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Research article

Intravitreal AAV-IKV mediated delivery of decorin inhibits choroidal neovascularization, fibrosis, inflammation and elevates autophagy

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A B S T R A C T

Age-related macular degeneration (AMD) is the most common cause of blindness in the elderly. The exudative or wet form of AMD is caused by choroidal neovascularization (CNV) and subsequently a macular edema. Wet AMD can be effectively treated with anti-vascular endothelial growth factor (VEGF) therapies. However, despite treatment, more than half of patients continue to lose vision due to a lack of compliance with frequent intravitreal injections, failure to adequately respond to anti-VEGF therapy and emergence of fibrotic scars underneath the retina. In this study we investigated the use of our retinal penetrating AAV for delivery of human decorin (AAV-IKV-Decorin) in a murine model of laser induced CNV. Our results indicate that following a single intravitreal injection, decorin is highly expressed in the outer retina of AAV-IKV-Decorin injected mice and such mice exhibit significantly less neovascularization in laser induced CNV relative to mice injected with an AAV-IKV-Aflibercept, an AAV expressing an anti-VEGF. AAV-IKV-Decorin also significantly inhibited fibrosis, reduced inflammatory markers and increased autophagy.

1. Introduction

Age-related macular degeneration (AMD) is the most common cause of blindness in the elderly (Wong et al., 2014; Rudnicka et al., 2015). The exudative or 'wet' form of the disease involves the growth of new blood vessels from the choroid into the subretinal space, a process referred to as choroidal neovascularization (CNV). These new blood vessels fail to mature, resulting in leakage of blood into the retinal matrix, generating a macular edema. If left untreated, the edema causes retinal degeneration and ultimately, blindness (reviewed in (Fleckenstein et al., 2021)). Currently available FDA-approved therapies for the 'wet' form of AMD include *ranibizumab* and *aflibercept* that target vascular endothelial growth factor (VEGF)- a key promoter of angiogenesis (Peden et al., 2015). These therapies have been extremely beneficial for patients. However, approximately half of anti-VEGF treated patients continue to lose vision (Peden et al., 2015; Munk et al., 2016; Kodjikian, 2018).

There are multiple proposed reasons for the continued loss of vision among wet AMD patients despite anti-VEGF treatment. Chief among these is lack of patient compliance (Boulanger-Scemama et al., 2015). Anti-VEGF treatment requires intraocular injections every four to six weeks. Studies show that elderly patients do not adhere to such a schedule (Boulanger-Scemama et al., 2015). For this reason, real-world outcomes of anti-VEGF treatments do not match the outcomes achieved in anti-VEGF clinical trials (Lupidi et al., 2021; Cheema et al., 2021; Okada et al., 2021a; Mones et al., 2020). In times of a pandemic, as occurred in 2020, this lack of compliance was exacerbated (Boulanger-Scemama et al., 2015; Valverde-Megias et al., 2021; Stattin et al., 2021; Weng et al., 2021; Okada et al., 2021b). This high frequency of injections may be addressed via the approach of gene therapy, that may theoretically enable treatment of AMD via a single intraocular injection during the lifetime of the patient.

Following anti-VEGF treatment, approximately half of patients develop a fibrotic scar underneath the retina within two years (Daniel et al., 2014). This pathophysiology is believed to be driven by transforming growth factor-β (TGF-β) that drives epithelial to mesenchymal transition (EMT) of retinal pigment epithelium (RPE) cells (Wang et al., 2019). As in other tissues, fibrosis interferes with proper functioning of the retina. Anti-VEGF therapies do not address the onset of fibrosis in the retina of AMD patients.

Finally, it is well known that while VEGF is a key player in neovascularization, other factors, e.g. Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Angiopoietin (ANG) in addition to many other molecules play a significant role in angiogenesis (reviewed in (Chung and Ferrara, 2011)). Thus, targeting VEGF exclusively, while clinically beneficial, is not effective in all patients (Suzuki et al., 2014), and is thus sub-optimal for the long-term treatment of 'wet' AMD. Indeed, apart from promoting angiogenesis, VEGF likely plays a key role in the maintenance of the choriocapillaris (Saint-Geniez et al., 2009). A healthy choriocapillaris is essential for maintenance of healthy

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Table 1
Primers.

Gene	Primers	Product length (basepair)
TGFβ2	5′-AGAAGGCGAATGGCTCTCC-3′ 5′-GATGGTACAAAAGTGCAAGAGG-3′	119bp
TNFα	5′-GATCGGTCCCCAAAGGGATG-3′ 5′-CCACTTGGTGGTTTGTGAGTG-3′	92bp
IL-1β	5′-GCCACCTTTTGACAGTGATGAG-3′ 5′-GACAGCCCAGGTCAAAGGTT-3′	95bp
IL-6	5′-CACTTCACAAGTCGGAGGCT-3′ 5′-CTGCAAGTGCATCATCGTTGT-3′	113bp
β-Actin	5′-CGGTTCCGATGCCCTGAGGCTCTT-3′ 5′-CGTCACACTTCATGATGGAATTGA-3′	99bp

photoreceptors and inhibition of VEGF may be responsible for the regression of the choriocapillaris and consequential retinal degeneration that is observed in AMD patients (Channa et al., 2015).

In this study, we examined the efficacy of adeno-associated virus (AAV) mediated gene transfer of human decorin in a murine model of laser induced CNV- the most commonly utilized animal model of 'wet' AMD. Decorin is known to play a key role in angiogenesis through the targeting of multiple factors beyond VEGF (Neill et al., 2012; Jarvelainen et al., 2015). Decorin is a potent inhibitor of TGF-β mediated EMT/fibrosis (Baghy et al., 2012), a regulator of autophagy (Schaefer and Dikic, 2021) and inflammation (Merline et al., 2011)- all key processes involved in the pathophysiology of AMD (Fleckenstein et al., 2021; Zhang et al., 2020; Kivinen, 2018; Anderson et al., 2002). Finally, decorin is also thought to play a role in neuroprotection (Ozay et al., 2017), and retinal degeneration or loss of photoreceptors is a key feature of AMD (Yang et al., 2016).

Delivery of gene therapy vectors to the outer retina generally requires the use of the surgical procedure known as a subretinal injection. Such an approach risks significant damage to the retina. Thus, in this study we utilized our recently developed AAV vector referred to as AAV-IKV (Kumar et al., 2024a) that can deliver transgenes to the outer retina via an intravitreal injection. AAV-IKV contains a nucleolin targeting peptide sequence referred to as Nuc1 in its capsid. Nuc1 behaves as a chaperone for macromolecule delivery to the outer retina following intravitreal delivery (Kumar et al., 2024b). Use of an intravitreal approach is key to the rapid adoption of gene therapy administered as an office procedure. Millions of AMD patients receive anti-VEGF therapy via an office procedure without any significant complications. Our results indicate that decorin delivered intravitreally using AAV-IKV leads to a highly significant inhibition of laser induced CNV as well as attenuation of fibrosis, inflammation and elevation of autophagy.

2. Materials and methods

2.1. Animals

This study was carried out in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research, set out by the Association of Research in Vision and Ophthalmology (ARVO) and was approved by Tufts University Institutional Animal Care and Use Committee (IACUC). Six-week-old C57Bl/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained under a 12 h light/dark cycle. Mice were acclimated to vivarium conditions for a minimum of three days prior to commencement of studies.

2.2. AAV purification

Human decorin, human TGFβ2, Aflibercept or poly-A (pA)- each regulated by the chicken beta actin promoter (Cashman et al., 2002, 2003) on an AAV-IKV 'backbone' (single-stranded AAVs as described previously (Kumar et al., 2024a)) were produced using HEK293T cells by a triple transfection method. The AAV-IKV vector containing a constitutively active TGFβ2 was constructed by mutating cysteine at positions 226 and 228 to serine (Brunner et al., 1989; Samuel et al., 1992) of the human TGFβ2 propeptide (NP_003229). Calcium phosphate transfection method was used for the plasmid transfection to the HEK293T cells (ATCC, Manassas, VA). Briefly, 24h after seeding HEK293T cells (5×10^6 cell/15 cm plate), the cell culture medium was replaced with serum free DMEM. Transfection mixture containing 2.5M $CaCl_2$, nuclease free water, and a 1:1:1 M ratio of the helper plasmid: cis plasmid: trans plasmid (0.4mg/plate), with equal volume of HEPES buffered saline (Boston Bioproducts, Ashland, MA). Transfection mixture (4 ml) was added in each plate, and one day post transfection, serum free medium containing transfection mixture was replaced with complete medium. After four days of transfection, cells were harvested using 0.5M EDTA solution. Cell pellet was harvested by centrifugation (1500 RPM) and resuspended in lysis buffer prior to three rounds of freeze/thawing (10 min each). Following the addition of Benzonase (50U/ml), the lysate was incubated at 37 °C for 60 min and centrifuged at 3400 RPM. After centrifugation, 10 ml of supernatant was transferred on to the top of 15–60% iodixanol (IDX) gradient (OptiPrep density gradient medium, Sigma, St. Louis, MO). Subsequently, tubes were sealed, and centrifuged at 60,000 RPM for 90 min using T70i rotor at 18 °C. After centrifugation, 40% gradient IDX was collected using a 16G needle. Pooled samples were concentrated using MWCO 100 kDa Amicon Ultra-15 filter unit (Millipore Sigma, Burlington, MA) and finally suspended in lactate Ringer solution. Titer of purified viruses were quantified using SYBR Green based real-time PCR (qPCR) assay in Bio-Rad thermocycler system (iQ5 Multicolor real time PCR detection system). A standard curve of cycle threshold (Ct), obtained from qPCR, against the copy number prepared using different dilutions of the respective plasmids (2×10^4 copies/μl to 2×10^8 copies/μl), The Ct-values obtained from the plasmid DNA isolated from the viruses were subsequently compared with the standard curve to quantify the number of viral genomes.

2.3. Intraocular injections

Mice were anesthetized by intraperitoneal injection of a mixture containing Ketamine (100 mg/kg) and Xylazine (10 mg/kg) followed by topical application of 0.5% proparacaine hydrochloride to the cornea. Intravitreal injections (1.1×10^9 vector genomes in a volume of approximately 1–1.5 μl) were performed using a 33-gauge needle and a 5 μl glass syringe (Hamilton, Reno, NV) in a trans scleral trans choroidal approach from the anterior segment of the eye to avoid penetrating the retina.

2.4. Tissue processing and decorin immunostaining

Six-weeks-old C57Bl/6J mice were infected with 1.7×10^9 vector genome AAVIKV-Decorin, and after two-weeks of infection the whole eyes were removed, and fixed in 4% paraformaldehyde for 6–8hrs at 4 °C. After fixing, the eyes washed with PBS and were placed in 15–30% sucrose (w/v) solution for 12hrs each at 4 °C, then embedded in OCT compound Tissue-Tek. Frozen sections were cut 14 μm thick, thawed at room temperature for 2min, and thereafter processed at room temperature. They were rehydrated in Phosphate Buffer saline (PBS) for 20min and processed for decorin staining using MOM immunodetection Kit (Vector Laboratories, Burlingame, CA). After 1 h of blocking, sections were incubated overnight at 4 °C with mouse anti-hDecorin (R&D, Minneapolis, MN) antibody, diluted in dilution buffer (1:200). Followed with the incubation at room temperature with Cy3-labelled anti-mouse secondary antibody for 2hrs, sections were washed with PBS and then mounted in DAPI containing mounting medium (Vectashield-DAPI; Vector Laboratories, Burlingame, CA). Images were examined and photographed with a Leica TCS-SPE confocal fluorescence microscope at

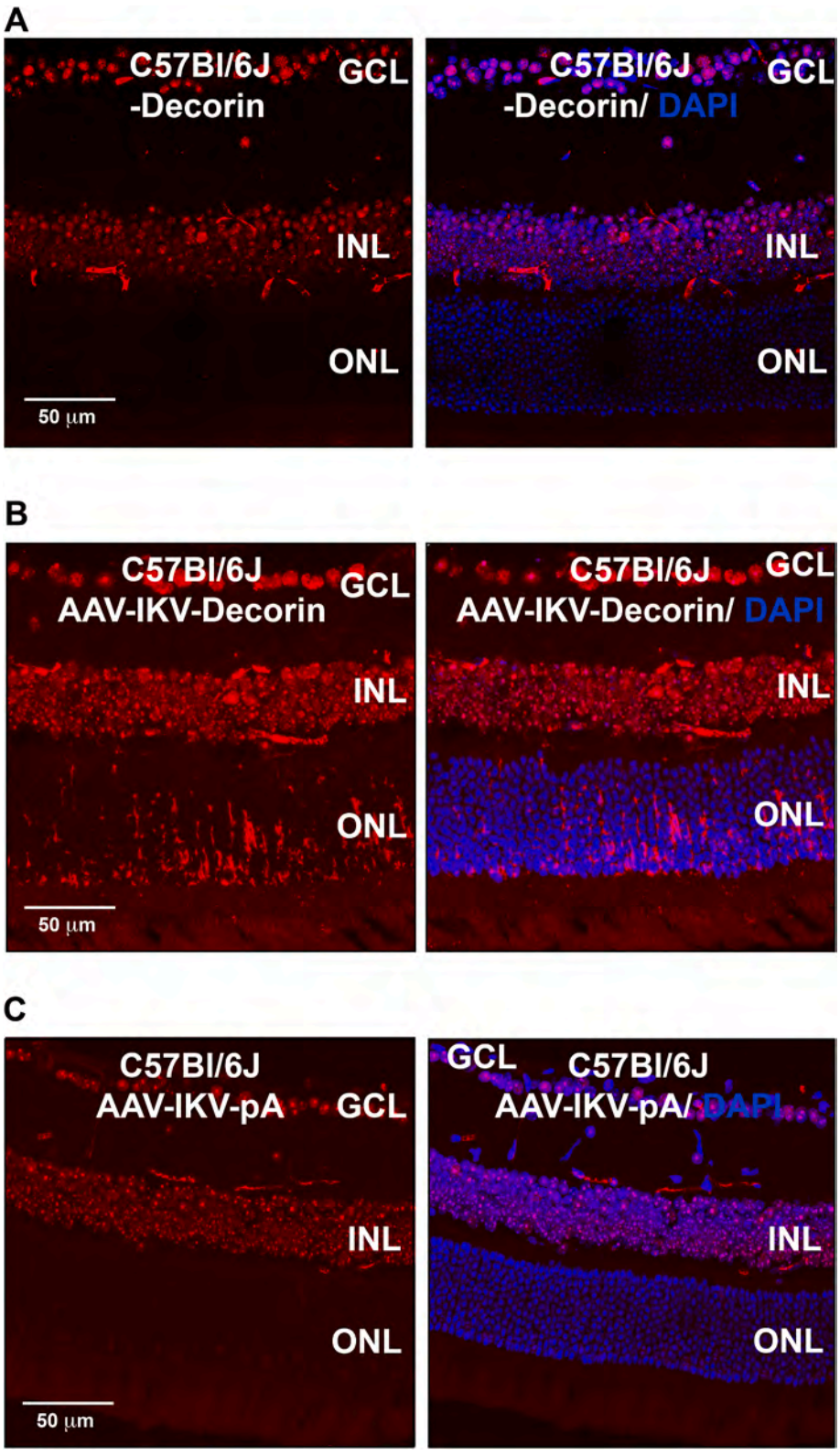

Fig. 1. AAV Mediated Delivery of Decorin to the Outer Retina Following Intravitreal AAV injection. A) Antibody staining in frozen sections from C57Bl/6J mice indicates that decorin is expressed primarily in the GCL, INL and blood vessels. There is no significant expression of decorin in the ONL. B) Antibody staining in frozen sections from C57Bl/6J mice injected two weeks prior with AAV-IKV-Decorin indicates that in addition to expression in the GCL, INL and blood vessels, there is now significant expression of decorin in the ONL. AAV-IKV-decorin also appears to elevate expression of decorin in the INL. C) Antibody staining in frozen sections from C57Bl/6J mice injected two weeks prior with AAV-IKV-pA indicates no expression of decorin in the ONL. GCL, ganglion cell layer; INL, inner nuclear layer, ONL, outer nuclear layer.

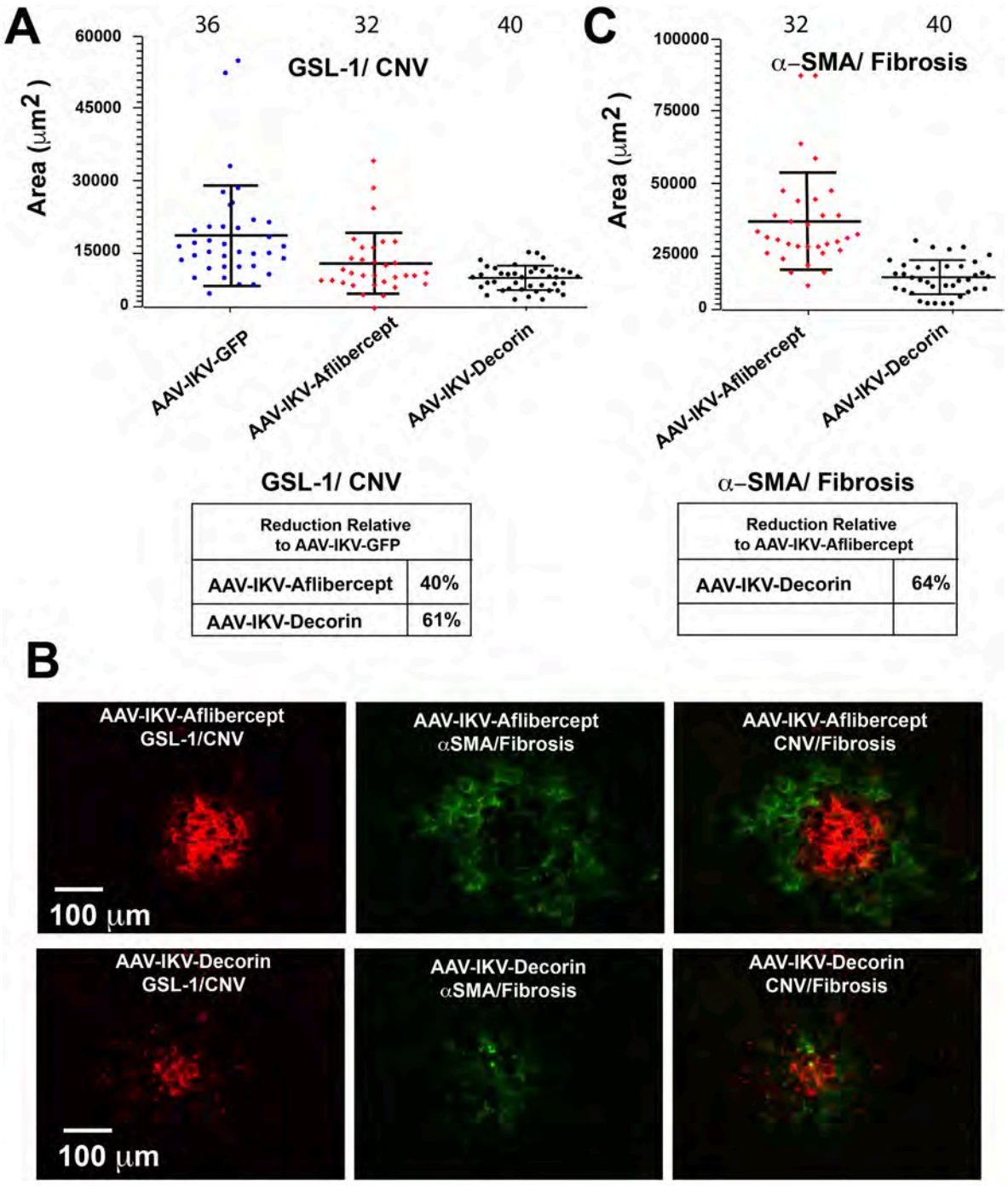

Fig. 2. Decorin inhibits laser induced CNV and fibrosis A) Choroidal neovascularization as measured by GSL-1 lectin staining of flat mounts from eyes injected with either AAV-IKV-GFP (negative control), AAV-IKV-Aflibercept and AAV-IKV-Decorin. B) Representative GSL-1 and α-SMA staining of flat mounts. C) α-SMA staining of flat mounts from AAV-IKV-Aflibercept and AAV-IKV-Decorin injected mice. The number of laser spots in each study is indicated. Because AAV-IKV-GFP injected mice express GFP, the channel used for α-SMA staining, fibrosis was not measured in those eyes. However, studies were also performed using an empty AAV vector, see Fig. 3.

40X magnification.

2.5. Laser induced choroidal neovascularization (CNV)

Mice were injected with one of AAV-IKV-Decorin, AAV-IKV-TGFβ2 or AAV-IKV-Aflibercept (1.1×10^9 vector genomes in a volume of approximately 1–1.5 μl) with Nuc1 cell penetrating peptide (1 μg). One week later, mice were anesthetized and four laser spots per eye were created around the optic nerve head using an argon laser (532 nm) set at 330 mW power and 100ms pulse (Phoenix Micron IV, Pleasanton, CA). After seven days, eyes were enucleated, retinas and corneas removed, and eyecups (RPE/choroid) fixed in 4% PFA in 0.1 M phosphate buffer (PB) for 6–8 h. The RPE/choroid tissue was incubated with FITC labelled mab α-SMA antibody at 4 °C overnight, followed with incubation with Cy3 labelled *griffonia simplicifolia* lectin I (GSL I; 10 mg/ml in PBS) for 2hrs at room temperature. Cy3-GSL I stained CNV lesions or Fluorescein-stained fibrotic spots were imaged on the RPE/choroid using a fluorescence microscope (Olympus IX51 inverted microscope) at 20X

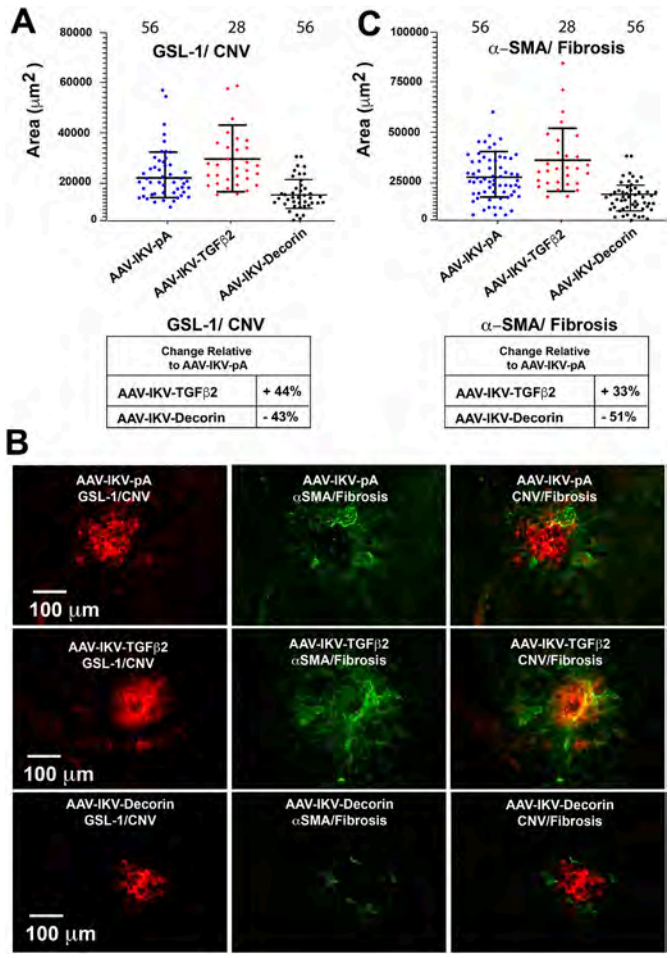

Fig. 3. Elevation of TGFβ2 exacerbates both neovascularization and fibrosis. A) Choroidal neovascularization as measured by GSL-1 lectin staining of flat mounts from eyes injected with either AAV-IKV-pA (negative control), AAV-IKV-TGFβ2 and AAV-IKV-Decorin. B) Representative GSL-1 and α-SMA staining of flat mounts. C) α-SMA staining of flat mounts from AAV-IKV-pA, AAV-IKV-TGFβ2 and AAV-IKV-Decorin injected mice. The number of laser spots in each study is indicated.

magnification, and the area of neovascularization and fibrosis were quantified using ImageJ software (http://rsbweb.nih.gov/ij/). Study samples were blindly randomized before sacrifice and the scoring investigators were not aware of the study group until scoring was complete.

2.6. Measurement of autophagy

ATG9A staining of the RPE/choroid flat mount was performed using a MOM-kit standard staining protocol. Briefly, following permeabilization with 0.1% PBST (0.1% Tween 20 in PBS) for 30 min, flat mounts were blocked in MOM™ Mouse IgG blocking reagent for 1h. After overnight immunostaining with anti-rabbit ATG9A antibody (Novus Biologicals, Littleton, CO) (1:100) at 4 °C, flat mounts were washed with PBS, and incubated with Cy3 labelled GSL1 for endothelial cell and FITC-labelled anti-rabbit secondary antibody, at room temperature for 2h. Finally, the samples were washed with PBS, and mounted with DAPI containing mounting media. Flat mounts were imaged under Fluorescence microscope at 20X magnification.

2.7. Real-time quantitative PCR

The effect of AAVs infection on the inflammatory response was measured by quantification of inflammatory markers in the RNA isolated from whole eye by qPCR using specific primers (Table 1). Following AAV infection, and after a week of laser treatment, total RNA from the whole eye was isolated (Qiagen, Hilden, Germany) and complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a one-step high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Expression of TNFα, IL-1β, IL-6 and TGFβ2, was quantified using qPCR. Reaction specificity was validated by a single peak in the melting curve. Values of the qPCR amplification were normalized to the Ct-value from the control group (uninfected and untreated), and those in cDNA to the Ct-values from β-Actin in the same sample.

3. Results

3.1. AAV mediated delivery of decorin to the outer retina following intravitreal AAV injection

CNV associated with the wet form of AMD occurs in the outer retina. Recently, we described an AAV vector (AAV-IKV) that can penetrate the retina following intravitreal injection (Kumar et al., 2024a). Maximum penetration of AAV-IKV was achieved when co-injected with a cell penetrating chaperone, Nuc1 (Kumar et al., 2024b). In order to determine whether decorin can be efficiently delivered and expressed in the outer retina of mice, we generated a recombinant AAV-IKV vector expressing human decorin (AAV-IKV-Decorin) and we co-injected 1.7×10^9 genome units of this virus with 1 μg Nuc1 into six-week-old C57Bl/6J mice via the intravitreal route. Two weeks later, eyes were harvested, fixed and the frozen retinal sections probed for expression of decorin using an anti-decorin antibody. We found that whereas uninjected eyes did not express any significant decorin in the outer retina relative to the inner retina (Fig. 1A), the eyes of AAV-IKV-Decorin injected animals exhibited readily detectable decorin in the outer retina, including the outer nuclear layer, i.e. the photoreceptor cell bodies (Fig. 1B). Furthermore, eyes injected with AAV-IKV-pA (a virus identical to AAV-IKV-Decorin without the decorin cDNA) did not exhibit any expression of Decorin in the ONL, confirming that expression of decorin in the ONL is not due to AAV-IKV virus or the injection procedure per se, but rather due to the expression of decorin from the transgene in AAV-IKV-Decorin (Fig. 1C) We conclude that intravitreal injection of AAV-IKV-Decorin enables significant expression of human decorin in the outer retina of mice.

3.2. Decorin inhibits laser induced CNV and fibrosis

One standard of care for the wet form of AMD is intravitreal injection of *Aflibercept*. Although Aflibercept, an anti-VEGF, is a recombinant protein, it too can be expressed from a recombinant transgene expression cassette from an AAV. AAV mediated delivery of *Aflibercept* is currently in clinical trials (NCT03748784) for the treatment of the wet form of AMD. In order to compare the potential efficacy of decorin relative to *Aflibercept* when delivered via an AAV vector intravitreally, we constructed a recombinant *Aflibercept* transgene expression cassette in a manner similar to that of AAV-IKV-Decorin. Adult C57Bl/6J Mice were injected intravitreally with either 1.1×10^9 genome units of AAV-IKV-Aflibercept or the same amount of AAV-IKV-Decorin or the control vector AAV-IKV-GFP, a virus expressing green fluorescent protein (GFP). In each case, and in all studies described below, 1 μg of Nuc1 was added to the injectate to enhance infection as described previously (Kumar et al., 2024a). One week post injection, mice were taken through a standard protocol of laser induced CNV as described in *Methods*. Seven days post laser, eyes were harvested, fixed and stained for endothelial cell migration in a flat mount using GSL1-isolectin or stained for fibrosis using an antibody against α-smooth muscle actin (α-SMA). We found that relative to AAV-IKV-GFP, AAV-IKV-Aflibercept reduced CNV by ~40% ($p < 0.008$) (Fig. 2A and B), whereas AAV-IKV-Decorin reduced

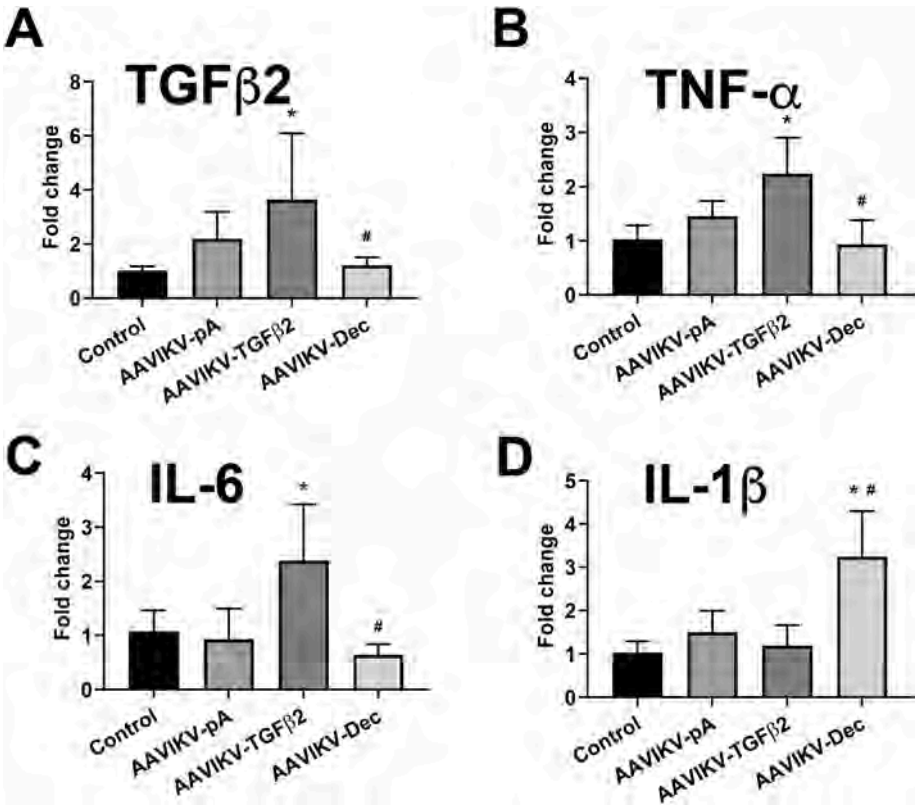

Fig. 4. Expression of constitutively active TGFβ2 induces inflammation in laser induced CNV. Animals injected with AAV-IKV-TGFβ2 had elevated levels of TGFβ2 as expected relative to both uninjected and AAV-IKV-pA injected animals. AAV-IKV-TGFβ2 led to an increase in expression of TNFα and IL-6 but not IL-1β. AAV-IKV-Decorin dampened the expression of TGFβ2, TNFα and IL-6 but surprisingly had elevated levels of IL-1β.

CNV by ~61% ($p < 0.0001$) (Fig. 2A and B). Relative to AAV-IKV-Aflibercept, AAV-IKV-Decorin had ~64% ($p < 0.0001$) less neovascularization. Furthermore, we found that relative to AAV-IKV-Aflibercept, AAV-IKV-Decorin had ~64% ($p < 0.0001$) less α-SMA staining (Fig. 2B and C). We conclude that AAV-IKV-Decorin is a potent inhibitor of CNV as well as fibrosis and it is more potent than AAV-IKV-Aflibercept in the murine model of laser induced CNV described herein.

3.3. Elevation of TGFβ2 exacerbates both neovascularization and fibrosis

The TGF-β superfamily is believed to play a significant role in wet AMD, including the modulation of angiogenesis, inflammation, fibrosis and immune pathways (Tosi et al., 2018). Decorin is a potent inhibitor of TGFβ2. In order to investigate a potential role for TGFβ2 in laser induced CNV, we generated an AAV-IKV vector expressing constitutively active human TGFβ2. Six-week-old mice were injected with either AAV-IKV-pA (an AAV with an expression cassette void of a cDNA), AAV-IKV-TGFβ2 or AAV-IKV-Decorin. As above, all eyes were co-injected with Nuc1. One week later, each group of mice were exposed to laser induced CNV. After a further one week, eyes were harvested and stained with GSL-1 lectin and α-SMA.

We found that relative to AAV-IKV-pA, AAV-IKV-TGFβ2 injected eyes exhibited 44% ($p = 0.002$) greater neovascularization (Fig. 3A and B). In contrast, relative to AAV-IKV-pA, AAV-IKV-Decorin injected eyes exhibited 43% ($p = 0.004$) less neovascularization (Fig. 3A and B). Relative to AAV-IKV-TGFβ2, AAV-IKV-Decorin injected eyes exhibited 60% ($p<.0001$) less neovascularization. Similarly, α-SMA staining of these flat mounts revealed that relative to AAV-IKV-pA, AAV-IKV-TGFβ2 injected eyes exhibited 33% ($p<.0001$) greater fibrosis (Fig. 3B, C). In contrast, relative to AAV-IKV-pA, AAV-IKV-Decorin injected eyes exhibited 51% ($p = 0.007$) less fibrosis (Fig. 3B and C). Relative to AAV-IKV-TGFβ2, AAV-IKV-Decorin injected eyes exhibited 64% ($p<.0001$) less fibrosis. We conclude that TGFβ2 significantly exacerbates laser induced CNV and fibrosis in mice and that decorin likely acts as a negative regulator of TGFβ2 in laser induced CNV and fibrosis.

3.4. Elevation of TGFβ2 induces inflammation in laser induced CNV

Inflammation is a key player in the pathophysiology of AMD (Curcio and Huisingh, 2017). TGF-β is known to have both pro and anti-inflammatory properties (Tosi et al., 2018). We investigated the impact of constitutively active TGFβ2 mediated inflammation in laser induced CNV. Specifically, we measured mRNA transcripts of tumor necrosis alpha (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β). We found that relative to injection of AAV-IKV-pA or uninjected controls, injection of AAV-IKV-TGFβ2 led to a significant increase in expression not only of TGFβ2 as expected (3.6-fold, $p < 0.002$ vs uninjected; 1.67-fold, $p < 0.15$ vs AAV-IKV-pA; Fig. 4A), but also TNFα (2.2 fold, $p < 0.0001$ vs uninjected; 1.54 fold, $p = 0.005$ vs AAV-IKV-pA, Fig. 4B) and IL-6 (2.22 fold, $p = 0.0009$ vs uninjected; 2.57 fold, $p = 0.0002$ vs AAV-IKV-pA injected, Fig. 4C). Expression of IL-1β was not significantly elevated relative to uninjected or AAV-IKV-pA injected animals (Fig. 4D). Interestingly, in contrast to TNFα and IL-6, expression of IL-1β was significantly increased in AAV-IKV-Decorin injected (3.18-fold, $p < 0.0001$ vs uninjected; 2.72-fold, $p < 0.0001$ vs AAV-IKV-TGFβ2, Fig. 4D) animals. Eyes injected with AAV-IKV-Decorin exhibited a reduction in expression of TNFα (2.41 fold, $p < 0.0001$, Fig. 4B), IL-6 (3.73 fold, $p < 0.0001$, Fig. 4C) and TGFβ2 (3.02 fold, $p < 0.003$, Fig. 4A) in comparison to the expression observed in AAV-IKV-TGFβ2 infected eyes. We conclude that active TGFβ2 exacerbates some key inflammatory markers in laser induced CNV and that AAV-IKV-Decorin injected eyes exhibit less inflammation and inhibit TGFβ2.

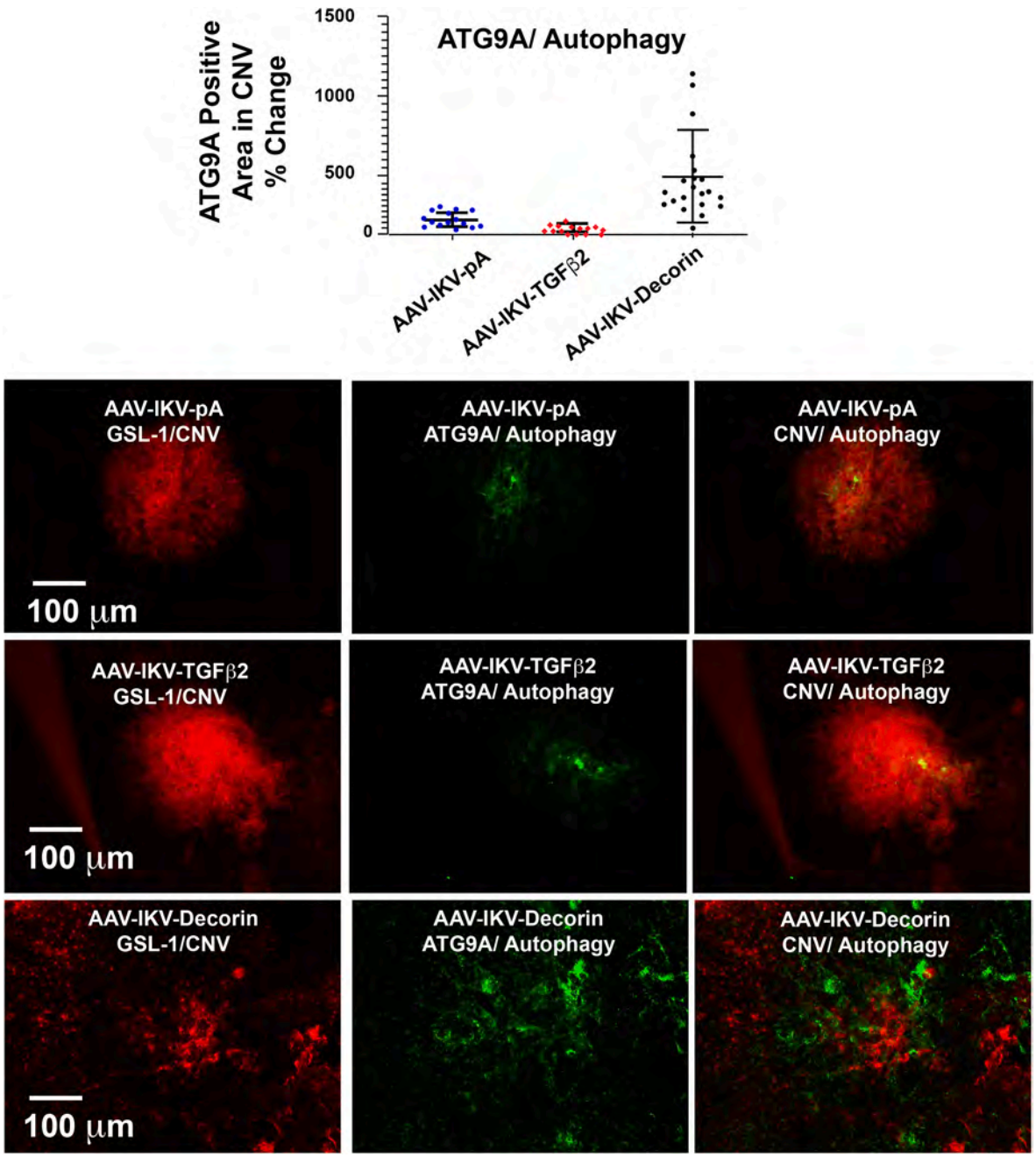

Fig. 5. AAV-IKV-Decorin Increases Autophagy in Endothelial cells. Representative and cumulative data of flat mounts from AAV-IKV-Decorin, AAV-IKV-TGF-β2 or AAV-IKV-pA injected adult C57Bl/6J mice stained with ATG9A and GSL1-isolectin at two weeks post injection (one week post laser induced CNV).

3.5. AAV-IKV-Decorin Increases Autophagy in Endothelial cells

One hallmark of AMD is the accumulation of lipofuscin, advanced glycation end products, misfolded protein deposits and dysfunctional mitochondria in the RPE due to impaired autophagy (Zhang et al., 2020; Kivinen, 2018). These undigested products elevate oxidative stress in the retina and contribute to retinal degeneration. Decorin is known to be an autophagy-inducible proteoglycan for endothelial cells (Schaefer and Dikic, 2021). In order to examine whether expression of decorin could induce autophagy in endothelial cells in laser induced CNV, we injected 1.7×10^9 AAV-IKV-Decorin or AAV-IKV-TGF-β2 or AAV-IKV-pA as negative control (with 1 μg Nuc1) into adult C57Bl/6J mice, and one week later, exposed these mice to the standard protocol of laser induced CNV. Seven days post laser, eyes were harvested and processed to prepare flat mounts that were stained with ATG9A and GSL1-isolectin. We found that AAV-IKV-Decorin led to a three-fold (299%, $p = 0.0005$) and thirteen-fold (1293%, $p < 0.0001$) increase in autophagy relative to AAV-IKV-pA and AAV-IKV-TGF-β respectively (Fig. 5). Although there was reduced autophagy observed in AAV-IKV-TGF-β2 relative to AAV-IKV-pA injected eyes, it was not statistically significant ($p = 0.62$) (Fig. 5). We conclude that decorin can significantly induce autophagy in endothelial cells in laser induced CNV.

4. Discussion

In this study we have shown that a retinal penetrating AAV-IKV virus can deliver human decorin to the outer murine retina via an intravitreal injection. AAV-IKV-Decorin could inhibit laser induced CNV at a level superior to that of AAV-IKV-Aflibercept in the study conditions described herein. Moreover, AAV-IKV-Decorin was capable of

attenuating fibrosis and inflammatory markers in the retina as well as elevating autophagy. Each of these properties of AAV-IKV-Decorin has potential implications for the treatment of AMD.

The wet form of AMD can be divided into subtypes depending on the origin and location of neovascular vessels, with type I CNV associated with vessels from the choriocapillaris growing into the subretinal space, type II CNV with blood vessels spreading into the subretinal space between the retina and RPE, and type III comprising proliferative blood vessels extending from the capillary plexus towards the outer retina. The Ang/Tie2 pathway complements VEGF-mediated activity in AMD by increasing neovascularization and increasing inflammatory signaling. *Faricimab* is a bispecific antibody that has shown promise as an inhibitor of both VEGF and Ang2 (reviewed in (Khanani et al., 2021)).

Decorin is a ligand for several tyrosine kinases, including VEGFR2 (Khan et al., 2011), the epidermal growth factor receptor (EGFR) (Iozzo et al., 1999), insulin-like growth factor 1 receptor (IGF-IR) (Schonherr et al., 2005) and MET, which is a receptor for hepatocyte growth factor (HGF) (Goldoni et al., 2009). Decorin may also bind platelet derived growth factor receptor (PDGF)(Baghy et al., 2013). Further, Decorin is known to impact the expression and bioavailability of multiple angiogenic growth factors, including VEGF (Grant et al., 2002), monocyte chemoattractant protein (MCP-1) and ANG (Mohan et al., 2011). Thus, unlike highly specific antagonists of VEGF, Decorin may be able to support homeostasis of the retina during pathological angiogenesis. This may be relevant for the treatment of AMD patients that do not adequately respond to anti-VEGF treatments.

Decorin binds and sequesters TGF-β in the extracellular matrix, preventing it from binding TGFR1 and TGFR2, thus blocking canonical and non-canonical signaling from these receptors. Thus, decorin acts 'upstream' relative to Smad 2/3; RhoA, PI3K and Ras. Signaling through these receptors via TGF-β plays a significant role not only in angiogenesis (Mahmoud et al., 2011) but also EMT/fibrosis (Piera-Velazquez and Jimenez, 2012; Rosenbloom et al., 2010). Approximately half of AMD patients being treated with anti-VEGF therapies develop fibrosis within two years (Daniel et al., 2014). Histopathological studies indicate that retinal degeneration is correlated with the diameter and thickness of the fibrotic lesion (Green and Enger, 1993). In surgically excised CNV from patients (and in laser induced CNV in mice), a significant proportion of cells are found to be positive for α-SMA (Lopez et al., 1996). Our results indicate that AAV-IKV-Decorin can potently limit CNV-associated α-SMA staining. Significant evidence supports the hypothesis that RPE is the origin of myofibroblastic cells through the development of EMT (Grisanti and Guidry, 1995). The role of the immune response in fibrosis is still being uncovered. Subretinal fibrous tissues are known to contain macrophages (Lopez et al., 1996). Macrophages secrete TGF-β (Wynn, 2007) and produce extracellular matrix including collagen I and fibronectin (Gratchev et al., 2001).

Among the multiple factors involved in the pathogenesis of AMD, a reduction in the level of cellular autophagy is considered as one of the critical factors in the progression of AMD. Autophagy is a cellular autodegradation process and is known to be impaired in aged RPE cells (Li et al., 2008). Autophagic flux is reduced in AMD RPE relative to normal RPE (Golestaneh et al., 2017). In addition, autophagy has also been found to inhibit EMT (Wang et al., 2017), which is also an important component in the progression of AMD, as fibrosis led by EMT causes RPE dysfunction. In this study we investigated the role of decorin on autophagy in neovascular cells in CNV. It is interesting to note that eyes infected with AAV-IKV-TGFβ2 exhibited exacerbated fibrosis and CNV along with reduced autophagy whereas eyes administered with AAV-IKV-Decorin exhibited significantly enhanced autophagy in neo-vascular cells and correspondingly less CNV and fibrosis. This suggests that autophagy plays an important role in transitioning of epithelial or endothelial cells to mesenchymal forms that lead to fibrosis in CNV.

Key to the observations documented in our study is the use of a retinal penetrating AAV vector, AAV-IKV (Kumar et al., 2024a). Currently, another retinal penetrating vector, 7m8 (Dalkara et al., 2013), expressing Aflibercept is being explored for the treatment of the 'wet' form of AMD. 7m8 was developed through library screening in murine retina. Although this vector performs extremely well in the context of retinal penetration in mice, the titers needed for observation of these properties in NHPs are deleterious to the retina, leading to significant immune responses (Ramachandran et al., 2017). We found that AAV-IKV-Decorin led to inhibition of some key markers of inflammation, justifying further study of decorin in the context of an intravitreally delivered AAV vector such as AAV-IKV or 7m8.

AAV-IKV was designed based on peptides known to bind human nucleolin, that is confirmed to be present on human photoreceptor cells (Leaderer et al., 2016). Whether AAV-IKV will potently infect NHP retina without any significant immune responses remains to be seen and are the subject of future studies.

CRediT authorship contribution statement

Manish Mishra: Writing – original draft, Methodology, Investigation, Formal analysis. **Siobhan M. Cashman:** Writing – review & editing, Writing – original draft, Funding acquisition. **Rajendra Kumar-Singh:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Conflict of interest statement

R.K-S is a listed inventor of US patent application US20220204562A1 and the data submitted in the current manuscript has been included in part in the patent application. Patent is assigned to Tufts University. R.K-S and MM serve as consultants for Visiogene LLC. SC does not have any conflicts of interest to declare.

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Data availability

No data was used for the research described in the article.

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