabbit	*in vivo*, subconjunctival injection	Reduction of IOP, cell proliferation, and scar tissue at the surgical site	[232]
Ad	RhoA	Human	*in vitro* injection through the cornea	Increased outflow facility in perfused human anterior segment cultures	[233]
AAV-2	GFP/LacZ	Rat, Monkey	*in vivo*, intracameral injection	Safe and prolonged transduction into the TM	[229]
AAV (Anc80L65)	CMV, WPRE	Human TM cells, Mouse	*in vitro, in vivo;* intracameral injection	Superior infectivity *in vitro* on HTM cells. Anc80L65 transduced all components of the mouse anterior segment with one injection	[230]
AAV-2/9	MMP-3	Mouse	*in vivo*, intracameral injection/ topical	Increased MMP-3 expression and decreased IOP.	[231]
AAV-2	C3 transferase	Mouse, monkey	*in vivo*, intracameral injection	Reduction of IOP and morphological changes in the TM	[238]
siRNA	RhoA	Mouse	*in vivo*, anterior chamber injection	Decreased expression of RhoA mRNA and protein in the TM and lowered IOP	[105]
siRNA	P2Y(2) receptor	Rat	*in vivo*, topical	Reduction in IOP	[235]
siRNA	β2 adrenergic receptor	Rat	*in vivo*, topical	Reduction in IOP	[236]
Dry Eye Disease					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**
Ad	TNF-α inhibitor	Rabbit	*in vivo*, lacrimal gland injection	Improvement in basal tear production, increase in tear stability, and reduction in ocular surface defects in an autoimmune dacryoadenitis model	[241]
AAV-2	IL-10	Rabbit	*in vivo*, inferior lacrimal gland injection	Suppression of lacrimal gland immunopathology of dacryoadenitis with decreased number of CD18+ cells	[242]
AAV-2	AQP1	Mouse	*in vivo*, submandibular salivary gland	Increase in secretory function of the lacrimal gland in a Sjögren's syndrome model *via* BMP6 regulation	[243]
Cationized gelatin-based nanoparticle	MUC5AC	Mouse	*in vivo*, topical	Restoration of MUC5AC levels and reduction in inflammation and increase in tear production in a dry eye model	[248]
Mucopolysaccharidosis					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**

Ad	β-glucuronidase (GUSB)	Mouse	*in vivo*, intrastromal injection	Significant reduction in the corneal clouding of MPSVII *via* B-glucuronidase expression	[252]
Ad	β-glucuronidase (GUSB)	Mouse, dog, lemur, human	*in vivo*, intrastromal injection, *ex vivo* cornea organ culture, cornea injection	Improved the pathology in the canine MPS VII in the cornea	[253]
AAV (AAV8G9 vector)	α-L-iduronidase (IUDA)	Human	*ex vivo*, cornea organ culture, intrastromal injection	Widespread transduction. IUDA therapy has a potential to reverse MPS-associated corneal blindness	[33]
Aniridia					
Vector	**Gene**	**Organism**	**Route of administration**	**Result**	**Reference**
CRISPR-Cas9	Pax6	Mouse	*in vivo*, microinjection	*Sey* mutation rescue by germline correction resulting in normalized eyes	[257]
CRISPR-Cas9	PAX6	Human	*in vitro*, human telomerase-immortalized LEC	Reversion of phenotypic defects in mutant LEC including normalized cell proliferation and migration	[258]