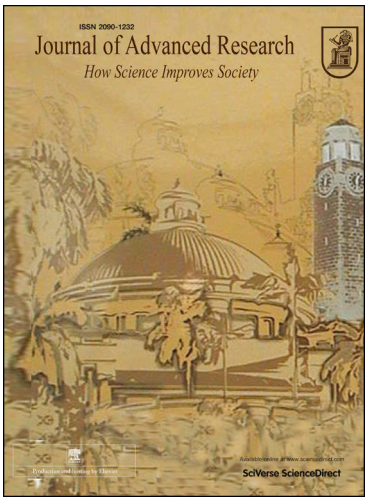
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Exploitation of enhanced prime editing for blocking aberrant angiogenesis

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Credit author statement

X.H., W.W. and H.Q., performed DNA preparation, cell culture, lentivirus production and infection, genomic DNA isolation, intravitreal injection, Western blot, whole mount retinal staining, analysis of the results, and wrote the manuscript; X.Y., L.D., Y.Y. and Q.Z. assisted on the *in vitro* and *in vivo* experiments; G.Z., G.M., and H.L. acquired funds,

conceived the experiments, analyzed the data and revised the manuscript. All the authors reviewed the manuscript.

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Abstract

Introduction: Aberrant angiogenesis plays an important part in the development of a variety of human diseases including proliferative diabetic retinopathy, with which there are still numerous patients remaining a therapeutically challenging condition. Prime editing (PE) is a versatile gene editing approach, which offers a novel opportunity to genetically correct challenging disorders.

Objectives: The goal of this study was to create a dominant-negative (DN) vascular endothelial growth factor receptor (VEGFR) 2 by editing genomic DNA with an advanced PE system to block aberrant retinal angiogenesis in a mouse model of oxygen-induced retinopathy.

Methods: An advanced PE system (referred to as PE6x) was established within two lentiviral vectors, with one carrying an enhanced PE guide RNA and a canonical Cas9 nickase fused with an optimized reversal transcriptase, and the other conveying a nicking guide RNA and a DN-MLH1 to improve PE efficiency. Dual non-integrating lentiviruses (NILVs) produced with the two lentiviral PE6x vectors were then employed to create a mutation of *VEGFR2* T17967A by editing the *Mus musculus VEGFR2* locus *in vitro* and *in vivo*, leading to generation of a premature stop codon (TAG, K796stop) to produce DN-VEGFR2, to interfere with the wild type VEGFR2 which is essential for angiogenesis.

Results: NILVs targeting *VEGFR2* delivered into cultured murine vascular endothelial cells led to 51.06% *VEGFR2* T17967A in the genome analyzed by next generation sequencing and the production of DN-VEGFR2, which was found to hamper VEGF-induced VEGFR2 phosphorylation, as demonstrated by Western blot analysis. Intravitreally injection of the dual NILVs into postnatal day 12 mice in a model of oxygen-induced retinopathy, led to production of retinal DN-VEGFR2 in postnatal day 17 mice which blocked retinal VEGFR2 expression and activation as well as abnormal retinal angiogenesis without interfering with retinal structure and function, as assessed by electroretinography, optical coherence tomography, fundus fluorescein angiography and histology.

Conclusion: DN-VEGFR2 resulted from editing genomic *VEGFR2* using the PE6x system can be harnessed to treat intraocular pathological angiogenesis.

Keywords: Enhanced prime editing; dominant-negative VEGFR2; angiogenesis; oxygen-induced retinopathy.

Introduction

Angiogenesis, a process by which new blood vessels sprout from pre-existing vessels[1-3], is associated with a variety of human diseases including proliferative diabetic retinopathy (PDR)[4, 5], retinopathy of prematurity (ROP)[6], and neovascular age-related macular degeneration (nAMD)[7, 8]. The signaling pathway of vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR)2 is essential for angiogenesis[9-12], and thus a number of antagonists such as ranibizumab, bevacizumab, and aflibercept, interrupting this pathway, have been developed for treating angiogenesis-associated human diseases[13-17]. Nevertheless, there are still myriad patients with these diseases resistant to such therapies[16-18]. Thereby, novel therapeutic approaches are urgently needed to angiogenesis-associated diseases including PDR, which occupies the highest number of acquired blindness in the working-age people[4, 5].

Genome editing provides novel therapeutic opportunities for human diseases that are currently challenging for therapy[19, 20]. Prime editing (PE) is a versatile gene editing system which consists of at least two components: a Cas9 nickase (n) including *Streptococcus pyogenes* (Sp) Cas9H840A(Cas9n) fused with an engineered reverse transcriptase (RT) (the PE1 protein) and a prime editing guide RNA (pegRNA). This pegRNA is composed of a single guide (sg) RNA and a 3' extension with a primer binding sequence (PBS) that is complementary to a portion of the DNA protospacer, and a RT template that contains the desired mutation(s)[21]. The pegRNA function can be much improved by fusion with a structured RNA motif such as tevopreQ$_1$ (trimmed evopreQ$_1$) to its 3'-terminus to enhance

its stability and protection from degradation designated as an enhanced (e) pegRNA[22], whereas the RT can be codon-optimized with D200N/L603W/T330P/T306K/W313F to improve PE1 activity, referred to as PE2[21]. Thereby, an epegRNA can guide the Cas9n-RT complex to bind one strand of a target DNA locus and to nick the opposite strand with a protospacer adjacent motif (PAM), resulting in exposure of a DNA end for the PBS to prime RT to reversely transcribe the RT template. As a consequence, the desired mutation(s) in the RT template are incorporated into the targeted genomic locus[23].

PE2 efficiency can be further improved to PE3 by using an additional sgRNA to direct Cas9n to nick the non-edited strand at a location about 50 base pair (bp) away from the pegRNA target[21]. Specific DNA mismatch repair (MMR) genes have been discovered that strongly suppress PE efficiency and promote formation of insertion/deletions (indels). To this end, co-expression of a dominant negative MMR protein (MLH1dn) with PE2 designated as PE4 or with PE3 as PE5 has been developed to further improve PE efficiency. In addition, synergizing with PE4, PE5 and epegRNAs, optimized prime editors including PE6a-g can further raise PE efficiency[24, 25]. Moreover, a truncated RT has also been demonstrated to be capable of enhancing PE efficiency[26]. To date, PE has been broadly applied to introduce genetic changes in a variety of animal models including flies[27], zebrafish[28], and mice[19, 25, 29-33].

Adeno-associated virus (AAV) is the most frequently used vector for *in vivo* gene editing due to its non-pathogenic, low immune response and sustained-expression in non-dividing cells[34, 35]. Nevertheless, in the clinical scenario, it would be ideal to have a gene editing system expressed transiently (hit-and-run) to reduce potentially detrimental effects. A non-integrating lentiviral vector (NILV) fits this purpose very well. The NILV system can be achieved by following approaches: (1) inactivating integrase by mutation of any amino acid of its catalytic triad (D64, D116, and E152)[36] or replacement of the ^{262}RRK motif with AAH[37]; (2) altering the integrase-recognition sequences (att) in the viral LTR (long-term repeats)[38]; and (3) ruining the active center of RT by changing the amino acid sequence YMDD to YMVV[39]. Such a NILV vector has the advantage of being able to

accommodate up to 8 kilobases (kb) of nucleotides of interest compared to an AAV vector, which has a 4.7-kb package limitation[37].

Genome editing other than PE has been leveraged to prevention of intraocular angiogenesis in animal models[40-43]. In comparison to other gene editing technologies, PE can precisely achieve all types of desired mutations in the genomic DNA of living cells including base pair substitutions, small insertion/deletions (indels) without requiring either a double-strand DNA break (DSB) or a foreign DNA template[44]; however, while this strategy has been harnessed to install or correct mutations in a number of mouse models[19, 25, 29-33, 45, 46], whether this strategy can be harnessed for preventing pathological retinal angiogenesis has not yet been explored.

The goal of this study was to create a dominant negative (DN) VEGFR2 by editing genomic DNA with an advanced PE system to block aberrant retinal angiogenesis in a mouse model of oxygen-induced retinopathy (OIR)[40, 47-49]. To achieve this aim, we designed a dual NILV system with a D64V mutation in integrase[50] carrying LTR- EF1α -SpCas9n-RT-U6-epegRNA-LTR (LV5) and LTR-EF1α-MLH1dn-U6-nicking sgRNA-LTR (LV6) (termed as PE6x) targeting genomic *VEGFR2* (Figure: Fig. 1), which would be delivered intravitreally to mice in the mouse OIR model[40, 47, 48] to suppress hypoxia-induced retinal angiogenesis.

Materials and Methods

Major reagents

Primary antibodies against VEGFR2 (Catalog #: 9698), and p-VEGFR2 (Y996) (Catalog #: 2474) were purchased from Cell Signaling Technology (Danvers, MA, USA); in addition, an antibody against VEGFR2 N-terminal (Catalog #: BS-10412R) was bought from Thermo Fisher Scientific (Waltham, MA, USA), and one against β-Actin (Catalog #: sc-47778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and anti-mouse IgG were

ordered from Santa Cruz Biotechnology. Enhanced chemiluminescent substrate for detection of HRP and Alexa fluorescence-594-conjugated mouse endothelial specific isolectin B4 (IB4) were purchased from Thermo Fisher Scientific. High-fidelity Herculase II DNA polymerases were from Agilent Technologies (Santa Clara, CA, USA).

DNA constructs

DNA sequences (TGCTTTCCAGGCCAATGAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTCTACAGTTCCCCTTCATTGGCCTGGAAAgcaCGCGGTTCTATCTAGTTACGCGTTAAACCAACTAGAAttttt. Spacer: TGCTTTCCAGGCCAATGAAG, Scaffold: GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC, RT template: GTCTAC AGTTCCCCTT, PBS: CATTGGCCTGGAAA, linker: gca, 3' motif: CGCGGTTCTATCTAGTTACGCGTTAAACCAACTAGAA, terminator: ttttt) (named VXPEG1) and TCATCCAAGGGCAATTCATC (named VX1) in the mouse *VEGFR2* genomic locus (NC_000071.6) (Table 1) were selected for generating enhanced prime editing single guide RNA (epegRNA) and nicking guide (ng)RNA for SpCas9 nickase (n) based on previous publications[22, 51, 52]. The control spacer sequence (5'-TGCGAATACGCCCACGCGATGGG -3') from the *lacZ* gene of *Escherichia coli* was designed for control epegRNA and nicking sgRNA[22, 51, 53-55].

The pLTR- EF1-SpCas9n (H840A)-eRT- U6-epegRNA-LTR (LV5) was derived from lent-crispr v2- (Cat. 52961, Addgene, Cambridge, MA, USA)[56] by replacing SpCas9 with SpCas9n (H840A)-eRT[21] and then inserting synthesized epegRNA; The pLTR- EF-1-MLH1dn- U6-sgRNA-LTR (LV6) was also derived from lenti-crispr v2 by replacing SpCas9 with MLH1dn[21, 24] and then inserting a ngRNA by *BsmB1*. All these constructs were confirmed by DNA sequencing.

To construct ngRNA, the top oligo 5' CACCG- TCATCCAAGGGCAATTCATC) and bottom oligo: 5'-AAAC-20nt-C-3'(20nt: complimentary top oligo sequences) were

annealed and cloned into the LV6 vector by *BsmB1*. All clones were confirmed by DNA sequencing using a primer 5'-GGACTATCATATGCTTACCG-3' from the sequence of U6 promoter driving expression of gRNAs. Both synthesis of oligos and Sanger DNA sequencing were performed by Tsingke Biotechnology Co., Ltd (Guangzhou, China).

Cell culture

Human embryonic kidney (HEK) 293T cells (containing SV40 T-antigen) from America Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in high-glucose (4.5 g/L) DMEM supplemented with 10% FBS. C57BL/6 mouse primary brain microvascular endothelial cells (MVECs) were purchased from CellBiologics (Chicago, IL, USA) and cultured in the endothelial cell medium with a kit (CellBiologics). All cells were cultured at 37°C in the humidified atmosphere with 5% CO_2[40, 57, 58].

Production of lentivirus and non-integrating lentivirus

A lentiviral vector (LV5 or LV6, 28 μg), a lentiviral envelope plasmid VSV-G (Catalog #: 8454, Addgene) (7 μg)[59], and a lentiviral packaging plasmid *psPAX2* (Catalog #: 12260, Addgene) (21 μg) or an integrase-deficient lentiviral packaging vector (21 μg) (Catalog #: 63586, Addgene)[50] were mixed together in DMEM without phenol (2 ml), and then a polyethylenimine (PEI) solution (220 μl, 1 μg/μl, PolySciences: #23966-2, IL, USA) was added to the mixtures. After thoroughly mixed, they were incubated at room temperature for 15 min and then respectively transferred into a 150-mm cell culture dish with HEK 293T cells, which were approximately 70% confluent without antibiotics. After 18 h (37°C, 5% CO_2), the medium was replaced with a fresh culture medium. At 24 h after changing the medium, lentiviruses were firstly harvested, and the viral harvest was repeated at 24-h intervals for 3 times. The virus-containing media were pooled and centrifuged at 800 ×g for 5 min; the supernatants were then subjected to a centrifugation at 25,000 ×g for 90 min at 4°C. Finally, the viral pellets were re-suspended in 500 μl of sterile TNE buffer (50 mM

Tris pH 7.8, 130 mM NaCl, 1 mM EDTA), which were then transferred into microtubes, and dissolved at 4°C with gentle rotation overnight. These dissolved lentiviruses were then titrated[60, 61] to infect MVECs in the presence of 8 μg/ml polybrene or kept at -80°C[54, 55, 57, 62].

Transduction of vascular endothelial cells in vitro

Culture media of MVECs at 80% confluence in 6-well plates were changed into the fresh culture, followed by addition of lentiviruses or NILVs [10 μl/well, 5 x10^9 viral genome-containing particles (vg)/ml] to the culture media supplemented with an additional 8 μg/ml polybrene. At desired periods after infection, the cells were harvested in 1 × extraction buffer (EB, 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) for Western blotting analysis, or in lysis buffer for isolation of genomic DNA[54, 55, 57, 62].

Western blotting

When infected MVECs reached 90% confluence, they were deprived of growth factors for 16 h, followed by treatment with or without VEGF (10 ng/ml) for 10 min. The cells were collected in 1 × EB and their protein concentrations in the EB were determined using a BCA protein assay kit (Thermo Fisher Scientific).

Proteins in the EB were then resolved in the 5 × protein sample buffer [25 mM EDTA (pH = 7.0), 10% sodium dodecyl sulfate (SDS), 500 mM dithiothreitol, 50% sucrose, 500 mM Tris HCl (pH 6.8), and 0.5% bromophenol blue], and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membranes and subjected to western blot analysis using desired antibodies. Experiments were repeated at least three times. Signal intensity was determined by densitometry using NIH ImageJ software (USA)[54, 55, 57, 62].

DNA sequencing

MVECs or mouse retinas were collected for genomic DNA extraction according to the manufacturer's protocol of the QuickExtract DNA Extraction from Epicenter (Chicago, IL, USA). Briefly, the cell pellets were re-suspended in the QuickExtract solution, vortexed, and incubated at 65°C for 6 min; the lysates were vortexed again and then incubated at 100°C for 10 min. DNA fragments from a mouse *VEGFR2* genomic locus with the anticipated mutation were PCR amplified with high-fidelity Herculase II DNA polymerases. Primers named as P51 were (forward P51F: 5'-AGCCAGATGAGCGG GTAAAA-3'), and (reverse P51R: 5'-TGAGATTATCATGAATGAGGCCC-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). Primer synthesis and DNA sequencing were conducted by Tsingke Biotechnology Co., Ltd.

Transduction of vascular endothelial cells in vivo

NILVs for *in vivo* use were produced by Shandong Vigene Biosciences, lnc. (Jinan, China). Briefly, lentiviral vectors including pLTR-EF1-SpCas9n (H840A)-eRT- U6-epegRNA-LTR (LV5), pLTR- EF-1-MLH1dn-U6-sgRNA-LTR (LV6) and pLTR-EF-1-EGFP-LTR, respectively with a vector expressing a lentiviral envelope protein (Catalog #: 8454, Addgene)[59] and a integrase-deficient lentiviral packaging vector (Catalog #: 63586, Addgene)[50] were transfected into HEK293T cells. NILVs were harvested from cultured media and then concentrated by centrifuge as described above. The concentrated NILVs were titrated, aliquoted, and kept at -80°C until use[54, 55, 62].

NILVs expressing enhanced green fluorescence protein (EGFP, 1 μl, 5 x 10^9 IU/ml) were intravitreally injected into 4-week-old mice; 5 days later, the eyeballs were isolated and fixed in 3.7% formaldehyde for 2 h. Whole-mount retinas were blocked in 5% normal goat serum for 30 min, stained overnight at 4°C with isolectin B4 (IB4) (1:100 dilution

(Thermo Fisher Scientific), a mouse endothelial cell marker[40]. Finally, the thoroughly-washed whole-mount retinas were subjected to photography in a confocal fluorescence microscope (Leica Microsystems Inc. Deerfield, IL, USA)[40, 55].

Genomic editing of retinal cells in a mouse model of oxygen-induced retinopathy

This experiment was performed as described previously[40, 47]. Briefly, C57BL/6J litters on postnatal day (P) 7 were exposed to 75% O_2 until P12 in an oxygen chamber (Central South University, Changsha, China). At P12, the anesthetized pups were intravitreally injected with NILVs-LV5 and NILVs-LV6 (PE6x) targeting *VEGFR2* or *lacZ* (1 μl, 5.0×10^9 vg/ml). After injection, the eyes were treated with a triple antibiotic (Neo/Poly/Bac) ointment and kept in room air (21% O_2). At P17, the mice were euthanized, and retinas were carefully removed for Western blot analysis, NGS or fixed in 3.7% paraformaldehyde. Mice under 6 g of total body weight were excluded from the experiments. Each experiment was at least repeated 2 times with more than 6 mice at each treatment in this OIR model. Retinal whole-mounts were stained overnight at 4°C with IB4-Alexa 594 (red). The images were taken in the Leica confocal fluorescence microscope.

Examination of toxicity of PE6x-VEGFR2 in mouse eyes

6-weeks-old C57BL/6J mice were anesthetized and intravitreally injected with 1 μl (5.0×10^9 vg/ml) of NILV-LV5 and NILV-LV6 (PE6x) targeting *VEGFR2* or *lacZ.* After 4 weeks of injection, electroretinography (ERG) (by light/dark adaptation) (Roland, Heidelberger Straße, Germany), optical coherence tomography (OCT) and fundus fluorescein angiography (FFA) were conducted using a Micron IV image system (Phoenix-Micron, Inc, Bend, OR. USA) as described previously[40, 58] and in brief below.

OCT. When mice were deeply anesthetized with an intraperitoneal injection of ketamine/xylazine (100-200 mg/kg Ketamine/20 mg/kg Xylazine), their pupils were dilated with topical 1% Tropicamide, and their eyes were covered with Genteal gel to prevent drying of the cornea. The fundus camera in the optical head of the apparatus

provided initial alignment, and final alignment was guided by monitoring the real time OCT image of the retina.

ERG. After overnight dark adaptation, mice were examined under dim red light for ERG. Under anesthesia, their pupils were dilated and eyes covered with Genteal. Subsequently, a drop of 0.9% sterile saline was put on the cornea of the treated eye to prevent dehydration and to allow electrical contact with the recording electrode (gold wire loop). A 25-gauge platinum needle, inserted subcutaneously in the forehead, served as reference electrode, while a needle inserted subcutaneously near the tail served as the ground electrode. A series of flash intensities was produced by a Ganzfeld controled by the Diagnosys Espion 3 to test both scotopic and photopic response.

FFA. After animals were anesthetized their pupils were dilated, a drop of sterile saline was placed on the experimental eye to remove any debris followed by Genteal. Then 0.01 ml of 25% sodium fluorescein (pharmaceutical grade sodium fluorescein; Akorn Inc) 5g body weight was injected intraperitoneally. The retinal vasculature was filled with dye in less than one min following injection. Photos were taken sequentially at 1 and 5 min post fluorescein injection.

After mice were examined by OCT, ERG and FFA, they were euthanized, and their eyeballs were carefully removed and fixed in 3.7% paraformaldehyde (PFA) for histological analysis as described previously [40, 58].

All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Investigators who conducted analysis were masked as to the treatment groups. All the mice were cared for by following the ACUC protocol approved by the Institutional Animal Ethics Committee of Peking University at Shenzhen (Shenzhen, China).

Statistics

The data from 3 independent experiments were analyzed using an unpaired t-test between two groups, and one-way ANOVA among more than two groups. For animal experiments the data from at least 6 mice were used for the statistical analysis. All data were analyzed using a masked procedure. P values < 0.05 were considered statistically significant.

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Animal Ethics Committee of Peking University at Shenzhen (Approval no. AP-2021-04).

Results

Creation of a dual NILV system for delivering PE6x

Lentiviral vectors (LVs) can transport large nucleotide fragments and preferentially transduce transcriptionally active cells including vascular endothelial cells （ECs） in the neo-vascular network[37, 63, 64, 65]. Retinal photoreceptor cells are permanently differentiated cells and thus are difficult to be targeted by LVs[37, 63, 64, 65], indicative of that the retinal neural cells are potentially protected when a NILV is employed as a gene editing vector for treating pathological intraocular angiogenesis such as PDR. We therefore aimed to develop a dual NILV system (Fig. 1A) for delivering PE6x intravitreally to create a T17967A mutation at the *Mus musculus VEGFR2* locus (NC_00071.6), resulting in a generation of an earlier stop codon (TAG, K796stop) from AAG (K796) when *VEGFR2* was transcribed and spliced in ECs during pathological angiogenesis (Fig. 1B). We envisioned that a dominant-negative (DN) VEGFR2 with 795 amino acids （aa） would be produced during translation as a result of the PE6x-generated stop codon （K796stop）, which is located at an intracellular juxtamembrane domain (Fig. 1C), and that this DN-

VEGFR2 would block VEGF/VEGFR2 signaling and hereby dampen pathological retinal angiogenesis.

Among the dual NILV system, LV5 was created to express a fusion protein of SpCas9n (H840A) with a codon-optimized RT driven by an EF1α core promoter and an epegRNA with a structured RNA sequence (TevopreQ1) driven by a U6 promoter[21, 56], and LV6 was to expresses a dominant-negative MLH1 (MLHdn)[24] and a nicking sgRNA(ngRNA)(Fig. 1A-B). To this end, three vectors: 1) LV5 or LV6 with an desired epgRNA or a ngRNA[56] targeting genomic *VEGFR2* or a bacterial gene *lacZ* as a control; 2) an integrase-deficient packaging vector expressing the genes of *Gag* and *Pol* encoding RT and integrase with a D64V mutation to inactivate it[50]; and 3) one expressing the VSV (vesicular stomatitis virus)-G protein[59], were together transfected into HEK293T cells for producing NILVs. NILV5 and NILV6 (Fig. 1A) were then concentrated and titrated for *in vitro* and *in vivo* use.

Generation of a dominant-negative VEGFR2 by PE6x in vitro

To assess if the dual NILV system was expressed transiently, we infected C57BL/6 mouse primary brain microvascular endothelial cells (MVECs)[40] with NILV5 or wild type (WT) LV5 for varying periods, and their lysates were subjected to a western blot analysis. As shown in Fig. 2, expression of a fusion protein with SpCas9n was detected at 6 h after NILV5 infection, and it reached the maximal at 48 h, which was maintained until 72 h after infection, and then dropped 51.2 ± 2.6 % down at 96 h, whereas SpCas9n/RT was kept to the almost same levels at this time when infected by WT LV5, indicating that SpCas9n/RT in PE6x is transiently expressed when cells are infected by the dual NILV system.

To explore whether DN-VEGFR2 was produced by PE6x, we infected MVECs with the dual NILV system of NILV5 and NILV6 targeting genomic *VEGFR2* or its control gene *lacZ* (Fig. 1). On day 3 after infection, genomic DNA and proteins isolated from the infected MVECs were subjected to PCR and western blot analyses, respectively. Sanger

DNA sequencing of the PCR products showed that there was a T17967A mutation at the *VEGFR2* locus (Fig. 3A), and next generation sequencing (NGS) demonstrated that the T17967A reached 51.06% among the PCR-amplified genomic fragments from the MVECs infected by the dual NILVs (Fig. 3B). This point mutation was designed to alter **A**AG (K796) to **T**AG (Stop codon), resulting in generation of DN-VEGFR2 by the dual NILV system (Fig. 1). Western blot analysis revealed that there was a smaller VEGFR2 fragment appeared with a molecular weight of around 88 kilodalton (kDa), with the level of WT VEGFR2 reduced 45.2 ± 3.6% in the MVECs infected by the dual NILV system targeting *VEGFR2* compared with those targeting *lacZ* (Fig. 3C). These results demonstrate that the T17967A mutation created by the PE6x in the genomic DNA of MVECs results in termination of translation of the whole-length *VEGFR2* mRNA at the earlier stop codon, and thus production of a new truncated protein: DN-VEGFR2 (88 kDa), as well as a decrease in WT VEGFR2 expression.

DN-VEGFR2 generated by PE6x blocks VEGF-induced VEGFR2 activation

DN-proteins can inhibit the function of their respective WT counterparts[24, 66-71]. We therefore next interrogated whether the DN-VEGFR2 generated by PE6x could block the activity of WT VEGFR2, which is essential for angiogenesis[13, 40, 69-71]. To answer this question, MVECs infected by the dual NILV system on day 3 were treated in the presence or absence of VEGF (10 ng/ml) for 10 min, and lysates subjected to a western blot analysis, which revealed that DN-VEGFR2 reduced VEGFR2 phosphorylation on Y951 by 79.8 ± 3.5% (Fig. 4).

DN-VEGFR2 generated by PE6x impedes pathological retinal angiogenesis in a mouse model of oxygen-induced retinopathy

Encouraged by the cell-based observation above, we next sought to examine whether DN-VEGFR2 could be generated by PE6x *in vivo* to inhibit retinal angiogenesis in a mouse

model of OIR. First, we assessed if NILV could infect retinal vascular ECs *in vivo* by intravitreal injection of NILV carrying CMV-GFP (NILV-GFP) into mice around 4-week-old. Whole-mount retinas from the mice at day 5 after injection were stained with isolectin 4 (IB4), a mouse endothelial specific marker. The results showed that GFP signal was co-localized with IB4 in whole-mount retinas in the NILV-GFP injected mice (Fig. 5A), indicating that NILVs are capable of transducing retinal vascular ECs.

We then intravitreally injected equal amounts of NILV5 and NILV6 targeting murine *VEGFR2* or *lacZ* into P12 mouse eyes within the OIR model. In this model, P7 mice are placed in a hyperoxic (75% oxygen) chamber for 5 days, during which central retinal vessel growth is inhibited; P12 mice are then returned to normal room air (21% oxygen). From this time, relative hypoxia induces central normal vessel regrowth and peripheral retinal abnormal angiogenesis, which is termed as preretinal tufts, which reach a maximal at P17. Whole-mount retinas from the P17 mice injected with PE6x targeting *VEGFR2* or *lacZ* were stained with IB4. As shown in Fig. 6, there was a 93.5 ± 2.1 % reduction in IB4-stained peripheral tufts and a 63.6 ± 3.2% decrease in the central retinal regrowth in mice injected with the dual NILVs targeting *VEGFR2* compared to those targeting *lacZ*, demonstrating that DN-VEGFR2 generated by PE6x in retinal vascular ECs impedes hypoxia-induced retinal angiogenesis in the mouse model of OIR.

To validate this speculation, the genomic DNA and proteins from the treated mouse retinas were subjected to next generation sequencing (NGS) and western blot analyses, respectively. The NGS results showed that there was about 11.77% of T17967A mutation from genomic DNA of the retinas treated with the dual NILVs targeting *VEGFR2*, but none with targeting *lacZ* (Fig. 7A); western blotting analysis results demonstrated that there were a smaller VEGFR2 protein appearing as well as a 22.6 ± 2.5% reduction in VEGFR2 from mice injected with the dual NILVs targeting *VEGFR2* compared to those targeting *lacZ* (Fig. 7B). Collectively, these data demonstrate that DN-VEGFR2 can be generated in genomic *VEGFR2* using PE6x *in vivo* and this DN-VEGFR2 is sufficient to block hypoxia-induced angiogenesis in the mouse model of OIR.

Notably, the dual NILVs targeting genomic *VEGFR2* did not cause detectable damage to the retinal morphology, structure, and function examined by histological staining with hematoxylin & eosin, OCT, ERG, and FFA at 4-weeks after intravitreal injection in adult mice (Fig. 8).

Discussion

In this article we document that a retinal *VEGFR2* T17967A mutation created by editing genomic *VEGFR2* locus with PE6x results in generation of an earlier stop codon (TAG, K796stop) and thus a retinal DN-VEGFR2 to block retinal VEGFR2 expression and activity as well as abnormal retinal angiogenesis in the mouse model of OIR. This PE6x system was delivered to retinas using dual NILVs via intravireal injection. Retroviral delivery of DN-VEGFR2 directly into animal models has been shown to suppress tumor angiogenesis[69, 70] and wound angiogenesis[71]. These prior investigations established a foundation for us to employ NILVs to deliver PE6x, an advanced PE system, for editing a genomic *VEGFR2* locus to produce an endogenous DN-VEGFR2, leading to inhibition of retinal angiogenesis. In comparison with retrovirus, NILVs exhibit obvious advantages of safety due to its non-integrative behavior and higher infection efficiency. In particular, our aim here was to abrogate pathological retinal angiogenesis by intravitreal delivery of NILVs, which are localized in the eye and thus whose potential immunological response is limited due to ocular immune privilege[72-74]. Similarly, when anti-VEGF antibodies were first used to treat cancer patients received severe side effects due to requirement of VEGF for normal physiological activity[75-78], whereas these agents including ranibizumab and aflibercept are the current standard of care for numerous blinding eye diseases such as nAMD and PDR[79-81]. Thereby, our data provide strong support to leverage PE6x-generated DN-VEGFR2 as a novel strategy to treat intraocular angiogenesis including PDR.

Upon the advent of PE in 2019[21], we transfected a PE2 system targeting genomic *VEGFR2* into 293T cells, but the results showed that only 0.5% edits were achieved (data not shown). This low editing efficiency might be due to an ineffective pegRNA or an MMR

effect. When PE5 emerged in 2021[24], we used a web tool[51] to design pegRNAs and then added a structured RNA motif tevopreQ$_1$ to its 3'-terminus as epegRNAs to prevent their degradation[22]; furthermore, we also employed a nicking RNA to nick a non-edited strand[21] and a DN-protein to suppress MMR[24]. To our pleasant surprise, these optimized methods remarkably increased PE efficiency, reaching more than 50% (Fig. 2A-B). Nevertheless, based on these results, we tried to further improve editing efficiency by modifying the lengths of PBS (Table 1), but these efforts did not lead to additional improvements (data not shown). Thereby, this dual-vector system for PE6x might be able to be generalized for broad applications.

Previous studies, which employed other gene editing strategies including AAV-mediated and endothelial specific promoter-driven SpCas9 targeting *VEGFR2* in the retina[40], lentiviral delivery of SpCas9 mRNA targeting *VEGF-A* in retinal pigment epithelial cells and AAV-mediated *Campylobacter jejuni* Cas9, a small Cas9) expression targeting *VEGF-A*[43], have been reported to inhibit intraocular angiogenesis in mouse models. Compared with these prior investigations prime editing is superior due to its non-breaking DNA double strands and thus increasing safety; compared with current intraocular anti-VEGF drugs, DN-VEGFR2 produced by prime editing may have higher efficiency in inhibiting angiogenesis due to its dual function of neutralizing VEGF and inhibiting VEGFR2 auto-phosphorylation. In addition, while anti-VEGF agents (e.g. ranibizumab and aflibercept) can suppress angiogenesis and vascular leakage associated with eye diseases (e.g. PDR and wet AMD), therapeutic challenges remain.

Notably, while genome editing strategies have their own advantages, they also bring their own set of limitations. For instance, it might result in certain degree of off-target effects and immune toxicity[44], which warrant further investigation when the PE6x is employed in clinical settings.

Conclusion

Our *in vitro* and *in vivo* work presented in this article demonstrates that PE6x-mediated expression of DN-VEGFR2 provides a novel opportunity for treating aberrant intraocular angiogenesis including PDR. Nonetheless, it is intriguing to explore whether this strategy can be harnessed to inhibit angiogenesis in other animal models (e.g., tumor angiogenesis, laser-induced choroid neovascularization). For these efforts will be necessary to expand its applications.

Conflict of Interest

The authors declare there is no conflict of interest in this work.

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Figure legends

Figure 1. Schematic of generating dominant-negative VEGFR2 with prime editing

A. *Schematic of dual integrase-deficient lentiviruses delivering PE6x*

1) A lentiviral vector (LV5) carrying DNA sequences for a fusion protein of SpCas9n (H840A) with RT (enhanced codon optimized reverse transcriptase) driven by a promoter of EF (elongation factor)-1α and U6-epgRNA. U6: U6 promoter, LTR: long terminal repeat.

2) A lentiviral vector (LV6) carrying EF-1α-MLHdn and U6-sgRNA for nicking (ngRNA). MLH1dn: MLH1 dominant negative.

3-4) Plasmids of an integrase-deficient packaging vector with a mutation D64V (Catalog #: 63586, Addgene), a vector expressing lentiviral envelop (Catalog #: 8454, Addgene), and LV5 or LV6 were transfected into 293T cells for production of integrase-deficient lentiviruses (LV5 or LV6). VSV-G: vesicular stomatitis virus–G protein.

B. *Schematic of generating a dominant negative VEGFR2 (K796stop) with PE6x*

1) Schematic of a *Mus musculus VEGFR2* locus (NC_00071.6) showing the 796th amino acid lysine (K) for mutation.

2) Schematic of creating *VEGFR2 T17967A* (K796stop) using PE6x

RT template (16 nucleotides: nt): template for reverse transcription, PBS (14 nt): primer binding sequence, PAM: protospacer adjacent motif, a protospacer sequence for generating a sgRNA, PBS: primer binding site.

3) An epegRNA sequence for generating *VEGFR2 T17967A* (K796stop) using PE6x. tevopreQ1: truncated evopreQ1 sequence for protection of epegRNA from degradation.

C*. Schematic of the full and dominant negative VEGFR2*

Full VEGFR2 (1345aa): an extracellular domain for ligand binding with 7 IgG domains linked by intra S-S bonds, a plasma membrane domain for crossing plasma membrane, and an intracellular domain with kinase domains; dominant negative (DN) VEGFR2 (795aa) with a mutation (K796stop) at intracellular juxtamembarane.

Figure 2. Expression of Cas9n-RT delivered by NILV is transient in mouse vascular endothelial cells

A. Western blotting analysis of clarified lysates from mouse vascular endothelial cells (MVECs) infected by non-integrated lentivirus (NILV: N) or lentiviruses (LV: L), which carried a fusion protein of SpCas9n (Cas9n) with enhanced reverse transcriptase (RT).

B. Quantification of western blotting band intensity. Data were from 3 representative independent experiments. N6 and L6: infection for 6 hours; N120 and L120: infection for 120 hours; ns: not significant; ****: significant with $p < 0.0001$ using one-way ANOVA.

Figure 3. Generation of dominant negative VEGFR2 by prime editing *in vitro*

A. Sanger DNA sequencing analysis of PCR products from genomic loci of MVECs transduced by dual NILVs, which carried a PE6x system consisting of 1) a fusion protein of SpCas9n with enhanced reverse transcriptase (RT) and epegRNA targeting *lacZ* (as a control) or *VEGFR2*, and 2) MLH1dn and nicking sgRNA targeting *lacZ* (as a control) or *VEGFR2*. There is a point mutation in genomic DNA transduced by PE6x-targeting *VEGFR2* (PE6x-*VEGFR2*), Stop: stop codon (TAG).

B. Next generation sequencing (NGS) analysis of PCR products from genomic loci of MVECs in A.

C. Western blotting analyses of lysates from transduced MVECs with indicated antibodies. A representative image shown on the left, and bar graphs were the data of western blot band intensity (VEGFR2 versus β-actin) from 3 representative experiments shown on the right; ****: significant with $p < 0.0001$ using an un-paired t test.

Figure 4. PE6x-created dominative-negative VEGFR2 attenuates VEGF-induced VEGFR2 activation *in vitro*

A. Western blotting analysis of lysates from mouse vascular endothelial cells, which were transduced by NILV-PE6x targeting *VEGFR2* or *lacZ* as a control, and stimulated by VEGF-A for 10 min after growth factors-deprivation. A representative image was shown. L: *lacZ*, V: *VEGFR2.*

B. Bar graphs were the data of western blot band intensity (p-VEGFR2 versus β-actin) from 3 representative experiments; ****: significant with $p < 0.0001$ using an un-paired t test.

Figure 5. NILVs transduce vascular endothelial cells *in vivo*

NILVs expressing EGFP (1μl, 5 x 10^9 IU/ml) were intravitreally injected into 4-week-old mice; 5 days later, the eyeballs were isolated and whole-mount retinas were stained with IB4, a mouse endothelial cell marker. The stained whole-mount retinas were photographed in a fluorescence microscope. Black thick arrows: amplification of square areas; thin white arrows: representative merge of GFP with IB4, scale bar: 200 μm.

Figure 6. DN-VEGFR2 generated by PE6x impedes pathological retinal angiogenesis in a mouse model of oxygen-induced retinopathy

Representative of IB4 staining analysis of retinal neovascularization in P17 mice of OIR after intraviteal injection of NILV-PE6x targeting *VEGFR2* or *lacZ* into P12 OIR mice (left). Bar graphs were the data of IB4-stained retinas (n = 6) from mice intravitreally injected with NILV-PE6x targeting *VEGFR2* or *lacZ*. ****: significant with $p < 0.0001$ using an un-paired t test.

Figure 7. Generation of *VEGFR2 T17967A* and DN-VEGFR2 by PE6x *in vivo*

A. NGS analysis of genomic DNA from mouse retinas. A mutation of *VEGFR2 T17967A* (K796stop) was generated in retinas from P17 OIR mice after intravitreal injection of NILV-PE6x targeting *VEGFR2* or *lacZ* into P12 mice.

B. Western blot analysis of retinal lysates with an antibody against N-terminal of VEGFR2. DN-VEGFR2 was generated in P17 mouse retinas after intraviteal injection of NILV-PE6x targeting *VEGFR2* or *lacZ* into P12 OIR mice. Bar graphs were the data of western blot band intensity (p-VEGFR2 or VEGFR2 versus β-actin) from 3 representative experiments; *** and ****: significant with $p < 0.001$ and $p < 0.0001$, respectively, using an un-paired t test.

Fig 8. Intravitreal injection of NILV-PE6x does not interfere with retinal structure and function

6-week-old mice were injected with the dual NILVs-PE6x targeting *VEGFR2* or *lacZ* into the left eye. After 4 weeks, the mice were examined by histology (H&E staining of eyeballs) (A), optical coherence tomography (OCT) (B), electroretinography (ERG) (C), and fundus fluorescein angiography (FFA) (D). NI: non-injection. PE6x-lacZ: dual NILVs targeting *lacZ*, PE6x-*VEGFR2*: dual NILVs targeting *VEGFR2*.

Figures

Figure 1

A. Schematic of dual integrase-deficient lenriviruses delivering PE6x

1) Lenti-EF-1-SpCas9n-RT-U6-epegRNA(**LV5**)

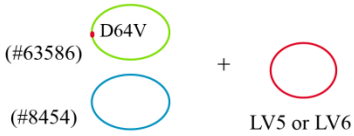


2) Lenti-EF-1-MLH1dn-U6-sgRNA(nicking)(**LV6**)



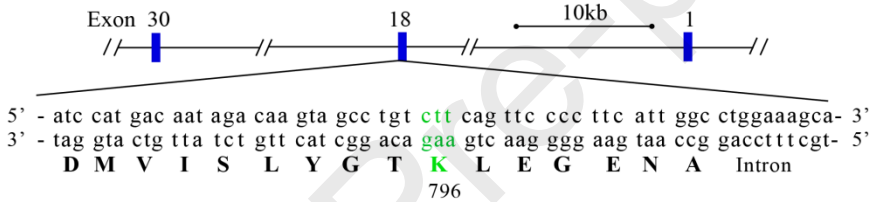
3) An integrase deficient packaging vector (#63586)

4) A VSV-G vector (#8454)

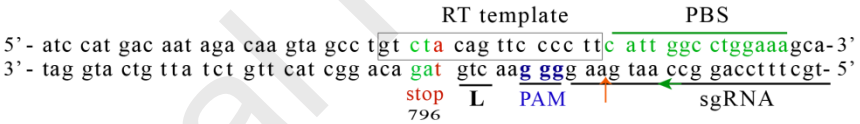


B. Schematic of generating a mutant of *VEGFR2* (K796stop) with PE6x

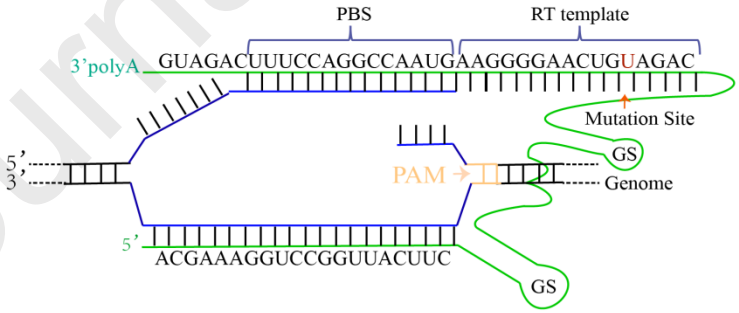
1) A Mus musculus *VEGFR2* locus (NC_000071.6)



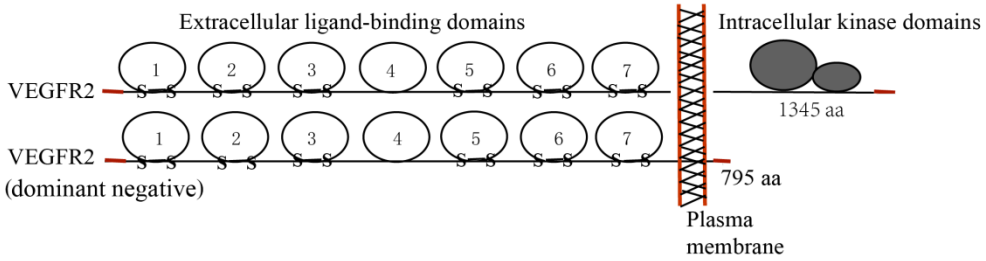
2) Schematic of creating *VEGFR2* T17967A(K796stop) using PE6x



3) The epegRNA sequence

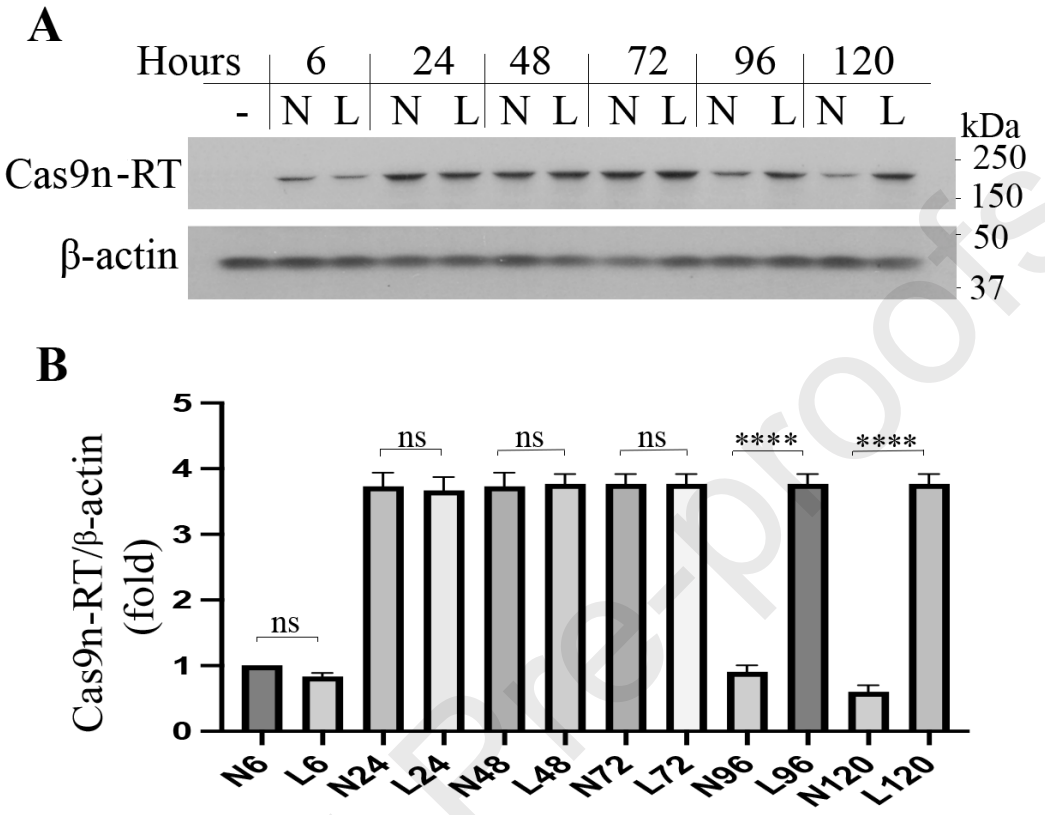


C. Schematic of the dominant negative VEGFR2



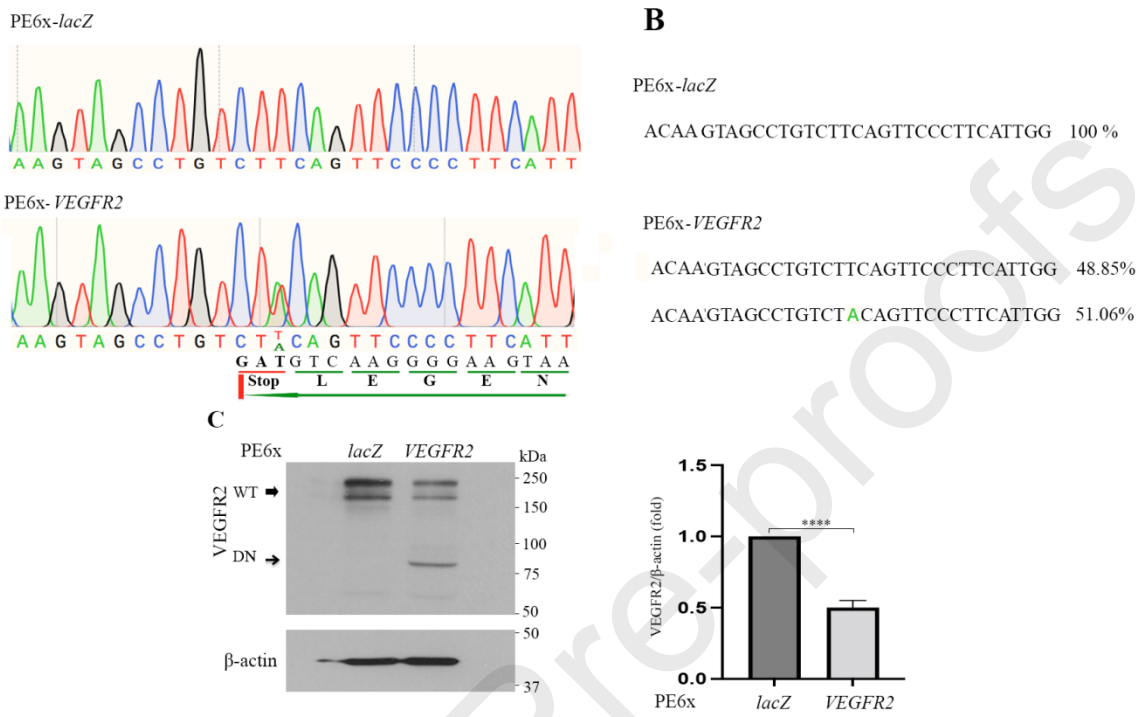
Journal Pre-proofs

Figure 2



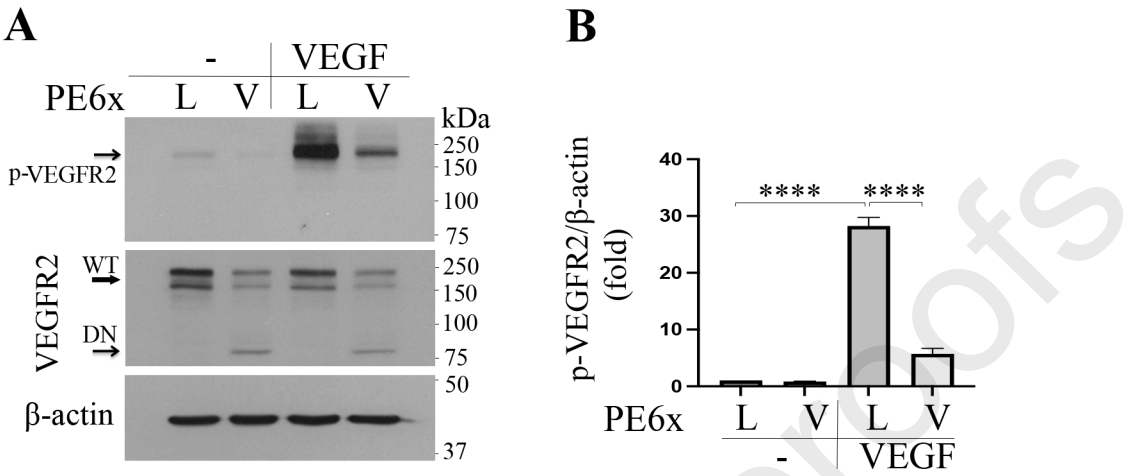
Journal Pre-proofs

Figure 3



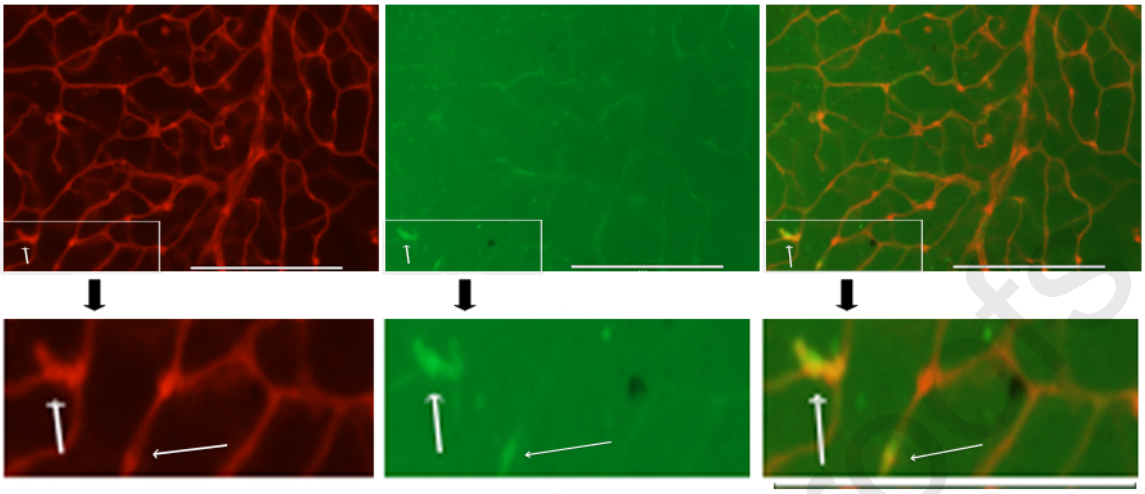
Journal Pre-proofs

Figure 4



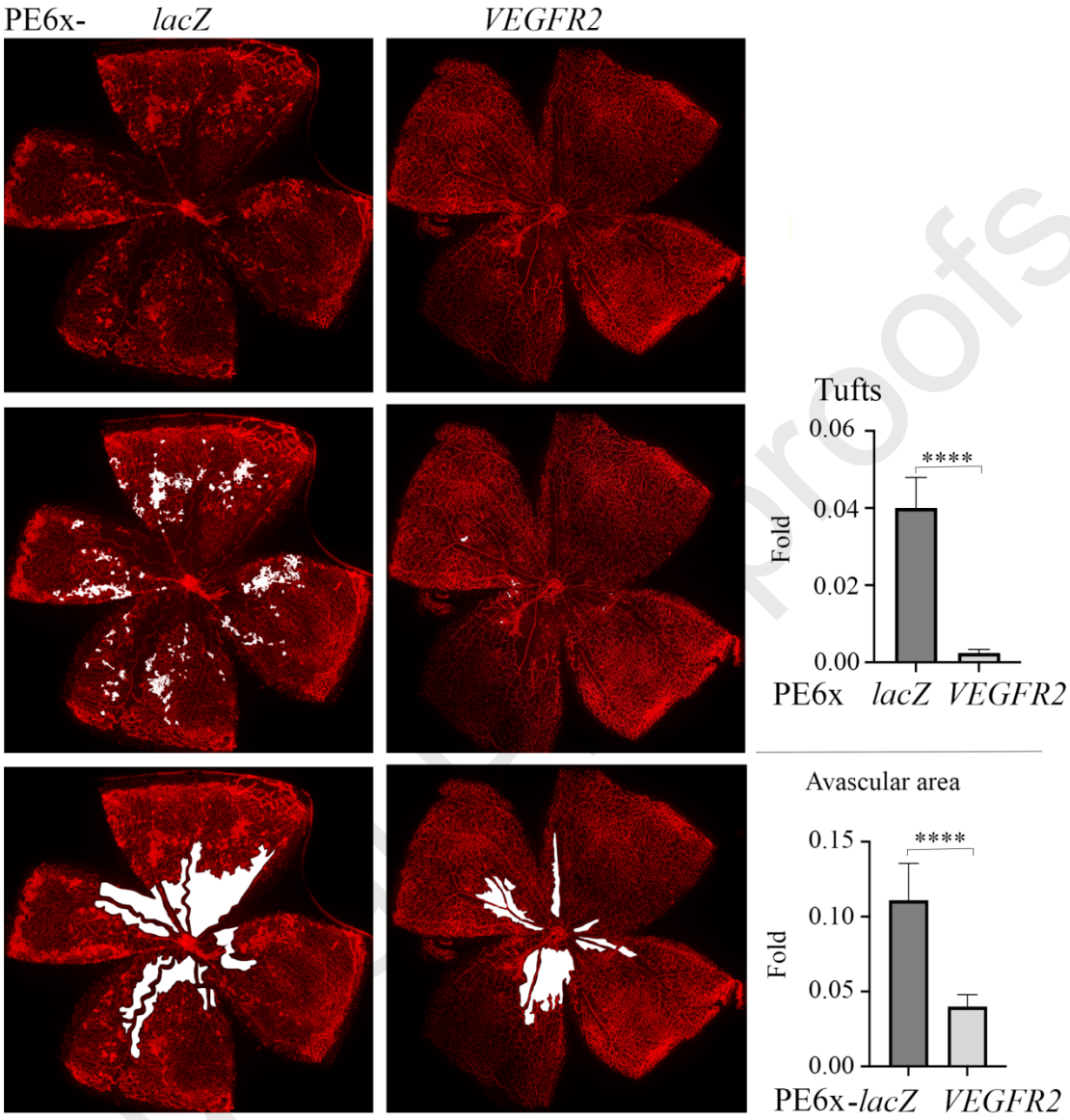
Journal Pre-proofs

Figure 5



Journal Pre-proofs

Figure 6



Journal Pre-proofs

Figure 7

A

PE6x-*LacZ*

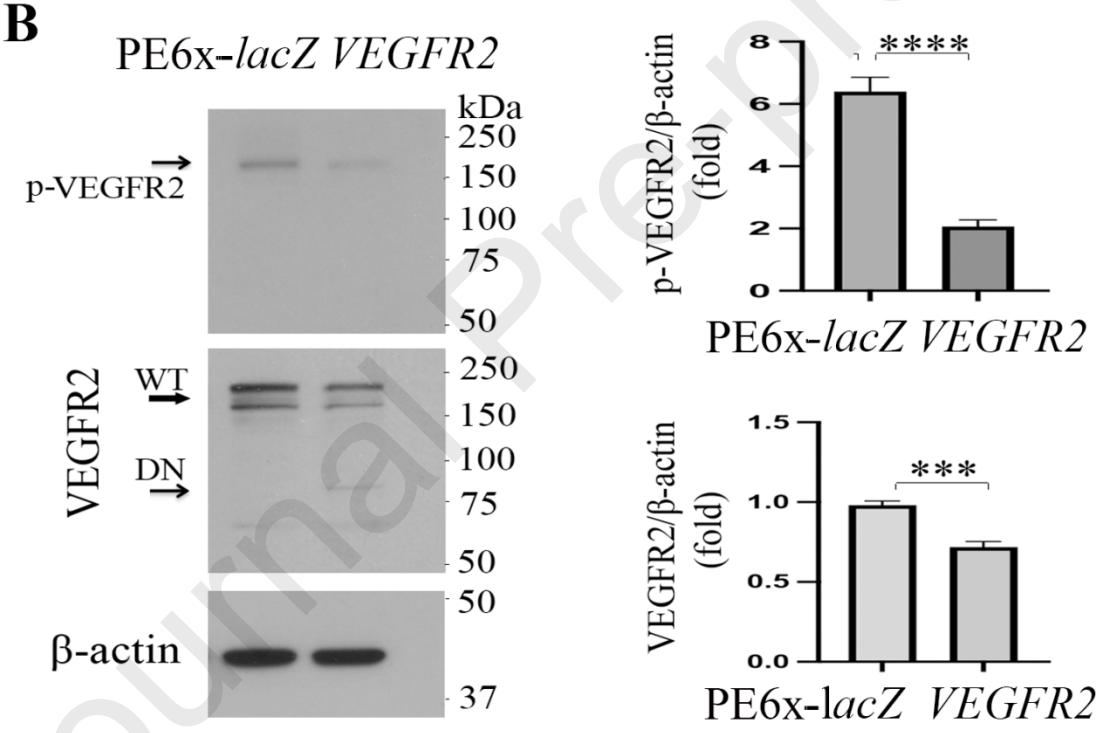
ACAA GTAGCCTGTCTTCAGTTCCCTTCATTGG 100%

PE6x-*VEGFR2*

ACAA GTAGCCTGTCTT CAGTTCCCTTCATTGG 88.16%

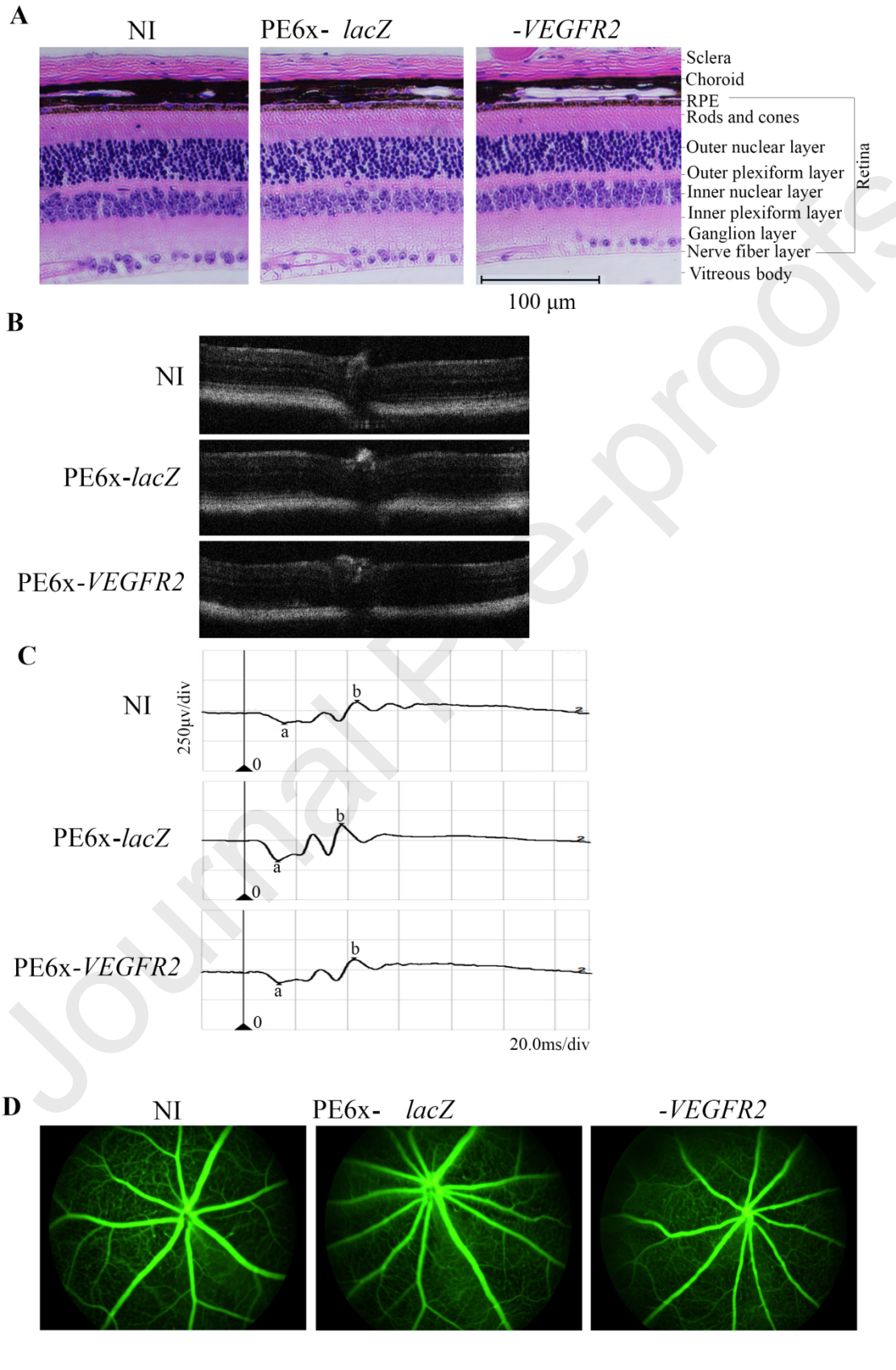
ACAA GTAGCCTGTCT A CAGTTCCCTTCATTGG 11.77%

B



Journal Pre-proofs

Figure 8



Journal Pre-proofs

Table 1 Selection of epegRNA for editing mouse *VEGFR2*

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epegRNA		Sequences
1	sgRNA	TGCTTTCCAGGCCAATGAAG
	RT template (16nt)	GTCTACAGTTCCCCTT
	PBS (14 nt)	CATTGGCCTGGAAA
2	sgRNA	TATGCTTTCCAGGCCAATGA
	RT template	TCTACAGTTCCCCTT
	PBS	CATTGGCCTGGAAAGC
3	sgRNA	GGGGAGGATTTATGCTTTCC
	RT template	TGTCTACAGTTCCCCTT
	PBS	CATTGGCCTGGAAAG

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EvopreQ1		CGCGGTTCTATCTAGTTACGC GTTAAACCAACTAGAA
ngRNA		TCATCCAAGGGCAATTCATC
PCR primers	P51F	GCCAGATGAGCGGGTAAAA
	P51R	TGAGATTATCATGAATGAGGCCC

Table 1.

Selection of epegRNA for editing mouse *VEGFR2*

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Journal Pre-proofs

Establishment of a novel prime editing (PE)6x system.

Creation of a dominant negative (DN) VEGFR2 with dual non-integrating lentivirus (NILV)-mediated PE6x.

Prevention of VEGF-induced VEGFR2 activation by PE6x-created DN-VEGFR2 *in vitro* and *in vivo.*

Inhibition of pathological retinal angiogenesis by PE6x-generated DN-VEGFR2 in a mouse model of oxygen-induced retinopathy.

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All the animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Investigators who conducted analysis were masked as to the treatment groups. All experiments involving animals were conducted according to the ethical policies and procedures approved by the laboratory animal ethics committee of Peking University at Shenzhen (Approval no. AP-2021-04).

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