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

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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 interspaced 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-CRISRP/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.[1] 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.[2,3] However, other receptors such as neuropilin/semaphorin on endothelial cell surfaces also affect angiogenesis. Neuropilin-1 can mediate vascular permeability independently of VEGFR2 activation[4] and semaphorin signaling can also influence angiogenesis.[5] Angiogenesis is critical for supporting the rapid growth of solid tumors beyond 1 to 2 mm^3 and for tumor metastasis.[6] 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).[7]

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.[8,9] 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.[10] 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).[11–13]

The CRISPR/Cas9 system is a powerful tool for the easy and highly specific targeting of eukaryotic genomes,[14] particularly human cells,[15] and subsequent gene insertion and deletion, resulting in reading frame shifts and protein depletion.[16] Importantly, the CRISPR-Cas9 system is superior to other gene manipulation tools in terms of reduced off-target effects.[17,18]

We have previously used a lentiviral vector to deliver the CRISPR-Cas9 to human retinal microvascular endothelial cells (HRECs) for depletion of VEGFR2.[13] 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).[13,19] The four target sequences were 5′-TCAGTTCCCCTTCATTGGCC-3′ (K7), 5′-AGGCTACTTGTC TATTGTCA-3′ (K8), 5′-TTCATCTGGATCCATGACGA -3′ (K9), and 5′-GGATCCAGATGAACTCCCAT-3′ (K10). The control sgRNA sequence (5′-TGCGAATACGCCCACGCGATGGG-3′) was designed to target the *lacZ* gene from *Escherichia coli*.[13,19,20] The lenti-crispr v2 vector (52961)[19] z, 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′-CACCG-20nt (target *VEGFR2* DNA sequences K7, K8, K9, K10 or the *lacZ* sgRNA sequence) and bottom oligos 5′-CAAC-20 nt (20nt: complementary target *VEGFR2* DNA sequences or *lacZ* sgRNA sequence) -C-3′ were annealed and cloned into the lenti-CRISPR v2 vector by *Bsm*B1, respectively.

The pAAV-U6-sgRNA-pICAM2-GFP vector originated from AAV-SpGuide (Cat. 60958; Addgene)[20] by replacing the hSyn with the PCR-amplified promoter of intercellular adhesion molecule 2 (ICAM2) from genomic DNA of HRECs, using *Xba*I/*Sal*I as described previously.[21] The PCR primers are P1F (forward), 5′ *Xba*I-*Kpn*I-*Apa*I-*Bam*HI-20nt- (5′-CGTCTAGAGG GTACCGGG GCCCGGGATCCTAGA ACGA GCTGGTGC ACGTGGC-3′); and P1R (reverse), 5′ *Sal*I-*Age*I-20nt- (5′-GGGTCGACgACCGGTCCAAGGGCTGCCTGGAGGGAG-3′). The pAAV-pICAM2-SpCas9 was derived from AAV-SpCas9 (Cat. 60957; Addgene) by replacing the pMecp2 with pICAM2, using *Xba*I/*Age*I. The DNA fragments of this ICAM2 (top: 5′-CTAGAgGGTACCgGGATCC TAGAACGAGCTGGTGCACG TGGCTTCCCAA AGATCTCTCAGATAATGAGAGGAAATGC AGTCATCAGTTTGCAGAAGGCT AGGGATTCTGGGCCATA GCTCAGACCTGCGCCCACCATCTCC CTCC AGGCAGC CCTTGGACCGGTgG-3′; bottom: 5′-TCGACcACCGGTCCAAGG GCTGCCTG GAGGGAGATGGTGGGCGCAGGTCTGAGCTAT GGCCCAGAATC CCTAGCCTTCTGCAAACTGATGACTGC ATTTCCTCTCATTATCTGAGAGATCTTTGGGAAGCCACGTGC ACCAGCTCGTTCTAGGATCCcGGTACCcT) in this vector were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

To express SpGuides in the targeted cells, the top oligos 5′-ACCG-K7-3′ and bottom oligos 5′-AAC-20nt (20nt: complementary target K7 *VEGFR2* DNA sequences or *lacZ* sgRNA sequence) -C-3′ were annealed and cloned into AAV-U6-sgRNA-pICAM2-CMV vector, respectively, by *Sap*I (New England Biolabs, Boston, MA, USA).[20] All clones were confirmed by DNA sequencing with a primer 5′-GGACTATCA TATGCTTACCG-3′ derived from U6 promoter that drives expression of sgRNAs. All primers and oligos were synthesized, and PCR products and clones were sequenced for confirmation at the Massachusetts General Hospital DNA Core Facility (Cambridge, MA, USA).

Cell Culture and Transfection

ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture (Thermo Fisher Scientific, Grand Island, NY, USA) with 10% inactivated fetal bovine serum (FBS; Lonza, Walkersville, MD). Primary HRECs were purchased from Cell Systems (Kirkland, WA, USA) and cultured in an endothelial growth medium (EGM)-2 kit (Lonza).[13] All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.[22] AAV serotype 5 (AAV5) of AAV-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.

Production of Lentivirus

The lentiCRISPR 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% CO_2), 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 800*g* 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 (7.5×10^9 GC/well in 24-well plates) for 3 days at 90% confluence in a 24-well plate deprived of serum and growth factors overnight were treated for 30 minutes with VEGF (20 ng/mL). After rinsing twice with ice-cold phosphate-buffered saline (PBS), cells were lysed in 1 × sample buffer that was diluted with extraction buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/mL aprotinin, 2 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride) from the 5 × protein sample buffer (25 mM EDTA [pH

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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 13,000*g*. Proteins in the samples were separated by 10% SDS- PAGE, transferred to polyvinylidene difluoride membranes, and subjected to Western blot analyses.[22] 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).[22]

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 Herculase II DNA polymerases (Agilent Technologies, Santa Clara, CA, USA). The PCR primers were P10F (5′-AATGCACCTAGCTTCAGCCG-3′) and P10R (5′-AGCACACAAAACAACAGCCAA-3′). The PCR products were separated in 2% agarose gel and purified with a gel extraction kit (Thermo Fisher Scientific) for Sanger DNA sequencing and next generation sequencing (NGS).[13]

NGS Analysis of Potential Off-targets

To find potential off-targets for the K7-targeted genes, the "CRISPR Design Tool" (http://crispr.mit.edu/; in the public domain) was used,[20] indicating that the most potential off-target sequence was ACAATTCCACTCCATTGGCCAAG, which was located at chr18:-9326920. From this information, we designed PCR primers (forward primer P30F: AGTAGT GAAAGGCCAGTGCAA; reverse primer P30R: ACACAGGGTT ACCACAAACCT) for PCR amplification of the DNA fragment covering the potential K7 off-targets for Sanger DNA sequencing and PCR primers (forward primer P31F: GGGTTTAAAGAGTGAGTAGAA; reverse primer P31R: GGTTACCACAAACCTTCAATT) for NGS. The PCR products from HRECs transduced by the dual AAV-CRISPR/Cas9 vectors either containing *lacZ*-sgRNA or *VEGFR2*-sgRNA K7 were sent for Sanger DNA sequencing and next generation sequencing (NGS).[13]

Cell Proliferation Assay

The infected HRECs were cultured into 24-well plates at a density of 30,000 cells/well in an EGM kit. After attachment, the cells were starved for growth factors for 7 hours. Then VEGF (20 ng/mL) was added into the wells. The treatment was repeated daily. After 48 hours, the cells were trypsin detached and then counted in a hemocytometer under a light microscope.[19,22,25]

Wound-Healing Assay

The wound-healing migration assay was performed as described previously.[19,26] Briefly, when the infected HRECs reached 80% confluence in 48-well plates, they were starved for growth factors for 8 hours, and the wells were scraped with a sterile pipette tip (200 μL). The cells were then washed twice to remove detached cells and treated with VEGF (20 ng/mL). The wound was photographed at 0 and 18 hours post wounding under a microcopy. Quantification was done by measuring the number of pixels in the wound area, using Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and analyzed by using ImageJ software.[19,25]

Tube Formation Assay

This assay was performed as described previously.[19,27–29] Briefly, Cultrex Basement Extract (BME) (Trevigen, Gaithersburg, MD, USA) from storage at –80°C was thawed overnight on ice. Then a 96-well plate was placed on ice for at least 10 to 15 minutes, and the solution of BME (80 μL) was transferred into each well. This plate was subsequently incubated at 37°C for 30 to 60 minutes to polymerize the gel. After 1 hour, HRECs infected with AAV5-SpCas9 together with AAV5-SpGuide (*VEGFR2*-sgRNA or *lacZ*-sgRNA) at a density of 2×10^4/well in 100 μL culture medium were plated on top of each polymerized BME gel. Specifically, the culture medium was EBM supplemented with 0.5% horse serum and 0.1% bovine brain extract supplemented with or without VEGF (20 ng/mL). 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.[13] 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[20,30] (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.[31]

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 HRECs, 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*[32] and cloned into the lentiCRISPR v2 vector by *Bsm*B1. The confirmed lentivectors by DNA sequencing were used to produce lentiviruses 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 lentiCRSIRP v2-K7-sgRNA and this

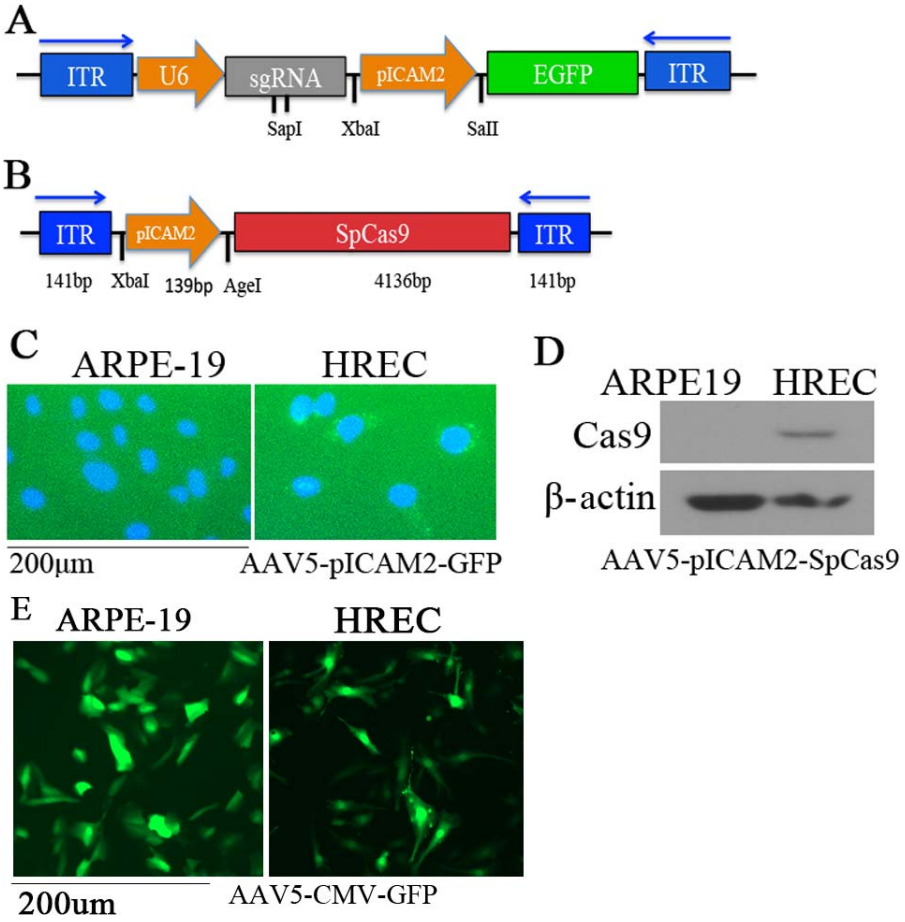

Figure 1. Establishment of dual AAV vectors for delivering CRISPR/Cas9 and confirmation of pICAM2 specificity in vascular ECs. (**A**) Schematic of AAV-U6-sgRNA-pICAM2-GFP. The hSyn promoter in AAV-SpGuide was substituted by pICAM2 by *Xba*I/*Sal*I. A protospacer sequence could be cloned into this vector by *Sap*I for synthesizing sgRNA. (**B**) Schematic of AAV-pICAM2-SpCas9. The ICAM2 promoter was used to replace the Mecp2 promoter in the AAV-SpCas9 by using *Xba*I/*Age*I for driving expression of SpCas9 specifically in vascular ECs. (**C**–**E**) ARPE-19 cells and HRECs were grown to 50% confluence in 48-well plates, AAV5-pICAM2-GFP, AAV5-pICAM2-SpCas9, or AAV5-CMV-GFP (3.75×10^{12} GC/mL) were added into the wells (2 μL/well). Two days later the cells were photographed by fluorescence microscopy (**C**, **E**), and the lysates of the cells transduced by AAV5-pICAM2-SpCas9 were subjected to Western blot analysis with antibodies against Cas9 and β-actin (**E**). These experiments were repeated at least three times.

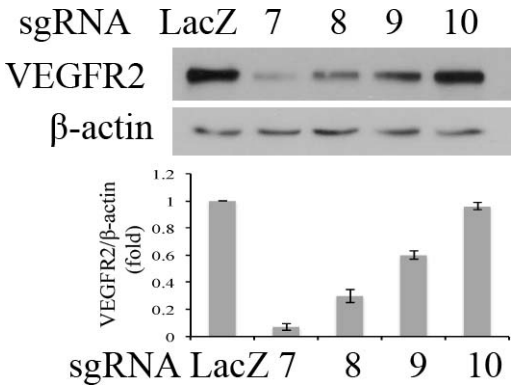

Figure 2. Selection of an effective sgRNA. Western blot analysis of VEGFR2 expression in the CRISPR/Cas9-engineered PAE-KDR cells, using indicated antibodies. *lacZ*-sgRNA served as a negative control sgRNA. Lanes of LacZ, K7, K8, K9, and K10 denote the protein samples from PAE-KDR cells, which were transduced by SpCas9 together with *lacZ*-, K7-, K8-, K9-, or K10-sgRNAs. "Fold" was calculated by first normalizing to the level of β-actin and then calculating the ratio of the K7, K8, K9, and K10 over the LacZ lane, respectively. Data of *bar graphs* are representative of three independent experiments and the *error bars* are standard derivation (SD).

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 *Sap*I (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-SpCas9 plus –*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-CRISRP/Cas9 system with K7-sgRNA efficiently induced mutations

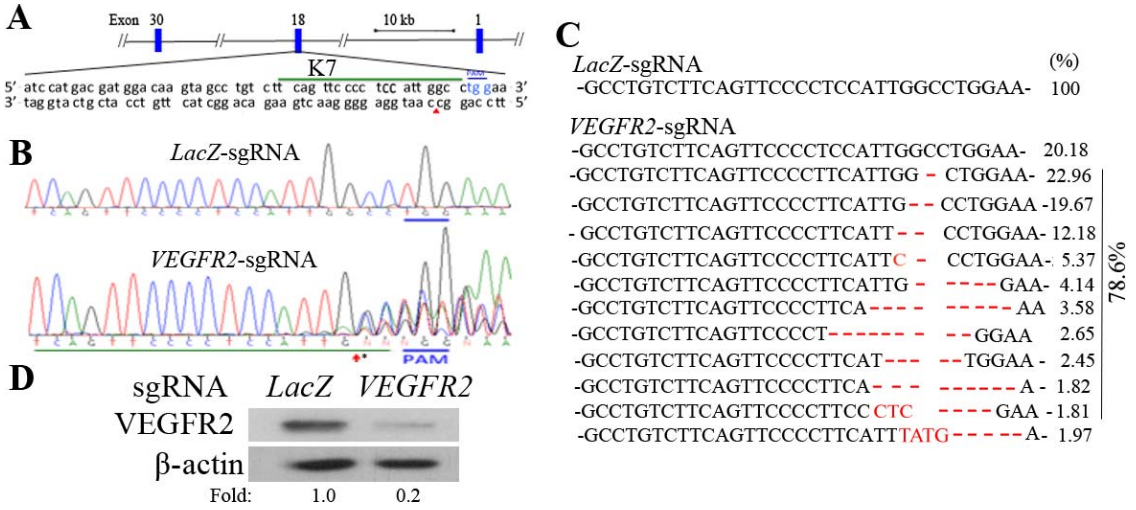

Figure 3. AAV5-CRISPR/Cas9–mediated depletion of VEGFR2. (**A**) Schematic of a target DNA sequence (K7) preceding a 5′-NGG PAM sequence at exon 18 in the genomic *VEGFR2* locus (NC_000071.6), which was selected for generating sgRNA. The *red triangle* points to an expected cleavage site of SpCas9 at 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.

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.[33] 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.[34,35] 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

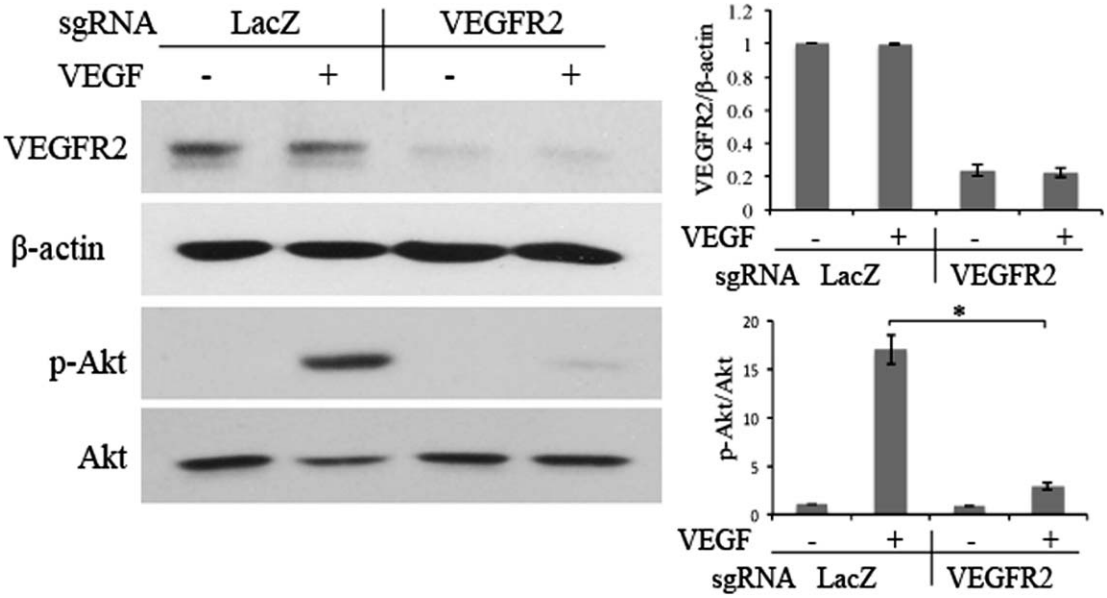

Figure 4. AAV-CRISPR/Cas9–mediated depletion of VEGFR2 prevents VEGF-induced activation of Akt. The HRECs transduced with the dual AAV vectors, as described in Figure 3, in growth factor–free medium overnight in wells of a 24-well plate, were treated with VEGF (20 ng/mL) for 30 minutes. The cell lysates 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.

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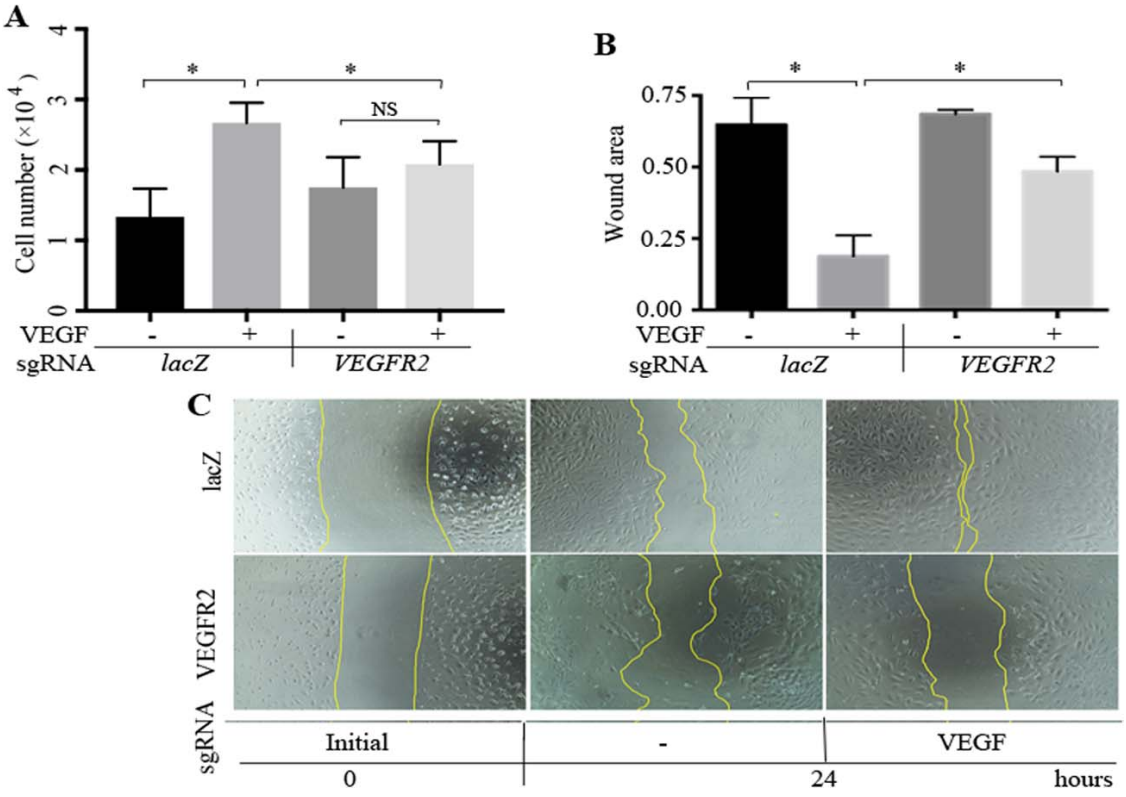

Figure 5. AAV-CRISPR/Cas9–mediated depletion of VEGFR2 prevented VEGF-induced proliferation and migration. (**A**) HRECs transduced with the dual AAV vectors, as described in Figure 3, were plated in wells of 48-well plates (15,000 cells per well). After cell attachment, they were switched to growth factor–free medium for 7 hours and VEGF (20 ng/mL) was added into the cells as indicated. After 48 hours, the cells were trypsin detached and then counted in a hemocytometer under a light microscope. (**B**) HRECs transduced with the dual AAV vectors (described in Fig. 3) and grown to near confluence in a 48-well plate were deprived of growth factors for 7 hours, and then scratched with a sterile 200-μL pipette tip. After washing twice with PBS, the cells were treated with VEGF (20 ng/mL). After 16 hours, the scratched area was photographed, the boundaries of scratches were outlined with the lines, and the scratched areas were analyzed with ImageJ software. 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. One representative experiment was shown below.

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 with SpCas9 plus *lacZ*-sgRNA, it failed to stimulate the migration of HRECs with SpCas9 plus *VEGFR2*-sgRNA (K7), indicating that editing genomic *VEGFR2*, using the dual AAV system, is able to block VEGF-induced endothelial cell proliferation and migration.

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

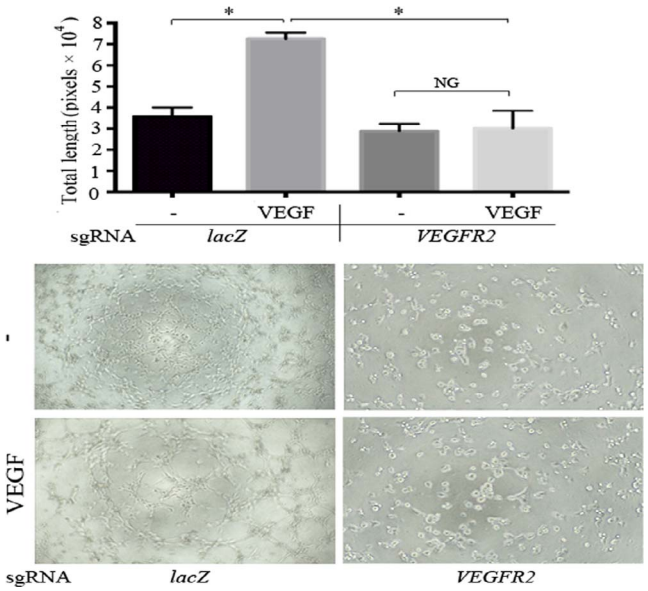

Figure 6. AAV-CRISPR/Cas9–mediated depletion of VEGFR2 blocked VEGF-induced tube formation. The solution of BME (80 μL) was transferred into each well of a 96-well plate, which was placed on ice for at least 10 to 15 minutes. The plate was then incubated at 37°C for 30 to 60 minutes to polymerize the gel. Then, HRECs transduced with AAV5-SpCas9 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*.

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 congenital amaurosis.[44,45] Thus, we adapted the dual AAV system for delivering CRISPR/Cas9 into HRECs. AAV5 has been shown to enter the cells via platelet-drived growth factor receptor (PDGFR),[46] but our results indicated that the PDGFRs are not essential for AAV5 to enter the cells because the cells engineered without PDGFRs could be transduced by AAV5 (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-CRIPSR/Cas9, may provide a potential novel alternative approach. However, VEGFR2 is essential for survival of vascular ECs[48,49]; 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[51] such as zinc finger nucleases,[52] transcription activator–like effector nucleases,[53] 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.[54,55] Rational engineering of SpCas9 would be expected to lead to improvement.

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