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Human Gene Therapy

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NEW FRONTIERS OF CORNEAL GENE THERAPY

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Abstract

Corneal diseases are among the most prevalent causes of blindness worldwide. The transparency and clarity of the cornea are guaranteed by a delicate physiological, anatomic and functional balance. For this reason, all the disorders, including those of genetic origin, which compromise this state of harmony can lead to opacity and eventually vision loss. Many corneal disorders have a genetic etiology and some are associated with rather rare and complex syndromes. Conventional treatments, such as corneal transplantation, are often ineffective and, to date, many of these disorders are still incurable. Gene therapy carries the promise to be a potential cure for many of these diseases, with solutions and strategies that did not seem possible until a few years ago. With its potential to treat genetic disease by means of deletion, replacement or editing of a defective gene, the challenge can also be extended to corneal disorders in order to achieve long-term, if not definitive, relief.

The aim of this paper is to review the state of the art of the different gene therapy approaches as potential treatments for corneal diseases and the future perspectives for the development of personalized gene-based medicine.

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Introduction

In the past decades, considerable progress has been made on understanding the altered genetic mechanisms underlying the pathogenesis of acquired or hereditary diseases of the eye. This knowledge has led to the identification of novel targets for therapeutic development. Among the various therapeutic approaches, ocular gene therapy seems to be the most promising one. The majority of eye diseases leading to blindness are caused by the degeneration of the retina. For this reason, retinal disorders have received much attention and so far over hundreds of experimental procedures have been performed for the identification of innovative gene-based interventions [1]. The progress achieved in this field culminated in successful clinical trials started in 2008 for Leber's Congenital Amaurosis (ClinicalTrials.gov number NCT00643747) and on December 19, 2017, the first FDA-approved gene therapy (Luxturna) was approved for the treatment of retinal diseases caused by mutations in the *RPE65* gene [2; 3]. However, gene therapy is suitable also to treat other forms of blindness and eye diseases. According to the World Health Organization, blindness of the cornea is the 4th leading cause of blindness globally, and is one of the major cause of visual deficiency after cataract, glaucoma, and age-related macular degeneration. The corneal tissue is widely studied for the easily monitoring in live animals or fixed samples using fluorescent genetic markers and for the possibility to topically apply drugs or therapeutic agents to the anterior or posterior segment using microinjection or surgery. Since the cornea can be cultured for a few weeks ex vivo, it becomes an appropriate model for *ex vivo* gene therapy as compared to other organs or tissues [4]. Tolerance to inflammation and high penetrating capacity of each layer, associated to immunological cells and soluble mediators, provide a strong immune privilege to the cornea [5; 6]. The corneal epithelial cells and keratocytes secrete cytokines creating a self-immune protection. Therefore, gene therapy represents an ideal strategy for the treatment of diseases related to the cornea, with some of them being very rare and having no current curative treatments available. Similarly to the retina, considerable steps forward have also been made in the field of corneal gene therapy, but not yet enough to reach the clinical application. Gene delivery of several therapeutic genes (see Box 1, Nucleic acids delivered for corneal gene therapy approaches) by means of viral, non-viral and hybrid vectors (see Box 2, Systems used to deliver nucleic acids for corneal gene

therapy), as well as DNA vaccination, enzyme modulation and various other innovative approaches have been proved to be useful in preclinical settings (see Table I for a summary). Lastly, gene editing is increasing its potential applicability and efficacy. In this paper, we critically review the gene administration and gene correction interventions attempted so far, and their effects or challenges posed for treating various corneal disorders.

1. Anatomy of the cornea

The adult human cornea is made up of five layers: (i) the epithelium, composed of 4-6 cell layers of non-keratinized, non-secretory, stratified squamous epithelium (40-50 µm); (ii) the Bowman's layer, an acellular structure of randomly arranged collagen fibrils (8-12 µm); (iii) the stroma, which makes up the bulk of the cornea and is essentially a collagenous matrix, with interspersed keratocytes; (iv) the Descemet membrane (DM), the basement membrane of the corneal endothelial cells; and (v) the endothelium, a single layer of polygonal cells made up of simple squamous epithelium.

From an embryological point of view, the corneal epithelium is derived from the surface ectoderm at approximately 5–6 weeks of gestation. Corneal epithelial cells undergo systematic evolution, apoptosis, and desquamation. Their physiological homeostasis occurs about every 5-7 days after terminal differentiation of epithelial stem cells (undifferentiated unipotent) located at the corneo-scleral limbus [7]. Alterations in epithelial cells cause at least three different diseases: Meesmann corneal dystrophy, aniridia and ectrodactyly-ectodermal dysplasia-clefting syndrome (EEC).

The major constituents of the stroma are the collagen fibers, which are disposed in regular lamellar sheets populated by keratocytes and mesenchymal cells and guarantee the high transparency of the cornea. An extremely rare cause of bilateral corneal opacity is the congenital stromal corneal dystrophy (CSCD), an autosomal dominant form of corneal dystrophy, characterized by progressively numerous opaque flaky or feathery areas of clouding in the stroma [8].

The epithelium and the stroma are usually considered as the most favorite locations for gene delivery.

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The endothelium has the vital function to keep the cornea transparent. In a normal cornea, the liquid equilibrium is guaranteed by the endothelial exchange/pumping excess of liquid and Na+-K+. Endothelium dysfunction results in excess accumulation of water in the corneal stroma, leading to the stromal swelling or edema resulting therefore in loss of transparency. The corneal endothelial dystrophies, comprising of (i) Fuchs endothelial corneal dystrophy, (ii) posterior polymorphous corneal dystrophy and (iii) congenital hereditary endothelial dystrophy, generally represent the defects of neural crest terminal differentiation. Fuchs endothelial corneal dystrophy is a degenerative, bilateral, often asymmetric and slowly progressive autosomal dominant disorder. It is characterized by a pleomorphic, attenuated, dysfunctional and degenerated corneal endothelium with the progressive formation of corneal guttae. Recent reports suggest that missense mutations in Zinc finger E-box-binding homeobox 1 (ZEB1) may be responsible for at least some cases of late-onset Fuchs [9].

2. Gene therapy approaches to corneal disorders

Examples of corneal disorders for which gene therapy has been considered as a potential treatment are described below.

Not inherited disorders:

a. Herpes simplex keratitis

Herpes simplex virus (HSV), after primary infection of mucous membranes, is able to establish lifelong infections by remaining latent in the peripheral nerve ganglia. During latency, the virus is maintained in an episomal form, and most of the viral genes are not expressed, with the exception of those belonging to the latency associated transcript (LAT) region. HSV can periodically reactivate, and new virions are produced that are transported back to the site of primary infection.

HSVs commonly cause oral and genital lesions, but HSV type 1 (HSV-1) may also be responsible for a variety of ocular diseases. Herpes simplex keratitis (HSK) is the most common infectious cause of blindness in the developed world, and it is also a major determinant of corneal graft rejection after transplantation. In the USA, the incidence of new cases of HSV eye infections is approximately 500,000 cases/year [10], even if clinical manifestations appear only in 20-30% of cases [11].

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The virus initially infects the corneal epithelium, then enters in sensory nerve endings and, traveling along axons, it reaches the trigeminal ganglion: the disease is most often due to reactivation of a latent infection of trigeminal sensory neurons. Differently from other infectious keratitis (e.g. fungal or bacterial keratitis), HSK may become chronic or recurrent [12], and the main source of recurrent disease is viral reactivation due to triggers such as UV exposure, stress, ocular surgery, hormonal factors [13; 14].

There are three main subtypes of HSK: epithelial, stromal and endothelial [15]. Epithelial HSK is the most common type, and it occurs after direct viral invasion. This form presents with dendritic lesions with swollen borders and intraepithelial cell infiltration. The stromal subtype is the consequence of an immune response against the virus and is characterized by corneal opacity and whitening. Lastly, endothelial keratitis may arise either from secondary inflammation caused by HSV-1 or from direct infection of endothelial cells, and manifests with keratic precipitates and iritis.

HSK symptoms include redness, discharge, lacrimation, irritation, itching, pain and photophobia [15]. The occurrence is usually unilateral, but a small fraction of affected people may also experience bilateral disease, especially younger and immunocompromised patients [16].

The treatment for HSK is mainly topical, and involves administration of antivirals such as acyclovir, ganciclovir and trifluridine, with acyclovir as the first-line treatment. Moreover, topical corticosteroids as adjuvant therapy may be employed [12]. Oral antivirals are also currently in use, and include acyclovir, valacyclovir and famciclovir [17]. Nevertheless, current antiviral treatments target HSV DNA polymerase, thus they are active in the replicative viral stage, but unable to clear latent HSV from the host. Moreover, any decrease in drug bioavailability and in virus sensitivity to the treatment may cause therapy failure. The study of molecular strategies that could be durably effective in the cells and avoid sensitivity issues led to the development of different gene therapy strategies targeting HSV.

Several approaches have been attempted focusing on the downregulation of viral or cellular gene expression, in order to affect viral replication and to prevent the development of herpetic lesions and diseases. In particular HSV-1 specific transcripts have been targeted by a plethora of approaches, including antisense phosphorotiates, peptide-

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conjugated phosphorodiamidate morpholino oligomers (PPMO), and by ribozymes [18; 19; 20; 21].

The first successful inhibition of HSV-1 replication by specific targeting of viral mRNA has been achieved by using antisense phosphorothioate oligonucleotides designed to inhibit the translation of HSV-1 transcripts. A screening of 100 phosphorothioate 20-mer oligonucleotides directed against different HSV-1 target genes led to the identification of 6 oligos significantly inhibiting viral replication, the most active being directed against the translation initiation site of the immediate early protein IE 110, involved in transcriptional activation of later viral genes [18].

Viral translation has subsequently been targeted also by PPMO, single-stranded nucleic acid analogues reducing gene expression through steric blockage of complementary RNA. Targeting the translation-start sites of HSV-1 ICP10 and ICP27 mRNA has been reported to decrease viral replication *in vitro* and to reduce ocular disease in HSV-1 infected mice [19].

Another possible approach is the degradation of HSV-1 transcripts by ribozymes, RNA enzymes cleaving and splicing RNA molecules. The HSV-1 late gene UL20 proved to be a valuable target for hammerhead ribozymes expressed by adenoviral vectors in both cell cultures and mice, suggesting that cleaving mRNA of essential late genes may represent an effective therapeutic strategy against HSV infection [20]. More recently, an adeno-associated virus (AAV) vector has been employed in eyes of rabbits with latent HSV-1 infection for the delivery of LAT-targeting ribozymes: this treatment appeared to block viral reactivation in more than 60% of infected eyes, thus showing the relevance of LAT for HSV reactivation and providing evidences of the potential of the approach for treatment of HSV infections [21].

As mentioned above, gene silencing has been employed not only for targeting the expression of viral genes, but also against pathways induced by HSV-1 infection. As demonstrated by Kim and colleagues, siRNA targeting vascular endothelial growth factor (VEGF) pathway genes may significantly inhibit excessive ocular angiogenesis, a phenomenon frequently resulting in pathological neovascularization in HSV-related eye disease [22]. Moreover, antisense oligonucleotides have been tested against IFN-γ mRNA: in a murine model of HSV-1 eye infection, a topical treatment with IFNγ-antisense oligonucleotides induced an improvement in incidence and progress of HSK, by reducing

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the number of inflammatory cells in the site of infection without affecting antiviral defenses [23].

Aptamers are functionally active nucleic acids that can bind to a wide range of molecules, included complex targets, and that are often been employed also against viral proteins. As demonstrated by *in vitro* antiviral assays, the gD protein of HSV-1, that mediates virus entry into host cells, may be bound with high affinity by an RNA aptamer, with consequent inhibition of viral entrance and replication. This finding suggests that the identified aptamer could be further investigated for the design of topical products reducing the risk of HSV-1 infection through physical contact [24].

Replication-defective and attenuated viruses have been frequently studied as potential vaccines in animal models. In 2004, Augustinova and colleagues generated a replication-defective, dominant- negative HSV-1 strain that acted as a replication inhibitor for itself and for wild-type HSV-1 in a mouse ocular model, showing a vaccine potential due to its capability of eliciting both innate and cell-mediated immunity [25].

Latent HSV-1 may be targeted by rare-cutting endonucleases such as meganucleases: these enzymes are able to recognize large DNA sequences (>12 bp) and can be engineered to obtain tailored specificity. The use of meganucleases to cleave regions of HSV genome has been reported by Grosse and colleagues, demonstrating that meganucleases engineered to target HSV-1 could prevent the infection of cultured cells by both recombinant and wild-type viral strains. Moreover, the cleavage could determine the consequent degradation and clearance of cleaved molecules, thus leading also to the disappearance of non-replicative episomal sequences in HSV-1 latency stage. The authors suggest that their strategy could be particularly useful for the prevention of recurrent infections during corneal transplantation, since introduction of meganucleases into the graft before transplantation could provide additional protection [26]. This concept has been furtherly highlighted in another study employing a recombinant adeno-associated virus to deliver anti-HSV-1 meganucleases to human corneas *ex vivo* before transplantation into HK patients to avoid reinfection and graft rejection [27].

The CRISPR/Cas9 system has been also tested as a strategy to target pathogenic viruses. Herpesviruses genomes may be altered in specific regions in order to impair viral replication, as demonstrated in a recent study showing a marked drop in production of

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HSV-1 infectious particles in cells carrying CRISPR/Cas9 systems directed against multiple essential HSV-1 protein-coding genes simultaneously. The system might also abrogate the replication of HSV-1 reactivated from quiescence, although it was not able to directly engineer the quiescent genome, due to the highly methylated state of latent HSV-1 DNA, that makes the genome unreachable by the CRISPR/Cas9 machinery [28].

The interest in developing many different gene therapy strategies against HSK throughout the years, spacing from the first gene silencing approaches up to CRISPR/Cas9 editing, highlights the clinical relevance of herpetic disease in the eye. Further studies focusing on the degradation of latent viral genomes or on the inhibition of HSV reactivation from latency are needed in order to eradicate chronic and recurrent forms of this pathology.

b. Dry Eyes – Sjögren's syndrome Dry Eyes

Described for the first time in 1933, the Sjogren Syndrome (SS) is a chronic systemic autoimmune disease, affecting the exocrine glands associated or not with autoimmune rheumatic disorders. The typical ocular and oral sickness is due to lymphocytic infiltrations of lacrimal and salivary glands [29; 30; 31]. Dry eye, or keratoconjunctivitis sicca, is associated to the dysfunction of the nasolacrimal unit composed by nasolacrimal glands, corneal surface and eye lids. Principal ocular symptoms are discomfort, visual disturbance and dryness, followed by photophobia, itching and tearing [32]. The prevalence of SS is estimated as 7 per 100000 people, more commonly in females and Caucasians [32; 33]. The pathogenic mechanism is based on an autoimmune response: the induced lacrimal gland dysfunction decreases the production of tear film, with a consequent corneal epithelial erosion. The epithelial erosion is unlighted by the reduction in Goblet cells and mucin production in conjunctiva, where artificial tear administration could provide comfort for patients and avoid corneal damage [29; 34]. Although the etiology is still unknown, different potential mechanisms have been revealed, involving genetic and environmental factors virus infection in susceptible subjects [32; 35]. Initially T cells were considered as the principal actors in the autoimmune process, but now there are evidence of an involvement of B cells in the pathophysiology of primary SS [36]. Antigens from various sources, like autoantigens, mimicry and apoptosis, presented to T cells, generate inflammation and immune response. It has been also demonstrated the dysfunction in B

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and Treg cells and the upregulation of HLA genes. Therapies, abrogating or modulating the inflammatory response, could play a key role in decreasing the inflammatory symptoms of the keratoconjunctivitis sicca.

Therefore, specific cytokines with anti-inflammatory effects could represent potential genes to be used in gene therapy approaches for SS treatment. In this context, the study of vector-specific reporter gene expression patterns allowed to conclude that vaccinia and adenovirus are efficient vectors for gene transfer into lacrimal gland tissue in primary culture [37]. Another study reported a partial, but transient, suppression of SS-like features of the disease in rabbit, after injection of an adenoviral vector carrying the vIL-10 gene [38]. Trousdale et al. showed that adenovirus-mediated gene therapy treatment of rabbits with established autoimmune dacryoadenitis, using AdTNFRIp55-Ig, resulted in improvement of clinical features, such as an increase in basal tear production, an increase in tear stability, and a reduction in surface corneal defects [39]. Different mouse models, treated with adenoviral administration of erythropoietin, known for its role in retinal protection and for its vasculo-proliferative characteristics (useful to facilitate wound healing in several diseases with neurotrophic or inflammatory keratopathy) [40] or mucin 5AC, fundamental for the lacrimal fluid stability [41], showed the tolerability and the success of gene transfer for SS. Zhang et al demonstrated the topical neutralization of IFN-γ produced by CD4+ T cells, thus preventing Goblet cells loss by modulating apoptosis and maintaining IL-13 levels in a mouse model dry eye model [42]. These preliminary results show the potential of gene therapy for the treatment of SS.

c. Gene therapy approaches for corneal graft survival

Even though corneal transplantation is the most successful example of tissue transplant worldwide, immunologic rejection remains a leading cause of graft failure, with a prevalence varying from 5% to 40%. To manage immune-mediated graft rejection, two main gene therapy-based strategies can be envisaged: (i) inhibiting or regressing corneal vascularization and (ii) preventing or reversing immune-mediated graft rejection. In addition, apoptosis has been identified as one of the mechanisms leading to corneal cell death and consequent diminished graft survival [43]. Again, approaches based on gene

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therapy might lead to treatments reducing the chances of graft failure after transplantation.

Anti-angiogenesis strategies. Vascularized beds are a serious hazard to grafting with the likely consequence that corneas are inundated with inflammatory cells, activated Antigen Presenting Cells and T cells that release cytokines and chemokines leading to a further amplification of the immune response and eventually graft rejection. Inhibition or regression of blood vessels is therefore the main strategy to maintain corneal clarity and survival. Improvements in graft survival have been achieved by knocking down neuropilin-2 through RNAi [44], targeting Flt23k [45], or, more generally, reducing the amount of free VEGF-A [46], thus leading to the inhibition of heme- and lymph-angiogenesis and eventually a decrease of activated T-cell influx into the corneal graft.

Immune response modulation. Since graft rejection is primarily due to an overwhelming immune response against antigens in the donor cornea, several molecular candidates have been tested in order to modulate the immune response. Adenovirus vector-mediated gene transfer of Cytotoxic T-Lymphocyte Antigen 4 protein (CTLA4-Ig) has been shown to prevent activation of T cells and effectively prolong graft survival [47; 48]. Similarly, adenoviral vectors have been used to deliver IL-10 and IL-12 to ovine corneas leading to higher rates of graft survival [49; 50; 51; 52]. However, the use of these vectors remains an issue, as highly immunogenic. A further strategy to modulate the immune response would be to induce or facilitate tolerance. Recently, lentivirus-mediated transduction of indoleamine 2,3 dioxygenase (thought to lead to T cell arrest in the G1 phase and therefore implicated in ocular immune privilege) to corneal ECs *ex vivo* prior to corneal transplantation was shown to prolong corneal graft survival in mice significantly [53].

Anti-apoptosis strategies. A further cause of corneal graft rejection is due to the loss of corneal endothelial cells (ECs) during storage in eye banks or after transplantation. Corneal ECs have limited proliferative capacity, therefore once lost, their contribution to maintaining corneal transparency is compromised. Cell apoptosis is believed to be a cause of EC loss and successful inhibition of the apoptotic pathways has proven to promote endothelial cell survival, thus suggesting a crucial role of apoptosis in graft survival/rejection [54]. Lentiviral vector-mediated gene delivery of baculoviral p35 or mammalian Bcl-xL to corneal endothelium during various storage conditions showed

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increased endothelial cell survival, thus indicating that the *ex vivo* modification of corneal allografts might increase the availability of tissues for transplantation [54]. Similarly, *ex vivo* transduction of rat corneas with lentiviral vectors expressing programmed death ligand-1 (PDL-1) significantly increased graft survival and led to a decrease in IL-6 and IFN-γ compared to controls [55]. As an alternative, a future possibility could envisage transfecting a cDNA encoding cytoprotective molecules for reduction of oxidation.

Considering that human donated corneas are stored in eye banks for some weeks before they are transplanted, *ex vivo* genetic modification during storage might represent a solution to minimize the chances of immunological rejection and therefore increase *in vivo* graft survival. This could eventually become a potential new treatment for high risk patients, who had previous and multiple graft rejections and are not responding to corneal HLA-matching either.

d. Corneal neovascularisation

Corneal neovascularisation (CNV) is a sight-threatening condition characterized by the ingrowth of new vessels from the limbus caused by the loss of the limbal stem cell barrier and it is becoming increasingly common worldwide with an estimated incidence rate of 1.4 million cases per year [56]. CNV occurs in a wide variety of corneal pathologies including congenital diseases, contact lens-related hypoxia, inflammatory disorders, chemical burns, limbal stem cell deficiency, allergy, trauma, infectious keratitis, autoimmune diseases, and corneal graft rejection [57]. CNV can lead to corneal scarring, stromal edema, lipidic deposition, and keratitis, resulting in significant visual impairment or blindness [58]. Angiogenesis is initiated when the balance between angiogenic and antiangiogenic factors is shifted toward angiogenic factors [57; 59; 60]. The clinical management of CNV represents a big challenge since current pharmacotherapeutic and surgical options are not always effective. Several approaches including amniotic membrane transplantation, topical nonsteroidal anti-inflammatory and corticosteroid medications, argon and yellow dye laser photocoagulation, photodynamic therapy, cautery, and diathermy have been used to shut new corneal vessels.

Various angiogenic factors mediate CNV, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), matrix metalloproteinase (MMP), platelet-

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derived growth factors (PDGFs), and interleukin-1 (IL-1). Among these, VEGF is reported to be the primary mediator of neovascularization in the eye and is elevated in inflamed vascularized corneas of rats and humans [61; 62]. For these reasons, many studies on gene delivery to the cornea have been focused on inhibiting the VEGF signaling pathway in animal models of chemical burn-induced corneal neovascularization. The intrastromal corneal injection of naked plasmid DNA encoding VEGF and the soluble receptor Flt-1 in mouse showed that the plasmid-injected corneas remained clear and free of inflammation, thus demonstrating that the expression of the genes injected into cornea is extraordinarily rapid [63]. In addition, a plasmid DNA encoding Kringle 5 of plasminogen and endostatin were effectively transferred to the rat cornea by subconjunctival injection with electroporation, showing the inhibition of alkali-induced corneal neovascularization [64]. Another study showed that Parstatin, a 41-mer peptide produced by proteolytic cleavage during activation of the PAR1 receptor, is believed to inhibit angiogenesis. Intravitreal or subconjunctival administration of Parstatin peptide reduced CNV in mice and interrupted the progress of CNV in rat corneas with chemical burns. It was demonstrated that combined blockage of VEGF and FGF2 receptor with Parstatin may have higher efficiency towards blocking ERK1/2. It was also reported that its application reduced the inflammatory cell count in rat corneas after chemical cauterization, thus suggesting that it could be a suppressant for inflammation in cornea [65]. The antiangiogenic effects of the brain-specific angiogenesis inhibitor 1 (BAI1-ECR) gene has been investigated in an *in vivo* rabbit model of corneal angiogenesis, by means of subconjunctival injection of the BAI1-ECR gene mixed with nonliposomal lipid, showing an effective reduction of corneal neovascularization induced by chemical and mechanical denudation and thus suggesting that the BAI1-ECR protein can be used as an angiogenesis suppressor in the eye [66].

Alternative approaches towards the treatments of CNV are represented by viral vectors encoding anti-angiogenic genes. The use of a recombinant adeno-associated viral (rAAV) vector carrying endostatin gene as an anti-angiogenic strategy was evaluated in a mouse model, where CNV was induced by silver nitrate cauterization [67]. The recombinant endostatin-AAV was administrated by subconjunctival injection and it proved to be an efficacious means of delivering the endostatin gene, since the transgene expression was stable for over 8 months with minimal immune reaction and successfully inhibited

neovascularization. Another study showed the delivery of the decorin, a small leucine-rich proteoglycan able to modulate angiogenesis in nonocular tissues, to the rabbit stroma with adeno-associated virus serotype 5 (AAV5), after CNV was induced with VEGF using a micro-pocket assay [68]. A significant inhibition of VEGF-induced corneal angiogenesis was observed, thus demonstrating the potential use of decorin gene therapy for treating corneal angiogenesis *in vivo*, without any major side effect.

Gene delivery by means of nanoparticles or nanomaterials also represents a promising method to treat CNV, as well as the polyplex micelles, obtained with the complex of plasmid DNA (pDNA) with synthetic cationic polymers [69]. A block copolymer, poly (ethylene glycol) (PEG)-block-polycation carrying ethylenediamine units in the side chain [PEG-b-P[Asp(DET)], generated a polyplex micelle via polyion complex formation with plasmid DNA. After injecting PEG-b-P[Asp(DET)] micelles in the subconjunctival space of mice, the reporter gene expression was monitored and was found prolonging gene expression with reduced cytotoxicity. Gene transfer by the same polyplex micelle carrying a sflt-1-containing plasmid also proved considerable inhibition of CNV in mice making it a potential source for treatment of CNV.

Another study demonstrated that intrastromal injection of a plasmid containing a small hairpin RNA cassette (pSEC.shRNA) against VEGF-A-loaded poly (lactic co-glycolic acid) nanoparticles was an effective, nonviral and nontoxic form of anti-angiogenic therapeutic strategy *in vivo* for murine CNV. This approach provides an attractive option for the treatment of human CNV [70].

Recently, Lu et al. identified potential anti-angiogenic corneal microRNAs (miRNAs) that can be delivered via recombinant adeno-associated viruses (rAAVs) into injured corneas to block angiogenesis. One of these, miR-204, showed strong anti-angiogenic properties and has proved to be effective in reducing vascolarization of injured mouse cornea [71].

In conclusion, gene therapy approaches by means of subconjunctival, intracorneal or topical delivery of anti-angiogenic genes using plasmids, viral vectors and nanoparticles have proven to be safe and effective in inhibiting experimental CNV, and pave the way for the treatment of patients with CNV.

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Inherited disorders:

e. Mucopolysaccharidosis (MPS)

Mucopolysaccharidosis (MPS) is an inherited group of disorders caused by defects in lysosomal enzymes responsible for the degradation of glycosaminoglycans (GAGs) and is characterized by a high range of clinical manifestations. MPS are classified on the basis of the enzymes which are dysregulated and include MPS I (Hurler, Scheie and Hurler/Scheie), MPS II (Hunter), MPS III (Sanfilippo A, B, C and D), MPS IV (Morquio A and Morquio B), MPS VI (Maroteaux-Lamy), MPS VII (Sly) and MPS IX Natowicz). Symptoms vary and include coarse face central nervous system (CNS) impairment, hearing loss, respiratory problems, valvular heart disease, hepatosplenomegaly, skeletal dysplasia, gait abnormality and corneal clouding.

Therapies like hematopoietic stem cell transplantation (HSCT), enzyme replacement therapies (ERT) and various surgical interventions are currently available for patients with MPS. While some advantages have been shown and a slower progression of the disease observed, limitations do exist. ERT requires weekly injections, is costly and has limited penetration to the bone and CNS. HSCT may not be applicable to all the patients because of the limited availability of matched donors and complications such as the graft-versus-host disease. Therefore, more effective and feasible therapies for MPS are urgently required.

Gene therapy might represent a promising approach for treating patients with MPS and has been under investigation for the last 3 decades. Phase I/II gene therapy clinical trials for some types of MPS (MPS I, II, IIIA, IIIB and VI) are ongoing or scheduled in the United States, some European countries and Australia (reviewed [72]). Despite all these studies, it remains controversial whether the ocular manifestation of MPS are influenced by HSCT, ERT or gene therapy [73; 74]. The proposed gene therapy-based strategies for the treatment of the ocular manifestations of MPS include both systemic and local approaches (i.e., delivered to the cornea or the retina), but findings are so far limited to experimental investigations in animal models (mainly mice, cats and dogs with MPS I and VII as they share many of the clinical, biochemical and histopathological features of humans).

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Systemic gene therapy approaches

Studies have been conducted in newborn and adult mice with MPS I or MPS VII using various vectors, dosages and outcome measures (reviewed [75]). High serum enzyme activity levels resulted in profound effects on the eyes of both newborn and adult mice at 6-8 months after treatment with retroviral vectors [76; 77]. Correction of the dark-adapted electroretinogram (ERG) and GAG storage in the corneal stroma and endothelium were normalized in a dose-dependent fashion [76]. Similar storage improvements in the cornea and retinal pigmented epithelium were found in MPS VII mice at 6 months after neonatal retroviral vector mediated gene transfer, which correlated with to the enzyme expression levels [77]. In contrast, intravenous administration of AAV vectors to MPS VII newborn mice resulted in low expression levels upon ageing, thus suggesting that only juvenile or adult mice should be treated in order to avoid the loss of extra-chromosomal AAV vector in the liver as hepatocyte divide during growth [78; 79; 80].

Success of systemic gene transfer in preventing the ocular manifestations of MPS was also demonstrated in studies with larger animal models. Ponder and colleagues showed that, when MPS VII dogs were injected intravenously at 2-3 days of age with a retroviral vector containing the human alpha1-antitrypsin promoter upstream of the canine beta-glucuronidase (GUSB) cDNA, little or no corneal clouding were observed. A likely explanation is that the prevention of corneal clouding is probably due to the uptake of enzyme from the blood via the prelimbic capillaries by the corneal stromal cells [81]. In conclusion, systemic gene therapy with integrating vectors could be an ideal treatment, as it would lead to widespread and persistent enzyme activity *in vivo*, even if high vector dosages might have to be used. However, safety concerns will have to be addressed before moving into clinical settings.

Local gene therapy approaches

Some of the concern of systemic gene therapy approaches could be overcome with local delivery of gene transfer agents to the eye. Examples of potential administration routes include the subretinal and intravitreal injections, or injections into the anterior chamber or periocularly. A few examples of such applications are reviewed below.

Vance and colleagues showed that AAV-mediated CMV-driven alpha-L-iduronidase (IDUA) administration to normal human donor cornea explants via intrastromal injection led to

widespread transduction with >10-fold increase in IDUA activity. Given the ability of secreted IDUA to cross-correct neighboring cells, these data suggest that the strategy adopted should result in normal levels of IDUA when MPS I patient corneas will be treated [82]. Since corneal transplants are met with high rejection rates in MPS I children [83], this strategy could prevent/reverse MPS I-associated cornea blindness, which is mainly caused by accumulation of chondroitin and dermatan sulphate GAGs, known to alter the uniform distribution, organization and size of collagen fibrils.

In animal models of MPS, all studies of local gene therapy have reported a reduction of histopathological aberrations and storage of GAGs in the cornea, ciliary body and retina. This was accomplished by benefits on corneal clouding and retinal function improvements, thus suggesting the efficacy of strategies based on local gene transfer, although longer term evaluations are needed [75]. In mice with MPS type VII, administration of an adenovirus expressing human GUSB under the control of the CAG promoter into the anterior chamber or within the intrastromal region of the cornea showed to be successful for the treatment of corneal clouding. Widespread transgene expression was observed when the adenoviral vector was administered inside the cornea after lamellar keratotomy and rapid elimination of the lysosomal storage in the corneal keratocytes was seen occurring. Furthermore, intrastromal vector administration did not generate significant levels of anti-adenovirus neutralizing antibodies, and secondary vector administration was effective, thus suggesting that a direct intraocular administration of adenoviral vectors could be beneficial for treatment of MPS VII [84]. In a more recent report, Serratrice and colleagues showed that the pathology in the canine MPS VII cornea improved after the injection in the cornea of a helper-dependent canine adenovirus type 2 vector carrying the human GUSB cDNA under the control of a Rous Sarcoma Virus promoter [85].

In conclusion, since the clinical trials with HST and/or ERT have not yet demonstrated whether there is any beneficial effect on the ocular manifestations of the disease, local delivery of gene therapeutics might become a supplemental therapy to reverse and/or prevent MPS-associated corneal blindness.

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f. Meesmann epithelial corneal dystrophy

Meesmann epithelial corneal dystrophy (MECD, OMIM: 122100) is a rare autosomal dominant inherited disease caused by heterozygous mutations in KRT3 (12q13.13) or KRT12 (17q21.2) genes, encoding respectively, corneal specific Keratins 3 and 12 (k3 and k12; [86]). K3 and k12 are the unit of the intermediate filament cytoskeleton of corneal epithelial cells, providing structure and stability [87] and consequently, their malfunction causes mechanical fragility of the anterior corneal epithelium. MECD, whose frequency is unknown, is characterized by the formation of myriads of intraepithelial microcysts of variable distribution and density in the outermost layer of the cornea that can appear early in life and increase in number with age [88]. In most cases, MECD remains asymptomatic while in some cases, microcysts can break, causing the typical symptoms, including photophobia, blepharospasm, increased tear production, intolerance to contact lenses, transient blurred vision, foreign body sensation, and irregular astigmatism. All mutations are found in the highly conserved helix initiation or termination motifs, located at the N- and C-termini of the keratin protein, and implicated in heterodimerization, protein coiling, and keratin fiber assembly [89; 90]. At least 24 mutations have been described for MECD (21 mutations in KRT12 gene and 3 in KRT3 gene), the majority of which are missense point mutations [86; 89; 91; 92; 93]. Nevertheless, the mechanism underlying the formation of corneal microcysts and the genotype phenotype correlation remain poorly understood. Stem cell transplantation and keratoplasty are the only options available when rare severe phenotypes with corneal scarring cause loss of visual acuity [94; 95]. Unfortunately, after penetrating keratoplasty, pathology reappearance can occur, due to resident mutant limbal stem cells that with time foster corneal surface regrowth [94]. To date, there is no therapy available to address MECD pathology. As a dominant-negative disease, gene therapy strategies for MECD should rely on mutant allele silencing or knock out. In fact, only expression of one K12 wild type allele, is required for normal corneal epithelial function, as demonstrated by studies on heterozygous mice (+/-) for Krtl.12 gene, which show a phenotype without corneal clinical manifestations [96]. Therefore, allele-specific small interference RNA (siRNA) therapy, designed to ablate the mutant allele, might represent a possible treatment. Importantly, allele-specific siRNAs have been successfully

developed against the severe heterozygous missense mutation Leu132Pro [89] and the milder and most common European mutation Arg135Thr [97; 98] in KRT12 gene. Both siRNAs specifically silenced the mutant alleles expression, without neither affecting the wild-type allele or other keratins. Cytoskeleton dysfunction was reversed both in a cell culture model system as well as in established cultures of MECD corneal epithelial limbal stem cells [97; 98; 99]. These inhibitors will have to be further tested before translating therapy into patients but might represent a viable treatment option for MECD. Recently, a mouse model of MECD with the severe Leu132Pro mutation has been generated by homologous recombination, providing an *in vivo* model to test potential therapies [90]. The phenotype of this model closely mirrors that of MECD patients: a corneal epithelium with cell fragility, de-stratification of the basal layer and the formation of micro-cysts that might rupture at the corneal surface. In this model, an alternative, but very innovative and efficacious approach for mutant allele ablation has been recently applied using the CRISPR/Cas9 technology [100]. In this pioneering work, the authors aimed at developing an allele-specific genome-editing strategy for the Leu132Pro mutation by exploiting the occurrence of a novel protospacer adjacent motif (PAM) site in the mutant allele, caused by the single base change T>C, and absent in the wild type allele, making this strategy very safe. This Cas9-based gene-editing system was found to be allele specific when injected intrastromally *in vivo*. The efficiency of non-homologous end joining (NHEJ) was of 38.5%, with deletions of up to 53 nucleotides, most of which were predicted cause frameshifts, thus demonstrating the potential of this approach for combined gene and cell therapy [100].

g. Ectrodactyly Ectodermal dysplasia Clefting (EEC) syndrome

Ectrodactyly Ectodermal dysplasia Clefting (EEC) syndrome is a rare autosomal dominant inherited disease characterized by ectrodactyly (split-hand-food malformation), ectodermal dysplasia and cleft lip and palate, affecting skin, nails, hair, teeth, sweat glands and the ocular ectodermal derivatives [101; 102]. The incidence in the population is about 1:900.000, according to the Italian Ministry of Health. While in childhood clefting and hand deformities are the main clinical features, during early adulthood ocular problems become the predominant clinical aspect of EEC syndrome [103; 104]. Patients often show ocular

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surface alterations such as recurrent blepharitis and conjunctivitis, superficial microlesions of the cornea, spontaneous corneal perforation and ulceration, defective regeneration and poor re-epithelialization following trauma or penetrating keratoplasty (PK). They develop progressive total bilateral limbal stem cell deficiency (LSCD), which eventually results in corneal blindness. The EEC syndrome is caused by mutations in the p63 gene, an important transcription factor (TF) for the normal development of orofacial ectoderma and limbs and for stem cell differentiation in stratified epithelia [101]. The p63 gene generates six isoforms and DNp63a is the predominant isoform in the human corneal epithelium [105]. The EEC syndrome is mainly caused by point mutations in the DNA binding domain of the p63 gene [106]. Five p63 DNA-binding mutations account for almost 90% of EEC patient cases. The five missense mutations affect arginine residues (R204, R227, R279, R280 and R304) and represent hotspot mutations for EEC [107]. In particular, mutations R379H and R304Q result as the most severe mutations affecting the cornea [106].

Despite the clinical and molecular knowledge accumulated, no definitive cures are currently available to treat the ocular alterations in EEC patients, and the biological knowledge of the disease has only recently been clarified [104; 108; 109]. Progressive limbal stem cell failure results in visual morbidity in EEC syndrome, ultimately leading to blindness [104]. Common therapeutic strategies, such as penetrating keratoplasty (PK), are not a solution since in case of limbal stem cell deficiency as soon as the donor epithelium is exhausted, conjunctivalisation occurs again [110]. As EEC syndrome results from heterozygous dominant-negative mutations in the p63 gene, therapeutic strategies based on allele-specific gene silencing through small interference RNA (siRNA) could specifically inhibit the expression of disease-associated alleles without suppressing the expression of the corresponding wild-type alleles to phenotypically correct the stem cell population. Recently, a systematic screening of locked nucleic acid (LNA)-siRNAs against R279H-p63 allele allowed to identify a number of potent siRNA inhibitors for the mutant allele, able to specifically downregulate the R279H-DNp63a mRNA by approximately 90%, without affecting the wild-type p63. Long-term treatment resulted in a longer acquired life span, thus counteracting premature stem cell aging in vitro, and finally showed to be effective in correcting the epithelial hypoplasia, giving rise to a full thickness stratified and differentiated epithelium [108].

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Similarly, specific siRNAs able to repress the EEC-causing p63 mutant R304W were identified, by using a systemic screening based on a dual-luciferase reported gene assay [111]. Upon siRNA treatment, the transcriptional function of DNp63-WT allele was restored in induced pluripotent stem cells derived from EEC patient biopsy.

These studies demonstrate the phenotypic correction of mutant stem cells in EEC syndrome by means of siRNA mediated allele specific silencing with restoration of function. Since EEC-related corneal pathology follows a clear clinical course with LSCD usually manifesting in the second to third decade leading to severe corneal failure in the fourth to fifth decade, this might provide a therapeutic window to correct the EEC defect using genetic preventive strategies. In early corneal disease, with limbal stem cells still present in the limbus, the use of eye drops containing mutant specific siRNAs may be a practical therapeutic option which could prevent blindness.

h. Aniridia

Aniridia is an infrequent, progressive panocular genetic disorder (incidence about 1:55000). It is characterized by a bilateral alteration in the development of the eye and it is associated with a noticeable foeval and iris hypoplasia, the latter also being the main diagnostic feature of the disease and usually leading to nystagmus. Patients usually develop a variable range of symptoms affecting a number of ocular structures, including cornea, iris, lens, fovea, and optic nerve. Therefore, a number of additional complications are common, including a few sights threatening ones such as glaucoma, cataracts and corneal opacification. Aniridia is a well-documented genetic anomaly that may appear sporadically (approximatively 33% of cases) or within families (approximatively 66% of cases), exhibiting a dominant autosomic inheritance pattern with variable expression amongst the members of a family. The disease is caused by an extremely heterogeneous number of different mutations leading to haploinsufficiency of *PAX6* gene, which is located in the short arm of chromosome 11^{p13} and is broadly expressed in the development of various eye structures, including the cornea [112].

Indeed, one of the causes of progressive loss of vision and morbidity in aniridia patients is aniridic keratopathy (AAK), which is caused by dysfunctioning limbal stem cells. PAX6 (+/-) small eye mice, despite they do not fully recapitulate the complexity of the phenotype

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observed in humans, have been used as an animal model for aniridia. In such disease model, altered expression of cytokeratin 12, the presence of goblet cells on the corneal surface, and modified migratory properties of corneal epithelium could be observed together with a depletion in limbal epithelial stem cells which caused a reduced production of progeny [113]. These experimental findings support the notion that limbal stem cell deficiency plays a causative role in ocular surface failure from aniridia [114; 115]. Hence, AAK is an example of primary, intrinsic limbal stem cell deficiency caused by mutations in *PAX6* gene.

Due to the progressive nature of the disease, the management depends on the clinical manifestations, and it is therefore very variable, mainly including the use of lenses as well as surgical procedures such as goniotomy, cataract extraction and keratoplasty, and it is generally associated with severe complications and unsatisfactory success rates [116]. No pharmacological treatment is available to date, and AAK remains a major issue. However, given the fact that the non-systemic forms of aniridia depend on defects of a single gene such as *PAX6*, combined stem cell-gene therapy approaches aimed at correcting *PAX6* function could represent an attractive treatment to be pursued [116].

A massive bulk of studies investigating the molecular mechanisms underlying the development of aniridia heterogeneous manifestations highlighted a prominent role for the alteration of *PAX6* expression levels in different eye districts. Importantly, around 50% of the more than 500 *PAX6* mutations causing aniridia are represented by in frame, nonsense mutations inserting premature termination codons (PTCs) within *PAX6* sequence [117]. This evidence, together with the autosomic dominant nature of aniridia, is consistent with alteration of PAX6 expression levels being responsible for the clinical manifestation, which implies the possibility to reverse the phenotype (i.e. cure the disease) by restoring protein expression in ocular tissues.

This goal could be reached using different strategies, depending on the mutations causing the disease. Indeed, the most promising approach for patients whereby PAX6 expression is altered by the presence of PTCs, is represented by the administration of aminoglycosides, or of small molecules capable of promoting ribosome read thorough at PTCs, and restoring wild-type levels of protein synthesis. A first important proof of principle of the feasibility of such approach came from studies performed using mice whereby one *pax6* allele bears a

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naturally occurring G194X stop codon mutation (UGA). This recapitulates a number of aniridia symptoms observed in humans, including corneal and retinal thickening, as well as underdeveloped lens and impaired vision. Remarkably, topical administration to the mice eyes of the so called START formulation (sodium chloride, Tween, small molecule ataluren – the compound acting on PTCs- and carboxymethylcellulose) postnatally from P14 to P60, restored PAX6 expression levels to wild-type and remarkably reduced the eye abnormalities (leading to a reduction of both retinal and corneal thickness, restoring normal lens size) and improved light stimuli. Very importantly, the treatment did not reverse the phenotype in mice bearing a splice site mutation in *pax6*, thus demonstrating the specificity of the mode of action of such formulation. A follow up study focused on testing the best Ataluren concentration and temporal treatment schedule using the very same small eye mouse model and a combination of histopathological, behavioral and electroretinographic tests [118]. The results confirmed the possibility to revert the aniridia phenotype postnatally and highlighted the importance in determining the most appropriate regimen to restore the physiological PAX6 expression levels in different eye districts. Indeed, eye development appears extremely sensitive to alterations in PAX6 expression levels, with just a 2.5-fold protein overexpression causing microphthalmia in mice [119]. Clearly, further studies are required for validating such results in humans. In this context, a phase II, interventional clinical trial aimed at assessing the safety and efficacy of ataluren in the management of aniridia patients, bearing a nonsense mutation in one allele of PAX6 gene started in 2015 (NCT02647359). However, ataluren appears particularly effective in promoting read-through of certain stop codons, also depending on nucleotides surrounding the stop codon itself and the location in the mRNA [120]. It is therefore hypothesizable that only a small percentage of aniridia patients bearing PTCs would benefit from such approach. For all the other patients, including the few ones bearing mutations in the *PAX6* regulatory regions [121], alternative approaches to restore physiological PAX6 levels would be required. One of them could be the controlled administration of human recombinant pax6 protein. Indeed, a recent report described the ability of bacterially expressed, recombinant human pax protein fused to a C-terminal tag of 11 arginine domain, which allows intracellular protein intake, to restore the phenotypic impairments observed in a cellular aniridia model. Such model is represented by

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telomerase immortalized limbal stem cells where one *PAX6* allele is edited by CRISPR/Cas9 technology to introduce a PTC mutation naturally occurring in aniridia patients, and exhibit defects in cell proliferation and migration [122].

Other approaches would involve the irreversible genetic modification of patient's cells to reverse the aniridia phenotype. This could be achieved by augmentation gene therapy, delivering exogenous genetic elements capable of correcting PAX6 expression levels in a spatiotemporal coordinated fashion. The main limitation would be represented by the difficulty to package into a viral vector all the regulatory elements required to ensure the proper PAX6 spatiotemporal expression profile in the patients. Indeed, *PAX6* gene expression is controlled by at least 39 cis-regulatory elements, including a rather large number of regulatory regions located even megabases away from the transcriptional start site [116]. To overcome this issue, a recent report described the genesis and characterization of PAX6 MiniPromoters. Their reduced size (less than 2 Kps) allowed insertion into rAAV, and regulated expression of a transgene in several mice ocular tissues after transduction [123]. Obviously, further optimization of such technology is required to ensure PAX6 expression in human aniridia patients. The last potential approach, i.e. genome editing of PAX6 mutated alleles by CRISPR/Cas9 technology, would have the advantage to potentially cure any aniridia patient regardless of the responsible mutation, but its use would be strongly limited by the necessity to design and optimize hundreds of guides specific to each mutation [117].

i. Fuchs endothelial corneal dystrophy (FECD)

Fuchs endothelial corneal dystrophy (FECD) is the most common form of posterior corneal dystrophy; it is characterized by the progression of focal excrescences in the Descemet membrane (also called the *guttae*) to endothelial cell degeneration and stromal edema. The clinical onset usually occurs during the fifth or sixth decade of life and FECD is slightly more common in women than in men. Patients experience discomfort and painful episodes related to corneal erosions, in association with gradual opacification that results in a visual veiling. Over time, discomfort may decrease, but severe visual acuity may occur, leading to blindness in older patients. The disease is often associated with cataract. Microbial keratitis and corneal neovascularization are very rare complications. Stromal

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edema causes an anterior bluish-gray opacification of the Descemet membrane, followed by a possible thickening of the cornea stroma, which can take on the ground-glass appearance. The etiology of FECD is not known, but appears to be a heterogeneous complex hereditary disease, caused by the interaction between genetic and environmental factors. There currently is no cure for FECD. In early stages of the disease, vision may be improved with a 5% salt solution. However, there is no treatment that can actually halt or reverse the course of the disease. Eventually, in some patients with FECD, surgery may be necessary. The two options currently available are: Penetrating Keratoplasty (PK) and Descemet's Stripping with Endothelial Keratoplasty (DSEK).

Rare cases with early onset were associated with mutations in the COL8A2, SLC4A11, ZEB1 and LOXHD1 genes [124]. Approximately 70% of FECD cases are caused by a trinucleotide repeat (TNR) expansion in the TCF4 gene [124] that leading to nuclear RNA foci, with the sequestration of splicing factor proteins (MBNL1 and MBNL2) to the foci and altered mRNA processing.

Studies using in vitro and animal models have shown that an antisense oligonucleotide (ASO) approach leads to a reduction in RNA foci and downstream markers of toxicity. This proof-of-concept study highlights the potential of a targeted ASO therapy to treat the accessible and tractable corneal tissue affected by this repeat expansion-mediated disease [125]. Further, with the latest evidence demonstrating pathogenic TCF4 trinucleotide repeat expansions causing defects in endothelial barrier function in a majority of FECD cases, an analogous application of CRISPR-Cas9 treatment for FECD holds significant promise [124].

Conclusions and future perspectives

Besides corneal transplantation, not many valuable therapies are available nowadays to treat corneal diseases. Thus, gene therapy can be considered as a promising biomedical approach.

Gene therapy is a new branch of regenerative medicine that has matured considerably in the last decade, as demonstrated by the results of several clinical trials for a number of different disorders of genetic origin and by means of a diverse range of gene transfer methods. The lentiviral and adeno-associated vectors are the main gene transfer vectors

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currently in use, but also other approaches, primarily non-vectorial, are under scrutiny because of their lower invasiveness and higher level of safety (see Box 2, Systems used to deliver nucleic acids for corneal gene therapy**)**. The experience and knowledge learnt from the successes, problems and obstacles encountered in recent studies will be indispensable for the innovation of gene therapy approaches of the future. New gene transfer protocols and tools are already under development, which will also contribute to expand the spectrum of diseases that can be treated with gene therapy. Continuous progress towards gene therapy of precision represented by genome editing is the next challenge.

The studies described in this review paper have proved to be valid and effective. Now it would be interesting to move these findings onto clinical settings, to see whether they are clinically valuable as well.

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REFERENCES

[1] D.A. Thompson, R.R. Ali, E. Banin, K.E. Branham, J.G. Flannery, D.M. Gamm, W.W. Hauswirth, J.R. Heckenlively, A. Iannaccone, K.T. Jayasundera, N.W. Khan, R.S. Molday, M.E. Pennesi, T.A. Reh, R.G. Weleber, D.N. Zacks, and C. Monaciano, Advancing therapeutic strategies for inherited retinal degeneration: recommendations from the Monaciano Symposium. Invest Ophthalmol Vis Sci 56 (2015) 918-31.

[2] N. Kumaran, A.T. Moore, R.G. Weleber, and M. Michaelides, Leber congenital amaurosis/early-onset severe retinal dystrophy: clinical features, molecular genetics and therapeutic interventions. Br J Ophthalmol 101 (2017) 1147-1154.

[3] S. Russell, J. Bennett, J.A. Wellman, D.C. Chung, Z.F. Yu, A. Tillman, J. Wittes, J. Pappas, O. Elci, S. McCague, D. Cross, K.A. Marshall, J. Walshire, T.L. Kehoe, H. Reichert, M. Davis, L. Raffini, L.A. George, F.P. Hudson, L. Dingfield, X. Zhu, J.A. Haller, E.H. Sohn, V.B. Mahajan, W. Pfeifer, M. Weckmann, C. Johnson, D. Gewaily, A. Drack, E. Stone, K. Wachtel, F. Simonelli, B.P. Leroy, J.F. Wright, K.A. High, and A.M. Maguire, Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet 390 (2017) 849-860.

[4] R.R. Mohan, A. Sharma, M.V. Netto, S. Sinha, and S.E. Wilson, Gene therapy in the cornea. Prog Retin Eye Res 24 (2005) 537-59.

[5] E.A. Klausner, D. Peer, R.L. Chapman, R.F. Multack, and S.V. Andurkar, Corneal gene therapy. J Control Release 124 (2007) 107-33.

[6] G.C. Smolin, C.S. Foster, D.T. Azar, and C.H. Dohlman, Smolin and Thoft's The Cornea: Scientific Foundations and Clinical Practice., LIPPINCOTT WILLIAMS & WILKINS, Philadelphia, 2005.

[7] C.P. Leblond, Classification of Cell Populations on the Basis of Their Proliferative Behavior. Natl Cancer Inst Monogr 14 (1964) 119-50.

[8] E. Rodahl, P.M. Knappskog, C. Bredrup, and H. Boman, Congenital Stromal Corneal Dystrophy. in: M.P. Adam, H.H. Ardinger, R.A. Pagon, S.E. Wallace, L.J.H. Bean, K. Stephens, and A. Amemiya, (Eds.), GeneReviews((R)), Seattle (WA), 1993.

[9] J. Lechner, D.P. Dash, D. Muszynska, M. Hosseini, F. Segev, S. George, D.G. Frazer, J.E. Moore, S.B. Kaye, T. Young, D.A. Simpson, A.J. Churchill, E. Heon, and C.E. Willoughby, Mutational spectrum of the ZEB1 gene in corneal dystrophies supports a genotype-phenotype correlation. Invest Ophthalmol Vis Sci 54 (2013) 3215-23.

[10] M. Tsatsos, C. MacGregor, I. Athanasiadis, M.M. Moschos, S. Jameel, P. Hossain, and D. Anderson, Herpes simplex virus keratitis: an update of the pathogenesis and current treatment with oral and topical antiviral agents - comment. Clin Exp Ophthalmol 45 (2017) 932.

[11] T.J. Liesegang, Classification of herpes simplex virus keratitis and anterior uveitis. Cornea 18 (1999) 127-43.

[12] A. Austin, T. Lietman, and J. Rose-Nussbaumer, Update on the Management of Infectious Keratitis. Ophthalmology 124 (2017) 1678-1689.

[13] P.A. Asbell, Valacyclovir for the prevention of recurrent herpes simplex virus eye disease after excimer laser photokeratectomy. Trans Am Ophthalmol Soc 98 (2000) 285-303.

[14] B.M. Brandt, J. Mandleblatt, and P.A. Asbell, Risk factors for herpes simplex-induced keratitis: a case-control study. Ann Ophthalmol 26 (1994) 12-6.

[15] T.N. Azher, X.T. Yin, D. Tajfirouz, A.J. Huang, and P.M. Stuart, Herpes simplex keratitis: challenges in diagnosis and clinical management. Clin Ophthalmol 11 (2017) 185-191.

[16] P.M. Souza, E.J. Holland, and A.J. Huang, Bilateral herpetic keratoconjunctivitis. Ophthalmology 110 (2003) 493-6.

[17] T. Kalezic, M. Mazen, E. Kuklinski, and P. Asbell, Herpetic eye disease study: lessons learned. Curr Opin Ophthalmol 29 (2018) 340-346.

[18] A. Peyman, M. Helsberg, G. Kretzschmar, M. Mag, S. Grabley, and E. Uhlmann, Inhibition of viral growth by antisense oligonucleotides directed against the IE110 and the UL30 mRNA of herpes simplex virus type-1. Biol Chem Hoppe Seyler 376 (1995) 195-8.

[19] M. Moerdyk-Schauwecker, D.A. Stein, K. Eide, R.E. Blouch, R. Bildfell, P. Iversen, and L. Jin, Inhibition of HSV-1 ocular infection with morpholino oligomers targeting ICP0 and ICP27. Antiviral Res 84 (2009) 131-41.
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Human Gene Therapy
New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
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[20] J. Liu, A.S. Lewin, S.S. Tuli, S.C. Ghivizzani, G.S. Schultz, and D.C. Bloom, Reduction in severity of a herpes simplex virus type 1 murine infection by treatment with a ribozyme targeting the UL20 gene RNA. J Virol 82 (2008) 7467-74.

[21] Z.L. Watson, S.D. Washington, D.M. Phelan, A.S. Lewin, S.S. Tuli, G.S. Schultz, D.M. Neumann, and D.C. Bloom, In Vivo Knockdown of the Herpes Simplex Virus 1 Latency-Associated Transcript Reduces Reactivation from Latency. J Virol 92 (2018).

[22] B. Kim, Q. Tang, P.S. Biswas, J. Xu, R.M. Schiffelers, F.Y. Xie, A.M. Ansari, P.V. Scaria, M.C. Woodle, P. Lu, and B.T. Rouse, Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. Am J Pathol 165 (2004) 2177-85.

[23] S. Wasmuth, D. Bauer, K.P. Steuhl, and A. Heiligenhaus, Topical antisense-oligonucleotides targeting IFN-gamma mRNA improve incidence and severity of herpetic stromal keratitis by cytokine specific and sequence unspecific effects. Graefes Arch Clin Exp Ophthalmol 246 (2008) 443-51.

[24] S.C. Gopinath, K. Hayashi, and P.K. Kumar, Aptamer that binds to the gD protein of herpes simplex virus 1 and efficiently inhibits viral entry. J Virol 86 (2012) 6732-44.

[25] H. Augustinova, D. Hoeller, and F. Yao, The dominant-negative herpes simplex virus type 1 (HSV-1) recombinant CJ83193 can serve as an effective vaccine against wild-type HSV-1 infection in mice. J Virol 78 (2004) 5756-65.

[26] S. Grosse, N. Huot, C. Mahiet, S. Arnould, S. Barradeau, D.L. Clerre, I. Chion-Sotinel, C. Jacqmarcq, B. Chapellier, A. Ergani, C. Desseaux, F. Cedrone, E. Conseiller, F. Paques, M. Labetoulle, and J. Smith, Meganuclease-mediated Inhibition of HSV1 Infection in Cultured Cells. Mol Ther 19 (2011) 694-702.

[27] H.M. Elbadawy, M. Gailledrat, C. Desseaux, G. Salvalaio, E. Di Iorio, B. Ferrari, M. Bertolin, V. Barbaro, M. Parekh, R. Gayon, D. Munegato, E. Franchin, A. Calistri, G. Palu, C. Parolin, D. Ponzin, and S. Ferrari, Gene transfer of integration defective anti-HSV-1 meganuclease to human corneas ex vivo. Gene Ther 21 (2014) 272-81.

[28] F.R. van Diemen, E.M. Kruse, M.J. Hooykaas, C.E. Bruggeling, A.C. Schurch, P.M. van Ham, S.M. Imhof, M. Nijhuis, E.J. Wiertz, and R.J. Lebbink, CRISPR/Cas9-Mediated Genome Editing of Herpesviruses Limits Productive and Latent Infections. PLoS Pathog 12 (2016) e1005701.

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New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[29] J.A. Gonzales, and T.M. Lietman, Ocular Involvement in Sjögren's Syndrome: Advances in Therapy. Current Treatment Options in Rheumatology 4 (2018) 99-109.

[30] I.S. Song, and S.-M. Lee, Ocular Manifestations of Sjögren Syndrome. Hanyang Med Rev 36 (2016) 161-167.

[31] B. Tashbayev, S. Rusthen, A. Young, B.B. Herlofson, L.H. Hove, P.B. Singh, M. Rykke, L.A. Aqrawi, X. Chen, O.A. Utheim, T.P. Utheim, O. Palm, and J.L. Jensen, Interdisciplinary, Comprehensive Oral and Ocular Evaluation of Patients with Primary Sjogren's Syndrome. Sci Rep 7 (2017) 10761.

[32] K.C. Liu, K. Huynh, J. Grubbs, Jr., and R.M. Davis, Autoimmunity in the pathogenesis and treatment of keratoconjunctivitis sicca. Curr Allergy Asthma Rep 14 (2014) 403.

[33] K.C. Shih, C.N. Lun, V. Jhanji, B.Y. Thong, and L. Tong, Systematic review of randomized controlled trials in the treatment of dry eye disease in Sjogren syndrome. J Inflamm (Lond) 14 (2017) 26.

[34] P.J. Venables, Sjogren's syndrome. Best Pract Res Clin Rheumatol 18 (2004) 313-29.

[35] I. Saito, B. Servenius, T. Compton, and R.I. Fox, Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. J Exp Med 169 (1989) 2191-8.

[36] T. Both, V.A. Dalm, P.M. van Hagen, and P.L. van Daele, Reviewing primary Sjogren's syndrome: beyond the dryness - From pathophysiology to diagnosis and treatment. Int J Med Sci 14 (2017) 191-200.

[37] E. Banin, A. Obolensky, E. Piontek, H. Falk, E. Pikarsky, J. Pe'er, A. Panet, and I. Chowers, Gene delivery by viral vectors in primary cultures of lacrimal gland tissue. Invest Ophthalmol Vis Sci 44 (2003) 1529-33.

[38] Z. Zhu, D. Stevenson, J.E. Schechter, A.K. Mircheff, T. Ritter, L. Labree, and M.D. Trousdale, Prophylactic effect of IL-10 gene transfer on induced autoimmune dacryoadenitis. Invest Ophthalmol Vis Sci 45 (2004) 1375-81.

[39] M.D. Trousdale, Z. Zhu, D. Stevenson, J.E. Schechter, T. Ritter, and A.K. Mircheff, Expression of TNF inhibitor gene in the lacrimal gland promotes recovery of tear production and tear stability and reduced immunopathology in rabbits with induced autoimmune dacryoadenitis. J Autoimmune Dis 2 (2005) 6.

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New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[40] E.M. Rocha, A.P. Cotrim, C. Zheng, P.P. Riveros, B.J. Baum, and J.A. Chiorini, Recovery of radiation-induced dry eye and corneal damage by pretreatment with adenoviral vector-mediated transfer of erythropoietin to the salivary glands in mice. Hum Gene Ther 24 (2013) 417-23.

[41] L. Contreras-Ruiz, G.K. Zorzi, D. Hileeto, A. Lopez-Garcia, M. Calonge, B. Seijo, A. Sanchez, and Y. Diebold, A nanomedicine to treat ocular surface inflammation: performance on an experimental dry eye murine model. Gene Ther 20 (2013) 467-77.

[42] X. Zhang, C.S. De Paiva, Z. Su, E.A. Volpe, D.Q. Li, and S.C. Pflugfelder, Topical interferon-gamma neutralization prevents conjunctival goblet cell loss in experimental murine dry eye. Exp Eye Res 118 (2014) 117-24.

[43] D.F. Larkin, R.A. Alexander, and I.A. Cree, Infiltrating inflammatory cell phenotypes and apoptosis in rejected human corneal allografts. Eye (Lond) 11 (Pt 1) (1997) 68-74.

[44] X.L. Tang, J.F. Sun, X.Y. Wang, L.L. Du, and P. Liu, Blocking neuropilin-2 enhances corneal allograft survival by selectively inhibiting lymphangiogenesis on vascularized beds. Mol Vis 16 (2010) 2354-61.

[45] Y.K. Cho, H. Uehara, J.R. Young, P. Tyagi, U.B. Kompella, X. Zhang, L. Luo, N. Singh, B. Archer, and B.K. Ambati, Flt23k nanoparticles offer additive benefit in graft survival and anti-angiogenic effects when combined with triamcinolone. Invest Ophthalmol Vis Sci 53 (2012) 2328-36.

[46] Y.K. Cho, X. Zhang, H. Uehara, J.R. Young, B. Archer, and B. Ambati, Vascular Endothelial Growth Factor Receptor 1 morpholino increases graft survival in a murine penetrating keratoplasty model. Invest Ophthalmol Vis Sci 53 (2012) 8458-71.

[47] R.M. Comer, W.J. King, N. Ardjomand, S. Theoharis, A.J. George, and D.F. Larkin, Effect of administration of CTLA4-Ig as protein or cDNA on corneal allograft survival. Invest Ophthalmol Vis Sci 43 (2002) 1095-103.

[48] N. Gong, U. Pleyer, J. Yang, K. Vogt, M. Hill, I. Anegon, H.D. Volk, and T. Ritter, Influence of local and systemic CTLA4Ig gene transfer on corneal allograft survival. J Gene Med 8 (2006) 459-67.

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New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[49] N. Gong, U. Pleyer, H.D. Volk, and T. Ritter, Effects of local and systemic viral interleukin-10 gene transfer on corneal allograft survival. Gene Ther 14 (2007) 484-90.

[50] S. Klebe, D.J. Coster, P.J. Sykes, S. Swinburne, P. Hallsworth, J.P. Scheerlinck, R. Krishnan, and K.A. Williams, Prolongation of sheep corneal allograft survival by transfer of the gene encoding ovine IL-12-p40 but not IL-4 to donor corneal endothelium. J Immunol 175 (2005) 2219-26.

[51] S. Klebe, P.J. Sykes, D.J. Coster, R. Krishnan, and K.A. Williams, Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. Transplantation 71 (2001) 1214-20.

[52] D.G. Parker, D.J. Coster, H.M. Brereton, P.H. Hart, R. Koldej, D.S. Anson, and K.A. Williams, Lentivirus-mediated gene transfer of interleukin 10 to the ovine and human cornea. Clin Exp Ophthalmol 38 (2010) 405-13.

[53] S.C. Beutelspacher, R. Pillai, M.P. Watson, P.H. Tan, J. Tsang, M.O. McClure, A.J. George, and D.F. Larkin, Function of indoleamine 2,3-dioxygenase in corneal allograft rejection and prolongation of allograft survival by over-expression. Eur J Immunol 36 (2006) 690-700.

[54] T.A. Fuchsluger, U. Jurkunas, A. Kazlauskas, and R. Dana, Anti-apoptotic gene therapy prolongs survival of corneal endothelial cells during storage. Gene Ther 18 (2011) 778-87.

[55] M. Nosov, M. Wilk, M. Morcos, M. Cregg, L. O'Flynn, O. Treacy, and T. Ritter, Role of lentivirus-mediated overexpression of programmed death-ligand 1 on corneal allograft survival. Am J Transplant 12 (2012) 1313-22.

[56] N.S. Abdelfattah, M. Amgad, A.A. Zayed, H. Salem, A.E. Elkhanany, H. Hussein, and N. Abd El-Baky, Clinical correlates of common corneal neovascular diseases: a literature review. Int J Ophthalmol 8 (2015) 182-93.

[57] J.H. Chang, E.E. Gabison, T. Kato, and D.T. Azar, Corneal neovascularization. Curr Opin Ophthalmol 12 (2001) 242-9.

[58] A. Kvanta, S. Sarman, P. Fagerholm, S. Seregard, and B. Steen, Expression of matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) in inflammation-associated corneal neovascularization. Exp Eye Res 70 (2000) 419-28.

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Human Gene Therapy
New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[59] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1 (1995) 27-31.

[60] E. Gabison, J.H. Chang, E. Hernandez-Quintela, J. Javier, P.C. Lu, H. Ye, T. Kure, T. Kato, and D.T. Azar, Anti-angiogenic role of angiostatin during corneal wound healing. Exp Eye Res 78 (2004) 579-89.

[61] C.M. Lai, K. Spilsbury, M. Brankov, T. Zaknich, and P.E. Rakoczy, Inhibition of corneal neovascularization by recombinant adenovirus mediated antisense VEGF RNA. Exp Eye Res 75 (2002) 625-34.

[62] J.H. Chang, N.K. Garg, E. Lunde, K.Y. Han, S. Jain, and D.T. Azar, Corneal neovascularization: an anti-VEGF therapy review. Surv Ophthalmol 57 (2012) 415-29.

[63] S.U. Stechschulte, A.M. Joussen, H.A. von Recum, V. Poulaki, Y. Moromizato, J. Yuan, R.J. D'Amato, C. Kuo, and A.P. Adamis, Rapid ocular angiogenic control via naked DNA delivery to cornea. Invest Ophthalmol Vis Sci 42 (2001) 1975-9.

[64] W.Z. Yu, X.X. Li, H.C. She, P.Y. He, J.Q. Dong, M. Rui, and D.L. Ma, Gene transfer of kringle 5 of plasminogen by electroporation inhibits corneal neovascularization. Ophthalmic Res 35 (2003) 239-46.

[65] H. Huang, P. Vasilakis, X. Zhong, J.K. Shen, K. Geronatsiou, H. Papadaki, M.E. Maragoudakis, S.P. Gartaganis, S.A. Vinores, and N.E. Tsopanoglou, Parstatin suppresses ocular neovascularization and inflammation. Invest Ophthalmol Vis Sci 51 (2010) 5825-32.

[66] K.C. Yoon, K.Y. Ahn, J.H. Lee, B.J. Chun, S.W. Park, M.S. Seo, Y.G. Park, and K.K. Kim, Lipid-mediated delivery of brain-specific angiogenesis inhibitor 1 gene reduces corneal neovascularization in an in vivo rabbit model. Gene Ther 12 (2005) 617-24.

[67] L.J. Lai, X. Xiao, and J.H. Wu, Inhibition of corneal neovascularization with endostatin delivered by adeno-associated viral (AAV) vector in a mouse corneal injury model. J Biomed Sci 14 (2007) 313-22.

[68] R.R. Mohan, J.C. Tovey, A. Sharma, G.S. Schultz, J.W. Cowden, and A. Tandon, Targeted decorin gene therapy delivered with adeno-associated virus effectively retards corneal neovascularization in vivo. PLoS One 6 (2011) e26432.

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New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[69] A. Iriyama, M. Oba, T. Ishii, N. Nishiyama, K. Kataoka, Y. Tamaki, and Y. Yanagi, Gene transfer using micellar nanovectors inhibits choroidal neovascularization in vivo. PLoS One 6 (2011) e28560.

[70] Y. Qazi, B. Stagg, N. Singh, S. Singh, X. Zhang, L. Luo, J. Simonis, U.B. Kompella, and B.K. Ambati, Nanoparticle-mediated delivery of shRNA.VEGF-a plasmids regresses corneal neovascularization. Invest Ophthalmol Vis Sci 53 (2012) 2837-44.

[71] Y. Lu, P.W.L. Tai, J. Ai, D.J. Gessler, Q. Su, X. Yao, Q. Zheng, P.D. Zamore, X. Xu, and G. Gao, Transcriptome Profiling of Neovascularized Corneas Reveals miR-204 as a Multi-target Biotherapy Deliverable by rAAVs. Mol Ther Nucleic Acids 10 (2018) 349-360.

[72] K. Sawamoto, H.H. Chen, C.J. Almeciga-Diaz, R.W. Mason, and S. Tomatsu, Gene therapy for Mucopolysaccharidoses. Mol Genet Metab 123 (2018) 59-68.

[73] J.L. Ashworth, S. Biswas, E. Wraith, and I.C. Lloyd, Mucopolysaccharidoses and the eye. Surv Ophthalmol 51 (2006) 1-17.

[74] S. Ferrari, D. Ponzin, J.L. Ashworth, K.T. Fahnehjelm, C.G. Summers, P.R. Harmatz, and M. Scarpa, Diagnosis and management of ophthalmological features in patients with mucopolysaccharidosis. Br J Ophthalmol 95 (2011) 613-9.

[75] K.P. Ponder, and A. A., Gene therapy for ocular problems in mucopolisaccharidosis: an experimental and promising approach with benefits in animal models – a review. Clinical and Experimental Ophthalmology 38 (2010) 43-51.

[76] Y. Liu, L. Xu, A.K. Hennig, A. Kovacs, A. Fu, S. Chung, D. Lee, B. Wang, R.S. Herati, J. Mosinger Ogilvie, S.R. Cai, and K. Parker Ponder, Liver-directed neonatal gene therapy prevents cardiac, bone, ear, and eye disease in mucopolysaccharidosis I mice. Mol Ther 11 (2005) 35-47.

[77] L. Xu, R.L. Mango, M.S. Sands, M.E. Haskins, N.M. Ellinwood, and K.P. Ponder, Evaluation of pathological manifestations of disease in mucopolysaccharidosis VII mice after neonatal hepatic gene therapy. Mol Ther 6 (2002) 745-58.

[78] T.M. Daly, C. Vogler, B. Levy, M.E. Haskins, and M.S. Sands, Neonatal gene transfer leads to widespread correction of pathology in a murine model of lysosomal storage disease. Proc Natl Acad Sci U S A 96 (1999) 2296-300.

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New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[79] T.M. Daly, K.K. Ohlemiller, M.S. Roberts, C.A. Vogler, and M.S. Sands, Prevention of systemic clinical disease in MPS VII mice following AAV-mediated neonatal gene transfer. Gene Ther 8 (2001) 1291-8.

[80] A. Donsante, B. Levy, C. Vogler, and M.S. Sands, Clinical response to persistent, low-level beta-glucuronidase expression in the murine model of mucopolysaccharidosis type VII. J Inherit Metab Dis 30 (2007) 227-38.

[81] K.P. Ponder, J.R. Melniczek, L. Xu, M.A. Weil, T.M. O'Malley, P.A. O'Donnell, V.W. Knox, G.D. Aguirre, H. Mazrier, N.M. Ellinwood, M. Sleeper, A.M. Maguire, S.W. Volk, R.L. Mango, J. Zweigle, J.H. Wolfe, and M.E. Haskins, Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. Proc Natl Acad Sci U S A 99 (2002) 13102-7.

[82] M. Vance, T. Llanga, W. Bennett, K. Woodard, G. Murlidharan, N. Chungfat, A. Asokan, B. Gilger, J. Kurtzberg, R.J. Samulski, and M.L. Hirsch, AAV Gene Therapy for MPS1-associated Corneal Blindness. Sci Rep 6 (2016) 22131.

[83] S.A. Harding, K.K. Nischal, A. Upponi-Patil, and D.J. Fowler, Indications and outcomes of deep anterior lamellar keratoplasty in children. Ophthalmology 117 (2010) 2191-5.

[84] Y. Kamata, T. Okuyama, M. Kosuga, A. O'Hira, A. Kanaji, K. Sasaki, M. Yamada, and N. Azuma, Adenovirus-mediated gene therapy for corneal clouding in mice with mucopolysaccharidosis type VII. Mol Ther 4 (2001) 307-12.

[85] N. Serratrice, A. Cubizolle, S. Ibanes, N. Mestre-Frances, N. Bayo-Puxan, S. Creyssels, A. Gennetier, F. Bernex, J.M. Verdier, M.E. Haskins, G. Couderc, F. Malecaze, V. Kalatzis, and E.J. Kremer, Corrective GUSB transfer to the canine mucopolysaccharidosis VII cornea using a helper-dependent canine adenovirus vector. J Control Release 181 (2014) 22-31.

[86] A.D. Irvine, L.D. Corden, O. Swensson, B. Swensson, J.E. Moore, D.G. Frazer, F.J. Smith, R.G. Knowlton, E. Christophers, R. Rochels, J. Uitto, and W.H. McLean, Mutations in cornea-specific keratin K3 or K12 genes cause Meesmann's corneal dystrophy. Nat Genet 16 (1997) 184-7.

[87] M.B. Omary, P.A. Coulombe, and W.H. McLean, Intermediate filament proteins and their associated diseases. N Engl J Med 351 (2004) 2087-100.

[88] J.S. Weiss, H.U. Moller, A.J. Aldave, B. Seitz, C. Bredrup, T. Kivela, F.L. Munier, C.J. Rapuano, K.K. Nischal, E.K. Kim, J. Sutphin, M. Busin, A. Labbe, K.R. Kenyon, S. Kinoshita, and W. Lisch, IC3D classification of corneal dystrophies--edition 2. Cornea 34 (2015) 117-59.

[89] H. Hassan, C. Thaung, N.D. Ebenezer, G. Larkin, A.J. Hardcastle, and S.J. Tuft, Severe Meesmann's epithelial corneal dystrophy phenotype due to a missense mutation in the helix-initiation motif of keratin 12. Eye (Lond) 27 (2013) 367-73.

[90] E.H. Allen, D.G. Courtney, S.D. Atkinson, J.E. Moore, L. Mairs, E.T. Poulsen, D. Schiroli, E. Maurizi, C. Cole, R.P. Hickerson, J. James, H. Murgatroyd, F.J. Smith, C. MacEwen, J.J. Enghild, M.A. Nesbit, D.M. Leslie Pedrioli, W.H. McLean, and C.B. Moore, Keratin 12 missense mutation induces the unfolded protein response and apoptosis in Meesmann epithelial corneal dystrophy. Hum Mol Genet 25 (2016) 1176-91.

[91] K. Nishida, Y. Honma, A. Dota, S. Kawasaki, W. Adachi, T. Nakamura, A.J. Quantock, H. Hosotani, S. Yamamoto, M. Okada, Y. Shimomura, and S. Kinoshita, Isolation and chromosomal localization of a cornea-specific human keratin 12 gene and detection of four mutations in Meesmann corneal epithelial dystrophy. Am J Hum Genet 61 (1997) 1268-75.

[92] N. Ehlers, J. Hjortdal, K. Nielsen, H.J. Thiel, and T. Orntoft, Phenotypic variability in Meesmann's dystrophy: clinical review of the literature and presentation of a family genetically identical to the original family. Acta Ophthalmol 86 (2008) 40-4.

[93] J.P. Szaflik, M. Oldak, R.B. Maksym, A. Kaminska, A. Pollak, M. Udziela, R. Ploski, and J. Szaflik, Genetics of Meesmann corneal dystrophy: a novel mutation in the keratin 3 gene in an asymptomatic family suggests genotype-phenotype correlation. Mol Vis 14 (2008) 1713-8.

[94] A.G. Chiou, G.J. Florakis, R.L. Copeland, V.A. Williams, S.A. McCormick, and R. Chiesa, Recurrent Meesmann's corneal epithelial dystrophy after penetrating keratoplasty. Cornea 17 (1998) 566-70.

[95] A. Goldberg, U. Schlotzer-Schrehardt, and T. Seiler, Unilateral Meesmann's dystrophy. Int Ophthalmol 21 (1997) 117-20.

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New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[96] W.W. Kao, C.Y. Liu, R.L. Converse, A. Shiraishi, C.W. Kao, M. Ishizaki, T. Doetschman, and J. Duffy, Keratin 12-deficient mice have fragile corneal epithelia. Invest Ophthalmol Vis Sci 37 (1996) 2572-84.

[97] H. Liao, A.D. Irvine, C.J. Macewen, K.H. Weed, L. Porter, L.D. Corden, A.B. Gibson, J.E. Moore, F.J. Smith, W.H. McLean, and C.B. Moore, Development of allele-specific therapeutic siRNA in Meesmann epithelial corneal dystrophy. PLoS One 6 (2011) e28582.

[98] E.H.A. Allen, S.D. Atkinson, H. Liao, J.E. Moore, D.M.L. Pedrioli, F.J.D. Smith, W.H.I. McLean, and C.B.T. Moore, Allele-specific siRNA silencing for the common keratin 12 founder mutation in Meesmann epithelial corneal dystrophy. Invest Ophthalmol Vis Sci 54 (2013) 494-502.

[99] D.G. Courtney, S.D. Atkinson, E.H. Allen, J.E. Moore, C.P. Walsh, D.M. Pedrioli, C.J. MacEwen, G. Pellegrini, E. Maurizi, C. Serafini, M. Fantacci, H. Liao, A.D. Irvine, W.H. McLean, and C.B. Moore, siRNA silencing of the mutant keratin 12 allele in corneal limbal epithelial cells grown from patients with Meesmann's epithelial corneal dystrophy. Invest Ophthalmol Vis Sci 55 (2014) 3352-60.

[100] D.G. Courtney, J.E. Moore, S.D. Atkinson, E. Maurizi, E.H. Allen, D.M. Pedrioli, W.H. McLean, M.A. Nesbit, and C.B. Moore, CRISPR/Cas9 DNA cleavage at SNP-derived PAM enables both in vitro and in vivo KRT12 mutation-specific targeting. Gene Ther 23 (2016) 108-12.

[101] T. Rinne, H.G. Brunner, and H. van Bokhoven, p63-associated disorders. Cell Cycle 6 (2007) 262-8.

[102] T.H. Celik, A. Buyukcam, P.O. Simsek-Kiper, G.E. Utine, S. Ersoy-Evans, A. Korkmaz, H.G. Yntema, K. Bodugroglu, and M. Yurdakok, A newborn with overlapping features of AEC and EEC syndromes. Am J Med Genet A 155A (2011) 3100-3.

[103] A.A. McNab, M.J. Potts, and R.A. Welham, The EEC syndrome and its ocular manifestations. Br J Ophthalmol 73 (1989) 261-4.

[104] E. Di Iorio, S.B. Kaye, D. Ponzin, V. Barbaro, S. Ferrari, E. Bohm, P. Nardiello, G. Castaldo, J.A. McGrath, and C.E. Willoughby, Limbal stem cell deficiency and ocular phenotype in ectrodactyly-ectodermal dysplasia-clefting syndrome caused by p63 mutations. Ophthalmology 119 (2012) 74-83.

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Human Gene Therapy
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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[105] E. Di Iorio, V. Barbaro, A. Ruzza, D. Ponzin, G. Pellegrini, and M. De Luca, Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc Natl Acad Sci U S A 102 (2005) 9523-8.

[106] V. Barbaro, L. Confalonieri, I. Vallini, S. Ferrari, D. Ponzin, G. Mantero, C.E. Willoughby, M. Parekh, and E. Di Iorio, Development of an allele-specific real-time PCR assay for discrimination and quantification of p63 R279H mutation in EEC syndrome. J Mol Diagn 14 (2012) 38-45.

[107] T. Hamada, I. Chan, C.E. Willoughby, D.R. Goudie, and J.A. McGrath, Common mutations in Arg304 of the p63 gene in ectrodactyly, ectodermal dysplasia, clefting syndrome: lack of genotype-phenotype correlation and implications for mutation detection strategies. J Invest Dermatol 119 (2002) 1202-3.

[108] V. Barbaro, A.A. Nasti, C. Del Vecchio, S. Ferrari, A. Migliorati, P. Raffa, V. Lariccia, P. Nespeca, M. Biasolo, C.E. Willoughby, D. Ponzin, G. Palu, C. Parolin, and E. Di Iorio, Correction of Mutant p63 in EEC Syndrome Using siRNA Mediated Allele-Specific Silencing Restores Defective Stem Cell Function. Stem Cells 34 (2016) 1588-600.

[109] V. Barbaro, A.A. Nasti, P. Raffa, A. Migliorati, P. Nespeca, S. Ferrari, E. Palumbo, M. Bertolin, C. Breda, F. Miceli, A. Russo, L. Caenazzo, D. Ponzin, G. Palu, C. Parolin, and E. Di Iorio, Personalized Stem Cell Therapy to Correct Corneal Defects Due to a Unique Homozygous-Heterozygous Mosaicism of Ectrodactyly-Ectodermal Dysplasia-Clefting Syndrome. Stem Cells Transl Med 5 (2016) 1098-105.

[110] A.J. Shortt, G.A. Secker, M.D. Notara, G.A. Limb, P.T. Khaw, S.J. Tuft, and J.T. Daniels, Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. Surv Ophthalmol 52 (2007) 483-502.

[111] F. Novelli, A.M. Lena, E. Panatta, W. Nasser, R. Shalom-Feuerstein, E. Candi, and G. Melino, Allele-specific silencing of EEC p63 mutant R304W restores p63 transcriptional activity. Cell Death Dis 7 (2016) e2227.

[112] T. Jordan, I. Hanson, D. Zaletayev, S. Hodgson, J. Prosser, A. Seawright, N. Hastie, and V. van Heyningen, The human PAX6 gene is mutated in two patients with aniridia. Nat Genet 1 (1992) 328-32.

[113] J.M. Collinson, S.A. Chanas, R.E. Hill, and J.D. West, Corneal development, limbal stem cell function, and corneal epithelial cell migration in the Pax6(+/-) mouse. Invest Ophthalmol Vis Sci 45 (2004) 1101-8.

[114] K. Nishida, S. Kinoshita, Y. Ohashi, Y. Kuwayama, and S. Yamamoto, Ocular surface abnormalities in aniridia. Am J Ophthalmol 120 (1995) 368-75.

[115] S.C. Tseng, Concept and application of limbal stem cells. Eye (Lond) 3 (Pt 2) (1989) 141-57.

[116] H.T. Lim, D.H. Kim, and H. Kim, PAX6 aniridia syndrome: clinics, genetics, and therapeutics. Curr Opin Ophthalmol 28 (2017) 436-447.

[117] I. Tzoulaki, I.M. White, and I.M. Hanson, PAX6 mutations: genotype-phenotype correlations. BMC Genet 6 (2005) 27.

[118] X. Wang, K. Gregory-Evans, K.M. Wasan, O. Sivak, X. Shan, and C.Y. Gregory-Evans, Efficacy of Postnatal In Vivo Nonsense Suppression Therapy in a Pax6 Mouse Model of Aniridia. Mol Ther Nucleic Acids 7 (2017) 417-428.

[119] A. Schedl, A. Ross, M. Lee, D. Engelkamp, P. Rashbass, V. van Heyningen, and N.D. Hastie, Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities. Cell 86 (1996) 71-82.

[120] S.W. Peltz, M. Morsy, E.M. Welch, and A. Jacobson, Ataluren as an agent for therapeutic nonsense suppression. Annu Rev Med 64 (2013) 407-25.

[121] D.A. Kleinjan, A. Seawright, S. Mella, C.B. Carr, D.A. Tyas, T.I. Simpson, J.O. Mason, D.J. Price, and V. van Heyningen, Long-range downstream enhancers are essential for Pax6 expression. Dev Biol 299 (2006) 563-81.

[122] L.N. Roux, I. Petit, R. Domart, J.P. Concordet, J. Qu, H. Zhou, A. Joliot, O. Ferrigno, and D. Aberdam, Modeling of Aniridia-Related Keratopathy by CRISPR/Cas9 Genome Editing of Human Limbal Epithelial Cells and Rescue by Recombinant PAX6 Protein. Stem Cells (2018).

[123] J.W. Hickmott, C.Y. Chen, D.J. Arenillas, A.J. Korecki, S.L. Lam, L.L. Molday, R.J. Bonaguro, M. Zhou, A.Y. Chou, A. Mathelier, S.L. Boye, W.W. Hauswirth, R.S. Molday, W.W. Wasserman, and E.M. Simpson, PAX6 MiniPromoters drive restricted expression from rAAV in the adult mouse retina. Mol Ther Methods Clin Dev 3 (2016) 16051.

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Human Gene Therapy
New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[124] E.D. Wieben, R.A. Aleff, X. Tang, M.L. Butz, K.R. Kalari, E.W. Highsmith, J. Jen, G. Vasmatzis, S.V. Patel, L.J. Maguire, K.H. Baratz, and M.P. Fautsch, Trinucleotide Repeat Expansion in the Transcription Factor 4 (TCF4) Gene Leads to Widespread mRNA Splicing Changes in Fuchs' Endothelial Corneal Dystrophy. Invest Ophthalmol Vis Sci 58 (2017) 343-352.

[125] C. Zarouchlioti, B. Sanchez-Pintado, N.J. Hafford Tear, P. Klein, P. Liskova, K. Dulla, M. Semo, A.A. Vugler, K. Muthusamy, L. Dudakova, H.J. Levis, P. Skalicka, P. Hysi, M.E. Cheetham, S.J. Tuft, P. Adamson, A.J. Hardcastle, and A.E. Davidson, Antisense Therapy for a Common Corneal Dystrophy Ameliorates TCF4 Repeat Expansion-Mediated Toxicity. Am J Hum Genet 102 (2018) 528-539.

[126] J.B. Opalinska, and A.M. Gewirtz, Nucleic-acid therapeutics: basic principles and recent applications. Nat Rev Drug Discov 1 (2002) 503-14.

[127] P.S. Eder, R.J. DeVine, J.M. Dagle, and J.A. Walder, Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3' exonuclease in plasma. Antisense Res Dev 1 (1991) 141-51.

[128] F. Eckstein, Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? Antisense Nucleic Acid Drug Dev 10 (2000) 117-21.

[129] J. Summerton, D. Stein, S.B. Huang, P. Matthews, D. Weller, and M. Partridge, Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. Antisense Nucleic Acid Drug Dev 7 (1997) 63-70.

[130] R.M. Hudziak, E. Barofsky, D.F. Barofsky, D.L. Weller, S.B. Huang, and D.D. Weller, Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev 6 (1996) 267-72.

[131] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411 (2001) 494-8.

[132] C.C. Mello, and D. Conte, Jr., Revealing the world of RNA interference. Nature 431 (2004) 338-42.

[133] S.M. Hammond, E. Bernstein, D. Beach, and G.J. Hannon, An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404 (2000) 293-6.

Downloaded by STOCKHOLMS UNIVERSITET from www.liebertpub.com at 04/27/19. For personal use only.
Human Gene Therapy
New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[134] P.D. Zamore, T. Tuschl, P.A. Sharp, and D.P. Bartel, RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101 (2000) 25-33.

[135] C. Wahlestedt, P. Salmi, L. Good, J. Kela, T. Johnsson, T. Hokfelt, C. Broberger, F. Porreca, J. Lai, K. Ren, M. Ossipov, A. Koshkin, N. Jakobsen, J. Skouv, H. Oerum, M.H. Jacobsen, and J. Wengel, Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. Proc Natl Acad Sci U S A 97 (2000) 5633-8.

[136] C.B. Moore, E.H. Guthrie, M.T. Huang, and D.J. Taxman, Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol Biol 629 (2010) 141-58.

[137] L.J. Scherer, and J.J. Rossi, Approaches for the sequence-specific knockdown of mRNA. Nat Biotechnol 21 (2003) 1457-65.

[138] P.R. Bouchard, R.M. Hutabarat, and K.M. Thompson, Discovery and development of therapeutic aptamers. Annu Rev Pharmacol Toxicol 50 (2010) 237-57.

[139] A.D. Keefe, S. Pai, and A. Ellington, Aptamers as therapeutics. Nat Rev Drug Discov 9 (2010) 537-50.

[140] A.D. Ellington, and J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands. Nature 346 (1990) 818-22.

[141] R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D.A. Romero, and P. Horvath, CRISPR provides acquired resistance against viruses in prokaryotes. Science 315 (2007) 1709-12.

[142] E. Deltcheva, K. Chylinski, C.M. Sharma, K. Gonzales, Y. Chao, Z.A. Pirzada, M.R. Eckert, J. Vogel, and E. Charpentier, CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471 (2011) 602-7.

[143] H. Deveau, R. Barrangou, J.E. Garneau, J. Labonte, C. Fremaux, P. Boyaval, D.A. Romero, P. Horvath, and S. Moineau, Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J Bacteriol 190 (2008) 1390-400.

[144] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, and F. Zhang, Multiplex genome engineering using CRISPR/Cas systems. Science 339 (2013) 819-23.

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Human Gene Therapy
New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[145] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, and E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337 (2012) 816-21.

[146] R.D. Johnson, and M. Jasin, Double-strand-break-induced homologous recombination in mammalian cells. Biochem Soc Trans 29 (2001) 196-201.

[147] F.S. Gimble, Invasion of a multitude of genetic niches by mobile endonuclease genes. FEMS Microbiol Lett 185 (2000) 99-107.

[148] A.J. Thompson, X. Yuan, W. Kudlicki, and D.L. Herrin, Cleavage and recognition pattern of a double-strand-specific endonuclease (I-creI) encoded by the chloroplast 23S rRNA intron of Chlamydomonas reinhardtii. Gene 119 (1992) 247-51.

[149] X. Duan, F.S. Gimble, and F.A. Quiocho, Crystal structure of PI-SceI, a homing endonuclease with protein splicing activity. Cell 89 (1997) 555-64.

[150] F.S. Gimble, C.M. Moure, and K.L. Posey, Assessing the plasticity of DNA target site recognition of the PI-SceI homing endonuclease using a bacterial two-hybrid selection system. J Mol Biol 334 (2003) 993-1008.

[151] B. Chevalier, M. Turmel, C. Lemieux, R.J. Monnat, Jr., and B.L. Stoddard, Flexible DNA target site recognition by divergent homing endonuclease isoschizomers I-CreI and I-MsoI. J Mol Biol 329 (2003) 253-69.

[152] F. Sonntag, K. Schmidt, and J.A. Kleinschmidt, A viral assembly factor promotes AAV2 capsid formation in the nucleolus. Proc Natl Acad Sci U S A 107 (2010) 10220-5.

[153] R.J. Samulski, L.S. Chang, and T. Shenk, Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J Virol 63 (1989) 3822-8.

[154] T.R. Flotte, Gene therapy progress and prospects: recombinant adeno-associated virus (rAAV) vectors. Gene Ther 11 (2004) 805-10.

[155] X. Wu, and S.M. Burgess, Integration target site selection for retroviruses and transposable elements. Cell Mol Life Sci 61 (2004) 2588-96.

[156] M.K. Lewinski, and F.D. Bushman, Retroviral DNA integration--mechanism and consequences. Adv Genet 55 (2005) 147-81.

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Human Gene Therapy
New frontiers of corneal gene therapy (DOI: 10.1089/hum.2019.026)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[157] A. Cereseto, and M. Giacca, Integration site selection by retroviruses. AIDS Rev 6 (2004) 13-21.

[158] S. Fukushima, K. Miyata, N. Nishiyama, N. Kanayama, Y. Yamasaki, and K. Kataoka, PEGylated polyplex micelles from triblock catiomers with spatially ordered layering of condensed pDNA and buffering units for enhanced intracellular gene delivery. J Am Chem Soc 127 (2005) 2810-1.

[159] J. Gehl, Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. Acta Physiol Scand 177 (2003) 437-47.

[160] M.L. Yarmush, A. Golberg, G. Sersa, T. Kotnik, and D. Miklavcic, Electroporation-based technologies for medicine: principles, applications, and challenges. Annu Rev Biomed Eng 16 (2014) 295-320.

[161] S. Bugeon, A. de Chevigny, C. Boutin, N. Core, S. Wild, A. Bosio, H. Cremer, and C. Beclin, Direct and efficient transfection of mouse neural stem cells and mature neurons by in vivo mRNA electroporation. Development 144 (2017) 3968-3977.

[162] R.R. Mohan, S. Sinha, A. Tandon, R. Gupta, J.C. Tovey, and A. Sharma, Efficacious and safe tissue-selective controlled gene therapy approaches for the cornea. PLoS One 6 (2011) e18771.

[163] C.Y. Gregory-Evans, X. Wang, K.M. Wasan, J. Zhao, A.L. Metcalfe, and K. Gregory-Evans, Postnatal manipulation of Pax6 dosage reverses congenital tissue malformation defects. J Clin Invest 124 (2014) 111-6.

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BOX1. Nucleic acids delivered for corneal gene therapy approaches.

Gene addition: the delivered nucleic acids are represented by the therapeutic gene (the wild type version of the gene that is usually mutated in the pathological situation) which is delivered into cells through different methods (see Box 2) in order to implement the physiological gene function.

Gene ablation: short nucleic acids sequences or programmable nucleases are delivered into cells in order to ablate the aberrant function of the mutated mRNAs (or proteins) or gene, respectively.

Antisense oligonucleotides (ASO): ASO are short (15 to 100 nt), chemically synthesized, single-stranded oligodeoxynucleotides which bind by complementary base pairing to a sequence of a target mRNA, leading to block of translation at the ribosome level, degradation by endonucleases and mediated transcript knockdown [126]. ASO might have a limited tissue distribution when injected in vivo; and due to their poor stability and rapid degradation by DNase and exonucleases might have a short half-life [127]. Chemical modifications such as in **antisense phosphorotioates**, in which sulfur replaces one oxygen in the phosphodiester bond, allow higher bioavailability due to an increased resistance against nuclease degradation [128]. Another modification is represented by **phosphorodiamidate morpholino (PMO) or morpholinos:** these are synthetic molecules of about 25nt, analog to DNA in which a morpholino ring replace the deoxyribose ring, and the phosphodiesteric bond is replaced by a phosphorodiamide bond [129]. Morpholinos inhibit gene expression by binding to the target mRNA and by blocking its processing in a steric manner, making them very resistant to nuclease and protease degradation [130].

Small interfering RNAs (siRNAs): siRNAs are 21-23nt long dsRNAs which recognize their mRNA targets by Watson-Crick base pairing and activate RNA interference (RNAi) pathway, leading to the degradation of the specific target mRNA [131]. siRNAs are produced by precursors consisting of long dsRNAs, either produced endogenously from the cell or supplied externally [132]. Once exogenously delivered into target cells, Dicer enzyme excise them from their precursors; one strand of the siRNA duplex (guide RNA) is then loaded into the RNA-induced silencing complex (RISCs, [133] and binds to target mRNA by

perfect or near-perfect base pairing, leading to its degradation with the assistance of Argonaute (Ago) proteins that support the silencing effector functions [134]. **Locked nucleic acid (LNA)-siRNAs** are nucleotide analogues-based siRNAs which confer higher affinity and nuclease resistance to RNA [135]. The most commonly used LNA contains a methylene bridge connecting the 2′-oxygen with the 4′-carbon of the ribose ring. **Short hairpin RNAs (shRNAs)** are another form of RNAi. They are transcribed within the cell from plasmids. shRNAs are composed by two complementary 19–22 bp RNA sequences linked by a short loop of 4–11 nt. Following transcription in the nucleus, the shRNA sequence enters the cytosol where it is recognized by Dicer, which processes it into the siRNA duplexes that binds to the target mRNA and are incorporated into the RISC complex for target-specific mRNA degradation [136].

Ribozymes: ribozymes are naturally occurring RNAs with enzymatic activity that can catalyze the cleavage of phosphodiesteric bonds present on RNA molecules. The catalytic core can be incorporated into an antisense molecule that directs its enzymatic function on specific cellular mRNAs, determining their degradation [137]. Ribozymes can be directly delivered into target cells or can be transcribed from exogenously delivered plasmids. Among the several types of ribozymes the most common used in therapy are derived from "hammerhead" motifs. Due to their poor stability *in vivo,* ribozymes often require chemical modifications.

Aptamers: aptamers are single stranded oligonucleotides (20-100nt) that exploit the secondary structure of nucleic acids rather than the complementarity of their sequence [138]. They bind with high affinity to a large variety of proteins subtracting them from their cellular functions, similarly to antibodies [139]. They are usually identified through multiple rounds of selection *in vitro* in a process known as systematic evolution of ligands by exponential enrichment (SELEX [140]). The applications are similar to those of antibodies but unlike these they are simpler to produce, they are not immunogenic and have greater stability, due to the possibility of modifications during chemical synthesis that allow to increase their resistance to nucleases and improve pharmacological properties.

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Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system: CRISPR/Cas9 system is a naturally occurring adaptive immune system that protects bacteria and archaea against invading conjugative plasmids, transposable elements, and viruses [141]. The type II CRISPR/Cas9 system incorporate sequences from invading DNA (termed protospacers) between CRISPR repeats encoded within the host genome. Transcripts from the CRISPR repeat arrays are processed into CRISPR RNAs (crRNAs), each harboring a variable sequence transcribed from the invading DNA. The crRNAs hybridized with another RNA, the trans-encoded small RNA (tracrRNA; [142]), and these complex with the CRISPR associated protein 9 (Cas9), the enzyme responsible for the cleavage of the target sequence. The protospacer encoded portion of the crRNA direct the Cas9 to exert DNA double strand brakes (DSBs) at the target-DNA sequence. The minimum requirement is the presence of a protospacer adjacent motif (PAM, NGG for *S. pyogenes*) at the 3' end of the DNA target site [143]. The CRISPR/Cas9 system can be adapted for genome editing purposes in eukaryotic cells [144]: a single guide RNA (sgRNA) complementary to the target site of interest is designed and inserted in a chimeric RNA, obtained by fusing the 3' end of crRNA to the 5' end of tracrRNA [145]. The system is then expressed in eukaryotic cells with the Cas9 enzyme. The formation of DNA DSBs is exploited to activate at the target site either Non Homologous end Joining (NHEJ) pathway or, in the presence of a template with a sequence homologous to the target sequence, Homologous direct repair (HDR) [146]. The former is an error-prone pathway that lead to the loss or acquisition of genetic material in the target region, which ultimately can generate frameshift and formation of premature stop codons. Consequently, nonsense-mediated RNA decay pathway is activated, leading to degradation of encoding mRNAs.

Meganucleases: meganucleases are homing endonucleases (HE) derived from microbial mobile genetic elements [147]. They are composed by a target recognition domain (14 to 40 bp) and an endonuclease domain, able to cleave the DNA and exert DNA DSBs which generates 3' overhangs (Marcaida et al. 2010). Engineered meganucleases are usually derived from members of the LAGLIDADG family (LHE) I-CreI and I-CeuI and recognize DNA palindromic sequences as homodimers [148]. I-SceI are variants that contain two motifs that act as monomers containing two nuclease domains and recognize DNA with

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symmetry-independent targeting [149]. Modifications of meganucleases's cleavage specificity are required in order to target endogenous sites in different genomes. *In vitro* selection methods such as bacterial two-hybrid screening strategy [150] or methods based on the redesign of the protein-DNA interface [151] allow the identification of mutated endonuclease constructs that displayed altered recognition specificity and that can be applied for targeted genome editing.

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BOX 2. Systems used to deliver nucleic acids for corneal gene therapy.

Delivery systems can be divided into viral and non-viral based methods. In order to obtain replication-defective viruses, viral genes, essential for replication and virulence, are replaced with the gene of interests (GOI) or non-coding nucleic acids. The non-viral based methods are mainly based on chemical or physical approaches applied to allow the nucleic acids, either free (short oligonucleotides) or inserted into expression plasmids, into the target cells.

Viral based methods:

Recombinant Adeno-Associated Viruses (rAAV):

Recombinant Adeno-Associated Viruses (rAAVs) are based on adeno-associated viruses (AAV), which belong to the *Parvoviridae* family, genus Dependovirus, as they rely on super infection with other viruses (such as Adenoviruses or Herpesviruses) to complete the replication cycle. AAVs are non-pathogenic non-enveloped viruses whose genome, a ssDNA molecule of about 4.7 Kb, is surrounded by a icosahedral capsid. The genome contains three open reading frames (ORFs) encoding for Rep, Cap and assembly-activating protein (AAP), that promotes capsid formation [152]. Rep genes are involved in viral replication, transcriptional regulation, genome integration and virion assembly while Cap gene encode for viral capsid proteins. The genome is flanked by 145 nucleotides-long inverted terminal repeats (ITRs), involved in many events of the replication cycle and the only elements necessary in *cis* for the construction of viral vectors [153]. rAAVs are generated by co-transfection of the packaging cell line with two components: the viral construct and an helper plasmid [154]. The first contains the AAV genome with the ITRs and the the gene of interest (GOI) under the control of a strong promoter that replace the viral ORFs. The latter is used to complement in *trans* Rep and Cap proteins as well as Adenovirus related genes, E1, E2, E4 and VA RNA, which are necessary for the formation of infectious particles. rAAVs can be produced at high titers, can transduce both dividing and non-dividing cells and their tropisms is dictated by the serotype employed. They have a high tropism for post-mitotic cells and this feature allow long lasting permanence of the viral vector that remains in an episomal form in non-proliferating cells. The limitations of

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rAAV are represented by the DNA packaging capacity (limited to about 4.5 Kb) and the possibility of pre-existing immunity that can lead to a fast clearance of the viral vectors.

Adenoviral vectors (AdVs):

Adenoviral vectors (AdVs) are based on Adenoviruses (Ads, usually on serotype 2 or 5) which belong to the *Adenoviridae* family and harbor a linear double stranded (ds) DNA genome of about 36Kb. The genome of Ads is flanked by Inverted terminal repeats (ITRs) and encodes for two classes of genes: the early genes (E1A, E1B, E2, E3 and E4), which are expressed during the early phases of infection before the viral replication take place and have regulatory functions and the late genes, which encode for structural proteins. In particular, E1A is a transcriptional regulating factor needed to begin the transcription of other early genes while E1B blocks apoptosis by binding to tumor suppressor protein tp53. E2 encodes for 3 proteins involved in DNA replication and transcription modulation (DNA polymerase, terminal protein and DNA-binding protein). E3 modulates the immune responses and E4 regulates transcription, transition of expression from early to late gene, viral replication, and virion assembly. The genome is surrounded by an icosahedral nucleocapsid composed by proteins called hexon and pentons. Each penton consists of a base and of a fiber projecting outwards and responsible for the binding to the cellular receptor for the entry of the virus, the Coxsackie Adenovirus Receptor (CAR). Adenoviruses are generally not highly pathogenic, except in the case of immunosuppressed patients. In first generations of AdVs, the essential E1A and E1B genes are deleted and complemented in *trans* in the packaging cell line and are replaced by an expression cassette with the GOI under the control of a strong promoter. E3 might be also deleted but its function does not need to be complemented. In second generation AdVs, besides E1A, E1B and/or E3 also E2 and E4 are deleted and complemented in *trans*. Finally, in the third generation AdVs, also called gutted vectors or helper-dependent adenoviral vectors, the only elements maintained in *cis* are the viral ITRs. In this case, a helper virus is needed in order to provide all the elements necessary for the packaging. AdVs have the advantages that can be grown at high titer; they have broad tropism and can transduce both dividing and non-dividing cells, providing a high and transient expression of the transgene; the DNA packaging

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capacity is high (from to in the gutted vectors). The limitations of AdVs is mainly imputable to the high immunogenicity.

Retroviral vectors:

Retroviruses are enveloped viruses belonging to the *Retroviridae* family. The genome is composed by two copies of single stranded (ss) positive sense RNA of about 9 Kb. Two long terminal repeats (LTRs) at the 5' and 3' ends of the genome, acting as promoters and regulating viral gene expression, flank three ORFs called *gag*, *pol* and *env*, encoding, respectively, the capsid proteins, replication enzymes, and envelope proteins. The peculiarity of this family of viruses is the fact that the viral RNA genome is converted by the reverse transcriptase to dsDNA, which is transported in the nucleus and randomly integrated into the host cell genome in a form of provirus [155]. Retroviral vectors are produced by co-transfecting into the packaging cell line the viral construct, containing the two LTRs, the packaging signal sequence (*Psi*) and the GOI, together with a plasmid complementing in *trans* gag, pol and env. This last one is usually substituted by pseudotyping the viral particle with the glycoprotein G of the Vesicular stomatitis virus (VSV-G), which allows a broader tropism, higher levels of transduction and the possibility to concentrate the virus by ultracentrifugation. Retroviral vectors, like the parental viruses, transduce target cells and allow for a stable expression of the transgene, which is integrated into the host cell genome. The disadvantages of using this type of vectors are that they can only transduce dividing cells and the risk of insertional mutagenesis, since they preferentially integrate close to cellular promoters and oncogenes [156].

Lentiviral vectors:

Lentiviral vectors are based on Human immunodeficiency virus 1 (HIV-1), belonging to the *Retroviridae* family. Beside the aforementioned canonical genes expressed by retroviruses, HIV-1 encodes six accessory proteins namely *tat*, *rev*, *vif*, *vpr*, *nef*, and *vpu* that encode for regulatory viral proteins. Lentiviral vectors lack all the viral sequences except the LTRs, rev responsive element (RRE), and cis -acting elements. The viral *gag*, *pol* and *env* (or VSV-G if the vector is pseudotyped) are complemented in *trans* together with *rev* whose product binds to RRE and facilitate the trafficking of viral genome in the nucleus. Lentiviral vectors

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have a broad tropism and randomly integrate into the host cell genome ensuring a long-lasting expression of the transgene. An advantage if comparing them to retroviral vectors is that they can transduce both dividing and non-dividing cells, and they have a lower risk of insertional mutagenesis as they tend to integrate in regions of actively transcribed genes away from cellular promoters [157].

Non viral based methods:

Synthetic cationic polymers:

Synthetic cationic polymers, such as Polyethylenimine (PEI) and chitosan, can complex plasmid DNA and condense it to form polyplex micelles that can enter the cell by endocytosis. Cationic polymers lack hydrophobic domains so they cannot fuse with endosomal membrane but once internalized into host cells within the endosome, acts as proton sponge causing its disruption and the release of the DNA. Synthetic cationic lipids can be modified with poly ethylene glycol (PEG) which enhance their transfection efficiency and half-life and can lead to high transgene expression [158].

Electroporation:

Electroporation is a physical method that uses physical force to perforate cell membrane and introduce nucleic acids into target cells [159]. In this method, an electric field is applied to change the permeability of cell membranes. According to the exposure duration and strength of the applied electric field, the effect on the cells can be reversible or irreversible. In the context of gene delivery for gene therapy purposes, electroporation is applied to cause a reversible poration [160]. Despite this method is mostly restricted to *in vitro* applications, it has been applied for successful gene transfer in vivo [161]. Limitations of electroporation are the efficiency of transfection that is different depending on the cell type and the low cell viability of electroporated cells.

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Table I: Gene terapy approaches for corneal disorders

Disease	Incidence	Cause	Clinical Manifestation	Therapy	Possible gene therapy approaches
Herpes simplex keratitis	500000 cases/year (US)	HSV-1 direct viral invasion or reactivation from latency	**Symptoms:** redness, discharge, lacrimation, irritation, itching, pain and photophobia **Clinical findings**: dendritic lesions, corneal opacity and whitening, keratic precipitates	Topical administration of antivirals and corticosteroids, oral antivirals	**Antisense oligonucleotides** [18]**, morpholinos** [19]**, siRNA** [22; 23], **ribozymes** [20; 21]**, aptamers** [24]**, replication-defective virus** [25]**, meganucleases** [26; 27]**, CRISPR/Cas9 editing** [28].
Sjogren Syndrome (#24355915)	About 7:100000	Etiology unknown (potential genetic and environme	**Dry eye:** ocular and oral dryness due to dysfunction of the	Artificial tears, anti-inflammatory agents (Lifitegrast, Cyclosprine A,	**Administration of cytokine using viral vectors** [38; 40; 41],

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		ntal factors involved)	nasolacrimal unit (nasolacrimal glands, corneal surface and eye lids)	rituximanb) and tears secretion stimulators	**TNFα inhibition by adenoviral transfer** [39].
Mucopolysaccharidosis type IH (Hurler), IH/S (Hurler-Scheie) and IS (Scheie) (#607014, #607015, #607016)	1:100.000	Mutations affecting the alpha-L-iduronidase activity (IDUA) on chromosome 4p16.3 (autosomic recessive)	Corneal clouding, glaucoma, retinopathy and optic nerve abnormalities	Haematopoietic Stem Cell Therapy; Enzyme Replacement Therapy (Laronidase also know with the brand name of Aldurazyme®)	**AAV mediated IDUA administration to human corneas via intrastromal injection** [82].
Mucopolysaccharidosis type VII (also known as Sly syndrome) (#253220)	1:250.000	Mutations affecting the beta-glucuronidase (GUSB) activity on chromosome 7q21.11 (autosomic	Corneal clouding and optic nerve abnormalities	Haematopoietic Stem Cell Therapy	**Adenovirus expressing human GUSB into the anterior chamber or within the intrastromal region** [84].

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		recessive)			
Increasing corneal graft survival	N/A	Immunologic rejection of the donor cornea	Graft rejection	Anti-inflammatory and immunosuppressive drugs, Donor cornea-HLA matching, Cornea re-grafting	**Anti-angiogenesis approaches (Inhibition or regression of blood vessels)** [44; 45; 46]. **Immune response modulation (Adenovirus vector-mediated gene transfer of CTLA4-Ig, interleukin-10 or interleukin-12)** [47; 48; 49; 50; 51; 52]. **Anti-apoptosis**

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					strategies (lentiviral vector-mediated gene delivery of baculoviral p35 or mammalian Bcl-xL or programmed death ligand-1) [54; 55].
Corneal Neovascularization	About 1.4 million cases per year	Congenital diseases, contact lens-related hypoxia, inflammatory disorders, chemical burns, limbal stem cell deficiency, allergy, trauma,	Corneal scarring, stromal edema, lipidic deposition, keratitis, visual impairment or blindness	Amniotic membrane transplantation, topical nonsteroidal anti-inflammatory and corticosteroid medications, argon and yellow dye laser photocoagulation, photodynamic	**Intrastromal corneal injection of naked plasmid DNA encoding VEGF** [61] **subconjunctival injection of the recombinant endostatin-AAV** [162],

		infectious keratitis, autoimmune diseases, corneal graft rejection		therapy, cautery, diathermy	**polyplex micelles** [69], **intrastromal injection of a plasmid containing a small hairpin RNA cassette (pSEC.shRNA) against VEGF-A** [70].
Meesmann epithelial corneal dystrophy (#122100)	N/A	Mutations affecting expression of KRT3 and KRT12 genes, on chromosome 12q13.13 and 17q21.2, respectively.	**Symptoms:** photophobia, blepharospasm, increased tear production, intolerance to contact lenses, transient blurred vision, foreign body sensation, and irregular astigmatism **Clinical**	Stem cell transplantation and Keratoplasty	**siRNA** [89; 97; 98]**, genome editing** [100].

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			findings: formation of multiple intraepithelial microcysts in the outermost layer of the cornea		
Ectrodactyly Ectodermal dysplasia Clefting (EEC) (#604292)	About 1/900000	Mutations affecting expression of the p63 gene on chromosome 3q27 (autosomic dominant)	Limbal stem cell deficiency characterized by conjunctival epithelial ingrowth, neovascularization, chronic inflammation, opacification, recurrent corneal erosion, persistent ulcers, destruction of basement membrane components, fibrous tissue ingrowth,	Scleral lenses, eye drops. Haematopoietic Stem Cell Therapy. Enzyme Replacement Therapy (Laronidase also know with the brand name of Aldurazyme®)	**Allele-specific gene silencing through small interference RNA (siRNA)** [109; 111].

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			progressive corneal clouding and eventually blindness.		
Aniridia (#24355915)	About 1:55000	Mutations affecting expression of the PAX6 gene on chromosome 11p13	**Ocular abnormalities**: foeval and iris hypoplasia, nystagmus, glaucoma, cataracts. **Corneal abnormalities**: AAK, corneal opacification	Lenses, surgical interventions	**PTCs read through** [118; 163], **recombinant pax6** [122], **augmentation therapy using viral vectors** [123], **genome editing.**
Fuchs endothelial corneal dystrophy (FECD) (#613267)	About 1: 2000	Trinucleotide repeat (TNR) expansion in the transcription factor 4 (TCF4) gene	Progression of focal excrescences in the Descemet membrane (guttae) to endothelial cell degeneration and stromal edema, corneal	5% salt solution, Penetrating Keratoplasty (PK) and Descemet's Stripping with Endothelial Keratoplasty (DSEK).	**Antisense oligonucleotide (ASO) approach** [125], **CRISPR-Cas9 treatment** [124].

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			erosions and gradual opacification that results in a visual veiling, cataract.		

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Figure

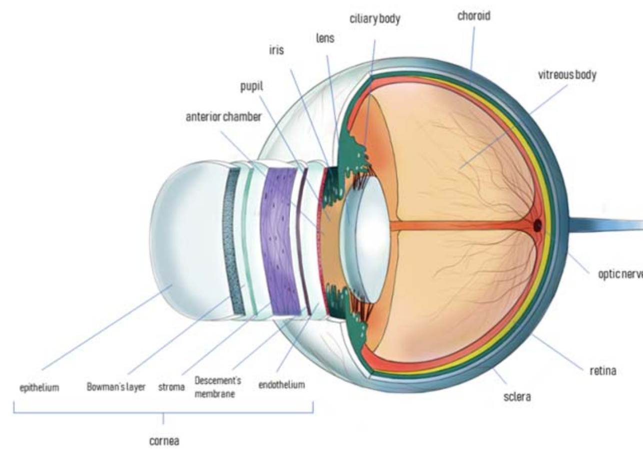

Figure 1. Anatomy of the cornea. Schematic representation of a section of the eye and of the five layers forming the adult human cornea

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