Long-Term Effects of In Vivo Genome Editing in the Mouse Retina Using Campylobacter jejuni Cas9 Expressed via Adeno-Associated Virus

Dong Hyun Jo,1,7 Taeyoung Koo,2,3,7 Chang Sik Cho,1 Jin Hyoung Kim,1 Jin-Soo Kim,2,4 and Jeong Hun Kim1,5,6

1Fight against Angiogenesis-Related Blindness (FARB) Laboratory, Clinical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea; 2Center for Genome Engineering, Institute for Basic Science, Seoul, Republic of Korea; 3Department of Pharmacy, Kyung Hee University, Seoul, Republic of Korea; 4Department of Chemistry, Seoul National University, Seoul, Republic of Korea; 5Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Republic of Korea; 6Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Republic of Korea

Genome editing with CRISPR systems provides an unprecedented opportunity to modulate cellular responses in pathological conditions by inactivating undruggable targets, such as transcription factors. Previously, we demonstrated that the smallest Cas9 ortholog characterized to date, from Campylobacter jejuni (CjCas9) targeted to Hif1a and delivered in an adeno-associated virus (AAV) vector, effectively suppressed pathological choroidal neovascularization in the mouse retina. Before implementation of CjCas9 as an in vivo therapeutic modality, it is essential to investigate the long-term effects of target gene disruption via AAV-mediated delivery of CjCas9 in vivo. In this study, histologic and electroretinographic analyses demonstrated that CjCas9 targeted to Hif1a did not induce any detectable toxicity in the retina, although the target gene was mutated with a frequency ranging from 45% to 79% in retinal or retinal pigment epithelial cells. Importantly, at 14 months after injection, no indels were detected at potential off-target sites identified using Digenome-seq and Cas-OFFinder, suggesting that long-term expression of CjCas9 does not aggravate off-target effects. Taken together, our results show that intravitreal injection of AAV encoding CjCas9 targeted to Hif1a effectively induced and maintained mutations in retinal tissues for more than 1 year and did not affect retinal histologic integrity or functions.

INTRODUCTION

Genome engineering tools such as CRISPR-Cas9 systems open an avenue for correcting pathogenic mutations and controlling gene expression.1–3 For example, in retinal diseases, subretinally or intravitreally administered Cas9 via adeno-associated viruses (AAVs), ribonucleoproteins, or plasmids can be used to cleave genetic sequences with mutations and suppress the expression of genes associated with pathological conditions.4–10 Besides correcting mutated gene segments, Cas9 can be applied therapeutically to disrupt disease-causing wild-type genes. Examples of such suppression include the depletion of vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1-alpha (HIF-1α), and VEGF receptor 2, which play roles in disease pathogenesis.4,7,8 In particular, the ability to target intracellular proteins (e.g., HIF-1α) and their coding genes (e.g., HIF1A), which are undruggable or untargetable by currently available therapeutic agents with high specificity, represents an additional advantage of CRISPR-Cas9-mediated therapeutic approaches.

Despite recent advances in genome engineering technologies, there are still concerns about in vivo genome editing regarding long-term or permanent genetic modification and unexpected off-target effects.1,2 Attempts have been made to use non-viral administration tools for the transient expression of Cas9,1,11 but viral vectors, including AAV, are still the most practical method to induce genome editing in vivo, especially in organs such as brain, liver, and eye, to which different serotypes of AAVs allow tissue-specific tropism.12–14 Although AAV or lentiviral vectors are utilized in many preclinical tests and clinical trials for gene therapy, it is essential to resolve concerns about the long-term effects of AAV encoding genome editing machinery, which results in prolonged expression of Cas9 in transfected cells.4

In our previous study, we showed that the efficient delivery of Cas9 derived from Campylobacter jejuni (CjCas9) with a single-guide RNA (sgRNA) in a single AAV vector resulted in the depletion of the Vegfa or Hif1a gene in murine retinal and retinal pigment epithelium (RPE) cells at 6 weeks post-injection.3 CjCas9-edited RPE reduced choroidal neovascularization, which is one of the major pathologic features of age-related macular degeneration (AMD).

In this study, we assessed the histologic integrity of retinal tissues and the electrical properties of retinal neurons of mice at 14 months after...
intravitreal injection of AAV encoding CjCas9 targeted to Hif1a, which effectively induced small insertions and deletions (indels) at the target site without detectable off-target effects. These long-term observations might provide support for in vivo genome editing of selected target genes associated with pathological conditions in various retinal diseases, including AMD, Leber congenital amaurosis, and retinitis pigmentosa.

RESULTS

Long-Term Effects of CjCas9 on Histologic Integrity and Functions of Retinal Tissues at 14 Months

We previously demonstrated that intravitreally administered AAV encoding CjCas9 targeted to Vegfa and Hif1a (AAV-CjCas9: Vegfa and AAV-CjCas9: Hif1a, respectively) resulted in 20–30% indels at target sites in RPE cells and effectively inhibited the formation of choroidal neovascular membranes compared to AAV encoding CjCas9 targeted to Rosa26 (AAV-CjCas9: Rosa26) or PBS.4 With the same procedure, mice were treated with intravitreal injections of AAV-CjCas9: Rosa26, AAV-CjCas9: Hif1a, and AAV-CjCas9: Vegfa at a concentration of 2 × 1010 viral genomes in 2 μL PBS. Interestingly, even at 14 months after the treatment, AAV-CjCas9: Rosa26 and AAV-CjCas9: Hif1a did not induce any definite changes in histologic integrity (Figure 1A), the number of apoptotic cells identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (Figure 1B), or the area of opsin positivity (Figure 1C). In contrast, AAV-CjCas9: Vegfa reduced the thickness of the retinal tissues (Figure S1), which was also seen in mice with an RPE-specific Vegfa deletion.15 There was an evident expression of the hemagglutinin (HA) epitope, which is conjugated to the C terminus of CjCas9 in AAV-CjCas9-treated tissues (Figure 1C).

In line with these results in histologic integrity, AAV-CjCas9: Rosa26 and AAV-CjCas9: Hif1a did not affect the scotopic and photopic responses upon electroretinogram (ERG) analyses (Figures 2A–2F). These ERG results suggest that there were no definite long-term functional changes in retinal tissues after intravitreal administration of AAV-CjCas9: Rosa26 and AAV-CjCas9: Hif1a. In addition, there were no significant differences in body weights among groups treated with AAV-CjCas9 targeting different target genes (Figure S2).

Long-Term Indel Rates and Off-Target Effects Induced by CjCas9 in the Mouse Retina

To assess the long-term genome editing effects of CjCas9, we investigated the presence of indels in the mouse retina at 14 months following AAV injection. At 6 weeks post-injection, Hif1a- and Vegfa-specific CjCas9 achieved indels with frequencies of 58 ± 5% and 20 ± 2% in the retina, respectively, and 31 ± 2% and 22 ± 3% in the RPE, respectively.4 At 14 months, CjCas9-induced indels were detected at the Rosa26, Hif1a, and Vegfa target sites with frequencies of 49 ± 14%, 79 ± 2%, and 49 ± 7% in retinal cells treated with AAV-CjCas9: Rosa26, AAV-CjCas9: Hif1a, and AAV-CjCas9: Vegfa, respectively (Figure 3A; Figure S3A). In RPE cells, indels were detected with frequencies of 28 ± 4%, 45 ± 7%, and 23 ± 5% at the Rosa26, Hif1a, and Vegfa target sites, respectively (Figure 3B; Figure S3B), indicating that indels were persistent and significantly

![Figure 1. Histologic Evaluation of Retinal Tissues at 14 Months after Intravitreal Injection of AAV-CjCas9 in Mice](image-url)
increased in the mouse retina for a period of up to 14 months after the initial injection. Accordingly, AAV-CjCas9: *Vegfa* decreased the VEGF levels in the retinal and RPE cells at 14 months after the initial injection (Figures S4A and S4B). In contrast, there was no definite change in the VEGF levels in the normal retinal and RPE tissues by treatment with AAV-CjCas9: *Hif1a* (Figures S4C and S4D). It is also remarkable that the major mutation patterns at the *Hif1a*, *Vegfa*, or *Rosa26* target sites were identical between 6 weeks and 14 months after post-intravitreal injection of AAV-CjCas9: *Hif1a*, *Vegfa*, or *Rosa26* (Figure S5; Tables S1–S3).

We next investigated whether the long-term expression of CjCas9 and its sgRNA might lead to off-target effects. In our previous report, two or one *in vitro* cleavage sites were identified by Digenome-seq using the *Hif1a*- or the *Vegfa*-specific sgRNA, respectively. Therefore, we performed targeted deep sequencing at these Digenome sequencing (Digenome-seq) captured potential off-target sites and found that CjCas9 did not cause any detectable off-target mutations at these sites (Figure 4A; Figure S6A). We further examined the genome-wide specificity of CjCas9 at potential off-target sites that differed from the on-target sites by up to 4 nt in the mouse genome, identified using the Cas-OFFinder algorithm (http://www.rgenome.net/cas-offinder/). CjCas9-edited retinal and RPE cells did not show any detectable off-target indels at 7 homologous sites for *Hif1a* and 14 homologous sites for *Vegfa* (Figure 4B; Figure S6B).

**DISCUSSION**

In this report, we describe the long-term effects of CjCas9 delivered via AAV to the mouse retina and RPE. Fourteen months after the successful targeting of *Hif1a* via intravitreal injection of AAV-CjCas9: *Hif1a*, we observed no definite histologic or functional abnormalities in the retinal tissues compared to age-matched controls. These data are in line with a previous report showing that there were no significant morphological, functional, or transcriptional abnormalities in RPE-specific *Hif1a* knockout mice.15

HIF-1α is a transcription factor that controls the expression of various angiogenesis-related genes under ischemic and/or hypoxic conditions. In particular, HIF-1α is upregulated in pathological states associated with various retinal vascular diseases, including AMD and diabetic retinopathy; a key effect is that HIF-1α promotes the expression of VEGF-A, one of the most potent players in pathological angiogenesis.15,17,18 Because there are several HIF-1α-independent pathways that also regulate VEGF-mediated physiological angiogenesis,19,20 HIF-1α is not required for the maintenance of physiological vasculature.15 In contrast, VEGF is a trophic factor for the maintenance of normal retinal neuronal cells, RPE cells, and vascular endothelial cells.5,21,22 As expected, AAV-CjCas9: *Vegfa* resulted in severe histologic changes of retinal tissues, while AAV-CjCas9: *Hif1a* induced no change in histologic integrity and retinal functions. These data demonstrated that targeting *Hif1a* with AAV-CjCas9 could be a safe way to address pathological angiogenesis without introducing unwanted toxicities related to histologic integrity, apoptotic activity, and off-target effects.

In the treatment of retinal diseases such as Leber congenital amaurosis, AAV is a widely utilized platform for therapeutic gene delivery and has demonstrated long-term efficacy and safety.23–25 Intravitreally administered AAVs do not significantly affect the gene expression in other organs.26–28 Similarly, both AAV-CjCas9: *Vegfa* and *Hif1a* did not induce any change in body weights of treated mice, regardless of their local effects. Nevertheless, there have been concerns about increased off-target effects associated with prolonged Cas9 expression. In our study, indels were detected...
at the on-target sites with frequencies of 79 ± 2% and 45 ± 7% in Hif1a-edited retinal and RPE cells, respectively. The indel rate increased at the Vegfa and Hif1a target sites in the Cas9-edited retina from 6 weeks to 14 months after the initial injection. Apparently, continuous Cas9 expression led to the induction of additional double-strand breaks and indels in cells, considering that most retinal cells are differentiated and non-transformed. Despite the increase in indel frequencies at on-target sites, there were no detectable off-target effects up to 14 months post-injection, suggesting that, because of high Cas9 specificity, long-term expression did not cause indels at potential off-target sites in vivo.

The lifespan of C57BL/6 mice is estimated to be less than 30 months (878 ± 10 days for male mice and 794 ± 6 days for female mice). Thus, our observation period (14 months) corresponds to half of the expected lifespan of the study animals. Our results show that intravitreally administered AAV-CjCas9 to a carefully selected target such as Hif1a effectively induced and maintained indels in retinal tissues for more than 1 year. Furthermore, this treatment did not affect retinal histologic integrity or functions and, importantly, did not aggravate off-target effects over this extended period of time. Taken together, our results provide evidence of long-term safety of CjCas9 expression in the eye, in support of in vivo genome editing for therapeutic treatments of various retinal diseases.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6 mice were purchased from Central Laboratory Animal and maintained under a 12-hr:12-hr dark:light cycle. All animal experiments were performed under the guidelines of the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research and approved by the institutional animal care and use committees of both Seoul National University and Seoul National University Hospital.

Construction of CjCas9 and sgRNA Plasmids

A human codon-optimized CjCas9 coding sequence, derived from Campylobacter jejuni subsp. jejuni NCTC 11168, was synthesized with a nuclear localization signal and an HA epitope at its C-terminal end (GeneArt Gene Synthesis, Thermo Fisher Scientific) and cloned into the p3s plasmid. The trans-activating CRISPR RNA sequence and the precursor CRISPR RNA sequence were fused with a TGAA linker to form an sgRNA sequence. sgRNAs were transcribed under the control of the U6 promoter.

AAV Vectors Encoding CjCas9 and sgRNAs

AAV inverted-terminal-repeat-based vector plasmids carrying an sgRNA sequence and the CjCas9 gene with a nuclear localization sequence and an HA tag at the C terminus were constructed. For retinal delivery, AAV vectors encoding CjCas9 under the control of the short form of elongation factor 1α (eEF1α) promoter; EGFP linked to the C terminus of CjCas9 with the self-cleaving T2A peptide; and a U6 promoter-driven sgRNA specific to the Rosa26, Vegfa, or Hif1a gene were constructed.

Production and Characterization of AAV Vectors

To produce AAV vectors, they were pseudotyped in AAV9 capsids. HEK293T cells were transfected with pAAV-ITR-CjCas9-sgRNA, pAAVED29, and a helper plasmid. HEK293T cells were cultured in DMEM with 2% fetal bovine serum (FBS). Recombinant pseudotyped AAV vector stocks were generated using PEI coprecipitation and triplex transfection with plasmids at a molar ratio of 1:1:1 in HEK293T cells. After 72 hr of incubation, cells were lysed and particles were purified by iodixanol (Sigma-Aldrich) step-gradient ultracentrifugation. The number of vector genomes was determined by qPCR.

Intravitreal Injection of AAV

After mice were deeply anesthetized, AAV9-CjCas9 (2 × 10^10 viral genomes in 2 μL PBS) was injected into the vitreous cavity of the retina using a customized Nanofil syringe with a 33G blunt needle (World Precision Instruments) under an operating microscope (Leica).
Histologic Evaluation
At 14 months after AAV-mediated delivery of CjCas9, paraffin blocks were prepared from enucleated eyes. Thin sections were prepared for H&E staining and TUNEL (Sigma) assays. TUNEL-positive cells were evaluated in 10 randomly selected fields in each slide at 400x magnification.

Immunofluorescence
At 14 months after AAV-mediated delivery of CjCas9, paraffin blocks were prepared from enucleated eyes. Thin sections were immunostained with anti-HA antibody (1:1,000; catalog no. 3F10, Roche) and anti-opsin antibody (1:1,000; catalog no. AB5405, Millipore), followed by treatment with Alexa Fluor 488 or 594 IgG (1:500; Thermo Fisher). Nuclear staining was performed using DAPI (Sigma). Then, the slides were observed under a fluorescence microscope (Leica).

Isolation of Retina and RPE Sheets
Enucleated eyes were incubated with 0.1% hyaluronidase (Sigma-Aldrich) for 45 min at 37°C, after removal of the lens, and then transferred in PBS for 30 min on ice. Next, the neural retina was completely removed from enucleated eyes, and only the RPE-choroid-scleral complex was incubated with Trypsin-EDTA solution for 45 min at 37°C. RPE cell sheets were isolated by shaking the eyecup using microforceps, and only monolayer RPE sheets were collected using a glass capillary on the microscope.

Genomic DNA Extraction and Mutation Analysis
The collected retina and RPE sheets were incubated with lysis buffer, and genomic DNA was extracted according to the manufacturer’s protocol (NucleoSpin Tissue, Macherey-Nagel). Genomic DNA was analyzed by targeted deep sequencing. On-target or off-target loci were amplified using 100 ng genomic DNA for targeted deep sequencing. Deep sequencing libraries were generated by PCR with the following primers: mouse Rosa26, 5'-CGGGAGTCTCTCGGCGAGCTTAA-3' (forward), 5'-CCGGCGATCAAGCAA-3' (reverse); mouse Hif1a, 5'-GTCCCCCATATATGAGAGGCCAC-3' (forward), 5'-CAATATCTGACTGAAAATCACCT-3' (reverse); mouse Vegfa, 5'-CCCTTGGGATCTTGCATC-3' (forward), 5'-TACTACGGAGCGAAAGACG-3' (reverse). TruSeq HT Dual Index primers were used to label each sample. Pooled libraries were subjected to paired-end sequencing using MiniSeq (Illumina). Indel frequencies were calculated as described previously.30

ERG
Mice were maintained in a dark-adapted state for over 16 hr. After deep anesthesia, pupils were dilated with an eye drop containing phenylephrine hydrochloride (5 mg/mL) and tropicamide (5 mg/mL). Full-field ERG was performed using the Universal Testing and Analysis System, Electrophysiologic 2000 (UTAS E-2000; LKC Technologies). The responses were recorded at a gain of 2 k, utilizing a notch filter at 60 Hz, and were bandpass filtered between 0.1 and 1500 Hz. In the light-adapted photopic state, with a 30-cd/m² background light to desensitize the rods and isolate cones, photopic cone responses were recorded in response to a single flash of 0 dB. The amplitudes of the a-wave were measured from the baseline to the lowest negative-going voltage, whereas peak b-wave amplitudes were estimated from the trough of the a-wave to the highest peak of the positive b-wave.

Statistics
No statistical methods were used to predetermine sample size for in vitro or in vivo experiments. All group results are expressed as
mean ± SEM, if not stated otherwise. Comparisons between groups were made using the two-tailed Student’s t test or one-way ANOVA and Tukey post hoc tests for multiple groups. Statistical analysis was performed in GraphPad Prism 5.

**Accession Numbers**
The accession number for the deep sequencing data reported in this paper is NCBI: PRJNA435661. Details of the primers used in this study are available on request.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes six figures and three tables and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.10.009.

**AUTHOR CONTRIBUTIONS**
D.H.J. and T.K. drafted the manuscript. D.H.J. performed experiments on the histologic evaluation of retinal tissues. T.K. performed experiments on the design of the AAV encoding CjCas9 and sgRNA, followed by genome analysis of on-target and off-target mutations. C.S.C. performed animal experiments. Jin Hyoung Kim, J.-S.K., and Jeong Hun Kim edited the manuscript. J.-S.K. and Jeong Hun Kim designed the study and revised the manuscript.

**CONFLICTS OF INTEREST**
J.-S.K. is a co-founder and shareholder of ToolGen.

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