Inhibition of vascular endothelial growth factor (VEGF) is the standard therapy for neovascular age-related macular degeneration (nAMD). However, anti-VEGF agents used in the clinic require repeated injections, causing adverse effects. Gene therapy could provide sustained anti-VEGF levels after a single injection, thereby drastically decreasing the treatment burden and improving visual outcomes. In this study, we developed a novel VEGF Trap, nVEGFi, containing domains 1 and 2 of VEGFR1 and domain 3 of VEGFR2 fused to the Fc portion of human IgG. The nVEGFi had a higher expression level than aflibercept under the same expression cassettes of adeno-associated virus (AAV8) in vitro and in vivo. nVEGFi was found to be noninferior to aflibercept in binding and blocking VEGF in vitro. AAV8-mediated expression of nVEGFi was maintained for at least 12 weeks by subretinal delivery in C57BL/6J mice. In a mouse laser-induced choroidal neovascularization (CNV) model, 4 × 10^9 genome copies of AAV8-nVEGFi exhibited a significantly increased reduction in the CNV area compared with AAV8-aflibercept (78.1% vs. 63.9%, p < 0.05), while causing no structural or functional changes to the retina. In conclusion, this preclinical study showed that subretinal injection of AAV8-nVEGFi was long lasting, well tolerated, and effective for nAMD treatment, supporting future translation to the clinic.

INTRODUCTION
Age-related macular degeneration (AMD) is a progressive macular disease. Late-stage AMD results in severe and permanent central vision impairment and legal blindness, which has an impact on quality of life. Neovascular AMD (nAMD) and atrophic AMD are two types of late-stage AMD. Because of the exponential population aging globally, the projected number of people with AMD in 2020 is 196 million, increasing to 288 million in 2040, thus posing a major public health issue with significant socioeconomic ramifications.

Neovascular AMD is characterized by choroidal neovascularization (CNV), incorporating with exudation, intraretinal and subretinal hemorrhage, retinal pigment epithelial detachment, hard exudate, or subretinal fibrous scar. Vascular endothelial growth factor (VEGF) is a major mediator in angiogenesis and CNV formation. The advent of anti-VEGF molecules drastically changed the treatment of nAMD, before which laser photocoagulation and photodynamic therapy with verteporfin were used. Intravitreal injection of anti-VEGF molecules demonstrates successful prevention of severe visual loss and is the current standard care for patients with nAMD. However, the widely used anti-VEGF agents in the clinic, including bevacizumab, ranibizumab, aflibercept, conbercept, and brolucizumab have short half-lives, thus requiring repeated injections, which can cause intraocular inflammation, retinal detachment, and ocular hemorrhage. Meanwhile, in the real world, patients receive insufficient injections and visits, resulting in a lower visual acuity gain than in phase III clinical trials. Gene therapy can solve this problem by two major strategies based on intraocular delivery of viral vector encoding antiangiogenic proteins or noncoding RNA interference targeting overexpression of VEGF, which gives a lifelong continuous supply of antiangiogenic proteins or small interfering RNA by a single injection, dramatically reducing injection frequency.

Antiangiogenic proteins, including pigment epithelium-derived factor, endostatin, and angiostatin, and anti-VEGF proteins, such as soluble fms-like tyrosine kinase-1, aflibercept, and ranibizumab, have been assessed for potential gene therapy for nAMD in clinical trials. Among them, aflibercept and ranibizumab delivered by adeno-associated virus (AAV) vectors showed optimistic results in reducing injection frequencies in clinical trials for nAMD treatment (ADV-M-022: AAV2.7m8-aflibercept, NCT03748784; RGX-314: AAV8-ranibizumab, NCT03066258). However, it is noteworthy that the high rescue injection-free rate is relevant to a high dose of AAV, that is 12 out of 16 patients at 6 × 10^11 genome copies (GC) and 8 out of 15 patients at 2 × 10^11 GC with ADVM-022, 9 out of 12 patients at 2.5 × 10^11 GC and 5 out of 12 patients at 1.6 × 10^11 GC, which laser photocoagulation and photodynamic therapy with verteporfin were used. The intravitreal injection of anti-VEGF molecules demonstrates successful prevention of severe visual loss and is the current standard care for patients with nAMD. However, the widely used anti-VEGF agents in the clinic, including bevacizumab, ranibizumab, aflibercept, conbercept, and brolucizumab have short half-lives, thus requiring repeated injections, which can cause intraocular inflammation, retinal detachment, and ocular hemorrhage. Meanwhile, in the real world, patients receive insufficient injections and visits, resulting in a lower visual acuity gain than in phase III clinical trials. Gene therapy can solve this problem by two major strategies based on intraocular delivery of viral vector encoding antiangiogenic proteins or noncoding RNA interference targeting overexpression of VEGF, which gives a lifelong continuous supply of antiangiogenic proteins or small interfering RNA by a single injection, dramatically reducing injection frequency.

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Received 2 August 2021; accepted 5 January 2022; https://doi.org/10.1016/j.omtn.2022.01.002.

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GC with RGX-314. However, a higher virus dose means a higher incidence of intraocular inflammation.17,18 In addition, a high dose of ADVM-022 (6 × 1011 GC/eye) showed toxicity when evaluated in patients with diabetic macular edema.19

All these findings highlight the importance of optimizing the anti-VEGF vector by enhancing the expression of the anti-VEGF molecule to reduce the effective dose of virus and to reduce toxicity and immunogenicity. We designed a novel VEGF Trap, nVEGFi, containing domains 1 and 2 of VEGFR1 and domain 3 of VEGFR2 fused to the Fc portion of human IgG to enhance the protein expression level without compromising its anti-VEGF efficacy. AAV is the most popular vector system for ocular gene therapy because of its nonintegrating nature, low immunogenicity, and potential long-term gene expression.20 When delivered subretinally, AAV8 demonstrated strong transduction of both RPE and photoreceptors.21 The use of AAV8 vectors to express nVEGFi may help to maximize long-term suppression of VEGF in the eye and overcome limitations of past gene transfer approaches in nAMD. In this study, we used a mouse laser-induced CNV model to test the efficacy of subretinal injection in a wide range of doses of an AAV8 vector containing an expression cassette for nVEGFi.

RESULTS

Novel nVEGFi provided a higher expression level in vitro

Afiblercept is a VEGF Trap comprising the second Ig domain of human VEGFR1 and the third Ig domain of human VEGFR2 expressed as an inline fusion with the Fc portion of human IgG, which binds to VEGF-A, VEGF-B, and placental growth factor (PIGF) and inhibits the activation of VEGFR1 and VEGFR2.22 We designed a novel VEGF Trap, named nVEGFi, containing the first and second Ig domains of human VEGFR1 as well as the third Ig domain of human VEGFR2 linked to the human IgG Fc region that resulted in the formation of a forced homodimer (Figure S1). nVEGFi had an identical sequence to afiblercept except for the addition of VEGFR1 Ig domain 1 in the N-terminus. The AAV cis plasmid pAAV-nVEGFi was then constructed to express nVEGFi (Figure 1A, upper). Meanwhile, pAAV-afiblercept-expressing afiblercept was constructed with the same other expression cassettes as pAAV-nVEGFi (Figure 1A, below). pAAV-EGFP serves as a negative control expressing EGFP. All three plasmids were under the control of a CB7 promoter and used for transfection of HEK293 cells at equimolar doses. ELISA showed that the concentration of VEGF Trap (ng/mL) in the cell supernatant transfected with pAAV-nVEGFi was 19.3 times higher than that transfected with pAAV-afiblercept (mean ± SD, pAAV-nVEGFi 709.0 ± 144.0 ng/mL, pAAV-afiblercept 36.7 ± 1.6 ng/mL) (Figure 1B). The VEGF Trap in cell supernatant and lysates was detected by western blot, demonstrating that the amount of VEGF Trap in cell supernatant transfected with pAAV-nVEGFi is around...
16-fold higher than that of pAAV-afiblerecept, and approximately 4 times in cell lysates normalized by beta-actin (Figure 1C). Next, relative nVEGFi mRNA or afiblerecept mRNA in transfected cells was measured, and it turned out that the transcription level of nVEGFi was 2.3 times higher than that of afiblerecept (p < 0.01) (Figure 1D). All these findings indicated that the addition of VEGFRI domain 1 can increase the mRNA and protein expression levels of VEGF Trap and, more importantly, can increase the secretion of VEGF Trap.

Then, the nVEGFi and afiblerecept expression cassettes were packaged into AAV8, with a strong CB7 promoter, a chicken β-actin intron, a kozak sequence, and a bGH polyadenylation sequence (Figure 1E). Except for the inclusion of VEGFRI Ig domain 1, AAV8-nVEGFi had the same sequences as AAV8-afiblerecept. Next, AAV8-nVEGFi and AAV8-afiblerecept were used to transduce HEK293 cells with the help of adenovirus under the same conditions. ELISA showed that the level of VEGF Trap (ng/10¹⁰ copies) in the cell supernatant transduced with AAV8-nVEGFi was 25.8 times higher than that transduced with AAV8-afiblerecept (mean ± SD, AAV8-nVEGFi 14.21 ± 1.68 ng/10¹⁰ copies, AAV8-afiblerecept 0.55 ± 0.09 ng/10¹⁰ copies) (Figure 1F).

### nVEGFi exhibited noninferior binding affinity and inhibition property to afiblerecept in vitro

Since the addition of VEGFRI domain 1 could significantly enhance the protein expression level, we first compared nVEGFi to afiblerecept for their ability to bind and block VEGF in vitro. We first synthesized and purified nVEGFi protein. The binding affinity of nVEGFi for VEGF family ligands, including VEGF-A₁₆₅ and PlGF, was measured with SPR-Biacore technology. Similar to afiblerecept, nVEGFi can target VEGF-A₁₆₅ and PlGF (Table 1). Meanwhile, the binding affinity was also measured by equilibrium binding assay, in which different concentrations of nVEGFi or afiblerecept were incubated with VEGF-A₁₆₅, and the amount of free VEGF-A₁₆₅ was measured, demonstrating that nVEGFi had a similar VEGF-binding affinity to afiblerecept (Figure 2A).

Next, the ability to block VEGF-stimulated human umbilical vein endothelial cell (HUVEC) proliferation was assessed by MTS assay for the nVEGFi protein and cell supernatant of pAAV-nVEGFi-transfected cells, with afiblerecept as a control. In agreement with the VEGF-binding affinity assay, all of them demonstrated an equal inhibitory effect on VEGF-dependent HUVEC proliferation (Figure 2B).

### In vivo dose-escalation efficacy study of AAV8-nVEGFi in comparison with AAV8-afiblerecept

According to the above studies, nVEGFi was at least as effective as afiblerecept as a VEGF blocker and had a significantly higher expression level, which made it more suitable for gene therapy. First, an in vivo dose-escalation efficacy study of AAV8-nVEGFi was carried out, in which 4-week-old C57BL/6J mice were subretinally injected with AAV8-nVEGFi at 4×10⁶, 4×10⁷, 4×10⁸ or 4×10⁹ GC/eye or PBS (vehicle of AAV or proteins) as a vehicle control. Retinal laser photoacoagulation was performed 4 weeks post injection to induce CNV (see Figure 3A for an illustration of AAV treatment timelines and a typical fundus image with an injection bleb). In addition, we established afiblerecept and nVEGFi protein treated groups. In these two groups, mice were intravitreally injected with 2.5 μg/eye afiblerecept or an equimolar dose of nVEGFi on the same day as the laser at 8 weeks old.

Six days after laser photoacoagulation, fundus fluorescein angiography (FFA) showed decreased leakage in AAV8-nVEGFi at 4×10⁶ and 4×10⁷ GC/eye, as well as afiblerecept and nVEGFi protein treated groups (representative pictures in Figure 3B). The retinal pigment epithelium (RPE)-choroid-sclera complexes were prepared for Isolec-tin-B4 (IB4) staining and evaluation of the CNV area 7 days after laser photoacoagulation. The CNV area was significantly reduced in groups treated with subretinal injection of AAV8-nVEGFi at 4×10⁶ (78.3%, p < 0.0001) and 4×10⁷ (80.4%, p < 0.0001) GC/eye and in groups treated with intravitreal injection of afiblerecept (79.7%, p < 0.0001) and nVEGFi protein (77.3%, p < 0.0001) compared to PBS control group, with no significant difference among these four groups (Figures 3C and 3D). These results demonstrated that intravitreal injection of nVEGFi protein and subretinal injection of AAV8-nVEGFi at 4×10⁶ and 4×10⁷ GC/eye had the same anti-VEGF ability as afiblerecept in vivo.

To compare AAV8-nVEGFi to AAV8-afiblerecept, mice were injected with AAV8-afiblerecept at 4×10⁶ or 4×10⁷ GC/eye and treated with the same procedure as before (Figure 3A). FFA showed that AAV8-afiblerecept at both doses reduced leakage (Figure 3B). Compared to the PBS-treated group, the CNV area was reduced 63.9% at 4×10⁶ GC/eye (p < 0.0001) and 67.8% at 4×10⁷ GC/eye (p < 0.0001) (Figures 3C and 3D). However, the reduction was significantly decreased compared with AAV8-nVEGFi at the same dose, 4×10⁶ GC/eye (p < 0.05) or 4×10⁷ GC/eye (p < 0.05).

### Long-term expression of AAV8-nVEGFi in vivo

To compare the expression of AAV8-nVEGFi and AAV8-afiblerecept in vivo, AAV8-nVEGFi vector at 4×10⁶, 4×10⁷, 4×10⁸, or 4×10⁹ GC/eye, or AAV8-afiblerecept vector at 4×10⁶ or 4×10⁷ GC/eye were administered by subretinal injection to 4-week-old C57BL/6J mice, with PBS as a negative control. Since the expression of AAV8-nVEGFi was stable from 4 weeks to 12 weeks post injection.

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**Table 1. Kinetic binding parameters for nVEGFi and afiblerecept binding to human VEGF family ligands determined by SPR-Biacore**

| VEGF inhibitor | Ligand | ka/10⁶ (M⁻¹ s⁻¹) | kd/10⁹ (s⁻¹) | KD (pM) |
|----------------|--------|------------------|--------------|---------|
| nVEGFi         | VEGF-A₁₆₅ | 46.6             | 12.6         | 27.1    |
|                | PlGF    | 6.19             | 78.4         | 1270    |
| Afiblerecept   | VEGF-A₁₆₅ | 116              | 17           | 14.7    |
|                | PlGF    | 8.11             | 1.41         | 1740    |

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In summary, dose levels of AAV8-nVEGFi from $4 \times 10^6$ to $4 \times 10^8$ GC/eye were delivered subretinally to C57BL/6J male mice at 4 weeks of age. Meanwhile, AAV8-afibercept or AAV8-EGFP at $4 \times 10^6$ or $4 \times 10^8$ GC/eye was injected as a control. Subsequently, optical coherence tomography (OCT) and field scotopic electroretinography (ERG) were performed at 4 weeks and 12 weeks after the injection, and mice were sacrificed for light microscopy evaluation immediately after the second ERG analysis.

Safety of AAV8-nVEGFi in C57BL/6J mice at different doses

We found a significant dose-related decrease in ERG amplitudes in eyes treated with AAV8-nVEGFi, AAV8-afibercept, and AAV8-EGFP. Eyes injected with AAV8-nVEGFi at the higher dose ($4 \times 10^8$ GC/eye) resulted in a decrease in both b-wave (−52.8%, p < 0.001) and a-wave amplitudes (−34.6%, p > 0.05) at 4 weeks post injection. The decrease in ERG was more pronounced at 12 weeks post injection, with a 60.1% (p < 0.01) decrease in b-wave amplitude and a 70.1% (p < 0.0001) decrease in a-wave amplitude. Eyes injected with AAV8-nVEGFi at $4 \times 10^6$, $4 \times 10^7$ or $4 \times 10^8$ GC/eye showed no deterioration in ERG (Figure 5A). However, eyes treated with AAV8-afibercept and AAV8-EGFP revealed a deteriorated ERG at both $4 \times 10^6$ and $4 \times 10^9$ GC/eye, with b-wave amplitude decreased by 66.4% in AAV8-afibercept at $4 \times 10^7$ and $4 \times 10^8$ GC/eye, 83.0% in AAV8-afibercept at $4 \times 10^7$ GC/eye, 85.5% in AAV8-EGFP at $4 \times 10^7$ GC/eye and 96.6% in AAV8-EGFP at $4 \times 10^9$ GC/eye 12 weeks post injection (Figure 5A).

Consistently, no significant retinal tissue abnormality was found in eyes treated with $4 \times 10^6$, $4 \times 10^7$, or $4 \times 10^8$ GC/eye AAV8-nVEGFi compared to PBS (Figures 5B and S3). However, eyes treated with $4 \times 10^8$ GC/eye presented with locally extensive ONL thinning, which was also present in eyes treated with AAV-afibercept at $4 \times 10^7$ GC/eye and AAV-EGFP at $4 \times 10^7$ and $4 \times 10^8$ GC/eye (Figures 5B and S4). In summary, dose levels of AAV8-nVEGFi from $4 \times 10^6$ to $4 \times 10^8$ GC/eye GC/eye were well tolerated. AAV8-nVEGFi at $4 \times 10^9$ GC/eye, AAV8-afibercept and AAV-EGFP at $4 \times 10^8$ and $4 \times 10^9$ GC/eye were associated with progressive reductions in ERG signals and retinal atrophy.
**DISCUSSION**

This study describes a novel VEGF Trap, nVEGFi, with comparable anti-VEGF efficacy to aflibercept. However, the addition of the first domain of VEGFR1 in nVEGFi resulted in a significantly higher amount of expression than aflibercept under the same conditions in vitro and in vivo. Because nVEGFi and aflibercept are secretory proteins that work extracellularly, the amount of VEGF Traps was first measured in the supernatant of cells transfected with plasmids or infected with AAV. When cells were transfected with plasmids, the amount of nVEGFi in the supernatant was 19.3 times higher than aflibercept, and when cells were infected with AAV with plasmids, it was 25.8 times higher. The expression of nVEGFi in cells in vitro was approximately 5.5 times higher than that of AAV8-aflibercept at $4 \times 10^5$ GC/eye, consistent with the in vitro results. The comparison of the expression level of VEGF Traps by the amounts of proteins, even transfected with the equal molar of plasmids or same copies of virus, is complicated by the different molecular weights of the two VEGF Traps. Aflibercept is a dimeric glycoprotein with a protein molecular weight of 97 kDa and contains glycosylation, constituting an additional 15% of the total molecular mass, resulting in a total molecular weight of 115 kDa. nVEGFi is also a dimeric protein with a protein molecular weight of approximately 125 kDa, which is approximately 1.3 times that of aflibercept. Even when the greater molecular weight is taken into consideration, adding domain 1 of VEGFR1 can boost the expression and, more predominantly, secretion of VEGF Trap. However, it is unknown why expression and secretion have improved.

The nVEGFi was designed based on the extracellular domains of VEGFR1, which have a higher VEGF-A binding affinity than VEGFR2 and can also bind to VEGF-B and PlGF.23 The second domain of VEGFR1 contains critical determinates required for the interaction with VEGF and PlGF, but full binding requires the additional presence of flanking domains 1 and 3, which can
be substituted by the homologous domains of other VEGF receptors. We reengineered VEGF-Traps and found that aflibercept, containing VEGFR1 domain 2 and VEGFR2 domain 3, was one of the most potent and effective VEGF blockers among a group of VEGF Traps because of its improved pharmacokinetic profile and high binding affinity to VEGF. VEGF-Grb, containing the second and third domains of VEGFR1 fused to human IgG1 Fc, was developed with stronger antiangiogenic efficacy than aflibercept. We designed nVEGFi, consisting of domains 1 and 2 of VEGFR1 and domain 3 of VEGFR2, and found that it had a higher expression and noninferior VEGF binding affinity and inhibition properties than aflibercept. Even though no pharmacokinetic evaluation was performed, the intravitreal injection of nVEGFi showed a similar CNV inhibition to aflibercept at the equimolar dose, which indicated that nVEGFi could be another potential anti-VEGF agent.

AAV8 was chosen to deliver nVEGFi because of its strong transduction of both RPE and photoreceptors. According to the fluorescence in situ hybridization results, the RPE is the first transduced cell at the lower dose of AAV8-nVEGFi. The whole retina could be transduced at a higher dose, which is in line with a previous study. Both retinal cell-type-specific promoters and ubiquitous promoters have been employed in gene therapy of ocular diseases. In most clinical trials of gene therapy for nAMD, ubiquitous promoters, including CB7/CAG, CMV and CAG, were used, which can drive much higher gene expression than many tissue-specific promoters. Tissue-specific expression has also been evaluated in mice to express miRNAs or proteins in specific cells, usually RPE, to downregulate angiogenesis to treat nAMD. In addition, inducible regulation of transgene expression could be a promising approach for regulating anti-VEGF agent dose to fit the patient-specific effective dose. Despite gene augmentation or gene silencing, gene knockout by the CRISPR/Cas system showed the potential to ablate pathologic angiogenesis and treat nAMD.

The two most common ways to deliver AAV are intravitreal and subretinal injections. Subretinal injection leads to superior retinal gene transfer compared with intravitreal injection in nonhuman primates. Moreover, intravitreal injection causes increased and persistent distribution of vector genomes in blood and lymphatic tissues, raising concerns regarding the immune response and off-target transduction. In this case, subretinal injection of AAV8-nVEGFi was used in this study. Currently, subretinal injection involves vitrectomy surgery, which is subject to potential complications. A novel method of subretinal injections using transscleral microneedles eliminates the need for vitrectomy surgery, simplifying the procedure and reducing the risks associated with the procedure, which could be considered for further experiments in nonhuman primates and clinical trials. Suprachoroidal injection could provide widespread transgene expression in the RPE but might lead to localized inflammation in rhesus macaques. This approach is now under evaluation in clinical trials for nAMD (NCT04514653) and diabetic retinopathy (NCT04567550).

The capsid, promoter, nature of the transgene, other cis regulatory factors and delivery approach all have a role in the effectiveness of transduction and transgenic expression. Thus, the effective dose threshold needs to be established for each vector, administered route, and target tissue. The increase in the expression level of AAV8-nVEGFi at the three lower doses showed a linear increasing trend; however, the nVEGFi level in eyes treated with 4 × 10⁸ GC was approximately 1.2 times higher than that of eyes treated with 4 × 10⁷ GC. Liu et al. showed a lower expression of a high dose of AAV8-antiVEGFab than a low dose. According to the toxicity results in this study, segmental dystrophy caused by a high AAV dose might be one of the explanations. Based on the expression assay and CNV area inhibition study, the minimum effective dose of subretinally injected AAV8-nVEGFi for nAMD treatment in mice should be in the range between 4 × 10⁷ and 4 × 10⁸ GC/eye. Although it is difficult to compare the effective doses from different experiments, this dose between 4 × 10⁷ and 4 × 10⁸ GC/eye for mice is relatively low, since the effective dose for subretinal injection in mice is typically 1 × 10⁸ to 1 × 10⁹ GC/eye. In this study, AAV8-aflibercept at 4 × 10⁸ GC/eye performed worse than AAV8-nVEGFi at 4 × 10⁸ GC/eye in reducing CNV areas (63.8% vs. 78.3%, p < 0.05), and it appeared that even AAV8-aflibercept at 4 × 10⁷ GC/eye performed worse than AAV8-nVEGFi at 4 × 10⁸ GC/eye (67.8% vs. 78.3%), even if the difference was not statistically significant. This is consistent with the expression results in vivo, where the expression of VEGF Trap in AAV8-aflibercept at 4 × 10⁸ GC/eye was much lower than that in AAV8-nVEGFi at 4 × 10⁸ GC/eye (1,309 ± 695.6 vs. 3,503 ± 856.9 ng/eye).

The use of AAV in the clinic demonstrated the virus’s safety profile. Nonetheless, it is known that AAV-mediated gene therapy of the retina can induce retinal toxicity at high doses. The nature of the transgene and the type of promoter, in addition to the AAV input dose, all have a role in determining the extent of retinal toxicity, which can be entirely avoided at low doses. Although subretinal injection can cause traumatic retinal lesions, it tends to cause focal retinal perforation with disorganization of the ONL to complete retinal rupture. Retinal thinning and retinal function impairment, observed in this study, have also been observed before in mice, dogs, and nonhuman primates and coincided with but not always limited to the region of the subretinal injection and appeared to be dose related. It is unclear how AAV-mediated gene therapy triggers retinal toxicity. A direct host cell response to transgene expression or vector uptake, indirectly from harmful immune responses to the vector or the transgene product, or a combination of both are hypotheses of the mechanism. High doses of AAV expressing no transgene (“null”) have been shown to lead to retinal toxicity. Different transgenes can cause varying degrees of damage to the retina, implying that both the capsid and the transgene play a role in toxicity. In this study, we evaluated the safety of AAV8-nVEGFi, AAV8-aflibercept and AAV8-EGFP by ERG and retinal histology. The dose of each AAV appeared to be the deciding factor in the level of retinal toxicity, demonstrating that lowering the effective dose of AAV by increasing transgene...
expression is an efficient strategy to reduce retinal toxicity. However, the damage caused by different AAVs at the same dose differed, and AAVs encoding EGFP were more toxic with deteriorated ERG and retinal thinning, which has been reported previously.47 Interestingly, AAV encoding aflibercept was more hazardous than AAV expressing nVEGF in according to ERG, although it had a lower expression level, implying that nVEGF is safer for the retina than aflibercept.

In conclusion, a novel VEGF Trap, nVEGF, was developed with a comparable capacity to bind and block VEGF in vitro and in vivo. The most notable feature of nVEGF is its significant expression augmentation, which reduces the effective dose when delivered by AAV in vivo and improves the safety profile of gene therapy. The preclinical dose-escalation study demonstrated that AAV8-nVEGF has long-term expression and can inhibit CNV safely and effectively in a mouse model.

MATERIALS AND METHODS

Plasmid construction

Aflibercept and the nVEGF gene were codon optimized and synthesized by Genewiz, and the sequences are presented in the supplemental information. To construct the AAV vector, the codon optimized aflibercept or nVEGF gene was subcloned into a parental cis plasmid containing the CB7 promoter, chicken β-actin intron, kozak sequence, and βGH polyadenylation sequence flanked by AAV2 ITRs, yielding the pAAV-aflibercept and pAAV-nVEGF plasmid vectors, respectively. The pAAV-EGFP plasmid was constructed by replacing the nVEGF gene of pAAV-nVEGF with EGFP, serving as a negative control. All constructed plasmids were verified by sequencing.

VEGF traps

The nVEGF gene was subcloned in pATX2 to construct an expression vector, pATX2-nVEGF. One liter of HEK293F cells was transfected with pATX2-nVEGF. Culture medium was collected on the sixth day post transfection. Then, the culture medium was pooled and filtered through a 0.22 μm membrane and loaded onto the column with Protein G resin (Smart Lifesciences). The resin was then washed with 10 column volumes of PBS (pH 7.5). The nVEGF protein was eluted from the resin with 0.1 M glycine (pH 2.7) and neutralized with 1 M Tris-HCl (pH 8.5). The commercial anti-VEGF antibody aflibercept (Eylea, 2 mg/0.05 mL) was purchased from Bayer Pharmaceuticals.

Plasmid transfection

HEK293 cells (ATCC) were cultured with DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (PAN Biotech) and 100 U/mL penicillin/streptomycin at 37°C with 5% CO2. One day before transfection, HEK293 cells were seeded into 6-well plates at

![Figure 4. Expression of nVEGF or aflibercept 12 weeks after subretinal injection of AAV8-nVEGF or AAV8-aflibercept in mice](image-url)
106 cells per well. Twenty-four hours later, cells were transfected with 360 fmol pAAV-aflibercept, pAAV-nVEGFi or pAAV-EGFP using polyethylenimine (PEI) at a 1:2 ratio of DNA:PEI in FBS-free DMEM with 100 U/mL penicillin/streptomycin. After 4 h, the medium was replaced with 1 mL FBS-free DMEM with 100 U/mL penicillin/streptomycin. For ELISA and western blot, culture medium or cells were harvested 48 h after transfection. For real-time qPCR, cells were harvested 24 h after transfection.

**Real-time qPCR**

Total RNA was extracted from cells with an RNAprep pure cell kit (Tiangen) according to the manufacturer’s instructions. Then, RNA was reverse transcribed to cDNA using the PrimeScript RT reagent Kit with gDNA eraser (perfect real time) (Takara) according to the manufacturer’s instructions. DNase was used in RNA extraction and transcription to ensure elimination of DNA contamination. Quantitative PCR was performed with TB Green Premix Ex Taq II (Takara). Primers for VEGF Trap (nVEGFi and aflibercept) and beta-actin genes are presented in the supplemental information. The qPCRs were run in triplicate for each gene per sample. The specificity of the qPCR was confirmed by detection of a single distinct peak on examination of the dissociation curve profile of the reaction product. The relative gene expression was calculated using the ∆∆Ct method. Target gene expression was normalized to the housekeeping reference gene beta-actin and then compared to the control (pAAV-aflibercept).

**ELISA**

Levels of VEGF-Traps in cell supernatant were measured 48 h after transfection. Levels of VEGF-Traps in eyes were measured 12 weeks after injection. Eyes were homogenized in 200 μL radioimmunoprecipitation assay buffer with a bead mill homogenizer (OMNI...
international, Inc.). The concentration of nVEGFi or aflibercept was measured by ELISA using known concentrations of purified nVEGFi or aflibercept (Bayer Pharmaceuticals) to generate standard curves. Briefly, ELISA plates were coated with 100 µL human VEGF-A165 (1 µg/mL) in carbonate/bicarbonate buffer at 4°C overnight. Plates were washed four times with PBST (0.1% Tween in PBS) and blocked with 5% BSA in PBST for 2 h at 37°C. Plates were washed four times with PBST, and samples were added to the wells and then incubated for 2 h at 37°C. After washing four times with PBST, plates were incubated with goat anti-human IgG Fc (HRP conjugate) (Sigma-Aldrich) diluted 1:30,000 at 37°C for 1.5 h. After 4 washes with PBST, the substrate was developed with 3,3′,5,5′-tetramethyl benzidine (NeoBio-science) at room temperature (RT) for 20 min, and then 100 µL ELISA stopped solution was added. Plates were read by Multiskan Sky (Thermo Fisher Scientific) at 450 nm and 570 nm.

**Western blot**

Cell supernatants (10 µL) and lysated (20 µg) from transfected HEK293 cells were analyzed by Western blot under nonreduced or reduced conditions. Aflibercept and nVEGFi proteins were detected by anti-VEGF receptor 1 antibody (Abcam) diluted 1:2,000. Beta-actin was detected by rabbit monoclonal anti-beta actin antibody (Proteintech) diluted 1:10,000. Briefly, samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST at RT for 2 h and then incubated with primary antibodies at RT for 2 h. Membranes were washed three times in TBST, and HRP-conjugated goat anti-rabbit IgG (Zsbio) diluted 1:10,000 was incubated. The protein bands were visualized via Immobilon western chemiluminescent HRP substrate (Millipore). Quantification of bands was performed using Image (Image Processing and Analysis in Java; the National Institutes of Health). The amount of nVEGFi in cell lysates was normalized to beta-actin.

**AAV8 vector production**

All AAV8 vectors were produced by triple plasmid transfection of HEK293 cells as previously described. The genome titer (GC/mL) of the AAV8 vector was determined by digital droplet polymerase chain reaction (ddPCR) using forward primer 5′-TAGTTGCCAGC-CATCTGTG-3′, reverse primer 5′-TAGGAAGAGCATGG-GAGT-3′, and probe 5′-Fam-CCTGTCCTTCTGACCT-T-BHQ-3′. All vectors used in this study passed the endotoxin assay using the endpoint chromogenic endotoxin test kit (Xiamen Bioendo Technology Co., Ltd.).

**AAV transduction in vitro**

HEK293 cells were seeded into 96-well plates at a density of 5 × 10^5 cells/well in 200 µL DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. The cells were allowed to adhere for 24 h. After 24 h, cells were infected with wild-type adenovirus (H5 serotype, ATCC) at a multiplicity of infection (MOI) of 30 GC/cell. Two hours post-infection with adenovirus, cells were transduced with AAV8 vectors at an MOI of 6 × 10^5 GC/cell in 100 µL FBS-free DMEM with 100 U/mL penicillin/streptomycin. After 4 h, the infection medium was replaced with 100 µL DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. Three days post AAV8 infection, all cell supernatant was collected, and the protein expression was determined by ELISA.

**Surface plasmon resonance**

SPR experiments were performed on a Biorex 8K (GE Healthcare) instrument using the Series S Sensor CM5 chip (GE Healthcare) at RT. The running buffer was filtered HBs-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% polysorbate 20, pH 7.4). The surface of the CM5 chip was activated with standard amine coupling reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. The ligands (nVEGFi and aflibercept) were diluted to 10 µg/mL with fixative reagent (10 mM sodium acetate, pH 5.0) and immobilized on the surface of a CM5 chip for 90 s. A range of concentrations of analytes (1.5625–25 nM for VEGF-A165 and 1.5625–50 nM for PlGF) were individually injected into the experimental channel and reference channel at a flow rate of 30 µL/min with a binding time of 120 s and a dissociation time of 600 s. The activated coupled chip surfaces were then washed with 10 mM glycine HCl, pH 1.5, to remove uncoupled residual proteins. The KD value of each antibody was calculated using analysis software.

**VEGF-binding assay**

The binding affinities of nVEGFi and aflibercept were measured by a human VEGF ELISA kit (Novus Biologicals) to detect residual unbound human VEGF in mixtures of nVEGFi or aflibercept (0.05–400 pM) with human VEGF-A165 (R&D Systems) at a final concentration of 10 pM and incubated at RT overnight. VEGF concentration (pM) was plotted as a function of increasing anti-VEGF molecule concentration (pM).

**HUVEC proliferation assay**

HUVECs (ScienCell Research Laboratories) were expanded through five passages in EGM-2 media (Lonza). HUVECs were seeded at 6 × 10^3 cells per well in a 96-well culture plate and incubated overnight in M199 (Gibco) starvation media (M199, 5% FBS). The following day, fresh M199 media supplemented with 20 ng/mL recombinant human VEGF-A165 (R&D Systems) and 1,100 pM aflibercept, nVEGFi protein or nVEGFi from the supernatant of HEK293 cells transfected with pAAV-nVEGFi were added. Absorbance was measured at OD490 with Multiskan Sky (Thermo Fisher Scientific).

**Animals**

Male C57BL/6J mice were purchased from Chengdu Dossy Experimental Animals Co., Ltd. and group-housed at four to six animals per cage in a temperature- and humidity-controlled, specific-pathogen-free animal facility at 25°C under a 12 h–12 h light–dark cycle with free access to food and water. Unless otherwise stated, mice were male.
anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (12 mg/kg) in this study. The pupils were dilated with an eye drop containing 0.5% tropicamide and 0.5% phenylephrine hydrochloride. Animal experiments were approved by the Institutional Animal Care and Concern Committee at Sichuan University, and animal care was in accordance with the committee’s guidelines.

Subretinal injection and intravitreal injection
Mice were subjected to bilateral, subretinal injection with AAV8-nVEGFi, AAV8-afibbercept, AAV8-EGFP, or PBS at 4 weeks of age. After anesthesia by isoflurane inhalation and pupil dilation, a limbal hole was made with a 31G needle under a stereomicroscope. Then, a blunt 33G needle (Hamilton) was inserted through the hole and directed toward the subretinal space, avoiding lens damage. Each eye was given 1 µL AAV8-nVEGFi at a titer of $4 \times 10^6$, $4 \times 10^7$, $4 \times 10^8$ or $4 \times 10^9$ GC/µL, AAV8-afibbercept or AAV8-EGFP at a titer of $4 \times 10^6$ or $4 \times 10^7$ GC/µL, or PBS. Immediately after injection, a retinal imaging microscope (Micron IV, Phoenix Research Labs) was used to observe the fundus. Injections creating subretinal blebs without massive vitreous or subretinal hemorrhage were considered to be successful. After the examination, olofoxacin eye ointment was applied to the cornea. For the afibbercept (2.5 µg, 2 µL) and nVEGFi protein (3.25 µg, 2 µL) treated groups, the mice were bilaterally intravitreally injected immediately after laser photocoagulation at 8 weeks of age. The process of intravitreal injection was similar to that of subretinal injection, except for injecting the medicine into the vitreous cavity rather than the subretinal space. All the AAV, afibbercept, and nVEGFi proteins used for intraocular injection were diluted with PBS.

Fluorescent in situ hybridization
Mice were euthanatized 12 weeks after injection. Eyes were enucleated and fixed in 4% paraformaldehyde (PFA)/PBS at RT for 6 h. PFA/PBS-fixed eyes were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. In situ hybridization was performed on paraffin-embedded retinal sections using the ViewRNA ISH Tissue Assay Kit (Thermo Fisher Scientific) with a custom designed nVEGFi probe (CVX-01, assay ID: VP47VRZ, Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, paraffin sections were deparaffinized at 60°C for 4 h and washed in xylene and 100% ethanol. Tissue was incubated with 1x pretreatment solution at 95°C for 5 min, followed by protease digestion at 40°C for 20 min. Sections were fixed with 4% PFA for 5 min and incubated with alkaline phosphatase-conjugated nVEGFi probes at 40°C for 2 h. Following a 2 h incubation, sections were washed and placed in storage buffer overnight. Fluorescently labeled substrate (Fast Red) was used to detect nVEGFi probes. Slides were costained with DAPI and imaged using a confocal laser microscope (Nikon).

Laser-induced CNV
Laser induction of CNV in mice was performed by an image-guided laser system (Micron IV, Phoenix Research Laboratories) in accordance with the method described by Gong et al. After anesthesia and pupil dilation, 2.5% Hypromellose was applied to the mouse cornea. The laser settings were as follows: wavelength, 532 nm; diameter, 50 µm; duration: 70 ms; and power, 260 mW. Three of four laser burns were induced around the optic disc. The distance between two laser burns and between the laser burn and the optic disc was approximately double the diameter of the optic disc. The success of the operation was confirmed by the formation of a bubble and haze area around the lesion immediately after laser photocoagulation. Eyes with significant subretinal or vitreous hemorrhage were excluded.

FFA
FFA was performed 6 days after laser photocoagulation with a retinal imaging microscope (Micron IV, Phoenix Research Laboratories). After anesthetization and pupil dilation, mice were intraperitoneally injected with 200 µL 1% fluorescein (Alcon). At 5 and 10 min after fluorescein injection, fluorescein fundus images were taken.

Immunostaining of RPE and choroidal flat-mounts
Mice were euthanized 7 days after laser photocoagulation. Eyes were enucleated and fixed in 4% PFA/PBS at RT for 1 h. RPE complexes (RPE/choroid/sclera) were prepared and permeabilized with 0.1% Triton X-100 at RT for 1 h. Then the RPE complexes were stained with 10 µg/mL IB4 (isolecitin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 594 conjugate, Thermo Fisher Scientific) overnight at RT. After washing with PBS 3 times, the RPE complexes were flat mounted with the scleral side down and viewed with a fluorescence microscope (DFC7000 T, Leica) at a magnification of 20×. The CNV area was measured using ImageJ (National Institutes of Health) by blind observers.

ERG
Scotopic ERG was measured 4 weeks and 12 weeks post subretinal injection. ERG was recorded under the manufacturer’s instructions of the Phoenix Ganzfeld ERG (Phoenix Research Labs). Briefly, mice were dark adapted for 16 h and then all the preparations were operated under dim red light. After anesthesia, mice were placed on a heating pad to maintain body temperature. The pupils were dilated. The reference electrode was placed subcutaneously in the forehead between the ears, and the ground electrode was placed subcutaneously in the tail. A corneal electrode was placed on the cornea after applying 2.5% Hypromellose. ERG was recorded with stimulus intensity at 1.2 log cd s/m².

Hematoxylin and eosin
Eyes were enucleated and fixed in 4% PFA/PBS at RT for 6 h. PFA/PBS-fixed eyes were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Paraffin-embedded retinas were sectioned at 5 µm and stained with hematoxylin and eosin according to standard protocols for light microscopy and photomicrography (Leica).

Statistical analysis
Data are presented as mean ± SD in Figures 1, 2 and 4, as median (P25, P75) in Figure 3, and as mean ± SEM in Figure 5. GraphPad Prism (University of California, San Diego, California) was used to perform statistical analysis and make figures. Comparisons were
Dunnett's test was used to analyze the difference between two groups in Figure 1. One-way ANOVA analysis with Tukey's post hoc test was used in Figures 2B and 4A. The nonparametric Kruskal-Wallis test with post hoc Dunnett’s test were used to analyze the differences in CNV area among different groups in Figure 3E. The comparison of ERG among different groups in Figure 5A was carried out by one-way ANOVA and post hoc Dunnett’s test.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.01.002.

ACKNOWLEDGMENTS
This work was supported by the Joint Funds of the National Natural Science Foundation of China (grant no. U19A2002), National Major Scientific and Technological Special Project for "Significant New Drugs Development" (no. 2018ZX09733001-005-002), and the Science and Technology Major Project of Sichuan Province (no. 2017SZDZX0011).

AUTHOR CONTRIBUTIONS
Y.Y., F.L., and Y.W. conceived this study and designed the experiments; K.S. and J.S. constructed the plasmid vectors and conducted transfection experiments; K.S. and X.Z. performed ddPCR and endotoxin assays; K.S., X.Z., Q.Z., and R.L. performed in vitro functional studies; K.S. wrote the manuscript; Y.Y., H.D., and F.L. edited the manuscript, and all authors read and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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