Genomic Disruption of VEGF-A Expression in Human Retinal Pigment Epithelial Cells Using CRISPR-Cas9 Endonuclease

Glenn Yiu,[1] Eric Tieu,[1] Anthony T. Nguyen,[1] Brittany Wong,[2] and Zeljka Smit-McBride[1]

[1]Department of Ophthalmology & Vision Science, University of California, Davis, Sacramento, California, United States
[2]Duke University, Durham, North Carolina, United States

Correspondence: Glenn Yiu, Department of Ophthalmology & Vision Science, University of California Davis Eye Center, 4860 Y Street, Suite 2400, Sacramento, CA 95817, USA; gyiu@ucdavis.edu.

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Purpose. To employ type II clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 endonuclease to suppress ocular angiogenesis by genomic disruption of VEGF-A in human RPE cells.

Methods. CRISPR sequences targeting exon 1 of human VEGF-A were computationally identified based on predicted Cas9 on- and off-target probabilities. Single guide RNA (gRNA) cassettes with these target sequences were cloned into lentiviral vectors encoding the *Streptococcus pyogenes* Cas9 endonuclease (SpCas9) gene. The lentiviral vectors were used to infect ARPE-19 cells, a human RPE cell line. Frequency of insertion or deletion (indel) mutations was assessed by T7 endonuclease 1 mismatch detection assay; mRNA levels were assessed with quantitative real-time PCR; and VEGF-A protein levels were determined by ELISA. In vitro angiogenesis was measured using an endothelial cell tube formation assay.

Results. Five gRNAs targeting VEGF-A were selected based on the highest predicted on-target probabilities, lowest off-target probabilities, or combined average of both scores. Lentiviral delivery of the top-scoring gRNAs with SpCas9 resulted in indel formation in the VEGF-A gene at frequencies up to 37.0% ± 4.0% with corresponding decreases in secreted VEGF-A protein up to 41.2% ± 7.4% ($P < 0.001$), and reduction of endothelial tube formation up to 39.4% ± 9.8% ($P = 0.02$). No significant indel formation in the top three putative off-target sites tested was detected.

Conclusions. The CRISPR-Cas9 endonuclease system may reduce VEGF-A secretion from human RPE cells and suppress angiogenesis, supporting the possibility of employing gene editing for antiangiogenesis therapy in ocular diseases.

Keywords: CRISPR-Cas9, gene editing, VEGF-A

In recent years, the advent of antiangiogenesis agents targeting VEGF has revolutionized the treatment of neovascular and exudative retinal diseases ranging from choroidal neovascularization in AMD to macular edema associated with diabetes mellitus and retinal vein occlusions. Current anti-VEGF therapies in clinical practice include humanized antibodies (bevacizumab); antibody fragments (ranibizumab); and recombinant decoy receptor proteins (aflibercept) that are directed against various secreted isoforms of VEGF-A.[1–3] However, frequent intravitreal injections of these drugs are a heavy burden to clinicians and patients, and pose significant costs to health care.

Viral-mediated gene therapy holds the promise for providing a longer duration of VEGF inhibition. Adeno-associated viral (AAV) expression of sFlt-1, a soluble form of the VEGF receptor, reduces laser-induced choroidal neovascularization in monkeys and is undergoing clinical trials for patients with neovascular AMD.[4–6] Gene silencing with small-interfering RNAs or microRNAs against VEGF has also demonstrated success in reducing ocular neovascularization.[7–9] However, these RNA-targeted strategies are susceptible to off-target effects, and the efficiency of gene knockdown is difficult to predict.[10] Moreover, the duration of viral-mediated gene therapy remains uncertain. Long-term follow-up of Leber congenital amaurosis type 2 patients who received the RPE65 gene via AAV-mediated transduction showed possible loss of efficacy after 2 to 3 years.[11,12]

Rather than targeting angiogenic factors at the protein or RNA level, gene editing technology using the type II clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 endonuclease system enables permanent disruption of the encoding genes at the DNA level. Derived from the adaptive immune system of bacteria, the CRISPR-associated Cas9 endonuclease has the ability to introduce double-strand breaks (DSB) at a specific locus in genomic DNA by recognition of a specific 20-nucleotide DNA sequence and a 5′-NGG-3′ protospacer adjacent motif by a CRISPR RNA sequence (crRNA) combined with a trans-activating crRNA (tracrRNA). By engineering a single guide RNA (gRNA) to mimic this two-RNA structure, the Cas9 endonuclease can be programmed to create precise DSBs that trigger error-prone nonhomologous end-joining repair pathways, resulting in insertions/deletions (indels) which can cause nonsense or frameshift mutations to permanently disrupt the target gene at the genomic level. In vivo ablation of a dominant rhodopsin mutation using CRISPR-Cas9 has shown anatomic and functional improvements in a rat

model of retinitis pigmentosa.[13] Gene editing has also been used to repair a point mutation associated with X-linked retinitis pigmentosa in patient-derived induced pluripotent stem cells.[14] However, the potential use of CRISPR technology to target angiogenic factors has not yet been explored.

In this study, we employed a CRISPR-Cas9 genome editing strategy to disrupt the VEGF-A gene in human RPE cells. We designed a set of five gRNAs to target exon 1 of VEGF-A based on computational predictions of on-target and off-target probabilities; then cloned these gRNAs into lentiviral vectors encoding the *Streptococcus pyogenes* Cas9 (SpCas9) endonuclease. We found that lentiviral delivery of select gRNAs with SpCas9 resulted in up to 37.0% ± 4.0% indel formation, with a corresponding reduction in secreted VEGF-A protein, suppression of angiogenesis in vitro, and no detectable off-target effects. These studies provide as proof of concept the possibility of employing genome engineering techniques for potential antiangiogenesis therapy in ocular diseases.

Methods

gRNA and Lentiviral Vector Design

CRISPR gRNAs were computationally designed to predict Cas9 activity, using gRNA design software (Benchling, San Francisco, CA, USA) to determine an on-target score that predicts Cas9 cleavage efficiency using algorithms previously described,[15,16] and CRISPR design software from Feng Zhang Lab (MIT, Cambridge, CA, USA) to calculate an off-target score that denotes an inverse probability of Cas9 off-target binding.[17] Both scores are presented on a scale from 1 to 100, with a higher on-target score predicting higher Cas9 activity and a higher off-target score predicting lower off-target binding. Two gRNAs were designed using commercial CRISPR design services from GeneArt (Thermo Fisher Scientific, Waltham, MA, USA). Target sequences were cloned into the lentiCRISPR v2 plasmid (Addgene plasmid# 52961) via BsmBI restriction enzyme sites upstream of the scaffold sequence of the U6-driven gRNA cassette. The single-vector lentiviral system also includes SpCas9 expressed from an elongation factor 1α short promoter with a FLAG octapeptide tag and a puromycin resistance selection marker linked by a 2A self-cleaving peptide.[18] Lentiviral vectors were produced using the triple transfection method with calcium phosphate as previously described,[19] and were packaged by the Univeristy of California, (UC) Davis Center for Vision Science Molecular Construct and Packaging core facility (Davis, CA, USA).

Cell Culture and Lentiviral Transduction

Human RPE cell line ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium:F12 (DMEM:F12, ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 0.45% glucose, and penicillin/streptomycin 100 units/mL; 100 µg/mL (Life Technologies, Benicia, CA, USA), in a humidified incubator with 5% CO_2 at 37°C. Cells were grown to 70% confluence and infected with lentiviral vectors at a multiplicity of infection (MOI) of 2 to 3. The media was changed 1 day after viral transduction and incubated for 3 days before selection with 1.5 µg/mL puromycin (EMD Millipore, Hayward, CA, USA) for 4 days. Genomic DNA, total mRNA, and conditioned media were collected at 14 days after viral transduction.

T7 Endonuclease I Assay

Genomic DNA from infected cells were extracted with a commercial kit (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany) following manufacturer's protocol, and quantified using a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific). The targeted regions in exon 1 of the VEGF-A gene were PCR-amplifed with high-fidelity DNA polymerase (Phusion; New England Biolabs, Ipswich, MA, USA) using primers flanking the target sites (Supplemental Table S1) and purified with a PCR purification kit (QIAquick; Qiagen). We denatured 200 ng of the PCR product then slowly hybridized to form heteroduplexes using the following program settings: 95°C for 5 minutes, 95° to 85°C at −2°C/second, 85° to 25°C at −0.1°C/second. Heteroduplexes were digested with T7 endonuclease I (New England Biolabs) at 37°C for 15 minutes. The reaction was stopped with 0.02 M EDTA, and the digested products were separated on a 2% TBE agarose gel for analysis. Band intensites were measured using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and the percentage of indel formation was calculated using the equation: indel% = $100 \times (1 - [1 - f_{cut}]^{1/2})$, where f_{cut} (cut fraction) is the total relative density of the cleavage bands divided by the sum of the relative density of the cleavage and uncut bands, as previously described.[20]

DNA Sequencing

Sanger sequencing of PCR products amplified from genomic DNA, using sequencing primers approximately 200 to 250 bp upstream from predicted cut sites (Supplemental Table S1), were performed by the UC Davis DNA sequencing facility. Deep sequencing of PCR products using primers flanking the target sites (Supplemental Table S1) was performed by the Massachusetts General Hospital DNA sequencing core facility (Cambridge, MA, USA).

Quantitative RT-PCR analysis

Virally transduced ARPE-19 cells were collected and stored (RNAlater; Thermo Fisher Scientific). Total RNA was then extracted and purified using an RNA purification kit (RNeasy Mini Kit; Qiagen) following manufacturer's protocol. Concentration of RNA was measured using a spectrophotometer (Thermo Fisher Scientific). Total RNA was reversed transcribed using a reverse transcription kit (QuantiTect; Qiagen), and qPCR performed using the following Taqman probes: Human VEGFA Hs00900055_m1, and three reference genes, Human GAPDH Hs99999905_m1, Human B2M Hs00984230, and Human HPRT1 Hs99999909_m1 (Thermo Fisher Scientific).

Enzyme-Linked Immunosorbent Assay

For enzyme-linked immunosorbent assays, infected cells were changed to low-serum media (DMEM:F12 with 1% FBS to preserve VEGF stability) and conditioned media collected over 3 hours. Protein secretion of VEGF was measured using a human VEGF ELISA kit (Quantikine; R&D systems, Minneapolis, MN, USA) following the manufacturer's protocol, normalized to total cell number per sample, and quantified using a microplate reader (Versamax; Molecular Devices, Sunnyvale, CA, USA).

Endothelial Cell Tube Formation

Human umbilical vascular endothelial cells (HUVECs) were cultured in Medium 200PRF (Thermo Fisher Scientific) supplemented with low serum growth supplement (Thermo Fisher Scientific), in a humified incubator with 5% CO_2 at 37°C. We coated 24-well plates with 10 mg/mL Matrigel (Corning, Corning, NY, USA) and incubated at 37°C for 30 minutes. We

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resuspended 25,000 HUVECs in unsupplemented media (Thermo Fisher Scientific) with conditioned ARPE-19 media in 200 μL total volume. The cells were incubated at 37°C for 4 to 8 hours, then imaged using an inverted microscope (Eclipse TE 200; Nikon Corp., Tokyo, Japan) at ×4 magnification. Tube formation was quantified from five random fields for each experiment using the Angiogenesis Analyzer for ImageJ tool, and reported as total length (mm) per unit area (mm^2).

Statistical Analysis

All results are presented as means ± standard deviation. We used 1-way ANOVA with Dunnett's posttests to compare mRNA levels, protein levels, and endothelial tube formation. Statistical analyses were conducted using statistical software (SPSS version 22.0; IBM Corp., Armonk, NY, USA).

Results

gRNA Design for VEGF-A Disruption

Vascular endothelial growth factor A is a diffusible mitogen that is secreted by RPE and other cells in the eye in response to hypoxic and inflammatory stimuli. Alternatively spliced transcript variants give rise to at least seven homodimeric isoforms, of which $VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$ represent the major isoforms and are differentially expressed in ocular neovascular conditions.[21] Exons 1 through 5 and 8 are conserved in all isoforms except $VEGF_{148}$, and encode the receptor-binding domain.[22] To enable genomic disruption of all VEGF isoforms, we designed CRISPR gRNAs to target locations in exon 1 of the human VEGF-A gene (Supplemental Figs. S1A, S1B), in which a frameshift mutation is most likely to result in truncated, nonfunctional gene products. Using computational methods to predict on-target Cas9 endonuclease activity and off-target Cas9 binding, we identified site 1 for the highest on-target and average on/off-target scores. Site 2 was selected for the second highest on-target score. Site 3 was chosen for the highest off-target score (lowest probability for off-target effects). Sites 4 and 5 were identified using commercial CRISPR design services (GeneArt; Thermo Fisher Scientific). These five gRNA target sequences were individually cloned into lentiCRISPR v2 constructs upstream of the gRNA scaffold sequence (Supplemental Fig. S1C), and used to produce lentiviral vectors that target VEGF-A. Viral vectors expressing Cas9 only without VEGF-targeted gRNA sequences were used as controls.

Genomic Disruption of VEGF-A Gene by Programmed Cas9

Human RPE cell line ARPE-19 is a spontaneously arising cell line that exhibits phenotypic characteristics of RPE such as pigmentation, barrier property, and polarity of secreted signals.[23] They also have a similar pattern of VEGF isoform expression as human RPE in vivo, including $VEGF_{165}$ as the predominant form, $VEGF_{121}$, and $VEGF_{189}$.[24,25] To demonstrate the possibility of genomic disruption of VEGF-A in human RPE cells, we infected ARPE-19 cells with Cas9-expressing lentiviral vectors to target VEGF-A, and showed frequencies of indel formation between 33.5% ± 3.8% and 37.0% ± 4.0% using the T7 endonuclease I (T7E1) mismatch detection assay (Fig. 1A). Formation of indel did not change significantly with higher viral concentrations (up to MOI of 10; Supplemental Fig. S2). Sanger sequencing confirmed that indel formation occurred at the predicted sites (Fig. 1B), although the chromatogram for target 5 suggests lower indel formation than the frequency indicated by the T7E1 assay. Deep sequencing also confirmed indel formation at the predicted sites, including a variety of frameshift and in-frame mutations (Fig. 1C, Supplemental Fig. S3). Interestingly, the gRNA with the highest on-target score (V-1) produced the highest frequency of indels, although on-target scores for other gRNAs did not always correlate directly with the indel frequency.

Reduction of VEGF-A Secretion by Programmed Cas9

To determine if genomic disruption of the VEGF-A gene resulted in changes in mRNA or protein levels, we performed quantitative RT-PCR on total RNA, and ELISA analysis on conditioned media from the infected cells. Since Cas9 endonuclease activity only alters the DNA gene sequence to introduce indel mutations, we would not expect significant alterations in VEGF-A mRNA levels unless a transcriptional stop or unstable RNA transcript has been created. Consistent with this prediction, quantitative RT-PCR showed no significant reduction in mRNA expression of VEGF-A after infection with any of the Cas9 constructs ($P = 0.583$; Fig. 2A). However, we did note significant variability in mRNA levels between individual experiments, suggesting possible effects on mRNA stability in some cell populations that may be stochastic in nature.

Using ELISA to measure VEGF-A protein levels, we found that gRNAs with the highest indel frequencies resulted in significant reduction of secreted VEGF-A, ranging from 20.8% ± 19.4% to 41.2% ± 7.4% ($P < 0.001$; Fig. 2B). Target V-5, which had a lower on-target score and lower indel frequency suggested by Sanger sequencing chromatograms, showed no significant reduction in VEGF-A. These results demonstrate that Cas9-mediated VEGF-A gene disruption can reduce VEGF secretion from human RPE cells, and that computational estimates of Cas9 activity may predict efficacy of gene product suppression.

Reduction of In Vitro Angiogenesis by Programmed Cas9

In addition to reducing the amount of secreted VEGF-A, Cas9-mediated indel mutations may also lead to functional disruption of the VEGF-A protein. For example, missense mutations may allow translation of full-length VEGF protein, but can cause protein misfolding and functional loss. To measure angiogenesis in vitro, we employed a human endothelial tube formation assay using conditioned culture media from the ARPE-19 cells. Consistent with the ELISA results, we detected a 30.5% ± 18.8% to 39.4% ± 9.8% decrease in tube formation ($P = 0.02$), with the most significant reduction using conditioned media that had the greatest reduction of VEGF-A levels (Fig. 3). Thus, genomic disruption of VEGF-A in human RPE cells using CRISPR-Cas9 may reduce angiogenesis in an in vitro model.

Off-Target Effects of Programmed VEGF-A Gene Disruption

Cas9-mediated gene disruption may be susceptible to off-target mutations if the gRNA sequences share homology with other loci in the genome. To investigate potential off-target effects of Cas9-mediated VEGF-A disruption, we investigated putative exonic off-target sites for each gRNA sequence, based on the level of homology with the target gRNA (Fig. 4A). Using the T7E1 mismatch assay, we did not detect any nonspecific cleavage activity at the three most probable off-target exonic loci for any of the gRNA target sequences (Fig. 4B). These

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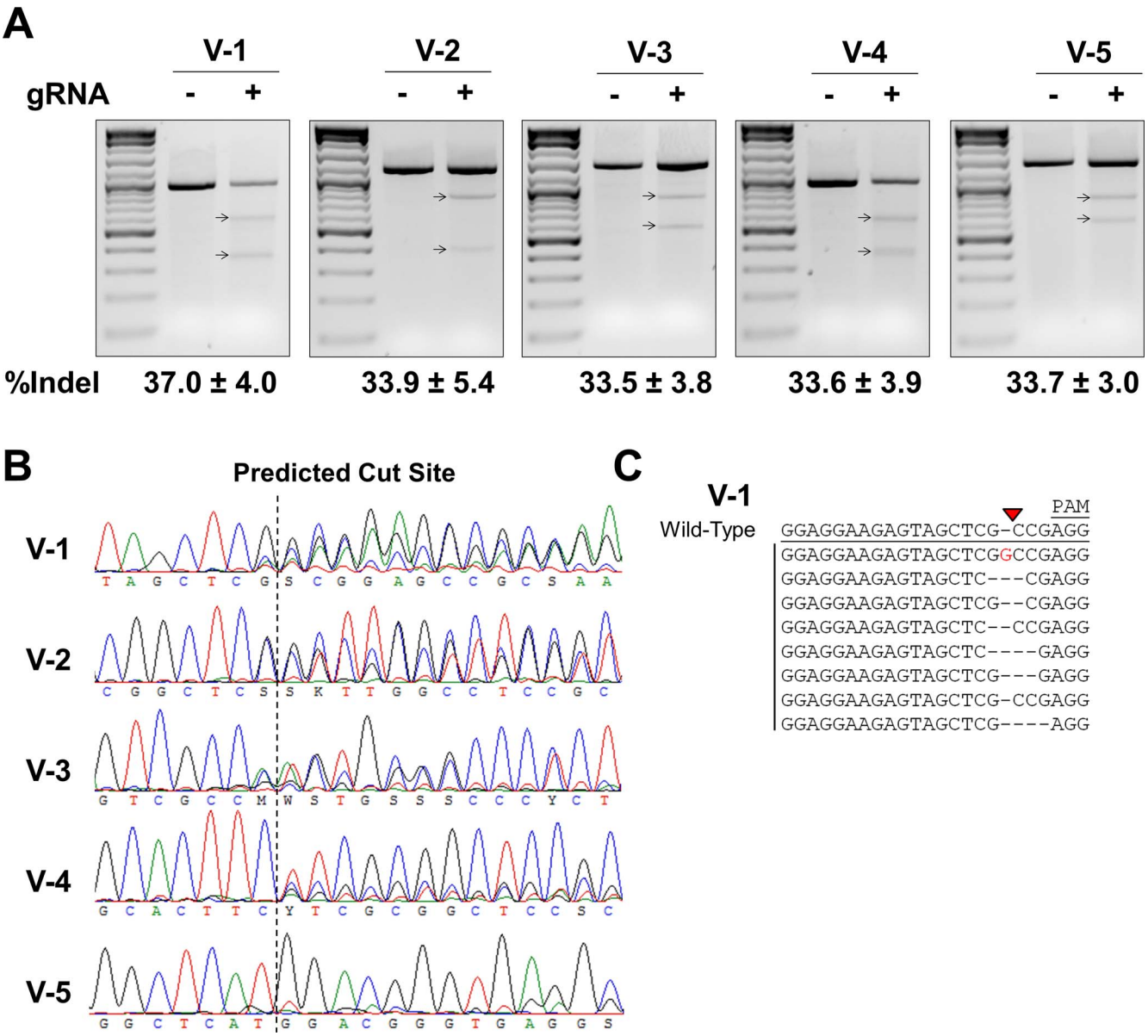

Figure 1. Cas9-mediated indel formation and DNA sequencing. (**A**) T7E1 mismatch detection assays demonstrating frequency of indel formation (%indel) for each gRNA targeting VEGF-A, expressed in mean ± standard deviation. Lentiviral vectors expressing SpCas9 without VEGF-targeted gRNAs were used as controls. Representative results are shown from four total independent experiments. (**B**) Sanger sequencing chromatograms showing indel formation at the predicted cut site for each target site. (**C**) Representative deep sequencing results for V-1 confirming indel formation at the predicted cut site (*red arrowhead*), including insertions (*bases in red*) and deletions (*dashes*).

results suggest that Cas9-mediated disruption of VEGF-A in RPE cells is highly selective with minimal off-target effects.

Discussion

Genome engineering using CRISPR-Cas9 technology has dramatically improved the efficiency and ease of creating targeted genetic modifications, revolutionizing many aspects of biologic and medical research in recent years. In this study, we successfully employed the CRISPR-Cas9 system to achieve genomic disruption of the VEGF-A gene in human RPE cells, with a corresponding reduction in secreted VEGF-A protein, suppression of angiogenesis in vitro, and no detectable off-target effects. These results demonstrate as proof of principle the feasibility of using CRISPR-Cas9 gene editing as potential antiangiogenesis therapy in ocular neovascular diseases.

Although gene therapy has been employed with some success in inherited retinal diseases that result from a single gene defect, the concept of employing gene therapy in complex, multifactorial diseases like AMD or diabetic retinopathy has not been explored at length due to the complex interactions of various genetic factors and molecular pathways involved in the pathogenesis of these conditions. However, CRISPR-Cas9 gene editing technology is particularly well-suited to address these conditions because multiplexed targeting of different genes can be achieved by expressing a battery of gRNAs with the Cas9 endonuclease in a single viral construct. In addition to reducing VEGF, for example, other angiogenic, exudative, and inflammatory pathways can be simultaneously

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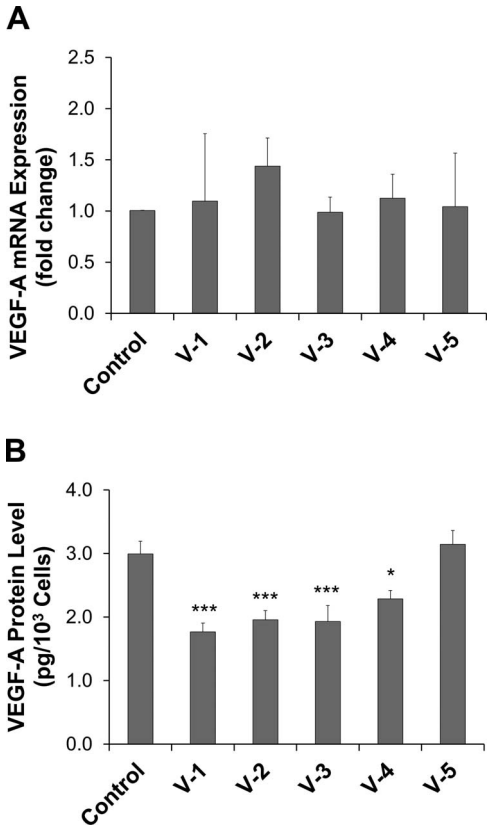

FIGURE 2. VEGF-A mRNA and protein levels after Cas9-mediated gene disruption. (**A**) Levels of VEGF-A mRNA measured from quantitative RT-PCR, showing no significant change in mRNA expression, following expression of Cas9 with gRNAs targeting VEGF-A, compared with Cas9-only controls. Results represent averaged values from three independent experiments, with three replicate samples each. (**B**) Protein levels of VEGF-A of conditioned media measured by ELISA, normalized to cell number, showing significant reduction of secreted VEGF-A after Cas9-mediated disruption of VEGF-A gene. Results represent averaged values from seven independent experiments, with two replicate samples each. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical values based on Dunnett's posttests.

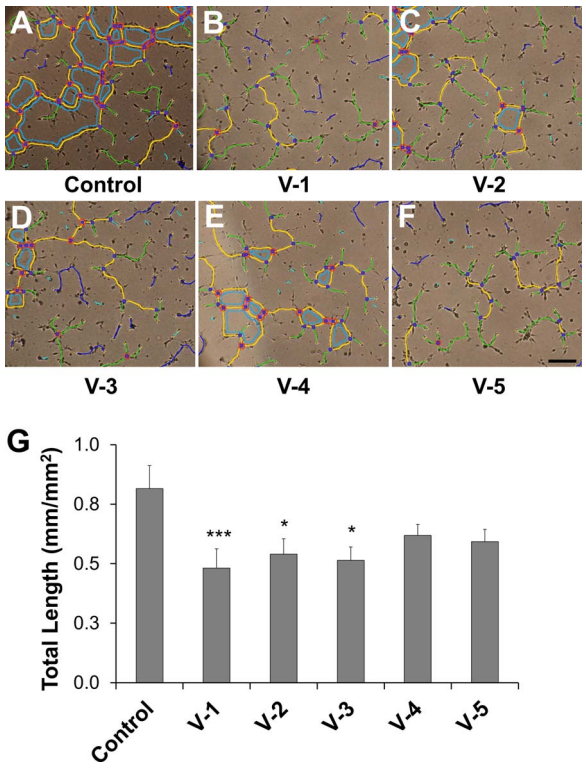

FIGURE 3. In vitro angiogenesis after Cas9-mediated VEGF-A disruption. (**A–F**) Representative phase contrast images of human endothelial cell tube formation after exposure to conditioned medium from ARPE-19 cells expressing Cas9 and guide RNAs targeting VEGF-A. Overlay was generated by the angiogenesis analyzer tool on ImageJ. *Scale bar*: 200 μm. (**G**) Quantification of HUVEC tube formation from experiments shown in (**A–F**) showing significant reduction in in vitro angiogenesis activity. Results represent averaged values from six independent experiments, with five random fields analyzed from each experiment. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical values based on Dunnett's posttests.

targeted in the context of different classes of retinal disorders. The system also affords tremendous specificity by selectively disrupting individual genes, in contrast to siRNA-mediated knockdown or dominant-negative protein expression that can unpredictably affect multiple targets at once. Importantly, manipulations at the genomic level lead to a permanent disruption of the target gene or genes, even if long-term viral expression of the Cas9 protein cannot be maintained. Hence, CRISPR-Cas9 gene editing has significant advantages as a strategy for human gene therapy.

In our studies, genomic disruption of VEGF-A with Cas9 using a single lentiviral system resulted in up to 41.2% reduction in VEGF secretion from RPE cells. The incomplete suppression may be due in part to incomplete Cas9 activity, since our results demonstrated only up to 37.0% indel formation, indicating that a portion of the cells did not undergo Cas9-mediated cleavage of the target site. Our data contrast with studies showing greater than 90% indel frequency after lentiviral transduction of gRNAs targeting enhanced green fluorescent protein (EGFP) in human embryonic kidney (HEK) 293T cells,[26] but are comparable to the 38.6% indel formation in VEGF-A after plasmid transfection of Cas9 with gRNAs in HEK293 cells.[27] Hence, Cas9 activity is not only determined by the specific target site, but also by the particular cell type, gene of interest, and mode of Cas9/gRNA delivery. Since RPE cells are not the only source of VEGF,[28] and other proangiogenic factors are involved in neovascular eye conditions, further studies to target additional angiogenic factors in other cell types may demonstrate more optimal Cas9 cleavage activity and greater suppression of angiogenesis.

Another reason for the incomplete reduction in VEGF secretion is the fact that not all indels result in biallelic null mutations. Rather than clonally expanding a single null mutant, as common in loss-of-function studies, our goal was to assess the direct application of this lentiviral CRISPR system to a population of RPE cells for potential translation to human therapy. We thus expect a mixture of biallellic null mutant, haploinsufficient, and wild-type cells, even within the population of cells that underwent Cas9 cleavage. Interestingly, despite the prediction that only approximately two-thirds of indels should lead to frameshift or nonsense mutations, and even fewer having these mutations in both alleles, we were surprised to detect similar decreases in both VEGF-A secretion and tube formation, compared with the estimated indel frequencies from the T7E1 assays. Based on the Sanger sequencing chromatograms, we suspect that mismatch detection with the T7E1 assay may reflect only a minimum estimate of actual indel frequencies.

Even if complete VEGF suppression cannot be achieved, partial reduction of VEGF may be sufficient to suppress neovascularization or exudation in vivo, or even just reduce

A

Site Name	Off-Target Site Name	Target Sequence	Off-Target Gene Name	On-Target Score
V-1	OTS 1-1	AGCGGAAGAGTAGCTCTCCG	SMYD3	1.5
	OTS 1-2	GGAGGAAGCGGAGCTCACCG	RNPEPL1	0.4
	OTS 1-3	GCAGGAAGACCAGCTCTCCG	NPFF	0.4
V-2	OTS 2-1	CCAATACTCGGGGCTCCGCT	C15ORF40	0.5
	OTS 2-2	GCCCGCCTCGCTGCTCCGCT	RP11-34016.1	0.4
	OTS 2-3	CCACCTCTCGCTGCTCAGCT	SRGAP3	0.3
V-3	OTS 3-1	TGCGCGCCGCGTCGCCTCTG	CAMKK2	0.7
	OTS 3-2	GGAGCCCGGAGTCGCCCCTG	GLT25D1	0.4
	OTS 3-3	CGCTCTCCGAGTCGCCCCTG	PLAA	0.4
V-4	OTS 4-1	ACCTGATCGAGCACTTCTCG	HNRNPA0	0.6
	OTS 4-2	GCCGCAGCGAGCTCTTCTCG	PKN1	0.4
	OTS 4-3	GCCCAAGCTGGGACTTCTCC	EPHB3	0.4
V-5	OTS 5-1	TGAGAGGCCTGGCTCATGGA	CTD-2130O13.1	0.8
	OTS 5-2	TGGGAAGCCGGGGTCAGGGA	RP11-552F3.9	0.6
	OTS 5-3	AGGGAAGCCGCGCTCAGGGA	IQSEC3	0.4

B

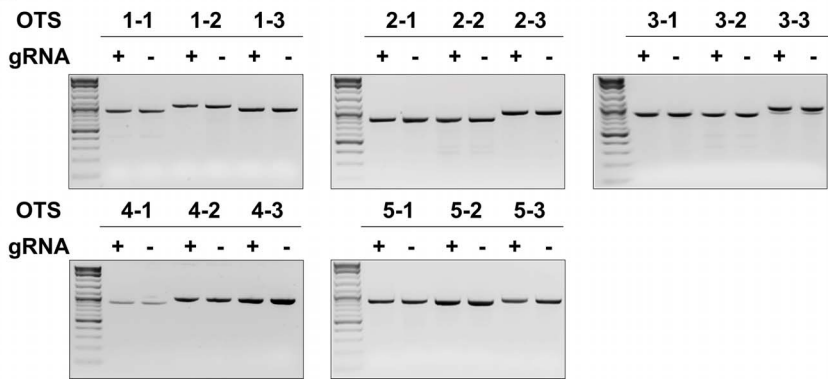

Figure 4. Off-target activity of Cas9-mediated VEGF-A gene disruption. (**A**) Top three putative off-target sites for each target CRISPR sequence for human VEGF-A, with respective on-target scores to predict off-target Cas9 activity at these locations. Only off-target sites located within gene exons were chosen. Base mismatches are shown in red. (**B**) T7E1 mismatch detection assays of putative off-target sites, showing no detectable indel formation at these locations.

the burden of frequent intravitreal injections of anti-VEGF agents. In fact, total VEGF suppression may not be optimal, as basal VEGF secretion is necessary for maintenance of the choriocapillaris,[29] and long-term VEGF suppression may be associated with progression of geographic atrophy in patients with AMD.[30,31]

The prospect of off-target mutations is of particular concern to researchers interested in using CRISPR-Cas9 technology in human therapeutic applications. The frequency of off-target mutagenesis is somewhat controversial, with some reports showing that Cas9 can efficiently discriminate on-target from off-target sites that differ by two bases,[32,33] and others noting off-target mutations even at sites that differ by 5 nucleotides from on-target sites.[16,27,34] We did not detect any indel formation at the three most probable off-target sites for each VEGF gRNA used in this study, even though these off-target sites differed by three or more base pairs and occur mainly in the 5′ upstream region of the protospacer, where Cas9 endonucleases are more likely to tolerate mismatches. Nevertheless, we cannot exclude the possibility of off-target mutations at very low frequencies that are below the detection limit of the T7EI assay or at other putative sites not examined in our studies, which could be revealed by deep sequencing of off-target sites or whole genome sequencing, respectively. Strategies to improve the specificity of the CRISPR-Cas9 system may include using a shorter 18 to 19 nt protospacer[35] or paired Cas9 nickases that generate two single-strand breaks on opposite DNA strands,[33,36] although the efficiency of Cas9 cleavage may be compromised. The use of an inducible[37–39] or cell-specific promoter to drive Cas9 expression may also further improve the temporal and spatial control of endonuclease activity, which will minimize the accumulation of random off-target mutations that may occur with long-term overexpression. Novel Cas9 variants with improved specificity are already in development,[40] and may further mitigate concerns of off-target mutagenesis.

Future studies will be necessary to assess the potential use of CRISPR-Cas9 gene editing to target VEGF as an ocular antiangiogenesis therapy. Use of primary human RPE cells, such as human fetal RPE, or demonstration of gene editing in vivo in a small animal model would further support the translational potential of this strategy to human therapy. We employed a lentiviral vector due to its efficiency in transducing RPE cells and the availability of well-characterized tools for predicting gRNA sequences for SpCas9. However, lentiviruses randomly integrate into the host genome, potentially triggering additional off-target effects such as activation of oncogenes or inactivation of tumor suppressor genes, which may have harmful consequences. Adeno-associated viruses have no association with human disease and are more amenable to

gene therapy, but have a limited packaging capacity. AAV-mediated expression of the *Staphylococcus aureus* Cas9 (SaCas9) endonuclease, which is 1 kb shorter than SpCas9, may provide a more feasible avenue for translational therapeutic applications.

In conclusion, we successfully employed a CRISPR-Cas9 system to disrupt VEGF secretion from human RPE cells and suppress angiogenesis in vitro, demonstrating as proof of concept the possibility of employing gene editing strategies for ocular antiangiogenesis therapy.

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