

Inhibition of mTOR via an AAV-Delivered shRNA Tested in a Rat OIR Model as a Potential Antiangiogenic Gene Therapy

Steven Hyun Seung Lee,^{1,2} HeeSoon Chang,³ Ji Hyun Kim,^{1,2} Hee Jong Kim,³ Jun-Sub Choi,³ Sunho Chung,⁴ Ha-Na Woo,^{1,2} Kyoung Jin Lee,^{1,2} Keerang Park,⁵ Joo Yong Lee,^{2,4} and Heuiran Lee^{1,2}

¹Department of Microbiology, University of Ulsan, College of Medicine, Seoul, Korea

²Bio-Medical Institute of Technology, University of Ulsan, College of Medicine, Seoul, Korea

³CuroGene Life Sciences Co., Ltd., Cheongju, Korea

⁴Department of Ophthalmology, Asan Medical Center, University of Ulsan, College of Medicine, Seoul, Korea

⁵Department of Biopharmacy, Chungbuk Health & Science University, Cheongju, Korea

Correspondence: Heuiran Lee, Department of Microbiology, Bio-Medical Institute of Technology, University of Ulsan, College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul, Republic of Korea 05505;

heuiran@amc.seoul.kr

Joo Yong Lee, Department of Ophthalmology, Asan Medical Center, University of Ulsan, College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul, Republic of Korea 05505;

ophthamo@amc.seoul.kr

SHSL, HC, and JHK are joint first authors.

Received: July 3, 2019

Accepted: November 27, 2019

Published: February 27, 2020

Citation: Lee SHS, Chang H, Kim JH, et al. Inhibition of mTOR via an AAV-Delivered shRNA tested in a rat OIR model as a potential antiangiogenic gene therapy. *Invest Ophthalmol Vis Sci.* 2020;61(2):45. <https://doi.org/10.1167/iovs.61.2.45>

PURPOSE. Recent studies have shown that inhibitors of the mechanistic target of rapamycin (mTOR) play important roles in proliferating endothelial cells within the retinal vasculature. Here we explore the effects of inhibiting mTOR as a potential gene therapeutic against pathological retinal angiogenesis in a rat model of oxygen-induced retinopathy (OIR).

METHODS. Sprague-Dawley pups were used to generate the OIR model, with a recombinant adeno-associated virus expressing an shRNA (rAAV2-shmTOR-GFP) being administered via intravitreal injection on returning the rats to normoxia, with appropriate controls. Immunohistochemistry and TUNEL assays, as well as fluorescein angiography, were performed on transverse retinal sections and flat mounts, respectively, to determine the in vivo effects of mTOR inhibition.

RESULTS. Compared with normal control rats, as well as OIR model animals that were either untreated (20.95 ± 6.85), mock-treated (14.50 ± 2.47), or injected with a control short hairpin RNA (shRNA)-containing virus vector (16.64 ± 4.92), rAAV2-shmTOR-GFP (4.28 ± 2.86 , $P = 0.00103$) treatment resulted in dramatically reduced neovascularization as a percentage of total retinal area. These results mirrored quantifications of retinal avascular area and vessel tortuosity, with rAAV2-shmTOR-GFP exhibiting significantly greater therapeutic efficacy than the other treatments. The virus vector was additionally shown to reduce inflammatory cell infiltration into retinal tissue and possess antiapoptotic properties, both these processes having been implicated in the pathophysiology of angiogenic retinal disorders.

CONCLUSIONS. Taken together, these results demonstrate the strong promise of rAAV2-shmTOR-GFP as an effective and convenient gene therapy for the treatment of neovascular retinal diseases.

Keywords: retinal neovascularization, pathological angiogenesis, oxygen-induced retinopathy, recombinant adeno-associated virus, mechanistic target of rapamycin

Angiogenic retinal pathologies, which include diabetic retinopathy (DR), retinal vein occlusion (RVO), and retinopathy of prematurity (RoP), are among the leading causes of vision loss in developed nations.¹ With DR currently affecting approximately 100 million patients worldwide and diabetes mellitus projected to have a global incidence rate of 592 million by 2035,² it can be seen that these conditions, of which RVO is the second most common neovascular retinal disease,³ constitute a growing health concern. The most salient characteristic shared among these disorders is retinal neovasculariza-

tion, a process for which vascular endothelial growth factor (VEGF) serves as a main driver.¹ Therefore anti-VEGF therapeutic strategies have been pursued and approved to treat these conditions,^{2,4} and are administered via repeated intravitreal injections, depending on the dosing schedule. Patient compliance is often negatively affected by the frequency of these injections,⁵ and with safety questions having been raised^{6,7} regarding the long-term inhibition of VEGF, the development of a gene therapeutic focused on a non-VEGF target may prove to be greatly beneficial.

One such therapeutic target that has previously been set forth is mechanistic target of rapamycin (mTOR),^{6,8} so named because it is targeted by the broadly antiproliferative rapamycin.⁹ A serine/threonine kinase, mTOR exists as a component member of three separate complexes, mTOR complex 1 (mTORC1), mTOR complex 2 (mTORC2),⁹ and the recently identified mTOR complex 3 (mTORC3).¹⁰ Acute rapamycin treatment has been shown to inhibit mTORC1 only, with mTORC2¹¹ and mTORC3 insensitive to its effects.¹⁰ Because of its integral role in various signaling pathways,⁹ mTOR dysfunction has been linked to a number of disease states, including ocular disorders.¹² Previous studies have demonstrated the ability of rapamycin to reduce retinal neovascularization *in vitro*¹³ and *in vivo*¹⁴ by inhibiting mTORC1, which acts through hypoxia inducible factor 1 α (HIF-1 α) to downregulate VEGF. However, as this VEGF downregulation is partial,¹⁵ developing a gene therapeutic that targets mTOR for the treatment of angiogenic retinal diseases necessitates the inhibition of all three complexes.

By employing ATP-competitive inhibitors of mTOR to block all downstream phosphorylation targets, several groups were able to successfully inhibit both mTORC1 and mTORC2.⁹ Unlike first-generation inhibitors, consisting of rapamycin and its structural analogues, called rapalogs, that only affect mTORC1,¹¹ these second-generation inhibitors were found to effectively block mTORC3 activity as well.¹⁰ However, we utilized CAPSID (Convenient Application Program for siRNA Design), a program developed in-house, to design a novel short hairpin RNA (shRNA) specifically targeting mTOR.¹⁶ By doing so, RNA interference is used to directly downregulate mTOR activity, rather than the inhibition being effected in a roundabout manner. To continue its development as a potential gene therapeutic, the mTOR-inhibiting shRNA was packaged alongside a GFP reporter in a recombinant adeno-associated virus (rAAV),¹⁷ which are capable of eliciting long-term transgene expression, are nonpathogenic in nature, and are able to transduce dividing and nondividing cells.¹⁸ US Food and Drug Administration approval of voretigene neparvovec-rzyl (Luxturna; Spark Therapeutics, West Philadelphia, PA) in late 2017, which delivers a human RPE65 cDNA via a rAAV2 to treat Leber's congenital amaurosis resulting from a mutation of the *RPE65* gene, confirmed the suitability of rAAVs for gene therapy applications involving ocular conditions.¹⁹

Previously, we explored the therapeutic efficacy of the shRNA-containing virus vector in a laser-induced mouse model of choroidal neovascularization, which models the wet subtype of age-related macular degeneration, and it was shown to reduce the extent to which neovascularization occurred.²⁰ To see if these effects were able to be recapitulated in an animal model of pathological retinal angiogenesis, we injected a rat oxygen-induced retinopathy (OIR) model with the virus vector, renamed recombinant adeno-associated virus expressing an shRNA (rAAV2-shmTOR-GFP), to explore its suitability as a potential gene therapeutic for neovascular retinal conditions. We show here that the direct inhibition of mTOR *in vivo* is antiangiogenic and that rAAV2-shmTOR-GFP reduces arterial tortuosity in the hypoxic retina, while also possessing anti-inflammatory and antiapoptotic capabilities. All of these aspects are associated with angiogenic retinal disorders and the ability of rAAV2-shmTOR-GFP to comprehensively address them demonstrates its promise as a gene therapeutic for the treatment of these conditions.

METHODS

Preparation of Virus Vectors

The experimental virus vectors, rAAV2-shmTOR-GFP (previously rAAV-mTOR shRNA-enhanced GFP (EGFP)) and rAAV2-shCon-GFP (previously rAAV-scrambled shRNA-EGFP), were prepared as previously described.²⁰ Briefly, the mTOR-inhibiting shRNA (5'-GAAUGUUGACCAAUGCUAU-3') or control shRNA (5'-AUUCUAUCACUAGCGUGAC-3'), both under the control of an H1 promoter, were inserted alongside an EGFP expression cassette driven by the cytomegalovirus promoter into a self-complementary AAV2 vector. All of the virus vectors used in this study were obtained from Cdmogen Co., Ltd. (Cheongju, Korea).

In Vitro Characterization

ARPE-19 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 2 mM GlutaMAX-1 (Thermo Fisher Scientific), and penicillin (100 IU/mL)/streptomycin (50 μ g/mL), and maintained at 37°C under a humidified 5% carbon dioxide atmosphere. Infections were performed in 6-well plates, in which 2.0×10^5 cells were treated with rAAV2-shCon-GFP or rAAV2-shmTOR-GFP at both 300 and 1500 multiplicity of infection (MOI), and whole-cell lysates prepared 48 hours later. The proteins were then resolved on reducing sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Primary antibodies specific to mTOR (2983S), GFP (332600), and β -actin (A5441) were obtained from Cell Signaling Technology (Danvers, MA, USA), Invitrogen, and Sigma-Aldrich (St. Louis, MO, USA), respectively, and protein bands detected using an enhanced chemiluminescence system.

Quantitative Reverse Transcription PCR (RT-qPCR)

TRIzol reagent (Invitrogen) was used to prepare total RNA from retinal tissue. Superscript III (Invitrogen) was used thereafter to reverse transcribe cDNA from RNA and a SYBR Green kit (Invitrogen) used to analyze mRNA levels. Primers specific for mTOR (forward, 5'-TTTGAGGTTGCTATGACCAG-3'; reverse, 5'-TCTATAGTTGCCATCGAGAC-3') and β -actin (forward, 5'-TGAAGATCAAGATCATTGCTC-3'; reverse, 5'-TGCTTGCTGATCCACATCTG-3') were used for amplification purposes.

Animal Care

Sprague-Dawley pups and dams (The Orient Bio, Sungnam, Korea) were used in this study, which was approved by the Internal Review Board for Animal Experiments at the Asan Institute for Life Sciences (University of Ulsan, College of Medicine). All animal care and experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research.

Rat Model of OIR

The well-established protocol for mice set forth by Smith et al.²¹ was generally followed to generate the rat OIR model. However, because of the lower levels of neovascularization observed in rats when compared with the mouse OIR model,²² Sprague-Dawley rats in particular,²³ pups were placed under hyperoxia at postnatal day 4 (P4) to take advantage of the less developed retinal vasculature and induce a more dramatic vaso-obliteration, as neovascularization depends on the extent to which vaso-obliteration occurs.²⁴ At P9, the pups were returned to normoxia and either intravitreally injected with their respective virus vectors, mock-treated, or left untreated before sacrifice on P14.

Intravitreal Injections

Prior to intravitreal injection, the rats were anesthetized with an intraperitoneally administered 4:1 mixture of 40 mg/kg of Zoletil (zolazepam/tiletamine) obtained from Virbac (Carros Cedex, France) and 5 mg/kg of Rompun (xylazine) sourced from Bayer Healthcare (Leverkusen, Germany). Their pupils were then dilated with Mydrin-P (0.5% tropicamide and 2.5% phenylephrine) supplied by Santen (Osaka, Japan) before being intravitreally injected with 1 μ L of the virus vectors at a concentration of 5.0×10^{10} viral genomes (v.g.)/mL.

Tissue Preparation

An intraperitoneal injection of a 4:1 mixture of Zoletil (80 mg/kg) and Rompun (10 mg/kg) was used to deeply anesthetize the rats, followed by an intracardial perfusion with 0.1 M phosphate-buffered saline (PBS) (7.4 pH) containing 150 U/mL heparin and infusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The eyeballs were enucleated and fixed for 1 hour in 4% PFA in 0.1 M PB and the anterior sections were then removed, including the cornea and lens, to generate eyecups. Flat mounts were generated by removing the retinal pigment epithelium (RPE)-choroid tissue, making four equidistant cuts, and mounting the retinal tissue between a microscope slide and cover slip. For frozen sectioned samples, eyecups were transferred to 30% sucrose in PBS overnight then embedded in Tissue-Tek, an optimum cutting temperature compound (Miles Scientific, Naperville, IL, USA), before 5 μ m-thick transverse retinal sections were prepared.

Fluorescein Angiography and Analysis

Retinal vessels were visualized by staining flat mounts with Alexa Fluor 568-conjugated isolectin GS-IB₄ (I21412; Thermo Fisher Scientific) for 1 day at 4°C prior to confocal microscopy. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to determine the effects of rAAV2-shmTOR-GFP activity on the rat OIR model. Neovascularization was quantified by manually outlining the areas wherein neovascular tufts were formed and comparing their sum amount with the total area of the flat mount. Calculating the percentage of avascular areas was carried out in a similar manner. Vessel tortuosity was determined by drawing a straight line from the terminus of a retinal artery to where it begins toward the center of the retina. Then, after manually drawing a line that follows the path of said artery, the two values were compared with yield a vessel tortuosity ratio.

Immunohistochemistry and TUNEL Assay

Frozen sections were stained for macrophage infiltration using anti-F4/80 (MCA497GA; Serotec, Oxford, UK); to visualize retinal transduction using anti-GFP (ab6556; Abcam, San Francisco, CA, USA) or anti-mTOR (AF15371; R&D Systems, Minneapolis, MN, USA); or to determine retinal tissue tropism of the virus vectors using anti-GFP alongside either anti-CD31 (550274; BD Pharmingen, San Diego, CA, USA), anti-gial fibrillary acidic protein (GFAP) (12389; Cell Signaling Technology), or anti-neuronal nuclei (NeuN) (MAB377, Millipore, Burlington, MA, USA) by incubating the samples overnight at 4°C with the diluted primary antibodies. They were then washed 3 times in PBS-Tween (PBST) for 10 minutes apiece, incubated for 2 hours at room temperature with the secondary antibodies Alexa Fluor 568 or 488 (Thermo Fisher Scientific), and stained with DAPI (D9542; Sigma-Aldrich) to visualize the cell nuclei. TUNEL assay was performed according to the manufacturer's protocol (12156792910; Roche Diagnostics, Indianapolis, IN, USA), the retinal sections were then washed 3 times in PBST for 10 minutes apiece and stained with DAPI.

Image Acquisition and Statistical Analysis

Samples were examined using an LSM 700 fluorescence confocal microscope (Carl Zeiss Microscopy, Jena, Germany) and images captured using ImageJ software. One-way ANOVA testing was used for statistical analysis, with significant difference determined at $P < 0.01$ or $P < 0.001$. The data were generally presented as dot plots with mean standard error of mean values also visualized.

RESULTS

In Vitro and In Vivo Efficacy of rAAV2-shmTOR-GFP

To characterize the experimental virus vectors in vitro, ARPE-19 cells were infected at 300 and 1500 MOI for 48 hours with either rAAV2-shmTOR-GFP or rAAV2-shCon-GFP (Fig. 1A). Western blotting revealed that the former markedly reduced mTOR expression in the RPE cell line, whereas it was high in cells infected with rAAV2-shCon-GFP (Fig. 1B). Greater levels of GFP protein were detected at the higher multiplicity of infection for both virus vectors. Immunohistochemistry performed on frozen sections using an antibody for GFP revealed that rAAVs delivered via intravitreal injections are able to effectively transduce rat retinas, as GFP was expressed in animals treated with the experimental virus vectors, whereas absent in the normal control and mock-treated groups. Immunostaining with anti-mTOR showed that the rat OIR model was successfully generated, with mock-treated and rAAV2-shCon-GFP-injected control groups exhibiting elevated mTOR protein levels compared with rats that were not exposed to hyperoxia. Additionally, the almost complete downregulation of mTOR expression confirmed the in vivo activity of rAAV2-shmTOR-GFP (Fig. 1C). RT-qPCR analysis showed significantly lower mTOR mRNA levels in retinal tissue sampled from rats administered with the therapeutic virus vector (Fig. 1D).

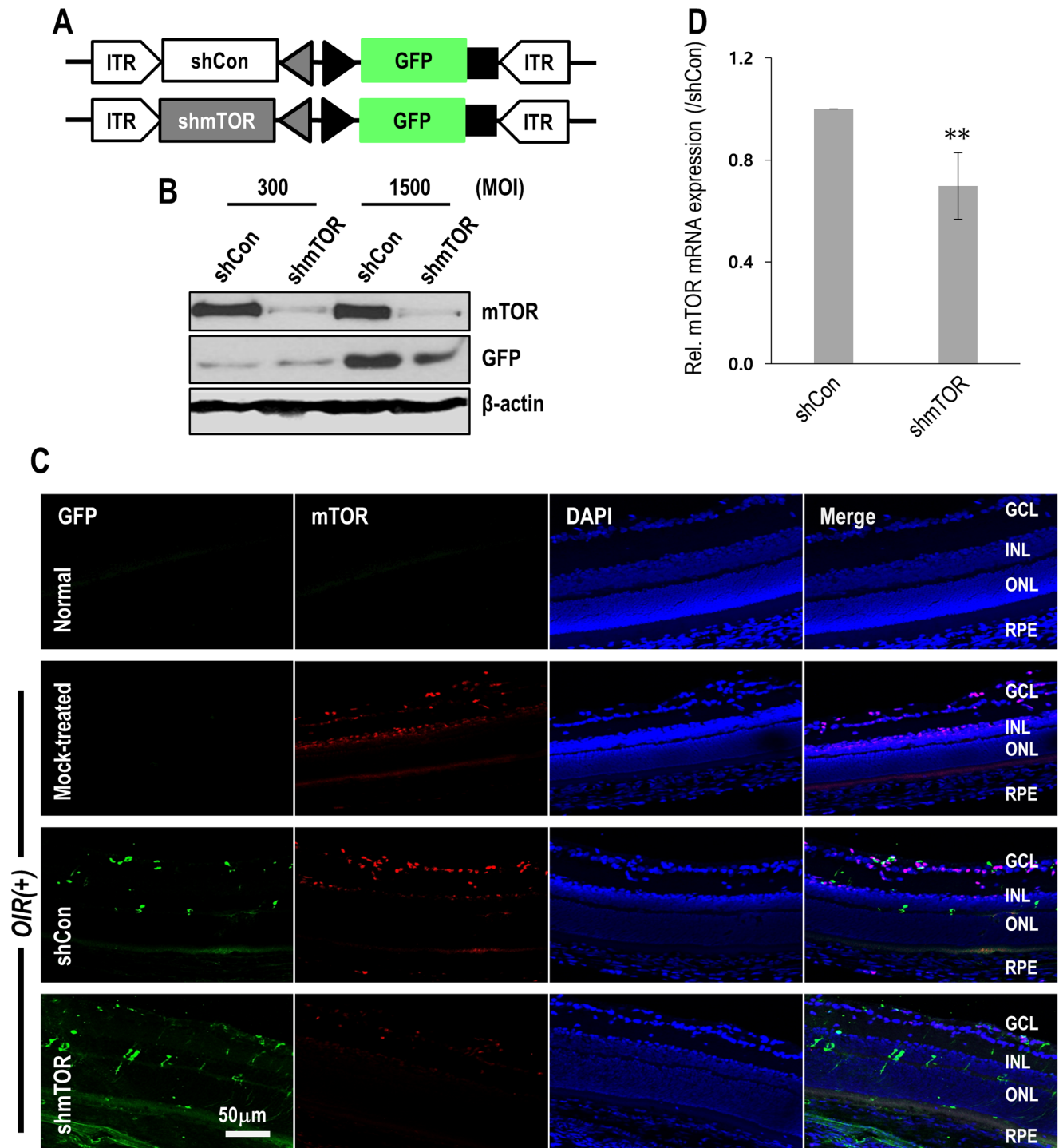


FIGURE 1. Characterization and retinal transduction of rAAV2-shmTOR-GFP. Schematic representation of rAAV2-shmTOR-GFP and rAAV2-shCon-GFP (A). ARPE-19 cells were infected with one of the experimental virus vectors, and western blotting performed thereafter showed that rAAV2-shmTOR-GFP treatment significantly reduced mTOR protein levels, whereas GFP expression correlated with virus amounts (B). β -actin was used here as a protein loading control. Compared with the normal control group, which did not undergo hyperoxic treatment, OIR model animals that were either mock-treated or injected with rAAV2-shCon-GFP exhibited elevated mTOR protein levels. This was almost completely abrogated in rats receiving rAAV2-shmTOR-GFP, which combined with GFP expression in vivo to reveal that the virus vectors successfully transduced the retinas of the rat OIR model (C). RT-qPCR showed markedly lower mTOR mRNA levels in retinas treated with the therapeutic virus vector, relative to rAAV2-shCon-GFP treatment (D). ITR, internal terminal repeat; GCL, ganglion cell layer; INL, inner nuclear layer (INL); ONL, outer nuclear layer.

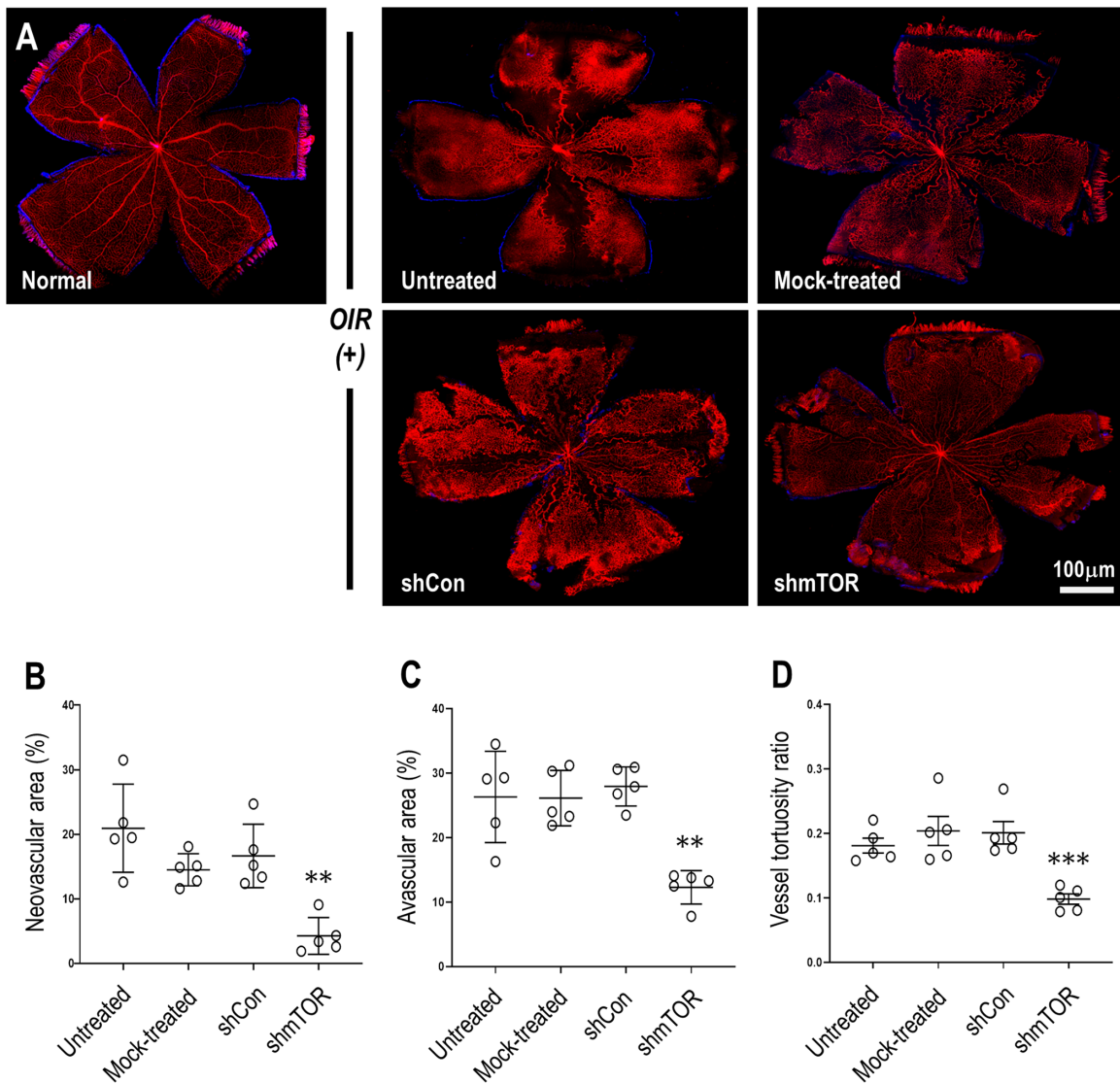


FIGURE 2. In vivo effects of rAAV2-shmTOR-GFP visualized via fluorescein angiography. Isolectin was used to visualize the extensiveness of the retinal vascular networks of normal control rats and the OIR model groups (A). Postsacrifice, ImageJ software was used to determine the extent to which neovascularization occurred (B) on returning the animal models to normoxia, as well as to measure the avascular area of the retinas (C) and arterial tortuosity (D). Among all of the groups, rAAV2-shmTOR-GFP treatment most limited neovascularization, while also resulting in the lowest ratio of vessel tortuosity and amount of avascular area (n = 5).

Effects of mTOR Inhibition on the Retinas of a Rat OIR Model

The implications of this mTOR-inhibiting activity, as they relate to neovascular retinal disorders, were examined via fluorescein angiography (Fig. 2A). In rat OIR models, returning the pups to normoxia after vaso-obliteration has occurred during the hyperoxic phase should result in the sprouting of new capillaries. However, abnormal vessels may arise in the form of neovascular tufting, called pathological angiogenesis.²⁵ As can be seen (Fig. 2B), neovascularization was least extensive in rats treated with rAAV2-shmTOR-GFP (4.28 ± 2.86 , $P = 0.00103$), whereas untreated, mock-treated, and rAAV2-shCon-GFP-injected animals had neovascularization present in 20.95 ± 6.85 , 14.50 ± 2.47 , and 16.64 ± 4.92 percent of their total retinal areas, respectively. During vaso-obliteration in the first part of OIR model generation, the creation of a capillary-free region in the rat retina results

in an increased amount of avascular areas,²⁶ and as vessels are regenerated in the second phase, an inverse relationship is found to exist between neovascular tufting and healthy vascular regeneration.²⁵ As such, a reduction of neovascularization correlates to increased normal revascularization, resulting in decreased avascularity. This was observed in the rat OIR model (Fig. 2C), in which untreated (26.31 ± 7.06), mock-treated (26.15 ± 4.30), and rAAV2-shCon-GFP-treated (27.96 ± 3.04) rats had greater percentages of avascular areas in their retinas compared with those injected with rAAV2-shmTOR-GFP (12.29 ± 2.57 , $P = 0.00310$). Hypoxia only affects retinal capillaries, leaving larger arteries and veins intact. Whereas vascular sprouting from the latter and any unaffected capillaries are responsible for regenerating the capillary network, with neovascular tufting being a dysfunction of this process, retinal arteries become tortuous, which is found in a number of retinopathic conditions.²⁵ Vessel tortuosity was least severe in rats treated with rAAV2-

shmTOR-GFP (0.098 ± 0.022 , $P = 0.00040$), whereas ratios of 0.181 ± 0.040 , 0.204 ± 0.074 , and 0.201 ± 0.043 were observed in untreated, mock-treated, and rAAV2-shCon-GFP-treated animals, respectively (Fig. 2D).

Anti-Inflammatory Effect of rAAV2-shmTOR-GFP

With the involvement of inflammation in the pathophysiology of angiogenic retinal conditions, DR in particular,^{27,28} inflammatory cell infiltration was examined in transverse retinal sections via anti-F4/80 immunostaining (Fig. 3A). Although macrophages were not readily detectable in normal control rats, they were found to have infiltrated the ganglion cell layers of the various OIR model control groups, with untreated (23.4 ± 7.3), mock-treated (26.8 ± 8.9), and rAAV2-siCon-GFP-injected (23.4 ± 6.1) rats all containing F4/80-positive cells in a given $100 \mu\text{m} \times 100 \mu\text{m}$ area of the retina. However, significantly fewer inflammatory cells (10.4 ± 2.7 , $P = 0.00236$) were found in rats treated with the mTOR-inhibiting shRNA (Fig. 3B).

Antiapoptotic Ability of rAAV2-shmTOR-GFP

Although the links between cell death and pathological retinal angiogenesis have yet to be fully elucidated, including the exact mechanism(s) involved, increased apoptosis has been implicated in the release of VEGF in proliferative DR (PDR).²⁹ TUNEL-positive cells were observed in the inner and outer nuclear layers of the untreated (16.6 ± 4.2), mock-treated (17.4 ± 5.6), and control shRNA-treated (17.6 ± 3.6) groups of the rat OIR model (Fig. 4A). The rAAV2-shmTOR-GFP administration resulted in a marked reduction of (5.8 ± 2.6 , $P = 0.00036$) apoptotic cells detected in the retina (Fig. 4B), suggesting that it has antiapoptotic properties, which may contribute to the therapeutic efficacy of the virus vector.

Retinal Tissue Tropism of rAAV2-shmTOR-GFP

Coimmunostaining with anti-GFP along with either anti-CD31, which indicates the presence of endothelial cells; anti-GFAP, which stains Müller cells (MCs) and other glial cells; or anti-NeuN, which localizes in the ganglion cell layer, was used to show which cell types supported the intravitreally injected virus vectors (Fig. 5A). This was visualized by a yellow signal marking the overlap of GFP-positive green signals with red signals of the cell types, and quantification of this overlap showed that rAAV2-shmTOR-GFP treatment resulted in lower ratios of colocalization than administration of the virus vector containing the control shRNA in all cases (Fig. 5B). Retinal neovascularization occurred in untreated and rAAV2-shCon-GFP-administered groups (0.567 ± 0.044), with the virus vector present in the endothelial cells of the newly formed vessels. Meanwhile, rats treated with rAAV2-shmTOR-GFP (0.376 ± 0.030 , $P = 0.004$) had significantly reduced levels of angiogenesis. The experimental virus vectors were additionally found to be located throughout the retinal layers. Similar results were seen with anti-GFAP and anti-GFP staining, with the virus vectors speckling all layers of the retina. The greatest amounts of glial cells were present in untreated rats and those treated with the control shRNA-containing virus vector (0.554 ± 0.101) (rAAV2-shmTOR-GFP: 0.378 ± 0.049 , $P = 0.014$), with highly overlapping yellow signals found in the latter. Finally, the virus vectors were generally less supported

by the ganglion cell layer (rAAV2-shCon-GFP: 0.342 ± 0.058 ; rAAV2-shmTOR-GFP: 0.266 ± 0.040 , $P = 0.064$).

DISCUSSION

Here we examined the effects of direct mTOR inhibition in a rat model of OIR with regard to aspects of various neovascular retinal pathologies, including PDR, RVO, and RoP. After confirming via western blotting from ARPE-19 cells that rAAV2-shmTOR-GFP elicited the desired effects in vitro, it was compared with untreated, mock-treated, and control shRNA-treated OIR model animals, as well as normal control rats. Among the therapeutically relevant results, rAAV2-shmTOR-GFP exhibited significantly reduced neovascularization and led to retinal arteries that had much lower ratios of vessel tortuosity, which directly relate to these angiogenic conditions. The virus vector was additionally shown to possess anti-inflammatory and antiapoptotic properties. Visualizing the retinal tissues wherein these effects were exerted was done by coimmunostaining anti-GFP with either anti-CD31, anti-GFAP, or anti-NeuN.

Although anti-VEGF therapeutic strategies have been employed thus far, the involvement of additional cellular factors³⁰ and symptoms beyond angiogenesis^{2,27,29} show that neovascular retinal diseases are multifactorial conditions for which currently available treatments may be inadequate to address. Additionally, these treatments require frequent administration on a long-term basis,³ from which other issues may arise, including safety concerns.³¹ For example, anti-VEGF therapy has been shown to accelerate fibrotic responses in PDR patients as a result of changes in the ratio of VEGF to connective tissue growth factor (CTGF) levels.³² Therefore³³ VEGF-independent treatment modalities that comprehensively address the various aspects of pathological angiogenesis may prove to be a more expedient solution to these disorders.²

One proposed therapeutic target is the mTOR.⁶ An essential constituent component of a number of cellular pathways,⁹ mTOR is well-suited to address various aspects of these conditions via the modulation of growth factors, inflammatory processes, and downstream regulators of angiogenesis.²⁷ Among the latter is HIF-1 α , which is mediated by mTORC1 to induce VEGF production³³ and subsequent neovascularization.^{27,34} Another inducer of HIF-1, of which HIF-1 α is a crucial subunit, is retinal hypoxia, a main feature of angiogenic retinal disorders.^{1,4} HIF-1, in turn, regulates a number of downstream factors that also contribute to these conditions,³⁵ including platelet-derived growth factor,⁴ and is absolutely required for the transcription of CTGF mRNA.³⁶ VEGF is also a target gene of HIF-2 α , whose expression is enhanced via the stabilization and activation of mTORC2,³⁷ showing that mTORC1 inhibition alone, although not ineffective, is limited in antiangiogenic activity. HIF-2 α has also been implicated in retinal angiogenesis³⁸ and the interplay among the HIF family of transcription factors,³⁰ mTOR complexes, and various proangiogenic factors, make mTOR a promising therapeutic target.

Until recently, studies involving mTOR inhibition to treat neovascular retinal pathologies have generally employed rapalogs,^{33,34,39,40} which only affect mTORC1. However, these treatments do not affect mTORC2, whose activation of the HIF-2 α pathway is also able to upregulate the expression of SLC7A5, an activator of mTORC1, thereby directly increasing mTORC1, HIF-1 α , and VEGF activity.⁴¹ Another

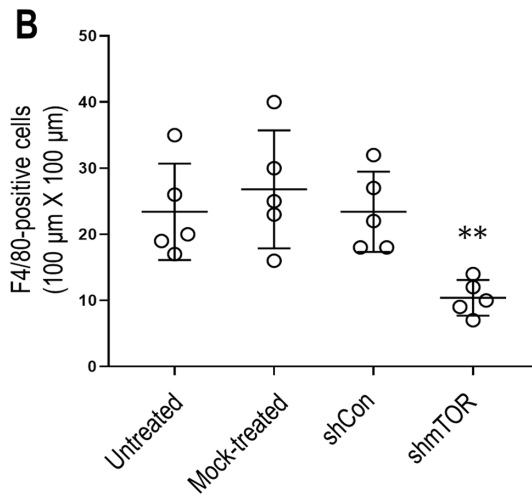
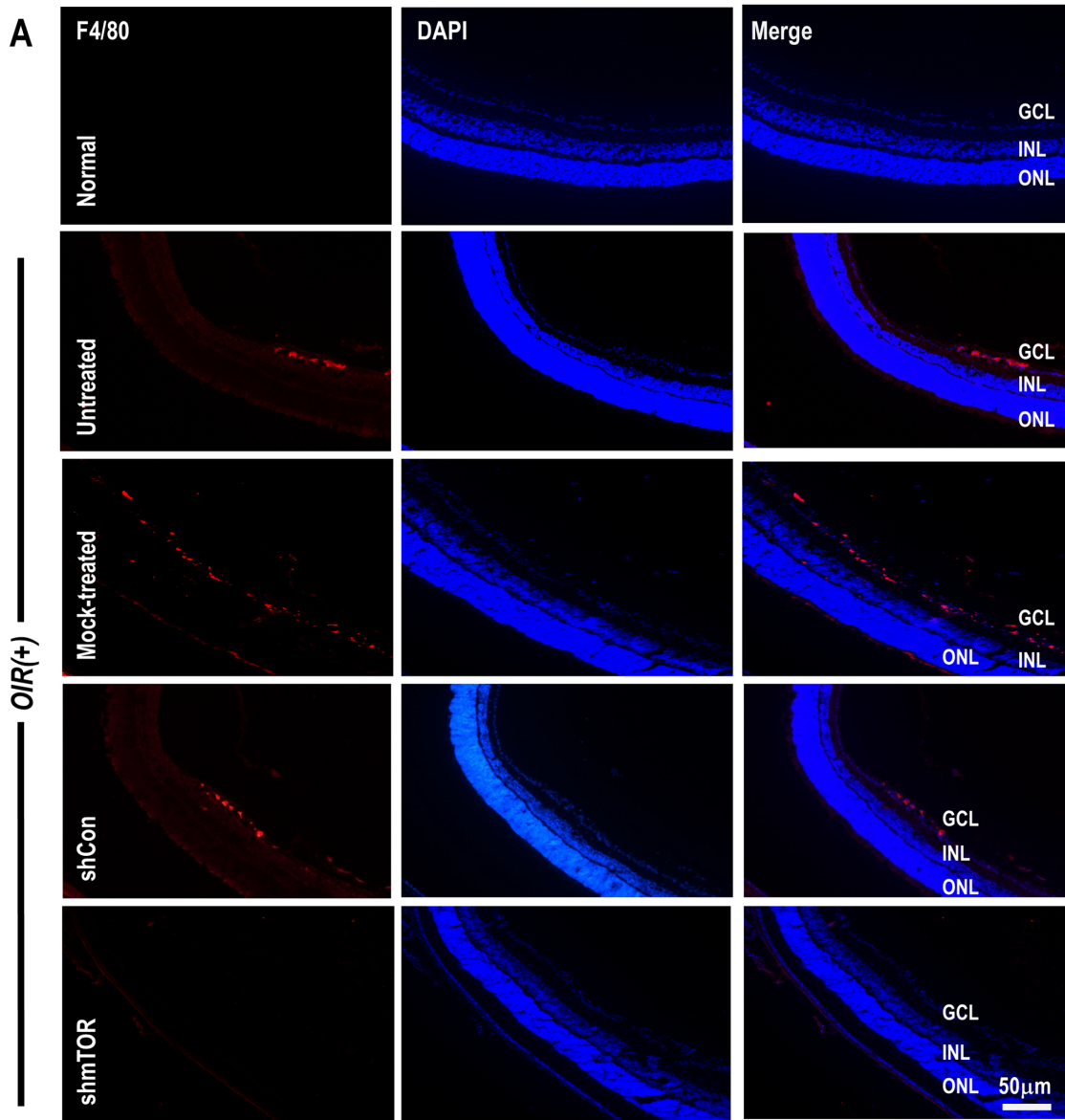


FIGURE 3. Immunostaining with F4/80 to demonstrate extensiveness of macrophage infiltration. Immunohistochemistry using anti-F4/80, which indicates the presence of macrophages, revealed significant inflammatory cell infiltration in the retinas of untreated, mock-treated, and rAAV2-shCon-GFP-treated OIR model groups. Macrophage detection was absent in normal control rats and markedly reduced in those intravitreally injected with rAAV2-shmTOR-GFP (A). Quantitative analysis of the data are visualized as well (B; n = 5). Outer nuclear layer (ONL).

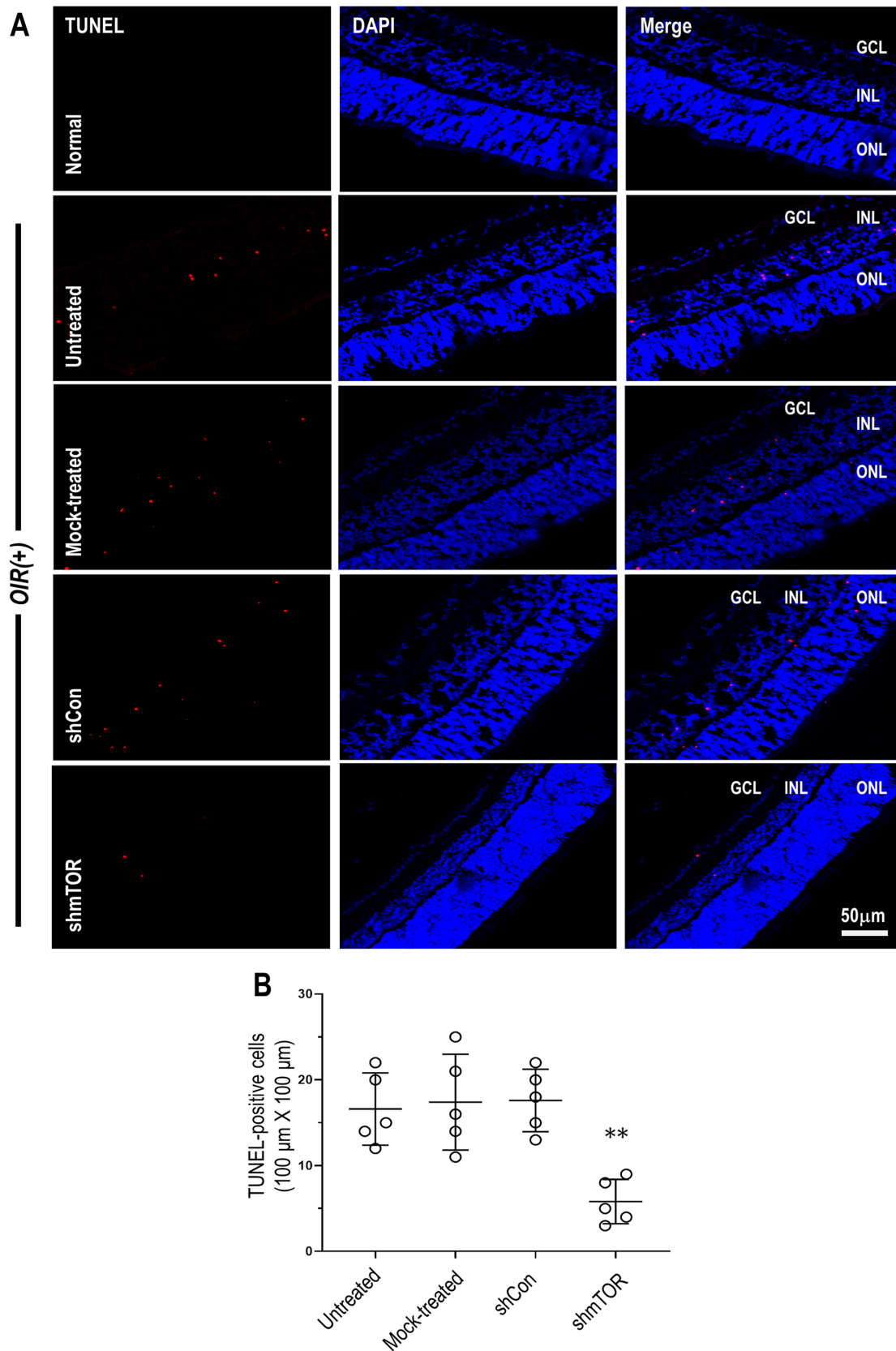


FIGURE 4. Antiapoptotic effect of rAAV2-shmTOR-GFP, as determined by TUNEL assay. TUNEL-positive cells could not be found in the normal control group, which was not exposed to hyperoxic conditions, but were detected in transverse retinal sections taken from rats that were untreated, mock-treated, or injected with rAAV2-shCon-GFP. Significantly fewer apoptotic cells were found in the OIR model group treated with rAAV2-shmTOR-GFP (A), which was quantified (B; n = 5), indicating that mTOR inhibition by RNA interference has antiapoptotic activity.

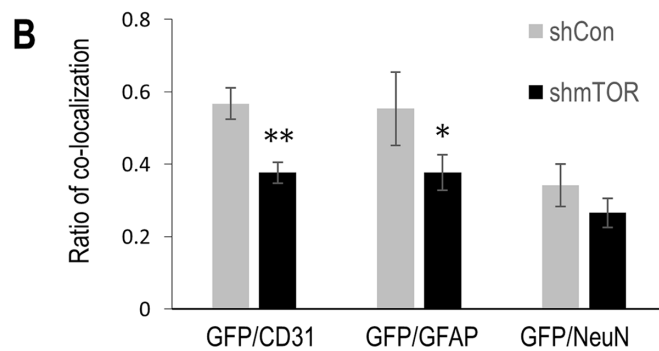
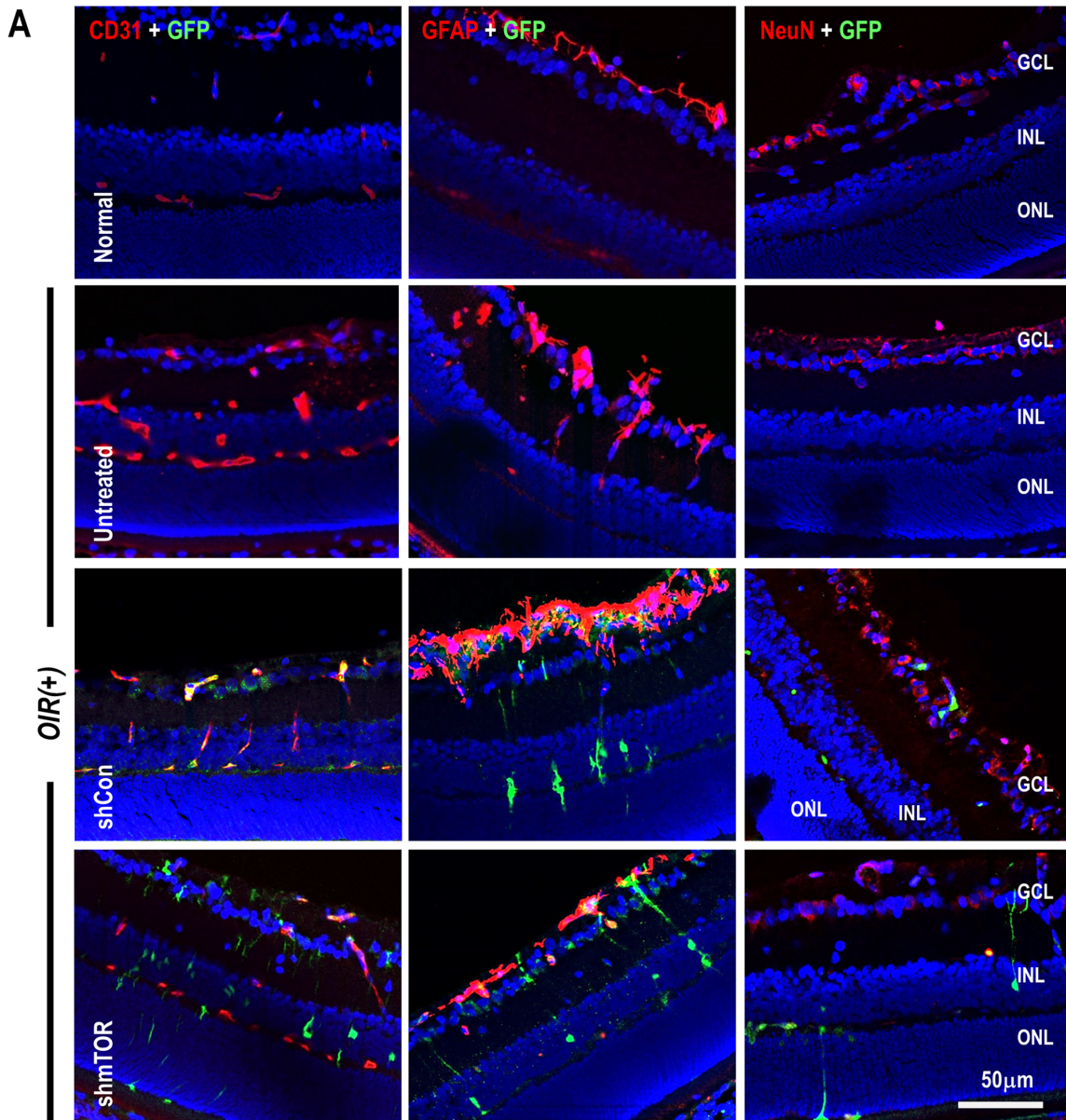


FIGURE 5. Retinal tissue tropism of the rat OIR model. Frozen section samples were stained with anti-GFP along with either anti-NeuN, anti-CD31, or anti-GFAP to determine the presence of the virus vectors in the ganglion cell layer, endothelial cells, or glial cells, including MCs, respectively, of the rat retinas. This is confirmed by an overlap of the green GFP signal with the red of the various cell types, which is visualized as a positive yellow signal (**A**), and the extent to which this overlap occurred was quantified (**B**; n = 5). It was seen that treatment with rAAV2-shmTOR-GFP significantly reduced retinal neovascularization and active MCs, both of which were present in rats administered with rAAV2-shCon-GFP, as well as the untreated group.

mechanism by which mTOR circumvents the inhibition of mTORC1 is through Akt, the major phosphorylation target of mTORC2.¹¹ Rapamycin and its analogues temsirolimus⁴² and everolimus⁴³ successfully inhibited mTORC1 both in vivo and in vitro, but this also led to the activation of Akt, a constituent part of the insulin/PI3K pathway responsible for mTORC1 activation.⁴⁴ Although second-generation mTOR inhibitors have shown their effectiveness in downregulating mTORC1, mTORC2, and even mTORC3 activity,¹⁰ long-term treatment is not able to nullify the eventual reactivation of Akt via the insulin/PI3K pathway.¹¹ Treatment with pp242, a second-generation mTOR inhibitor, significantly reduces both HIF-2 α mRNA levels, as well as VEGF expression,³⁷ and previous studies have suggested the potential of second-generation mTOR inhibitors in treating PDR²⁷ and RoP.³⁰

However, the recent discovery of mTORC3 means it is uncertain if any heretofore uncharacterized cellular processes involving mTOR exist, as well as the effect this complex has on previously well-established mechanisms. As such, direct targeting via RNA interference is the most certain method to ensure the comprehensive inhibition of mTOR. This ability was demonstrated in both a laser-induced mouse model of choroidal neovascularization²⁰ and here by rAAV2-shmTOR-GFP in a rat OIR model. Through this inhibitory activity, the virus vector was also able to exert therapeutically relevant effects with regard to angiogenic retinal disorders. This is owing to the ability of rAAV2-shmTOR-GFP to transduce retinal cell types that are involved in various aspects of these conditions, glial cells in particular, as visualized by coimmunostaining with anti-GFP and anti-GFAP.

The VEGF that drives PDR is mainly derived from MCs, a type of glial cell,²⁹ making it a primary cellular target²⁸ for rAAV2-shmTOR-GFP. This VEGF release results from autophagy dysfunction in MCs, which is a part of the pathogenesis of PDR.²⁹ As an inducer of the autophagy pathway,¹¹ mTOR inhibition may be therapeutically beneficial, and indeed, rapamycin treatment was found to improve autophagy and mitigate VEGF production.²⁹ Improved autophagy function was also suggested to be crucial in protecting against retinal inflammation,⁴⁵ a PDR-related process for which MCs play a major role,²⁸ and a phenotype that was suppressed by mTOR inhibition.²⁷ Inflammation was also shown in mouse OIR models to be involved in neovascular tufting, as the extent to which it occurred was reduced via anti-inflammatory treatments.²⁵ Through the NLRP3 inflammasome, reactive oxygen species (ROS) produce the proinflammatory cytokine IL-1 β , one of the three major cytokines responsible for the inflammatory activity of macrophages. ROS and oxidative stress processes are also involved in the progression of PDR,^{27,33} whereas F4/80 immunostaining for macrophages demonstrated the anti-inflammatory ability of rAAV2-shmTOR-GFP. In all, the therapeutic virus vector was able to address multiple aspects of PDR, an exemplification of neovascular retinal pathologies.

CONCLUSIONS

As neovascular retinal diseases consist of multiple conditions with multiple factors driving their pathophysiologies, any potential solution would involve addressing various aspects of the disorders in a comprehensive manner. As we have shown here, mTOR inhibition via RNA interference exhibits great promise in this respect. In the future, neutralizing

antibody, off-target, and long-term safety and efficacy studies will be performed to further pursue rAAV2-shmTOR-GFP as a human gene therapeutic against angiogenic retinal pathologies, and the growing global health concerns they represent.

Acknowledgments

The authors thank Paula Khim for her proofreading and editing. Author contributions: Conceptualization, HL and JYL; Methodology, HL, JYL, SHSL, HC, JHK, HJK, SC, JSC, HNW, KP, and KJL; Investigation, HL, JYL, SHSL, HC, JHK, HJK, SC, HNW, and KJL; Writing—Original Draft; HL, JYL, SHSL, HC, and KP; Writing—Review and Editing HL, JYL, SHSL, HC, and KP.

Supported by CuroGene Life Sciences Co., Ltd., and by grants from Asan Institute for Life Sciences, Asan Medical Center (2019-287, to H. Lee), and the Ministry of Health & Welfare (HI15C2599, 2016 to JY Lee), Republic of Korea.

Disclosure: **S.H.S. Lee**, None; **H. Chang**, None; **J.H. Kim**, None; **H.J. Kim**, None; **J.S. Choi**, None; **S. Chung**, None; **H.N. Woo**, None; **K.J. Lee**, None; **K. Park**, None; **J.Y. Lee**, None; **H. Lee**, None

References

1. Campochiaro PA. Ocular neovascularization. *J Mol Med (Berl)*. 2013;91:311–321.
2. Duh EJ, Sun JK, Stitt AW. Diabetic retinopathy: current understanding, mechanisms, and treatment strategies. *JCI Insight*. 2017;2:e93751.
3. Cehofski LJ, Honoré B, Vorum H. A review: proteomics in retinal artery occlusion, retinal vein occlusion, diabetic retinopathy and acquired macular disorders. *Int J Mol Sci*. 2017;18:E907.
4. Rubio RG, Adamis AP. Ocular angiogenesis: vascular endothelial growth factor and other factors. *Dev Ophthalmol*. 2016;55:28–37.
5. Stewart MW. Extended duration vascular endothelial growth factor inhibition in the eye: failures, successes, and future possibilities. *Pharmaceutics*. 2018;10:E21.
6. Nakahara T, Morita A, Yagasaki R, Mori A, Sakamoto K. Mammalian target of rapamycin (mTOR) as a potential therapeutic target in pathological ocular angiogenesis. *Biol Pharm Bull*. 2017;40:2045–2049.
7. Nishijima K, Ng YS, Zhong L, et al. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to adaptive injury. *Am J Pathol*. 2007;171:53–67.
8. Demidenko ZN, Blagosklonny MV. The purpose of the HIF-1/PHD feedback loop: to limit mTOR-induced HIF-1 α . *Cell Cycle*. 2011;10:1557–1562.
9. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274–293.
10. Harwood FC, Klein Geltink RI, O'Hara BP, et al. ETV7 is an essential component of rapamycin-insensitive mTOR complex in cancer. *Sci Adv*. 2018;4:eaar3938.
11. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell*. 2017;168:960–976.
12. Liegl R, Koenig S, Siedlecki J, Haritoglou C, Kampik A, Kernt M. Temsirolimus inhibits proliferation and migration in retinal pigment epithelial and endothelial cells via mTOR inhibition and decreases VEGF and PDGF expression. *PLoS One*. 2014;9:e88203.
13. Stahl A, Paschek L, Martin G, et al. Rapamycin reduces VEGF expression in retinal pigment epithelium (RPE) and

- inhibits RPE-induced sprouting angiogenesis in vitro. *FEBS Lett.* 2008;582:3097–3102.
14. Dejneka NS, Kuroki AM, Fosnot J, Tang W, Tolentino MJ, Bennett J. Systemic rapamycin inhibits retinal and choroidal neovascularization in mice. *Mol Vis.* 2004;10:964–972.
 15. Brugarolas JB, Vazquez F, Reddy A, Sellers WR, Kaelin WG, Jr. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell.* 2003;4:147–158.
 16. Lee HS, Ahn J, Jun EJ, et al. A novel program to design siRNAs simultaneously effective to highly variable virus genomes. *Biochem Biophys Res Commun.* 2009;384:431–435.
 17. Ahn J, Woo HN, Ko A, et al. Multispecies-compatible antitumor effects of a cross-species small-interfering RNA against mammalian target of rapamycin. *Cell Mol Life Sci.* 2012;69:3147–3158.
 18. Park K, Kim WJ, Cho YH, et al. Cancer gene therapy using adeno-associated virus vectors. *Front Biosci.* 2008;13:2653–2659.
 19. Ameri H. Prospect of retinal gene therapy following commercialization of voretigene neparvovec-rzyl for retinal dystrophy mediated by RPE65 mutation. *J Curr Ophthalmol.* 2018;30:1–2.
 20. Park TK, Lee SH, Choi JS, et al. Adeno-associated viral vector-mediated mTOR inhibition by short hairpin RNA suppresses laser-induced choroidal neovascularization. *Mol Ther Nucleic Acids.* 2017;8:26–35.
 21. Smith LEH, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101–111.
 22. Liu CH, Wang Z, Sun Y, Chen J. Animal models of ocular angiogenesis: from development to pathologies. *FASEB J.* 2017;31:4665–4681.
 23. Gao G, Li Y, Fant J, Crosson CE, Becerra SP, Ma JX. Difference in ischemic regulation of vascular endothelial growth factor and pigment epithelium-derived factor in Brown Norway and Sprague Dawley rats contributing to different susceptibilities to retinal neovascularization. *Diabetes.* 2002;51:1218–1225.
 24. Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc.* 2009;4:1565–1573.
 25. Scott A, Fruttiger M. Oxygen-induced retinopathy: a model for vascular pathology in the retina. *Eye (Lond).* 2010;24:416–421.
 26. Stahl A, Connor KM, Sapiha P, et al. The mouse retina as an angiogenesis model. *Invest Ophthalmol Vis Sci.* 2010;51:2813–2826.
 27. Jacot JL, Sherris D. Potential therapeutic roles for inhibition of the PI3K/Akt/mTOR pathway in the pathophysiology of diabetic retinopathy. *J Ophthalmol.* 2011;2011:e589813.
 28. Wang J, Xu X, Elliott MH, Zhu M, Le YZ. Müller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage. *Diabetes.* 2010;59:2297–2305.
 29. Lopes de Faria JM, Duarte DA, Montemurro C, Papadimitriou A, Consonni SR, Lopes de Faria JB. Defective autophagy in diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2016;57:4356–4366.
 30. Rivera JC, Holm M, Austeng D, et al. Retinopathy of prematurity: inflammation, choroidal degeneration, and novel promising therapeutic strategies. *J Neuroinflammation.* 2017;14:e165.
 31. Lim LS, Cheung CMG, Mitchell P, Wong TY. Emerging evidence concerning systemic safety of anti-VEGF agents—should ophthalmologists be concerned? *Am J Ophthalmol.* 2011;152:329–331.
 32. Van Geest RJ, Lesnik-Oberstein SY, Tan HS, et al. A shift in the balance of vascular endothelial growth factor and connective tissue growth factor by bevacizumab causes the angiofibrotic switch in proliferative diabetic retinopathy. *Br J Ophthalmol.* 2012;96:587–590.
 33. Heng LZ, Comyn O, Peto T, et al. Diabetic retinopathy: pathogenesis, clinical grading, management and future developments. *Diabet Med.* 2013;30:640–650.
 34. Wei J, Jiang H, Gao H, Wang G. Blocking mammalian target of rapamycin (mTOR) attenuates HIF-1 α pathways engaged-vascular endothelial growth factor (VEGF) in diabetic retinopathy. *Cell Physiol Biochem.* 2016;40:1570–1577.
 35. Dong A, Seidel C, Snell D, et al. Antagonism of PDGF-BB suppresses subretinal neovascularization and enhances the effects of blocking VEGF-A. *Angiogenesis.* 2014;17:553–562.
 36. Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH. Hypoxic induction of *Ctgf* is directly mediated by Hif-1. *Am J Physiol Renal Physiol.* 2004;287:F1223–F1232.
 37. Nayak BK, Feliers D, Sudarshan S, et al. Stabilization of HIF-2 α through redox regulation of mTORC2 activation and initiation of mRNA translation. *Oncogene.* 2013;32:3147–3155.
 38. Nakajima T, Nakajima E, Shearer TR, Azuma M. Concerted inhibition of HIF-1 α and -2 α expression markedly suppresses angiogenesis in cultured RPE cells. *Mol Cell Biochem.* 2013;383:113–122.
 39. Yagasaki R, Nakahara T, Ushikubo H, Mori A, Sakamoto K, Ishii K. Anti-angiogenic effects of mammalian target of rapamycin inhibitors in a mouse model of oxygen-induced retinopathy. *Biol Pharm Bull.* 2014;37:1838–1842.
 40. Dugel PU, Blumenkranz MS, Haller JA, et al. A randomized, dose-escalation study of subconjunctival and intravitreal injections of sirolimus in patients with diabetic macular edema. *Ophthalmology.* 2012;119:124–131.
 41. Elorza A, Soro-Arnáiz I, Meléndez-Rodríguez F, et al. HIF2 α acts as an mTORC1 activator through the amino acid carrier SLC7A5. *Mol Cell.* 2012;48:681–691.
 42. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene.* 2007;26:1932–1940.
 43. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 2006;66:1500–1508.
 44. Feldman ME, Apsel B, Uotila A, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol.* 2009;7:e38.
 45. Lin W, Xu G. Autophagy: a role in the apoptosis, survival, inflammation, and development of the retina. *Ophthalmic Res.* 2019;61:65–72.