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/supernumerary microvascular endothelial cell surface 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 (Cas9) 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 depletion. 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 to 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 chemiluminescence of the horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG and anti-mouse IgG were 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′-TCAGTTCCCTCTATTGGCC-3′ (K7), 5′-AGGCCTACCTGTC TATGTGC-3′ (K8), 5′-TCTATCTGGATCACTGACG-3′ (K9), and 5′-GGATCCAGATTCAATCCCAT-3′ (K10). The control sgRNA sequence (5′-TGCGGATACGGCGACCGTGGG-3′) was designed to target the lacZ gene from Escherichia coli. The lenti-crispr-v2 vector (59261) was used in the dual AAV vectors (AAV-SpCas9: 60957 and AAV-SpGuide: 60958) targets for generating sgRNA for SpCas9 and pICAM2, using the CRISPR design Web site (http://crispr.mit.edu; in the public domain). 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 using the CRISPR design Web site (http://crispr.mit.edu; in the public domain). The four target sequences were 5′-TCAGTTCCCTCTATTGGCC-3′ (K7), 5′-AGGCCTACCTGTC TATGTGC-3′ (K8), 5′-TCTATCTGGATCACTGACG-3′ (K9), and 5′-GGATCCAGATTCAATCCCAT-3′ (K10). The control sgRNA sequence (5′-TGCGGATACGGCGACCGTGGG-3′) was designed to target the lacZ gene from Escherichia coli. The lenti-crispr-v2 vector (59261) was used in the dual AAV vectors (AAV-SpCas9: 60957 and AAV-SpGuide: 60958) targets for generating sgRNA for SpCas9 and pICAM2, using the CRISPR design Web site (http://crispr.mit.edu; in the public domain).

**Production of Lentivirus**

The lentiviral vector v2 vector inserted with sgRNA (K7, K8, K9, or K10) (2000 ng) and the packaging plasmid psPLX2 (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 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.

**Western Blot Analysis**

PAEC-KDR cells infected with the lentivirus or HRECs with AAV5-SpGuide and -SpCas9 (7.5 × 10⁶ 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 Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) from the 5× protein sample buffer (25 mM EDTA [pH 8.0] Downloaded From: http://tvst.arvojournals.org/ on 12/31/2017
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Tube Formation Assay

This assay was performed as described previously. Briefly, Cultrex Basement Extract (BME) (Trevigen, Gaithersburg, MD, USA) was added to 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 tubes 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 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 and cloned into the lentCRISPR v2 vector by BsmBI. The confirmed lentivectors by DNA sequencing were used to produce lentivirus in HEK 293T cells for infecting PEC-KDR cells. Western blot analysis showed that expression of VEGFR2 was reduced approximately 90% in the PEC-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, 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 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.

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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. 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. As expected, VEGF stimulated proliferation of HRECs transduced with SpCas9 plus 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, 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 migration 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, 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. 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

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VEGFR2 expression in HRECs. Not all sgRNAs designed by using the online tool would be expected to have the same efficiency in guiding SpCas9, so we designed four sgRNAs from exon 18 of the human genomic VEGF, 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. 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. Thus, we adapted the dual AAV system for delivering CRISPR/Cas9 into HRECs. AAV5 has been shown to enter the cells via platelet-driven growth factor system for delivering CRISPR/ Cas9 into HRECs. AAV5 has been shown to effectively transduce vascular ECs. 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 VEGF and VEGFR2 in vascular ECs, using AAV-CRISPR/Cas9, may provide a potential novel alternative approach. However, VEGFR2 is essential for survival of vascular ECs; 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. Furthermore, we have previously reported that AAV1-mediated CRISPR/Cas9 abrogates angiogenesis in mouse models of oxygen-induced retinopathy and laser-induced choroidal neovascularization. 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. 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. Rational engineering of SpCas9 would be expected to lead to improvement.

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