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이학박사 학위논문

mTOR 발현 저해를 통한 당뇨망막병증 치료 가능성 연구

Inhibiting the mechanistic target of rapamycin (mTOR) via RNA interference as a novel human gene therapeutic strategy for the treatment of diabetic retinopathy

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2021 년　8 월

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2021 년 8 월

Abstract

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Purpose: The standard of care for diabetic retinopathy (DR) has heretofore consisted of laser photocoagulation and anti-VEGF therapeutics. However, drawbacks exist for both forms of treatment, with the short durations of activity for the latter being of particular import as they necessitate frequent administration via intravitreal injections. The treatments are additionally narrowly focused in scope and may be limited in their abilities in comprehensively addressing the multifactorial nature of DR. Due to the complex pathophysiology of DR, a therapeutic target with broad efficacy, specifically the mechanistic target of rapamycin (mTOR), may prove to be beneficial in helping to address these concerns. Furthermore, the utilization of gene therapy as a treatment strategy would provide long-lasting effect in a patient-friendly manner, thereby overcome the most significant limitation of currently-used therapeutics.

Methods: A single high-dose of streptozotocin (STZ) was administered to 7-week old male C57/BL6 mice via intraperitoneal injection to generate a diabetic animal model, with intravitreal injection of the control and therapeutic virus vectors, the latter consisting of a mTOR-inhibiting shRNA packaged in a recombinant adeno-associated virus (rAAV2-shmTOR-SD), occurring one month later. Assays performed on mouse retinas isolated post-sacrifice two months thereafter include trypsin digest preparations to examine the retinal

vasculature, FITC-dextran straining, TUNEL assay, and immunohistochemistry to determine virus vector tropism. Additionally, H&E staining was performed on retinal samples taken from mice sacrificed six months after STZ administration to visualize the cell layers of the retina.

Results: Previously, it was demonstrated in a rat model of oxygen-induced retinopathy that the therapeutic virus vector is able to address the vascular aspect of DR via its anti-angiogenic activity, whereas rAAV2-shmTOR-SD was shown here to exert a therapeutic effect upon early DR processes. Intravitreally injected rAAV2-shmTOR-SD effectively transduced the mouse retinas and markedly downregulated mTOR expression, as compared to sham-treated and control virus vector-injected (rAAV2-shCon-SD) control groups. This lead to significant reductions in pericyte loss, a consequential early histopathological characteristic of DR, while also reducing acellular capillary formation, vascular permeability, and the thinning of retinal cell layers, with all of these processes being key contributors to DR progression. rAAV2-shmTOR-SD also reduced the pathogenic activation and proliferation of Müller cells while being anti-apoptotic and decreasing retinal ganglion cell loss, with this data combining to suggesting that the therapeutic virus vector possesses neuroprotective qualities as well.

Conclusions: Taken together, these results continue to demonstrate the great promise of rAAV2-shmTOR-SD as a potential human gene therapeutic versus DR due to its broad ability to address various aspects of DR pathophysiology.

Keywords

Diabetic retinopathy, gene therapy, recombinant adeno-associated virus, mechanistic target of rapamycin, RNA interference

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Introduction

A frequently occurring complication of diabetes mellitus (DM), diabetic retinopathy (DR) is currently among the leading causes of blindness worldwide and a looming threat to develop into a global health crisis in the near future. This is due to the predicted development of DR to levels that would threaten the sight of one quarter[1] of the 592 million DM patients projected by 2035.[2] Presenting initially in a non-proliferative form (NPDR), DR is a degenerative condition that later progresses to proliferative diabetic retinopathy (PDR). PDR is the form of the disease most directly linked to vision loss and is characterized by neovascularization (NV), with vascular endothelial growth factor (VEGF) being the major driver behind this process.[3] VEGF is also closely associated with diabetic macular edema (DME), a thickening or swelling of the macula[4] that can develop during either form of DR.[2] DME is itself a complication of DR, as well as being the most prevalent cause of blindness related to DR.[2-4]

With the involvement of VEGF as a central factor in both PDR and DME, which are most directly linked with threatening DR patients' vision, anti-VEGF strategies are unsurprisingly employed to treat both conditions.[2] The standard of care additionally includes the use of laser photocoagulation,[5] although questions have been raised regarding its safety and efficacy. These include the development of processes that contribute to the progression of DR and its

pathophysiology. Namely, vascular leakage,[6] newly arising choroidal NV, and the development of subretinal fibrosis.[7] Contrastingly, the major limitation of currently-used anti-VEGF therapeutics delivered intravitreally is inherent to the treatments themselves. Being protein-based, they are susceptible to degradation and have relatively short durations of therapeutic efficacy. Therefore, sustained VEGF suppression is only achievable via frequent intravitreal administration,[2,5] which may be difficult for the patient in both the economic and procedural senses. This has been shown to reduce patient compliance,[8] and therefore, patient outcomes, as DR is a condition that is both degenerative[5] and progressive.[3] Questions regarding the safety of suppressing VEGF on a long-term basis have also been raised,[9,10] as well as the potential for adverse effects due to injection frequency.[5] Furthermore, a large proportion of patients has been shown to be unresponsive to currently-used anti-VEGF treatments.[3,4]

An additional limitation of these therapeutics is that they focus on a single target, despite DR being a highly multifactorial condition.[3,5] This can be seen in the variety of factors that are involved in its pathoprogression, which include higher-level processes such as inflammation, oxidative stress, hyperglycemia resulting from DM, and subsequent effects due to hypoxia upon retinal vessels. These processes are also highly involved in the progression of DME, angiogenic activity[11] and Müller cell activation in particular.[12] As such, a treatment strategy focusing on a therapeutic target with comprehensive efficacy in addressing the many aspects

of DR may prove to be a beneficial solution for overcoming the limitations of currently-used therapies. One such potential target is the mechanistic target of rapamycin (mTOR), a serine/threonine kinase. Due to its involvement in various signaling pathways and cellular processes,[13] it may be possible to address a number of aspects of DR by targeting mTOR. This approach has been identified as a potential therapeutic strategy for various angiogenic retinal disorders,[10] PDR among them,[14] whereas mTOR dysfunction has been linked to a number of ocular conditions.[15]

With current DR treatments having been shown to be limited in target and scope, the potential use of a gene therapeutic strategy via mTOR inhibition may prove to be advantageous in many ways, especially in its ability for long-term efficacy without frequent intravitreal injections. To this end, the utilization of RNA interference (RNAi) via a novel short hairpin RNA (shRNA) that directly inhibits mTOR was explored for its therapeutic efficacy. The shRNA, which has multi-species activity, was developed using CAPSID (Convenient Application Program for siRNA Design), a program created in-house.[16] The therapeutic virus vector was then generated by packaging the shRNA in a recombinant adeno-associated virus vector (rAAV),[17] a well-established gene therapeutic delivery vector as a result of its non-pathogenicity, long-term expression of the encoded transgene, and its ability to transduce both non-dividing and dividing cells.[18]

Following the overall developmental trajectory of anti-VEGF drugs used to treat angiogenic ocular conditions,[8] the mTOR-inhibiting shRNA was then examined using a laser-induced mouse model of choroidal NV within the context of the wet subtype of age-related macular degeneration (wAMD).[19] This was followed by demonstrating the efficacy of this RNAi-based treatment as a potential gene therapeutic strategy for a number of angiogenic retinal conditions, including DR and retinopathy of prematurity, in a rat model of oxygen-induced retinopathy (OIR).[20] Up to this point, the therapeutic virus vector contained a GFP reporter transgene to enhance its utility as a research vector. However, due to the immunogenicity and cytotoxic effects elicited by GFP,[21] its inclusion would have been inappropriate for a human gene therapeutic. As such, the GFP expression cassette was replaced with a stuffer DNA shown to have no expression[22] to generate the current therapeutic virus vector, rAAV2-shmTOR-SD. rAAV2-shmTOR-SD was then compared to the construct containing GFP, rAAV2-shmTOR-GFP, in a laser-induced mouse model of choroidal NV, where it was shown to be comparable in its in vivo efficacies.[23]

In order to further explore the therapeutic potential of rAAV2-shmTOR-SD versus DR, it was tested here in a STZ-induced diabetic mouse model, which recapitulates early aspects of the condition.[24] Administered via intravitreal injection, rAAV2-shmTOR-SD was able to effectively transduce the mouse retinas and reduce mTOR expression therein. The therapeutic virus vector was additionally shown to significantly reduce the occurrence of a

number of early DR processes, including pericyte loss, acellular capillary formation, the development of leaky vessels, and retinal cell layer thinning, all of which are consequential in the progression of the disease. rAAV2-shmTOR-SD was also noted for its activity in a number of retinal cell types linked to DR pathophysiology, as well as its anti-apoptotic activity. Combined with previous explorations of the mTOR-inhibiting construct,[19,20,23] the results here further demonstrate the promise of rAAV2-shmTOR-SD as a potential human gene therapeutic versus a number of angiogenic ocular conditions, including DR.

Materials and Methods

Experimental virus vector development

The generation of rAAV2-shmTOR-SD and rAAV2-shCon-SD[23] from pAAV-shmTOR-SD and pAAV-shCon-SD, their respective precursor plasmids,[19] was previously described. Briefly, the mTOR-inhibiting shRNA (5'-GAAUGUUGACCAAUGCUAU-3') or control shRNA (5'-AUUCUAUCACUAGCGUGAC-3') was inserted alongside a stuffer DNA derived from the human *UBE3A* gene into a self-complementary AAV2 vector under the control of an H1 promoter. All virus vectors used in this study were provided by CdmoGen Co., Ltd. (Cheongju, Korea).

STZ-induced diabetic mouse model and animal care

The well-established protocol set forth by Furman[25] for inducing diabetes mellitus in mice using a single high-dose of streptozotocin was followed, with minor modifications, with 7-week-old male C57/BL6 mice sourced from The Orient Bio (Sungnam, Korea). All animal care and experiments were overseen by the Internal Review Board for Animal Experiments at the Asan Institute for Life Sciences (University of Ulsan, College of Medicine) and performed according to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research. Mice were intraperitoneally injected with 150 mg/kg of streptozotocin (Sigma-Aldrich, St. Louis, MO) rather than the 200 mg/kg specified to mitigate the potential for deaths as a result of STZ-induced toxicity. Additionally, 10% sucrose water was only provided to mice whose condition was shown to deteriorate significantly, as determined via reductions in body weight. Successfully generated diabetic mouse models were selected using Accu-Chek (Roche Diagnostics, Indianapolis, IN) for those animals whose tail vein blood samples had

blood glucose levels exceeding 300 mg/dL at 1 week post-STZ treatment.

Intravitreal injections and sacrifice

1 month after the administration of STZ, an intraperitoneally administered 4:1 mixture of 40 mg/kg Zoletil (zolazepam/tiletamine) obtained from Virbac (Carros Cedex, France) and 5 mg/kg of Rompun (xylazine) sourced from Bayer Healthcare (Leverkusen, Germany) was used to anesthetize the mice, and their pupils were dilated using Mydrin-P (0.5% tropicamide and 2.5% phenylephrine) from Santen (Osaka, Japan). Both eyes were intravitreally injected with 1 μL of the virus vectors at a concentration of 5.0 x 10^{10} viral genomes (v.g.)/mL. Sacrifice was performed at either 2 or 5 months post-intravitreal injection, wherein the mice were deeply anesthetized using a 4:1 mixture of Zoletil (80 mg/kg) and Rompun (10 mg/kg) prior to intracardial perfusion with 0.1 M PBS (7.4 pH) containing 150 U/mL heparin and infusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Eyecups were then generated by enucleating the eyeballs, which were fixed for 1 hour in 4% PBA in 0.1 M PB and the anterior sections removed, including the cornea and lens.

Tissue preparation and immunohistochemistry

The eyecups were then placed overnight in 30% sucrose in PBS, after which they were embedded in Tissue-Tek (Miles Scientific, Napierville, IL), an optimal cutting temperature compound, and 5-10 μm-thick frozen transverse retinal sections prepared. The in vivo efficacy of the mTOR-inhibiting shRNA was visualized by staining the section samples with anti-mTOR (AF15371; R&D Systems, Minneapolis, MN). Tissue tropism of the virus vector was determined via anti-NeuN (MAB377; Millipore, Burlington, MA), anti-GFAP (12389; Cell Signaling Technology, Danvers, MA), or anti-GS (MAB302; Millipore) staining. The samples were incubated overnight with the diluted primary antibodies at 4°C, washed in PBST 3 times for 10 minutes apiece, incubated with the secondary antibodies Alexa Fluor

568 or 488 (Thermo Fisher Scientific, Waltham, MA) for 2 hours at room temperature, and then stained with DAPI (D9542; Sigma-Aldrich) to visualize cell nuclei. A LSM 710 fluorescence confocal microscope (Carl Zeiss Microscopy, Jena, Germany) was used to examine the section samples. Images were captured using the black edition of Zeiss Zen software (Carl Zeiss Microscopy), which were then analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

Retinal trypsin digest and FITC-dextran staining

After fixing the enucleated eyeballs for 24 hours in a 10% formalin solution, they were washed in PBS and the retina isolated. Trypsin digestion was then performed in general accordance with a protocol set forth by Chou et al.[26] The retina was then incubated in a 3% trypsin solution (15090046; Thermo Fisher Scientific) in 0.1 M Tris buffer (pH 7.8) with gentle shaking at 37°C for 1 hour. After washing with water, the retina was stained using conventional hematoxylin and eosin (H&E) methods, and examined via light microscopy. To visualize the occurrence of vascular leakage, 50 mg/mL of FITC-dextran (FD2000S; Sigma-Aldrich) was administered to mice via tail vein injection 30 minutes prior to sacrifice, after which the enucleated eyeballs were fixed in 10% formalin solution for 1 hour. After washing with PBS, the retina was isolated from the RPE-choroid complex and flat mounts generated using four equidistant cuts for observation via fluorescence microscopy (Eclipse Ti-U; Nikon, Tokyo, Japan).

Determination of retinal cell layer thinning and TUNEL assay

H&E staining was performed on frozen transverse sections, including the optic nerve head, and ImageJ used to visualize the retinal cell layers. Changes to the inner retina from the nerve fiber layer to the inner nuclear layer were calculated to determine the extent to which retinal cell layer thinning occurred and expressed as a ratio. The formula defining this ratio is

as follows: [mean(ratio of inner retina) = (thickness from nerve fiber layer to inner nuclear layer) / (thickness from nerve fiber layer to outer nuclear layer)]. TUNEL assay was performed in accordance with the manufacturer's protocol (12156792910; Roche Diagnostics), after which the frozen sections were washed in PBST 3 times for 10 minutes apiece and then stained with DAPI.

Statistical analysis

Statistical analysis was performed using one-way ANOVA testing, with significant difference determined at *: $p < 0.05$; **: $p < 0.01$; or ***: $p < 0.001$. The data were visualized using dot plot graphs, which include significance and mean standard error of mean values.

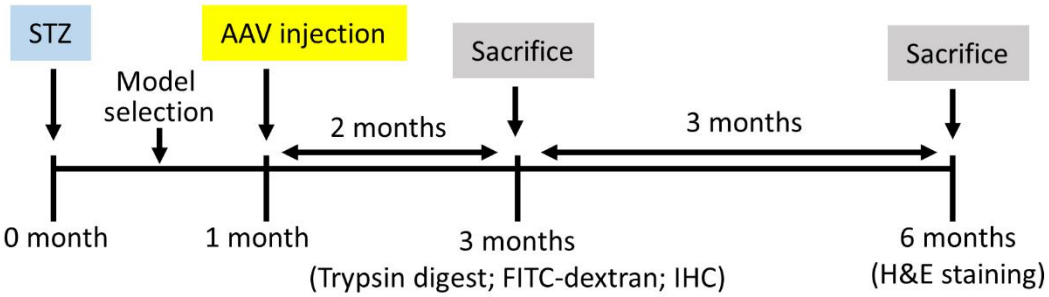

Figure 1. Experimental schematic.

Figure 1. Experimental schematic.

A single high dose of STZ was administered to C57/BL6 mice to generate the diabetic mouse model, the establishment of which was confirmed after 1 month of selection. The mice were then injected with the therapeutic virus vector or a control virus vector before being sacrificed at 2 or 5 months post-injection, dependent upon the assay to be performed upon the retinal samples isolated from the mice.

Results

In vivo efficacy of rAAV2-shmTOR-SD

After confirming that the diabetic mouse model was successfully generated (Fig. 1), the in vivo efficacy of the therapeutic virus vector was determined by comparing its ability to inhibit mTOR versus the sham-treated STZ-induced model group, mice injected with rAAV2-shCon-SD, and a normal control group that had not been treated with STZ (Fig. 2A). Relative to the latter group, mTOR expression was shown to be significantly upregulated in the sham- (3.898 ± 0.209) and control virus vector-treated (3.510 ± 0.385) groups via anti-mTOR immunostaining performed on frozen transverse retinal section samples prepared 2 months after intravitreal injection. On the other hand, the administration of rAAV2-shmTOR-SD (1.146 ± 0.197; $p < 0.001$) led to mTOR levels that were similar to normal control mice (1.000 ± 0.114) (Fig. 2B). As such, the ability of the therapeutic virus vector to transduce mouse retinas was demonstrated, as well as its capacity to inhibit mTOR in vivo.

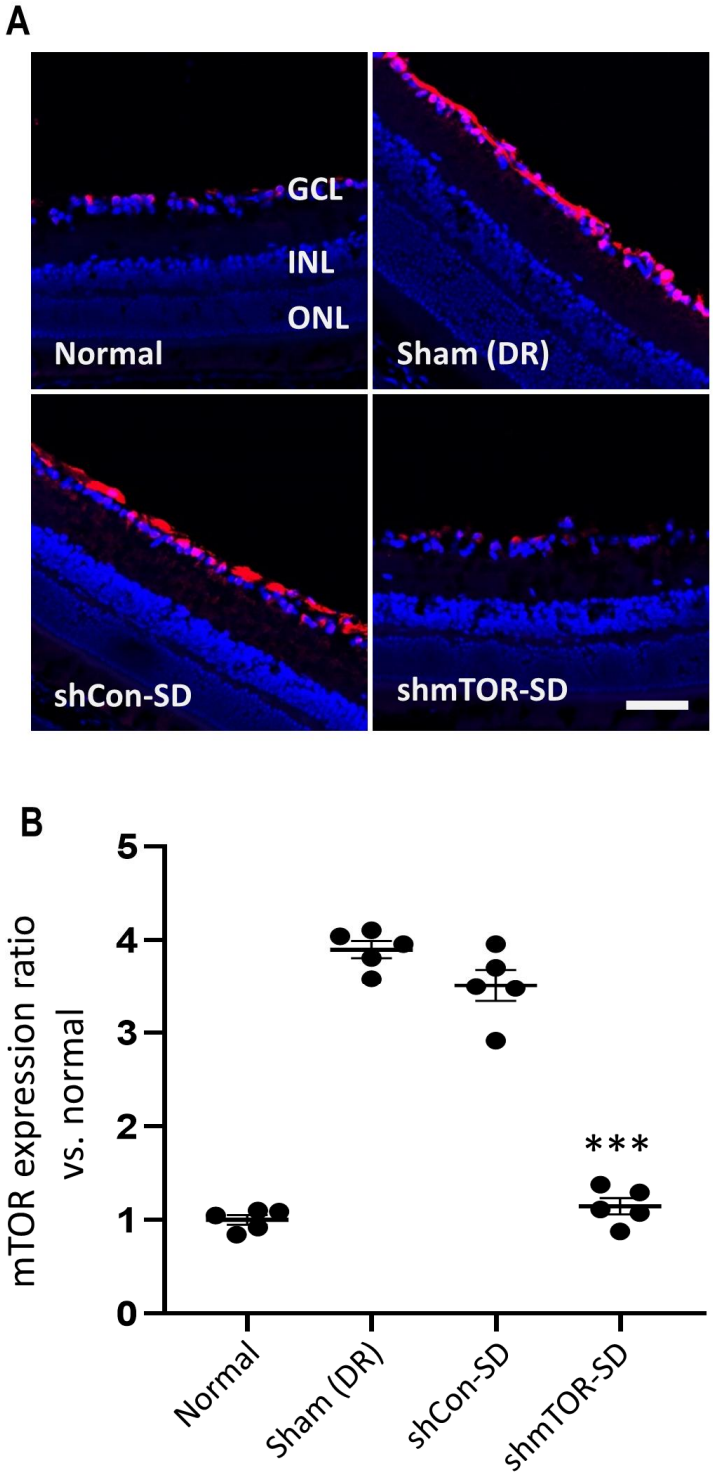

Figure 2. Characterization of rAAV2-shmTOR-SD and retinal transduction of the STZ mouse model.

Figure 2. Characterization of rAAV2-shmTOR-SD and retinal transduction of the STZ mouse model.

Immunohistochemistry performed on transverse retinal sections sampled two months post-intravitreal injection showed that the therapeutic virus vector is able to effect the long-term inhibition of mTOR, which was elevated in the diabetic mouse model and those injected with rAAV2-shCon-SD. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μm (A). Quantification of mTOR expression levels, relative to normal mice. $p < 0.001$. Data represented as mean ± SEM (n = 5) (B).

Retinal pericyte loss and acellular capillary formation are reduced by rAAV2-shmTOR-SD

The loss of pericytes (Fig. 3, white arrowheads) is among the earliest manifestations of DR pathophysiology[4] and was shown to readily occur in the sham-treated control group (0.320 ± 0.067) and mice injected with rAAV2-shCon-SD (0.280 ± 0.045). Meanwhile, the administration of the therapeutic virus vector (0.750 ± 0.072; $p < 0.001$) significantly reduced the incidence of this key histopathological marker of DR,[27] with the determination occurring 3 months after the establishment of the STZ-induced diabetic mouse model and 2 months post-intravitreal administration. Finally, the quantitation of the data (Fig. 4A) was performed relative to normal control mice (1.000 ± 0.082).

Due to the loss of pericytes, retinal non-perfusion may occur,[4,12] which can be visualized in diabetic animal models as acellular capillary development using trypsin digest preparations.[12] Acellular capillaries (Fig. 3, black arrows) were readily observed in sham- (10.600 ± 2.302) and control virus vector-treated (11.200 ± 2.588) mice, but almost wholly absent in the normal control group (0.600 ± 0.548). Meanwhile, rAAV2-shmTOR-SD administration (2.600 ± 1.140; $p < 0.001$) led to a significant reduction (Fig. 4B) in acellular capillary formation.

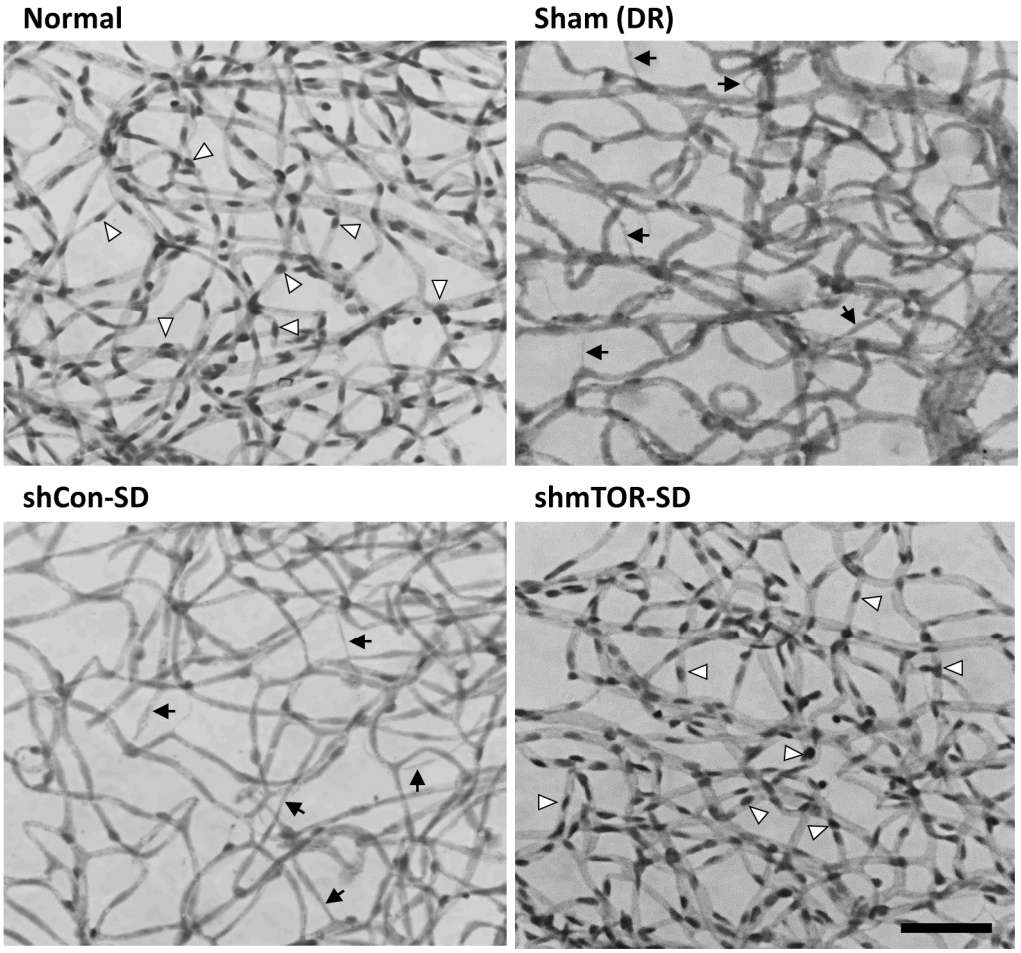

Figure 3. rAAV2-shmTOR-SD reduces pericyte loss and the formation of acellular capillaries.

Figure 3. rAAV2-shmTOR-SD reduces pericyte loss and the formation of acellular capillaries

rAAV2-shmTOR-SD administration was shown to reduce pericyte loss, visualized here using white arrowheads, in the diabetic retina. This activity corresponded to a reduction of the formation of acellular capillaries, which are indicated by black arrows, with these two processes related to DR-related vasodegeneration. An influential key early characteristic of DR, pericyte loss was not observed in normal mice but readily detectable in mice treated with rAAV2-shCon-SD and sham-treated control mice, which also held true for observed acellular capillary formation. Scale bar = 50 μm.

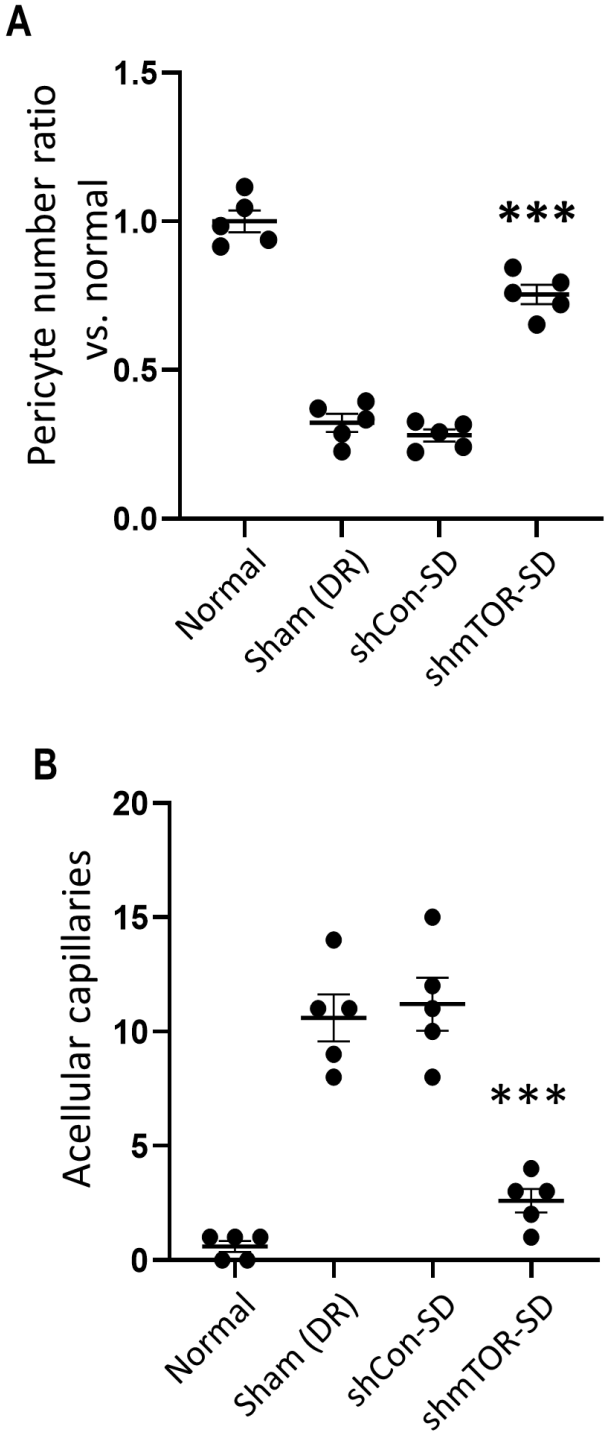

Figure 4. Quantification of pericyte loss and acellular capillary formation.

Figure 4. Quantification of pericyte loss and acellular capillary formation.

Quantified relative to the normal control group, rAAV2-shmTOR-SD administration protected against the loss of pericytes compared to the sham- and control virus vector-treated mice. $p < 0.001$. Data represented as mean ± SEM (n = 5) (A). On the other hand, acellular capillaries were generally absent in mice treated with the therapeutic virus vector and the normal control group, while being readily observed in the STZ-induced diabetic model and mice injected with rAAV2-shCon-SD. $p < 0.001$. Data represented as mean ± SEM (n = 5) (B).

rAAV2-shmTOR-SD reduces retinal vessel leakage

As a result of the non-perfusion of retinal vessels, vasodegeneration may occur,[12,28] as well as the subsequent development of vascular leakage, a process directly linked to DR-associated blindness and a hallmark of the disease. This was visualized (Fig. 5) in retinal flat mounts via FITC-dextran staining. Quantified (Fig. 6) relative to normal control mice (1.000 ± 0.142), it was demonstrated that treatment with rAAV2-shmTOR-SD (1.143 ± 0.096; $p < 0.001$) resulted in a marked suppression of vascular permeability to levels similar to mice that had not been treated with STZ. This was in contrast to sham- (1.928 ± 0.252) and control virus vector-treated (1.974 ± 0.165) mice, in whose retinas vascular leakage, a process that contributes significantly to DR pathophysiology, was observed at elevated levels.

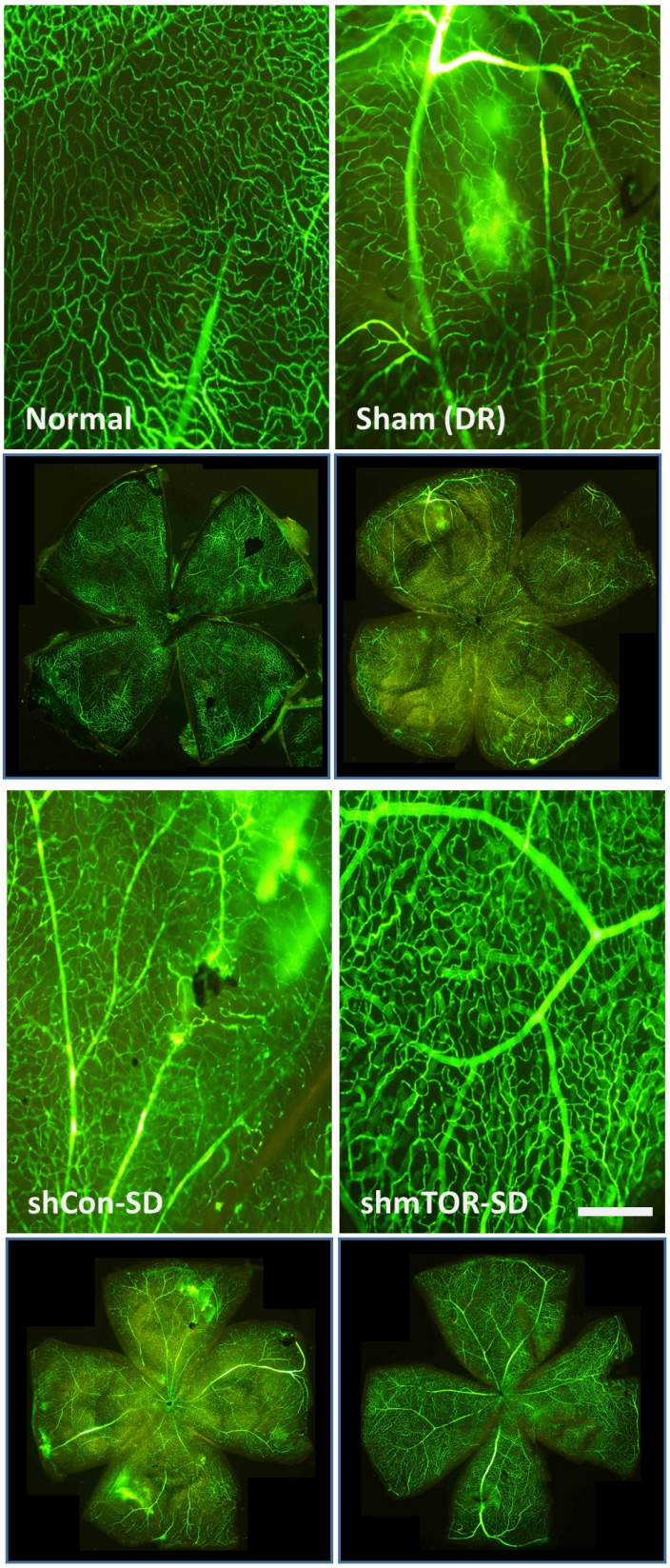

Figure 5. Visualization of vascular leakage via FITC-dextran staining.

Figure 5. Visualization of vascular leakage via FITC-dextran staining.

Retinal vessel leakage, a process directly associated with blindness due to DR, is visualized here as indistinct regions of blurred staining in both the flat mounts and the magnified images. Vascular leakage was found to occur throughout the retinas of sham-treated mice, as well as those administered with the virus vector containing a control shRNA, whereas rAAV2-shmTOR-SD resulted in a marked decrease in vascular permeability. Scale bar = 50 μm.

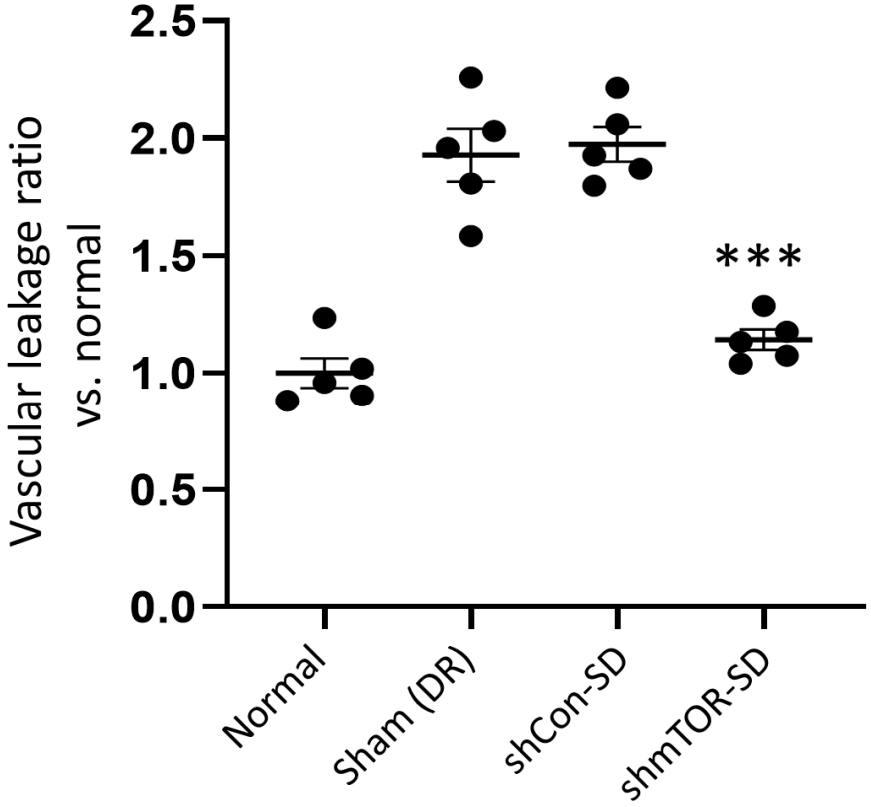

Figure 6. Vessel leakage quantification.

Figure 6. Vessel leakage quantification.

Vascular leakage levels were calculated by randomly sampling 5 areas of the retinal flat mounts and normalizing the quantifications relative to the normal control group, with the data revealing a marked lack of leaky vessel development in mice administered with the therapeutic virus vector. $p < 0.001$. Data represented as mean ± SEM ($n = 5$).

rAAV2-shmTOR-SD protects against retinal cell layer thinning

Due to the multifactorial nature of DR,[3] a variety of neuronal and vascular cells in the retina,[5] as well as pericytes, are involved in the development and progression of the condition. Among these are ganglion cells, glial cells, and endothelial cells, which combine with pericytes, among others, to form the neurovascular unit.[2,3] Neurodegeneration of the NVU, occurring via an increase in apoptotic activity, is in turn considered to a major aspect of DR pathology[29] and an important contributor to its pathoprogression. This process is exemplified in the significant thinning of the retinal cell layers observed (Fig. 7) in the STZ-induced diabetic mouse model (0.659 ± 0.038) and animals injected with the virus vector containing the control shRNA (0.642 ± 0.042). Specifically affected areas included the ganglion cell layer, which is particularly susceptible to neurodegeneration,[30] and the inner nuclear layer. In contrast, administration of the therapeutic virus vector (0.920 ± 0.074; $p < 0.001$) led to a marked reduction of retinal cell layer thinning, with all of the data quantified (Fig. 8) relative to normal control mice (1.000 ± 0.089).

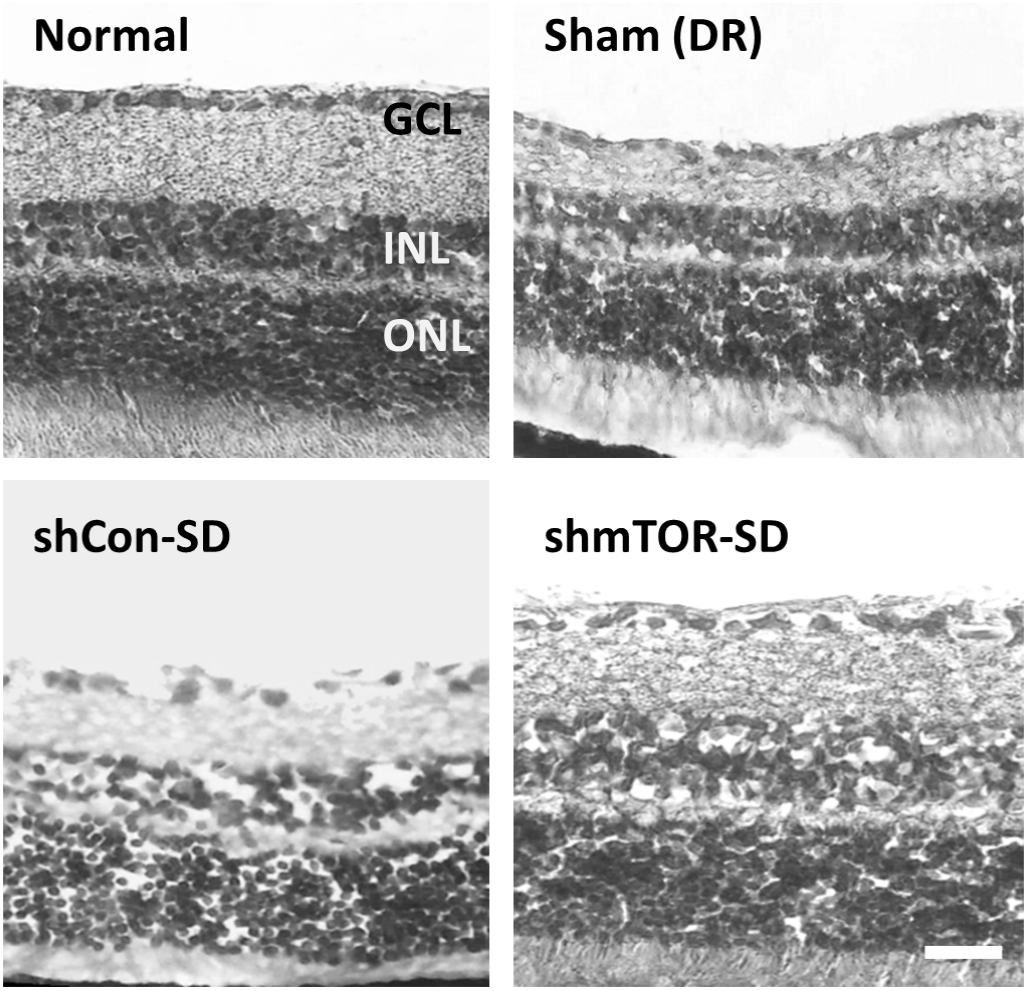

Figure 7. rAAV2-shmTOR-SD reduces retinal cell layer thinning.

Figure 7. rAAV2-shmTOR-SD reduces retinal cell layer thinning.

H&E staining demonstrated that rAAV2-shmTOR-SD significantly reduced the thinning of retinal cell layers, particularly in the ganglion cell layer, which is especially susceptible to neurodegeneration. On the other hand, cell layer thinning noticeably occurred in mice administered with a control shRNA-containing virus vector and sham-treated mice. These results suggest that the therapeutic virus vector may be neuroprotective. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μm.

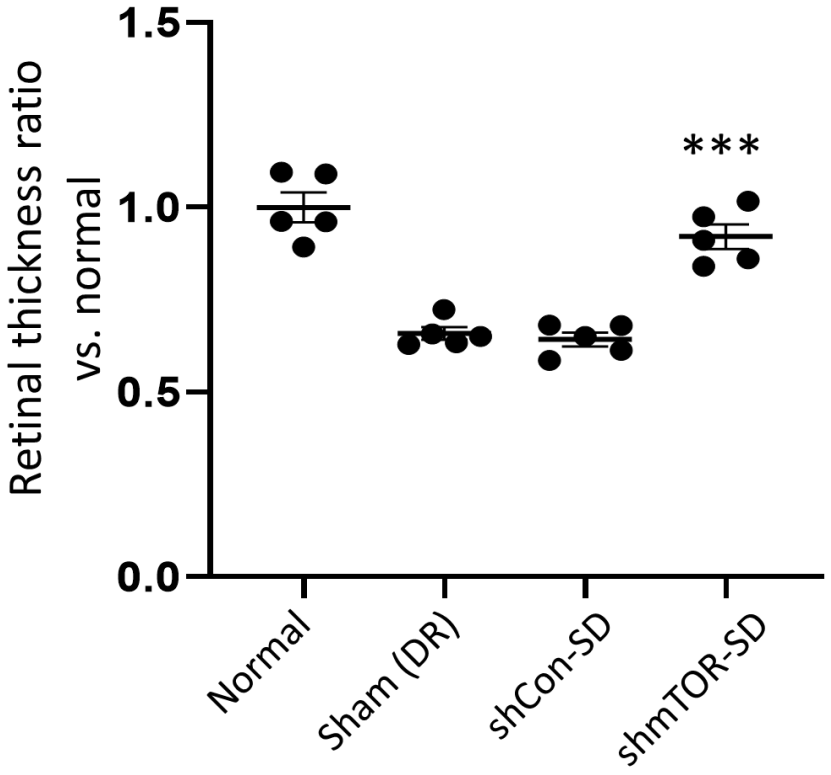

Figure 8. Quantification of retinal cell layers.

Figure 8. Quantification of retinal cell layers.

Retinal cell layer thicknesses for the mice were determined by calculating, as a ratio, the changes of the inner retina from the nerve fiber layer to the inner nuclear layer, and then normalizing the data relative to normal control mice. $p < 0.001$. Data represented as mean ± SEM ($n = 5$).

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rAAV2-shmTOR-SD has an anti-apoptotic effect

The mTOR-inhibiting shRNA has been demonstrated throughout its development to possess anti-apoptotic qualities,[19,20,23] which specifically addresses the mechanism by which cell death occurs in DR-related neurodegeneration.[27,29] This ability was confirmed here, with TUNEL-positive cells being readily observed speckling the various cells layers throughout the retinas (Fig. 9) of the sham-treated control group (7.600 ± 2.074) and mice injected with the control virus vector (7.200 ± 1.924). Apoptotic cells, on the other hand, were generally absent in the retinas of the normal control group (0.400 ± 0.548) and those treated with rAAV2-shmTOR-SD (1.800 ± 0.837; $p < 0.001$) (Fig. 10).

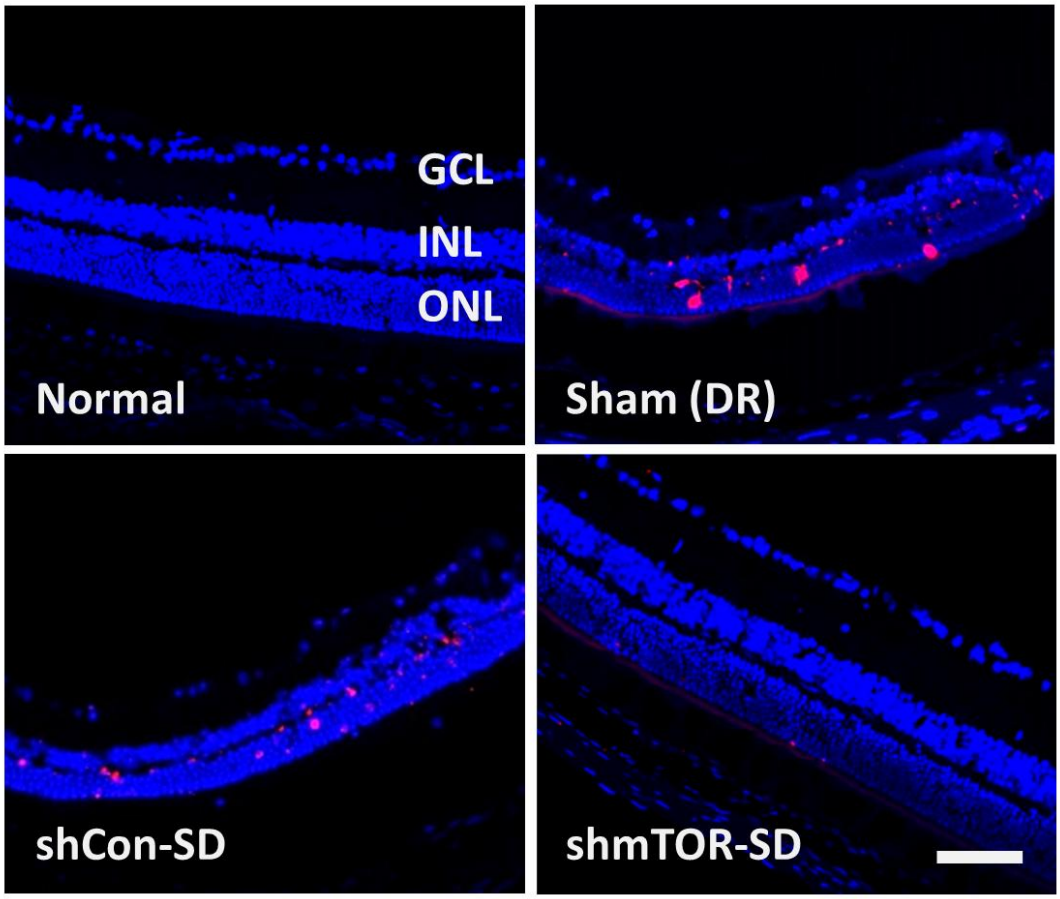

Figure 9. Anti-apoptotic activity of rAAV2-shmTOR-SD.

Figure 9. Anti-apoptotic activity of rAAV2-shmTOR-SD.

TUNEL-positive cells were not detected in the frozen transverse sections of the normal control group, but were found throughout the retinas of sham-treated control mice and those injected with the control virus vector. Compared to these latter groups, rAAV2-shmTOR-SD administration resulted in a marked reduction in apoptotic cells. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μm.

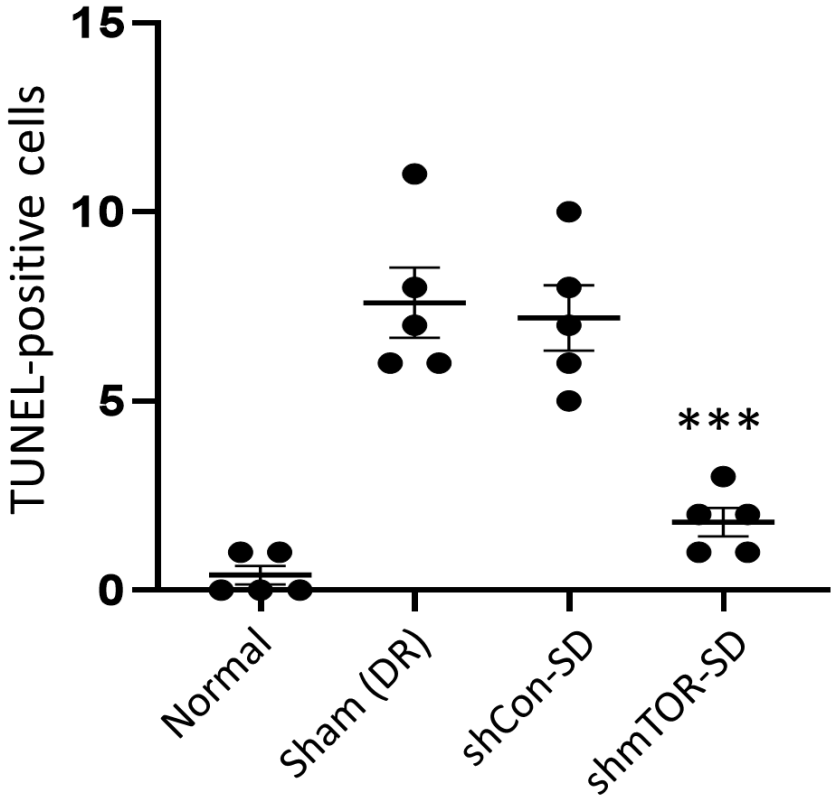

Figure 10. TUNEL assay results.

Figure 10. TUNEL assay results.

Quantification of the number of TUNEL-positive cells, which helps demonstrate the anti-apoptotic effect of the therapeutic virus vector. $p < 0.001$. Data represented as mean ± SEM ($n = 5$).

Effects of rAAV2-shmTOR-SD on retinal cells

Having demonstrated the transduction ability of the therapeutic virus vector into the retinas of the STZ-induced diabetic mouse model, where it was shown to downregulate mTOR expression and reduce retinal cell death, immunohistochemistry was used to visualize the effects of rAAV2-shmTOR-SD on a number of types of retinal cells. This was done using anti-NeuN, anti-GFAP, and anti-GS, which stain the ganglion cell layer, glial cells, and activated Müller cells, respectively. As shown here (Fig. 11), mice injected with rAAV2-shmTOR-SD had similar results, in terms of activity in retinal cell types, with normal control mice, whereas the sham- and rAAV2-shCon-SD-treated groups yielded profiles similar to one another. Further suggesting that the therapeutic virus vector possesses neuroprotective properties, rAAV2-shmTOR-SD specifically reduced ganglion cell loss (Fig. 12A). The therapeutic virus vector additionally shown to have reduced the activity of glial cells (Fig. 12B) and Müller cells (Fig. 12C) in particular.

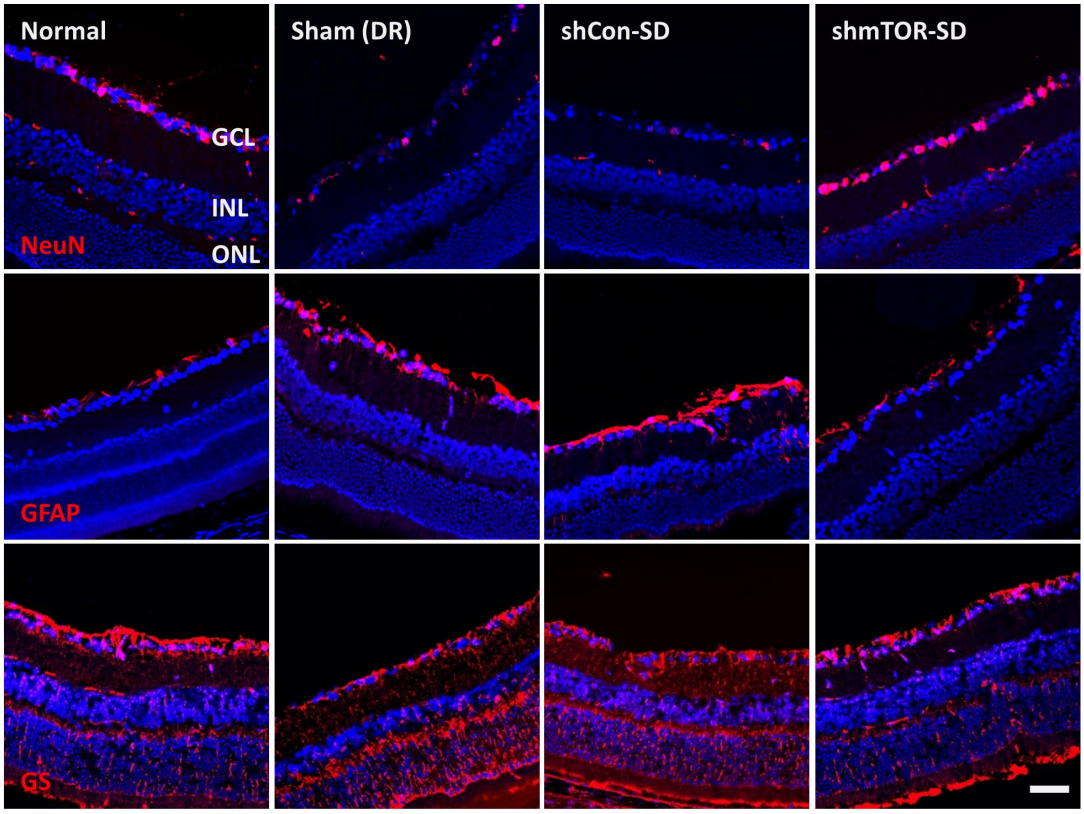

Figure 11. Localization of rAAV2-shmTOR-SD in various retinal cells.

Figure 11. Localization of rAAV2-shmTOR-SD in various retinal cells.

Anti-NeuN, anti-GFAP, and anti-GS to were used to visualize the ganglion cell layer, glial cells, and activated Müller cells, respectively, in the retinas of the STZ-induced mouse model. Revealed here is that rAAV2-shmTOR-SD treatment elicited similar staining patterns to normal control mice, whereas administration of the control virus vector resulted in patterns corresponding to the sham-treated group. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μm.

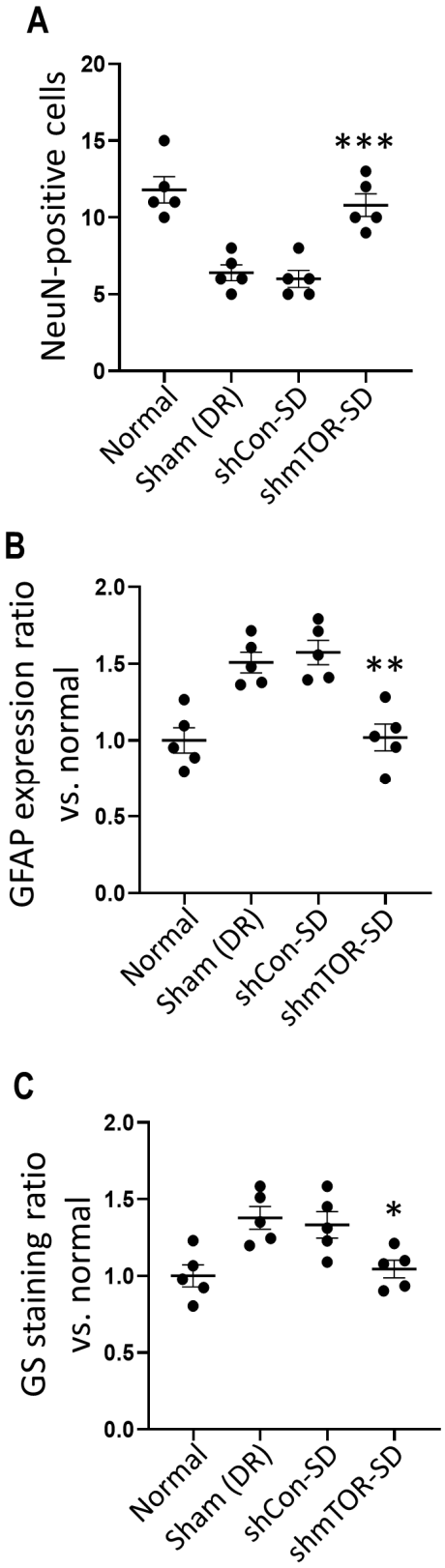

Figure 12. Immunohistochemistry data.

Figure 12. Immunohistochemistry data.

Quantification of NeuN-positive cells showed that rAAV2-shmTOR-SD treatment resulted in mouse retinas maintaining retinal ganglion cells at a level similar to the normal control group. $p < 0.001$. Data represented as mean ± SEM (n = 5) (A). Relative to normal control mice, GFAP expression was elevated in sham-treated mice and those injected with rAAV2-shCon-SD, and reduced in the group administered with the therapeutic virus vector. $p < 0.01$. Data represented as mean ± SEM (n = 5) (B). Determined relative to the normal control group, GS expression showed that rAAV2-shmTOR-SD treatment resulted in markedly reduced levels of active Müller cells. $p < 0.05$. Data represented as mean ± SEM (n = 5) (C).

Discussion

Here, a recombinant adeno-associated virus vector encoding a shRNA designed to inhibit mTOR directly was examined in a STZ-induced diabetic mouse model for its ability to address various early aspects of DR. Post-intravitreal injection, rAAV2-shmTOR-SD was shown to effectively transduce the mouse retinas and therein reduce mTOR expression while being active in retinal cell types important to DR pathophysiology. Treatment with the therapeutic virus vector additionally led to marked reductions in the loss of pericytes, acellular capillary formation, and the development of leaky vessels, all of which are heavily involved in the progression of DR. rAAV2-shmTOR-SD administration had an anti-apoptotic effect while reducing the thinning of retinal cell layers, suggesting that it may be neuroprotective.

Due to it being a complication of DM, aspects of the general condition can be seen to influence the pathophysiology of DR. In the diabetic retina, for instance, hyperglycemia has been linked to the loss of pericytes,[4] which was shown to be reduced by the administration of rAAV2-shmTOR-SD (Fig. 3). Pericytes are crucial to the normal functioning of the retina,[27] and their loss is a consequential early process that affects the progression of the disease. Pericytes also play an important structural role with regard to maintaining vessel integrity in the retina,[12] with their loss being associated with the development of non-perfused areas and

other abnormalities of the vessels. Specifically, these may include ischemic activity and/or the occlusion of capillaries.[4] As a result, the retinal environment may become hypoxic, leading to hypoxia inducible factor 1 (HIF-1) activation and the upregulation of VEGF, its target gene. A major driver of the NV that characterizes PDR, VEGF is a major contributor to the overall pathoprogression of DR. Pericytes have additionally been linked to the proper functioning of tight junctions in the retina,[12] while several studies have suggested that conformational changes of the tight junctions may be induced by VEGF.[5] Therefore, the loss of pericytes, which leads to the development of a hypoxic retina and the upregulation of VEGF, both directly and indirectly may lead to the development of leaky vessels in the retina. A significant aspect of DR that may directly lead to DR-associated blindness, vascular leakage was shown by FITC-dextran staining to be markedly attenuated by rAAV2-shmTOR-SD treatment (Fig. 5).

A significant aspect of DM, inflammation is an influential process in the development of DR pathophysiology, as can be seen in the numerous examinations of various potential anti-inflammatory therapeutics for the treatment of DR.[31] Müller cells, which are induced by hyperglycemia into expressing a variety of pro-inflammatory cytokines, are especially associated with inflammation occurring as a result of DR.[4] Among these pro-inflammatory cytokines are TNF-α and IL-1β, which act in concert to upregulate the expression of IL-8.[31] Within DR pathophysiology, Müller cells are also the primary source for secreted VEGF.[32]

In addition to influencing tight junction conformational changes and driving the NV that characterizes PDR, VEGF is pro-inflammatory,[5,33] while also increasing vascular permeability.[5] Furthermore, hyperglycemia has been shown to result in Müller cell activation and proliferation,[34] which helps to amplify its effects with regard to DR through the creation of a positive feedback loop. As a result of the variety of roles Müller cells play in the development and pathoprogression of DR, they are a major target for rAAV2-shmTOR-SD with regard to providing therapeutic effect, and anti-GS staining was used to show (Fig. 11) that administration of the therapeutic virus vector led to a significant reduction of Müller cell activity.

DR-related inflammation has additionally been implicated with the induction of apoptosis,[27] whose activity within the pathophysiology of the condition is associated with leaky vessel development[4,27] and the retinal neurodegeneration[3,30] of the NVU.[3,4,32] In fact, due to the importance of the latter process, which occurs via apoptotic cell death,[27,29] DR can be considered to be a disease of the NVU.[3] Therefore, the ability rAAV2-shmTOR-SD to significantly reduce the thinning of retinal cell layers (Fig. 7) while additionally being anti-apoptotic (Fig. 9), suggests that it may possess neuroprotective qualities. Additional evidence was provided by anti-NeuN staining, which demonstrated that the therapeutic virus vector was able to mitigate the loss of ganglion cells in the retinas of the STZ-induced diabetic mouse model (Fig. 12A).

As a result of the number of the processes implicated in the complex pathophysiology of DR and involved in its development, focusing on a single therapeutic target or pathway, such as VEGF and neovascularization, respectively, may prove to be limited as a treatment strategy. Therefore, a different target with broader effects, mTOR, for instance, may prove to be more efficacious in terms of a developing a comprehensively effective therapeutic. mTOR associates with a number of additional factors to form mTOR complex 1 (mTORC1), mTOR complex 2 (mTORC2)[13], and mTOR complex 3 (mTORC3)[35], the latter having been identified recently. As a component of these complexes, mTOR is involved in the modulation of a number of cellular processes and signaling pathways.[13] mTOR received its name due to its being targeted by rapamycin. Rapamycin and its structural analogues, called rapalogs, have previously been set forth as potential therapeutics for the treatment of DR.[15,36]

First-generation mTOR inhibitors, consisting of rapamycin and rapalogs, were identified thusly due to their anti-angiogenic properties, which they effect by targeting mTORC1[13] to downregulate VEGF and reduce neovascularization. This is done through HIF-1α, a downstream target of mTORC1,[37] yet VEGF is also a target gene of HIF-2α, a target gene of mTORC2,[38] thereby reducing the potential therapeutic efficacy of first-generation mTOR inhibitors versus DR. Second-generation mTOR inhibitors, which act by blocking the phosphorylation of the downstream targets of mTOR, have also been examined as potential DR treatments,[14] and they have demonstrated their efficacy in downregulating mTORC1,

mTORC2,[13] and mTORC3 activity.[35] However, various paths to reactivation exist for the three complexes through a number of mechanisms,[13,20,39] limiting their long-term efficacy in terms of inhibiting VEGF.[13] As the mechanisms by which mTORC3 act have yet to be elucidated, along with the potential existence of additional mTOR complexes, directly inhibiting mTOR itself via RNA interference may prove to be the most efficacious solution for the development of a therapeutic for the treatment of DR.

On the other hand, the most commonly used therapeutics currently available for DR are anti-VEGF drugs that were originally indicated for wAMD, as it, like PDR, is characterized by neovascularization driven by VEGF leading to the loss of patients' vision.[8] Use of these therapeutics was then expanded to DME and PDR before eventually being approved for all stages of DR.[40,41] The mTOR-inhibiting shRNA has followed a similar developmental trajectory, having previously been explored in a laser-induced mouse model of choroidal NV, a wAMD animal model system, where it was demonstrated to possess anti-angiogenic properties. Additional effects included a reduction in inflammatory cell infiltration into the choroidal NV lesions, anti-apoptotic activity, and a reduction of vascular leakage.[19] A rat OIR model was then used to determine its therapeutic efficacy with regard to angiogenic retinal conditions, including retinopathy of prematurity and DR, where it was shown to reduce retinal NV, inflammatory cell infiltration, and apoptotic activity.[20] The laser-induced mouse model was also used to compare the therapeutic effects of the original GFP-

containing construct versus the current virus vector containing a stuffer DNA. There, it was shown that rAAV2-shmTOR-SD, which is more appropriate for use as a human gene therapeutic, is as effective as rAAV2-shmTOR-GFP in terms of all the performed assays.[23]

Having previously followed the developmental trajectory of approved therapeutics for angiogenic ocular conditions, rAAV2-shmTOR-SD was examined here in a STZ-induced diabetic mouse model. Although this model system does not recapitulate the pathological proliferative aspect of PDR,[44] early DR processes that drive the development of the conditions are observed.[42,43,44] Included among these are pericyte loss leading to retinal vessel dysfunctions,[24] neurodegeneration,[30] and chronic inflammation.[45] As such, the OIR and STZ-induced diabetic models can combine to provide significant information regarding the therapeutic efficacy of a potential DR treatment. Therefore, after examining the effects of rAAV2-shmTOR-SD upon the angiogenic processes that occur later during DR pathoprogression, here it was possible to demonstrate its therapeutic efficacy with regard to early DR processes that influence the overall development of the condition.

As such, the transduction ability of rAAV2-shmTOR-SD was demonstrated in a STZ-induced diabetic mouse model, where the therapeutic virus vector was shown to downregulate mTOR expression (Fig. 2). rAAV2-shmTOR-SD was also shown to be active in cell types important to DR pathophysiology, including retinal ganglion cells and Müller cells (Fig. 11). The reduction of both retinal cell layer thinning (Fig. 7) and apoptotic activity

(Fig. 9), as well as the activity of the therapeutic virus vector in the ganglion cell layer suggest that rAAV2-shmTOR-SD is neuroprotective. The therapeutic virus vector was additionally shown to reduce pericyte loss, acellular capillary formation (Fig. 3), and vascular leakage (Fig. 5). These results (Fig. 13) combine with those of previous explorations of the mTOR-inhibiting shRNA to demonstrate the promise of rAAV2-shmTOR-SD as a potential human gene therapeutic for a variety of angiogenic retinal conditions and DR in particular.

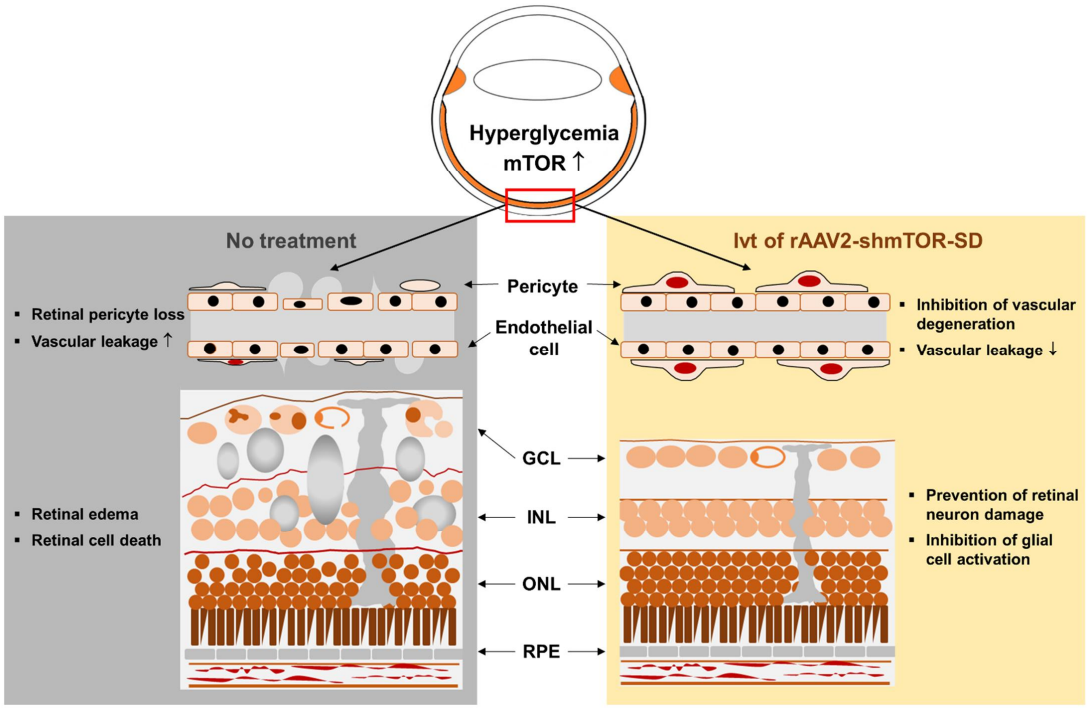

Figure 13. Conclusions.

Figure 13. Conclusions.

As depicted here, rAAV2-shmTOR-SD demonstrates broad efficacy against processes relating to DR pathophysiology. Among the early processes are pericyte loss and vascular leakage, which, if left untreated, may lead to the development of edema, cell death, and disruptions of the retinal cell layers. Intravitreal administration of the therapeutic virus vector was shown to effectively address these aspects of DR in a STZ-induced diabetic mouse model, further demonstrating its potential as a human gene therapeutic.

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국문 요약

목적: 당뇨망막병증의 치료제로는 레이저 광촉매와 anti-VEGF 치료가 있다. 그러나 두가지 치료 형태 모두 단점이 존재하며, 특히 안구 내 주사를 통한 적용방법은 짧은 활성 때문에 빈번한 관리가 필요하다. 또한 이 두가지 치료법은 적용되는 병증이 매우 제한적으로, 이는 당뇨망막병증의 다원적 발병 특성을 포괄적으로 다루는 능력에 한계가 될 수 있다. 당뇨망막병증의 복잡한 병리생리학 때문에 광범위한 효능을 가진 치료 대상인 mTOR 가 이러한 문제를 해결하는데 도움이 될 수 있다. 게다가 유전자 치료를 치료전략으로 활용하는 것은 환자 친화적인 방식으로 오래 지속되는 효과를 제공하여 현재 사용되는 치료법의 가장 중요한 한계를 극복해 줄 것이 기대된다.

방법: 당뇨병 동물 모델을 만들기 위해 7 주령의 C57/BL6 생쥐 복강 내로 고농도의 스트렙토조토신(STZ)를 단 회 투여하였다. 그리고 대조군 및 rAAV2-shmTOR-SD 인 치료 바이러스 벡터를 안구 내로 주사하였다. 이 후 생쥐를 희생하여 망막을 분리하였고 망막 혈관 구조를 확인하기위한 trypsin digest, 바이러스 벡터 tropism 을 확인하기 위한 FITC-dextran 염색, TUNEL assay, IHC 를 진행하였다. 게다가 STZ 적용 후 6 개월 뒤 H&E 염색을 통하여 망막의 세포 층들을 관찰하였다.

결과: 이전에 치료 바이러스 벡터는 항 혈관 신생 활성을 통해 당뇨망막병증의 혈관을 치료 할 수 있다는 것이 산소 유도에 의한 망막증 랫드 모델에서 밝혀졌었다. 반면에 rAAV2-shmTOR-SD 는 DR 의 초기 과정에 치료 효과를 나타냄을 이번 연구에서 확인하였다. 실제로 마우스에 주입된 rAAV2-shmTOR-SD 는 sham 및 rAAV2-shCon-SD 처치 그룹에 비해 현저하게 mTOR 발현이 낮아지는 것을 확인하였다. 이는 DR 의 초기 병리학적 특성인 혈관주변세포 손실을 크게 억제시키는 동시에 세포 모세관 형성, 혈관 투과성 및 망막 세포층의 얇아지는 것을 억제하였다. 또한 rAAV2-shmTOR-SD 는 Müller 세포의 병원성 활성화 및 증식을 감소시켰으며, 세포 사멸과 망막신경절세포의 감소를 억제하였다. 이러한 결과는 치료 바이러스 벡터가 신경 보호 특징도 가지고있음을 확인하였다.

결론: 본 연구결과를 통해, 당뇨망막병증 치료에 대한 rAAV2-shmTOR-SD 의 광범위한 능력을 병리생리학적 관점에서 다각도로 검증했으며, 이에 따라 rAAV2-shmTOR-SD 가 갖는 당뇨망막병증 치료용 유전자 치료제로서의 미래 가능성을 성공적으로 제시할 수 있었다.