

Reduced Expression of VEGF-A in Human Retinal Pigment Epithelial Cells and Human Muller Cells Following CRISPR-Cas9 Ribonucleoprotein-Mediated Gene Disruption

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Purpose: To evaluate the effects of vascular endothelial growth factor-A (VEGF-A) gene editing in human retinal pigment epithelial (RPE) cells and human Muller cells, which are the main VEGF-A producing cells in the eye.

Methods: CRISPR-Cas9 ribonucleoprotein was used to target exon 1 in VEGF-A gene. Lipofectamine CRISPRMAX was used as a vehicle. In vitro gene editing efficiency was assessed on oligonucleotides and genomic DNAs. Sanger sequencing was performed to detect indels. VEGF-A messenger RNA and protein expressions were assessed using quantitative polymerase chain reaction and enzyme-linked immunosorbent assay.

Results: In vitro cleavage assay on a 60-nucleotide DNA duplex showed 88% cleavage of the precursor. The cleavage efficiency was 40% in RPE cells and 32% in Muller cells. Sanger sequencing in the CRISPR-Cas9 treated RPE and Muller cells showed indels at the predicted cut site in both cells. After the VEGF-A gene disruption, VEGF-A protein levels decreased 43% in RPE cells ($P < 0.0001$) and 38% in Muller cells ($P < 0.0001$).

Conclusions: CRISPR-Cas9-mediated gene disruption resulted in a significant decrease in the VEGF-A gene protein expression in human RPE and Muller cells. CRISPR-Cas9 ribonucleoprotein may allow simultaneous targeting of multiple VEGF-A producing cells.

Translational Relevance: VEGF-A gene disruption using CRISPR-Cas9 ribonucleoprotein has a potential in treating retinal vascular diseases.

Introduction

Vascular endothelial growth factor-A (VEGF-A), with its potent vasopermeability and vasoproliferative effects, is an important mediator in retinal vascular diseases. The VEGF-A gene, located on chromosome 6 (6p21.1), is a member of the platelet-derived growth factor/VEGF growth factor family. Alternative splicing results in multiple isoforms of which VEGF₁₆₅ is the most abundant isoform found in the eye.¹

Intravitreal injection of anti-VEGF medications is commonly used to temporarily decrease intraocular VEGF levels in the treatment of macular edema and retinal or choroidal neovascularization associated

with common eye diseases such as age-related macular degeneration, diabetic retinopathy, retinal vein occlusion, and many less common retinal vascular diseases. Current anti-VEGF medications include bevacizumab, ranibizumab, aflibercept, and brolucizumab, all of which bind to and block the actions of VEGF-A.

Although these medications have tremendously improved visual outcomes for patients with retinal vascular diseases, frequent intraocular injections are often needed, and in some patients injections may reach frequencies of up to every four weeks indefinitely. Frequent intravitreal injections increase the risk of complications such as endophthalmitis, are inconvenient to patients, and place a significant financial burden on health care systems in the United States^{2,3}

and throughout the world. Developing slow release formulations and devices as well as longer lasting medications are among some of approaches taken to address these problems. However, there has not been any indication that any of these methods would lead to a permanent treatment.

Gene therapy has the potential to permanently decrease VEGF-A levels and eliminate the need for frequent intravitreal injections. Both gene augmentation and gene silencing methods have been used to decrease VEGF-A levels.⁴⁻⁶ Gene silencing has not yet reached human clinical trials, but in vitro and in vivo studies using microRNA and shRNA have demonstrated some success in VEGF reduction.⁷⁻⁹ In recent years, clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) has been used to disrupt VEGF-A gene in retinal pigment epithelial (RPE) cells and in mouse retina.¹⁰⁻¹² Within the retina, VEGF is constitutively expressed in Muller cells, RPE cells, ganglion cells, and retinal and choroidal vasculature; this expression is significantly increased in pathologic angiogenic states.^{13,14} The purpose of our study was to evaluate the effects of VEGF-A gene disruption in Muller cells as well as in RPE cells, both of which are major VEGF producers in the eye. We used CRISPR-Cas9 ribonucleoprotein (RNP) delivered via lipofectamine CRISPRMAX (LCM).

Methods

Cell Culture and Transfection Using LCM Nanoparticle

RPE cells (ARPE-19, ATCC CRL-2302, Manassas, VA) were grown in T75 flasks using Dulbecco's Modified Eagle's Medium: F-12 (ATCC 30-2006) with 10% fetal bovine serum (ATCC 30-2020). Muller cells (MIO-M1) were purchased from XIP (London, UK). Dulbecco's Modified Eagle's Medium (ATCC 30-2002) with 10% fetal bovine serum (ATCC 30-2020) was used to grow Muller cells in T75 flasks. Primary human retinal microvascular endothelial cells (ACBRI 181) purchased from Cell Systems (Kirkland, WA) were grown in T75 flasks using Complete Classic Medium with Serum and CultureBoost (4Z0-500, Cell Systems, Kirkland, WA).

For transfection with LCM, the manufacturer's protocol (Invitrogen TrueGuide Synthetic gRNA - Thermo Fisher Scientific, Waltham, MA) was followed. The cells were seeded into a 6-well plate the day before transfection so that they reached 30% to 70% confluence at the time of transfection. On

the day of transfection, 125 μ L of Opti-MEM I Medium (Gibco, Thermo Fisher Scientific), 37.5 pmol of TrueCut Cas9 Protein v2 (Invitrogen by Thermo Fisher Scientific), 37.5 pmol of sgRNA and 12.5 μ L of Lipofectamine Cas9 Plus Reagent (Invitrogen by Thermo Fisher Scientific), were added to a 1.7-mL sterile microcentrifuge tube (tube 1). Meanwhile, 125 μ L of Opti-MEM I Medium and 7.5 μ L of LCM Transfection Reagent (Invitrogen by Thermo Fisher Scientific), were added to another sterile tube (tube 2). Tube 2 was incubated for 1 minute at room temperature, then mixed well into tube 1 by frequent pipetting. The mixture was incubated for 15 minutes at room temperature to allow the formation of CRISPR-Cas9 RNPs. We added 250 μ L of the transfection complex to each of the wells, after which the cells were incubated at 37°C in the presence of 5% CO₂. The cells were analyzed 48 hours after transfection.

Designing a Specific Single-guide RNA (sgRNA) Targeting the Human VEGF-A Gene

Guide RNA (gRNA) was designed using Benchling CRISPR gRNA design software (Benchling, San Francisco, CA). A protospacer adjacent motif (PAM) sequence of 5'-NGG-3' for *Streptococcus pyogenes* Cas9 (SpCas9) and a guide sequence length of 20 nucleotides were selected.

The analysis showed the guide sequence of 5'-GGAGGAAGAGTAGCTCGCCG-3' with the PAM sequence of 5'-AGG-3' on exon 1, encoding for amino acids 146 to 152, to have the best on-target and off-target scores of 74.3 and 85.1, respectively. Search of the entire VEGF-A gene revealed that this same area on exon 1 had the best overall on-target and off-target scores.

In Vitro CRISPR-Cas9-Mediated Cleavage of DNA Oligonucleotide Duplex

The target DNA duplex and the sgRNAs were obtained commercially from Integrated DNA Technology (Skokie, IL) and Invitrogen by Thermo Fisher Scientific (Waltham, MA), respectively. The sequences are listed in Figure 1A. DNA cleavage was monitored with 5' ³²P-labels at both strands of the DNA duplex following a previously reported protocol.¹⁵ A typical 10 μ L ³²P-labeling reaction contained 10 μ M double-stranded DNA, 8 μ L ³²P γ -ATP (MP Biomedicals, Irvine, CA; 6000 Ci/mmol), 10 units T4 polynucleotide kinase (New England Biolabs, Ipswich, MA; #M0201), and 1X polynucleotide kinase Buffer (New England Biolabs, 70 mM Tris-HCl, 10 mM MgCl₂, 5 mM DTT, pH 7.6). The reaction mixture was

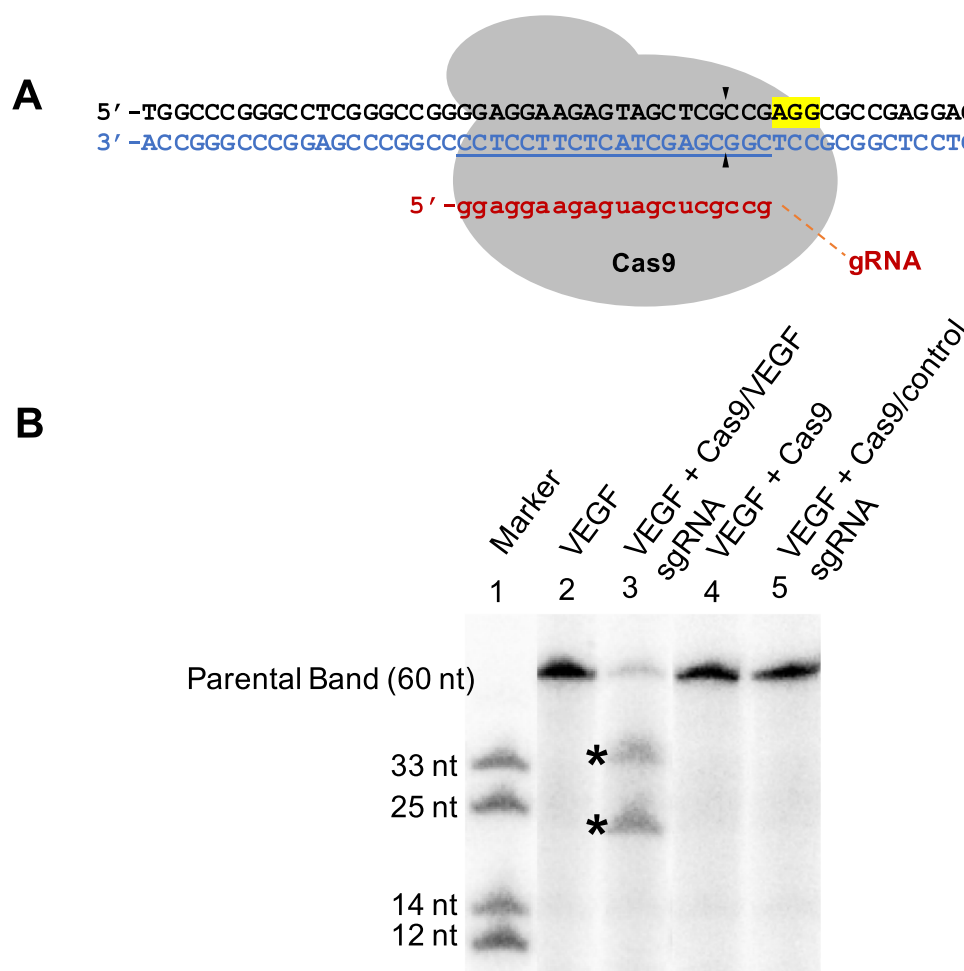


Figure 1. In vitro DNA cleavage by Cas9 effector complex. (A) Schematic of DNA/Cas9/sgRNA system. Cas9 is presented by the gray lobe, and the 20-nt guide segment of the sgRNA is shown in red. The DNA target strand is shown in blue, the nontarget strand is in black, and the 20-nt protospacer is underlined. The PAM is highlighted in yellow. The cleavage sites are marked by solid wedges. (B) Cleavage of duplex VEGF DNA monitored by 5'-³²P labels on both strands. The Cas9/RNA used was 10 nM, and the DNA duplex was 1 nM. Asterisks show the two cleaved bands at the expected locations (37 nt and 23 nt) along the length of the gel in lane 3.

incubated at 37°C for 30 minutes, then the T4 polynucleotide kinase was deactivated by heating at 65°C for 20 minutes. The cleavage reaction was carried out at single-turnover condition: ³²P-labeled DNA duplex (1 nM) was subjected to cleavage by a pre-formed Cas9/sgRNA effector complex, with the concentration of the complex at least 10 times higher than that of the DNA. To preform the effector complex, an appropriate amount of RNA (Invitrogen) was first heated at 95°C for 1 minutes, then incubated in a reaction buffer (20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl₂, 5% [v/v] glycerol, and 0.5 mM TCEP) for 10 minutes. Then desired amount of Cas9 (Invitrogen) was added to obtain a final ratio of RNA/Cas9 approximately 1.5: 1. The Cas9/RNA mixture was incubated at room temperature for 15 minutes, then an appropriate

amount of DNA substrate was added, and the mixture was incubated at 37°C for 30 minutes. To terminate the reaction, an equal amount of denaturing solution (8 M urea, 20 mM EDTA, 20% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) was added and the mixture was heated at 95°C for 1 minute to deactivate the Cas9 enzyme. The cleavage reaction was resolved by 20% denaturing PAGE, and DNA species were visualized by autoradiography using a Personal Molecular Imager (Bio-Rad, Hercules, CA).

To quantify the reaction, the signal of the individual precursor and product band was corrected for background according to: $I = I_0 - S_0 \times a_{bg}$, where I_0 is the raw measured signal, S_0 is area of the band, and a_{bg} is the average intensity per unit area obtained from multiple sections of the gel between the

precursor and product bands. The reaction product was then computed as:

$$\% \text{Product} = [I_{\text{product}} / (I_{\text{precursor}} + I_{\text{product}})] \times 100$$

where $I_{\text{precursor}}$ is the intensity of DNA precursor signal and I_{product} is the sum of the products signal.

Genomic Cleavage Assay

The genome editing efficiency was determined by the GeneArt Genomic Cleavage Detection Kit (Life Technologies, Thermo Fisher Scientific). The sgRNA targeting sequence 5'- GGAGGAAGAG-TAGCTCGCCG -3' was used to edit the VEGF-A gene. At 48 hours after transfection, the culture medium was removed and the cells were rinsed twice with 500 μL phosphate-buffered saline. The cells were detached by adding 500 μL of Trypsin/EDTA to the selected well of a 6-well plate. Transfected cells were spun down at 200g for 5 minutes at 4°C. The supernatant was carefully removed, after which cell lysis proceeded. We mixed 50 μL of Cell Lysis Buffer and 2 μL of Protein Degradar in a microcentrifuge tube and 50 μL of the Cell Lysis Buffer/Protein Degradar mixture was added to the cell pellet. The pellet was resuspended by frequent pipetting and transferred to a polymerase chain reaction (PCR) tube. The PCR program was set to 68°C for 15 minutes and 95°C for 10 minutes. The cell lysate was vortexed briefly and the following components were added to a PCR tube: sample tube containing 2 μL of Cell Lysate, 1 μL of each forward and reverse primers, 25 μL of AmpliTaq Gold 360 Master Mix and 21 μL of water. For best results, 5 μL of 360 GC Enhancer per 50 μL PCR reaction was added to the sample tube. For the VEGF-A gene target, a forward primer, 5'-TGTGCGCAGACAGTGCTCCA-3', and a reverse primer, 5'-CCAGATCGTACGTGCGGTGACT-3', were used. The second PCR reaction was run with the following conditions: 95°C for 10 minutes for one cycle, then 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for a total of 40 cycles. The final extension was set at 72°C for 7 minutes for one cycle. Three microliters of the resulting PCR product were mixed with 1 μL of 10 \times Detection Reaction Buffer and 5 μL water, then subjected to denaturing and re-annealing at 95°C for 5 minutes, 95°C to 85°C (2°C/sec) and 85°C to 25°C (0.1°C/sec). Finally, 1 μL of detection enzyme was added to the test sample and then incubated at 37°C for 1 hour. The digested product was analyzed with a 2% E-Gel EX agarose gel on E-Gel iBase Power System (Thermo Fisher Scientific). The following equation was used to calculate the

genomic cleavage efficiency:

$$\text{Cleavage efficiency} = 1 - [(1 - \text{fraction cleaved})^{1/2}],$$

$$\text{Fraction cleaved} = \text{sum of cleaved band intensities} / (\text{sum of the cleaved and parental band intensities})$$

DNA Sequencing

To assess the effects of the CRISPR-Cas9 gene editing on the DNA sequence, a Sanger sequencing assay of a 284 base pair (bp) amplicon flanking the target area on the VEGF-A gene was performed by Genewiz (South Plainfield, NJ). The assay included TA cloning and DNA amplification. The traces were compared with the reference wild-type sequence to detect any insertion, deletion, or substitution.

Enzyme-Linked Immunosorbent Assay

The Human VEGF Enzyme-Linked Immunosorbent Assay Kit (Abcam, Cambridge, MA) was used to measure VEGF-A protein. Cell culture medium was centrifuged at 2000g for 10 minutes. The top portion of the supernatant was collected and the remainder was discarded. Standard solutions were prepared according to the protocol and serial standard dilutions were made. Samples and antibody cocktail were prepared using appropriate diluents. Samples and standards were loaded on a coated microplate and antibody cocktail was added. After following other steps, including incubation on a plate shaker the samples were read using SpectraMax iD3 (Molecular Devices, San Jose, CA) and the protein concentration was calculated based on the standard curve.

Quantitative RT-PCR Assay

A Purelink RNA Mini Kit (ThermoFisher Scientific) was used and the manufacturer's protocol was followed to extract total RNA from cell culture. Reverse transcription was performed using 1 μg of total RNA per 50 μL of reaction volume of TaqMan Reverse Transcription Reagents (ThermoFisher Scientific). Complementary DNA was obtained by incubating the reaction in thermal cycler (Veriti, Applied Biosystem, ThermoFisher Scientific). Real-time quantitative PCR was performed using TaqMan Fast Advanced Master Mix solution (ThermoFisher Scientific) and amplification was achieved using QuantStudio 6. GAPDH expression was used as an internal control. The probes included Hs00900055_m1 (ThermoFisher Scientific) for VEGF-A and Hs02758991_g1 (ThermoFisher Scientific) for GAPDH.

Statistical Analysis

Data are presented as mean \pm standard error of the mean. A two-tailed Student *t*-test for independent samples was used for VEGF-A messenger RNA (mRNA) and protein analysis.

Results

In Vitro Cleavage of Synthetic VEGF-A DNA Oligonucleotide Duplex by CRISPR-Cas9 RNP Complex is Highly Efficient

In vitro cleavage studies were carried out with catalytically active Cas9 using a synthetic DNA duplex (designated as “VEGF”), mimicking a segment of the VEGF-A gene (Fig. 1A). Figure 1B shows a representative gel monitoring cleavage of 5'-³²P-labeled VEGF duplex by Cas9 effector complexes. When the Cas9 effector was assembled using an sgRNA containing the correct guide segment (Fig. 1A, red font), both the target and nontarget strands were cleaved to near completion with $88 \pm 2\%$ of products observed (Fig. 1B, lane 3). Based on marker DNA strands with a known length (Fig. 1B, lane 1), the target strand was cleaved at the 23rd nucleotide from the 5' terminus, and the nontarget strand was cleaved at the 37th nucleotide from the 5' terminus. Both were consistent with the expected Cas9 cleavage sites. In addition, control experiments showed no VEGF DNA cleavage if only Cas9 was presented (Fig. 1B, lane 4) or if Cas9 was assembled with a sgRNA whose 20-nt guide did not match the VEGF DNA protospacer (Fig. 1B, lane 5). Overall, the data demonstrated successful in vitro cleavage of VEGF DNA by Cas9 with high efficiency.

CRISPR-Cas9 RNP Complex Delivered via LCM Can Target and Disrupt the VEGF-A Gene In Vitro in RPE and Muller Cells

First, to demonstrate the ability of LCM in transfecting target cells, we used green fluorescent protein (GFP) mRNA (TriLink, San Diego, CA) and performed fluorescent microscopy. When GFP mRNA was combined with LCM, it produced green fluorescence, whereas no fluorescence was noted in the absence of LCM (Fig. 2A).

To evaluate the efficiency of CRISPR-Cas9 RNP in VEGF-A gene editing via LCM, a cleavage detection assay was performed on RPE and Muller cells subjected to CRISPR-Cas9 RNP. Selecting an amplicon length of 487 bp for the genomic cleavage assay, two bands of 408 bp and 79 bp were detected on

the agarose gel (Fig. 2B). The presence of the two expected bands confirmed that the VEGF-A gene was successfully disrupted at the desired region. The genomic cleavage efficiency was quantified using E-Gel GelQuant Express Analysis Software (ThermoFisher), which estimated a cleavage rate of 40% ($n = 8$) for RPE cells and 32% ($n = 9$) for Muller cells.

Sanger sequencing showed a complex mixture of insertion and deletion events at the predicted site in both RPE (Fig. 2C) and Muller cells treated with CRISPR-Cas9 RNP. In treated RPE cells, TA cloning of 20 randomly selected colonies produced readable results in 18. All 18 colonies showed indels at the predicted cut site defined by the guide sequence. The indels ranged from 1 to 148 nucleotides. In treated Muller cells, TA cloning of 25 randomly selected colonies produced readable results in 19. Seventeen of 19 colonies showed indels at the predicted cut site. The indels ranged from 1 to 81 nucleotides. In untreated cells, TA cloning of 32 randomly selected colonies from RPE and Muller cells, produced 20 readable results in each. No indels were found at the predicted cut site in Muller cells; however, there was a single nucleotide substitution at the predicted cut site in one colony from RPE cells. In all CRISPR-Cas9-treated and untreated RPE cells and Muller cells there were random mutations outside of the guide area in some colonies. These mutations were generally single nucleotide substitutions (Supplementary Fig. 1). Overall, the data indicated that LCM delivery of the CRISPR-Cas9 RNP yielded efficient editing at the target site, on the VEGF-A gene, in both RPE and Muller cells.

The Effects of the VEGF-A Gene Disruption on the VEGF-A mRNA and Protein Expression

To assess whether CRISPR-Cas9, delivered as an RNP complex, can affect VEGF-A expression, RPE and Muller cells were transfected with CRISPR-Cas9 targeting the exon 1 of the VEGF-A gene. The transfection was performed 1 day after seeding, and expression assays were performed 2 days after transfection. Quantitative RT-PCR showed no significant reduction in the VEGF-A mRNA expression in RPE cells, but a modest decrease in Muller cells that underwent VEGF-A gene disruption (Fig. 3). Comparing CRISPR-Cas9 treated cells to untreated cells, the VEGF-A mRNA expression was 5% less in RPE cells ($P = .297$) and 17% less in Muller cells ($P < .0001$). In contrast, VEGF-A gene disruption resulted in robust reduction of the VEGF-A protein expression in both RPE and Muller cells (Fig. 3). Comparing the CRISPR-Cas9-treated

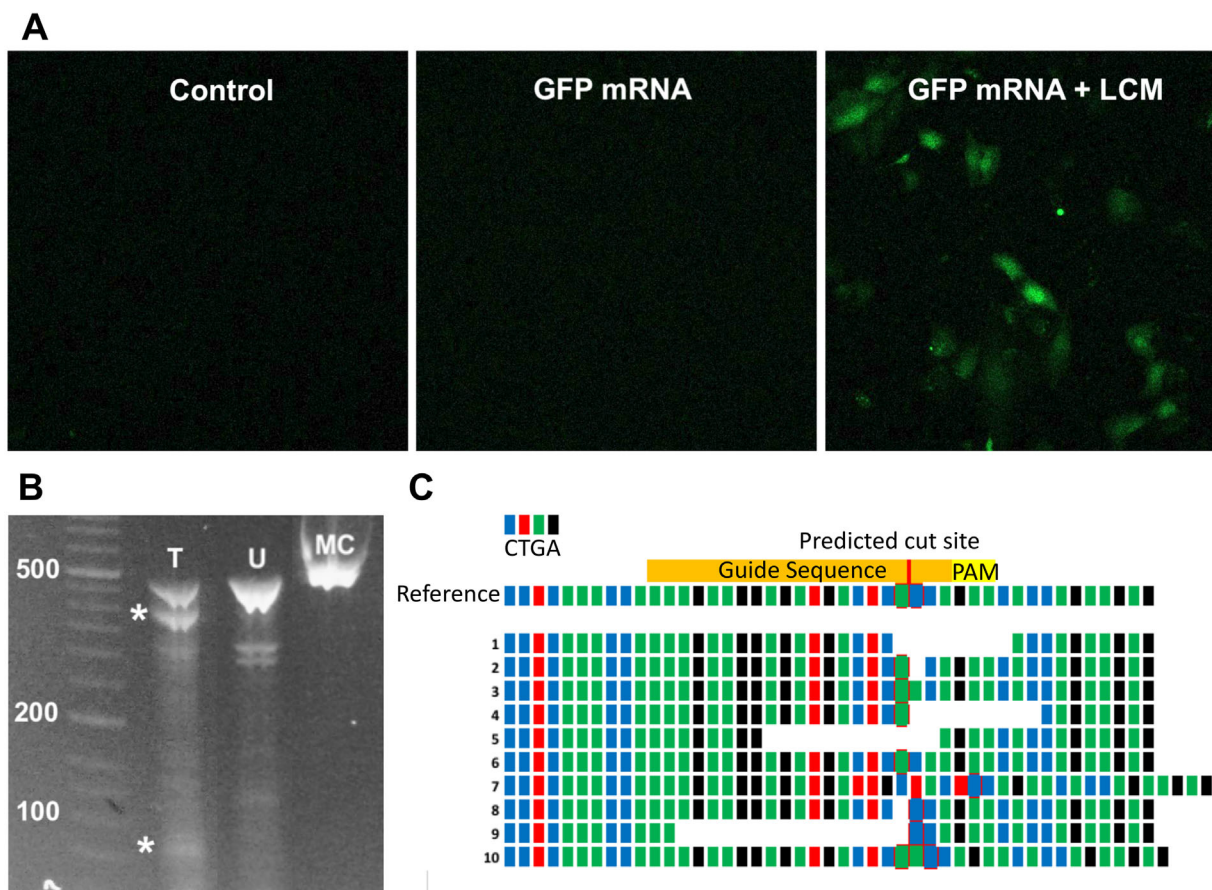


Figure 2. LCM-mediated transfection and VEGF-A gene disruption after CRISPR-Cas9 RNP treatment in RPE cells. (A) LCM-mediated transfection of RPE cells. At 48 hours after transfection, RPE cells treated with GFP mRNA combined with LCM show green fluorescence; no fluorescence is seen in untreated cells (control) and in cells treated with GFP mRNA alone. (B) Representative agarose gel in genomic cleavage detection assay using transfected RPE cells. The CRISPR-Cas9 treated (T) and untreated (U) cells as well as the manufacturer control (MC) were PCR amplified using the same set of primers flanking the region of interest. Note the presence of the parental band (*top band*) in all groups; however, only CRISPR-Cas9 treated cells (T) show the two cleaved bands (asterisks) at the expected locations (408 and 79) along the length of the gel. Other bands, which correspond in both groups, are likely artefactual. (C) Diagram of representative colonies showing indels at the predicted cut site detected by Sanger sequencing. The guide sequence, PAM and adjacent nucleotides of the normal VEGF-A gene are shown as a reference. The border of nucleotides adjacent to the predicted cut site is shown in red. In eight colonies (not shown here) large deletions involved other areas in addition to the guide sequence.

cells with untreated cells, the VEGF-A protein level was 43% less in RPE cells ($P < .0001$) and 38% less in Muller cells ($P < .0001$).

CRISPR-Cas9 RNP Complexed with LCM Does Not Result in Cell Loss

To assess whether CRISPR-Cas9 RNP delivery via LCM caused cell loss, CRISPR-Cas9-treated cells were compared with untreated cells using hemocytometer manual cell counting system. No significant difference was noted for RPE ($P = .42$) or Muller cells ($P = .9$) between the two groups (Fig. 4).

Discussion

Frequent anti-VEGF injection, although effective, is a burden to patients and to the health care system. Gene therapy has the potential to decrease this burden by potentially eliminating the need for, or decreasing the frequency of, intravitreal anti-VEGF injections. We successfully employed CRISPR-Cas9 RNP to disrupt the VEGF-A gene in RPE and Muller cells, which are major VEGF-A-producing cells in the eye. We further established that VEGF-A gene disruption achieved with this method can successfully decrease VEGF-A expression. The results of this study demonstrate the feasibility of CRISPR-Cas9 RNP in nonspecifically

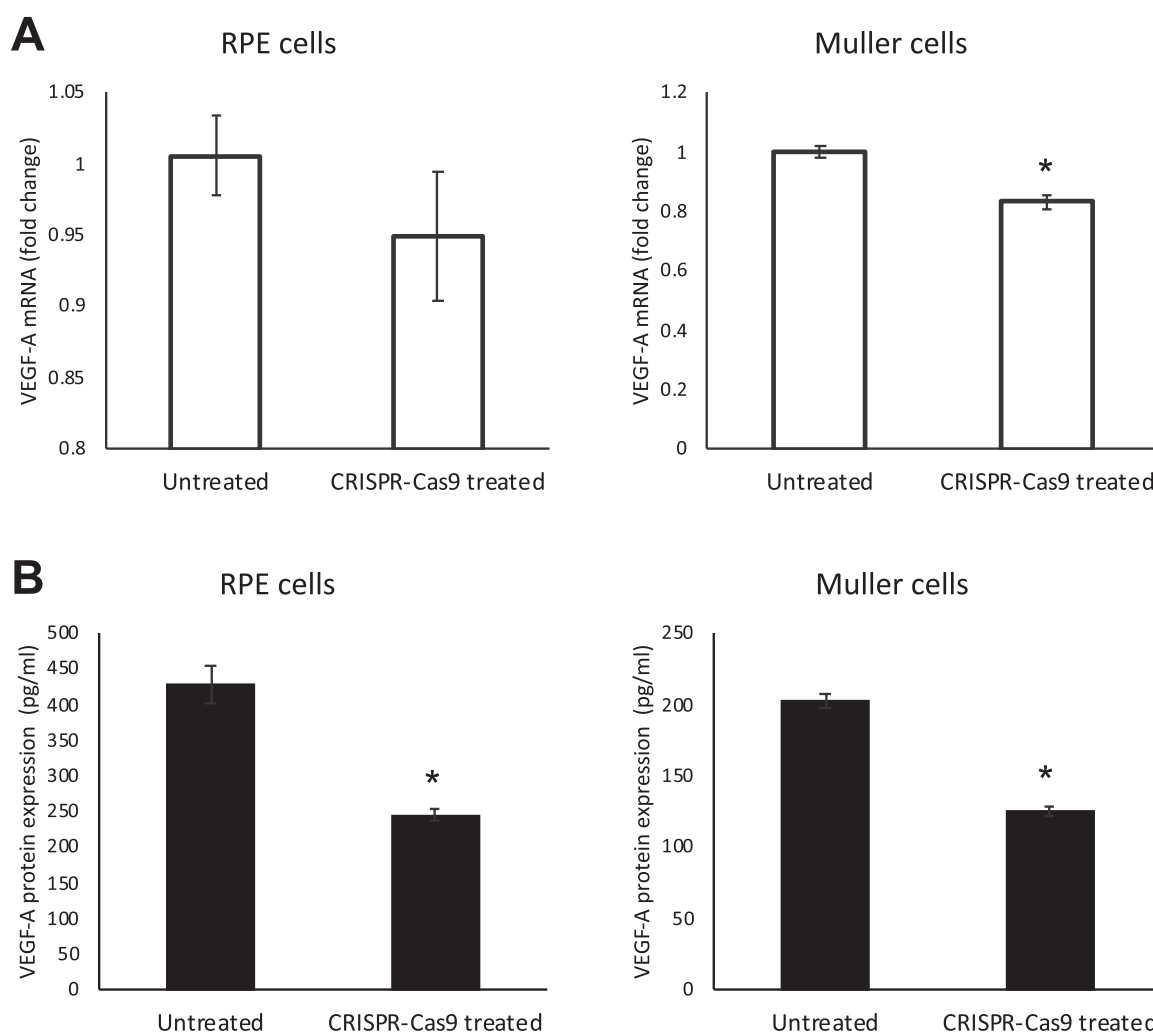


Figure 3. VEGF-A expression after VEGF-A gene disruption in RPE and Muller cells. CRISPR-Cas9-treated cells were compared with untreated cells. Data are presented as mean \pm standard error of the mean. * $P < 0.0001$. (A) VEGF-A mRNA measured using quantitative RT-PCR showing lower expression in CRISPR-Cas9-treated cells in both RPE and Muller cells; however, it is only statistically significant for Muller cells. Results are composed of 15 independent experiments for RPE and nine independent experiments for Muller cells, with three replicate samples for each experiment. (B) VEGF-A protein level in cell culture medium measured using enzyme-linked immunosorbent assay showing lower expression in CRISPR-Cas9-treated cells in both RPE and Muller cells. Results composed of nine independent experiments for both RPE and Muller cells, with two replicate samples for each.

targeting more than one cell type. This method could potentially be used to simultaneously disrupt the VEGF-A gene in all VEGF-A-producing cells of the eye to significantly reduce the intraocular VEGF-A level and treat retinal vascular diseases such as age-related macular degeneration, diabetic retinopathy, and retinal vein occlusion.

A major advantage of LCM delivery of CRISPR-Cas9 RNP is its lack of specificity in cell transfection. Genomic insertion of CRISPR-Cas9 via viral vectors may be helpful when the target is a specific cell type. However, when nonspecific targeting of several cell types is desirable, as is the case for the VEGF-A gene disruption, this limitation could become significant.

For example, the use of lentivirus after subretinal injection limits the delivery to the RPE cells only. In contrast, CRISPR-Cas9 RNP delivery via lipid nanoparticles is not cell specific and several cell types could be targeted simultaneously. For the VEGF-A gene disruption, this advantage is significant because all the VEGF-A-producing cells in the retina, including RPE cells and Muller cells, could be targeted simultaneously. Based on morphometric studies, the cell density of Muller cells seems to be approximately five times higher than RPE cells in the human retina.^{16–19} Therefore, to achieve a significant decrease in intraocular VEGF, it is important to target Muller cells as well as RPE cells.

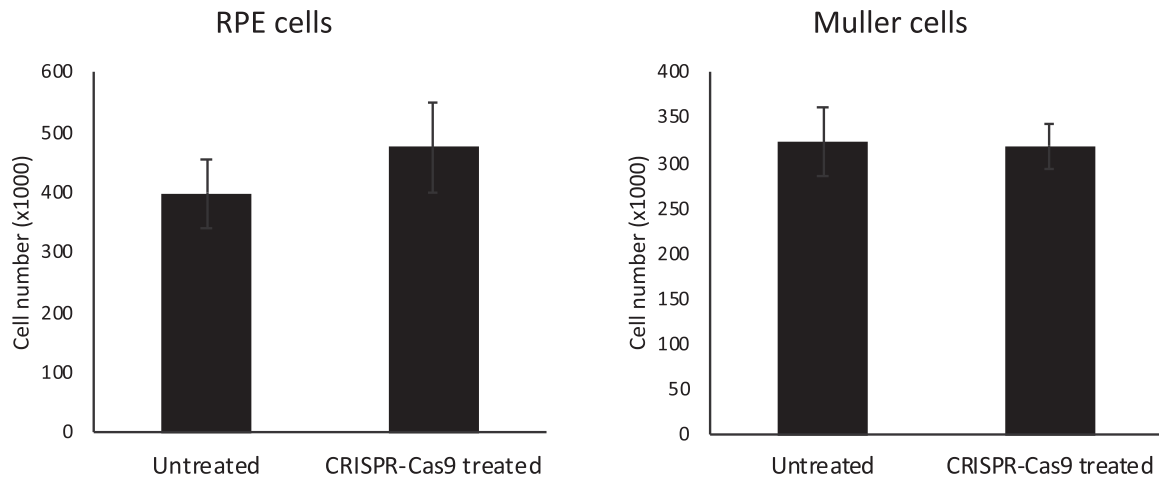


Figure 4. Cell count results comparing CRISPR-Cas9 treated and untreated cells shows no statically significant change in either RPE or Muller cells. Data are presented as mean \pm standard error of the mean. Results composed of 12 independent experiments for RPE and nine independent experiments for Muller cells.

Another advantage of using the CRISPR-Cas9 RNP is that it has a short half-life²⁰ and has a short-term effect. This property is in contrast with the genomic insertion of CRISPR-Cas9, which would continuously produce Cas9 and sgRNA, and may potentially increase the rate of off-target effects. This property is particularly more concerning when lentivirus is used, because the transgene integrates into the human genome.²¹ We chose LCM for our study because it has been shown to be superior to other nanoparticles for delivering CRISPR-Cas9 RNP into mammalian cells.²² Lipofectamine 2000, another lipid nanoparticle, has been reported to cause cell toxicity.²³ Although no such toxicity has been reported with LCM, we evaluated its potential toxicity in RPE and Muller cells and did not find any toxicity in either cell types.

We demonstrated here that VEGF-A gene disruption using CRISPR-Cas9 RNP significantly decreased VEGF-A protein expression in both RPE and Muller cells. Our results are similar to previous studies on RPE cells,^{10–12} but to the best of our knowledge this is the first time that Muller cells have been studied. Yiu et al.¹⁰ used genomic insertion of CRISPR-Cas9 delivered via lentivirus to target VEGF-A gene in RPE cells. Although this approach was effective in disrupting the VEGF-A gene in RPE cells, it is associated with some challenges when it comes to human treatment. Lentivirus has a good tropism for RPE cells, but only when it is delivered into the subretinal space.²⁴ Subretinal injection of genomic CRISPR-Cas9 via lentivirus has been shown to successfully transfect RPE cells and disrupt the VEGF-A gene in mice.¹¹ In addition,

subretinal injection of CRISPR-Cas9 RNP has been shown to decrease the size of the choroidal neovascular membrane in mice.¹² This success is less likely to apply to human retina, because the target area is limited to the RPE cells within the bleb created during the subretinal injection, and the bleb formed in human is much smaller than the one in mice. In humans, the bleb is contained in the posterior pole, and this would result in the transfection of only a fraction of RPE cells; it is unclear whether this would be sufficient for significant decrease of intraocular VEGF.

Unlike subretinal injection, intravitreal injection can distribute CRISPR-Cas9 to the entire retinal surface; however, it is unclear whether lipid nanoparticle transportation can result in efficient delivery to all VEGF producing cells. The internal limiting membrane is a barrier for viral vectors²⁵; however, whether the internal limiting membrane limits the passage of LCM remains to be determined. Apart from the limited surface area of subretinal injection, it is plausible that RPE transfection is more efficient with subretinal injection within the bleb area. For Muller cells, whose cell bodies are located in the inner nuclear layer, and their projections span across the retina, efficient transfection may be possible via both intravitreal and subretinal approaches. Given the expression of VEGF in ganglion cells and retinal vasculature, in addition to RPE cells and Muller cells, *in vivo* studies are needed to determine which approach—intravitreal or subretinal—can transfect more cells and result in a greater decrease in the global intraocular VEGF level.

Although there was a minimal but statistically significant reduction in VEGF-A mRNA expression

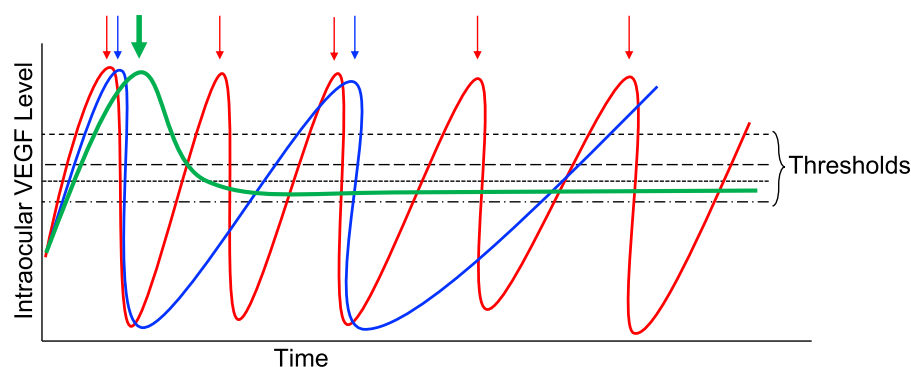


Figure 5. A diagram presumptively comparing the effects of CRISPR-Cas9 RNP gene editing (*green arrow*) with intravitreal anti-VEGF injections (*red and blue arrows*). The threshold above which pathologic effects of VEGF occur varies in different diseases and among different individuals; in addition, the speed of VEGF increase may depend on disease severity. Because of these factors, patients require anti-VEGF injection at different frequencies (*red and blue lines* represent VEGF levels in two different individuals with different needs for anti-VEGF injections). CRISPR-Cas9 RNP gene editing could potentially decrease the VEGF level by a single treatment, but its therapeutic effects would depend on the VEGF threshold. Although in some patients a single treatment may be all that they need, others may still require anti-VEGF injection, but at a lower frequency.

in Muller cells, the decrease in RPE cells did not reach statistical significance. A lack of decrease in VEGF-A mRNA after CRISPR-Cas9 gene editing has been previously reported by Yiu et al.,¹⁰ and it has been postulated that a change in mRNA expression would depend on the amount of transcriptional stop or creation of unstable RNA. Although this explanation is plausible, Kim et al.¹² showed a statistically significant decrease in VEGF-A mRNA expression in RPE cells.

We showed that CRISPR-Cas9-mediated VEGF-A gene disruption induces various indels at the predicted cut site in both RPE cells and Muller cells. We also noted unexpected mutations outside the target area. These mutations, which almost exclusively were single nucleotide substitutions, were also seen in control cells that did not receive VEGF-A gene disruption. These mutations, as well as a single nucleotide substitution seen at the predicted cut site in the control RPE cells, likely represent the well-established phenomenon of single nucleotide variants that result from clonal expansion of human cells.²⁶

VEGF is constitutively expressed in the eye and there is a concern that gene therapy may eliminate intraocular VEGF and result in unforeseen consequences. Systemic neutralization of VEGF using gene augmentation of sFlt-1, a soluble VEGF receptor, via adenovirus in mice showed no effects on the normal vasculature but a significant cell loss in the inner and outer nuclear layers.²⁷ However, a long-term study on intraocular delivery of the same factor, sFlt-1 via adeno-associated virus in mice and monkeys did not show any toxicity.²⁸ In addition, phase I and IIa

human clinical trials have demonstrated the safety of adeno-associated virus-mediated gene augmentation of sFlt-1 in the treatment of wet age-related macular degeneration, although only a portion of patients showed some response to treatment in these trials.⁴⁻⁶ Although gene augmentation of a soluble VEGF receptor may theoretically result in the production of enough receptor to potentially block all the released VEGF, gene disruption through CRISPR-Cas9 is not efficient enough to eliminate VEGF release from all VEGF-producing cells and is unlikely to result in total lack of VEGF (Fig. 5). A limitation of VEGF-A gene disruption in decreasing its level is that it may not reach therapeutic level for some patients (Fig. 5).

Based on previous reports that retinal vessels express VEGF,^{13,14} we used human retinal microvascular endothelial cells to study endothelial cells as well. However, our preliminary assessments revealed that VEGF-A protein expression in human retinal microvascular endothelial cells was almost undetectable by enzyme-linked immunosorbent assay. Perhaps other cellular components of the vasculature may be responsible for VEGF expression in normal vessels. Ganglion cells have also been reported to express VEGF.^{29,30} However, we did not study ganglion cells because they have numerous subtypes and are very difficult to culture.

In summary, we demonstrated that VEGF-A gene disruption using CRISPR-Cas9 RNP results in significant reduction in VEGF-A expression in both human RPE and Muller cells. Given the abundance of Muller cells in human retina, it is imperative to use therapeutic approaches that target these cells as well as RPE cells.

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