Targeting vascular endothelial growth factor using retinal gene therapy

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Contributions: (I) Conception and design: SH Chung, G Yiu; (II) Administrative support: G Yiu; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: SH Chung, SL Frick; (V) Data analysis and interpretation: SH Chung, G Yiu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Pharmacotherapies targeting vascular endothelial growth factor (VEGF) have revolutionized the management for neovascular retinal disorders including diabetic retinopathy and neovascular age-related macular degeneration. However, the burden of frequent injections, high cost, and treatment resistance in some patients remain unresolved. To overcome these challenges, newer generations of anti-angiogenic biological therapies, engineered proteins, implantable delivery systems, and biopolymers are currently being developed to enable more sustained, longer-lasting treatments. The use of gene therapies for pathologic angiogenesis has garnered renewed interests since the first FDA-approval of a gene therapy to treat inherited retinal diseases associated with biallelic *RPE65* mutations. Newer generations of viral vectors and novel methods of intraocular injections helped overcome ocular barriers, improving the efficiency of transduction as well as safety profile. In addition, unlike current anti-VEGF gene therapy strategies which employ a biofactory approach to mimic existing pharmacotherapies, novel genome editing strategies that target pro-angiogenic factors at the DNA level offer a unique and distinct mechanistic approach that can potentially be more precise and lead to a permanent cure. Here, we review current anti-VEGF therapies and newer pharmacologic agents under development, examine technologies and progress in adapting anti-VEGF gene therapies, and explore the future application of CRISPR-Cas9 technology to suppress ocular angiogenesis.

Keywords: Angiogenesis; vascular endothelial growth factor (VEGF); gene therapy; neovascular age-related macular degeneration (nAMD); neovascularization

Vascular endothelial growth factor (VEGF) is a proangiogenic cytokine that has been implicated in a variety of retinal disorders including diabetic retinopathy, retinal vein occlusion (RVO), and neovascular age-related macular degeneration (nAMD). It is encoded in 8 exons located on chromosome 6, and its functions include stimulating endothelial cell migration, proliferation and tube formation, all of which lead to neovascularization in the eye (1). The VEGF family consists of 7 members (VEGFa–VEGFe and placental growth factor or PGF), among which the secreted isoforms of VEGFa (VEGF121 and VEGF165) are the most potent factors associated with pathologic angiogenesis (1,2). VEGF is primarily an endothelial cell mitogen, but may also be secreted by retinal pigmented epithelium (RPE), Muller glia, and astrocytes (3-6). Early success in pre-clinical studies using intravitreal injections of anti-VEGF antibody in the 1990s led to clinical trials and subsequent approval for human use, enabling anti-VEGF therapies to become the standard of care for various ocular angiogenic disorders (7-10). The challenges with current anti-VEGF pharmacotherapies, however, include short durability requiring frequent injections, limited efficacy in some patients, as well as infection risk and high costs of multiple treatments. In this review, we review current anti-

Table 1 Summary of Anti-VEGF drugs

Generic name	Bevacizumab	Ranibizumab	Aflibercept	Brolucizumab
Trade name	Avastin	Lucentis	Eylea	Beovu
Structure	Full length humanized monoclonal antibody	Fragmented humanized monoclonal antibody	Fusion protein containing domains from VEGFR-1 and VEGFR-2	Humanized single-chain antibody fragment
Molecular mass (kDa)	149	48	115	26
Mechanism of action	Binds all isoforms of VEGF-A	Binds all isoforms VEGF-A	Binds all isoforms of VEGF-A, VEGF-B, and PIGF	Binds all isoforms of VEGF-A
Clinical development status	Off-label use; not FDA approved for ophthalmic use	FDA approval for nAMD [2006], DME [2012], mCNV [2017], DR [2017]	FDA approval for nAMD [2011], DME [2014], DR [2019]	FDA approval for nAMD [2019]
Ocular half-life in humans (days)	4.9	7.19	11	4.3

VEGF, vascular endothelial growth factor; PIGF, placental growth factor; nAMD, neovascular age related macular degeneration; DME, diabetic macular edema; mCNV, myopic choroidal neovascularization; DR, diabetic retinopathy.

VEGF treatments and existing strategies to improve drug efficacy and durability, then discuss the use of gene therapy and potential applications of genome editing technology for treatment of neovascular retinal conditions.

Current anti-angiogenic therapies for retinal diseases

Prior to the advent of pharmacologic interventions, the primary mode of inhibiting retinal angiogenesis was with thermal laser. Eyes with proliferative diabetic retinopathy could be treated with pan retinal photocoagulation, while choroidal neovascularization (CNV) in nAMD could be treated with laser ablation if the fovea were spared, then later with photodynamic therapy (PDT) using a porphyrin-based photosensitizer (verteporfin) if the fovea were involved. These destructive laser treatments were designed to halt the disease progression but can cause permanent damage of target retina and subsequent vision loss. In the early 2000s, intravitreal injections of agents targeting VEGF, known previously as "factor X", led to a fundamental paradigm shift in the management of neovascular retinal conditions, enabling regression of aberrant neovessels. Above, we summarize the current generation of anti-VEGF pharmacotherapies (*Table 1*).

Bevacizumab

Bevacizumab is a humanized full-length VEGF monoclonal antibody (149 kDa) that binds to all isoforms of VEGFa (11). Initially approved for metastatic colon cancer, bevacizumab given systemically demonstrated benefit in patients with nAMD, enabling not only a reduction in CNV and macular thickness, but also significant improvement in visual acuity (12). When given intravitreally off-label, the treatment continued to show significant anatomic and functional benefits while avoiding systemic side effects (13). Although it never received approval by the Food and Drug Administration (FDA), bevacizumab remains one of the most commonly used treatments due to its clinical benefits and lower cost compared to approved therapies. A randomized controlled trial directly comparing 1.25 mg bevacizumab with the FDA-approved ranibizumab, which is many times more expensive, demonstrated similar benefits, supporting the use of bevacizumab as first-line therapy in most clinical scenarios (14).

Ranibizumab

Ranibizumab is a humanized monoclonal antibody fragment similar to the binding (Fab) region of bevacizumab that was developed specifically for intraocular use (15). The smaller

size of ranibizumab (48 kDa) compared to bevacizumab (149 kDa) was designed to enable better penetration through the neurosensory retina and reduce systemic exposure or potential adverse effects, making it safer for ophthalmic use (16). Its efficacy was demonstrated in two phase III clinical trials (MARINA, NCT02147067 and ANCHOR, NCT00061594) which demonstrated efficacy for treating different types of CNV in nAMD as compared with observation or PDT (17,18), resulting in its approval for nAMD in 2006. The drug was subsequently found to benefit eyes with RVO-related macular edema in the BRAVO (NCT00486018) and CRUISE (NCT00485836) studies (19,20), and eyes with diabetic macular edema in the RIDE (NCT00473382) and RISE (NCT00473330) trials (21), resulting in its approval for these two conditions in 2010 and 2012, respectively. Due to small potential safety concern in cardiovascular endpoints, ranibizumab was approved at 0.3 mg for diabetic patients, compared to the 0.5 mg dose for nAMD and RVO patients. More recently, ranibizumab has also been approved for the treatment of myopic CNV as well as diabetic retinopathy in 2017.

Aflibercept

Aflibercept is a recombinant chimeric fusion protein that consists of key domains of human VEGF receptors 1 and 2 with the constant region (Fc) of human IgG (14,22). It functions as a decoy receptor that can bind to VEGFa and PGF and hinder their functions (23). Its higher binding affinity and longer half-life in the vitreous was believed to improve the efficacy and durability of the drug (24). The phase III VIEW 1 and 2 trials (NCT00509795 and NCT00637377) demonstrated non-inferiority of aflibercept given every 8 weeks as compared to ranibizumab given monthly for nAMD, although an equivalent head-to-head comparison was not conducted (22,25). Based on this data, 2-mg aflibercept was FDA-approved for nAMD in 2011. Subsequent completion of the COPERNICUS (NCT00943072), GALILEO (NCT01012973), and VIBRANT (NCT01521559) trials, as well as the VIVID (NCT01331681) and VISTA (NCT02299336) studies (26-29), led to the drug's approval for RVO-related and diabetic macular edema in 2014. Following ranibizumab, aflibercept was also later approved for diabetic retinopathy in 2019. Despite the presumed greater efficacy and durability of aflibercept, long-term studies have not shown a difference in visual benefits (30).

Brolucizumab

Brolucizumab is a single-chain antibody fragment targeting VEGFa, which due to its much smaller size (23 kDa), allows a much higher dose to be delivered intravitreally (31,32). A comparison between brolucizumab and other anti-VEGF drugs suggests that it has similar or superior binding affinity to human VEGF (33), and showed early promise in phase II studies (34,35). The HAWK (NCT02307682) and HARRIER (NCT02434328) phase III trials found that over 50% of the patients that received intravitreal injections of 6-mg brolucizumab were able to maintain 3-month intervals for additional treatment through week 48 suggesting improved durability, resulting in the drug's approval by the FDA in 2019. Some reports of an obstructive vasculitis distinct from the mild intraocular inflammation typically seen in early deployment of other anti-VEGF therapies have been reported after brolucizumab treatment, raising some concerns (36). Further studies are required to confirm the long-term safety of this treatment.

Emerging anti-angiogenic therapies

Despite the successes of current anti-VEGF therapies, their limited durability and treatment resistance in subsets of patients remain areas of unmet needs. Frequent injections increase the risk of endophthalmitis, retinal detachment, elevated ocular pressure, and vitreous hemorrhage (37). In particular, endophthalmitis is associated with poor visual prognosis (38,39). Thus, new technologies aimed at greater efficacy and durability are under development, as summarized below (Table 2).

Port delivery system (PDS)

The PDS is a refillable, non-biodegradable implant that enables continuous delivery of drug to the vitreous (40). It can be permanently inserted via a small incision through the sclera with a self-sealing valve, and releases drugs into the vitreous through passive diffusion. A phase II clinical trial delivering ranibizumab through the PDS demonstrated safety and a median refill time of 15 months (NCT02510794), suggesting that sustained VEGF suppression may be therapeutic at lower doses than the pulsatile dosing from multiple intravitreal injections (40). Although a high rate of vitreous hemorrhage at the incision site was noted in earlier stages of the trial, the surgical procedure has been optimized and hemorrhage occurrence

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Table 2 Anti-angiogenic therapies for increased durability

Delivery method	Studies/clinical trials	Study target	Anti-VEGF agents used	Duration/results	References
Port delivery system (PDS)	Phase II clinical trial	Patients with nAMD	Ranibizumab	Average 15-month refill time	(40)
	Phase III clinical trial	Patients with nAMD	Ranibizumab	Currently under trial	NCT03677934
Designed ankyrin repeat proteins (DARPins)	In vitro	HUVECs	Abicipar pegol (abicipar)	Binding affinity (K_d) of 394 fM for human VEGF-A$_{165}$, 386 fM for rab VEGF-A$_{164}$, and 8.49 pM for rabbit VEGF-A$_{165}$	(41)
	In vivo	Mouse model of chronic RNV	Abicipar pegol (abicipar)	84% suppression of vessel growth	(41)
	In vivo	Rabbit model of chronic RNV	Abicipar pegol (abicipar)	Suppressed angiogenesis 2 weeks longer than ranibizumab	(41)
	Phase II clinical trial (REACH)	Patients with nAMD	Abicipar pegol (abicipar)	Abicipar pegol showed longer durability effect than ranibizumab in BCVA and CRT measurements	(42)
	Phase III clinical trials (SEQUOIA, CEDAR)	Patients with nAMD	Abicipar pegol (abicipar)	Abicipar (2 mg) delivered every 8 weeks or every 12 weeks showed non-inferior results compared to ranibizumab (0.5 mg) delivered every 4 weeks	(43)
Thermosensitive hydrogel	In vitro	ARPE-19	Bevacizumab	Bevacizumab was released at a constant rate for 11 days with no cytotoxicity	(44)
	In vitro	–	Ranibizumab	Controlled release of ranibizumab for 6 months	(45)
	In vitro	HUVECs	Aflibercept	Controlled release of aflibercept for 6 months with no cytotoxicity	(46)
	In vivo	Nonhuman primate model	Aflibercept	Sustained aflibercept level was detectable for 6 months post injection	(47)
Micro- and nanoparticles	Phase III clinical trials	Patients with DME	Dexamethasone (DEX implant)	Patients with DEX showed ≥15-letter improvement in BCVA and −107.9 to −111.6 µm reduction in CRT	(48–50)
	Phase IIb clinical trial (ALTISSIMO)	Patients with nAMD	Anti-VEGF sunitinib malate (GB-102)	Currently under trial	NCT03953079
	In vivo	Rat model of CNV	Bevacizumab	Reduced 2 neovascularization with nanoparticles	(51)

nAMD, neovascular age-related macular degeneration; HUBECs, human umbilical vein endothelial cells; RNV, retinal neovascularization; DME, diabetic macular edema; CNV, corneal neovascularization; VEGF, vascular endothelial growth factor; BVCA, best-corrected visual acuity; CRT, central retinal thickness.

has decreased (40). A phase III trial assessing its efficacy and pharmacokinetics is currently underway in patients with nAMD (NCT03677934).

Designed ankyrin repeat proteins (DARPins)

DARPins are genetically engineered proteins that mimic antibodies with equal or superior binding affinity and specificity (52). They are smaller in size, enabling better penetration with a longer half-life (>13 days) than ranibizumab (7.2 days) (53-55). For ophthalmic applications, abicipar pegol, an anti-VEGF DARPin, demonstrated effective suppression of vascular leak and neovascularization in animal models of corneal neovascularization and retinal vasculopathy (41). Phase III clinical trials (SEQUOIA (NCT02462486) and CEDAR (NCT02462928)] evaluating abicipar pegol given every 2–3 months in nAMD patients demonstrated non-inferior in improving visual acuity compared with monthly treatment of ranibizumab (41-43).

Thermosensitive hydrogel

Hydrogels are three-dimensional networks of hydrophilic polymers that can hold large quantity of water content similar to natural tissue (56,57). Thermosensitive hydrogels are liquid at room temperature, but becomes solid at body temperature, allowing the drug-carrying polymer to be injected intravitreally, but serve as a reservoir for slow, sustained drug delivery inside the eye (58,59). For ophthalmic use, Wang *et al*. evaluated a biocompatible material composed of an amphiphilic triblock of copolymer of poly(2-ethyl-2-oxazoline)-b-poly(ε-caprolactone)-b-poly(2-ethyl-2-oxazoline) (PEOz-PCL-PEOz) and demonstrated extended release of bevacizumab with no significant toxicity in the rabbit retina (44). More recently, hydrogels also demonstrated controlled release of ranibizumab and aflibercept *in vitro* (45,46), and when injected intravitreally showed sustained release of aflibercept over 6 months in nonhuman primates without significant adverse effects (47).

Micro- and nanoparticles

Biodegradable microparticles and nanoparticles also hold great potential for sustained delivery of drugs. The use of engineered polymeric microparticles of poly lactic-co-glycolic acid (PLGA) was previously approved by the FDA for inflammatory diseases. For ocular delivery, the microparticles are designed to aggregate upon exposure to vitreous fluid to form a depot at the bottom of the eye to gradually release the loaded compound. The PLGA-based intravitreal implant for dexamethasone has been used successfully in patients with diabetic macular edema with good efficacy (Ozurdex, AbbVie-Allergan) (48-50). The ongoing ALTISSIMO phase IIb trial (NCT03953079) is designed to evaluate PLGA microparticles carrying the anti-VEGF sunitinib malate (GB-102) in eyes with diabetic macular edema. Similar to microparticles, nanoparticles are also synthetic polymeric drug carriers but are nanometer in size. Nanoparticles have mainly been tested in preclinical studies in rats and mice, and showed promise suppressing ocular angiogenesis by topical application (51,60).

Gene therapy considerations for neovascular retinal diseases

Gene therapy has several advantages over pharmacological treatments including long-term therapeutic effects without repeated treatments, and the capacity for cell-targeted delivery using cell-specific promotors. There has been tremendous excitement surrounding the first FDA-approval of a retinal gene therapy for retinal degenerations associated with biallelic loss of the *RPE65* gene (61,62). However, unlike most inherited retinal diseases which are caused by single gene mutations, retinal angiogenesis involves a complex network of many different pro-angiogenic and anti-angiogenic factors. Also, while no current therapies are available for most inherited retinal degenerations, many treatments already exist for neovascular retinal diseases. While gene therapies for inherited conditions target younger patients before the onset of blindness, degenerative conditions such as AMD primarily impact older adults, where the quality-adjusted life-year gain may not necessarily justify the cost and risks of a new therapy. Nevertheless, given the tremendous burden of pharmacologic anti-VEGF treatments, gene therapy holds the promise for long-term suppression of VEGF in neovascular retinal diseases.

Gene therapy involves the delivery of a therapeutic gene into retinal cells using a gene-carrying vector. However, the specific vector and mode of delivery depends on the choice of the therapeutic gene, target cell type, and target region of transduction. Here we discuss gene delivery vectors and modes of intraocular delivery in designing a gene therapy strategy for neovascular retinal diseases.

Table 3 Types of transgene delivery vehicles for ocular gene therapy

Vehicle property	Lentivirus	Adenovirus	AAV	Synthetic
Expression level	High	High	High	Low
Expression duration	Long-term	Transient	Long-term	Variable
Packaging capacity	~8 kb	~9 kb	~5 kb	Variable
Immunogenicity	High	High	Low	Low
Risk of mutation	High	Low	Low	Low

AAV, adeno-associated virus.

Gene delivery vectors

The most common carriers for delivering genetic material are viral vectors. Although synthetic polymers such as PLGA nanoparticles have been extensively evaluated as gene-carrying vectors, and are generally safe with low immunogenicity (63), these non-viral carriers are generally less efficient at transducing retinal cells compared with viral vectors. Common viral delivery platforms include lentivirus, adenovirus, and adeno-associated virus (AAV), each of which has distinct advantages and disadvantages (*Table 3*).

Lentiviral vectors

Lentiviral vectors are single-stranded RNA viruses that can deliver ~8-kb long transgene to both dividing and non-dividing cells. They integrate into the host genome to enable sustained and long-lasting transgene expression, but carries a risk of mutagenesis if it integrates into a tumor suppressor gene. To overcome this issue, inactivation at the 3' long terminal repeat (LTR) has been developed for self-inactivation vectors (64).

Adenoviruses

Adenoviruses are double-stranded DNA viruses that can package larger genes (9 kb), but its infectivity is limited to postmitotic cells. While adenoviruses can efficiently infect a variety of retinal cell types, they have largely been abandoned due to significant host immune responses which results in a loss of therapeutic effect.

Adeno-associated virus

AAV vectors are the leading platform for *in vivo* gene delivery as they have been engineered to exclude intrinsic viral sequences resulting in low immunogenicity and cytotoxicity (65). Despite its limited genome packaging size (~4.7 kb), AAV has been widely-used both in preclinical and clinical studies as recombinant vectors with pseudo-typed capsids that can achieve cell-specific therapy. For example, retinal ganglion neurons can be infected with AAV2 and AAV8 given intravitreally, while photoreceptors and RPE cells can be efficiently transduced with AAV2, AAV5, AAV7, AAV8, and AAV9 after subretinal delivery (66-68). Typically, AAV vectors in the vitreous cavity cannot transduce photoreceptors or RPE due to the internal limiting membrane (ILM) barrier that is formed by the foot plates of Muller glia. Using a method of "directed evolution" where libraries of AAV variants are rapidly screened *in vivo* for cell-type tropism and transduction efficiency, newer generations of AAV such as the AAV2-7m8 vector have been developed to overcome the ILM barrier to transduce outer retinal neurons after intravitreal injection (69).

Modes of vector delivery

Intraocular delivery of viral vectors enables localized transduction of different retinal cell types with minimal systemic exposure. Intraocular injections require a smaller amount of virus to be delivered than intravenous delivery, and due to the immune privileged status of the eye, can limit host immune responses that could otherwise cause cellular damage or reduce transduction efficiency. However, different modes of intraocular delivery vary with regard to ease of application, biodistribution, and immunogenicity. Here, we discuss 3 major modes of vector delivery (*Figure 1*).

Subretinal injections are the most common route of viral delivery in the eye because it readily bypasses the ILM barrier and produces reliable, robust transgene expression in outer retinal cells that are the target of most gene therapy strategies. Because the viral particles are confined to the immune-privileged subretinal space, host immune responses are minimal, and the degree of intraocular inflammation is very mild. However, subretinal injections are performed using a transretinal cannula that must be inserted through
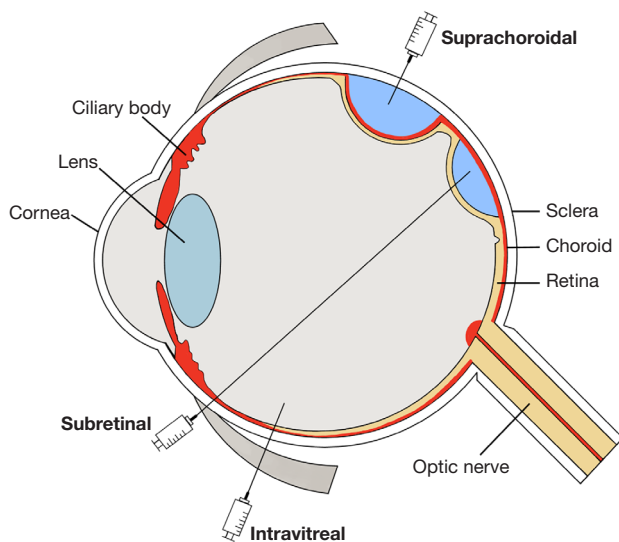
Figure 1 A schematic diagram illustrating 3 different intraocular injection methods; subretinal, intravitreal and suprachoroidal injections.

the neurosensory retina to create a retinotomy—a technique that generally requires complex vitrectomy surgery and has a risk of retinal detachment. Moreover, the transduction area is limited to the small area created by the injected fluid bleb, so widespread expression is difficult to achieve. The first FDA-approved ocular gene therapy for *RPE65* employs subretinal AAV2 delivery, and has demonstrated both safety and efficacy in clinical trials.

Intravitreal injections are commonly performed by retinal specialist for delivering pharmacotherapies such as steroids and anti-VEGF agents. These injections are easy to perform in an office setting, have low risks of endophthalmitis or retinal detachment, and can be repeatedly given. Unlike subretinal injections, the injected viral particles can distribute broadly to transduce the entire retina, rather than just a small region. Although efficient transduction of outer retinal layers is limited by the ILM barrier, surgical removal of ILM may improve transduction efficiency, although it still requires intraocular surgery (70). Newer generations of AAV such as AAV2-7m8 and some tyrosine mutants have been shown to exhibit better penetration and transduction efficiency when given intravitreally (69). However, intravitreal viral injections generally cause more intraocular inflammation than subretinal delivery, possibly due to the greater degree of trabecular outflow to the systemic circulation as compared to uveoscleral outflow.

Suprachoroidal injections are a novel mode of intraocular delivery that uses microneedles or microcatheters to access a potential space between the choroid and the scleral wall of the eye (71-73). Suprachoroidal delivery of triamcinolone are effective in treating macular edema resulting from RVO and uveitis (74,75). Recent studies of suprachoroidal AAV delivery demonstrated widespread transgene expression and greater vector coverage in outer retinal cells, although mostly confined to the peripheral retina (76,77). Compared to methods using microcatheters (78,79), custom microneedles can be used in office settings similar to intravitreal injections (77). However, because the suprachoroidal space is outside the blood-retinal barrier delimiting the zone of immune privilege, there is a higher potential risk of inflammation or host immune responses.

The choice of viral vector and delivery mode depends significantly on the choice of the therapeutic transgene, the target cell type, and target region of transduction. For example, for gene replacement strategies, the cell types that natively produce the mutated gene product should at least be transduced. However, if the transgene is a secreted protein such as an anti-VEGF antibody or decoy VEGF receptor, the identity of the transduced cells may not be important, as they essentially serve as a "biofactory". For diseases such as nAMD, the treatment effect only needs to be localized to the area of the CNV, while global ischemic conditions such as proliferative diabetic retinopathy may require broader areas of therapeutic effect. However, if the therapeutic transgene can exert its effect at a distance, it may be more beneficial to transduce cells farther away from the pathologic region or macula area to minimize any potential damage from the injection procedure itself. Since anti-angiogenesis gene therapy strategies vary widely, we review several approaches most actively under investigation.

Anti-VEGF gene therapy strategies

rAAV-sFlt1

One of the first anti-VEGF gene therapy strategies employed subretinal injections of recombinant AAV2 vector expressing soluble VEGF receptor 1, sFlt-1. Several pre-clinical studies using a transgenic mouse model and non-human primates have shown that a single subretinal injection of rAAV-sFlt1 was well tolerated, and suppressed angiogenesis effectively without significant adverse effects or host immune responses (80-82). However, although phase I studies (NCT01494805) in nAMD patients demonstrated

safety (83), the phase IIa randomized clinical trial with 32 patients showed no clear benefit in visual acuity or anatomy compared with baseline and control eyes (84). Another phase I clinical trial with AAV2-sFlt01 (Sanofi Genzyme) was conducted (NCT01024998) with 19 nAMD patients, which proved its safety, but showed high variability in sFlt expression and anti-permeability between patients (85). Interestingly, 5 of 10 patients did not express sFlt01 after receiving AAV2-sFlt01 (2×10^{10} vg), and 4 of these 5 non-expressors had serum neutralizing antibody titer greater than 1:400 (85), suggesting that humoral immune responses impacted efficacy. These small early studies enrolled patients with chronic nAMD who received previous anti-VEGF injections or showed minimal response to anti-VEGF agents at baseline. Thus, the potential effectiveness of gene therapy strategies for neovascular retinal conditions was not fully evaluated.

ADVM-022

To overcome the difficulties of subretinal injections, ADVM-022 employs the AAV2-7m8 vector to encode aflibercept to be given as an intravitreal injection. A preclinical study with laser-induced CNV in non-human primates found ADVM-022 effective at maintaining high aflibercept levels in the vitreous for 3–9 months with no serious adverse effects (86), and prevented laser-induced CNV at levels comparable to a single intravitreal aflibercept at the time of CNV induction (86). The multicenter, open-label, dose-ranging phase I clinical trial (OPTIC trial, NCT03748784) is currently ongoing, and early reports found 10 out of 12 patients did not require a rescue injection for 24 weeks while maintaining visual acuity and reduction in central retinal thickness (87). A more recent report showed that the 6 patients who received the higher 6×10^{11} vg dose of ADVM-022 maintained vision and anatomy on optical coherence tomography (OCT) without rescue injections through a median of 34 weeks (range, 24–44 weeks) (88).

RGX-314

RGX-314 is an AAV8 vector that expresses an anti-VEGF antibody fragment (Fab) that selectively binds to human VEGF. Preclinical studies with a transgenic mouse model of retinal neovascularization showed that the subretinal injection of RGX-314 resulted in significant reduction of neovascularization (89). More recently,

Ding *et al.* compared the effects of a suprachoroidal and subretinal injection of RGX-314 in rat, and suggested that suprachoroidal delivery of RGX-314 produced comparable anti-VEGF Fab expression and suppression of VEGF-induced hemorrhage (76). A phase I/IIa clinical trial delivering RGX-314 by subretinal injections into nAMD patients is currently ongoing (NCT03066258), and the interim assessment found that RGX-314 was well tolerated and continued to produce anti-VEGF Fab for 2 years (90,91). Interestingly, more than 50% of patients did not require anti-VEGF injection for as long as 2 years with improved visual acuity and central retinal thickness (90).

RNA interference (RNAi)

RNAi is a gene silencing mechanism found in eukaryotic cells, in which small interfering RNA (siRNA) guides the cleavage of multiple mRNAs resulting in gene silencing (92). For anti-VEGF therapy, a synthetic siRNA, bevasiranib, suppressed VEGF and laser-induced CNV in human cells lines and mice (93,94). However, the phase III clinical trial (COBALT, NCT00499590) comparing the safety and efficacy of combining bevasiranib with ranibizumab in nAMD patients was terminated due to adverse events including decreased visual acuity, endophthalmitis, uveitis, and cataract formation.

Clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing

Despite the early successes of viral-mediated gene therapies, the long-term durability of these treatments remains unclear. Follow-up of Leber congenital amaurosis type 2 patients who received the *RPE65* gene via subretinal AAV2 showed possible loss of efficacy after 2 to 3 years (95,96), although these findings have not been substantiated in later follow-up studies. Nevertheless, new strategies using genome editing have the potential to permanently interrupt pro-angiogenic pathways. CRISPR and CRISPR-associated protein (CRISPR-Cas) systems were discovered as part of bacterial adaptive immunity against viral infection, and are also effective in eukaryotic cells as tools for genome engineering. CRISPR-associated endonucleases such as Cas9 can be programmed using a single guide RNA (gRNA) to target specific sequences in the host genome and create double-strand DNA breaks to create insertions/deletions (indels) of nucleotides that cause frameshift mutations, or enable homology

directed repair when paired with an additional donor DNA template (97,98). The fast-moving technology has resulted in the rapid discovery of different Cas9 orthologs as well as various editing methods and targets ranging from single base editing to RNA editing that can be utilized in mammalian cells (99,100). While the Cas9 from *Streptococcus pyogenes* (SpCas9, 4.1 kb) has been the best characterized, smaller orthologs from *Staphylococcus aureus* (SaCas9, 3.1 kb) and *Campylobacter jejuni* (CjCas9, 3 kb) have been employed with some success (101). Due to their smaller size, these newer Cas9 variants may have better translational potential when packaged into AAV with gRNAs as "all-in-one" vector systems. The first human clinical trial using subretinal AAV delivery of the *CEP290* gene for treatment of Leber congenital amaurosis 10 (LCA10) commenced in late 2019 to determine the safety of this approach (NCT03872479).

Early applications of CRISPR-based genome editing for ocular angiogenesis involved the direct subretinal delivery of CRISPR ribonucleoproteins (RNP) combining both Cas9 endonuclease and gRNA into mouse eyes to ablate the *VEGF* gene. This approach successfully suppressed laser-induced CNV without significant off-target effects, although the specific cell type targeted was unclear (102). Subsequent studies packaged smaller CRISPR endonucleases such as CjCas9 and *Prevoltella* and *Francisella* I (Cpf1, Cas12a) along with respective gRNAs into AAV9 (101,103), and found that targeting either *HIF-1α* (the upstream transcriptional regulator of VEGF), or *VEGFa* resulted in similar levels of CNV suppression when compared with aflibercept, suggesting the potential of CRISPR-based strategies for permanent VEGF suppression and a possible true cure for nAMD (103). In addition, rAAV1 expressing SpCas9 has been used to deplete *VEGFR2* in vascular endothelial cells, and successfully suppressed mouse models of oxygen-induced retinopathy (OIR) and laser-induced CNV (104). Efficient suppression of angiogenesis was found despite employing intravitreal delivery of a dual AAV vector system, where Cas9 and the sgRNA were packaged separately.

To evaluate the translational potential of CRISPR-based genome editing for neovascular retinal diseases, our group was the first to demonstrate effective suppression of VEGF secretion from human cells *in vitro* using a lentiviral vector to express SpCas9 and gRNAs (105). Due to the large size of SpCas9, we subsequently compared subretinal delivery of a dual-AAV vector system to deliver SpCas9 and gRNAs separately, versus a single-AAV vector system to express both SaCas9 and gRNA in mouse eyes *in vivo* (106). Interestingly, despite similar cutting efficiency *in vitro* and viral transduction efficiency *in vivo* between the two platforms, we found that the dual-vector SpCas9 system was more effective in suppressing VEGF than the single-vector SaCas9, suggesting that *in vivo* performance may be dictated more by the Cas9 ortholog type than the efficiency of viral transduction (106).

Despite early successes in using genome editing to treat ocular angiogenesis, these approaches must be carefully optimized prior to clinical translation. Because genome editing is permanent, and VEGF play physiologic roles in maintaining the health of the retinal and choroidal vasculature, over-suppression may result in harmful adverse effects. Also, the potential for off-target effects as well as the potential for host immune responses due to the exogenous nature of bacterial Cas9 proteins may further pose additional barriers to success. Yet, CRISPR-based strategies may also be particularly suited to address complex, multifactorial processes such as angiogenesis, as it enables multiplexing, where a collection of different gRNAs can be designed to target multiple loci within VEGF or several pro-angiogenic pathways simultaneously (*Figure 2*). Cell-specific promoters may also be directed to suppress VEGF from more disease-relevant cellular sources, while sparing more physiologic VEGF sources. Thus, future studies to enhance the specificity of CRISPR-based strategies may provide a pathway to enable translation of genome editing to the management of retinal neovascular conditions.

Conclusions

VEGF plays a significant role in ocular angiogenesis, and anti-VEGF pharmacotherapies have revolutionized the management of neovascular diseases of the retina and enabled the restoration of vision for many patients. However, the frequent need for intraocular injections remain a clinical and financial burden. Recent advances in gene therapy have the potential to improve both the efficacy and durability of anti-VEGF therapies, resolve current unmet needs, and improve patients' quality of life.
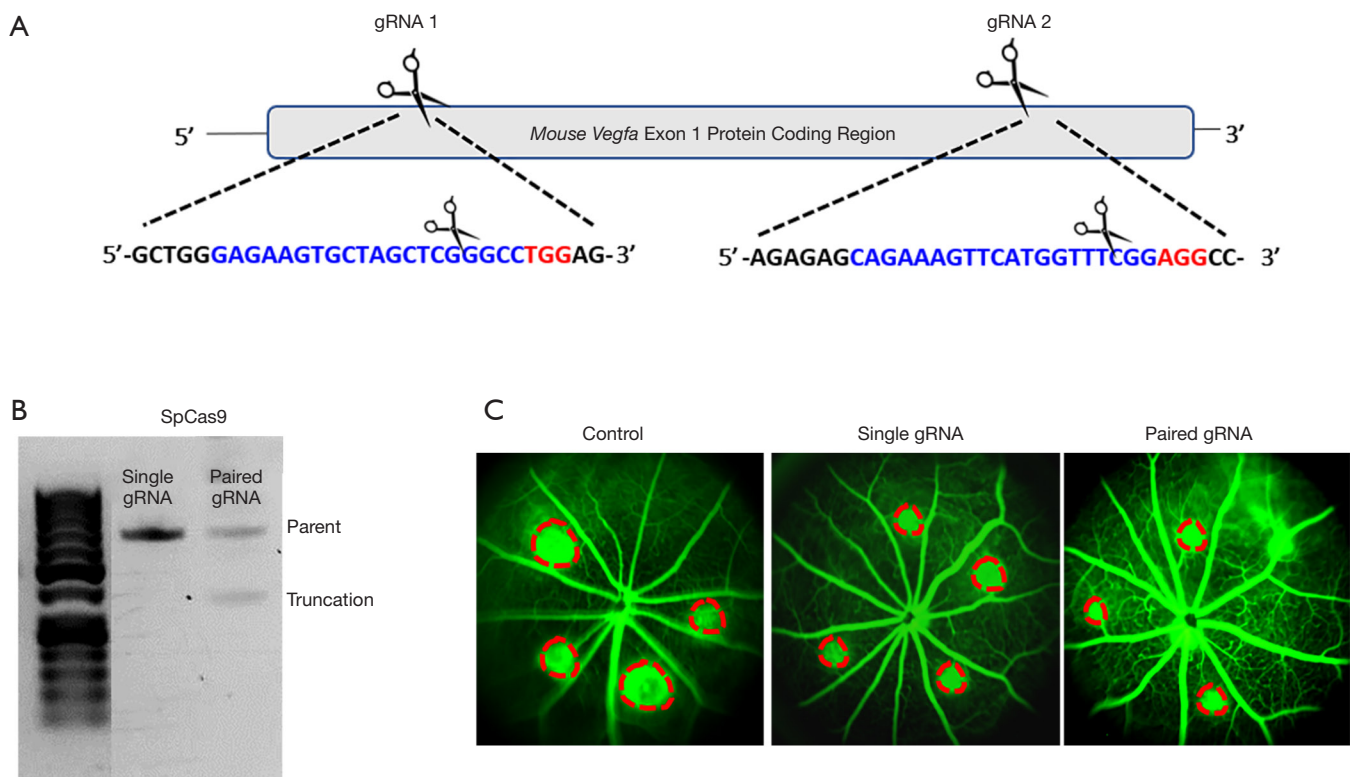
Figure 2 CRISPR technology targeting mouse VEGFa. (A) A schematic diagram of CRISPR genome editing within exon 1 of mouse VEGFa gene. CRISPR with single gRNA targets gRNA1, and CRISPR with paired gRNA system edits both gRNA1 and gRNA2 simultaneously. (B) Genomic analysis showing gene truncation in CRISPR with paired gRNA system. (C) Fluorescence angiography showing suppression of lase-induced CNV after CRISPR delivery in mouse retina. CRISPR, clustered regularly interspaced short palindromic repeats; VEGF, vascular endothelial growth factor; gRNA, guide RNA; CNV, choroidal neovascularization.

Acknowledgments

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editor (Susanna S. Park) for the series "Novel Tools and Therapies for Ocular Regeneration" published in *Annals of Translational Medicine*. The article has undergone external peer review.

Peer Review File: Available at https://dx.doi.org/10.21037/atm-20-4417

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi. org/10.21037/atm-20-4417). The series "Novel Tools and Therapies for Ocular Regeneration" was commissioned by the editorial office without any funding or sponsorship. GY reports personal fees from Allergan, personal fees and non-financial support from Clearside Biomedical, personal fees from Genentech, grants and personal fees from Iridex, outside the submitted work. The other authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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