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AAV5-mediated sFLT01 gene therapy arrests retinal lesions in *Ccl2*^{-/-} /*Cx3cr1*^{-/-} mice

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Abstract

To test the effects of adeno-associated virus encoding sFLT01 (AAV5.sFLT01) on the retinal lesions in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice, a model for age-related macular degeneration (AMD), AAV5.sFLT01 was injected into the subretinal space of the right eyes and the left eye was served as controls. Histology found no retinal toxicity due to the treatment after 3 months. The treated eyes showed lesion arrest compared to lesion progression in the left eyes by fundus monitoring monthly and histological evaluation 3 months after treatment. Retinal ultrastructure showed fewer lipofuscin and better preserved photoreceptors after the treatment. A2E, a major component of lipofuscin, was lower in the treated eyes than in the control eyes. Molecular analysis showed that AAV5.sFLT01 lowered retinal extracellular signal-regulated kinase (ERK) phosphorylation and iNOS expression, which suggested the involvement of reactive nitrogen species in the retinal lesions of *Ccl2*^{-/-}/*Cx3cr1*^{-/-}. We concluded that local delivery of AAV5.sFLT01 can stabilize retinal lesions in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. The findings provide further support for the potential beneficial effects of sFLT01 gene therapy for AMD.

Keywords

soluble VEGF receptor-1; age-related macular degeneration; animal model; *Ccl2*; *Cx3cr1*; adeno-associated virus, gene therapy; retina

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1. Introduction

Age-related macular degeneration (AMD) is the most common cause of irreversible legal blindness of the elderly in the world (Gehrs et al., 2006). The pathological features of AMD include degeneration and atrophy of the photoreceptors and the retinal pigment epithelium (RPE) in the macula. There are two types of AMD, the exudative or “wet” form, with choroidal neovascularization, and the atrophic or “dry” form, with geographic atrophy of the photoreceptors and RPE. Except for the suppression of choroidal neovascularization, there is no definitive treatment for AMD. Care for intermediate AMD and dry AMD is limited to risk factor management. Smoking cessation, body mass reduction, and specific vitamins and nutrient supplements have been reported to slow disease progression (Smith and Lee, 2007)

We have reported that *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice developed a broad spectrum of AMD-like pathology with early onset and high penetrance (Chan et al., 2008; Tuo et al., 2007). The retinal lesions are generally symmetric in both eyes and deteriorates with age. Using this model, we have successfully demonstrated the beneficial effects of long-term dietary intake of long chain omega-3 polyunsaturated fatty acids (n-3) to alleviate the retinal lesions of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. Chow rich in n-3 was able to decelerate the retina lesion observed by funduscopy, reserve the retinal structure observed by histopathology, and reduce the A2E level (retina autofluorescence component) measured by HPLC (Tuo et al., 2009). The study indicated that the retinal lesions of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice could be arrested with proper intervention, which encouraged us to test other potential options for AMD therapy.

Enhanced expression of vascular endothelial growth factor A (VEGFA) has been detected in the retinas of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (Herzlich et al., 2009). VEGFA is one of the major stimulators in neovascularization and a defining molecule of wet AMD. The biological activities of VEGF are mediated by its receptors including Flt-1 (VEGF receptor 1), Flk-1 (VEGFR2), and neuropilin-1 (NRP-1). The receptor binding of VEGF activates both the Extracellular Signal-Regulated Kinase (ERK) and Akt signaling pathways (Basu et al., 2010; Narasimhan et al., 2009). ERK activation by VEGF has demonstrated an increase of apoptosis in cerebral endothelial cells (Narasimhan et al., 2009). This data provides an intriguing hypothesis that overexpressed VEGF at sites of chronic inflammation may be one of the causes to continuous degeneration of the mouse retina. Moreover, a broad role for VEGF in immunological regulation has been observed, including enhanced production of cytokines in Th1, Th2, and Th17 responses (Kim et al., 2009; Lee et al., 2004; Mor et al., 2004).

Flt-1 is a potent natural VEGF binder. A soluble form of Flt-1 (sFlt-1) originated from an alternatively splicing contains only the extracellular domains of the Flt-1 protein. The sFlt-1 protein has the similar VEGF-binding affinity as the full-length protein (Shibuya et al., 1990). Binding between sFlt-1 and VEGF neutralizes various ligands for VEGF receptors without triggering any receptor-mediated signal transduction pathways. To obtain small molecule only containing necessary portions of the protein for VEGF binding, a novel soluble chimeric VEGFR-1 molecule, sFLT01, which consist of the second immunoglobulin (IgG)-like domain of Flt-1 fused to a human IgG1 Fc through a polyglycine linker 9Gly has been previously generated (Pechan et al., 2009).

Given the possible role of enhanced VEGFA expression in the development of retinal lesions in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice, we explored stable expression and secretion of sFLT01 peptide in the retina to neutralize the excessive VEGFA as a potential treatment. We adopted a previously reported approach using subretinal injection of a recombinant adeno-associated virus (rAAV) vector in which a portion of the sFlt-1 gene was cloned (Lai et al., 2005).

Funduscopy, histopathology, the level of retina lipofuscin and other AMD-relevant molecules were used to evaluate the effects of the intervention.

2. Methods

2.1. Animals and treatment

Ccl2^{-/-}/*Cx3cr1*^{-/-} mice and wild type control (C57BL/6) were bred in-house. The study was conducted in compliance with the ARVO statement for the use of animals, and all animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of National Eye Institute, National Institutes of Health, USA.

The generation of sFLT01 open reading frame (ORF), where the Flt-1 signal peptide sequence was fused directly to Flt-1 domain 2 and linked to human IgG1-Fc region through the polyglycine 9-mer (9Gly), as described by Pechan et al. (Pechan et al., 2009). The sFLT01 ORF was then cloned into an AAV previral plasmid vector, pTR-UF5, containing hybrid chicken β -actin (CBA) promoter and SV40 polyadenylation signal sequence (SV40 poly A) and AAV2 inverted terminal repeats (ITRs) (Flannery et al., 1997). The AAV5.sFLT01 vector was produced in 293 cells by triple-transfection method after co-transfection of pTR-UF-SB.sFLT01, AAV helper plasmid with AAV5 cap gene and adenovirus helper plasmid, further purified and titered as described previously (Pechan et al., 2009).

One μ l of the construct, containing 5.82×10^{12} drps (DNase resistant particles) per ml, was injected into the subretinal space of the right eyes of 4-week-old *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (n=50). Subretinal injections were performed as previously described (Pang et al., 2005; Pang et al., 2006). Subretinal injection of AAV5.GFP showed neither therapeutic nor toxic effects in mouse retina (Pang et al., 2006). Therefore, the left eyes were untreated and served as controls. Eyes were harvested 3 months after the injection for various measurements.

2.2. Detection of transgene by real time RT-PCR

Total RNA from mouse retina/RPE was used for cDNA synthesis using Trizol (Invitrogen, Carlsbad, CA). cDNA was synthesized by Reverse Transcriptase using 3 μ g total RNA in a total volume of 20 μ l (Superscript II RNase H Reverse Transcriptase, Invitrogen, Grand Island, NY). The ocular expression of human *sFLT01* transgene was detected by real-time RT-PCR using ABI 7500 Real-Time PCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene, CA). The primers for the transgene are forward primer: CAGGGGAACGTCTTCTCATG; Reverse primer: GCATTTTTTTTCACTGCATTC, which amplifies a 169 bp target bridging the junction of the 3' end of human sFLT01 and the SV40 polyA. For the internal control, β -actin was amplified using primers 5'-CCCAGCACAAATGAAGATCAA-3' and 5'-ACATCTGCTGGAAGGTGGAC-3'. Following PCR, a thermal melt profile was performed for amplicon identification.

2.3. Fundus Photography

A Karl Storz veterinary ophthalmoscope coupled with a Nikon D90 digital camera was modified and used for taking mouse fundus photographs before the injection and every month after the injection for 3 months (Paques et al., 2007). The fundus photograph was taken after pupil dilation (1% tropicamide ophthalmic solution, Alcon Inc, Fort Worth, Texas) and intraperitoneal injection of ketamine (1.4 mg/mouse) and xylazine (0.12 mg/mouse) for systemic anesthesia. We evaluated the lesion changes by comparing sequential photos taken in the same fundus area of each eye. Progression was defined as a >10% increase the number of the retinal lesions, a >50% increase in the lesion size in at least 1/3 of the lesions, >5 fused lesions, or the appearance of >2 chorioretinal scars in comparison with

the previous observation. Regression was defined as a >10% decrease in the number of retinal lesions or a >50% decrease in lesion size in at least 1/3 of the lesions. To avoid bias, evaluation of the pictures was conducted by a masked observer.

2.4. Histopathology

Whole eyes were fixed in 4% glutaraldehyde-10% formalin and then embedded in methacrylate. The eyes were serially sectioned in the vertical pupillary-optic nerve plane. Each eye was cut into 6 sections. All sections were stained with hematoxylin and eosin. If an ocular lesion was observed, another 6-12 sections were cut through the lesion. These slides were also stained with Periodic Acid Schiff (PAS) to highlight Bruch's membrane and the basement membrane of small neovascular vessels.

2.5. Transmission Electron Microscopy

The whole eyes were fixed in 2% glutaraldehyde and 2% paraformaldehyde. The tissue was embedded in Durcupan epoxy resin. Six 1µm-thick sections stained with toluidine blue were examined under light microscopy. Based on the lesions shown on the thick sections, ultrathin sections of these lesions were taken and were stained with uranyl acetate and lead citrate for examination under a JEOL1010 microscope. Four eyes from two mice were used for transmission electron microscopy.

2.6. Retinal lipofuscin extraction and quantification

A2E ([2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetra-enyl]-1-(2-hydroxyethyl)-4-[4-methyl-6(2,6,6-trimethyl-1-cyclohexen-1-yl) 1E,3E,5E,7E-hexatrienyl]-pyridinium) is the major component of lipofuscin fluorophores generated from the visual cycle flux of all-trans-retinal. The molecule is particularly relevant to aging and AMD pathogenesis (Ben-Shabat et al., 2001). The mice were kept in the dark for >12 hours before being sacrificed. Whole eyes were removed in a dark room under dim red light and homogenized. A2E was extracted with chloroform/methanol as previously described (Karan et al., 2005). The extracts dissolved in methanol were separated by HPLC (Agilent 1100 LC, Wilmington, DE) and detected by an ultraviolet detector at a wavelength of 435 nm. A gradient of 40-95% acetonitrile/H₂O in 0.1% trifluoroacetic acid was used to elute A2E on a reverse-phase C18 column (Agilent, eclipse XD8-C18, 5 µm, 4.6X150 mm) at a flow-rate of 1.0 ml/min. A2E was quantified using external A2E standards (Parish et al., 1998).

2.7. Quantification of gene expression by RT-PCR

cDNA synthesis was described as above. The primers/probes for *Vefga*, *Tnf-α*, *Il-6*, *Chop*, *Cxcl12*, *Cxcr4*, *Pon2*, *Gpx3* and *iNos* and *Gapdh* were purchased from ABI as inventoried TaqMan gene expression reagents. Relative quantitative real time PCR was performed according to ABI's instructions. To determine the Ct values, the threshold level of fluorescence was set manually in the early phase of PCR amplification. ABI SDS 1.3.1 software and the $2^{-\Delta\Delta Ct}$ analysis method were used to determine relative amounts of product using *GAPDH* as an endogenous control. The fold change was normalized first by the level of *GAPDH* from same cDNA sample. The average fold change due to treatment was again normalized to the transcript level in untreated eyes. Each sample was analyzed in triplicate.

2.8. Western Blot Analysis

The mouse retina/RPE was lysed in the buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM Na₃VO₄, and 1 mM NaF. The lysate was homogenized, and the supernatant was collected after centrifugation at 12,000g for 15 minutes at 4°C. The protein mixture was separated by SDS-polyacrylamide gel

electrophoresis, transferred to polyvinylidene difluoride membranes (Invitrogen), and the following proteins were detected with anti-mouse human antibody: actin (Santa Cruz, Rabbit, 1:3000), ERK (Cell Signaling, Rabbit, 1:2000), pERK (Cell Signaling, Rabbit, 1:2000), iNOS (BD, Mouse, 1:20000, and VEGF (ABBIOTEC, rabbit, 1:2000). The blots were subsequently incubated with goat anti-rabbit or goat anti-mouse secondary antibody conjugated to alkaline phosphatase. Images were developed using the WesternBreeze Chromogenic Immunodetection Kit (Invitrogen).

2.9. Statistical Analysis

The rates of progression and regression between groups were compared by the Chi-square test. Multiple means were compared by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for post hoc comparison of means. Differences were considered significant when $p < 0.05$.

3. Results

3.1. AAV5.sFLT01 transgene and mRNA expression

The majority of the treated eyes showed detachment of >50% of the retina during subretinal injection, indicating a successful surgical procedure. The lesion due to the injection healed after 1 month, which was identified by the funduscopy. The overall structure of retina was intact as determined by funduscopy and histology. The detection of sFLT01 transcripts in the retinal tissues by real time RT-PCR indicated successful transduction and expression of the vector (data not shown). Similar results were reported previously in subretinal injection of AAV.sFLT-1 using mice and monkey (Lai et al., 2005).

3.2. sFLT01 gene therapy arrested retinal lesions

The right eyes that received AAV5.sFLT01 treatment showed retinal lesion arrests as compared to the contralateral left eyes (control), in which the retinal lesions progressed 3 months after injection (Figure 1 (A)). The plot of 33 pairs of eyes showed that 16 treated eyes were healthier, 13 treated eyes remained at same lesion scales and 4 treated eyes became worse relative to the contralateral eye by funduscopy examination 3 months after injection (Figure 1B). The natural course of the retinal lesions in *Ccl2^{-/-}/Cx3cr1^{-/-}* mice is to worsen with time (Tuo et al., 2007), therefore, most untreated left eyes showed lesion worsen. The average lesion score changes were 0.23 ± 0.14 (mean $\pm$ SD) in the treated right eyes compared to 0.85 ± 0.19 in the control left eyes ($p < 0.01$) (Figure 1C).

In general, the histological observations indicated better retinal morphology in the treated eyes than in the non-treated ones, characterized with less photoreceptor atrophy, smaller retinal lesions and thicker retinal outer layers (Figure 2). The histological scores between the right and left eyes from 10 pairs of eyes revealed a decreased lesion severity in 6 pairs, similar in 2 pairs, and an increased lesion in only 2 pairs three months after injection (Table 1). Retinal ultrastructures also illustrated fewer lipofuscin granules in the RPE cells, better preserved photoreceptors and outer plexiform layer in the right eyes compared with the left eyes (Figure 3).

3.3. sFLT01 intervention decreased the accumulation of retina lipofuscin

A2E eluted at 31.3 minutes with the method described below. A 71.3% decrease in the amount of A2E was detected in the treated eyes (17.3 ± 8.7 pmol/eye) relative to the untreated eyes (60.3 ± 4.9 pmol/eye) ($p < 0.05$) (Figure 4).

3.4. sFLT01 intervention selectively decreased iNos mRNA expression

The transcripts of *Vefga*, *Tnf- α* , *Il-6*, *Chop*, *Cxcl12*, *Cxcr4*, *Pon2*, *Gpx3* and *iNos* were selected to measure because our previous work demonstrated altered mRNA expression of those genes in the eyes of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (Cao et al., 2010; Herzlich et al., 2009; Tuo et al., 2007). RT-PCR data demonstrated a significant reduction of only ocular *iNOS* in the treated eyes as compared to the untreated eyes (Figure 5A). The reduction was confirmed by the end-point PCR gel analysis (Figure 5B) and gel image quantification (Figure 5C). We did not detect significant change of the expression of other 8 genes between the control and treated eyes (Figure 5A).

3.5. sFLT01 intervention reduced ERK phosphorylation, VEGF and iNOS protein

Western blot detected enhanced ERK phosphorylation along with elevated amount of VEGF and iNOS protein in the ocular tissues of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (Figure 6A). Subretinal delivery of sFLT01 eliminated ERK phosphorylation and reduced the VEGF and iNOS protein (Figure 6B). ERK phosphorylation and iNOS levels were positively associated (Figure 6C).

4. Discussion

We find that subretinal injection of AAV5-CBA.sFLT01 can efficiently and locally express sFLT01 and reduced the amount of VEGF protein in the retina. The intervention can stabilize the progression of retinal lesions in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice, shown through better preserved morphology of retinal structure and lower retinal level of lipofuscin comparing to untreated control eyes. Trapping excess VEGFA in the retina using sFLT01 may abolish enhanced ERK phosphorylation in the retinal tissues of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. The decreases in ERK phosphorylation and iNOS expression in the retinal tissues suggest the involvement of reactive nitrogen species in the retina lesions that can be reduced by AAV5-CBA.sFLT01. No histological toxicity due to sFLT01 treatment was observed for up to 3 months following subretinal delivery. These findings indicate the potentially beneficial effects of sFLT01 gene therapy for AMD.

Gene therapy refers to the introduction of gene into cells for the purpose of treating disease by restoring gene expression. AAV vectors are the most commonly used delivery system for ocular gene therapy (Liu M.M. et al., 2010). AAV is a small virus, which infects humans and some other primate species. The virus is not currently known to cause any disease but only a very mild immune response (Grieger and Samulski, 2005). AAV can infect both dividing and non-dividing cells including photoreceptors and RPE cells. These characteristics make it particularly suited for applications in a variety of chronic ocular diseases (Buch et al., 2008). Because the genes for all virally encoded proteins have also been deleted in AAVs used in gene therapy (Carter, 2005), it added more safety and specificity in its application. In this study, we observed continuous better-off in the retinal lesion three months after a single local injection, which complied a stable expression of the inserted gene.

AAV8 might be a better choice than AAV5 for the delivery of therapeutic gene because of its higher transduction efficiencies in photoreceptor cells (Igarashi et al., 2010; Jing et al., 2010). This specific property may be vital in some early onset mouse models of retinal degeneration (Jing et al., 2010). However, this is not the case for our AMD model, in which retinal lesions have already developed. Furthermore, transduction efficiency is related to not only serotype but also other factors, such as titer in the sense that serotype-related transduction differences are more evident when the titer is low. In our experience, the titer level (10^{13} vector genomes/ml) used in most of our previous studies is sufficient to rescue

most RPE and/or photoreceptor function in many different mouse models (Pang et al., 2005; Pang et al., 2006). AAV5 viral vectors have been shown to be safe and efficient for retinal gene therapy targeting the RPE or photoreceptor cells, which is critical for our DKO model.

Enhanced local expression of sFLT01 significantly decreased the elevated VEGF protein levels but not the transcript levels in the retinal tissues of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. This is expected because sFLT01 neutralizes VEGF protein but has no impact on gene transcription. Physiological levels of VEGF have been demonstrated to be neuroprotective (Gora-Kupilas and Josko, 2005). However, the beneficial effects after sFLT01 mediated neutralization of VEGF indicate that the overexpression of VEGF was probably hazardous to the retinal tissues in this animal model by promoting retinal degeneration in addition to angiogenesis.

AAV.sFLT-1 has been tested for the potential treatment of ocular neovascularization due to its anti-angiogenic features (Lai et al., 2009). In 1991, Rakoczy and her group reported successful retardation or inhibition of VEGF-induced corneal angiogenic changes via the injection of AAV.sFLT-1 into the anterior chamber of the rat (Lai et al., 2001). They also applied rAAV2.sFLT-1 gene therapy for VEGF-induced retinal neovascularization in mice (Lai et al., 2009). Ocular administration of AAV-sFLT-1 slowed the progression of diabetic retinopathy in diabetic rat model by inhibiting the angiogenesis (Ideno et al., 2007). We were unable to evaluate whether the beneficial effects to retinal structure after administration of AAV.sFLT01 were attributed to the blockage of angiogenesis. *Ccl2*^{-/-}/*Cx3cr1*^{-/-} has a broad spectrum of retinal lesions including approximately 15% incidence of subretinal neovascularization (Tuo et al., 2007). However, with currently available techniques, we are not able to determine whether subretinal neovascularization will develop before the mice are grouped. Random grouping would require a much large number of mice to achieve enough statistical power.

Given the role of oxidative stress and inflammation in the retinal tissues of AMD patients as well evidence collected in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (Cao et al., 2010; Herzlich et al., 2009; Ross et al., 2008; Tuo et al., 2009), we sought to measure the changes in the ocular expression of *Tnf-α*, *Il-6*, *Pon2*, *Gpx3* and *iNos* when AAV-sFLT01 treatment was incorporated. We examined the expression of *Chop*, a chaperone gene, because an altered endoplasmic reticulum/chaperone system was proposed as one of the mechanisms for the formation of retinal lesions in this mouse model (Cao et al., 2010; Herzlich et al., 2009; Ross et al., 2008; Tuo et al., 2009). Moreover, we reported an upregulation of *Vegf* in the retinal tissues of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice through PPAR (Herzlich et al., 2009; Ma et al., 2009). We and others have reported that microglial activation is involved in the formation of retinal lesions in several animal AMD models (Combadiere et al., 2007; Ross et al., 2008). We evaluated *Cxcl12* and *Cxcr4* transcription because they were reported to be correlated with microglial activation (Shimoji et al., 2009).

Among those tested, only *iNOS* was significantly down-regulated after the treatment. We observed a relationship between VEGF level, ERK phosphorylation and iNOS level in this study. It is known that the activation of ERK signaling pathway stimulates the iNOS expression (Caivano, 1998; Zhang et al., 2009), and that VEGF activates three mitogen-activated protein kinases including ERK by phosphorylation (Kroll and Waltenberger, 1997; Rousseau et al., 2000; Son et al., 2009). The retinal degeneration of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice might be related to ERK activation by VEGF-ligand binding, followed by an increase in iNOS expression. This pathway can be, at least partly, blocked by VEGFA trapping (Figure 7).

Our data did not support a direct link between A2E and the VEGF-ERK-iNOS pathway. However, as a major component of lipofuscin, A2E is generated by a series of oxidative

stress mediated redox reactions (Sparrow and Boulton, 2005) and is high in our mice (Chan et al., 2008; Tuo et al., 2007). An *in vitro* study has shown that A2E is an endogenous ligand for retinoic acid receptor and induces VEGF expression (Iriyama et al., 2008). An *in vivo* study has shown that subretinal injection of A2E induces RPE cell apoptosis and concomitant upregulation of VEGF in RPE and choroid (Iriyama et al., 2009). Subretinal injection of AAV5.sFLT01 decreases retinal VEGF levels, which subsequently decreased ERK activation and iNOS levels, thereby improving the retinal lesions. The lower iNOS levels causes less oxidative stress, which consequently decreases A2E production. The overall decrease in retinal lesions also results in lower A2E levels, which we have observed in this study (Figure 7).

In summary, subretinal injection of AAV5.sFLT01 to trap excess VEGFA either stabilizes or arrests the progression of retinal lesions in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. The changes in VEGFA, ERK phosphorylation and iNOS in the retinal tissues suggested the involvement of reactive nitrogen species in the retinal lesions. The findings indicate the potentially beneficial effects associated with sFLT01 gene therapy for AMD patients in terms of oxidative stress control in addition to angiogenesis.

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References

- Basu A, Hoerning A, Datta D, Edelbauer M, Stack MP, Calzadilla K, Pal S, Briscoe DM. Cutting edge: Vascular endothelial growth factor-mediated signaling in human CD45RO+ CD4+ T cells promotes Akt and ERK activation and costimulates IFN-gamma production. *J Immunol.* 2010; 184:545–549. [PubMed: 20008289]
- Ben-Shabat S, Parish CA, Hashimoto M, Liu J, Nakanishi K, Sparrow JR. Fluorescent pigments of the retinal pigment epithelium and age-related macular degeneration. *Bioorg Med Chem Lett.* 2001; 11:1533–1540. [PubMed: 11412975]
- Buch PK, Bainbridge JW, Ali RR. AAV-mediated gene therapy for retinal disorders: from mouse to man. *Gene Ther.* 2008; 15:849–857. [PubMed: 18418417]
- Caivano M. Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. *FEBS Lett.* 1998; 429:249–253. [PubMed: 9662426]
- Cao X, Liu M, Tuo J, Shen D, Chan CC. The effects of quercetin in cultured human RPE cells under oxidative stress and in *Ccl2/Cx3cr1* double deficient mice. *Exp Eye Res.* 2010; 91:15–25. [PubMed: 20361964]
- Carter BJ. Adeno-associated virus vectors in clinical trials. *Hum Gene Ther.* 2005; 16:541–550. [PubMed: 15916479]
- Chan CC, Ross RJ, Shen D, Ding X, Majumdar Z, Bojanowski CM, Zhou M, Salem N Jr, Bonner R, Tuo J. *Ccl2/Cx3cr1*-deficient mice: an animal model for age-related macular degeneration. *Ophthalmic Res.* 2008; 40:124–128. [PubMed: 18421225]
- Combadiere C, Feumi C, Raoul W, Keller N, Rodero M, Pezard A, Lavalette S, Houssier M, Jonet L, Picard E, Debre P, Sirinyan M, Deterre P, Ferroukhi T, Cohen SY, Chauvaud D, Jeanny JC, Chemtob S, Behar-Cohen F, Sennlaub F. CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration. *Journal of Clinical Investigation.* 2007; 117:2920–2928. [PubMed: 17909628]
- Flannery JG, Zolotukhin S, Vaquero MI, LaVail MM, Muzyczka N, Hauswirth WW. Efficient photoreceptor-targeted gene expression *in vivo* by recombinant adeno-associated virus. *Proc Natl Acad Sci USA.* 1997; 94:6916–6921. [PubMed: 9192666]
- Gehrs KM, Anderson DH, Johnson LV, Hageman GS. Age-related macular degeneration--emerging pathogenetic and therapeutic concepts. *Ann Med.* 2006; 38:450–471. [PubMed: 17101537]

- Gora-Kupilas K, Josko J. The neuroprotective function of vascular endothelial growth factor (VEGF). *Folia Neuropathol.* 2005; 43:31–39. [PubMed: 15827888]
- Grieger JC, Samulski RJ. Adeno-associated virus as a gene therapy vector: vector development, production and clinical applications. *Adv Biochem Eng Biotechnol.* 2005; 99:119–145. [PubMed: 16568890]
- Herzlich AA, Ding X, Shen D, Ross RJ, Tuo J, Chan CC. Peroxisome proliferator-activated receptor expression in murine models and humans with age-related macular degeneration. *Open Biol J.* 2009; 2:141–148. [PubMed: 21152244]
- Ideno J, Mizukami H, Kakehashi A, Saito Y, Okada T, Urabe M, Kume A, Kuroki M, Kawakami M, Ishibashi S, Ozawa K. Prevention of diabetic retinopathy by intraocular soluble flt-1 gene transfer in a spontaneously diabetic rat model. *Int J Mol Med.* 2007; 19:75–79. [PubMed: 17143550]
- Igarashi T, Miyake K, Masuda I, Takahashi H, Shimada T. Adeno-associated vector (type 8)-mediated expression of soluble Flt-1 efficiently inhibits neovascularization in a murine choroidal neovascularization model. *Hum Gene Ther.* 2010; 21:631–637. [PubMed: 20053138]
- Iriyama A, Fujiki R, Inoue Y, Takahashi H, Tamaki Y, Takezawa S, Takeyama K, Jang WD, Kato S, Yanagi Y. A2E, a pigment of the lipofuscin of retinal pigment epithelial cells, is an endogenous ligand for retinoic acid receptor. *J Biol Chem.* 2008; 283:11947–11953. [PubMed: 18326047]
- Iriyama A, Inoue Y, Takahashi H, Tamaki Y, Jang WD, Yanagi Y. A2E, a component of lipofuscin, is pro-angiogenic in vivo. *J Cell Physiol.* 2009; 220:469–475. [PubMed: 19418485]
- Jing PJ, Dai X, Boye SE, Barone I, Boye SL, Mao S, Everhart D, Dinculescu A, Liu L, Umino Y, Lei B, Chang B, Barlow R, Strettoi E, Hauswirth WW. Long-term Retinal Function and Structure Rescue Using Capsid Mutant AAV8 Vector in the rd10 Mouse, a Model of Recessive Retinitis Pigmentosa. *Mol Ther.* 2010
- Karan G, Lillo C, Yang Z, Cameron DJ, Locke KG, Zhao Y, Thirumalaichary S, Li C, Birch DG, Vollmer-Snarr HR, Williams DS, Zhang K. Lipofuscin accumulation, abnormal electrophysiology, and photoreceptor degeneration in mutant ELOVL4 transgenic mice: a model for macular degeneration. *Proc Natl Acad Sci USA.* 2005; 102:4164–4169. [PubMed: 15749821]
- Kim YS, Hong SW, Choi JP, Shin TS, Moon HG, Choi EJ, Jeon SG, Oh SY, Gho YS, Zhu Z, Kim YK. Vascular endothelial growth factor is a key mediator in the development of T cell priming and its polarization to type 1 and type 17 T helper cells in the airways. *J Immunol.* 2009; 183:5113–5120. [PubMed: 19786548]
- Kroll J, Waltenberger J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. *J Biol Chem.* 1997; 272:32521–32527. [PubMed: 9405464]
- Lai CM, Brankov M, Zaknich T, Lai YK, Shen WY, Constable IJ, Kovesdi I, Rakoczy PE. Inhibition of angiogenesis by adenovirus-mediated sFlt-1 expression in a rat model of corneal neovascularization. *Hum Gene Ther.* 2001; 12:1299–1310. [PubMed: 11440623]
- Lai CM, Estcourt MJ, Wikstrom M, Himbeck RP, Barnett NL, Brankov M, Tee LB, Dunlop SA, gli-Esposti MA, Rakoczy EP. rAAV.sFlt-1 gene therapy achieves lasting reversal of retinal neovascularization in the absence of a strong immune response to the viral vector. *Invest Ophthalmol Vis Sci.* 2009; 50:4279–4287. [PubMed: 19357358]
- Lai CM, Shen WY, Brankov M, Lai YK, Barnett NL, Lee SY, Yeo IY, Mathur R, Ho JE, Pineda P, Barathi A, Ang CL, Constable IJ, Rakoczy EP. Long-term evaluation of AAV-mediated sFlt-1 gene therapy for ocular neovascularization in mice and monkeys. *Mol Ther.* 2005; 12:659–668. [PubMed: 16023893]
- Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, Cohn L, Kim YK, McDonald DM, Elias JA. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med.* 2004; 10:1095–1103. [PubMed: 15378055]
- Liu MM, Tuo J, Chan CC. Gene therapy for ocular diseases. *Br J Ophthalmol.* 2010 in press.
- Ma W, Zhao L, Fontainhas AM, Fariss RN, Wong WT. Microglia in the mouse retina alter the structure and function of retinal pigmented epithelial cells: a potential cellular interaction relevant to AMD. *PLoS ONE.* 2009; 4:e7945. [PubMed: 19936204]

- Mor F, Quintana FJ, Cohen IR. Angiogenesis-inflammation cross-talk: vascular endothelial growth factor is secreted by activated T cells and induces Th1 polarization. *J Immunol.* 2004; 172:4618–4623. [PubMed: 15034080]
- Narasimhan P, Liu J, Song YS, Massengale JL, Chan PH. VEGF Stimulates the ERK 1/2 signaling pathway and apoptosis in cerebral endothelial cells after ischemic conditions. *Stroke.* 2009; 40:1467–1473. [PubMed: 19228841]
- Pang JJ, Chang B, Hawes NL, Hurd RE, Davisson MT, Li J, Noorwez SM, Malhotra R, McDowell JH, Kaushal S, Hauswirth WW, Nusinowitz S, Thompson DA, Heckenlively JR. Retinal degeneration 12 (rd12): a new, spontaneously arising mouse model for human Leber congenital amaurosis (LCA). *Mol Vis.* 2005; 11:152–162. [PubMed: 15765048]
- Pang JJ, Chang B, Kumar A, Nusinowitz S, Noorwez SM, Li J, Rani A, Foster TC, Chiodo VA, Doyle T, Li H, Malhotra R, Teusner JT, McDowell JH, Min SH, Li Q, Kaushal S, Hauswirth WW. Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis. *Mol Ther.* 2006; 13:565–572. [PubMed: 16223604]
- Paques M, Guyomard JL, Simonutti M, Roux MJ, Picaud S, LeGargasson JF, Sahel JA. Panretinal, High-Resolution Color Photography of the Mouse Fundus. *Invest Ophthalmol Vis Sci.* 2007; 48:2769–2774. [PubMed: 17525211]
- Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow J. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci USA.* 1998; 95:14609–14613. [PubMed: 9843937]
- Pechan P, Rubin H, Lukason M, Ardinger J, DuFresne E, Hauswirth WW, Wadsworth SC, Scaria A. Novel anti-VEGF chimeric molecules delivered by AAV vectors for inhibition of retinal neovascularization. *Gene Ther.* 2009; 16:10–16. [PubMed: 18633446]
- Ross RJ, Zhou M, Shen D, Fariss RN, Ding X, Bojanowski CM, Tuo J, Chan CC. Immunological protein expression profile in Ccl2/Cx3cr1 deficient mice with lesions similar to age-related macular degeneration. *Exp Eye Res.* 2008; 86:675–683. [PubMed: 18308304]
- Rousseau S, Houle F, Kotanides H, Witte L, Waltenberger J, Landry J, Huot J. Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. *J Biol Chem.* 2000; 275:10661–10672. [PubMed: 10744763]
- Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene.* 1990; 5:519–524. [PubMed: 2158038]
- Shimoji M, Pagan F, Heaton EB, Mocchetti I. CXCR4 and CXCL12 expression is increased in the nigro-striatal system of Parkinson's disease. *Neurotox Res.* 2009; 16:318–328. [PubMed: 19551455]
- Smith TC, Lee L. Age related macular degeneration - new developments in treatment. *Aust Fam Physician.* 2007; 36:359–361. [PubMed: 17492074]
- Son SH, Kim MJ, Chung WY, Son JA, Kim YS, Kim YC, Kang SS, Lee SK, Park KK. Decursin and decursinol inhibit VEGF-induced angiogenesis by blocking the activation of extracellular signal-regulated kinase and c-Jun N-terminal kinase. *Cancer Lett.* 2009; 280:86–92. [PubMed: 19307054]
- Sparrow JR, Boulton M. RPE lipofuscin and its role in retinal pathobiology. *Experimental Eye Research.* 2005; 80:595–606. [PubMed: 15862166]
- Tuo J, Bojanowski CM, Zhou M, Shen D, Ross RJ, Rosenberg KI, Cameron DJ, Yin C, Kowalak JA, Zhuang Z, Zhang K, Chan CC. Murine ccl2/cx3cr1 deficiency results in retinal lesions mimicking human age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2007; 48:3827–3836. [PubMed: 17652758]
- Tuo J, Ross RJ, Herzlich AA, Shen D, Ding X, Zhou M, Coon SL, Hussein N, Salem N Jr, Chan CC. A high omega-3 fatty acid diet reduces retinal lesions in a murine model of macular degeneration. *Am J Pathol.* 2009; 175:799–807. [PubMed: 19608872]
- Zhang X, Li H, Feng H, Xiong H, Zhang L, Song Y, Yu L, Deng X. Valnemulin downregulates nitric oxide, prostaglandin E2, and cytokine production via inhibition of NF-kappaB and MAPK activity. *Int Immunopharmacol.* 2009; 9:810–816. [PubMed: 19293003]

Figure 1 (A)

Figure 1 (B)

Figure 1 (C)

Fig. 1. Continuous fundoscopic monitoring

(A) Representative fundoscopic photographs of a pair of eyes in one mouse monitored for 3 months. The eye given AAV5-CBA-sFLT01 (OD) showed fewer retinal lesions than the control eye (OS) after 3 month.

(B) Pairwise plotting of 33 pairs of eyes.

(C) The average scores of retinal lesion in treated and untreated eyes.

Fig. 2. Histological examination

Representative histological micrographs of the retina from a pair of eyes in one mouse: Healthier retinal structure was shown by less photoreceptor loss, less photoreceptor atrophy and a thicker retinal outer layer in the treated eye (right) in comparison to the untreated eye (left) three month after AAV5-CBA- sFLT01 subretinal injection. (hematoxylin & eosin, original magnification, $\times 200$)

Fig. 3. Transmission electron micrographs of outer retina

Left panel: The retina of the untreated eye shows degeneration of RPE and photoreceptors. Right panel: The retina of the treated, contralateral eye tends toward normal organization, structure of RPE and photoreceptors, fewer lipofuscin granules in the RPE cells, better preserved photoreceptors and outer plexiform layer

Fig. 4. Quantification of A2E in eyes

A2E was lower in the retinas of AAV5-CBA-sFLT01 treated eyes (n=7) compared to the untreated eyes (n=7) at 3 months after treatment (* $p < 0.05$).

Figure 5 (A)

*

Figure 5 (B)

Figure 5 (C)

Fig. 5. Gene transcripts in the retina

A: The graph is plotted as mean $\pm$ SD. Among nine genes being measured, only *iNOS* mRNA was found to be lower in AAV5-CBA- sFLT01 treated eyes (n=5) than in untreated eyes (n=5). The data were obtained by quantitative RT-PCR. *: p < 0.05

B: The transcription of *iNos* was further visualized by gel imaging of end-point RT-PCR

C: Quantification of band density of the gel from end-point RT-PCR

Figure 6 (A)

Figure 6 (B)

Figure 6 (C)

Fig. 6. Western blot of retinal tissue lysate

(A) Protein levels of ERK, pERK, iNOS and VEGF in the retinas of normal control and *Ccl2^{-/-}/Cx3cr1^{-/-}* mice

(B) Protein levels of ERK, pERK, iNOS and VEGF in the retinas of AAV5-CBA- sFLT01 treated and untreated *Ccl2^{-/-}/Cx3cr1^{-/-}* eyes

(C) Quantification of band density of Western blot

Fig. 7.

The schematic diagram of the working model

The interaction of various components and pathways in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice after AAV5-CBA.sFLT01 therapy

Table 1

The summary of histological examination on retinal lesion between treated and untreated eyes

Treated/Control	Pair of eyes	Healthier	Similar Scale	Worsened
OD/OS	10	6	2	2