AAV-CRISPR/Cas9–Mediated Depletion of VEGFR2 Blocks Angiogenesis In Vitro

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation
Wu, Wenyi, Yajian Duan, Gaoen Ma, Guohong Zhou, Cindy Park-Windhol, Patricia A. D’Amore, and Hetian Lei. 2017. “AAV-CRISPR/Cas9–Mediated Depletion of VEGFR2 Blocks Angiogenesis In Vitro.” Investigative Ophthalmology & Visual Science 58 (14): 6082-6090. doi:10.1167/iovs.17-21902. http://dx.doi.org/10.1167/iovs.17-21902.

Published Version
doi:10.1167/iovs.17-21902

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:34651754

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Angiogenesis In Vitro

Wenyi Wu,1,2 Yajian Duan,1,3 Gaoen Ma,1,4 Guohong Zhou,1,3 Cindy Park-Windhol,1 Patricia A. D’Amore,1 and Hetian Lei1

1Schepens Eye Research Institute of Massachusetts Eye and Ear; Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, United States
2Department of Ophthalmology, Second Xiangya Hospital, Central South University, Changsha, Hunan, China
3Shanxi Eye Hospital, Taiyuan City, Shanxi Province, China
4Department of Ophthalmology, The Third Affiliated Hospital of Xinxiang Medical University, Eye Hospital of Xinxiang Medical University, Xinxiang, Henan Province, China

Correspondence: Hetian Lei, Schepens Eye Research Institute of Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, 20 Stanford Street, Boston, MA 02114, USA; Hetian Lei@meei.harvard.edu.

Submitted: March 20, 2017
Accepted: October 29, 2017
Citation: Wu W, Duan Y, Ma G, et al. AAV-CRISPR/Cas9–mediated depletion of VEGFR2 blocks angiogenesis in vitro. Invest Ophthalmol Vis Sci. 2017;58:6082–6090. DOI:10.1167/iovs.17-21902

PURPOSE. Pathologic angiogenesis is a component of many diseases, including neovascular age-related macular degeneration, proliferation diabetic retinopathy, as well as tumor growth and metastasis. The purpose of this project was to examine whether the system of adeno-associated viral (AAV)-mediated CRISPR (clustered regularly interspersed short palindromic repeats)-associated endonuclease (Cas)9 can be used to deplete expression of VEGF receptor 2 (VEGFR2) in human vascular endothelial cells in vitro and thus suppress its downstream signaling events.

METHODS. The dual AAV system of CRISPR/Cas9 from Streptococcus pyogenes (AAV-SpGuide and SpCas9) was adapted to edit genomic VEGFR2 in primary human retinal microvascular endothelial cells (HRECs). In this system, the endothelial-specific promoter for intercellular adhesion molecule 2 (ICAM2) was cloned into the dual AAV vectors of SpGuide and SpCas9 for driving expression of green fluorescence protein (GFP) and SpCas9, respectively. These two AAV vectors were applied to production of recombinant AAV serotype 5 (rAAV5), which were used to infect HRECs for depletion of VEGFR2. Protein expression was determined by Western blot; and cell proliferation, migration, as well as tube formation were examined.

RESULTS. AAV5 effectively infected vascular endothelial cells (ECs) and retinal pigment epithelial (RPE) cells; the ICAM2 promoter drove expression of GFP and SpCas9 in HRECs, but not in RPE cells. The results showed that the rAAV5-CRISPR/Cas9 depleted VEGFR2 by 80% and completely blocked VEGF-induced activation of Akt, and proliferation, migration as well as tube formation of HRECs.

CONCLUSIONS. AAV-CRISPR/Cas9-mediated depletion of VEGFR2 is a potential therapeutic strategy for pathologic angiogenesis.

Keywords: AAV5, CRISPR/Cas9, VEGFR2, angiogenesis

Vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis, the process by which new blood vessels grow from preexisting vessels. Among the VEGF receptors 1, 2, and 3 (VEGFR1, VEGFR2, and VEGFR3), VEGFR2 mediates nearly all the known VEGF-induced angiogenesis effect, including microvascular permeability and neovascularization. However, other receptors such as neuropilin/semaphorin on endothelial cell surfaces also affect angiogenesis. Neuropilin-1 can mediate vascular permeability independently of VEGFR2 activation and semaphorin signaling can also influence angiogenesis. Angiogenesis is critical for supporting the rapid growth of solid tumors beyond 1 to 2 mm3 and for tumor metastasis. Abnormal angiogenesis is also associated with a variety of other human diseases such as arthritis, proliferative diabetic retinopathy (PDR), and wet age-related macular degeneration (AMD). Adeno-associated viruses (AAVs) are small viruses that are not currently known to cause any disease, and AAV-derived vectors show promise in human gene therapy, especially for eye disease. AAV-mediated gene therapy has been reported to be both safe and effective in the treatment of a monogenic disorder like Leber’s congenital amaurosis type 2. The clustered regularly interspersed palindromic repeats (CRISPR)-associated DNA endonuclease (Cas)9 in Streptococcus pyogenes (SpCas9) processes pre-CRISPR RNA (pre-crRNA) transcribed from the repeat spacers into crRNA and cleaves invading nucleic acids on the direction of crRNA and trans-activating crRNA (tracrRNA). A single-guide RNA (sgRNA) engineered as a crRNA-tracrRNA chimeric RNA can direct sequence-specific SpCas9 cleavage of double-stranded DNA containing an adjacent “NGG” protospacer-adjacent motif (PAM). The CRISPR/Cas9 system is a powerful tool for the easy and highly specific targeting of eukaryotic genomes, particularly human cells, and subsequent gene insertion and deletion, resulting in reading frame shifts and protein deletion. Importantly, the CRISPR-Cas9 system is superior to other gene manipulation tools in terms of reduced off-target effects.
We have previously used a lentiviral vector to deliver the CRISPR-Cas9 system targeting human retinal microvascular endothelial cells (HRECs) for depletion of VEGFR2. Here, we used a dual AAV vector system to deliver CRISPR-Cas9 for depletion of VEGFR2 in HRECs and found that AAV5-CRISPR-Cas9-mediated depletion of VEGFR2 was able to block VEGF-induced activation of Akt and proliferation, migration, as well as tube formation of HRECs.

**Material and Methods**

**Major Reagents**

VEGF was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against VEGFR2, Akt, and p-Akt (S473) were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibody against β-actin and secondary antibodies of the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescent substrate for detection of HRP was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

**DNA Constructs**

The four 20-nt target DNA sequences preceding a 5'-NGG PAM sequence at exon 18 in the genomic VEGFR2 locus (NC_000071.6) were selected for generating sgRNA for SpCas9 targets using the CRISPR design Web site (http://crispr.mit.edu; in the public domain). The four target sequences were 5'-TACGTTCCTTCTATGGGC-3' (K7), 5'-AGGCTACTTTGTGCATTTGTA-3' (K8), 5'-TCTATCTGATGATCAAGCA-3' (K9), and 5'-GGATCTGATGATCAAGCA-3' (K10). The control sgRNA sequence (5'-TGGCAATTACGCCACGAGATGGG-3') was designed to target the lacZ gene from Escherichia coli. The lenti-crispr-v2 vector (52961)9,10 and the dual AAV vectors (AAV-SpCas9: 60957 and AAV-SpGuide: 60958) were purchased from Addgene (Cambridge, MA, USA).13,20

To select the most efficient sgRNA among the four sgRNAs, the top oligos 5'-ACCG-20nt (target VEGFR2 DNA sequences K7, K8, K9, K10 or the lacZ sgRNA sequence) and bottom oligos 5'-CAAC-20 nt (complementary target VEGFR2 DNA sequences or lacZ sgRNA sequence) -C-3' were annealed and cloned into the lenti-CRISPR v2 vector by BsmB1, respectively.

The pAAV-U6-sgRNA-psiCMV-2FP vector originated from AAV-SpGuide (Cat. 60958; Addgene)20 by replacing the hSyn with the PCAplamid promoter of intercellular adhesion molecule 2 (ICAM2) from genomic DNA of HRECs, using XbaI/Sall as described previously.21 The PCR primers are P1F (forward), 5’ XbaI-Kpn1-Apal-BamHI-20nt (5’-CGTCTAGAAG GTACCGGG GGGCCGGATCAGGAC-3’); and P1R (reverse), 5’ Sall-Agel-20nt- (5’-GGTTGCAGACCCGCTAGGAGCGAGGAG-3’). The pAAV/pCMV2-SpCas9 was derived from AAV-SpCas9 (Cat. 60957; Addgene) by replacing the pMEcp2 with pCMC2, using XbaI/Agel. The DNA fragments of this ICAM2 (top: 5’- CTAGAAGGTACCGGGATCAGGAC-3’) were ligated into AAV-U6-sgRNA-psiCMV-2FP vector, respectively.

**Production of Lentivirus**

The lentiviral vector v2 vector inserted with sgRNA (K7, K8, K9, or K10) (2000 ng), the packaging plasmid psPAX2 (12260; Addgene) (900 ng), and the envelope plasmid VSV-G (8454; Addgene) (100 ng) were mixed together with P3000 (Thermo Fisher Scientific) and then added to a mixture of lipofectamine 3000 (Thermo Fisher Scientific) 6 μL with OPTI-MEM (Thermo Fisher Scientific) 90 μL. This transfection mix was incubated at room temperature for 30 minutes and then carefully transferred into a 60-mm cell culture dish with human embryonic kidney 293T cells that were approximately 70% confluent without antibiotics. After 18 hours (37°C, 5% CO2), the medium was replaced with growth medium supplemented with 30% FBS, and at 24 hours after the medium change, lentiviruses were harvested. The viral harvest was repeated at 24-hour intervals three times. The virus-containing media were pooled, centrifuged at 8000 for 5 minutes, and the supernatant was used to infect porcine aortic endothelial cells (PAECs) overexpressing VEGFR2 (PAEC-KDR), supplemented with 8 μg/mL polybrene (Sigma-Aldrich Corp., St. Louis, MO, USA). The infected cells were selected in media by using puromycin (Sigma-Aldrich Corp.) (0.5 μg/mL) and the resulting cells were examined by Western blot.19,22-24

**Western Blot Analysis**

PAEC-KDR infected with the lentivirus or HRECs with AAV5-SpGuide and -SpCas9 were produced and the titers of AAV5 were determined by real-time PCR by Gene Transfer Vector Core at Schepens Eye Research Institute of Massachusetts Eye and Ear.
CRISPR/Cas9 Blocks Angiogenesis In Vitro

7.0), 10% sodium dodecyl sulfate (SDS) (Sigma-Aldrich Corp.), 500 mM dithiothreitol, 50% sucrose, 500 mM Tris HCl [(pH 6.8), and 0.5% bromophenol blue]. After boiling for 5 minutes, the samples were centrifuged for 5 minutes at 13,000g. Proteins in the samples were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to Western blot analyses. Experiments were repeated at least three times. Signal intensity was determined by densitometry with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Sanger DNA Sequencing

Genomic DNA was extracted from the transduced cells with the QuickExtract DNA Extraction Solution (Epicenter, Chicago, IL, USA) by following the manufacturer’s protocol. The genomic fragment approximately 200 bp around the PAM was PCR amplified with high-fidelity Hercule II DNA polymerases (Agilent Technologies, Santa Clara, CA, USA). The PCR primers were P10F (5′-ATGACACTAGTTAGCCGCG-3′) and P10R (5′-GGCACGACAAAACAGCCGAG-3′). The PCR products were separated in 2% agarose gel and purified with a gel extraction kit (Thermo Fisher Scientific) for Sanger DNA products. Images of the tubes were photographed at 6 hours post assay under a light microscope. The data were imported as a TIFF file into ImageJ software for calculating the total length of all tubing with each field, using angiogenesis analysis module. The data of three independent experiments were analyzed with Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

For statistical analysis, data from the three independent experiments were analyzed by using an unpaired t-test in Prism 6 software. P values of less than 0.05 were considered statistically significant.

RESULTS

Establishment of Dual AAV Vectors for Delivering CRISPR/Cas9

Recombinant AAV (rAAV) vectors are the most suitable candidates for virus-based gene therapy because of their broad tissue tropism, nonpathogenic nature, and low immunogenicity (Deyle, 2009, No. 412; Pillay, 2016, No. 801). In our study, we adapted a dual AAV vector system packaging SpCas9 and SpGuide. In the SpGuide vector the Syn promoter was replaced with ICAM2 promoter (pICAM2), an endothelial-specific promoter (Fig. 1A), for driving green fluorescence protein (GFP) expression; Mecp2 promoter was substituted for pICAM2 for driving SpCas9 expression in the AAV-SpCas9 (Fig. 1B). Subsequently, the dual AAV vectors of AAV-SpGuide with the lacZ-sgRNA or K7-sgRNA and AAV-SpCas9 were used to produce rAAV5 in the 293T cells because rAAV5 has been shown to infect endothelial cells (ECs) at high efficiency.

To demonstrate whether the ICAM2 promoter could drive protein expression specifically in vascular ECs, we infected ARPE-19 cells, a spontaneously immortalized cell line of retinal pigment epithelial cells, and HRECs with rAAV5-pICAM2-GFP and rAAV5-pICAM2-SpCas9, respectively. As shown in Figures 1C and 1D, expression of GFP and SpCas9 was detected in rAAV5 structures, but not in ARPE-19 cells, but the cytomegalovirus (CMV) promoter-driven GFP expression in rAAV5-CMV-GFP was able to be detected in both ARPE-19 cells and HRECs (Fig. 1E). Taken together, these results demonstrated that the dual AAV-CRISPR/Cas9 system is able to specifically target genomic loci of vascular ECs.

AAV5-CRISPR/Cas9–Mediated Depletion of VEGFR2

To identify sgRNAs to effectively guide SpCas9 to edit the genomic VEGFR2 locus, four protospacers were selected from exon 18 of human VEGFR2 and cloned into the lentCRISPR v2 vector by BsmB1. The confirmed lentivectors by DNA sequencing were used to produce lentivirus in HEK 293T cells for infecting PAEC-KDR cells. Western blot analysis showed that expression of VEGFR2 was reduced approximately 90% in the PAEC-KDR cells infected by lentCRISPR v2-K7-sgRNA and this
was the most effective sgRNA among the four sgRNAs in depleting VEGFR2 (Fig. 2). Therefore, the K7-sgRNA and lacZ-sgRNA were cloned into the SpGuide vector by SapI (Fig. 1A) for production of rAAV5, respectively; in addition, the vector of AAV-SpCas9 (Fig. 1B) was also subjected to generation of rAAV5. To assess the editing efficiency, we infected HRECs by using rAAV5-SpCas9 with rAAV5-VEGFR2 (K7) or rAAV5-lacZ. Seven days post infection, the genomic DNA was isolated for PCR. Sanger DNA sequencing results showed that there were mutations around the PAM sequence of PCR products from HRECs transduced with rAAV5-SpCas9 plus –VEGFR2-sgRNA (K7), but not from those with rAAV5-lacZ-sgRNA (Fig. 3B), indicating that the K7 sgRNA-guided SpCas9 cleaved the VEGFR2 locus at the expected site. NGS indicated that there were approximately 80% indels generated from the loci around the PAM of K7 sgRNA (Fig. 3C), but we did not find any indels among the most possible off-target by Sanger DNA sequencing and NGS; Western blot analysis of the infected cell lysates demonstrated an 80% decrease in VEGFR2 in the HRECs infected with the dual rAAV5 of SpCas9/VEGFR2-sgRNA (K7), compared with those transduced with SpCas9/lacZ-sgRNA (Fig. 3D). These results demonstrated that the AAV-CRISPR/Cas9 system with K7-sgRNA efficiently induced mutations
within the VEGFR2 locus and subsequent protein depletion in HRECs.

**Editing VEGFR2 Using CRISPR/Cas9 Blocked VEGF-Induced Activation of Akt**

VEGF binding to VEGFR2 can trigger the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt signaling pathway. Akt, also known as protein kinase B, is a serine/threonine kinase that plays a key role in multiple cellular responses including proliferation and migration, which are all intrinsic to angiogenesis. To evaluate whether AAV-CRISPR/Cas9-mediated depletion of VEGFR2 prevented VEGF-induced Akt, we cultured HRECs to approximately 80% confluence in the growth factor–free medium and then treated them with VEGF for 30 minutes. Western blot analysis showed that VEGF-induced activation of Akt was nearly completely blocked by AAV5-CRISPR/Cas9-mediated depletion of VEGFR2 (Fig. 4). These data indicate that the AAV5-CRISPR/Cas9-mediated

---

**Figure 3.** AAV5-CRISPR/Cas9-mediated depletion of VEGFR2. (A) Schematic of a target DNA sequence (K7) preceding a 5'-NGG PAM sequence at exon 18 in the genomic VEGFR2 locus (NC_000071.6), which was selected for generating sgRNA. The red triangle points to an expected cleavage site of SpCas9 at the human genomic VEGFR2 locus. (B, C) Purified PCR products from the CRISPR/Cas9-engineered HRECs were subjected to Sanger DNA sequencing. The DNA sequencing results, indicated by lacZ-sgRNA and K7-sgRNA, were derived from the HRECs transduced by SpCas9 together with lacZ-sgRNA or K7-sgRNA. The PAMs are indicated above a thick blue line and the expected cleavage site of SpCas9 is indicated by a red triangle. (D) Western blot analysis of VEGFR2 expression in the AAV-CRISPR/Cas9-edited HRECs, using indicated antibodies. lacZ-sgRNA served as a negative sgRNA control. “Fold” was calculated by first normalizing to the level of β-actin and then calculating the ratio of the VEGFR2 over the LacZ lane. This is representative of three independent experiments and error bars are SD.

**Figure 4.** AAV-CRISPR/Cas9-mediated depletion of VEGFR2 prevents VEGF-induced activation of Akt. The HRECs transduced with the dual AAV vectors, as described in Figure 3, in growth factor–free medium overnight in wells of a 24-well plate, were treated with VEGF (20 ng/mL) for 30 minutes. The cell lysates were then subjected to Western blot by using the indicated antibodies. The lanes of LacZ and VEGFR2 represent the cell lysates from HRECs transduced by SpCas9 together with lacZ-sgRNA or VEGFR2-sgRNA. “Fold” was calculated by first normalizing to the level of β-actin or Akt and then calculating the ratio of the VEGFR2 and other lanes over the LacZ lane. Data of bar graphs are representative of three independent experiments.
depletion of VEGFR2 has potential to block VEGF-induced cellular responses via blocking VEGF-induced activation of Akt.

**Editing VEGFR2 Using CRISPR/Cas9 Prevented VEGF-Induced Proliferation and Migration**

VEGF-induced autophosphorylation of VEGFR2 stimulates cellular responses including cell proliferation and migration.35–37 To examine VEGF-induced proliferation of HRECs that had been transduced by SpCas9 together with VEGFR2-sgRNA or lacZ-sgRNA, these HRECs were deprived of the growth factors overnight and then treated with VEGF (20 ng/mL) for 48 hours. 22,38 As expected, VEGF stimulated proliferation of HRECs transduced by SpCas9 with lacZ-sgRNA but failed to induce the proliferation of HRECs transduced by SpCas9 with VEGFR2-sgRNA (Fig. 5A).

Migration is one of the important cellular events in the process of angiogenesis,39 so we investigated whether AAV-CRISPR/Cas9-mediated depletion of VEGFR2 prevented VEGF-induced migration of HRECs. HRECs infected with the dual AAV vectors of AAV5-SpCas9 plus VEGFR2-sgRNA (K7) or lacZ-sgRNA were examined in a scratch wound-healing assay. As shown in Figure 5B, whereas VEGF induced migration of HRECs transduced by SpCas9 with lacZ-sgRNA but failed to induce the proliferation of HRECs transduced by SpCas9 with VEGFR2-sgRNA (Fig. 5A).

Migration is one of the important cellular events in the process of angiogenesis,39 so we investigated whether AAV-CRISPR/Cas9-mediated depletion of VEGFR2 prevented VEGF-induced migration of HRECs. HRECs infected with the dual AAV vectors of AAV5-SpCas9 plus VEGFR2-sgRNA (K7) or lacZ-sgRNA were examined in a scratch wound-healing assay. As shown in Figure 5B, whereas VEGF induced migration of HRECs transduced by SpCas9 with lacZ-sgRNA but failed to induce the proliferation of HRECs transduced by SpCas9 with VEGFR2-sgRNA (Fig. 5A).

Migration is one of the important cellular events in the process of angiogenesis,39 so we investigated whether AAV-CRISPR/Cas9-mediated depletion of VEGFR2 prevented VEGF-induced migration of HRECs. HRECs infected with the dual AAV vectors of AAV5-SpCas9 plus VEGFR2-sgRNA (K7) or lacZ-sgRNA were examined in a scratch wound-healing assay. As shown in Figure 5B, whereas VEGF induced migration of HRECs transduced by SpCas9 with lacZ-sgRNA but failed to induce the proliferation of HRECs transduced by SpCas9 with VEGFR2-sgRNA (Fig. 5A).

**AAV-CRISPR/Cas9-Mediated Depletion of VEGFR2 Blocked VEGF-Induced Tube Formation**

To evaluate whether AAV-CRISPR/Cas9-mediated depletion of VEGFR2 prevented VEGF-induced tube formation, an in vitro model of endothelial morphogenesis,19,29,38,40,41 HRECs infected with the dual AAV system of AAV-SpCas9 with VEGFR2-sgRNA (K7) or lacZ-sgRNA were used in a collagen-based tube formation assay. As shown in Figure 6, VEGF stimulated tube formation in the HRECs transduced by SpCas9 with lacZ-sgRNA, but it did not induce this reaction in those that were transduced by VEGFR2-sgRNA (K7)/SpCas9. These results suggest that editing genomic VEGFR2 with VEGFR2-sgRNA (K7)/SpCas9 is a potentially powerful therapeutic approach to the treatment of abnormal angiogenesis.

**DISCUSSION**

Herein we reported that AAV-CRISPR/Cas9-mediated depletion of VEGFR2 in HRECs blocked VEGF-stimulated Akt activation and cellular responses intrinsic to angiogenesis. Angiogenesis plays a significant role in a number of pathologic conditions, such as PDR and wet AMD.42 VEGFR2-sgRNA (K7) was used in this study to efficiently guide SpCas9 to cleave the double DNA strands in exon 18 of the human genomic VEGFR2, leading to a nonhomologous end joining (NHEJ) repair and resulting in indels in the VEGFR2 locus and subsequent depletion of
VEGFR2 expression in HRECs. Not all sgRNAs designed by using the online tool would be expected to have the same efficiency in guiding SpCas9,43 so we designed four sgRNAs from exon 18 of the human genomic VEGFR; the K7-sgRNA was the most efficient among the four sgRNAs. Previously, we have reported that an sgRNA generated from a protospacer in exon 3 of the human genomic VEGFR2 also efficiently guides SpCas9 to cleave its target around the PAM with subsequent depletion of VEGFR2 in lentivirally infected HRECs.19 In this report, we extended the study by using the dual AAV5-CRISPR/Cas9-infected HRECs and explored a potential opportunity with this sgRNA to prevent angiogenesis-related diseases.

AAV vectors have been used in clinical trials for gene transfer in liver (Wang, 2000, No. 692) and to retina for Leber’s with this sgRNA to prevent angiogenesis-related diseases. Cas9–infected HRECs and explored a potential opportunity report, we extended the study by using the dual AAV5-CRISPR/Cas9 system for delivering CRISPR/Cas9 into HRECs. AAV5 has been shown to enter the cells via platelet-drived growth factor system for delivering CRISPR/Cas9 into HRECs. AAV5 has been shown previously to effectively transduce vascular ECs.47 Our results (data not shown). In addition, AAV5 has been shown previously to effectively transduce vascular ECs.47 Our results showed that rAAV5-VEGFR2-sgRNA (K7)/SpCas9 efficiently depleted VEGFR2 expression in HRECs, suggesting that this dual AAV system has potential to be translated into a therapeutic purpose for pathologic angiogenesis. At present, anti-VEGF agents (e.g., ranibizumab and aflibercept) are the standard care for intraocular neovascularization such as PDR and wet AMD. While these anti-VEGF agents can reduce angiogenesis and vascular leakage, therapeutic challenges remain, including the need for chronic treatment and the fact that a significant number of patients do not respond. Gene therapy targeting genomic VEGFR2 in vascular ECs, using AAV-CRISPR/Cas9, may provide a potential novel alternative approach. However, VEGFR2 is essential for survival of vascular ECs48,49; in addition, it has been shown that conditional knockout of VEGF in retinal pigment epithelial cells leads to death of choroid capillaries and to cone photoreceptor degeneration.50 Furthermore, we have previously reported that AAV1-mediated CRISPR/Cas9 abrogates angiogenesis in mouse models of oxygen-induced retinopathy and laser-induced choroidal neovascularization.13 Whether AAV5-mediated CRISPR/Cas9 could block angiogenesis in vivo and whether AAV5 is superior to AAV1 for delivering CRISPR/Cas9 into pathologic angiogenic ECs need further investigation.

Technologies used for genome editing based on programmable nucleases such as zinc finger nucleases, transcription activator–like effector nucleases and CRISPR/Cas9 are opening up the possibility of achieving therapeutic genome editing in diseased cells and tissues.54 CRISPR/Cas9 technology enables especially precise genome editing by introducing DNA double-strand breaks at specific genomic loci with subsequent protein depletion. However, genetic modifications are permanent, and deleterious off-target mutations could reduce cellular integrity, leading to functional impairment or creating cells with oncogenic potential.55 Rational engineering of SpCas9 would be expected to lead to improvement.

**Acknowledgments**

Supported by the National Eye Institute of the National Institutes of Health under Award No. R01EY012509 (HL), China Scholarship Council (WW), and Central South University Student Innovation Project 2016zts148 (WW).

Disclosure: W. Wu, None; Y. Duan, None; G. Ma, None; G. Zhou, None; C. Park-Windhol, None; P.A. D’Amore, None; H. Lei, None

**References**

1. Millauer B, Wizigmann-Voos S, Schnurch H, et al. High affinity VEGF binding and developmental expression suggest FkI-1 as a major regulator of vasculogenesis and angiogenesis. *Cell. 1993;72:835–846.*

2. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol. 2000;2:737–744.*

3. Wise LM, Veikola T, Mercer AA, et al. Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. *Proc Natl Acad Sci U S A. 1999;96:3071–3076.*

4. Roth L, Prahlst C, Ruckdeschel T, et al. Neuropilin-1 mediates vascular permeability independently of vascular endothelial growth factor receptor-2 activation. *Sci Signal. 2016;9:ra42.*

5. Sakurai A, Doci CL, Gutfind JS. Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. *Cell Res. 2012;22:23–32.*

6. Hetian L, Ping A, Shumee S, et al. A novel peptide isolated from a phage display library inhibits tumor growth and metastasis by blocking the binding of vascular endothelial growth factor to its kinase domain receptor. *J Biol Chem. 2002;277:43157–43142.*

7. Timar J, Dome B, Fazeekas K, Janovics A, Paku S. Angiogenesis-dependent diseases and angiogenesis therapy. *Patol Oncol Res. 2001;7:85–94.*

8. Flootte TR, Barraza-Otiz X, Solow R, Aflone SA, Carter BJ, Guggino WB. An improved system for packaging recombinant

---

**FIGURE 6.** AAV-CRISPR/Cas9-mediated depletion of VEGFR2 blocked VEGF-induced tube formation. The solution of BME (80 μL) was transferred into each well of a 96-well plate, which was placed on ice for at least 10 to 15 minutes. The plate was then incubated at 37°C for 30 to 60 minutes to polymerize the gel. Then, HRECs transduced with AAV5SpCas9 together with AAV5-SpGuide (VEGFR2-sgRNA or lacZ-sgRNA) at a density of 20,000 cells per well in the 100-μL culture medium, with or without VEGF (20 ng/mL), were plated on top of each polymerized BME gel. After 6 hours, the cells were photographed under a light microscope, and the data were imported as a TIFF file into ImageJ software for calculating the total length of all tubing with each field, using angiogenesis analysis module. Each bar graph indicates mean ± SD of three independent experiments. **"*** denotes a significant difference in results between the two compared groups. *P* < 0.05 using an unpaired *t*-test. Images of one representative experiment are shown below the *bar graphs.*
CRISPR/Cas9 Blocks Angiogenesis In Vitro

IOVS  December 2017  Vol. 58  No. 14  6089

9. Hussain SP, Amstad P, He P, et al. p53-induced up-regulation of MnSOD and GPx but not catalase increases oxidative stress and apoptosis. Cancer Res. 2004;64:2350-2356.

10. Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber’s congenital amaurosis. New Engl J Med. 2008;358:2240-2248.

11. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014;157:1262-1278.

12. Gao J, Kang AJ, Lin S, et al. Association between MDM2 rs227974 polymorphism and breast cancer susceptibility: a meta-analysis based on 9,788 cases and 11,195 controls. Tiber Clin Risk Manag. 2014;10:269-277.

13. Huang X, Zhou G, Wu W, et al. Genome editing abrogates gene transfer for Leber’s congenital amaurosis. NEnglJMed. 2014;371:1007-1016.

14. Doudna JA, Charpentier E. Genome editing: the new frontier. Science. 2014;348:1258096.

15. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods. 2014;11:783-784.

16. Perez-Pinera P, Kocak DD, Vockley CM, et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat Methods. 2013;10:973-976.

17. Heler R, Wright AV, Vucelja M, Bikard D, Doudna JA, Marraffini LA. Structures of the CRISPR genome integration complex. Science. 2013;341:1204.

18. Wright AV, Liu JJ, Knott GJ, Doxzen KW, Nogales E, Doudna JA. Structures of the CRISPR genome integration complex. Science. 2017;357:1113-1118.

19. Duan Y, Ma G, Huang X, D’Amore PA, Zhang F, Lei H. The p110delta phosphoinositide 3-kinase and regulation of Akt/PKB by the rictor-mTOR complex 2. Mol Cell. 2013;50:636-647.

20. Swiech L, Heidenreich M, Banerjee A, et al. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nature Biotechnol. 2015;33:102-106.

21. Duan Y, Ma G, Huang X, D’Amore PA, Zhang F, Lei H. The clustered, regularly interspaced, short palindromic repeats-associated endonuclease 9 (CRISPR/Cas9)-created MDM2 T309G mutation enhances vitreous-induced expression of MDM2 and proliferation and survival of cells. J Biol Chem. 2016;291:16339-16347.

22. Lei H, Qian CX, Lei J, Haddock LJ, Mukai S, Kazlauskas A. RasGAP promotes autophagy and thereby suppresses platelet-derived growth factor receptor-mediated signaling events, cellular responses, and pathology. Mol Cell Biol. 2015;35:1673-1685.

23. Lei H, Kazlauskas A. Growth factors outside of the PDGF family employ ROS/SFKs to activate PDGF receptor alpha and thereby promote proliferation and survival of cells. J Biol Chem. 2009;284:6329-6336.

24. Lei H, Velez G, Cui J, et al. N-acetylcysteine suppresses retinal migration in an experimental model of proliferative vitreoretinopathy. Am J Pathol. 2010;177:132-140.

25. Ruan GX, Kazlauskas A. Axl is essential for VEGF-A-dependent migration and regulation of Akt/PKB by the rictor-mTOR complex 2. Mol Cell. 2013;50:636-647.

26. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc. 2007;2:329-335.

27. De Francesco EM, Pellegrino M, Santolla MF, et al. GPER mediates activation of HIF1α/VEGF signaling by estrogens. Cancer Res. 2014;74:4053-4064.

28. Caceres PS, Mendez M, Ortiz PA. Vesicle-associated membrane protein 2 (VAMP2) but not VAMP3 mediates cAMP-stimulated trafficking of the renal Na+K+-2Cl– co-transporter NKCC2 in thick ascending limbs. J Biol Chem. 2014;289:23951-23962.

29. Amaoutova I, Kleinman HK. In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc. 2010;5:628-635.

30. Ali K, Bilancio A, Thomas M, et al. Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. Nature. 2004;431:1007-1011.

31. Ellis BL, Hirsch ML, Barker JC, Connelly JP, Steininger RJ III, Porteus MH. A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with nine natural adeno-associated viruses (AAV-1-9) and one engineered adeno-associated virus serotype. Virol J. 2013;10:74.

32. Patterson C, Perrella MA, Hsieh C-M, Yoshizumi M, Lee M-E, Haber E. Cloning and functional analysis of the promoter for KDR/flk-1, a receptor for vascular endothelial growth factor. J Biol Chem. 1995;270:23111-23118.

33. Ruan GX, Kazlauskas A. Lactate engages receptor tyrosine kinases Axl, Tie2, and vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis. J Biol Chem. 2013;288:21161-21172.

34. Franke TF, Yang SL, Chan TO, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell. 1995;81:727-756.

35. Chen J, Somanath PR, Razorenova O, et al. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nat Med. 2005;11:1188-1196.

36. Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science. 1997;275:665-668.

37. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/mTOR complex 1. Science. 2005;307:1098-1101.

38. Ruan GX, Zhang DQ, Zhou T, Yamazaki S, McMahon DG. Circadian organization of the mammalian retina. Proc Natl Acad Sci U S A. 2006;103:9703-9708.

39. Holmes K, Roberts OL, Thomas AM, Cross MJ. Vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis. J Biol Chem. 2009;284:29122-29129.

40. Im E, Kazlauskas A. Regulating angiogenesis at the level of PtdIns-4,5-P2. EMBO J. 2006;25:2075-2082.

41. Im E, Kazlauskas A. Src family kinases promote vessel stability by antagonizing the Rho/ROCK pathway. J Biol Chem. 2007;282:29122-29129.

42. Bradley J, Ju M, Robinson GS. Combination therapy for the treatment of ocular neovascularization. Angiogenesis. 2007;10:141-148.

43. Ran FA, Hsu PD, Lin CY, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell. 2013;154:1380-1389.

44. Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber’s congenital amaurosis. N Engl J Med. 2008;359:2240-2248.

45. Bainbridge JW, Smith AJ, Barker SS, et al. Effect of gene therapy on visual function in Leber’s congenital amaurosis. N Engl J Med. 2008;358:2251-2259.

46. Di Pasquale G, Davidson BL, Stein CS, et al. Identification of PDGFR as a receptor for AAV-5 transduction. Nat Med. 2005;11:1306-1312.

47. Chen S, Kapturczak M, Loiler SA, et al. Efficient transduction of vascular endothelial cells with recombinant adeno-associ-
ated virus serotype 1 and 5 vectors. *Hum Gene Ther.* 2005;16:235–247.

48. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med.* 2012;2:a006502.

49. Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat Rev Mol Cell Biol.* 2016;17:611–625.

50. Le YZ, Bai Y, Zhu M, Zheng L. Temporal requirement of RPE-derived VEGF in the development of choroidal vasculature. *J Neurochem.* 2010;112:1584–1592.

51. Stoddard BL. Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Structure.* 2011;19:7–15.

52. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet.* 2010;11:636–646.

53. Bogdanove AJ, Voytas DF. TAL effectors: customizable proteins for DNA targeting. *Science.* 2011;333:1843–1846.

54. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat Med.* 2015;21:121–131.

55. Zhang F. CRISPR-Cas9: prospects and challenges. *Hum Gene Ther.* 2015;26:409–410.