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

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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 (Cas9) in Streptococcus pyogenes 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.
CRISPR/Cas9 Blocks Angiogenesis In Vitro

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 chemiluminescent substrate for detection of HRP was purchased from Promega (Madison, WI, USA). 3,3′-Diaminobenzidine (DAB) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Enhanced chemiluminescent substrate for HRP was 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 (NM_000719.6) 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′-TCGTTTCCCCCTCTGGCC-3′ (K7), 5′-AGGCCATGTTGC TATTGCTCA-3′ (K8), 5′-TCTATCTGAGTATCTAGGCA-3′ (K9), and 5′-GGATGGATAGGAACTCTCAT-3′ (K10). The control sgRNA sequence (5′-TGCGATACGCCCACGGAGTG-3′) was designed to target the lacZ gene from Escherichia coli.13-15 The lenti-crispr v2 vector (52961) was used to transduce target K7, K8, K9, K10 or the lacZ sgRNA sequence) -C-3′ were annealed and cloned into the lenti-CRISPR v2 vector by BsmBI, respectively.

**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% 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.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.

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). All cells were cultured at 37°C in a humidified 5% CO₂ atmosphere. All 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.
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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'-AGCACACAAACACAGCCCA-3') and P10R (5'-GGTGTTAAAGAGTGAGTAGAA; reverse primer P31R: ACACAGGGTT ACCAACAACCT) for PCR amplification of the DNA fragment covering the potential K7 off-targets 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, indicating that the most potential off-target sequence was ACAATTCACCTCAATTTGGCACAAG, which was located at chr18:9326920. From this information, we designed PCR primers (forward primer P30F: AGTATG GAAAGCCAGTGCAA; reverse primer P30R: ACACAGGGTT ACCAACAACCT) for PCR amplification of the DNA fragment covering the potential K7 off-targets for Sanger DNA sequencing and PCR primers (forward primer P31F: GGGTATTAAGAGTGAGTAGAA; reverse primer P31R: GGTTACACAAACACCTTAAT) 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 50,000 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.13,22,25

Wound-Healing Assay

The wound-healing migration assay was performed as described previously. 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 microscope. 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.19,23

Tube Formation Assay

This assay was performed as described previously. 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 tubules with each condition, 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, non-pathogenic 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 expression (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.30

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 VEGFR225 and cloned into the lentCRISPR v2 vector by BsmBI. 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 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-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-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...
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 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, 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
congenital amaurosis. Thus, we adapted the dual AAV with this sgRNA to prevent angiogenesis-related diseases. Cas9–infected HRECs and explored a potential opportunity. In a previous 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-derived growth factor receptor (PDGFR), but our results indicated that the PDGFRs were not depleted in the cells transduced by AAV5. VEGFR2 showed that rAAV5-CRISPR/Cas9–mediated depletion of VEGFR2 blocked angiogenesis in vitro. The solution of BME (80 μL) was aspirated into 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, 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. 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-derived growth factor receptor (PDGFR), 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. 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 ECs, 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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