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Molecular Therapy
Review

Gene Therapy Intervention in Neovascular Eye Disease: A Recent Update

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Aberrant growth of blood vessels (neovascularization) is a key feature of severe eye diseases that can cause legal blindness, including neovascular age-related macular degeneration (nAMD) and diabetic retinopathy (DR). The development of anti-vascular endothelial growth factor (VEGF) agents has revolutionized the treatment of ocular neovascularization. Novel proangiogenic targets, such as angiopoietin and platelet-derived growth factor (PDGF), are under development for patients who respond poorly to anti-VEGF therapy and to reduce adverse effects from long-term VEGF inhibition. A rapidly advancing area is gene therapy, which may provide significant therapeutic benefits. Viral vector-mediated transgene delivery provides the potential for continuous production of antiangiogenic proteins, which would avoid the need for repeated anti-VEGF injections. Gene silencing with RNA interference to target ocular angiogenesis has been investigated in clinical trials. Proof-of-concept gene therapy studies using gene-editing tools such as CRISPR-Cas have already been shown to be effective in suppressing neovascularization in animal models, highlighting the therapeutic potential of the system for treatment of aberrant ocular angiogenesis. This review provides updates on the development of anti-VEGF agents and novel antiangiogenic targets. We also summarize current gene therapy strategies already in clinical trials and those with the latest approaches utilizing CRISPR-Cas gene editing against aberrant ocular neovascularization.

Angiogenesis is the formation of new blood vessels from preexisting vasculature, a process regulated by a dynamic balance between endogenous proangiogenic and antiangiogenic factors. Stimuli, such as biomechanical stress, hypoxia, ischemia, immune or inflammatory responses, and genetic variations, can disturb the balance in favor of angiogenesis.[1] Ocular angiogenesis can occur in the retina, choroid, as well as cornea, and unchecked pathological blood vessel formation (neovascularization) can lead to severe visual impairment. These newly formed blood vessels are exudative, resulting in the accumulation of extracellular fluid subsequent to impairment of retinal function. Moreover, the aberrant growth of new vessels interferes with normal tissue structure and corneal transparency.[2,3] Neovascularization is associated with a range of ocular disorders, including neovascular age-related macular degeneration (nAMD), diabetic retinopathy (DR), retinopathy of prematurity (ROP), corneal neovascularization, retinal vessel occlusion, and neovascular glaucoma (reviewed in Campochiaro[4] and Usui et al.[5]). Among these neovascularization-related diseases, nAMD and DR are leading causes of visual impairment in the developed world.[6]

AMD occurs in patients $\geq$65 years of age. It is predicted that the number of individuals affected globally by AMD will reach 288 million by 2040.[7] AMD can be classified as non-neovascular (dry form AMD [dAMD]) or neovascular forms (nAMD). nAMD, an advanced stage of AMD, is characterized by the pathologic growth of blood vessels from the choroid, beneath the macula, which is also termed choroidal neovascularization (CNV). The vessel outgrowth from the choroidal lacks normal vascular structure and function, thus resulting in fluid leakage and ultimately hemorrhagic or exudative retinal detachment. The growth of new vessels is often accompanied by fibrosis, which causes further damage to retina cells, particularly photoreceptors.[8,9] Despite a variety of available treatments, nearly half of patients develop retinal pigment epithelium (RPE) atrophy or macular scarring within 2 years, a major cause of permanent vision loss.[10] The most susceptible genetic loci relevant to AMD are *CFH* (complement factor H) and *ARMS2* (age-related maculopathy susceptibility 2). Non-genetic risk factors for AMD include smoking and deficiency in dietary intake of antioxidants such as carotenoids and zinc.[11,12] During the last two decades, therapeutic intervention for nAMD has shifted from laser

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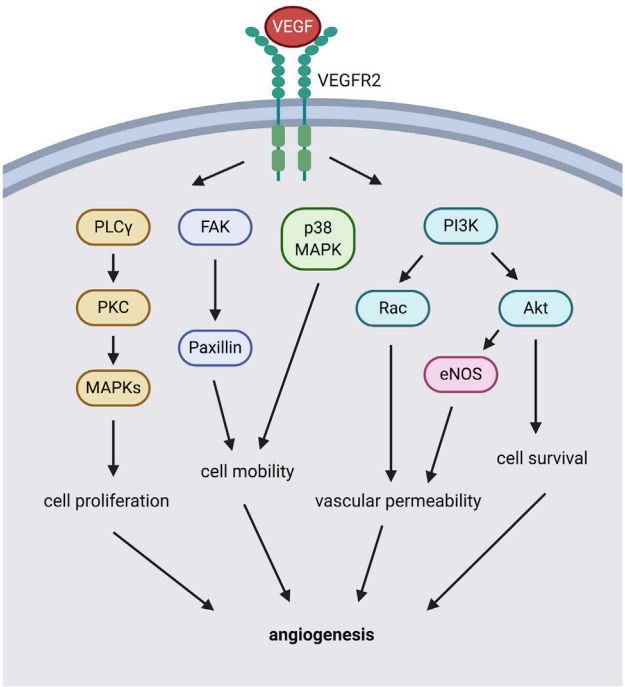

Figure 1. VEGF/VEGFR2 Signaling Pathway in Endothelial Cell Relevant to Angiogenesis

The VEGF-VEGFR2 system engages activation of PI3K, p38MAPK, FAK, and PLCγ. PI3K, in turn, activates AKT and Rac, resulting in cell survival and increased vascular permeability, respectively. PLCγ, in turn, activates PKC with the downstream MAPK cascade facilitating cell proliferation and cell motility. Both p38MAPK and FAK mediate cell mobility. The combined effects of these pathways on the vascular endothelium result in angiogenesis. VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; PLCγ, phospholipase C gamma; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; eNOS, endothelial nitric oxide synthase.

photocoagulation to block leaky vessels to pharmacotherapeutic intervention, particularly anti-vascular endothelial growth factor (VEGF) therapy.[13] Anti-VEGF therapy has also become a mainstay for the treatment of neovascularization and vascular hyperpermeability in DR.

DR is a leading cause of legal blindness in those of working age (20–65 years).[14] According to the International Diabetes Federation (IDF), in 2017 there were an estimated 451 million people with diabetes mellitus (DM), a number projected to increase to 693 million by 2045.[15] The prevalence of any form of retinal pathology (retinopathy) in DM patients is approximately 35%, and the number of people with DR is estimated to increase to 191 million in 2030.[16,17] Around 7%–10% patients with DM will develop vision-threatening (proliferative) retinopathy, an advanced stage of DR.[17,18] Diabetes-associated hyperglycemia damages the retinal vasculature vascular, including endothelial cells, basement membrane, and supporting pericytes.[19] Non-proliferative DR (NPDR) is the initial stage of DR characterized by mild changes such as microaneurysms, microhemorrhages, hard exudates, and cotton wool spots, which can be detected clinically using ophthalmoscopy or fundus imaging. Diabetic macular edema (DME) is a frequent cause of vision loss, resulting from the breakdown of the outer blood-retinal barrier due to damaged endothelial tight junctions that allow salts, proteins, and water to accumulate within the retina.[20,21] DME can occur at any stage of DR. Proliferative DR (PDR), or advanced DR, is characterized by neovascularization with or without pre-retinal or vitreal hemorrhages. Retinal neovascularization along with fibrosis can promote tractional retinal detachment, a severe complication of PDR that requires surgical interference. Retinal neovascularization can also occur on the iris and fluid drainage angle at the anterior eye, promoting the risk of neovascular glaucoma (reviewed in Rodríguez et al.[22]). For these reasons, those patients that progress to the PDR stage are at high risk of vision loss. Anti-VEGF therapies, along with laser photocoagulation, are currently the first-line treatment for DR.[21,22]

VEGF, Vascular Injury, and Current Anti-VEGF Agents

A vasoactive molecule that drives neovascularization common to corneal, retinal, and choroidal vascular beds is VEGF.[23,24] VEGF is considered to be the most critical regulator of ocular angiogenesis, where it regulates cellular proliferation, survival, motility, and vascular permeability.[25,26] VEGF is released either as soluble or extracellular matrix-bound forms to interact with VEGF receptor 2 (VEGFR2), which are found on vascular endothelial cells.[27] Activation of VEGFR2 modulates a number of downstream signaling cascades, one of which is receptor tyrosine kinases. Engagement of VEGFR2 by VEGF activates phosphatidylinositol 3-kinase (PI3K), phospholipase C γ (PLCγ), focal adhesion kinase (FAK), and p38 mitogen-activated protein kinase (p38MAPK) (Figure 1). PI3K, in turn, activates AKT and Rac, resulting in inhibition of apoptotic signaling (promote survival) and decreased cellular adhesion (increase vascular permeability), respectively. PLCγ activates protein kinase C (PKC) and subsequently the MAPK cascade, which promotes cell proliferation and cytoskeletal reorganization (increase motility).[26] Both FAK and p38MAPK are known as the key mediators of endothelial cell adhesion and migration (increase motility).[28] The combined effects of increased VEGF contribute to several important steps in angiogenesis such as endothelial cell mitogenesis, migration, sprouting, and tube formation.[29] Clinical studies have shown that abnormally high VEGF levels are found in several ocular diseases, including nAMD, DR, DME, ROP, and neovascular glaucoma.[4] Intravitreal injection of anti-VEGF agents significantly improves visual function in patients with nAMD and DR (Table 1).[8,30–34]

The VEGF aptamer pegaptanib (Macugen; Eyetech Pharmaceuticals/Pfizer) was the first antiangiogenic agent approved at a recommended dosage of 0.3 mg, administered by intravitreal injection every 6 weeks by the US Food and Drug Administration (FDA) for the treatment of nAMD.[46,35] Pegaptanib specifically inhibits $VEGF_{165}$, the dominant isoform of VEGF-A. This selectivity limits its efficacy compared with non-selective VEGF inhibitors (e.g., bevacizumab) for nAMD treatment.[8,47] Due to its long-term safety profile, pegaptanib has been suggested for patients who are prone to the development of thromboembolic events (e.g., diabetes, cardiovascular disease) or

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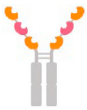
Table 1. Summary of Available Anti-VEGF Therapies

Generic Name	Pegaptanib	Bevacizumab	Ranibizumab	Aflibercept	Brolucizumab
Brand name	Macugen	Avastin	Lucentis	Eylea	Beovu
Targets	only one VEGF-A isoform ($VEGF_{165}$)	all VEGF-A isoform	all VEGF-A isoform	all VEGF-A/VEGF-B/PlGF isoforms	all VEGF-A isoform
Format	aptamer	full monoclonal antibody	antibody fragment	VEGFR1/2 recombinant fusion protein	single-chain antibody fragment
Function	VEGF inhibitor	anti-VEGF antibody	anti-VEGF antibody	VEGF trap	anti-VEGF antibody
Molecular mass	49 kDa	149 kDa	48 kDa	115 kDa	26 kDa
FDA-approved indications	nAMD	no FDA approval for ophthalmic use	nAMD; DR; DME; macular edema after RVO; mCNV	nAMD; DR; DME; macular edema after RVO	nAMD
Clinical dosage regimen	0.3 mg every 6 weeks[35]	1.25 mg every 4 weeks[36]	0.5 mg every 4 weeks for nAMD[a] or 0.3 mg every 4 weeks for DR or DME[37,36]	2.0 mg every 4 weeks for first three injections, then every 8 weeks for nAMD[b] or 2.0 mg every 4 weeks for the first five injections, then every 8 weeks for DR or DME[36]	6.0 mg every 4 weeks for the first three injections, then every 8–12 weeks[38]
Intraocular half-lives	4 days[39]	6.7 days[40] 4.9 days[44]	7.19 days[41] 9 days[45]	11 days[42]	4.3 days[43]
Molecular structure					

VEGF, vascular endothelial growth factor; PlGF, placental growth factor; nAMD, neovascular age-related macular degeneration; DR, diabetic retinopathy; DME, diabetic macular edema; mCNV, myopic choroidal neovascularization; RVO, retinal vein occlusion.
[a]May give every 12 weeks after 3 or 4 monthly injections for nAMD, but is less effective than once monthly dosing.
[b]May give every 12 weeks for selected patients after first year.

geographic atrophy.[48,49] Other anti-VEGF agents, including ranibizumab, aflibercept, and bevacizumab, are widely used to inhibit ocular neovascularization.[50] Bevacizumab (Avastin) and ranibizumab (Lucentis) are immunoglobulin antibodies (monoclonal and fragment, respectively) both targeting VEGF-A. Ranibizumab is 5- to 30-fold more potent at neutralizing VEGF-A than is bevacizumab.[51] Ranibizumab was shown to improve vision and was approved at a dose of 0.5 mg every 4 weeks for nAMD treatment in 2006,[52,53] and 0.3 mg every 4 weeks for DR and DME in 2012.[37] Bevacizumab was originally approved for treatment of metastatic colorectal cancer but has been used since 2005 as an off-label treatment for nAMD at a dose of 1.25 mg every 4 weeks.[54] Because of its lower cost and greater availability, bevacizumab has become the first option for many patients.[55,56] Aflibercept (Eylea) is a recombinant fusion protein that forms a VEGF trap, which targets VEGF-A, VEGF-B, and placental growth factor (PlGF).[57] PlGF is another member of the VEGF family that activates VEGFR1.[58] PlGF was reported to potentiate angiogenic signaling by forming heterodimers with VEGF-A, or by displacing VEGF-A from VEGFR1, and contributes to the development of neovascularization.[59] Aflibercept is 100-fold higher than both bevacizumab and ranibizumab,[60] and as such aflibercept provides comparable effectivity but requires less frequent injection compared with ranibizumab and bevacizumab. Aflibercept was approved for nAMD, DME, and DR treatment in 2011, 2014, and 2019, respectively,[55,61] with a recommended dose of 2 mg for three to five initial monthly doses followed by doses every 8 weeks. Ziv-aflibercept (Zaltrap) contains the same aflibercept molecule but with higher osmolarity (1,000 mOsm/kg compared to 300 mOsm/kg for aflibercept), which helps to achieve iso-osmolarity for intravitreal injection. Ziv-aflibercept was approved in 2012 for treatment of metastatic colorectal carcinoma. Several cases have demonstrated that off-label, intravitreal ziv-aflibercept treatment is safe and effective in various chorioretinal vascular diseases.[55] As its cost is comparable to bevacizumab (in US dollars, $50 for bevacizumab versus $2,000 for aflibercept), ziv-aflibercept is an attractive treatment option in low- to middle-income countries.[62] Similar to aflibercept, conbercept is a recombinant fusion protein that targets VEGF-A, VEGF-B, VEGF-C, and also PlGF.[63] In 2013, conbercept was approved for nAMD treatment and had been extensively used in China, and FDA-approved phase III clinical trials are currently well underway in the US (ClinicalTrials.gov: NCT03577899 and NCT03630952). Brolucizumab (Beovu), a humanized single-chain antibody fragment inhibitor of VEGF-A, is the most recent (October 2019) FDA-approved agent for nAMD at a recommended dosage of 6 mg.[64] Its small molecular mass (26 kDa) allows for high solubility, extended duration of action, and improved ocular tissue penetration.[55] Brolucizumab is the first drug to offer less frequent dosing (3-month dosing interval) in the first year of therapy while maintaining comparable effectiveness. Indeed, brolucizumab-treated patients showed greater fluid reduction, as evidenced by larger decreases in retinal thickness compared

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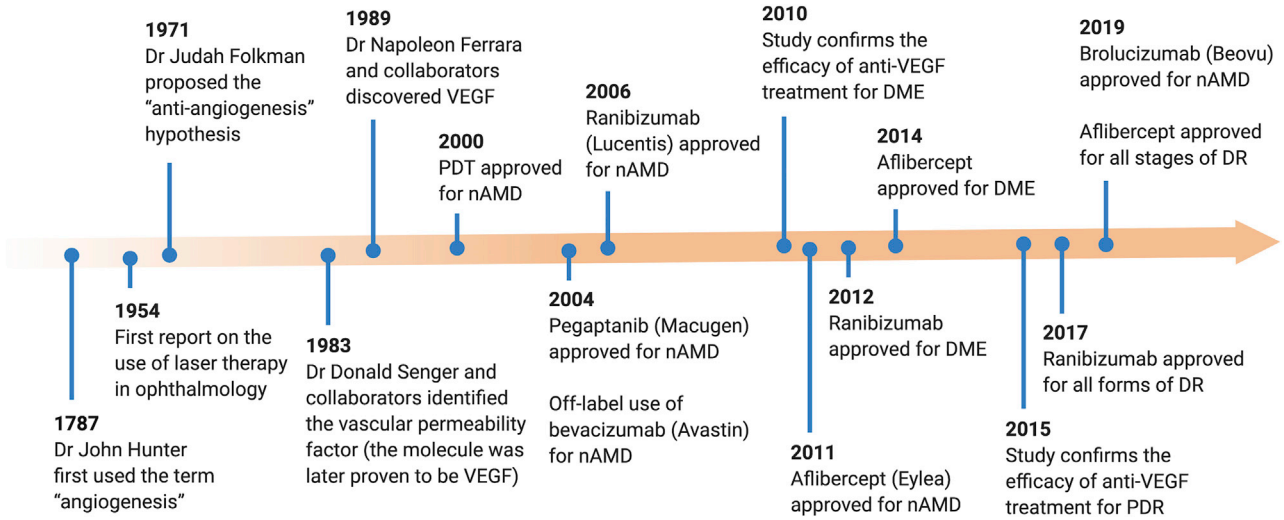

Figure 2. Graphical Timeline of Major Advances in Discovery of VEGF and the Developments of Anti-VEGF Therapy for Neovascular Eye Disease
For each drug, the time indicates the year of FDA approval (except bevacizumab). DR, diabetic retinopathy; DME, diabetic macular edema; nAMD, neovascular age-related macular degeneration; PDT, photodynamic therapy; VEGF, vascular endothelial growth factor.

with aflibercept.[38] A timeline of major advances in understanding ocular angiogenesis and the developments of antiangiogenesis therapy is shown in Figure 2 (reviewed in Yang et al.,[65] Ferrara,[66] and Shah and Gardner[67]).

Novel Ocular Angiogenesis-Related Targets in Clinical Trials

Although anti-VEGF therapy is now the mainstream therapy for ocular neovascularization, its application is not without challenges. First, some patients still show a progression of neovascular pathology despite aggressive anti-VEGF treatment.[68] Second, systemic side effects from prolonged use of anti-VEGF associated with thromboembolic complications such as myocardial infarction, stroke, and non-ocular hemorrhage can still occur even though anti-VEGF therapy is locally administered into the eye.[69] Finally, as VEGF plays an important role in maintaining healthy choroidal vasculature, corneal nerves, and retinal neurons,[70–72] chronic suppression of VEGF may lead to inhibitory trophic effects. Given these challenges, novel molecular targets and delivery methods are being sought.[73] Currently, new treatments targeting angiopoietins (Angs)/Tie-2 or platelet-derived growth factors (PDGFs) are being examined in a number of clinical trials for nAMD and DME.[50,73,74] Other novel therapeutic targets are summarized in Table S1.

Angiopoietins/Tie-2

The Tie-2 tyrosine kinase receptor is primarily expressed in vascular endothelial cells and can be found in other cell types, including epithelial cells, smooth muscle cells, fibroblasts, glial cells, neutrophils, and monocytes. The activation of Tie-2 receptors in endothelial cells reinforces junctional proteins and stabilizes the vasculature to limit permeability.[75,76] While VEGF promotes sprouting and tube formation from primitive vessels in the early stage of angiogenesis, the angiopoietins/Tie-2 system impacts the late stages of angiogenesis by recruiting mural cells (mainly pericytes), mediating interactions between endothelium cells and pericytes, to stabilize new vessels through the formation of endothelial tight junctions.[77] Angiopoietins are ligands that bind to Tie-2, regulating vascular development, maintenance, and permeability. Angiopoietin-1 (Ang-1) has been shown to activate the Tie-2 receptor, thereby reducing vascular leakage and thus inhibiting CNV formation.[78] Ang-1 is also shown to be upregulated by VEGF stimulation in RPE cells, indicating that VEGF may selectively regulate the expression of Ang-1 to induce the structural and functional maturation of new blood vessels in the late stage of CNV.[79] Angiopoietin-2 (Ang-2) acts as a competitive antagonist to Ang-1, promoting destabilization of vessels and causing vascular leakage.[80] Both Ang-2 and VEGF were found to be upregulated in highly vascular areas of surgically excised choroidal neovascular membranes[81] and in vitreal fluids from patients with PDR.[82] A study has shown that simultaneous VEGF and Ang-2 inhibition reduces vascular lesion size, permeability, retinal edema, and neuron loss in a spontaneous mouse CNV model.[83] Faricimab is a bispecific antibody manufactured using CrossMab technology, which is designed to simultaneously neutralize both VEGF-A and Ang-2.[84] Two phase II clinical trials (BOULEVARD, ClinicalTrials.gov: NCT02699450 and STAIRWAY, ClinicalTrials.gov: NCT03038880) show that faricimab is safe and effective in treating patients with nAMD and DME, providing evidence that simultaneous inhibition of VEGF and Ang-2 could be a viable therapeutic strategy against ocular angiogenesis.[84,85]

PDGF

Angiogenesis is associated with the recruitment of pericytes and smooth muscle cells to blood vessels and subsequent extracellular matrix production. Pericytes could directly contact endothelial cells and contribute to vessel maturation through the release of angiogenic

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growth factors, including VEGF.[86,87] PDGF is critical for pericyte recruitment, maturation, and survival;[88] thus, a combined therapy with anti-PDGF agents may overcome limitations of anti-VEGF monotherapy. Studies using a range of preclinical ocular neovascularization models have demonstrated that simultaneous inhibition of VEGF and PDGF, especially PDGF-B, results in an enhanced antiangiogenic effect.[89,90] However, clinical studies report that combining pegpleranib (Fovista) or rinucumab (a neutralizing monoclonal antibody to human PDGF-B) with anti-VEGF therapy showed no further benefits in treating nAMD.[91] Results from two phase III clinical trials, OPH1002 (ClinicalTrials.gov: NCT01944839) and OPH1003 (ClinicalTrials.gov: NCT01940900), revealed that combined pegpleranib and ranibizumab therapy failed to improve vision at 12 months when compared with ranibizumab monotherapy.[92,93] Further studies and subgroup analyses of these data may reveal specific groups of retinal or CNV patients who may benefit from anti-PDGF and anti-VEGF combined therapy.[50,91]

Drawback of Current Antiangiogenic Therapy

Although intravitreal injections of anti-VEGF agents are effective and safe,[25] they do not cure the fundamental problem, and most patients will require repeated intravitreal injections to sustain therapeutic efficacy due to the relatively short half-life of these agents.[94,95] Following intravitreal injections (0.5 mg), the half-life of ranibizumab is approximately 9 and 7 days in the vitreous humor and aqueous humor, respectively.[41,45] Clinically, a meaningful improvement in visual acuity was only seen in patients receiving monthly ranibizumab injections, rather than those who had received injections every 3 months.[96,97] The vitreous half-life of bevacizumab is between 4.9 and 6.7 days after the intravitreal administration (1.25 mg), as reported by two studies in human subjects.[40,44] Aflibercept has a longer half-life of 11 days, suggesting that intravitreal aflibercept administration could have a longer dosing interval than either ranibizumab or bevacizumab.[42,98] The need for injections every 1–2 months can be a cause of discomfort and anxiety, and this together with the need for repeated office visits with time and cost implications all impact patient compliance with treatment regimens.[99] Additionally, frequent intravitreal injections can increase the risk of complications, including submacular hemorrhage, intraocular hypertension, endophthalmitis, and retinal detachment.[100–102] Given this, researchers have sought to develop long-acting anti-VEGF modalities, sustained-release formulation, and device, and they have looked to gene therapies to address the abovementioned issues.[73]

Gene Therapy for Ocular Disease

Gene therapy designed to directly repair or compensate for defective genes has shown promise for a range of retinal disorders. Clinical success has been realized for Leber's congenital amaurosis, with voretigene neparvovec-rzyl (Luxturna) becoming the first-ever FDA-approved gene therapy in 2017. The agent is directly administered into the eye to correct a deficiency caused by mutations in the *RPE65* gene.[103] There are currently more than 60 gene therapy trials ongoing for retinal diseases.[104] Hereditary conditions currently being considered for gene therapy include achromatopsia, choroideremia, Leber's congenital amaurosis, Leber's hereditary optic neuropathy, retinitis pigmentosa, X-linked retinoschisis, Usher syndrome, and Stargardt's disease. Gene therapy is also being considered for non-hereditary ocular diseases such as AMD (both dAMD and nAMD), DME, and glaucoma.

Advantage in Using Gene Therapy for Ocular Disease

The eye has advantages as a target organ for gene therapy, including high accessibility, a relative immune-privilege, and relative compartmentalization away from other organs.[105] Ease of access and optical clarity facilitate direct visualization of the microsurgical delivery of genetic material to the retina.[106,107] These characteristics mean that imaging and functional tools are readily available for quantification of the safety and efficacy of gene therapy. The blood-retinal barrier provides the retina with a relative immune-privilege, thereby blocking the trafficking of immune cells from the systemic circulation to the eye, which dampens inflammatory responses. This functional barrier also prevents the leakage of genetic material into the systemic circulation and thus localizes the expression of the therapeutic gene to the eye.[108,109] Given that genetic material can be largely compartmentalized to the eye, smaller doses of genetic material can be delivered.[110] Moreover, target cells in the retina such as photoreceptors and RPE do not divide, and as such gene therapy produces prolonged effects.[111] Thus, gene therapy provides the possibility for targeted, localized, and sustained delivery of therapeutic genetic material into specific intraocular sites.[112]

Types of Gene Therapy for Ocular Disease

Gene therapy requires the introduction of exogenous genetic material, such as DNA, RNA, small interfering (siRNA), microRNA (miRNA), and antisense oligonucleotides (synthesized nucleic acid sequence complementary to mRNA), into cells via viral or non-viral vectors to regulate, replace, or modulate specific gene functions.[113] Gene therapy approaches are generally categorized into gene augmentation, gene-specific targeting, or genome editing.[104] Gene augmentation therapy refers to the introduction of correct copies of genes into the host genome to compensate for the faulty gene, as is the case with Luxturna. Mutations in the *RPE65* gene impair the activity of a protein integral to photopigment recycling (visual cycle), leading to the death of photoreceptors and thereby impaired vision. Gene augmentation therapy through the addition of a normal copy of the *RPE65* gene delivered by subretinal adeno-associated virus (AAV) vector injection is effective at improving vision.[103] AAVs are low in toxicity (minimal pathogenicity, immunogenicity, and capacity for self-replication) and are high-yield single-stranded DNA viruses with a cloning capacity of ~4.7 kb. AAVs are currently the most commonly used vector for retinal gene transfer in both preclinical studies and clinical trials.[114] Although AAV has an excellent safety profile, mild and temporary inflammatory responses have been reported with higher ocular AAV vector doses.[115–117] Recently, Timmers et al.[117] studied the contribution of the AAV vector genome and capsid in triggering ocular inflammatory responses. They suggested that lowering the total capsid dose by removing empty AAV capsids could decrease inflammation and improve viral transduction.

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Gene augmentation therapy is useful for inherited retinal degenerations where mutations produce loss of function and is a good approach for X-linked or autosomal recessive mutations.[118] Gene-specific targeting represents the introduction of genetic materials into a target cell or tissue to specifically alter or turn off a gene responsible for the disease, or the addition of copies of genes for protective or regenerative purposes.[104,119] Targeted therapies to modify pathological molecular pathways at the gene level are proving to be beneficial for long-term treatment of both non-genetic diseases and autosomal dominant genetic diseases.[104] Genome editing, also known as genome surgery, seeks to excise or correct the faulty gene at a specific genomic location. This approach fundamentally repairs faulty genes rather than just adding normal copies of genes by gene augmentation therapy.[120] Genome editing uses programmable site-specific endonucleases to target specific endogenous loci and thus corrects specific pathogenic alleles.[121] Endonucleases used for genome editing include meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALENs), targetrons, and, most recently, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) systems.[122] The CRISPR-Cas system has several potential advantages over other editing tools such as simplicity of target design, ease of generating large-scale libraries, and relatively low cost.[122,123] Moreover, genome editing with CRISPR-Cas9 enables multiplex editing through the engagement of multiple guide RNAs (gRNAs).[124,125] In addition to gene knockout or transcriptional regulation, base editing of DNA or RNA at specific positions is feasible using the CRISPR-Cas system or Cas variants engineered with cytidine deaminases.[126,127] Recently, an upgraded CRISPR-Cas-based technique called prime editing was developed, and it is able to introduce indels and enables all 12 base-to-base conversions (both transitions and transversions) without inducing a double-strand break (DSB).[128] Moreover, the action of the CRISPR-Cas system is not restricted to DNA. Recently developed CRISPR-Cas-based RNA editing technology is capable of recognizing and cleaving RNA sequences without altering the sequence or integrity of genomic DNA.[129] Compare to other existing RNA interrupting approaches, this system possessed higher efficiency and specificity.[130] CRISPR-Cas-based gene therapies have shown promise in a number of animal models of inherited retinal degenerations and non-inherited ocular diseases such as AMD, highlighting their therapeutic potential for ocular diseases.[131–134]

Current Gene Therapy Trial for Neovascular Eye Disease

Success in gene therapy has encouraged scientists and clinicians to use this approach for neovascular eye diseases such as nAMD and DR. Gene therapy is particularly attractive, as it has the potential to provide long-term efficacy, thereby eliminating the need for ongoing frequent intraocular injections. Current gene therapy clinical trials for nAMD or DME use two major strategies based on intraocular injection of viral vectors encoding antiangiogenic proteins or non-coding RNA interference molecules that target overexpression of VEGF (Tables 2 and 3). Several antiangiogenic proteins, such as endostatin, angiostatin, pigment epithelium-derived factor (PEDF), and secreted extracellular domain of VEGFR1, soluble fms-like tyrosine kinase-1 (sFLT-1), have been assessed for potential gene therapy. These studies have significantly contributed to our understanding of the safety profile, feasibility (protein expression levels), as well as the optimal evaluation methods for assessing the biological activity of ocular gene therapy.

PEDF

GenVec's phase I clinical trial (ClinicalTrials.gov: NCT00109499) assessed the safety of AdGVPEDF.11D in patients with advanced nAMD and was one of the earliest human applications of intraocular gene therapy. The replication-deficient adenoviral vector (E1, E3, and E4 deleted) was used to deliver the PEDF gene.[140] Adenovirus is a double-stranded DNA virus with the highest loading capacity of up to 37 kb for transgene delivery.[145,146] PEDF is a potent endogenous antiangiogenic protein, the levels of which are decreased in patients with nAMD.[147,148] Participants received an intravitreal injection of AdGVPEDF.11 ranging in dose from 1E6 to 1E9 particle units (PU). The trial reported that 25% of patients experienced mild, transient intraocular inflammation, but there were no serious adverse events related to AdGVPEDF.11. Although therapeutic efficacy cannot be concluded from this phase I trial, patients receiving 1E8 PU or greater appeared to show stable or a reduction in CNV lesion size compared with those receiving doses lower than 1E8 PU.[140]

Anti-VEGF

Currently, most clinical trials of gene therapy for AMD predominantly focus on the application of viral vector-delivered FLT-1 (also known as VEGFR-1). The Flt-1 receptor gene is upregulated by hypoxia. As an endogenous inhibitor of VEGF-A, increased sFLT-1 protein neutralizes VEGF-A and prevents heterodimerization with its membrane-bound receptor VEGFR-2, thereby preventing VEGF-A-mediated angiogenesis.[149–151] Several preclinical studies show that increasing levels of sFLT-1 via ocular injection (intravitreal or subretinal) of AAV.sFlt-1 inhibits laser-induced CNV in rodent and non-human primate models.[152–154] These results led to the initiation of a phase I clinical trial (ClinicalTrials.gov: NCT01024998, Sanofi Genzyme) and a phase I/IIa clinical trial (ClinicalTrials.gov: NCT01494805, Avalanche Biotechnologies) in which AAV2-sFLT01 (encodes a fusion protein of the sFLT-01 domain 2 with the Fc domain of immunoglobulin [Ig]G_1) and recombinant AAV (rAAV).sFLT-1 (encodes naturally occurring sFlt-1) were intravitreally and subretinally delivered in patients with nAMD, respectively.

Follow-up results released by Sanofi Genzyme in 2017 showed that the AAV2-sFLT01 vector was not detectable systematically and there was no immunogenicity to the vector. sFLT01 was detectable in aqueous humor in 5 out of 10 patients in the highest dose group (2E10 vector genomes [VG]) within 52 weeks. Expression was dose-related but was variable between participants. It was of interest that four of the five non-expressers of sFLT01 had detectable baseline anti-AAV2 antibodies titers of 1:400 or greater, which provides insight into patient-specific factors that can impact efficacy. While the therapy seemed to be safe and well tolerated at all doses, there was no significant improvement in retinal thickness (or central subfield thickness) and best-corrected visual acuity (BCVA).[139]

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Table 2. Clinical Trials Investigating Human Gene Therapy for Ocular Angiogenesis Diseases Based on Viral Vectors Encoding Antiangiogenic Proteins

Conditions	Identifier: ClinicalTrials.gov	Development Status	Drug	Vector	Mechanism	Route	Company	Study Start Date	Outcome
nAMD	NCT03999801	prospective, observational study: enrolling by invitation	RGX-314	AAV8	encoding for anti-VEGF monoclonal antibody fragment	subretinal	REGENXBIO	May 2019	a long-term follow-up study for RGX-314; no study results posted.
nAMD	NCT03585556	phase I: active, not recruiting	HMR59	AAV2	encoding for sCD59	IVT	Hemera Biosciences	July 2018	evaluated the efficacy and safety of HMR59 with anti-VEGF treatment in nAMD; no study results posted
nAMD	NCT03748784	phase I: recruiting	ADVM-022	AAV2.7m8	encoding for aflibercept	IVT	Adverum Biotechnologies	November 2018	32-week data showed a maintenance of VA with an improvement of retina structural parameters; no rescue injections were needed to control AMD disease activity (S. Kiss, 2019, Amer. Acad. Ophthal., conference; S. Kiss, 2019, Eur. Soc. Gene Cell. Ther., conference)
nAMD	NCT03066258	phase I/IIa: active, not recruiting	RGX-314	AAV8	encoding for anti-VEGF monoclonal antibody fragment	subretinal	REGENXBIO	March 2017	improvement both in VA and retina structural parameters was noted; most patients remained rescue injection-free[135]
nAMD	NCT01678872	phase I: active, not recruiting	RetinoStat	lenti-EIAV	encoding for endostatin and angiostatin	subretinal	Oxford Biomedica	August 2012	a follow-up study to evaluate the safety of RetinoStat; no study results posted
nAMD	NCT01494805	phase I/II: completed	rAAV.sFlt-1	rAAV	encoding for VEGF-neutralizing protein sFLT-1	subretinal	Avalanche Biotechnologies	December 2011	rAAV.sFLT-1 was safe and well tolerated; none of the exploratory endpoints (anti-VEGF retreatment injections, BCVA, and CPT) were statistically significant when rAAV.sFLT-1 was administered alone[136,137]
nAMD	NCT01301443	phase I: completed	RetinoStat	lenti-EIAV	encoding for endostatin and angiostatin	subretinal	Oxford Biomedica	February 2011	EIAV vectors provide a safe platform with robust and sustained transgene expression; no significant change in mean lesion size[138]
nAMD	NCT01024998	phase I: completed	AAV2-sFLT01	AAV2	encoding for VEGF-neutralizing protein sFLT01	IVT	Genzyme (Sanofi)	January 2010	AAV2-sFLT01 was safe and well tolerated; further studies are required to identify sources of variability in expression and anti-permeability activity[139]
nAMD	NCT00109499	phase I: completed	AdGVPEDF.11D	adenovirus	encoding for PEDF	IVT	GenVec	April 2005	no serious adverse events were related to AdGVPEDF.11; a possible dose-escalation response was reported[140]

nAMD, neovascular age-related macular degeneration; AAV, adeno-associated virus; LTFU, long-term follow-up; IVT, intravitreal injection; VA, visual acuity; PEDF, pigment epithelium-derived factor.

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Table 3. Clinical Trials Investigating Human Gene Therapy for Ocular Angiogenesis Diseases Based on Ocular Injection of Non-coding RNA Interference Molecules

Conditions	Identifier: ClinicalTrials.gov	Development Status	Drug	Vector	Mechanism	Route	Company	Study Start Date	Outcome
DME	NCT01445899	phase II: completed	PF-04523655	siRNA	siRNA targeting hypoxia-inducible gene *RTP801*	IVT	Quark	February 2012	evaluated the effect of PF-04523655 with/without ranibizumab in DME; no study results posted
nAMD	NCT00713518	phase II: completed	PF-04523655	siRNA	siRNA targeting hypoxia-inducible gene *RTP801*	IVT	Quark/ Pfizer	November 2009	PF-04523655 monotherapy did not improve AMD as compared with ranibizumab monotherapy; combination of PF-04523655 with ranibizumab provided a synergic therapeutic benefit in visual acuity[141]
nAMD	NCT00557791	phase III: withdrawn	bevasiranib (Cand5)	siRNA	siRNA against *VEGF*	IVT	OPKO Health	November 2009	study not initiated
DME	NCT00701181	phase II: terminated	PF-04523655	siRNA	siRNA targeting hypoxia-inducible gene *RTP801*	IVT	Quark/ Pfizer	June 2008	a dose-dependent improvement in VA was observed in PF-04523655 therapy compared with laser treatment; the study was terminated due to an unexpectedly high discontinuation rate[142]
nAMD	NCT00499590	phase III: terminated	bevasiranib (Cand5)	siRNA	siRNA against *VEGF*	IVT	OPKO Health	August 2007	bevasiranib-ranibizumab combination therapy showed a possible benefit for AMD, but the trial was unlikely to meet its primary endpoint and was terminated
nAMD	NCT00725686	phase I: completed	PF-04523655	siRNA	siRNA targeting hypoxia-inducible gene *RTP801*	IVT	Quark/ Pfizer	February 2007	intravitreal injection with PF-04523655 appeared to be tolerable and safe[143]
nAMD	NCT00395057	phase II: terminated	AGN211745 (Sirna-027)	siRNA	siRNA targeting *VEGFR-1* mRNA	IVT	Allergan	January 2007	failed to meet therapeutic criteria
DME	NCT00306904	phase II: completed	bevasiranib (Cand5)	siRNA	siRNA against *VEGF*	IVT	OPKO Health	January 2006	an improved anatomic outcome and stabilization in VA were observed within 4 months
nAMD	NCT00259753	phase II: completed	bevasiranib (Cand5)	siRNA	siRNA against *VEGF*	IVT	OPKO Health	July 2005	bevasiranib-treated patients showed vision loss and increased lesion size, suggesting an insufficient efficacy with bevasiranib monotherapy
nAMD	NCT00363714	phase I/II: completed	AGN211745 (Sirna-027)	siRNA	siRNA targeting *VEGFR-1* mRNA	IVT	Allergan	November 2004	AGN211745 was well tolerated in patients; stabilization or improvement in visual acuity and retinal structure was observed[144]
nAMD	NCT00722384	phase I: completed	bevasiranib (Cand5)	siRNA	siRNA against *VEGF*	IVT	OPKO Health	August 2004	the safety data of five dosing groups was encouraging

DME, diabetic macular edema; siRNA, small interfering RNA; IVT, intravitreal injection; nAMD, neovascular age-related macular degeneration; VA, visual acuity.

In parallel, a clinical trial sought to assess baseline safety and efficacy of rAAV.sFlt-1 in nAMD patients conducted by Avalanche Biotechnologies (ClinicalTrials.gov: NCT01494805). Similar to AAV2-sFLT01, a subretinal injection of the rAAV.sFlt-1 vector was used to deliver naturally occurring sFlt-1. In phase I and phase IIa clinical trials, 40 participants with nAMD were assigned to low-dose (1E10 VG), high-dose (1E11 VG), or control (no vector, ranibizumab only) groups. During the treatment period, rescue treatments with

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the traditional anti-VEGF drug ranibizumab were given to patients as needed based on vision loss quantified using Early Treatment Diabetic Retinopathy Study (ETDRS) letter scores, increases in intraretinal or subretinal fluid on optical coherence tomography (OCT), and increased leakage on fluorescein angiography. Endpoints including the number of intravitreal anti-VEGF retreatment injections, BCVA, and central point thickness (CPT) were followed during 36 months. The phase I/IIa clinical trial was completed in 2017 with 3-year follow-up results reported in 2019. The authors demonstrated that rAAV.sFLT-1 subretinal gene therapy was safe and tolerable (particularly among the elderly), highly reproducible, and may reduce anti-VEGF drug retreatments for nAMD; however, none of the exploratory endpoints (anti-VEGF retreatment injections, BCVA, and CPT) was statistically significant.[136,155,156]

Based on encouraging results from studies in the laser-induced CNV model in non-human primates,[157] a phase I clinical trial (ClinicalTrials.gov: NCT03748784, Adverum Biotechnologies) assessed the safety and tolerability of ADVM-022 gene therapy for nAMD in 2018. In this study, an AAV2-derived vector, 7m8 (AAV.7m8-aflibercept), was intravitreally injected to produce long-term, robust expression of aflibercept. Other outcome measures included BCVA, anatomic outcomes assessed by OCT, and the needs for rescue aflibercept injections. A novel vector, AAV.7m8, was designed by directed evolution and optimized for intravitreal gene delivery to the outer retina. Eighteen patients with nAMD who were responsive to anti-VEGF treatment were enrolled in this phase I, dose-escalation trial, where three doses of ADVM-022 (2E11, 6E11, and 6E12 VG/eye) were evaluated. The latest data are available on the Adverum Biotechnologies corporate website. No serious adverse events and no drug-related systemic adverse events were noted, and mild inflammatory events responded well to topical steroid drops. The median 44-week data showed general maintenance of BCVA with an improvement in retinal thickness (measured using OCT) in 12 patients injected with 2E11 or 6E11 ADVM-022; most significantly, 10 of 12 (83%) patients did not need rescue injections that help to control AMD disease activity for about 11 months after a single injection of ADVM-022.[158]

Another encouraging anti-VEGF gene therapy approach is RGX-314 (REGENXBIO). A phase I/IIa clinical trial (ClinicalTrials.gov: NCT03066258, REGENXBIO) was started to evaluate the safety and tolerability of RGX-314 gene therapy in nAMD patients previously treated with VEGF injections in 2017. A novel NAV AAV8 vector was subretinally administered to deliver the gene encoding a monoclonal antibody fragment similar to ranibizumab. Forty-two patients were enrolled and divided into five groups with different viral doses (3E9, 1E10, 6E10, 1.6E11, and 2.5E11 gene copies [GC]). Rescue anti-VEGF treatment was given when patients showed increased, new, or persistent fluid, vision loss of five or more ETDRS letters, or occurrence of new ocular hemorrhages. Results released in October 2019 revealed that RGX-314 was well tolerated in all cohorts with no severe adverse effects. RGX-314 protein levels increased in aqueous humor in a dose-dependent manner across the five cohorts. In comparison to a mean sFLT01 protein level of 73.7 ng/mL at week 26 (peak concentration) after treatment with AAV2-sFLT01,[139] RGX-314 protein concentration in cohort 3 (6E10 GC) reached 260.5 ng/mL at 1 year post-injection. The 1.5-year data from cohort 3 showed an improvement both in BCVA (+9 letters) and central retinal thickness (−40 μm thinner) compared with baseline. Approximately 6 months of data from cohorts 4 (1.6E11 GC) and 5 (2.5E11 GC) showed on average a stable improvement in BCVA and central retinal thickness, with 42% and 75% of patients remaining anti-VEGF rescue injection-free, respectively.[135] A longer-term follow-up study for RGX-314 (ClinicalTrials.gov: NCT03999801, REGENXBIO) has recently started. Plans are in place to initiate a phase IIb clinical trial for nAMD and also an investigational new drug (IND) phase II clinical trial for DR.

Endogenous Inhibitor of Angiogenesis

Endostatin and angiostatin, cleavage products of collagen VII and plasminogen, respectively, are endogenous inhibitors of angiogenesis.[159,160] A number of preclinical studies showed that ocular injection of rAAV-endostatin, rAAV-angiostatin, or lentiviral vector (Lenti)-angiostatin is able to efficiently suppress pathological corneal or retinal angiogenesis.[161–163] Subretinal injection of an EIAV (VSV-G pseudotyped equine infectious anemia virus) lentiviral vector encoding angiostatin and/or endostatin significantly reduced lesion size in a mouse model of laser-induced CNV.[164,165] These results led to a phase I clinical study (ClinicalTrials.gov: NCT01301443, Oxford Biomedica) to assess the dose-escalation safety of RetinoStat in patients with advanced AMD, with a long-term follow-up cohort (ClinicalTrials.gov: NCT01678872) initiated in 2012. In these studies, the non-replicating bicistronic EIAV vector encoding both endostatin and angiostatin was subretinally injected to encode both endostatin and angiostatin in 21 participants with advanced nAMD (three cohorts of 2.4E4, 2.4E5, and 8E5 transduction units [TU]). There were no detectable levels of endostatin at baseline in aqueous humor samples from participants. Long-term and stable expression of angiostatin and endostatin were noted for eight subjects after 2.5 years, and two subjects showed stable expression for more than 4 years. At completion, the results suggest that this EIAV lentivector-based gene therapy was safe and well tolerated and that it was clinically feasible to use a cytomegalovirus (CMV) promoter to express two secreted proteins. However, the data failed to show therapeutic benefit for advanced nAMD, although there was a reduction in fluorescein angiographic leakage.[138]

Targeting the Complement Cascade

Recent evidence implicates an overactivation of the complement cascade in the development of AMD. Activation of the complement cascade leading to membrane attack complex (MAC) accumulation on cell surfaces leads to cell damage and death, causing the clinical features seen in AMD (reviewed in Anderson et al.[166]). Accumulation of MAC can be found in RPE and choriocapillaris of AMD patients, suggesting a possible role for MAC in the pathogenesis of geographic atrophy or CNV (reviewed in Kumar-Singh[167]). CD59 is a naturally occurring membrane-bound inhibitor of MAC formation.[168] CD59

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targets the final step of activation of complement by preventing MAC formation in the cell membrane. In order to enable CD59 to block the formation of MAC at sites distal, a soluble non-membrane binding form of CD59 (sCD59)[169,170] has been developed and used for targeted gene therapy. Two phase I clinical trials sponsored by Hemera Biosciences (ClinicalTrials.gov: NCT03144999 and NCT03585556) were initiated in 2017 and 2018 to investigate the efficacy and safety of AAVCAGsCD59 (HMR59) gene therapy for patients with dAMD and nAMD, respectively; however, the results have not been released.

RNA Interference in Ocular Angiogenesis

Synthetic siRNA is usually 19–22 bp long and can selectively complex with complementary mRNA through the RNA-induced silencing complex.[171,172] Post-transcriptional gene silencing with siRNAs targeting VEGF and its receptor have demonstrated success in reducing ocular angiogenesis in experimental models of nAMD,[173–175] DR,[176] and corneal neovascularization.[176,177] These developments have led to clinical trials for bevasiranib (formerly Cand5) (ClinicalTrials.gov: NCT00722384, OPKO Health) and AGN 211745 (formerly Sirna 027) (ClinicalTrials.gov: NCT00363714, Allergan).

Bevasiranib, a complex of two 21-nt RNA molecules that selectively silence mRNA encoding VEGF, was the first siRNA protocol granted IND status and tested in a clinical trial for treating nAMD via intravenous administration.[178] The safety data for five dosing groups (0.1, 0.33, 1, 1.5, and 3 mg) in a phase I clinical trial (ClinicalTrials.gov: NCT00722384) was encouraging.[179] However, in the phase II study (ClinicalTrials.gov: NCT00259753) comparing three doses (0.2, 1.5, or 3.0 mg) in patients with CNV secondary to AMD, efficacy could not be shown with bevasiranib monotherapy treatment. A phase II study (ClinicalTrials.gov: NCT00306904) evaluating bevasiranib's safety and efficacy in patients with DME reported improved anatomic outcomes between weeks 8 and 12, with 91% of patients showing stabilization of BCVA through 6–12 weeks. The detailed results of these three clinical trials have not yet been published.[180–183] As bevasiranib may only inhibit new VEGF synthesis, without impacting existing VEGF levels, a phase III trial (ClinicalTrials.gov: NCT00499590) was performed to assess the efficacy of combining bevasiranib and ranibizumab therapy for nAMD treatment. Although bevasiranib-ranibizumab combination therapy showed a possible benefit in AMD treatment, the trial was unlikely to meet its primary endpoint and thus was terminated.

A subsequent phase III trial (ClinicalTrials.gov: NCT00557791) evaluating the efficacy of combination bevasiranib and ranibizumab treatment in nAMD was withdrawn prior to enrolment, following the discovery that angiogenesis suppression by siRNA agents such as bevasiranib could be acting through non-specific activation of Toll-like receptor (TLR)3 rather than the presumed sequence-dependent property of the siRNA.[184,185] Specifically, Kleinman et al.[184] demonstrated that suppression of CNV was only observed in $Tlr3^{+/+}$ but not $Tlr3^{-/-}$ mice after intravitreal injection of anti-VEGF siRNA, suggesting the involvement of TLR3 in CNV. Subsequent studies also confirmed that regardless of their targeting sequences, 21-nt siRNAs can inhibit experimental CNV.[186,187] Retinal toxicity remains a concern, as 21-nt siRNAs were found to induce apoptosis in mouse retinal pigmented epithelial cells through TLR3 and interferon regulatory factor 3.[188]

AGN211745 is a 21-nt RNA duplex designed to target FLT1 (VEGFR1) mRNA. Despite initial positive reports from a phase I trial (ClinicalTrials.gov: NCT00363714),[144] the phase II trial (ClinicalTrials.gov: NCT00395057) for treatment of CNV associated with AMD was halted due to failure to meet therapeutic criteria and uncertainty around possible widespread involvement of TLR3. To avoid TLR3 activation and thus enhance therapeutic specificity,[189] PF-04523655 (developed by Quark Pharmaceuticals, licensed to Pfizer) targeting the hypoxia-inducible *RTP801* gene (also known as *DDIT4*) was designed as a 19-nt siRNA with 2′-*O*-methylation in every pair of oligonucleotides and was successfully tested clinically in CNV or DME with/without ranibizumab. The phase I trial (ClinicalTrials.gov: NCT00725686) reported that intravitreal injection with PF-04523655 ($\leq$3,000 μg) was tolerable and safe in patients with nAMD.[143] The phase II MONET study (ClinicalTrials.gov: NCT00713518) demonstrated that PF-04523655 monotherapy did not improve visual acuity as compared with ranibizumab monotherapy, but there was the suggestion of a synergetic therapeutic benefit for visual acuity when PF-04523655 was combined with ranibizumab.[141] Concurrently, the phase II DEGAS study compared PF-04523655 with laser photocoagulation therapy in patients with DME (ClinicalTrials.gov: NCT00701181). Improvement in visual acuity was dose related and was more evident in PF-04523655 than in the laser therapy group; however, the study was terminated due to an unexpected high discontinuation rate.[142] The latest MATISSE study (ClinicalTrials.gov: NCT01445899) assesses the effect of PF-04523655 with/without ranibizumab in DME, with detailed results not yet made public.

Although siRNA-based therapies showed some benefits for patients with nAMD and DME, this approach has no advantage over conventional anti-VEGF treatments since repeated injections are still needed to deliver the siRNA. Furthermore, the gene-silencing effect of siRNAs is transient, typically effective only for 3–7 days, as siRNAs are degraded by tissue nucleases. Nevertheless, prolonged effects could be achieved by chemical modifications or using viral vectors to sustain the effectiveness of RNA interference-based therapies.[182] Short hairpin RNAs (shRNAs), structurally similar to cellular miRNAs, are processed to generate siRNAs.[190] A number of studies have demonstrated that subretinal administration of viral vectors encoding VEGF-targeting shRNAs can reduce CNV lesions in VEGF-induced or laser-injured CNV mouse models.[191,192] Recently, Kaadt et al.[193] demonstrated a potentially safer and more efficient strategy for gene knockdown by Dicer-independent shRNAs expressed from miRNA scaffolds, which produce no passenger strand activity and remain active in Dicer-knockout cells. Given encouraging results from clinical studies of dual therapy using siRNA and ranibizumab,[24,183,141] there has been interest in investigating the efficacy of combination therapy. Askou et al.[194,195] have developed multigenic

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lentiviral vectors, enabling the cell- or tissue-specific simultaneously expression of anti-VEGF miRNAs and antiangiogenic factors. Later, the authors proved that dual-acting therapy using AAV encoded for anti-VEGF miRNAs and secreted PEDF protects against CNV *in vivo*.[196] As multiple dysregulated pathways are involved in ocular angiogenesis and pathogenesis,[24,183] an ideal drug candidate might combine antiangiogenic, anti-inflammatory, or neuroprotective genes. Given the feasibility of loading multiple transgenes into one vector, such a combined approach may be an important future advance.

Preclinical Studies of CRISPR-Cas Gene Editing to Treat Ocular Angiogenesis

Currently, more than 20 clinical trials are underway to test the therapeutic effects of CRISPR-Cas systems in diseases, including cancers and thalassemia, or to develop this approach as a diagnostic tool for tuberculosis and sepsis (reviewed in ClinicalTrials.gov). Of particular note in the field of genome editing for eye diseases, a phase I/II clinical trial has been approved by the FDA (ClinicalTrials.gov: NCT03872479, Allergan/Editas Medicine) to assess AGN-151587 (EDIT-101), a CRISPR-Cas-based genome editing approach for the treatment of Leber's congenital amaurosis type 10 (the most common form of inherited childhood blindness).[197] In addition to those inherited retinal diseases caused by specific gene mutations,[132,198] numerous preclinical studies had investigated CRISPR-Cas applications for complex retinal diseases with multiple risk factors such as AMD (Table 4).

CRISPR-SpCas9 System

Streptococcus pyogenes Cas9 (SpCas9) was first applied in human cells and has become one of the most commonly used Cas endonuclease variants.[124] Huang et al.[201] adapted CRISPR-Cas9-mediated disruption of *VEGFR2* in both the mouse model of oxygen-induced retinopathy (OIR) mice and laser-induced CNV. The authors first tested the rAAV1-CRISPR-SpCas9 systems to target human *VEGFR2* in C57BL/6 mouse primary brain microvascular endothelial cells (MVECs) using a dual AAV vector system. An 80% decrease in VEGFR2 production in CRISPR-SpCas9-edited MVECs was detected by western blot. Equal amounts of rAAV1-SpCas9 and rAAV1-single guide RNA (sgRNA) targeting *VEGFR2* were intravitreally administered in OIR or laser-induced CNV mice. In CRISPR-edited OIR mice retina, there was approximately a 30% reduction in VEGFR2 production with significant inhibition of retinal neovascularization. Similarly, CRISPR-SpCas9-mediated disruption of VEGFR2 reduced CNV size compared with vehicle control laser-induced CNV mice. The authors speculate that rAAV1 preferentially transduces pathological vascular endothelial cells over healthy cells, as a way to account for the limited efficacy of *in vivo* VEGFR2 disruption compared with its effect *in vitro*. Holmgaard et al.[202] examined *in vivo* knockout of the *VEGF* gene by lentiviral delivery of SpCas9 with sgRNA targeting exon 3 of murine *VEGF-A*. Five weeks after subretinal administration of the lentivirus in mouse eyes, robust expression of sgRNA with SpCas9 and successful indel formation in the *VEGF-A* gene were found specifically in the RPE cell layer.

Smaller CRISPR-Cas System

AAV-mediated delivery of CRISPR-Cas appears to be a safer, more efficient, and precise tool for retinal genome editing.[206] However, the relatively small packaging capacity (<4.7 kb) of AAVs means that the most frequently used SpCas9 gene (~4.10 kb)[207] and sgRNA sequences cannot be packaged into the same AAV vector.[208] As compared with AAV, lentiviral vectors have larger payload capacity (8–10 kb) but limited ability to transduce outer retina,[209] whereas EIAV-based lentiviral vector was found to efficiently transduce both RPE and photoreceptors in the macaque.[210] In addition, dual-vector AAV systems,[201,211] AAV-split-Cas9 systems,[212,213] smaller Cas9 orthologs (such as *Staphylococcus aureus* [SaCas9; 3.16 kb],[214] *Campylobacter jejuni* [CjCas9; 2.95 kb],[203] *Neisseria meningitidis* [NmCas9; 3.25 kb],[203] and *Streptococcus thermophilus* [StCas9][215]), or other class 2, type V nucleases such as Cpf1 (also known as Cas12a) from *Acidaminococcus* sp. (AsCpf1) or *Lachnospiraceae bacterium* (LbCpf1)[134,216] have been developed. Despite successful gene knockout using dual-vector AAV systems, the need for co-transduction could be a drawback for their application.[202] For example, the co-delivery of two AAV vectors has been reported to be less efficient than delivery by a single AAV vector *in vivo*.[203,217] Alternatively, a split SpCas9 approach is less active compared with the delivery of intact SpCas9.[212,218] Other Cas endonucleases, such as NmCas9 and StCas9, have received less attention, as their longer protospacer-adjacent motif (PAM) regions (5′-NNNNGATT-3′ and 5′-NNAGAAW-3′, respectively) limit sequences available for targeting.[208]

Kim et al.[203] employed intravitreal injections of AAV9 vector encoding for CjCas9 and sgRNA targeting *VEGF-A* or *HIF-1a* genes in a laser-induced CNV mouse model. They show that partial knockdown of either *VEGF-A* or *HIF-1α* in the mouse retina, particularly in RPE cells, suppressed CNV by approximately 20% compared with the control group. Of note, cone dysfunction was observed in the *VEGF-A*-edited group but not in the *HIF-1α*-edited group. This might suggest that *HIF-1α* is a safer therapeutic target for CNV. The authors reported that there were no detectable off-target indels 6 weeks after AAV9-CjCas9 injection.[203] Later, the authors reported that 14 months after gene editing with AAV9-CjCas9, *HIF-1α* indels reached frequencies of 79% ± 2%. Importantly, after 14 months, there were no indels at potential off-target sites, and the treatment did not affect retinal function or histology. These results indicated that a single intravitreal injection of AAV9-CjCas9 could induce long-term gene editing without significant genotoxic risk, supporting the contention that AAV-CRISPR-CjCas9-mediated gene therapy is long-lasting, effective, and safe in eyes.[204] The same team also considered whether LbCpf1-based gene surgery could be used in the treatment of CNV. Following laser-induced CNV in the mouse retina, intravitreal injection of AAV2/9-expressing *VEGF-A* or *HIF-1a*-specific LbCpf1 reduced CNV area by 42% ± 4% and 34% ± 5%, respectively. The antiangiogenic effects of CRISPR-LbCpf1 were comparable to an aflibercept-injected group and more effective than the aforementioned CjCas9-based gene therapy.[134,203] In addition, no deleterious effects on

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Table 4. Preclinical Genome Surgery with CRISPR-Cas to Treat Ocular Angiogenesis

Target Gene	Experiment		Specific Nuclease	Delivery Method	Outcome	References
	In Vivo	*In Vitro*				
VEGF-A		human ARPE-19 cell line	SpCas9	lentivirus (single vector)	CRISPR-Cas9 reduced VEGF-A secretion from human RPE cells and suppressed angiogenesis	199
VEGFR2		primary human retinal microvascular endothelial cell (HREC)	SpCas9	rAAV5 (dual vector)	CRISPR-Cas9-mediated depletion of *VEGFR2* prevented VEGF-induced Akt activation, as well as proliferation, migration, and tube formation in HRECs	131
VEGFR2		primary human retinal microvascular endothelial cell (HREC)	SpCas9	lentivirus (single vector)	CRISPR-Cas9 decreased VEGF-induced Akt activation, as well as proliferation, tube formation, and migration in *VEGFR2*-depleted HRECs, and did so to a greater extent than ranibizumab and aflibercept	200
VEGFR2	oxygen-induced retinopathy and laser-induced CNV	C57BL/6 mouse primary brain microvascular endothelial cell (MVEC)	SpCas9	rAAV1 (dual vector)	CRISPR-Cas9-edited MVECs significantly reduced VEGFR2 production *in vitro*; intravitreal administration of CRISPR-Cas9 targeting *VEGFR2* reduced CNV area in both animal models	201
VEGF-A	non-disease mouse		SpCas9	lentivirus (single vector)	*in vivo* subretinal administration of CRISPR-Cas9 disrupted *VEGF-A* gene specifically in mouse RPE	202
VEGF-A and *HIF-1α*	mouse mode of laser-induced CNV		CjCas9	AAV9 (single vector)	intravitreal injection of CRISPR-Cas9 targeting either *VEGF-A* or *HIF-1α* suppressed CNV, particular in the RPE; cone dysfunction was observed in the *VEGF-A*-edited but not in the *HIF-1α*-edited group; no detectable off-target indels were noted 6 weeks after AAV9-CjCas9 injection	203
HIF-1α	mouse mode of laser-induced CNV		CjCas9	AAV9 (single vector)	intravitreal injection of AAV9-CjCas9 targeting *HIF-1α* induced long-term gene disruption by constitutive expression of CjCas9 nuclease without detectable off-target indels 14 months after injection; the treatment did not affect retina function or histology	204
VEGF-A and *HIF-1α*	mouse mode of laser-induced CNV		LbCpf1	AAV2/9 (single vector)	intravitreal injection of AAV2/9-LbCpf1 targeting either *VEGF-A* or *HIF-1α* reduced CNV area and did not affect cone function; the antiangiogenic effects of CRISPR-LbCpf1 were comparable to the aflibercept group	134
VEGF-A	mouse mode of laser-induced CNV	human ARPE-19 cell line	SpCas9	Cas9 ribonucleoproteins (RNPs)	Cas9 RNP reduced the VEGFA mRNA and protein levels in ARPE-19 *in vitro*; subretinal injection of Cas9 RNPs induces indels specific in RPE cells, resulting in the reduction of VEGF-A protein and CNV; Cas9 protein was completely degraded 3 days post-injection, and no cone dysfunction was observed 7 days post-injection	205

CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; VEGF, vascular endothelial growth factor; rAAV, recombinant adeno-associated virus; CNV, choroidal neovascularization; NV, neovascularization; RPE, retinal pigment epithelium; HIF, hypoxia-induced factor.

cones were observed either with *VEGF-A*- or *HIF-1a*-specific LbCpf1-edited groups, providing further support for CRISPR-LbCpf1 as a safer therapeutic approach. Cpf1 has the advantage of higher genome-wide specificity in human cells with minimum off-target potential compared with SpCas9, as well as the capacity for streamlined multiplex genome editing.[219,220] These results support the potential application of LbCpf1-based gene editing for pathologic angiogenesis diseases in the eye.

CRISPR-Cas Ribonucleoprotein

Significant challenges for the field remain, namely viral vector-evoked host immune responses and constitutively expressed Cas9-related off-target events.[208,221–223] Kim et al.[205] thus tested a non-viral approach by delivering preassembled *VEGF-A*-specific Cas9 ribonucleoproteins (RNPs) in human RPE cells and a mouse model of CNV. The recombinant Cas9 protein complexed with sgRNA targeting *VEGF-A* was delivered via transfection using cationic lipids. The subretinal

injection of RNPs induced indels specifically in RPE cells near the injection site, resulting in a significant reduction of VEGF-A protein levels and CNV area. The Cas9 protein was completely degraded 3 days after subretinal injection, and no cone dysfunction was observed 7 days post-injection. The rapid turnover of Cas9 RNP is less likely to elicit host immune responses and to produce off-target effects. Also, note that large amounts of recombinant Cas9 (8 μg) and sgRNA (4.5 μg) were used in the RNPs, but the authors had previously shown that RNP delivery was safer than plasmid delivery in human embryonic cells.[224] These results highlight the possibilities of locally modifying *VEGF-A* genes using Cas9 RNPs.

Improving the Safety of the CRISPR-Cas System

It is clear that therapeutic applications of CRISPR-Cas in ocular angiogenesis are an active area of research, particularly to limit off-target effects, which is a significant concern to scientists and clinicians. Higher-fidelity CRISPR-Cas9 nucleases such as SpCas9-HF1, HypaCas9, and eSpCas9 have been developed to address these issues.[225–227] Moreover, AAV-based CRISPR-Cas delivery system raises concern around the effect of persistent *in vivo* expression of the bacterially derived Cas enzyme. Systems including anti-CRISPR proteins, inducible promoters, and split-intein Cas9 are being developed to modulate Cas9 cleavage activity (not remove the Cas9 protein).[212,228,229] Other approaches involving lentiviral vector-based KamiCas9 and lentiSLiCES, as well as a self-deleting AAV-CRISPR-Cas system, have been exploited to remove the Cas protein to circumvent problems related to long-term Cas expression.[230–232]

Conclusion

There has been a rapid expansion of efforts to explore the potential benefits of ocular gene therapy. Current clinical results suggest that viral vector-mediated gene augmentation holds the potential to provide long-lasting therapeutic benefits for patients with nAMD or DME. This stands to diminish the a need for repeated intraocular injections. Although post-transcriptional gene silencing with RNA interference has also shown some promise as an adjunct to anti-VEGF therapies, there is need for further study. CRISPR-Cas-based gene editing may be on the cusp of clinical application for ocular neovascularization. Further developments in vector design, genetic material modification, and delivery strategies are needed to improve the safety, specificity, and therapeutic efficacy of gene transfer. Reliable tools for assessment of *in vivo* genotoxicity risks such as insertional mutagenesis or off-target activity are also needed to monitor the biosafety of ocular gene therapy. Furthermore, the measurement of treatment outcomes should also be a key consideration to assess and monitor the efficacy and safety of gene therapy in clinical trials. With continued development in both preclinical and clinical settings, gene therapy may be a viable alternative approach for the treatment of ocular neovascularization.

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization: F.-L.L. and G.-S.L. Writing – Original Draft: F.-L.L. and G.-S.L. Writing – Review & Editing: P.-Y.W., J.-H.W., B.V.B., Y.-F.C., and V.H.Y.W. Funding Acquisition: P.-Y.W. and G.-S.L.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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