Intravitreal Injection of AAV Expressing Soluble VEGF Receptor-1 Variant Induces Anti-VEGF Activity and Suppresses Choroidal Neovascularization

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METHODS. C57/B6 mice were intravitreally administered with rAAV2-sVEGFRv-1, rAAV2-GFP, or clinically used bevacizumab after CNV lesions were induced via laser photocoagulation. Immunostaining was performed with phalloidin and CD31 to measure CNV extensiveness, F4/80 and CD11b for inflammatory cell infiltration, and pan-cytokeratin to visualize fibrotic progression.

RESULTS. rAAV2-sVEGFRv-1 (5.0 × 10^7 viral genomes) possesses antiangiogenic, anti-inflammatory, and antifibrotic properties. rAAV2-sVEGFRv-1 was demonstrated to significantly decrease retinal CNV lesion size (1336 ± 186) when compared to rAAV2-GFP-treated (2949 ± 437, P = 0.0043), mock-treated (3075 ± 265, P = 0.0013), and bevacizumab-treated models (995 ± 234). Infiltration by inflammatory cells significantly decreased with rAAV2-sVEGFRv-1 administration, while groups treated with rAAV2-GFP did not. Additionally, antiapoptotic activity was observed via TUNEL assay in rAAV2-sVEGFRv-1 (16.0 ± 3.6) and bevacizumab-treated groups (23.6 ± 7.5, P = 0.003). Overall, the rAAV2-sVEGFRv-1 viral vector was positively comparable to bevacizumab, indicating it as effective as approved therapeutics.

CONCLUSIONS. The ability of a low dose of rAAV2-sVEGFRv-1 to exert a therapeutically relevant anti-VEGF effect in a CNV model is demonstrated, and strongly suggests gene therapy as an effective and convenient treatment for sustained VEGF suppression.

Keywords: wet age-related macular degeneration, choroidal neovascularization, recombinant adeno-associated virus, vascular endothelial growth factor, soluble VEGF receptor 1

Purpose. With anti-VEGF-based treatments for wet AMD requiring frequent injections, it is often burdensome to both patients and healthcare providers. To explore its possibility as a desirable alternative, we investigated the therapeutic potential of a recombinant adenovirus 2 expressing a soluble variant of VEGF receptor-1 (rAAV2-sVEGFRv-1) in a laser-induced choroidal neovascularization (CNV) model, as CNV is a defining feature of AMD progression.

Methods. C57/B6 mice were intravitreally administered with rAAV2-sVEGFRv-1, rAAV2-GFP, or clinically used bevacizumab after CNV lesions were induced via laser photocoagulation. Immunostaining was performed with phalloidin and CD31 to measure CNV extensiveness, F4/80 and CD11b for inflammatory cell infiltration, and pan-cytokeratin to visualize fibrotic progression.

Results. rAAV2-sVEGFRv-1 (5.0 × 10^7 viral genomes) possesses antiangiogenic, anti-inflammatory, and antifibrotic properties. rAAV2-sVEGFRv-1 was demonstrated to significantly decrease retinal CNV lesion size (1336 ± 186) when compared to rAAV2-GFP-treated (2949 ± 437, P = 0.0043), mock-treated (3075 ± 265, P = 0.0013), and bevacizumab-treated models (995 ± 234). Infiltration by inflammatory cells significantly decreased with rAAV2-sVEGFRv-1 administration, while groups treated with rAAV2-GFP did not. Additionally, antiapoptotic activity was observed via TUNEL assay in rAAV2-sVEGFRv-1 (16.0 ± 3.6) and bevacizumab-treated groups (23.6 ± 7.5, P = 0.003). Overall, the rAAV2-sVEGFRv-1 viral vector was positively comparable to bevacizumab, indicating it as effective as approved therapeutics.

Conclusions. The ability of a low dose of rAAV2-sVEGFRv-1 to exert a therapeutically relevant anti-VEGF effect in a CNV model is demonstrated, and strongly suggests gene therapy as an effective and convenient treatment for sustained VEGF suppression.

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AMD is a leading cause of central vision loss in the elderly populations of the developed world. Furthermore, due to accelerating trends in population aging, AMD is projected to affect 196 million people worldwide by 2020 and 288 million by 2040. The wet subtype of AMD is progressive and distinguished primarily by choroidal neovascularization (CNV). Other characteristics are the development of fibrotic tissue, retinal bleeding, and/or fluid leakage from permeable, newly formed vessels. As such, wet AMD can lead to permanent blindness if left untreated. VEGF has been implicated as a major driving force of CNV in wet AMD, leading to anti-VEGF treatments becoming widely used as an interventional strategy. Conventional anti-VEGF treatments include monoclonal antibodies (bevacizumab), Fab fragments (ranibizumab), and recombinant fusion proteins ( aflibercept). However, these therapies need to be administered relatively frequently via intravitreal injection to maintain anti-VEGF activity, which makes treatment burdensome, both procedurally and economically. This has been proven to have a negative effect on patient compliance, which in turn reduces treatment efficacy and disease prognosis. As wet AMD is a progressive disease and requires sustained, long-term treatment, the negative effects are amplified, posing a serious threat to patient outcomes. Thus, the need to develop a convenient
Therapeutic Ability of AAV-Soluble VEGFR-1 Variant

AAV works by binding to a pair of fms-like tyrosine kinase receptors, Flt-1 and KDR/Flk-1, of which the latter interaction drives the angiogenic process. Soluble Flt-1 (sFLT-1) is a naturally occurring, alternatively spliced soluble variant of the receptor which lacks the membrane-spanning domain present in Flt-1. sFLT-1 directly binds to VEGF with high affinity, thereby sequestering VEGF from interacting with KDR/Flk-1 and providing anti-VEGF activity.\textsuperscript{6} sFLT-1 also associates with KDR/Flk-1 itself to form inactive heterodimers.\textsuperscript{9}

Here, we demonstrate that a variant of soluble VEGF receptor-1 (sVEGFRv-1), a truncated form of sFLT-1 packaged in a recombinant adeno-associated virus 2 (rAAV2) delivery vehicle, may serve as the basis for developing gene therapy modalities against wet AMD. rAAV2 vectors are particularly attractive for this purpose due to their ability to elicit long-term transgene expression and transduce non-dividing cells, in addition to being non-pathogenic by nature.\textsuperscript{10} Furthermore, a number of clinical trials utilizing gene therapy constructs delivered via AAV against a range of pathogenic conditions, including ocular diseases, have been conducted with good prognosis and efficacy.

The virus vector, rAAV2-sVEGFRv-1, was administered by intravitreal injection (5.0 × 10^7 viral genomes [vg]) into the retinas of a mouse model of CNV induced via laser photocoagulation. Subsequent immunostaining of whole mounts of RPE-choroid mounts revealed that rAAV2-sVEGFRv-1 has antiangiogenic, anti-inflammatory, antifibrotic, and antiapoptotic properties. In fact, when the transgene construct was expressed, rAAV2-sVEGFRv-1 protected the retina against CNV at levels comparable to treatment using bevacizumab.

**Methods**

**Cell Culture and Preparation of rAAV2s**

HeLa (ATCC, Manassas, VA, USA) cells were cultured in RPMI media (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Invitrogen), 15 mM HEPES (Sigma-Aldrich Corp., St. Louis, MO, USA), GlutaMAX-1 (2 mM), and penicillin (100 IU/mL) streptomycin (50 μg/mL), and then maintained at 37°C under a humidified 5% CO2. The soluble variant of VEGF receptor-1 (sVEGFRv-1) was designed from human vascular endothelial growth factor receptor (VEGFR) 1 gene, also called Flt-1 (XM_017020485.1, NCBI Reference Sequence, NIH). sVEGFRv-1 consists of the first six of 7 extracellular immunoglobulin-like domains of the Flt-1 receptor and lacks the 51-amino acid tail found on sFLT-1, which is a result of alternative splicing of the Flt-1 mRNA\textsuperscript{6} (Fig. 1A). The region spanning from nucleotide position 282 to 2,253 of the Flt-1 mRNA sequence (total 1972 base pairs) was inserted into a pAAV-F. IX cis plasmid (US Patent 6,093,392) containing a CMV promoter, SV40 polyadenylation signal, and both ITRs to generate rAAV2-sVEGFRv-1. For a negative control, GFP gene was inserted to generate rAAV2-GFP. rAAV2 was produced using a triple cotransfection method negative control, GFP gene was inserted to generate rAAV2-GFP. rAAV2-sVEGFRv-1 at MOI 200,000. After 48 hours of incubating, a wound is introduced by manually scratching along the cells with a cell scraper, which is then washed two times. VEGF (Calbiochem, Cat# 676472, Walkersville, MA) at a final concentration of 10 ng/mL is then introduced to the virus vector-treated sample and the untreated control sample before incubating the samples again. This allows cells to regrow into the wound, the numbers of which were counted.

**Animals**

We used 8-week-old male C57/BL6 mice (The Orient Bio, Inc., Sungnam, Korea) in this study. All animal care and experiments were performed in accordance with the guidelines available through the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and was overseen by the Institutional Animal Care and Use Committee of Soonchunhyang University Hospital Bucheon. For intravitreal injections, 5-day post-laser coagulation, 1 μL of the viral vectors (5.0 × 10^10 vg/mL) was injected into the right eye of each mouse, under anesthesia and with pupil dilation, as described above.

**Migration Assay**

To verify soluble VEGF receptor activity, a HUVEC line (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) was cultured in EGM-2 complete media (Cat# CC-3162, Cambrex Bio Science Walkersville, Inc.), and either mock-treated, treated with rAAV2-GFP as a negative control, or rAAV2-sVEGFRv-1 at MOI 200,000. After 48 hours of incubating, a wound is introduced by manually scratching along the cells with a cell scraper, which is then washed two times. VEGF (Calbiochem, Cat# 676472, Walkersville, MA) at a final concentration of 10 ng/mL is then introduced to the virus vector-treated sample and the untreated control sample before incubating the samples again. This allows cells to regrow into the wound, the numbers of which were counted.

**Western Blot Analysis and ELISA Assay**

In the western blot analysis, 1 × 10^6 HeLa cells were treated for 2 days with either rAAV2-sVEGFRv-1, rAAV2-GFP, or mock-treated at 10,000 MOI, along with adenovirus 5 at 5 MOI. The cells were lysed after collecting cell supernatants. Considering that a sequence of 10 basic amino acids forms a binding for the anticoagulant heparin, sVEGFRv-1 in the culture media was concentrated using heparin-sepharose beads (Biovision, Milpitas, CA, USA) based on the heparin-binding feature of sVEGFRv-1. The proteins were then resolved on reducing sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. An enhanced chemiluminescence (ECL) system was used to detect the bands. Primary antibodies for sVEGFRv-1 (AF321) and β-actin (YIF-LF-PA0209A) were purchased from R&D Systems (Minneapolis, MN, USA) and AbFrontier (Seoul, Korea), respectively.

For ELISA analysis, a Human VEGF-R1/Flt-1 Quantikine ELISA kit (R&D systems) was used, with the assays performed according to the manufacturer’s instructions using either cell lysates or cell supernatants. The human VEGF-R1 standard provided in the kit was used to calculate sVEGFRv-1 concentrations.

**Tissue Preparation**

A 4:1 mixture of zolazeptam/tiletamine (80 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally was used to deeply anesthetize the mice, which were then perfused intracardially with 0.1 M PBS (7.4 pH), followed by 4% paraformaldehyde.
in 0.1 M PBS. For RPE whole mounts, the eyeballs were enucleated. In addition, the anterior segments, including the cornea, lens, and neural retina, were removed to generate eyecups. The cornea and lens were removed from the eyeball, and the eye cups embedded in an optimum temperature cutting compound (Tissue-Tek; Miles Scientific, Napierville, IL, USA) before generating 5 µm-thick frozen section samples.

Immunohistochemistry and TUNEL Assay

Whole mounts were immunostained by incubating the RPE-choroid tissues overnight at 4°C in mouse anti-CD31 (550274; BD Pharmingen, Inc., San Diego, CA, USA), diluted in PBS containing 1% Triton X-100 (PBST). They were then washed three times in PBST for 10 minutes apiece, and incubated with AlexaFluor 532 conjugated goat anti-mouse (A21270; Thermo Fisher Scientific) and rhodamine conjugated phalloidin (R415; Thermo Fisher Scientific) for 2 hours at room temperature. Meanwhile, frozen sections were stained for leukocytes with anti-CD11b, macrophages with anti-F4/80 (MCA497GA; Serotec, Oxford, UK), or fiber cells with anti-pan cytokeratin (ab27988; Abcam, San Francisco, CA, USA). These sections were incubated with diluted primary antibodies overnight at 4°C, after which they were washed 3 times in PBST. The frozen sections were then incubated with secondary antibodies AlexaFluor 568 or 488 (Thermo Fisher Scientific) for 2 hours at room temperature and stained with DAPI for nuclear visualization purposes. Fluorescence confocal microscopy (LSM 700; Carl Zeiss Microscopy GmbH, Jena, Germany) was used to examine both the whole mounts and frozen sections, and the images were captured using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was performed using 1-way ANOVA testing, with the significant difference determined at $P < 0.05$, $P < 0.01$, or $P < 0.001$. The data is visualized through box plot
**Antiangiogenic Effect of rAAV2-sVEGFRv-1**

Fourteen days after retinal injury induced via laser photocoagulation, whole mounts of RPE-choroid tissue were prepared and immunostained with phalloidin specific to F actin for RPE cells and anti-CD31 for endothelial cells, to visualize the extensiveness of the resultant CNV (Fig. 2). In mock-treated control mice and those injected intravitreally with rAAV2-GFP, the damage to the RPE and the formation of penetrating new blood vessels was readily observed. This contrasts with intravitreal injections of rAAV2-sVEGFRv-1, in which CNV formation was markedly suppressed by the injection. The antiangiogenic effect of rAAV2-sVEGFRv-1 was comparable to that of bevacizumab, a widely used drug for the treatment of wet AMD. Quantitatively determined by phalloidin, the CNV area was observed as 1336 ± 114.4 μm², 186 ± 6.9 μm², and 9.2 μm² for rAAV2-sVEGFRv-1, rAAV2-GFP, and bevacizumab-treated groups, respectively (Fig. 2M; n = 5). These data strongly suggest that rAAV2-sVEGFRv-1 yields a functional soluble variable VEGF receptor-1 product.

**Anti-Inflammatory Effect of rAAV2-sVEGFRv-1**

The diagnosis of wet AMD has an established association with the infiltration and proliferation of inflammatory cells during CNV development. Thus, the presence of inflammatory cells was determined using anti-F4/80 (Fig. 3; n = 5) and anti-CD11b (Fig. 4; n = 5) specific to macrophages and leukocytes, respectively, for an immunohistochemistry evaluation. Both macrophages and leukocytes were readily detectable in the mock-treated control and rAAV2-GFP-treated groups (anti-F4/80: 77.0 ± 9.2 and 82.0 ± 9.0, respectively; anti-CD11b: 72.0 ± 7.3 and 75.6 ± 10.5, respectively). In contrast, inflammatory cell infiltration was dramatically reduced in mice treated with rAAV2-sVEGFRv-1 (anti-F4/80: 35.0 ± 6.9; anti-CD11b: 42.2 ± 8.7), which is also at a greater rate than its bevacizumab correspondent (anti-CD11b: 49.6 ± 8.8, Fig. 4Q), though not statistically significant.

**Antifibrotic Effect of rAAV2-sVEGFRv-1**

To observe the antifibrotic effects of rAAV2-sVEGFRv-1, transverse retinal sections were immunostained with anti-pan cytokeratin, an indicator of retinal pigment epithelial cell fibrosis. Figure 4 shows that subretinal fibrosis was readily observable alongside CNV in the retinas of the mock-treated control and rAAV2-GFP-injected mice (4553.0 ± 462.0 and 4429.3 ± 671.5 μm² per pan cytokeratin-positive area, respectively; n = 3). Of noteworthiness, rAAV2-sVEGFRv-1 (2534.7 ± 703.5; n = 3), like bevacizumab (2957.0 ± 585.4; n = 5), had an inhibitory effect on the development of subretinal fibrosis, which characteristically occurs in wet AMD (Fig. 4R).

**Antiapoptotic Effect of rAAV2-sVEGFRv-1**

Significantly fewer TUNEL-positive cells were seen in the samples taken from mice treated with the construct, while apoptotic cells were readily observed in the outer nuclear layer (ONL) and around CNV regions in mock-treated or GFP-treated animals (P = 0.001 and P = 0.003, respectively; n = 5). Again, the observed reduction of apoptotic cells due to viral vector treatment was comparable to that resulting from treatment with bevacizumab (Fig. 5). The number of TUNEL-positive cells were 16.0 ± 3.6 for rAAV2-sVEGFRv-1, 46.0 ± 7.5 for rAAV2-GFP, 47.0 ± 5.0 in mock-treated control retinas, and 18.3 ± 2.1 for bevacizumab (Fig. 5M).

**DISCUSSION**

Here, we show that rAAV2-sVEGFRv-1 is very capable of exerting anti-VEGF effects to address the major symptoms of wet AMD. This was initially determined via a wound migration in vitro assay, where HUVECs expressing the viral vector in the presence of VEGF effectively resisted cell regrowth into the scratch wound. Anti-VEGF activity was reinforced in vivo with the intravitreal injection of rAAV2-sVEGFRv-1 into the retinas of a laser-induced CNV mouse model. The transgene construct encodes a truncated form of sFLT-1 that works mechanistically in a similar manner to ranibizumab and bevacizumab, which both bind VEGF isoforms associated with the Flt-1 receptor, whereas aflibercept, a fusion protein, interacts with both Flt-1 and KDR/Flk-1.4 In addition to the wet AMD therapeutics currently in use, all investigational products evaluated thus far have had anti-VEGF principles in common.

Previous attempts at developing gene therapeutics to treat wet AMD have used sFLT-1-based constructs as well.12,13 An Australian group inserted a natural form of sFLT-1 under the control of a CMV promoter into a rAAV2 delivery vehicle (rAAV2-sFLT-1) that was administered via subretinal injection.14 Structurally very similar to rAAV2-sVEGFRv-1, rAAV2-sFLT-1 completed phase I clinical trials,14,15 but was no longer pursued after phase II clinical trials indicated that rAAV2-sFLT1 treatment did not result in significant visual gain.16 A separate group based in the United States utilized intravitreal injections as the treatment paradigm to deliver a
chimeric molecule containing Flt-1 domain 2, which is responsible for VEGF binding. Packaged into an AAV2 vehicle (AAV2-sFLT01) and expressed from a chicken β-actin promoter, AAV2-sFLT01 completed phase I clinical trials. Further trials were not conducted, but rather, the construct was packaged into AAV9 as a possible treatment for brain angiogenesis.

Early animal model studies for rAAV2-sVEGFRv-1 focused almost exclusively on the effects of the viral vectors on CNV to appraise its potential suitability as a wet AMD gene therapeutic, which is appropriate considering the relevance of CNV to AMD. However, rAAV2-sVEGFRv-1 treatment in a mouse model proved rAAV2-sVEGFRv-1 to be effective in addressing other major symptoms of wet AMD as

![Figure 2](image-url)

**Figure 2.** Visualization of CNV resulting from laser photocoagulation in RPE-choroid tissue using phalloidin and CD31. The extensiveness to which laser-induced CNV occurred in the mice was revealed by immunostaining whole mounts with phalloidin (red) and CD31 (yellow). While CNV was widespread in the mock-treated control mice (A–C) and the group injected with rAAV2-GFP (D–F), CNV was substantially reduced in the groups treated with rAAV2-sVEGFRv-1 (G–I) and bevacizumab (J–L). This can be seen in the statistical analysis, which used the control group as the comparative basis (M); n = 4. Beva, bevacizumab. **P < 0.01.
Whole mounts of RPE-choroid tissues immunostained with phalloidin and CD31 revealed the antiangiogenic properties of the viral vector. Meanwhile, immunohistochemistry performed on transverse retinal sections using F4/80 and CD11b, respective markers for macrophages and leukocytes, demonstrated that rAAV2-sVEGFRv-1 had an anti-inflammatory effect. Additionally, pan-cytokeratin immunostaining provided evidence for the viral vector’s antifibrotic activity.

On a symptom-by-symptom basis, rAAV2-sVEGFRv-1 was comparable to bevacizumab in addressing many aspects of wet AMD, and in fact, was likely to be more efficacious than bevacizumab in reducing leukocyte infiltration into the CNV lesions. Were rAAV2-sVEGFRv-1 administered to additional model animals, resulting datasets could suggest that the effects of the viral vector are virtually indistinguishable from bevacizumab. Bevacizumab was used as a positive control comparator, despite officially being a cancer treatment, because of its widespread use as an off-label therapeutic due to the significant cost advantages it has over other drugs.20 Furthermore, bevacizumab has been shown to have similar

**Figure 3.** Macrophage infiltration analysis via immunostaining with F4/80. Immunohistochemistry with F4/80 performed on transverse sections showed that macrophage cells heavily infiltrated the retinas of the mice in the control (A–C) and rAAV2-GFP-treated (D–F) groups, whereas significantly fewer F4/80-positive cells could be found in the mice treated with rAAV2-sVEGFRv-1 (G–I) and bevacizumab (J–L), as can be seen (M); n = 5. **P < 0.01.
FIGURE 4. Immunohistochemistry of CD11b and pan cytokeratin for transverse retinal sections. CD11b and pan cytokeratin expression, which indicate the presence of inflammatory cells and the progression of fibrosis, respectively, was readily detectable in the mock- (A–D) and rAAV2-GFP-treated (E–H) groups. The expression of both CD11b (n = 5) and pan cytokeratin (n = 5) were reduced in mice injected with rAAV2-sVEGFRv-1 (I–L) and bevacizumab (M–P), as can be also seen (Q, R). *P < 0.05. **P < 0.01.
effects to ranibizumab on wet AMD visual acuity outcomes over a long period. Little separates bevacizumab, ranibizumab, and aflibercept overall in terms of therapeutic efficacy, though the latter two are the approved therapeutics for wet AMD.

Negative effects on vascular health are observed in the previously mentioned three drugs. This cannot be overlooked, as wet AMD patients are generally more likely to be at risk for vascular events due to their age. Significant systemic safety concerns were also noted, which is concerning because VEGF is involved in many of cellular processes, and long-term treatment is necessary for wet AMD. While a meta-analysis was inconclusive on the latter issue, treating wet AMD with conventional drugs may be potentially deleterious, making gene therapy an attractive alternative. Being nonpathogenic and capable of providing long-term expression for

**Figure 5.** Antiapoptotic effect of rAAV2-sVEGFRv-1, exhibited via TUNEL assay. TUNEL-positive cells were found in the control group (A–C), as well as the mice treated with rAAV2-GFP (D–F). A marked reduction of apoptotic cells was seen in sections sampled from mice injected with rAAV2-sVEGFRv-1 (G–I) and bevacizumab (J–L), with the fewest TUNEL-positive cells being detected in the mice subjected to rAAV2-sVEGFRv-1 treatment (M); n = 3. **P < 0.01.
sustained VEGF suppression, rAAVs are particularly well-suited for this application. Furthermore, previous investigations utilizing rAAV-mediated gene therapy to treat ocular conditions have yielded positive results on its safety profile.\textsuperscript{15,16} This can be seen in the development of voricogene neparvovec (Luxturna; Spark Therapeutics, Inc., Philadelphia, PA, USA)\textsuperscript{23–25} a treatment for Leber congenital amaurosis. Approved in late 2017, voricogene neparvovec is also delivered via a rAAV2, a well-characterized serotype, and is the first in vivo gene therapeutic targeting a disease caused by genetic alteration.

While wet AMD drugs are delivered intravitreally, both Luxturna and rAAV2.sFLT-1 utilize subretinal injections. The advantages of subretinal injections are its efficient uptake and transduction, enabled by the proximity of RPE and photoreceptors to the delivery site,\textsuperscript{16} the bypassing of immune surveillance in the retina, and limited potential for systemic biodistribution of the therapeutic.\textsuperscript{14} However, a phase II study of rAAV2.sFLT1 directly linked the injection method and the attendant vitrectomies to significant adverse events,\textsuperscript{16} including cataracts.\textsuperscript{26} In fact, subretinal injections carry a 60% risk of cataract progression to the point that cataract surgery is necessitated and usually occurs within a year.\textsuperscript{18} By comparison, intravitreal injections are significantly less invasive, more convenient for the patient, and proven to effectively transduce the retina when using rAAV2.\textsuperscript{27} Suggestions have also been made that rAAV transduction is actually enhanced in retinas that are inflamed or in a pathological state, both which occur in wet AMD.\textsuperscript{28,29}

Finally, the strength of rAAV2-sVEGFRv-1 as a potential wet AMD therapeutic becomes evident when comparing this study to other CNV mouse model characterizations, particularly those that led to clinical trials. Specifically, \(8.0 \times 10^7\) vg of rAAV2.sFLT1 was delivered subretinally into a transgenic mouse model,\textsuperscript{30} and \(1.1 \times 10^7\) vg of AAV2-sFLT1 injected intravitreally\textsuperscript{31} into a mouse OIR model.\textsuperscript{31} Both groups used mice significantly younger than the 8-week old C57/B6 mice used in this study. Despite rAAV2-sVEGFR1 being administered at levels that were magnitudes lower (5.0 \(\times 10^7\) vg), rAAV2-sVEGFRv-1 had therapeutic effects that rivaled bevacizumab. rAAV2-sVEGFRv-1 was also magnitudes more efficient than a similar construct delivered via rAAV8 (1.0 \(\times 10^7\) vg), a serotype that transduces retinal tissues more readily than AAV2.\textsuperscript{32} Additionally, rAAV2-sVEGFR1 also surpasses in efficiency a potential rAAV- and sFLT1-based gene therapeutic for diabetic retinopathy (4.0 \(\times 10^{10}\) vg).\textsuperscript{33}

Looking ahead, the next steps in characterizing rAAV2-sVEGFRv-1 for potential development as a wet AMD therapeutic involve performing dose-escalation and safety studies, both of which are already in progress. Containing significant and competitive anti-VEGF effects that address major aspects of wet AMD, gene therapy via rAAV-sVEGFRv-1 appears highly attractive, especially considering the exceedingly low amounts of viral vector utilized in this study. When its optimal dosage is identified, gene therapy via rAAV-sVEGFRv-1 appears highly attractive, especially considering the exceedingly low amounts of viral vector utilized in this study. When its optimal dosage is identified, gene therapy via rAAV-sVEGFRv-1 may ultimately outperform bevacizumab and other approved treatments. There is a suggestion that high anti-AAV2 titers negatively affects transgene expression,\textsuperscript{38} meaning seropositive members of the patient population may not be eligible this type of gene therapy. Further investigation will be necessary to elucidate if the suggested negative transgene expression is truly a limiting factor of rAAV-mediated gene therapy.

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