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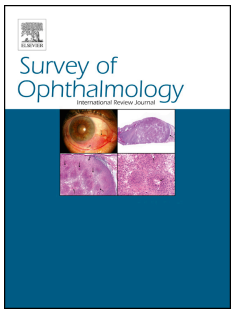

Gene-Based Antiangiogenic Applications for Corneal Neovascularization

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Abstract

Corneal avascularity is maintained by angiogenic privilege, an active process involving the production of higher level of angiostatic factors to offset the effect of angiogenic factors. A wide range of pathological insults to the cornea can disrupt this intricate equilibrium and promote angiogenesis and corneal neovascularization with resultant visual impairment. Corneal neovascularization is also a major risk factor for graft failure post-keratoplasty. Current treatment options for corneal neovascularization are restricted by limited efficacy, adverse effects, and a short duration of action. The unique anatomical position and relative immune-privilege of cornea make it an ideal tissue for gene-based therapies. Gene transfer vectors have been used to deliver or target genes involved in the pathogenesis of corneal neovascularization in animal models. Several pro-angiogenic and anti-angiogenic factors have been targeted and assessed in experimentally- induced corneal neovascularization. Antisense oligonucleotides targeting corneal neovascularization have entered human clinical trials and have not required vector delivery systems. The emergence of these RNA-based strategies heralds a new era in the management of corneal neovascularization and ocular therapeutics.

Key Words

cornea, neovascularization, angiogenesis, gene therapy, vascular endothelial growth factor, antisense oligonucleotide, miRNA

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I. Introduction

The healthy cornea is avascular and nourished by diffusion from the aqueous humor and tear film-supported circular pericorneal plexus derived from the anterior ciliary arteries that surrounds the cornea in the limbal region. The maintenance of corneal avascularity is termed 'angiogenic privilege,'[13] and in its resting state, this is an active process of homeostasis between the low level of angiogenic and high level of antiangiogenic factors[87].

A wide range of external insults to the cornea can disturb the delicate equilibrium required for angiogenic privilege by increasing the production of angiogenic factors, which lead to corneal neovascularization with resultant loss in corneal transparency and visual acuity from scarring, stromal edema, lipid deposition, and inflammation. Currently there is no epidemiological study that provide an accurate estimate of the incidence and prevalence of corneal neovascularization in the general population[164]. Many of the conditions resulting in corneal neovascularization eventually require a penetrating or lamellar keratoplasty to restore vision; however, graft rejection rates are higher in vascularized corneal beds even with systemic immunosuppression , and post-transplant vision is often compromised[44,64,73]. Many risk factors for corneal graft rejection are recognized, such as recipient age, previous rejection episodes, previous grafts, gender matching, and timing of the graft[116,263,278]. Corneal neovascularization, however, also develops in 41% of eyes after penetrating keratoplasty, even without pre-existing corneal angiogenesis[73]. Corneal neovascularization is therefore a risk factor for graft failure post-keratoplasty and also a major complication following the surgical procedure itself. Successful keratoplasty is attributed to corneal 'immune privilege', the suppressed corneal inflammation induced by the lack of afferent lymphatic and efferent blood vessels in the recipient cornea, lack of major histocompatibility antigens class II, and the anterior chamber associated immune deviation[266,288]. Lymphatic vessels and associated blood vessels are found in neovascularized cornea[65]. The presence of corneal neovascularization, therefore, enables access of antigenic material to regional lymph node, completes the 'immune reflex arc' in cornea, and compromises its immune privilege.

Current treatment options for corneal neovascularization include topical application of steroids[25,36,44,220] or surgical interventions: laser ablation[14,49,203,234], photodynamic therapy[4,94] and fine-needle diathermy[221,238,240,261]. Targeting pro-angiogenic molecules with topical or

subconjunctival use of vascular endothelial growth factor (VEGF) inhibitors such as bevacizumab has been reported[45]. Despite some degree of success, the current treatment options are restricted by adverse effects[23,143,146,175,236,279]. Gene-based therapy might be able to circumvent these shortcomings and improve the duration of therapeutic effect. The unique anatomical and immune characteristics of the cornea along with the relative ease of access make it an ideal candidate for gene-based therapy; however, gene-based therapies for corneal neovascularization are still largely at the preclinical stage [98,162,244,287,308]. Herein, we provide a comprehensive review on therapeutic target genes and potential vectors available to treat corneal neovascularization.

II. Pathophysiology of Corneal Angiogenesis

Clinically, corneal neovascularization is subdivided into three groups based on the pattern of angiogenic invasion: 1) superficial neovascularization, new vessels that invade just below the corneal epithelium into the stroma; this is commonly seen in stromal keratitis, 2) vascular pannus involves both the extension of vessels and fibrous tissue onto the peripheral cornea and is mainly seen in ocular surface disorders, and 3) interstitial and deep neovascularization consists of lamina of new vessels in stroma as seen in herpetic and luetic interstitial keratitis. Deep neovascularization is a specific interstitial neovascularization in which there is angiogenesis between the stroma and Descemet membrane[55,69].

The progression of corneal neovascularization is broadly divided into three phases: a latent pre-vascular phase, an active neovascularization phase, and lastly a maturation phase[248] (Figure 1). Upon exposure to a stimulus such as injury or hypoxia, the corneal epithelium, leukocytes, pericorneal blood vessels and extracellular matrix (ECM) release angiogenic growth factors which bind to receptors on the vascular endothelial cells of pericorneal vessels[150]. These vessels dilate, their permeability increases, and leukocytes migrate into the surrounding corneal stroma, resulting in inflammatory edema and opacification[95]. Subsequently, these endothelial cells are “activated”, characterized by decreased cell junction integrity and degradation of the endothelial lamina[136,172,245]. Matrix metalloproteinases (MMP) released by endothelial cells and migrating leukocytes degrade the surrounding ECM, paving the way for invasion and proliferation of vascular endothelial cells[172]. This is followed by the endothelial cell migration toward the angiogenic stimulus source[150]. The endothelial migration and proliferation from parent vascular structures is facilitated by altered

expression of adhesive proteins, such as integrins and selectins, and cytoskeletal reorganization[225]. Finally, the formation of vascular lumen and anastomosis ensues as supporting pericytes are recruited, marking the maturation of vessels do not require the stimulus of pro-angiogenic factors for survival.

III. Cause of Corneal Neovascularization

A wide range of clinical conditions can cause corneal neovascularization. Most of these conditions induce corneal neovascularization via three broad pathological mechanisms: hypoxia, inflammation, and limbal barrier dysfunction[181]. Hypoxia, one of the pathological mechanisms that drives corneal neovascularization, is commonly seen in contact lens use[47]. Contact lens use is the leading cause of corneal neovascularization in the USA[164]; and 20% of contact lens users suffer from corneal neovascularization[168]. Contact lenses reduce by 8-14% the oxygen delivered to the cornea, and this hypoxic condition leads to the downregulation of antiangiogenic factors (e.g., pigment epithelium-derived factor) and an upregulation of angiogenic factors (principally VEGF, mediated by hypoxia-inducible factor 1-alpha[252]), initiating the neovascularization process to deliver oxygen to the hypoxic cornea[168,230].

Infections, inflammation, and corneal transplant can all cause corneal neovascularization via upregulation of inflammatory cytokines, which attract myeloid cells into the cornea[230]. These myeloid cells establish a cycle of cytokine secretion and further myeloid cell recruitment in the cornea[230]. There are significant alterations in multiple cytokines which increase the inflammatory status of cornea and lead to corneal neovascularization[54,230]. In human herpes simplex-1 (HSV-1) infection, the HSV-1 virus migrates from initial infection site of cornea to the trigeminal ganglia where it lies dormant[298]. The virus replication cycle is reactivated upon stress and immunosuppression, during which the virus travels back to the corneal epithelial surfaces via the trigeminal nerve, which leads the elevation of VEGF-A and MMPs levels, and reduced expression of anti-angiogenic soluble VEGF receptor-1 (sFlt-1)[139,298]. MMPs are secreted by the neutrophils recruited through inflammatory cytokines, and they contribute to corneal neovascularization by degrading the remaining little amount of sFlt-1 produced[270].

IV. Clinical Assessment of Corneal Neovascularization

An essential requirement for evaluating the efficacy of any potential treatment for corneal neovascularization and performing clinical trials is the ability to grade and quantify corneal

neovascularization before and after intervention (Figure 2). Numerous modalities have been employed to evaluate corneal neovascularization. Historically the most common method is to examine photographic images of corneas taken by slit lamp biomicroscopy[35,66,90,91], but more advanced imaging techniques have been developed[75,83,94,169].

Biomicroscopic examination of corneal neovascularization is limited by inconsistent vessel delineation from frequently coexisting corneal opacification, poor standardization and the inability to perform quantitative measurements[9] (Figure 2). Additionally, it is difficult to distinguish afferent from efferent vessels visually even with the aid of the patient's pulse[59,240]. Therefore, ancillary techniques were needed for the clinical characterization of corneal neovascularization[83]. This was recognized by Bron and Easty, who, in the 1970s, used angiography to study corneal neovascularization in more than 250 patients[83]. As acknowledged by these authors, relying solely on clinical assessment with biomicroscopic photography to estimate vessel leakage is unreliable;[83] therefore, improved imaging techniques that makes identification of small vessels more evident are essential.

Angiography using fluorescein and indocyanine green is an objective tool to measure corneal neovascularization[9,148]. Both techniques allow the characterization of corneal neovascularization (Figure 2) based on the assessment of both morphological parameters (such as diameter, length tortuosity, area etc.) and functional parameters (such as flow and time to leakage) that are indicators of vessel maturity and disease activity[9,238,261,264]. With angiography, the anatomy of the marginal corneal and limbal vascular arcades can be elucidated, which is important for assessing progression of corneal neovascularization and limbal disorders[9]. Angiography, even in the presence of exudate and scarring, allows precise detection of the afferent stems of the vessel, which is helpful for guiding surgical treatment of corneal neovascularization[261,264]. Therefore, angiography provides an objective evaluation of corneal neovascularization to plan surgical treatment and monitor treatment responses. In addition, digital subtraction analysis of corneal angiograms depicts and characterizes clinically invisible corneal lymphatic new vessels[241]. The in vivo depiction of corneal lymphatic vessels is of great importance, as it has been shown that lymphatic rather than hematic corneal new vessels are the primary mediators of immunological graft rejection in vascularized corneas[80,118].

Optical coherence tomography angiography (OCTA) is another promising method for the assessment of corneal neovascularization[8]. This relatively new modality is not yet widely

used in part because of current limitations in the definition of images produced, lack of functional information, and inability to detect vessels without red-cell flow. In vivo confocal microscopy (IVCM) has been used to visualize presumed lymph vessels in a case of corneal transplantation[217], and a novel non-invasive in vivo technique for the quantification of leukocyte rolling and extravasation at sites of inflammation in human patients has been reported[147]. More recently, IVCM has been used to demonstrate acellular perfusion of ghost vessels, intravascular cellular traffic and corneal lymphatic new vessels[241]. The emerging IVCM and OCTA techniques have the advantage of being non-invasive, but refinement of these imaging techniques is still needed. Regardless of the modalities used, further development of a standardized measurement procedure is still necessary to allow consensus in measuring and comparing efficacy of new treatments for corneal neovascularization.

V. Current treatments of Corneal Neovascularization

Many treatment options for the management of corneal neovascularization are currently available, and various degrees of success have been reported. These therapeutic interventions are either medical or surgical. We present an overview of these modalities to place in context the emergence of gene-based anti-angiogenic applications for corneal neovascularization.

A. Pharmacologic Treatment

Glucocorticosteroids (also called glucocorticoids, corticosteroids, or steroids) have traditionally been the mainstay of managing corneal neovascularization; however, the complete suppression of corneal neovascularization with topical steroids is not possible, as glucocorticosteroids do not cause established corneal neovascularization to regress[163,166]. Topical steroid treatment also has side effects such as glaucoma, cataracts, super-infection, and herpes simplex recurrence, which further hamper the clinical utility of steroid treatment[236]. Moreover, despite the widespread use of topical steroids, the mechanism of their anti-angiogenic action is not fully understood[197]. The antiangiogenic effect of steroid is proposed to result from their anti-inflammatory properties, via inhibition of neutrophilic cell chemotaxis[95,194,220,237,246], modulation of the proteolytic activities of vascular endothelial cells [25,36,180,194], inhibition of pro-inflammatory cytokines[28,29,84,117,260,284], inhibition of plasminogen activator (PA) including the stimulation of PA inhibitors[180] and altered prostaglandin synthesis[130,237]. Non-steroidal anti-inflammatory agents have been used clinically to treat corneal neovascularization because of their ability to target prostaglandins synthesis, but they are not considered sufficiently effective[79,203,271]. Given the known side effects and variable

clinical effects of these anti-inflammatory agents, more targeted treatments have been evaluated.

Anti-VEGF agents (e.g. bevacizumab; Avastin®) have been utilized to treat corneal neovascularization[19,174]. Bevacizumab has been delivered topically (dose range: 5–25 mg/mL, 2–5 times per day)[34,75,78,280], by subconjunctival injection (dose range: 1.25 mg/0.05 mL to 5 mg/0.2 mL)[12,15,16,72,82,89,99,145,173,231,303], or using pre-soaked corneal collagen shields (1.25 mg/0.05 mL for 20 minutes for 11 weeks[173]. Most of the anti-VEGF trials were uncontrolled studies conducted with small sample size, and the reported reduction in corneal neovascularization appeared to be only transient and incomplete[12,75,303]. Ultimately, anti-VEGF therapies may be safer than steroid-based approaches, but prospective multi-center clinical trials are required to prove the efficacy of anti-VEGF treatments in corneal neovascularization.

Thus far, studies that investigated the efficacy of anti-VEGF agents only achieved incomplete regression of corneal neovascularization[45]. This can be attributed to the fact that anti-VEGF therapy is only effective against newer, actively growing blood vessels. These new vessels go through a period when vascular endothelial cell survival depends on the presence of pro-angiogenic factors, like VEGF. After two weeks, most of these vessels are covered by pericytes, which marks the end of the pro-angiogenic factors dependence period for endothelial cells, and treating these mature vessels with anti-VEGF agents is often less than satisfactory[67]. Targeting VEGF in isolation may also be ineffective because of redundancy in the pro-angiogenic cascade, with other pro-angionic factors driving corneal neovascularization[1,140,141]. Acquired resistance to anti-VEGF and anti-angiogenic drugs represents a further mechanism limiting the efficacy of anti-VEGF therapies[178,222,299].

B. Surgical Treatments for Corneal Neovascularization

Argon laser photocoagulation is an established treatment for retinal neovascularization[259]. Hemoglobin has a high absorption rate of argon energy, and laser treatment can coagulate hemoglobin-filled corneal vessels[234]. Yellow laser and neodymium-doped yttrium aluminum garnet laser were also suggested for the treatment of corneal neovascularization, but neither are routinely used clinically[154,202]. Despite successful outcomes with argon laser treatment of lipid keratopathy[104], several reasons have restricted argon laser usage for corneal

neovascularization. Specifically, the procedure is technically difficult to perform as corneal vessels are difficult to visualize and have a rapid pulsatile flow.[221] The occlusive effect was shown to be transient.[49,256] The laser-induced thermal damage to the vessels might lead to upregulation of inflammatory mediators and VEGF in the surrounding stroma, which may paradoxically lead to more neovascularization[99]. In addition, high laser energy predisposes to complications such as iris atrophy, corneal thinning, pupillary ectasia, peripheral corneal hemorrhage,[88,104,203] and necrotizing scleritis[211]. Because of these technical difficulties and related side effects, laser ablation of corneal neovascularization has not gained widespread acceptance.

Photodynamic therapy (PDT) has also been used as a treatment for corneal neovascularization[4]. The effect of PDT is based on the combination of a photosensitizing compound (verteporfin), light, and oxygen, which together produce cytotoxic free radicals that cause vascular endothelial damage and intravascular thrombosis likely involving both apoptosis and necrosis[102,114,204,233,235]. The photo-oxidative effect of PDT is confined within the blood vessels in a non-inflamed cornea; however, the higher permeability of blood vessels in an inflamed cornea results in dye leakage that extends the photo-oxidative damage into stroma[60]. The collateral damage of stromal tissues may exacerbate an inflammatory reaction increasing the risk of reperfusion and angiogenesis[60]. PDT has not been widely used for the treatment of corneal neovascularization because of the aforementioned potential complications[221].

Fine needle diathermy (FND) occlusion of corneal neovascularization is a technique that involves a stainless steel 3/8 circle side-cutting, single-armed needle inserted into the limbus at the level of the blood vessel to be occluded or into the vessels lumen directly if the vessel is large. A unipolar diathermy unit set to coagulation mode is then connected with the needle at the cornea to start the occlusion process[221]. FND has been reported as an effective and relatively easy procedure to perform;[221,261]however, diathermy should only be applied to the afferent vessels (selective FND), and the potential adverse effects of FND should be taken into account[238–240,261]. There may be collagen shrinkage and damage to the adjacent stroma of the diathermy site .[20] Heat applied to cornea also alters corneal curvature[20,85]. Long-term effects of FND to the cornea are not yet clear. Moreover, FND itself may stimulate further corneal neovascularization by triggering secondary release of pro-angiogenic factors[33]. It would, therefore, be reasonable to minimize the application of FND and to selective FND on the afferent vessels, as arterioles only comprise less than 1% of total corneal

neovascularization[67]. Combining angiographic guidance to target the afferent vessels, FND is a promising procedure in reducing the area of corneal neovascularization[238,261].

VI. Gene Delivery to the Cornea

Gene therapy refers to the transfer of nucleic acids into cells using viral and non-viral vectors to correct cellular dysfunction or restore cellular function[188,192]. As such, gene therapy is a type of molecular medicine that targets the underlying molecular basis of disease. Compared to drug or antibody-based treatments for corneal neovascularization that only provide short-term benefits and require repeated applications, a gene-based approach offers targeted treatments providing long term therapeutic correction[149,185,188]. The cornea has properties that makes it an attractive target for gene-based manipulations: relative immune-privilege and ease of access [131,149,185,188]. Corneal transparency allows live tracking of labelled molecules in animal studies[149,185]. The cornea is easily accessible to administer gene therapy reagents, and the ability to maintain the cornea in culture for several weeks permits *ex vivo* gene therapy approaches[185,216]. A variety of vectors have been used forgene-based therapies for corneal angiogenesis, including viral vectors, lipid-based vectors, nanoparticles, polymers or naked plasmid--each with their own advantages and limitations[57,110]. Viral-based vectors are well-established and effective, but can induce immune responses, whereas non-viral delivery methods are less likely to induce an immune response, but only produce short-term gene expression[57,103,274]. These vectors are normally delivered to the cornea by subconjunctival, intrastromal, or intracameral injection[188,228]. In some cases, topical application or *ex vivo* incubation of cornea buttons were also employed[71,216].

A. Adenoviral Vector

The adenoviral vector, the first viral vector used for direct gene transfer to the cornea, is capable of infecting both mitotically active and mitotically quiescence cells and can carry large gene inserts with no risk of insertional mutagenesis [176]. The use of adenoviral vector for corneal gene transfer has been tested extensively in animal and *ex vivo* studies[10,39,232,277]. Recombinant adenovirus encoding vasohinbin-1 was injected was subconjunctivally into mouse to suppress corneal neovascularization[308]. Adenovirus is a 35kb double-stranded DNA virus, and the wild type virus causes a benign respiratory tract infection in humans. Over 40 serotypes of wild-type adenovirus have been discovered,[283] and the recombinant vector commonly employs adenovirus serotype 2 and 5 genetically engineered to remove their replicating ability[283]. Later generations of adenovirus vector have been modified by deleting

its entire viral genome, leaving only the inverted terminal repeats and packaging genes behind, thus reducing its immune response and enabling it to carry larger gene inserts[151]. As a result of this extensive deletion, a helper virus is required for adenoviral vectors to be propagated, and there is a risk that helper virus contamination could induce a strong inflammatory reaction, limiting their clinical use and safety. The adenoviral vector enters cells through coxsackie-adenovirus receptors and integrin-mediated endocytosis [293]. Once inside the cytoplasm, the virus undergoes endosomal lysis and releases its genome. As there is no integration into the host genome, transgene expression is usually only short-term. This caveat necessitates repeat treatment if a sustained effect is to be achieved, which can limit clinical utility and result in higher cumulative risks of immune responses.

B. Lentiviral Vectors

Lentivirus belongs to the retroviridae family with its single-stranded viral mRNA, which possesses the enzyme reverse transcriptase that transcribes its RNA into double-stranded DNA[53]. Lentiviral vectors have been able to transduce a gene of interest to corneal epithelium, stroma and endothelium in both animal and *ex vivo* studies[17,215,290]. Upon association with specific surface receptors of the host cell, the viral envelope fuses with the cytoplasmic membrane and ejects its cylindrical core into the cytoplasm[184]. DNA is generated from mRNA by viral reverse transcriptase, and the DNA subsequently migrates into the nucleus. At this point, wild-type lentiviral DNA usually integrates into the host genome[61]. Lentivirus vectors are mostly derived from equine infectious anemia virus and the human immunodeficiency virus 1[281]. Unlike other retroviruses, lentivirus is able to infect non-dividing cells,[228] and compared to adenovirus, lentiviruses also appears to be less immunogenic[294]. To generate the lentiviral vector, the viral genome is engineered to remove its self-replicating capabilities[188]. As lentiviral vectors integrate into the host genome, sustained transgene expression is achieved; however, the risk of insertional mutagenesis remains prohibitive for testing of lentiviral vectors in clinical trials[108,295]. To combat this limitation, non-integrating lentiviral vectors are being developed[201].

C. Adeno-Associated Viral (AAV) Vectors

AAV is a small single-stranded DNA virus belonging to the parvoviridae family that is non-pathogenic to humans, making AAV a safe option for gene delivery[188]. AAV-based gene delivery has been able to transfect corneal stroma and endothelium in both *in vivo* and *ex vivo* studies without apparent toxicity[119,186,276]. Subconjunctival injection of AAV vector carrying

endostatin and angiostatin genes to the corneal epithelium inhibits corneal neovascularization[48,161]. Owing to its simple genomic structure, the presence of a helper virus such as adenovirus or herpes simplex virus is necessary for replication. Upon binding to primary cell surface and integrin, AAV is internalized, [282]and its single stranded DNA is released. This single-stranded DNA anneals to a complementary strand from another AAV or through host DNA polymerase. On reaching the nucleus, the therapeutic gene is integrated into the host genome[247]. The first generation of recombinant AAV lacked the genes needed for replication because they were replaced with the therapeutic gene; therefore, co-infection with adenoviral and AAV helper plasmids carrying genes encoding for replication were necessary[196]. In order to avoid helper virus contamination, AAV- and adenoviral-helper genes were subsequently combined into a single plasmid in newer generation AAVs [106].

To further dampen immune response, hybrid vectors have been developed by combining the genome of one serotype and the capsid of another serotype retrieved from the Rhesus monkey[96]. Another breakthrough was the development of self-complimentary AAV, which allows for more rapid gene transfection as, under normal conditions, there is a delay in the single stranded viral genome to spontaneously anneal to its complimentary strand[179]. A further development is the generation of tyrosine mutant AAV vector resistant to proteasome-mediated degradation, allowing more efficient gene delivery with reduced loading titers[219]. AAV is an attractive option as a viral vector for gene-based therapies for corneal neovascularization because of its safety profile and sustained longer-term gene expression [253]. One limitation, however, is its incapability to incorporate large DNA constructs. Nonetheless, AAV has been used clinically in the gene therapy for Leber congenital amaurosis to restore RPE65 function[124,258].

D. Nanoparticles

Cationic polymers have been widely used as non-viral vectors *in vitro* as they can form association with DNA and promote induction of the DNA into cells[210,285]. The positively charged surface of cationic polymers, however, are potentially cytotoxic, and the clinical application of these molecules might therefore be limited[206,209]. Development of biocompatible polymeric micelles from newly designed cationic block polymer resolves this issue[2]. Polymeric micelles are nanoparticles that self-assemble as a result of amphiphilic interaction[133]. One example is polyethyleneglyco -b-P[Asp(DET)], which has a hydrophilic polyethylene glycol segment that forms the non-cationic shell of a micelle[183,269]. As a vector,

polymeric micelles form a hydrophobic core containing the therapeutic gene and a hydrophilic shell which interacts with solvent. The unique core-shell architecture of polymeric micelles allows therapeutic genes to be protected and therefore making delivery more efficient[112]. Nanoparticles have a large vector-carrying capacity and are able to exhibit sustained gene expression after transfection [92,109]. Subconjunctival and intrastromal injection of nanoparticles-based vector carrying a sFlt-1 plasmid and shRNA against VEGF-A respectively were able to suppress corneal neovascularization[123,229]. Further development of more biocompatible nanovectors could lead to widespread use of polymeric micelles for gene delivery.

Another non-cationic nanovector, polylactic-co-glycolic acid (PLGA), is a biodegradable copolymer used as a therapeutic device for many FDA-approved drug delivery systems[103]. Previously PLGA nanoparticles were shown to increase delivery of plasmids into cells or the cornea in a non-toxic fashion, with enhanced uptake at the site of administration[11,125]. Sustained release of small interfering RNAs or pharmacologic agents were also observed[18,56,97]. The non-cationic properties of PLGA nanoparticles also avoid the toxicity issues associated with cationic polymers. These biologically desirable properties make PLGA a promising vector system for the delivery of genes to the cornea. Liposomes are composed of a lipid bilayer and an aqueous compartment, forming vesicles that are able to encapsulate both hydrophilic and lipophilic therapeutic agents[275]. They have shown stability and good capacity for transfection *in vitro* and *in vivo*[275]. In a recent modification of the vector construct, dextran and protamine were added to improve cellular uptake of the vector and aid translocation of the plasmid DNA into the nucleus respectively[227]. As the cornea is negatively charged, positively charged liposomes enhance the absorption and transfection of the encapsulated agents. Short storage time and limited carrying capacity, however, pose challenges for the clinical application of liposomes[103].

Albumin is a naturally nano-sized biodegradable particles that allow drugs or plasmids to be encapsulated and released in a sustained manner[11,153]. This common serum protein forms dimer with the drug and facilitate entrance into cells via a vesicle-forming process called transcytosis, therefore increasing the efficacy of the drugs[77,105]. In the context of corneal gene delivery, plasmid-linked to albumin nanoparticles persist in the stromal space for extended period of time[125]. As a non-viral, non-immunogenic and biodegradable

nanoparticle, albumin appears to have the attributes required for delivering therapeutic genes to the cornea.

E. Gene Silencing Methods

Gene silencing methods such as antisense oligonucleotides, morpholino oligomers, small interfering RNAs (siRNAs), or short hairpin RNA (shRNA) can be useful for targeting pro-angiogenic factors in the cornea. Antisense oligonucleotides are single-stranded RNA or DNA that can prevent protein translation by specific binding to a complementary RNA sequence. Morpholino oligomers are synthetically produced antisense reagents similar to DNA oligonucleotides, but possess morpholine ring backbones[191]. They are effective in blocking mRNA translation and alternative splicing without causing it to degrade. siRNAs are double-stranded RNA that can initiate RNA-induced silencing complex-mediated binding to specific RNA and induce nonsense mediated decay[107]. Rather than directly targeting the transcribed RNA, shRNA integrates into the host nucleus where the host machinery produces the encoded siRNA[189]. The silencing effect of siRNA is transient owing to intra-cellular degradation, whereas shRNA that is constantly produced by the host provides a continuous silencing effect. Further improvements on the safety and efficacy of gene silencing techniques are likely to maximize its application for the treatment of corneal neovascularization.

F. Alternative Delivery Methods

Other methods for transferring therapeutic genes to the cornea include the injection of naked DNA or plasmid[302], electroporation[208], iontophoresis[27] and the use of a gene gun[272]. These modalities have had various degree of success for corneal gene delivery. Injection of naked plasmid is not associated with risk of immune response, but produces only transient transgene expression.[262] Electroporation alone to deliver genes into the cornea was considered ineffective, and there are potential risks of tissue injury from the electric current[32,111]. Iontophoresis performed under correct electric current conditions with short-duration is considered to be safe, but is unable to deliver larger molecules such as plasmid DNA.[27,120,300] Transfection using gene guns is restricted to the corneal epithelium and produces mild corneal inflammation[22,272].

VII. Target Genes and Therapeutic Application

Our increasing understanding of the mechanisms underlying angiogenic privilege in the cornea has facilitated the development of gene therapy approaches for corneal neovascularization (Table 1). Two therapeutic approaches are described: either transgenic expression of an anti-angiogenic factor or inactivation of a pro-angiogenic factor via gene silencing.

A. Vascular Endothelial Growth Factor (VEGF)

VEGFs production is increased in pro-angiogenic environments such as hypoxia, inflammation, and tumor cell proliferation[42,43,307]. VEGF is the most common therapeutic target in corneal neovascularization[45]. Specifically, VEGF-A is expressed in embryonic, physiological and pathologic neovascularization and is considered to be the major factor involved in angiogenesis[37,95]. There is upregulation expression of VEGF-A in vascularized corneas[45]. Upon binding to the cell surface membrane-bound VEGF receptor 1(VEGFR1; mbFlt-1), VEGF-A is activated and promotes proteolysis of ECM, vascular endothelial cell proliferation, migration and tube formation[218]; all of which are essential steps of angiogenesis. Other VEGF isoform bind to different VEGF receptors and have different functions. VEGF-C and D, for example, were implicated as mediators of lymphangiogenesis - the growth of new lymphatic vessels in the cornea[62].

Paradoxically, VEGF-A, a potent pro-angiogenic molecule, is also found in the avascular cornea under normal conditions[5]. It is proposed that the angiogenic effect of VEGF-A in the corneal stroma is antagonized by sFlt-1, an alternatively spliced isoform of mbFlt-1, which can act as an endogenous VEGF-A "trap" [5]. sFlt-1 is present extracellularly in the cornea, and a reduction of sFlt-1 leads to corneal vascularization[5,6]. There is strong evidence that sFlt-1 plays an angiogenic role in the normal cornea, and it is therefore a popular target for gene-based therapies for corneal neovascularization. In one study, only 18% of mouse eyes injected with complimentary DNA (cDNA) of sFlt-1 in adenoviral vectors intracamerally developed corneal neovascularization after silver nitrate cauterization, as compared to 100% of the untreated mouse[158]. Intracameral injection of an AAV sFlt-1 expressing vector into mice eyes reduced silver nitrate-induced corneal neovascularization by 36% compared to controls[162]. The non-viral vectors, PLGA[51] and polymeric micelles[123], were also able to deliver a plasmid DNA encoding sFlt-1 or Flt23K (a recombinant construct of sFlt-1 domains 2 and 3 and endoplasmic reticulum-retaining peptide) to the mouse cornea via subconjunctival injection and achieve prolonged expression[51,123]. Injecting naked plasmid-

containing sFlt-1 cDNA to rabbit corneal stroma also reduced angiogenesis by 23.6% compared to controls, although tissue specificity is questionable as gene expression was also observed in the posterior segment[262]. A Flt-1 specific morpholino oligomers targeting the exon 13/intron 13 junction of the murine Flt-1 transcript was also successful in modulating the alternative splicing process and promoting the production of sFlt-1 instead of mbFlt-1, and thus showed 22.78% less angiogenesis compared to controls in murine cornea associated with penetrating keratoplasty[52].

As well as acting through VEGFR-1, VEGF-A can bind and activate VEGFR-2 promoting angiogenesis[257]. A soluble form of VEGFR2 prevents lymphangiogenesis in the cornea[5] and has immunosuppressive effects after corneal transplantation[3]. Administratation of sVEGFR-2 in murine models (corneal suture or transplantation) reduced lymphangiogensis but not hemangiogenesis suggesting sVEGFR2 is not a major contributor to corneal neovascularization[3]. However, subsequent studies have reported that a soluble VEGFR2/Fc chimera protein has a significant inhibitory effect on angiogenesis and lymphangiogenesis[113].

RNA interference-mediated silencing of VEGF-A is an alternative approach . Subconjunctival injection of synthetic siRNAs were able to silence VEGF-A sequences and inhibit mouse corneal angiogenesis induced by alkali burn, showing 2.34mm^2 less neovascularised area than the uninjected controls[310]. Utilizing a similar approach, shRNA or antisense oligonucleotides-mediated silencing of VEGF-A also effectively supressed corneal neovascularization in murine models[159,229]. Although clinical trials of VEGF siRNA for corneal neovascularization have not been reported, this approach is currently undergoing clinical evaluation to treat age-related macular degeneration[132,198,199]. Silencing VEGF may also affect cell death, however, as some studies have shown that VEGF can be neuroprotective for corneal innervation[212,265,304].

Vascular endothelial cell growth inhibitor (VEGI), an endothelial cell-specific tumor necrosis factor, inhibits endothelial cell growth and induces apoptosis[305]. Using a positively charged lipid vector, VEGFI cDNA was successfully delivered into all layers of the cornea and produced 13.8mm^2 less rabbit corneal neovascularization after a silk suture was placed, compared to controls[287]. Another family member of VEGF, placental growth factor (PlGF), shares biochemical similarities with VEGF-A. In addition to having the same receptor of Flt-1, PlGF can also form a heterodimer with VEGF-A. These similarities were utilized to generate a PlGF variant, termed PlGF1-DE, that is unable to bind Flt-1, but is still able to

hetero-dimerize with VEGF-A[273]. Heterodimerized VEGF-A is unable to bind and activate mbFlt-1, and hence the angiogenic effect of VEGF-A is suppressed[273]. Injection of PlGF1-DE cDNA carried by AAV vectors into the corneal stroma immediately after suture placement every three days for 14 days in a murine model resulted in 37% reduction of neovascularised area, which was significant[273].

B. Vasohibin

Vasohibin-1 is a novel endothelium-specific negative feedback mediator of angiogenesis that is upregulated when VEGFs are present[291]. The anti-angiogenic role of the vasohibin-1 protein was demonstrated by its ability to block neovascularization in the retina and the cornea (murine bFGF micropocket-induced corneal angiogenesis model)[254,291]. Vasohibin-1 acts as a negative feedback mediator of angiogenesis since its expression is usually low in vascular endothelial cells, but increased when stimulated by VEGF and FGF during neovascularization. It was able to inhibit VEGF- and FGF-driven proliferation, migration, and tube formation by vascular endothelial cells[142,291]. In murine models subjected to alkali burns , subconjunctival injection of vasohibin-1 cDNA incorporated within an adenovirus vector was able to reduce neovascularised area to 45.2% of the cornea on day 9 after the alkali burn, in contrast to 66.24% in controls, though the therapeutic effect was delayed and transgene expression was transient[308].

C. Angiostatin and Endostatin

Endostatin, a cleavage fragment in the NC1 domain of type XVIII collagen, and angiostatin, a proteolytic fragment in the kringle domains 1-4 of plasminogen, were identified as potent anti-angiogenic factors via their inhibition of VEGF- and bFGF-mediated vascular endothelial cell proliferation and migration[40,41,81]; their anti-angiogenic effect in tumor suppression had also been investigated in clinical trials.[115,157]. Kringle 5 of plasminogen (K5), a relative of angiostatin,has been shown to inhibit vascular endothelial cell activities[171]. Electroporation combined with injection of naked plasmid containing K5 cDNA reduced corneal neovascularization induced by alkali burns in the rat cornea[306]. Wild-type endostatin and modified RGDRGF-endostatin (mutated native sequence of RGIRGAD into RGDRGD) gene have also been evaluated for their anti-angiogenic effect on corneal neovascularization induced by alkaline burn in the rabbit cornea[98]. Subconjunctival injection of both genes resulted in suppression of corneal neovascularization; however, the modified endostatin gene was more effective, resulting in a 58% reduction in corneal new vessels compared to the wild type[98].

Several studies have assessed the efficacy of multigene-based therapy involving endostatin and angiostatin. Two studies investigated the possibility of preventing transplant-induced corneal neovascularization, a common sign of graft rejection, by transferring a fusion of endostatin and angiostatin or K5 cDNA via lentiviral vector to corneal buttons in a rabbit *in vivo* model[195,216]. In both studies, the transgenes were stably expressed after incubating the corneal buttons ex vivo with the cDNA-lentivirus before transplantation[195,216]. Subsequent examination and immunostaining showed that corneal neovascularization was suppressed and vessels did not cross the donor-recipient margin after gene transfer. Another multigene-based therapy for corneal neovascularization comprised of endostatin, sFlt-2 and sTie2 (a soluble "sink" for angiopoietin, another vascular growth factor) was shown to be therapeutically superior in inhibiting vascular endothelial cell proliferation *in vitro*, as compared to mono-gene modulation[46].

D. Peroxisome Proliferator-Activated Receptor Gamma (PPARγ)

PPARγ is a nuclear receptor involved in modulation of adipose metabolism, inflammatory cell function, and cell proliferation[50]. The PPARγ signal can suppress inflammation-mediated neovascularization by negatively regulating pro-inflammatory responses from macrophages[50]. Topical application of a solution of adenoviral construct carrying the PPARγ gene on murine corneas caused overexpression of PPARγ and substantially reduced corneal neovascularization induced by alkaline burn[244]. The upregulation of inflammation-related growth factors related to the insult was also suppressed. These results demonstrated the therapeutic potential of PPARγ gene delivery in treating corneal neovascularization by manipulating the inflammation pathway[127,244].

E. Decorin

Decorin is a small leucine-rich proteoglycan expressed in the cornea that plays a major role in angiogenesis regulation by suppressing endothelial cell migration and tube formation[76]. Topical application of decorin cDNA in the AAV5 vector on the corneal stroma after removal of epithelium was an effective genetic modulation for inhibiting neovascularization in a rabbit model[187]. In this study, implantation of a VEGF micro-pocket was performed on rabbit corneas to induce neovascularization. Compared to controls, the decorin-delivered corneas showed over 60% less neovascularization. Moreover, on an mRNA level, expression of angiogenic factors such as VEGF and angiopoietin were downregulated while anti-angiogenic factors were upregulated[187].

F. Brain-Specific Angiogenesis Inhibitor 1 (BAI1)

BAI1 is a transmembrane protein that has an anti-proliferative function by blocking αvβ5 integrin in vascular endothelial cells. Its middle extracellular region contains five thrombospondin-1 repeats[152]. As thrombospondin-1 is known to play a potent anti-angiogenic role in some tumor cells[251], the anti-angiogenic effect of BAI1 is mediated by its thrombospondin-1 functional domain[152]. Injection of BAI1 gene mixed with a non-liposomal lipid and delivered subconjunctively in a rabbit model reduces corneal neovascularization induced by epithelial debridement with heptanol by 51.1%, compared to the untreated eyes [301]. The reduction in the area of corneal neovascularization in the BAI1 gene-delivered eyes was comparable to corneas treated with anti-VEGF antibody[301]. Despite effective anti-angiogenic function reported by this study, further research investigating the translational potential and safety profile of BAI1 gene-based therapies for corneal neovascularization is required.

G. Cannabinoid Receptor Type 1 (CB1) Receptor

The endocannabinoid system is a well-established regulator in a range of neurologic and psychiatric diseases [122].Pharmacological blockade of the CB1 receptor, a component of the endocannabinoid system, can inhibit tumor angiogenesis by interrupting the VEGF signaling pathway and inducing endothelial cell apoptosis[226]. siRNA-mediated silencing of the CB1 gene can inhibit bFGF and VEGF-stimulated vascular endothelial proliferation, migration and tube formation[223]. Utilizing an *in vivo* rabbit and mouse model, bFGF micropocket-induced corneal neovascularization and hypoxia-induced retinal neovascularization were also effectively inhibited by a CB1 antagonist[223]. Moreover, the *in vitro* inhibition of endothelial cells proliferation only occurs in the presence of pro-angiogenic factors, which suggests a low risk of non-specific cytotoxic effects[223]. Given its specific anti-angiogenic effect, the CB1 receptor might have high translational potential as an interesting target of gene therapy in corneal neovascularization.

H. Cytochrome P450 4B1 (CYP4B1)

CYP4B1 expression is markedly increased in the cornea and tear film in the presence of ocular inflammation[58]. Its metabolite was shown to have pro-angiogenic effects in a VEGF-dependent manner[177]. Gene silencing of CYP4B1 by subconjunctival injection of its siRNA reduces VEGF mRNA and silk suture-induced corneal neovascularization by more than 50% compared to controls in the rabbit cornea[249]. This supports a role for CYP4B1 in the

inflammatory and neovascularization cascade and that gene silencing of CYP4B1 gene might be a useful approach for inhibiting corneal neovascularization *in vivo*[249].

I. GA-Binding Protein (GABP)

GABP is a nuclear transcription factor that has 3 subunits: α, β and γ[242]. The α subunit of GABP forms a heterodimer with the subunit β to suppress VEGF transcription[126]. *In vivo* subconjunctival injection of a plasmid DNA-encoding GABP in a lipid-based vector decreased VEGF gene expression after a mouse cornea was subjected to an alkaline insult[302]. Roundabout 4 (Robo4), a well-established guidance receptor in the nervous system[144,170], is also involved in pathological angiogenesis and transcriptionally regulated by GABP[207]. Robo4 is expressed in the endothelial cells of blood vessels of tissues with angiogenic process, such as tumors[250], placenta[121], heart[214] and developing embryos[214]. Slit is a family of neuronal guidance cues that regulate monodirectionally in nervous system[267], which interacts with Robo4 to mediate axonal repulsion[38], leukocyte migration[296] and neovascularization[24]. Slit also inhibited neovascularization and vascular leakage in mice with oxygen-induced retinal and laser-induced choroidal vascular disease, whereas deletion of Robo4 enhanced these pathologic processes[129]. More recently, Robo4 knockout mice were shown to produce more corneal neovascularization after HSV-1 ocular infection, compared to infected wild type controls[101]. Despite present evidence, the roles of Robo4 in neovascularization remain ill defined[137,214,250,268,286]. Transgene overexpression of GABP in the mouse cornea suppressed Robo4 mRNA expression and subsequent microscopic and histologic examination also showed 20.3% less neovascularised corneal area than did experimental control eyes[302]. However, the anti-angiogenic effect of GABP gene delivery only lasted for two weeks in this model and , this relatively short-term transgene expression is not ideal for clinical application[302].

J. Pigment Epithelium-Derived Factor (PEDF)

PEDF, a 50 kDa glycoprotein, is a potent anti-angiogenic factor, inhibiting vascular endothelial cell proliferation and migration mediated by the VEGF and bFGF pathways[182]. Subconjunctival transplantation of transfected retinal pigment epithelial cells that secret PEDF inhibited corneal neovascularization elicited by alkaline burn in a rabbit model [155]. Similarly, subconjunctival injection of SAINT-18 (a cationic synthetic vector) carrying plasmid DNA of PEDF was capable of inhibiting corneal neovascularization induced by stromal implantation of micropocket containing bFGF *in vivo* in a murine model, with $3001\times10^{-4}mm^2$ less neovascularised areas than the control group[156]. In this model transgene

expression commenced on day 3 after gene transfer and lasted for 3 months. The delivery vehicle used, SAINT-18, is safe, low toxicity and efficient for *in vivo* gene delivery. This study indicates the clinical feasibility of this gene-based therapy for corneal neovascularization by overexpressing PEDF via the SAINT-18 vector to provide a sustained anti-angiogenic effect.

K. Insulin Receptor Substrate-1 (IRS-1)

To date, the only gene therapy option for corneal angiogenesis that has reached the clinical trial stage is aganirsen (GS-101, Gene Signal), an antisense oligonucleotide targeting insulin receptor substrate-1(IRS-1)[63,71]. IRS-1 is a cytosolic adapter protein that plays an important role in ocular neovascularization by regulating VEGF and other proangiogenic cytokines, as well as interacting with integrins[128,292]. Using a rat *in vivo* model, with corneal neovascularization induced by the removal of the limbus strip, silencing the IRS-1 gene with specific antisense oligonucleotides was able to reduce IRS-1 production and regress corneal neovascularization[26]. Subsequently, these experimental findings were translated into application on human subjects[63]. In randomized clinical trials, a solution of GS-101 was administered topically on the corneas of patients with ongoing keratitis-related corneal neovascularization[63,71]. After treatment, twice a day for 90 days, GS-101 significantly reduced corneal neovascularization by 26.2% and the therapeutic effect lasted more than 180 days[71]. Other benefits included the lowered need of transplantation for patients with viral keratitis and central neovascularization and improved quality of life[71]. Moreover, the eye drops were safe and well tolerated. GS-101 is the first clinical trial-tested gene therapy for corneal neovascularization and has shown promising results.

L. MicroRNA (miRNA)

miRNAs are naturally occurring, 21-25-nucleotide, non-coding molecules that regulate gene expression at the post-transcriptional level[7,160]. Mature miRNAs are derived from a one arm of a larger imperfect stem-loop precursor hairpin, and are released by ribonuclease-III enzymes[21,160,165]. Thereafter, miRNAs form RNA-induced silencing complexes to repress translation by imperfect base-pairing with the three-prime untranslated region of messenger RNA (mRNA) promoting RNA degradation[21,297]. Several miRNAs have been associated with angiogenic processes, some are pro-angiogenic[93,100,200,289], while the others are anti-angiogenic[134,193,213,224,255]. The levels of miRNA-31, -150 and -184 are reduced during the formation of choroidal neovascularization induced by retinal ischemia, while their levels are high in cornea and lens, suggesting these miRNAs maintain the avascularity of these tissues

and are as such antiangiogenic [255]. The target genes for these three miRNAs were identified as genes encoding for pro-angiogenic proteins: platelet-derived growth factor-B (PDGF-B) and hypoxia-inducible factor 1- α for miRNA-31, VEGF and PDGF-B for miRNA-150, and Frizzled4 for miR-184[255]. Moreover, intraocular injection of miRNA-31, -150, and -184 significantly reduced retinal and choroidal neovascularization in mice.[255] This approach could also be applied to corneal neovascularization.

A few miRNAs are potential targets for gene silencing as their expression is upregulated in corneal neovascularization. miRNA-132 triggers vascular endothelial cells to undergo vasculogenesis, and antagomir nanomolecules targeting miRNA-132 can inhibit tumor angiogenesis. In the cornea miRNA-132 showed different levels of augmented expression across different time points after herpes simplex virus-1 (HSV-1) infection[193]. Subconjunctival injection of antagomir-132 nanoparticles (a single-stranded small RNA targeting miRNA-132) to mice effectively controlled corneal neovascularization induced by HSV-1[193]. This antiangiogenic effect was evident even if the treatment was administered 7 days post-infection[193]. The expression of miRNA-155, a molecule known to be involved in inflammatory processes[30,205], was also upregulated after corneal HSV-1 infection, mainly in macrophages and $CD4^+$ cells[31]. Similarly, silencing of miRNA-155 by subconjunctival injection of antagomir-155 nanoparticles in mice with HSV-1 infection diminished stromal keratitis and corneal neovascularization[31]. Another non-coding miRNA expressed in the cornea, miRNA-206, was upregulated after chemical injury[167]. Intrastromal injection of oligonucleotides inhibitor targeting miRNA-206 one hour after alkali burn in mice significantly reduced corneal neovascularization[167]. The molecular target of miRNA-206 was identified to be the gene for connexin43 (Cx43)[167], a trans-membrane protein that facilitate wound healing in damaged cornea[190]. Inhibition of miRNA-206 therefore upregulated the expression of Cx43, thus augmenting the wound healing process in chemically-injured cornea. Two miRNAs have been implicated as specifically involved in anti-angiogenesis and using the mimic of these miRNAs may suppress corneal neovascularization.

miRNA-184 is most abundantly expressed in the corneal epithelium[243]. miRNA-184 was shown to negatively regulate pro-angiogenic factors such as VEGF, PDGF and MMPs.[213] Transfection of miRNA-184 also suppressed the proliferation, migration, and tube formation of both macro- and micro- vascular endothelial cells[213]. The expression of miRNA-184 was reduced in the cornea of rats with suture-induced neovascularization, but topical administration of miRNA-184 reduced the neovascularization on day 7 after suture[309]. miR-

204 has also been studied in corneal neovascularization. The expression of angiopoietin-1 (a pro-angiogenic factor) increased during the neovascularization in the dystrophic corneas of KLEIP$^{-/-}$ mice, while the level of miRNA-204 was strongly downregulated[134]. Angiopoietin-1 was identified as a molecular target of miRNA-204, and endothelial cells transfected with miRNA-204 mimic produced less angiopoietin-1 protein[134]. Based on the above evidence, miRNAs are important regulatory factors in corneal neovascularization. The therapeutic strategies utilizing miRNAs with either antagomirs (inhibition) or miRNA mimics to suppress or augment the expression of miRNA could be used as therapeutic strategies to modulate corneal neovascularization. Further studies are required to investigate miRNA-based therapies for corneal neovascularization.

To date, most studies of gene therapy for corneal neovascularization are still in the pre-clinical experimental stages using trauma-induced neovascularization in animal models. Corneal neovascularization induced by external injury is generally linked to an inflammatory process[74]. Recently, genetically-engineered mice that develop spontaneous corneal neovascularization were used for studying pathologic angiogenesis[135]. While the traditional trauma-induced approach initiates a cascade of healing process whose involvement in corneal neovascularization is not well understood, the corneal neovascularization in transgenic models takes place as part of a clear pathological pathway[68,70,134,138] In the future, transgenic corneal neovascularization models will complement the existing models for investigating the mechanisms of corneal neovascularization.

VIII. Conclusion

Corneal neovascularization is a vision-impairing condition and a leading risk factor for corneal graft rejection. Current therapeutic options may be associated with significant side effects, limited efficacy, and a short duration of action. The immune-privileged nature and accessibility of the cornea makes it an attractive target for gene therapy, an alternative to pharmacological treatment that could provide non-toxic and long-term benefits. Additionally, progress of gene therapy to the cornea can be monitored visually and using several imaging modalities. Gene therapy seems to be effective in animal studies, although safety issues arising from the vectors, and transgenic overexpression may limit clinical utility. In addition, the mode of delivery requires further refinement. The success of gene therapy seen in some animal studies is accomplished by early and frequent administration, which is far from ideal

for treating on-going corneal neovascularization. As clinical trials of GS-101 have recently approached the phase III stage[71], however, the first non-invasive gene therapy that can provide a sustained anti-angiogenic effect is about to be applied clinically. With more target genes and biocompatible vectors being developed, more studies are needed to develop safe gene therapy that can not only prevent, but also regress, on-going corneal neovascularization without the need of frequent and invasive administration. Failing this approach, using *ex vivo* incubation of the donor cornea button with therapeutic genes has been successful experimentally in both animal and human models to limit post-corneal transplant angiogenesis. Clinically, this may be a novel and safe approach to treat donor button in eye banks before transplantation into a high risk vascularized corneal bed[86,195].

IX. Method of Literature Search

All studies included in this review were collated through online databases PubMed using the search terms “cornea”, “gene therapy”, “angiogenesis”, “neovascularizaiton” and “vectors”. Promising studies listed in selected publications were also reviewed for potential inclusion in our article. Inclusion criteria includes availability in English full text, relevancy to genetic therapy and its application in corneal neovascularization, quality of the source published and whether the articles has been cited by other studies.

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Figure 1. Angiogenic process leading to neovascularization. (A) At quiescent state, angiogenic privilege is maintained in pre-existing blood vessels. After being exposed to hypoxic injury or inflammation, endothelial cells are activated by pro-angiogenic factors such as VEGF and bFGF; (B) extracellular matrix and basement membrane are destabilize by MMPs. (C) Endothelial cells are converted into tip cells which invade the surrounding environment in the form of endothelial sprout. (D) Endothelial cells continue to migrate, proliferate and form vascular tube following the tip cells. (E) Newly formed vessels are stabilized by pericyte coverage, marking the maturation of these new vessels.
**VEGF inhibitors: sFlt, heparan sulfate proteoglycan, placental growth factor, decorin, cannabinoid receptor type-1*.

Figure 2. Corneal neovascularization under different imaging modalities. (A) Biomicroscopic photography. (B) Indocyanine green angiography. (C) Fluorescein angiography.

Table 1. Gene therapy approaches for corneal neovascularization.

Abbreviations: AAV, adeno-associated virus; BAI1-ECR, brain-specific angiogenesis inhibitor 1 – extracellular region; bFGF, basic fibroblast growth factor; CB1, Cannabinoid Receptor; CMV, Cytomegalovirus; CYP4B1, Cytochrome P450 4B1; IRS-1, insulin receptor substrate-1; K5, kringle 5 of plasminogen; PEDF, pigment epithelium-derived factor; PFU, plaque-forming units; PLGA, poly(lactic-co-glycolic acid); PlGF1-DE, placental growth factor 1-DE; PPARγ, peroxisome proliferator-activated receptor gamma; RGDRGD, arginine-glycin-aspartic-arginine-glycin-aspartic; sFlt-1, soluble Flt-1; shRNA, short hairpin RNA; siRNA, small interfering RNA;TU, Transducing Units; VEGF, vascular endothelial growth factor; VEGI, vascular endothelial cell growth inhibitor; vg, vector genomes.

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Target gene	Vector	Delivery	Subjects	Models	Dose	Result	Reference
IRS-1 mRNA	Antisense oligonucleotide	topical	Human	Keratitis	86µg/day for 90 days	Reduced corneal neovascularization by 26.2%	71
Endostatin and Angiostatin	Lentivirus	*Ex vivo* incubation	Rabbit	Transplant	2.10^6 TU/ml overnight for 37°C	Neovascularization failed to cross the donor-recipient margin in 50% of treated cornea	216
PlGF1-DE	Adeno-associated virus	Sub-retinal injection	Mouse	Nylon suture	5ng in 5µL of PBS post-insult, then every 3 days for 14 days	Reduced corneal neovascularization by 37.2%	273
Flt-1	Morpholino	Sub-conjunctival injection	Mouse	Transplant	15µL(40 ng/µL) post-transplant weekly for 7 weeks	Reduced corneal neovascularization by 22.8%	52
VEGF-A	PLGA	Stromal injection	Mouse	Alkaline	2µg plasmid 4 weeks post-injury	Reduced corneal neovascularization by 43.0%	229
Flt23k	PLGA	Sub-conjunctival injection	Mouse	Transplant	10µL of plasmid (0.1µg/µL) at day 0 and 4 weeks post-transplant	Reduced corneal neovascularization by 71.0%	51
Flt-1	PEG-b-P[Asp(DET)] polyplex micelle	Sub-conjunctival injection	Mouse	Nylon suture	1mg in 5µL post-insult	Reduced corneal neovascularization by 45.0%	123
Decorin	Adeno-associated virus	Topical-stromal	Rabbit	Pocket pellet	100µl($5x10^{12}$vg/ml) 1-day post-pellet implantation	Reduced corneal neovascularization by 60.0%	187
RGDRGD endostatin	CMV	Sub-conjunctival injection	Rabbit	Alkaline	5µg twice a week for two weeks post-insult	Reduced corneal neovascularization by 58.0%	98
CB1 receptor	siRNA KD	Endothelial cell transfection	*In vitro*	bFGF	100nM	Inhibition of endothelial proliferation, migration, tube-formation	223
VEGI	Lipofectine	Sub-conjunctival injection	Rabbit	Suture	20µl post-insult	Reduced corneal neovascularization by $13.8mm^2$	287
VEGF-A	siRNA KD	Sub-conjunctival injection	Mouse	Alkaline	10µl(10µg/10µl) days 1, 3, 5 post-insult	Reduced corneal neovascularization by $2.34mm^2$	310
Vasohibin-1	Adenovirus	Sub-conjunctival injection	Mouse	Alkaline	5µl containing 109 viral particles 5 days pre-insult	Reduced corneal neovascularization by 21.22%	308
PEDF	SAINT-18	Sub-conjunctival injection	Mouse	Pocket pellet	10µg post-insult	Reduced corneal neovascularization by $3001x10^{-4}mm^2$	156
GA-binding protein	Lipoplexes	Sub-conjunctival injection	Mouse	Alkaline	2µg in 20µl post-insult	Reduced corneal neovascularization by 20.3%	302
CYP4B1	siRNA	Sub-conjunctival injection	Rabbit	Suture	20µl (200µM) day 2, 4 post-insult	Reduced corneal neovascularization by 50%	249
PPARγ	Adenovirus	Topical	Mouse	Alkaline	1.0x107PFU/µL day 1, 5, 10 post-insult	Reduced corneal neovascularization	244
BAI1-ECR	CMV	Sub-conjunctival injection	Rabbit	Epithelial removal	5 mg(0.4 ml) twice post-insult at 1 week interval	Reduced corneal neovascularization by 51.1%	301
Endostatin K5	Lentivirus	*Ex vivo* incubation	Rabbit	Transplant	50µL for 18hours at 37°C	Neovascularization failed to cross the donor-recipient margin in all treated cornea	195
K5	Electroporation	Sub-conjunctival injection	Mouse	Alkaline	50µg	Neovascularization score of treated eyes was lower than controls	306
VEGF	Adenovirus	Sub-conjunctival injection	Mouse	Cautery	2µL($2x10^8$PFU/µL) 24 hours pre-insult	Less treated eyes developed neovascularization than controls	159
Flt-1	AAV-CMV	Intra-cameral	Mouse	Cautery	2µL(10^{11}PFU/ml) 3 weeks pre-insult	Reduced corneal neovascularization by 36%	162
Flt-1	Naked plasmids	Stromal injection	Mouse	Pocket pellet	2µL 24 hours pre-insult	Reduced corneal neovascularization by 23.6%	262
Flt-1	Adenovirus	Intra-cameral	Mouse	Cautery	2µL(10^{11}PFU/ml) 24 hours pre-insult	18% of treated eyes developed neovascularization compared to 100% in controls	158

Abbreviations: AAV, adeno-associated virus; BAI1-ECR, brain-specific angiogenesis inhibitor 1 – extracellular region; bFGF, basic fibroblast growth factor; CB1, Cannabinoid Receptor; CMV, Cytomegalovirus; CYP4B1, Cytochrome P450 4B1; IRS-1, insulin receptor substrate-1; K5, kringle 5 of plasminogen; PEDF, pigment epithelium-derived factor; PFU, plaque-forming units; PLGA, poly(lactic-co-glycolic acid); PlGF1-DE, placental growth factor 1-DE; PPARγ, peroxisome proliferator-activated receptor gamma; RGDRGD, arginine-glycin-aspartic-arginine-glycin-aspartic; sFlt-1, soluble Flt-1; shRNA, short hairpin RNA; siRNA, small interfering RNA;TU, Transducing Units; VEGF, vascular endothelial growth factor; VEGI, vascular endothelial cell growth inhibitor; vg, vector genomes.

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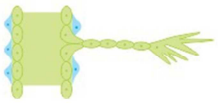
	Pro-angiogenic	Anti-angiogenic
A. Capillary vessel	Hypoxia-inducible factors (HIF) IL-17A Insulin Receptor Substrate-1 VEGF	VEGF inhibitors* Apoptosis inducing factor Endostatin Thrombospondin-1 Fas ligand

Hypoxia
Inflammation

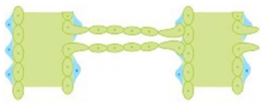
Loss of angiogenic privilege/balance

B. Destabilize	+VEGF + bFGF + MMPs + Angiopoietin	VEGF inhibitors* Vasohibin-1 Endostatin Angiostatin PPARγ Pigment epithelium-derived factor



C. Sprouting	+ VEGF + bFGF + MMPs + Angiopoietin	VEGF inhibitors* Vasohibin-1 Endostatin Angiostatin PPARγ Pigment epithelium-derived factor

D. Lumen formation	+ VEGF + Integrins + MMPs + bFGF	VEGF inhibitors* Vasohibin-1 Endostatin Angiostatin PPARγ Pigment epithelium-derived factor



E. Stabilization	Angiopoietin PDGF Integrins O2 Blood flow

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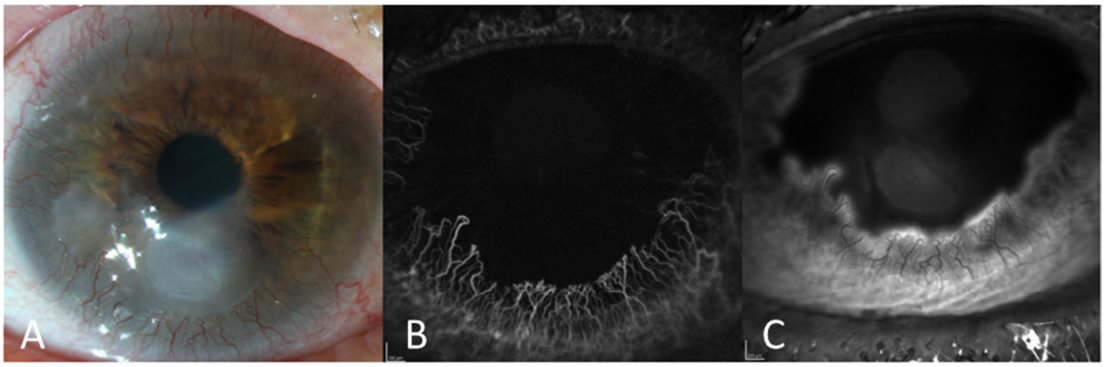

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