Designing Adenoviral Vectors for Gene Therapy

A dissertation submitted by

John Harry Sweigard

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

August, 2011

ADVISER:
Rajendra Kumar-Singh, PhD

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abstract

The eye represents an isolated and immune privileged region of the body where multiple gene polymorphisms have been identified. Therefore, gene therapy is an attractive therapeutic approach to ameliorate disease. Adenovirus has been used successfully in multiple clinical trials, has a large cloning capacity, does not integrate into genomic DNA, and is capable of expressing for long periods of time. The capsid of adenovirus has a fiber extending from the viral coat with a penton structure at the base of the fiber. These two structures are mainly what determine binding and entry of the virus into cells. Altering the fiber has been a successful way to re-direct the tropism of adenovirus towards the cell type intended for gene therapy.

Loss of RPE cells is characteristic of age related macular degeneration (AMD), the leading cause of blindness in people over 65. A low level but chronic inflammatory response of the innate immune system has been implicated in the progression of AMD. Specifically, activity of the alternative arm of the complement pathway has been observed. Genetic studies have identified that polymorphisms in complement regulatory proteins place individuals at an elevated risk for developing AMD. CD46 is a membrane bound complement regulator that is normally expressed on RPE cells with expression levels reduced in AMD patients.

A limitation to using a membrane bound complement inhibitor for gene therapy in AMD is that protection is limited to the site of expression. This can be overcome by designing

an inhibitor that is secreted from cells. The optimal secreted inhibitor must be highly effective at blocking complement so that protection occurs even when the concentration of inhibitor is low, at sites distant from initial expression. It must also be small enough to readily diffuse through the eye.

Here we describe how we re-directed the tropism of adenovirus to specifically express in photoreceptors, the retinal cell type with the most polymorphisms, by altering the fiber/penton base and using a cell specific promoter. We also describe the first gene therapy approach to treating AMD using CD46 along with the first description of a novel secreted complement regulator.

Chapter 1:

Viral Vectors for Gene Delivery and Current Strategies for Treating Age-related Macular Degeneration

I. Visual Processing

The human eye is a highly specialized structure capable of converting packages of light (photons) into electrochemical energy that is then relayed to the brain and processed into an image. This involves the interplay of a complex yet highly organized network of cells that are capable of capturing, converting, and relaying the signal to the visual cortex of the brain so it can be deciphered into an image.

Anatomy

The cornea is the outermost covering of the eye offering a protective yet sensitive barrier. After passing through the cornea light enters the lens where it is focused onto the retina (figure 1). There it is captured by photoreceptors and converted from photons into electrochemical energy that is eventually perceived as vision. There are two types of photoreceptors designed to capture light, rods and cones. Rods are responsible for vision under low light conditions while cones are adept at determining color and visual acuity. At the center of the retina lies a region of densely packed photoreceptors, mainly cones, referred to as the macula. The majority of light is focused here making it a highly active region for visual processing. At the macula the photoreceptor cell bodies are shifted to the side so light proceeds unimpeded to the photoreceptor outer segments, where it is captured. Additionally, the inner nuclear layers of the retina are clear to facilitate light transmission to the outer segments. The inner nuclear layer contains bipolar and horizontal cells involved in transmitting the action potential generated from the photoreceptors to the optic nerve. Müller cells also reside in the inner nuclear layer and are the resident glial cells of the eye. The retinal pigment epithelium (RPE) is juxtaposed

to the outer segments making it the outer most layer of the retina. The RPE captures any light that is scattered by the photoreceptors. It is also intimately involved in phototransduction.

Phototransduction

Phototransduction is the process of converting light energy into chemical energy and is accomplished mainly by the photoreceptors[1]. Photons enter the outer segment discs of the photoreceptors where they initiate the isomerization of 11-cis-retinal, a chromophore covalently bonded to the photoreceptor G-coupled protein rhodopsin[2-5]. The 11-cis retinal is converted to all-trans retinal then finally to all-trans retinol. The all-trans retinol diffuses to the RPE where enzymes convert it back into 11-cis-retinal so that it can be taken back in to the outer segments to regenerate rhodopsin.

Activated rhodopsin further activates the G-coupled protein transducin. The alpha subunit of transducin activates cGMP-phosphodiesterase which hydrolizes cytoplasmic cGMP[6-8]. This causes closure of cation channels on the plasma membrane and the photoreceptor cells become hyperpolarized, releasing glutamate at their synapses[9-12]. Bipolar cells respond to the glutamate by generating an action potential that is transmitted to the ganglion cells and relayed to the optic nerve[13-17]. The optic nerve terminates at the lateral geniculate nucleus of the thalamus and from there the signal is sent to the visual cortex for image processing[18].

II. Genetic diseases originating in distinct retinal cell layers

Since the eye is a highly integrated system loss of any single cell type can impede vision. Many diseases that lead to vision loss have been linked to polymorphisms in genes that

play a critical role in specific cell types of the retina. Whether the cell plays a direct or indirect role in phototransduction the end result of the defect is often blindness. Below is a brief description of the more commonly known polymorphisms found in several cell layers of the retina.

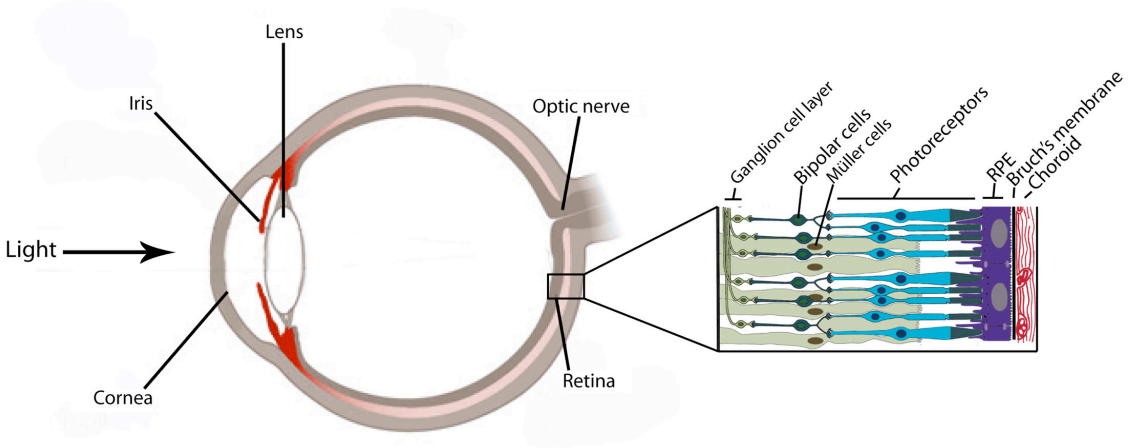


Figure 1: Anatomy of the eye

Illustrated cross section of a human eye showing the path light follows. The boxed region of the retina is enlarged to illustrate the retinal cell layers.

Photoreceptors

In the photoreceptors, the principle cell type involved in phototransduction, multiple polymorphisms either in the same or different genes have been identified that lead to vision loss. The majority of these polymorphisms, over 100 to date (retnet.com), have

been mapped to the rhodopsin gene. Since rhodopsin plays a critical role in the initiation of phototransduction loss of function either results in lack of photoreceptor outer segment formation or progressive degeneration[13, 19-22]. Peripherin is another gene found in photoreceptors that is involved in phototransduction, acting as a cGMP phosphodiesterase. Several point mutations have been identified, mainly in the promoter region, that lead to photoreceptor degeneration[23-32]. Peripherin 2 is a protein found in the rim of the flattened discs of the photoreceptor outer segments. It has 4 membrane spanning domains, tetraspanin, and serves as an adhesion molecule to maintain the morphology of the outer disc. Several mutations have been shown to either lead to the inability to form outer segments or to the formation of highly disorganized structures[33-36].

Müller cells

In the late 1990's a detailed family history of blindness linked a genetic defect in müller cells to inner limiting membrane dysfunction, later termed müller cell sheen dystrophy[37]. A critical function of the müller cells is to synthesize the collagenous inner limiting membrane found on the anterior most portion of the retina. Traditionally, müller cells have been considered the resident glial cells of the eye, responding to injury and providing neurotrophic support. More recently, they have been shown to have the ability to dedifferentiate into multipotent progenitor cells allowing them to replace dying photoreceptors[38]. While müller cells have not been shown to play a direct role in phototransduction their loss critically affects vision.

RPE cells

One of the most well studied mutations in the RPE cells is found in the RPE65 gene. RPE65 is a protein that serves as the palmitoyl donor for lecithin retinol acyltransferase (LRAT), the enzyme involved in converting vitamin A to all trans retinol. A mutation in RPE65 prevents the regeneration of rhodopsin, impeding phototranssduction. This leads to the clinical disease Leber's congenital amaurosis (LCA) and will be talked about in more detail in the next section[39, 40].

III. Ocular gene therapy using viral vectors

Because of the multitude of genetic mutations that have been linked to eye diseases the prospect of delivering the correct copy of the gene is a promising therapeutic. Additionally, the eye represents a unique opportunity for gene therapy as it is compartmentalized and an immune privileged region of the body. Evidence for the efforts to develop ocular gene therapy approaches can be seen through the multiple ongoing clinical trials (clinicaltrials.gov) currently underway. At this moment the most widely accepted mode of delivery in the clinic are viral vectors, as seen by the choice of vectors used in clinical trials. Efforts have been made to use non-viral vectors to deliver DNA using lipofection, electroporation[41] and more successfully the use of nanoparticles to carry compacted DNA into retinal cells[42]. While these approaches are appealing since they circumvent the potential hazards associated with viral vectors their clinical application is still in its infancy. Therefore, below is a summary of the most current and clinically relevant viral vectors along with some of the benefits and drawbacks associated with each.

<u>Lentivirus</u>

Lentivirus is able to offer stable long-term gene expression[43], a major advantage to using this vector. The reason this is achieved is due to integration into genomic DNA. Therefore, serious safety concerns must be considered to prevent insertional oncogenesis. Several advances have been made towards this end, including self-inactivating[44] and non-integrating[45] lentivirus. While these approached seem promising a complete safety evaluation has not yet been conducted. At this point lentivirus is a great research tool but likely needs to be refined before clinical application.

Adeno-associated virus (AAV)

AAV is one of the most commonly used viral vectors to date. This is primarily due to several safety advantages that it offers[46]. AAV is non-integrating, eliminating concerns for insertional oncogenesis[47]. Even though it remains as extragenomic DNA gene expression is stable and long-lasting. One sub-retinal injection has been shown to last for more than 5 years in dogs[48], the longest time point examined to date. Furthermore, it has the ability to transduce a variety of cell types in the eye. More recently, it has been shown that cell transduction can be somewhat directed by pseudotyping the capsid, changing the tropism within the retina[49]. The clinical efficacy of using AAV has been observed, in 2009, by the success of delivering the correct copy of the RPE65 gene to LCA-2 patients[50]. Aside from a clean safety profile significant vision improvements were observed in nearly all patients. While this was a clinical success the major limitation of using AAV for gene therapy is that the cloning capacity is limited to only 4.2kb. Therefore, the use of AAV is restricted to relatively small genes.

Adenovirus

Several major benefits of adenovirus are that it does not integrate into DNA, it is able to transduce a variety of dividing and non-dividing cell types, and has a large cloning capacity[51, 52]. There have been many generations of adenovirus developed that mainly include variations on the amount of adenoviral genetic material that has been deleted. The main limiting factor for adenovirus is that it is recognized by the host immune system. The immune response is believed to be the cause for the relatively short-lived episomal gene expression, usually of several months. Although, recombinant adenoviral constructs have been developed that have been shown to persist in rodents for at least one year (latest time point examined)[53] or potentially for the "lifetime" of the animal[54]. In addition, helper-dependent adenovirus vectors can accommodate transgene expression cassettes up to 36 Kb[51, 55]. This is a major benefit and it allows for the expression of multiple transgenes as well as the inclusion of native gene regulatory elements. Gene transfer approaches to ocular tissues using adenovirus as the gene delivery vector have been found to be safe in a number of clinical trials[56, 57]. Campochiaro performed phase 1 clinical trials in wet AMD using adenovirus expressing pigement epithelial derived factor (PEDF)[58]. There was slight transient inflammation but reduction in choroidal neovascularization (CNV) was seen in all treated eyes. There were no safety issues but efficacy could not be evaluated because negative control subjects were not used. Conditionally replicating Ad coupled with a tumor specific promoter to deliver tyrosine kinase (TK) specifically to retinoblastoma cells has been generated[59]. This may be a promising alternative to patients that have failed surgical removal and chemotherapy for retinoblastoma.

IV. Designing vectors for targeting specific retinal layers

As the safety profile of viral vectors continues to improve and with so many genetic mutations occurring in specific ocular cell types the next goal for vector development is to tailor transduction to only those cells in need of the correct gene copy. For the benefits listed above adenovirus is a well-suited vector to use for cell targeting.

Adenoviral structure

The adenoviral structure consists of double stranded DNA that is surrounded by a protective capsid. Aside from protecting the DNA the capsid also serves as a means to direct viral entry into host cells. The capsid has projections (fibers) that extend from the surface that facilitate binding to specific receptors on the surface of host cells. The fiber consists of an RGD (argenine, glycine, aspartic acid) motif at the base and a knob at the apex. By design the knob is the first structure to contact the host cell. In doing so it recognizes specific receptors for initial binding. The RGD domain at the base of the fiber then interacts with integrins and pulls the capsid close to the surface of the cell[60-64]. Internalization of the viral particle is accomplished through the formation of clathrin-coated pits[65]. Once inside the cell the pH within the vessicle causes disassembly of the capsid releasing the viral DNA. The DNA is then transported to the nucleus by binding to the actin cytoskeleton and trafficked by actin motors (Fig. 2).

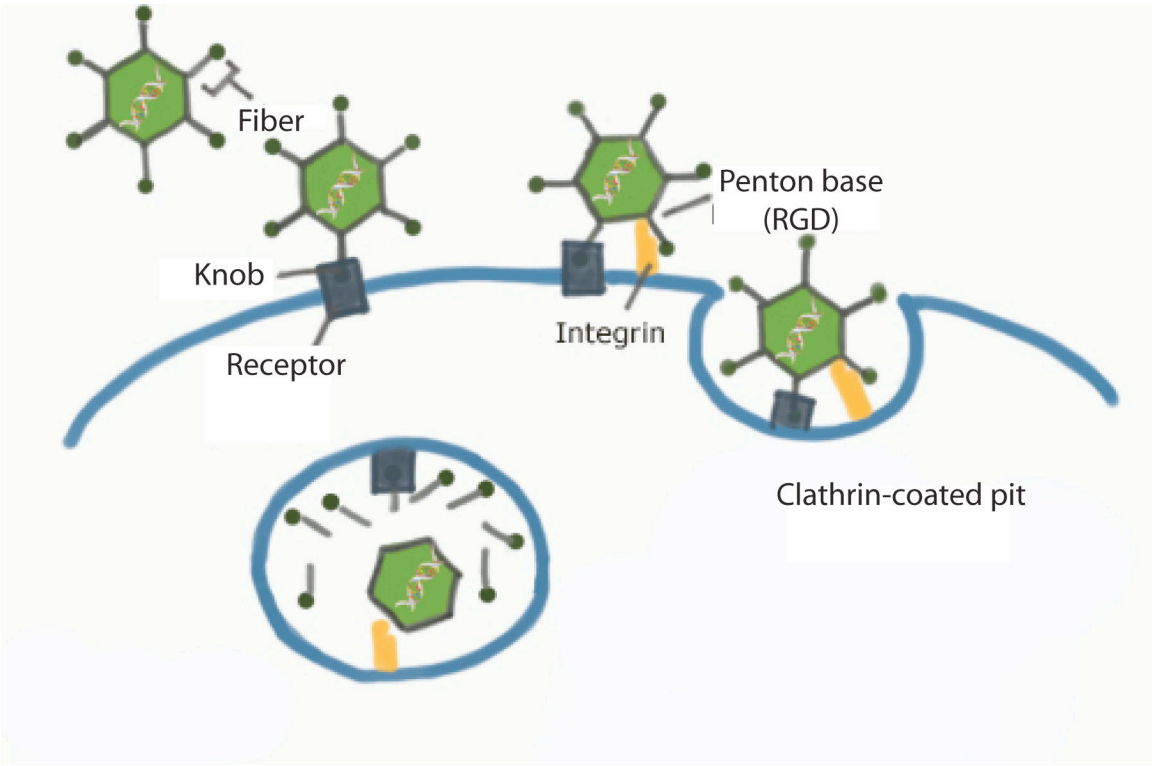


Figure 2: Adenovirus binding and entry into host cell

Adenoviral targeting

What has become more evident is that there are different serotypes of adenovirus that exist. The main difference among serotypes lies in structural variations of the fiber. This results in the use of different receptors for initial viral binding and cell entry[66]. The most commonly used adenovirus for gene therapy is adenovirus serotype 5 (Ad5). Ad5 infects cells by initial binding of the Ad fiber protein to the membrane associated coxsackie adenovirus receptor (CAR). In contrast, Ad35 and Ad37 fibers have been shown to interact with CD46 and sialic acid, respectively[67, 68]. Therefore, psuedotyping the Ad5 virus with a fiber from another serotype is one way to direct the tropism of adenovirus to cell types that express high levels of the respective receptor for each fiber. Another way to redirect tropism is by taking advantage of the integrin

interaction between the RGD domain. Interrupting RGD binding to integrins limits viral entry in cells that express high levels of integrins allowing more 'free' viral particles to enter neighboring cells. The layers of cells in the retina each express different levels of the receptors used by each adenoviral vector. Therefore, a way to direct the tropism to specific cells would be to exchange the fiber to one that binds to the receptor expressed at the highest levels on the cell type desired. Afterwards, specificity can be finely tuned by using a cell specific promoter.

V. Age-related macular degeneration (AMD)

Pathogenesis

As improvements in medical care allow the population to live longer the incidence of age-related diseases is rising. One disease affecting the aging population is age-related macular degeneration (AMD)[69]. There are two types of AMD termed 'wet' and 'dry'. Roughly 15% of AMD patients have the wet form of the disease. This is associated with the growth of new blood vessels that penetrate from the vascular choroid into the retina. When this happens vision loss is rapid, over the course of 2-3 years. However, the majority of AMD patients, 85%, have the dry form. In dry AMD new vessel growth does not occur but geographic atrophy begins to develop leading to progressive vision loss. This may happen over the course of 10-15 years, during which performing daily tasks becomes more difficult and often requires assisted living. One common feature to both wet and dry AMD is the presence of extra-cellular deposits in the back of the eye termed 'drusen'[69]. Drusen are considered the hallmark pathological feature defining AMD (Fig. 3A). They are composed of a variety of proteins and lipids and form between the

RPE cell layer and the Bruch's membrane[70]. At this point it is not yet clear whether drusen precede AMD or whether they are a manifestation of the disease. What is known is that they are present in all confirmed AMD diagnosis.

Risk factors

As the name suggests the biggest risk factor for developing AMD is age. While only 2% of Americans are at risk for developing AMD in their 50's the incidence increases to 30% for those between the age of 75 and 85. Other risk factors include smoking, cardiovascular disease, high blood pressure, family history, and obesity[71-73]. Many of these risk factors can be managed through life style changes but one that cannot is family history. There is a growing body of evidence linking genetic polymorphisms to AMD. The majority of these polymorphisms are found in proteins that are involved in the complement component of innate immunity[74]. Evidence for this comes from genetic association studies, complement proteins levels in peripheral blood, and immunohistochemical studies. One of the strongest pieces of evidence for linking AMD to complement activation comes from a genome wide screen where the results reveal there is a 7.4 fold increase in risk of developing AMD for individuals that have a tyrosine to histidine change at amino acid 402 of factor H, a protein involved in regulating complement activity[75].

Complement activation in AMD

The complement system is a part of innate immunity, the most evolutionarily conserved aspect of the immune system. Rather than generating antibodies like the adaptive immune system it is designed to respond immediately by discriminating between the host and an invading pathogen. The complement system is divided into 3 arms; lectin,

classical, and alternative. The lectin arm recognizes specific carbohydrate motifs unique to pathogens, the classical recognizes antigens presented on the cell surface, and the alternative arm is constantly 'ticking' over by hydrolysis. When one or several arms of complement become activated a series of proteolytic events occur among the complement proteins, normally present in blood serum. The cleavage products deposit on the cell surface, ultimately leading to the formation of a lytic pore termed a membrane attack complex (MAC). When enough MAC has deposited on a pathogen the membrane becomes permeabilized, resulting in lysis. Although each arm of the complement system is initiated by a different mechanism they all converge at a central C3 convertase. The C3 convertase acts as an enzyme capable of actively cleaving C3, the starting product of the alternative pathway. Therefore when C3 convertase forms the alternative arm is no longer ticking over through hydrolysis but becomes actively engaged[76]. Mammals have evolved several proteins that are capable of preventing complement activation on the surface of their own cells so that only the invading pathogen is destroyed. These proteins act at distinct steps of the complement cascade with the key regulators being factor H, CD46, CD55, complement receptor I, and CD59 (Fig. 3B).

The current model for AMD progression suggests that drusen are recognized by the complement system as a foreign pathogen. This causes activation of complement on and around drusen. Since drusen are located between the RPE cell layer and the collagenous Bruch's membrane this low level yet chronic activation of complement eventually overcomes the regulators that are found on host RPE cells causing them to succumb to lysis. IHC studies looking at complement cleavage products reveal they are mainly associated with the alternative pathway suggesting it is over-active in AMD[57, 77].

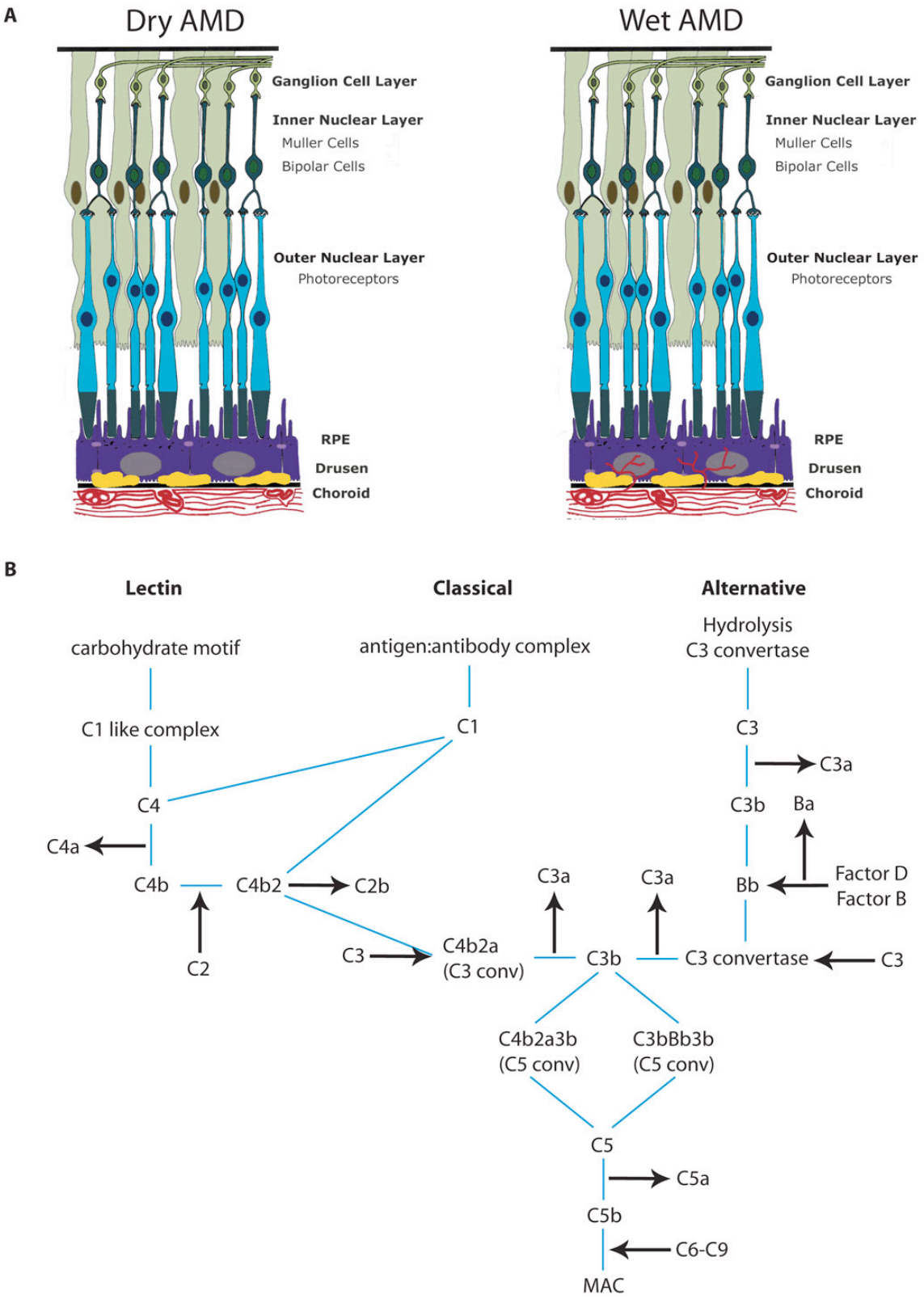


Figure 3: A) Dry and wet AMD. B) Complement cascade.

Treatment options

Currently there are several treatment options available for wet AMD, the most invasive type of AMD. The first approved pharmacologic therapy is photodynamic therapy (PDT), showing modest results in the clinic[78]. Although, the most success to date for treating wet AMD has come from the advent of anti vascular endothelial growth factor (antiVEGF) treatment. Several mechanisms have been used to block VEGF including the use an RNA aptamer (Mucagen)[79], antibody fragments (lucentis), and full-length antibodies (avastin)[80]. The anti VEGF therapies have shown great success in the clinic with nearly all patients either maintaining or improving vision following treatment[81]. New formulations and combination therapies are underway in an attempt to improve upon what has already been a breakthrough for treating wet AMD.

The majority of AMD patients, 85%, have the dry form. While dry AMD is less invasive the impact it has on quality of life can be just as damaging. At this point, unfortunately, there are no treatment options available. Due to the unequivocal link between complement activation and AMD progression there are several emerging products in clinical trials that aim to block complement. POT-4 (Potentia pharmaceuticals/Alcon) is a synthetic analogue of compstatin, an inhibitor of the complement protein C3. It is delivered by injection into the vitreous. Eculizumab (Solaris, Alexion pharmaceuticals) is a monoclonal antibody that binds to C5 and is delivered by intravenous infusion. ARC1905 (Ophthotech corporation) is an aptamer that binds to C5. It is currently in clinical trials as a combination therapy used with the anti VEGF agent ranibizumab. FCFD4514S (Genentech/Roche) is an antibody fragment that inhibits factor D from forming the C3 convertase[74].

VI Summary and strategy for developing adenovirus vectors for retinal gene therapy and treating AMD

The aim of the following studies was to address two specific areas in order to move the field of ocular gene therapy forward; 1) To develop adenoviral vectors that are able to tranduce specific cell layers within the retina and 2) Design and test an anti-complement based therapy for potentially treating AMD using a gene therapy approach. Below is a summary of the reasoning and approach taken to accomplish each goal based on the background provided in the previous paragraphs.

Adenoviral targeting in the retina

The capsid of adenovirus has a fiber extending from the viral coat with a penton structure at the base of the fiber. These two structures are mainly what determine binding and entry of the virus into cells. Two different fibers (serotypes) and one virus with a deletion in the penton base have been reported with enhanced transduction of photoreceptors[82]. These studies cannot be compared because each was carried out using a different transgene, promoter, or detection method. We have compared each of these vectors under the same conditions. Additionally, we used the most appropriate vector for photoreceptors and introduced a cell specific promoter to demonstrate that expression can be refined to only one cell type.

CD46 as a potential gene therapy for AMD

Loss of RPE cells is characteristic of age related macular degeneration (AMD), the leading cause of blindness in people over 65[83]. The hallmark feature of AMD is drusen, composed mainly of lipids and proteins, located near the RPE and Bruch's

membrane. A low level but chronic inflammatory response of the innate immune system has been implicated in the development of AMD. Specifically, activity of the alternative arm of the complement pathway has been observed. Genetic studies have identified that polymorphisms in complement regulatory proteins place individuals at an elevated risk for developing AMD[75, 84-92]. CD46 is a complement regulatory protein that has been shown to have an affinity for regulating the alternative arm of complement. In humans, it is normally expressed on the basal and lateral surface of RPE cells, placing it near the site of alternative pathway activity[93]. Importantly, decreased expression of CD46 has been observed in AMD patients[115]. While a direct link has not been established between CD46 and AMD it has been implicated in the development of other immune diseases, such as atypical haemolytic uraemic syndrome (aHUS)[94, 95]. In aHUS, polymorphisms in CD46 lead to kidney failure due to disregulated activity of the alternative pathway. Restoring wild type CD46 has been an effective way to treat aHUS. Therefore, we delivered CD46 to the RPE using adenovirus to dampen activation of the alternative complement pathway hence providing a possible means for slowing the progression of AMD.

Designing a novel inhibitor of complement

A limitation to using a membrane bound complement inhibitor for gene therapy in AMD is that protection is limited to the site of expression. One way to circumvent this limitation by using a complement regulator that is secreted so that protection is extended away from the initial site of expression. There are two main obstacles that need to be overcome in developing an effective secreted complement regulator: 1) Because the majority of the complement cascade is occurring on the surface of cells it is more

efficient to have the regulator located on the membrane, placing it at the site of activity. Therefore, the ability of the soluble inhibitor to block the cascade must be enhanced so that less inhibitor is needed to have the same effect. 2) The size of the protein must be small enough so that the ability for diffusion in the eye is not hindered. With these limitations in mind we designed a novel protein that contains the functional complement regulatory domains of CD46, CD55, and CD59. Each of these complement regulators blocks activation at a different part of the cascade. Therefore, the effectiveness of the protein should be enhanced since it has the functional capability of 3 complement inhibitors. We have previously confirmed the efficacy of CD46, CD55, and CD59 individually as 'membrane' bound regulators. Furthermore, several studies have identified the essential binding regions in each of these for complement regulation. Therefore, as a means to minimize the size of the protein we designed a truncated chimeric protein that contains the minimum functional domain of each regulator. We then tested the efficacy of the novel protein at blocking complement activity first *in-vitro* and ultimately in an *in-vivo* model.

The following chapters describe the detailed studies along with any publications that were a result.

Chapter 2:

Adenovirus Vectors Targeting Distinct Cell Types in the Retina

J. Harry Sweigard[1], Siobhan M. Cashman[2], and Rajendra Kumar-Singh[1,2]

Published

Investigative Ophthalmology and Visual Science, 2010 Apr;51(4):2219-28

[1]Program in Cell, Molecular and Developmental Biology

Tufts Sackler School of Graduate Biomedical Sciences, 136 Harrison Avenue, Boston, Massachusetts 02111, USA.

[2]Department of Ophthalmology

Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111, USA.

2.1 Abstract

Gene therapy for a number of retinal diseases necessitates efficient transduction of photoreceptor cells. Whereas adenovirus (Ad) serotype 5 (Ad5) does not transduce photoreceptors efficiently, prior studies have demonstrated improved photoreceptor transduction by Ad5 pseudotyped with Ad35 (Ad5/F35) or Ad37 (Ad5/F37) fiber or by the deletion of the RGD domain in Ad5 penton base (Ad5ΔRGD). However, each of these constructs contained a different transgene cassette, preventing the evaluation of the relative performance of these vectors- an important consideration prior to the use of these vectors in the clinic. The aim of this study was to evaluate the above vectors in the retina and to attempt photoreceptor-specific transgene expression.

2.2 Introduction

Loss of any one of the multiple cell types found in the retina can lead to blindness, the second most feared condition after cancer amongst Americans. The human retina is a laminated tissue comprised of light-sensitive photoreceptors in the outer retina that modulate current with a complex array of inner retinal neurons. The photoreceptors rely on the juxtaposing retinal pigment epithelium (RPE) for generation and recycling of *retinal* (Vitamin A), the chromophore that absorbs light to initiate the phototransduction cascade that is eventually perceived as vision. Retinal neurons are supported by müller glia, which provide critical support for maintenance and functioning of the retina.

Loss of RPE cells is characteristic of age related macular degeneration (AMD), the most common cause of blindness in elderly individuals in the United States[96]. Loss of photoreceptors may lead to retinitis pigmentosa (RP), the most common cause of inherited blindness in the working population, affecting approximately 1 in 3000 individuals[97]. The rare hereditary disease referred to as müller cell sheen dystrophy is due to primary müller cell dysfunction[98]. Hence, there is a substantial need for gene transfer vectors that effectively transduce each of the different cell types in the retina. To date, all gene transfer vectors injected in the outer retina, in a region referred to as the sub-retinal space, infect primarily the RPE. However, through vector modifications, such as pseudotyping or deletion of integrin binding motifs in the viral capsids, other cell types in the retina such as photoreceptors may also be transduced, although still not as effectively as the RPE.

Human adenovirus (Ad) has been previously utilized in two clinical trials for eye diseases without any serious adverse events[56, 58]. Short-term transgene expression typically observed from Ad vectors may be overcome by the use of the helper-dependent Ad vectors[51, 52, 55]. Therefore, a clear understanding of the tropism of different Ad serotypes in the retina would greatly enhance the progression of Ad-mediated ocular gene therapy studies. Ad serotype 5 (Ad5) infects cells by initial binding of the Ad fiber protein to the membrane associated coxsackie adenovirus receptor (CAR), followed by internalization of the virus via an interaction between the RGD motif in Ad penton base with $\alpha_v\beta_{3/5}$ integrins[61, 63, 99]. In contrast, Ad35 and Ad37 fibers have been shown to interact with CD46 and sialic acid, respectively[67, 68]. Previously it has been shown that transduction of photoreceptors can be enhanced either by deletion of the RGD motif in the penton base or by replacement of the Ad5 fiber with that of Ad35 or Ad37[82, 100, 101].

While each of the above studies described improved photoreceptor transduction, the data from these studies cannot be directly compared due to the different genes, promoters, and/or transgene detection methods utilized. Hence, the relative performance between these vectors is still an unanswered and relevant question for the advancement of Ad-mediated retinal gene therapy. One goal of the current study was to compare the performance of these different Ad vectors under conditions that permit their direct comparison in the retina, i.e. the use of the same transgene and promoter combination as well as a similar detection method for the presence of the gene product. A second aim of this study was to utilize the most potent vector identified herein to attempt photoreceptor

specific transgene expression in the retina through the use of cell-specific promoters. Significant photoreceptor-specific transgene expression has not thus far been described using Ad vectors. Our results and conclusions differ significantly from prior studies and hence highlight the need for continued evaluation of Ad tropism in the retina prior to their advancement into the clinic.

2.3 Results

Ad Vectors

In order to compare Ad tropism in the retina, we investigated the performance of four different Ad vectors, each of which expressed GFP under the control of a chicken beta actin promoter/CMV enhancer/rabbit globin intron. We have previously demonstrated that this promoter yields significantly greater transgene expression in all layers of the retina and RPE relative to the CMV promoter[82]. In each vector, the transgene expression cassette was cloned in the anti-sense orientation with respect to the deleted E1 region. Each vector backbone was based on Ad5. Two of the vectors were modified either by replacement of the Ad5 fiber with that of Ad35 or Ad37, and in the third vector the RGD (Arg-Gly-Asp) motif in the Ad5 penton base was deleted (Fig. 4A). Hereafter each vector is referred to as Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD, respectively. In order to confirm expression and incorporation of the modified fibers, denatured samples of purified virus preparations were immunoblotted using an antibody raised against the first 17 amino acids at the N terminus of the Ad5 fiber. The immunoblots correlated with the predicted sizes according to amino acid sequence analysis for the fiber monomers of the denatured samples of Ad5 (64kDa), Ad5/F35 (35kDa), and Ad5/F37 (38kDa) (Fig.

4B). Trimerization of the fibers was observed in the samples that were not denatured for western analysis and also correlate to the predicted sizes for Ad5 (192kDa), Ad5/F35 (105kDa), and Ad5/F37 (114kDa).

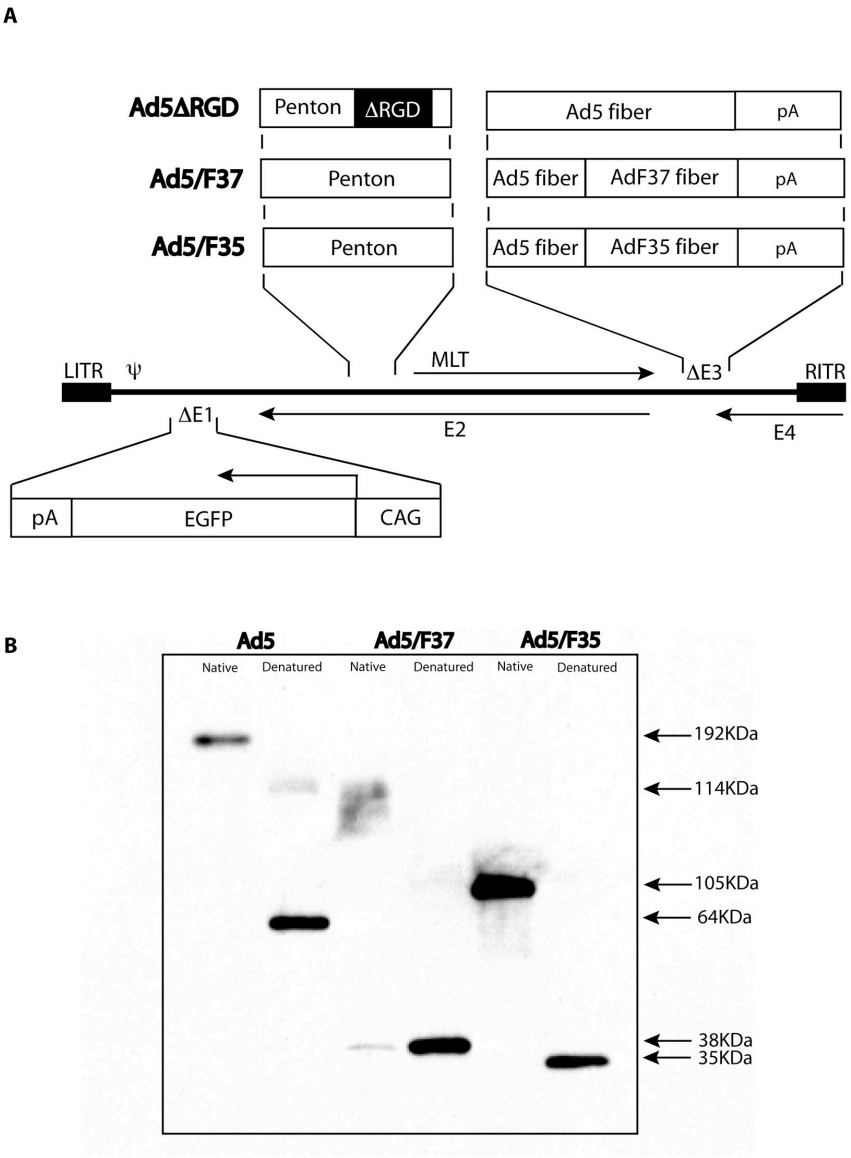


Figure 4: Structure of Ad5ΔRGD, Ad5/F37, and Ad5/F35.

(A)The RGD domain is deleted in the penton base of Ad5ΔRGD. The Ad5/F37 hybrid fiber is composed of the first 17 amino acids of the Ad5 fiber followed by amino acids 18-365 of the wild-type Ad37 fiber and a BGH pA signal. The Ad5/F35 hybrid fiber is composed of the first 44 amino acids of the Ad5 fiber followed by amino acids 132-991 of the wild type Ad35 fiber and the Ad5 pA signal (aa 32775-33651). An EGFP expression cassette was cloned in the antisense orientation into the E1 region. Expression is driven by a chicken beta actin promoter/CMV enhancer/rabbit globin intron (CAG). (B) Western analysis of the native and denatured fiber proteins of Ad5, Ad5/F37, and Ad5/F35 reveal the expected trimer/monomer band sizes of 192/64, 114/38, and 105/35 KDa, respectively, using a monoclonal antibody to the retained N-terminus of the Ad5 fiber. (ΔRGD=deleted amino acids argenine, glycine, aspartic acid; pA= poly A tail;

LITR=left inverted terminal repeat; RITR=right inverted terminal repeat; Ψ=packaging signal; MLT=major late transcript; E1-E4=early regions 1-4)

Ad5/F35 Vector

Prior to examination of the tropism of each of the above vectors in the retina, we confirmed that each vector binds its respective receptor in cell culture. In order to examine whether our novel Ad5/F35 vector was redirected from CAR to CD46, we infected murine HEPA 1c1c7 cells with either Ad5 or Ad5/F35. The multiplicity of infection (MOI) for each vector was adjusted to attain a GFP-positive cell population of approximately 10%. For Ad5 this was achieved at an MOI of 20 whereas for Ad5/F35 an MOI of 750 was necessary for the same transduction frequency. When murine HEPA 1c1c7 cells were pre-transfected with a cassette expressing human CD46, infection by the Ad5/F35 virus increased by nearly 3 fold relative to pre-transfection with a control plasmid, that expresses LacZ. In contrast, a decrease in infection by the Ad5 virus was observed when cells were pre-transfected with human CD46 relative to Lac Z (Fig. 5A). We also noted that the process of transfection alone (with a control plasmid) was sufficient to enhance infection by both viruses but transfection with CD46 had a significantly greater effect on Ad5/F35 than on Ad5.

Ad35 fiber-binding has previously been shown to occur through the short consensus repeat 1 (SCR1) and 2 (SCR2) region of CD46[102]. In order to examine the binding of Ad5/F35 to SCR1/2, we pre-incubated murine HEPA 1c1c7 cells with the antibody MEM258, which is known to bind SCR1. Rabbit anti sheep IgG was used as a non-specific control. As above, cells were also pre-transfected with either a human CD46 or lac Z-expressing plasmid. We found that whereas there was no significant change in Ad5

infection following blocking of CD46 with MEM258, Ad5/F35 infection was reduced by more than 2 fold (Fig. 5B). A detailed characterization of the Ad5/F37 virus utilized in this study and specifically its ability to utilize sialic acid as a receptor has been described by us previously[100].

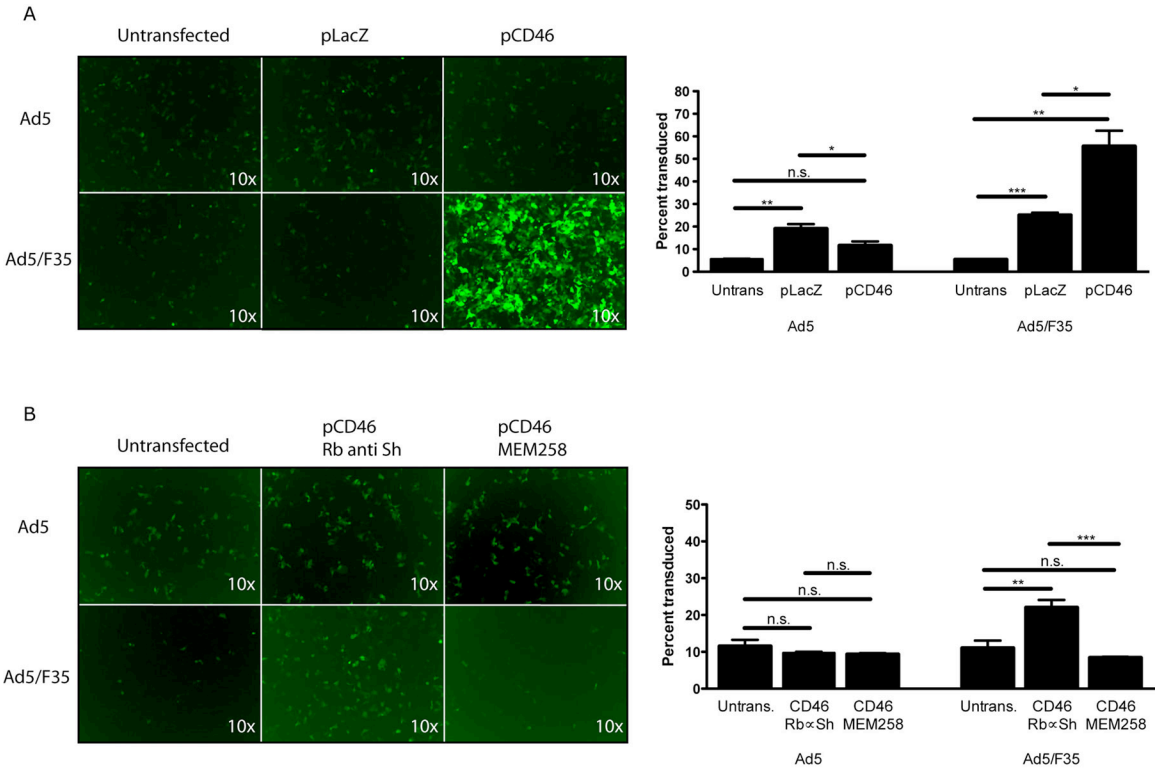


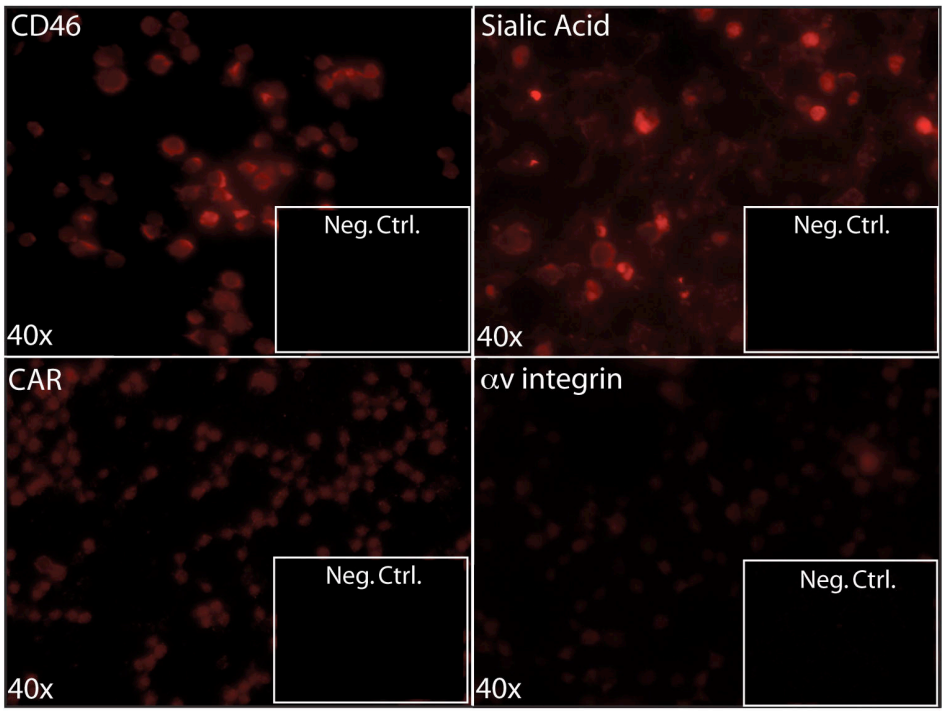
Figure 5: CD46 expression enhances Ad5/F35 transduction of cells.

(A) Transfecting HEPA 1c1c7 cells with a plasmid expressing CD46 improved transduction of Ad5/F35 but not Ad5. (B) Pre-transfection of HEPA 1c1c7 cells with a plasmid that expresses CD46 followed by blocking with an antibody that binds to the SCR1 extracellular domain of CD46 reduces the efficiency of Ad5/F35 viral cell entry. (*p≤0.05, **p≤0.01, ***p≤0.001)

Ad5/F35 transduces human retinoblastoma cells (Y79) more efficiently than Ad5, Ad5/F37, or Ad5ΔRGD

Previous studies have shown that CD46 is expressed on human retinoblastoma (Y79) cells, allowing for efficient transduction of these cells by Ad5/F35 viruses[101]. We confirmed that Y79 cells express CD46 and sialic acid along with moderate amounts of CAR but very little α_v integrin by immunohistochemistry (Fig. 6A). Y79 cells were infected at an MOI of 500 with each recombinant virus for 24 hours and analyzed for GFP expression by FACS. The number of GFP-expressing cells for Ad5, Ad5/F35, Ad5/F37, and Ad5/ΔRGD was determined to be 10±1%, 31±2%, 2±1%, and 12±1% respectively (Fig 6B). Collectively, these data suggest that our recombinant Ad5/F35 construct binds CD46 and efficiently transduces cells that express CD46 on their surface. We determined that sialic acid is also expressed on Y79 cells, yet Ad5/F37 did not transduce Y79 cells efficiently, perhaps because Y79 cells express very little integrin for which Ad37 viruses have been shown to have a high affinity[64]. Interestingly, Ad5 and Ad5ΔRGD had nearly identical transduction efficiency in Y79 cells.

A



B

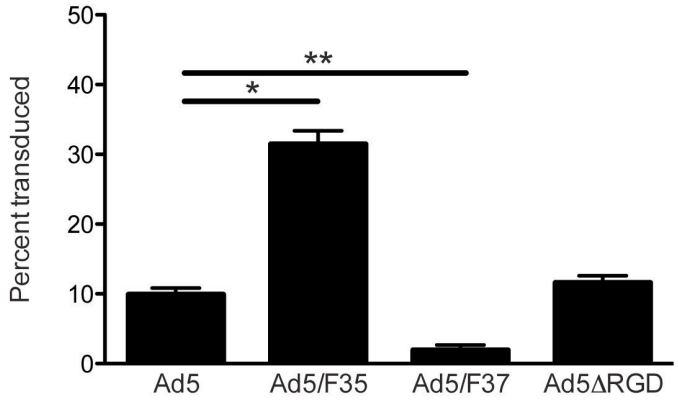


Figure 6: Y79 retinoblastoma cells express CD46 and are more efficiently transduced by Ad5/F35 than Ad5, Ad5/F37, or Ad5ΔRGD.

(A) Immunostaining indicates that Y79 cells express CD46 and some sialic acid while CAR and αv integrin are expressed at very low levels. (B) FACS analysis of GFP expression following infection at an MOI of 500 reveals that Ad5/F35 more readily transduces Y79 cells than Ad5, Ad5/F37, or Ad5ΔRGD. (*p≤0.05, **p≤0.01)

In Vivo Studies

In order to study the kinetics of transgene expression from each of the viral constructs in the retina *in vivo*, 10^9 viral particles were injected into the sub-retinal space of six week old C57BL6J mice. Six days later, the eyes were harvested, flat mounted, and quantitated for GFP fluorescence using Image J (nih.gov/ij). Diffusion of the virus through the retina was measured as the total GFP-positive surface area relative to the surface area of the total eyecup. The transduced area for each virus varied slightly and for Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD was determined to be 9±1%, 5±1%, 6±1%, and 9±2%, respectively (Fig 7A and B). The mean GFP pixel intensity for Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD was 12±1, 5±0.4, 8±1, and 61±4 relative units, respectively (Fig. 7A and C). To differentiate gene expression levels between retina and RPE, quantitative RT-PCR was performed separately on retina and RPE/choroid. We found that relative to Ad5, transgene expression from Ad5ΔRGD was *increased* 12±3 fold in the retina, whereas transgene expression from Ad5/F35 and Ad5/F37 *decreased* 9±1 and 3±1 fold in the retina, respectively (Fig. 7D). Although Ad5ΔRGD was originally designed to have reduced tropism for RPE and hence potentially redirect viral tropism towards photoreceptors, we found that relative to Ad5, transgene expression from Ad5ΔRGD-transduced RPE increased 11±7 fold (Fig. 7E). Hence, higher transduction of photoreceptors may have been achieved in part by greater infection by the Ad5ΔRGD virus overall in the retina. This is surprising given that an important component of Ad entry, the integrin binding RGD domain in the penton base, is deleted in this virus. In contrast, transgene expression in Ad5/F35 and Ad5/F37 infected RPE was reduced 160±38 and 4±2 fold, respectively (Fig. 7E).

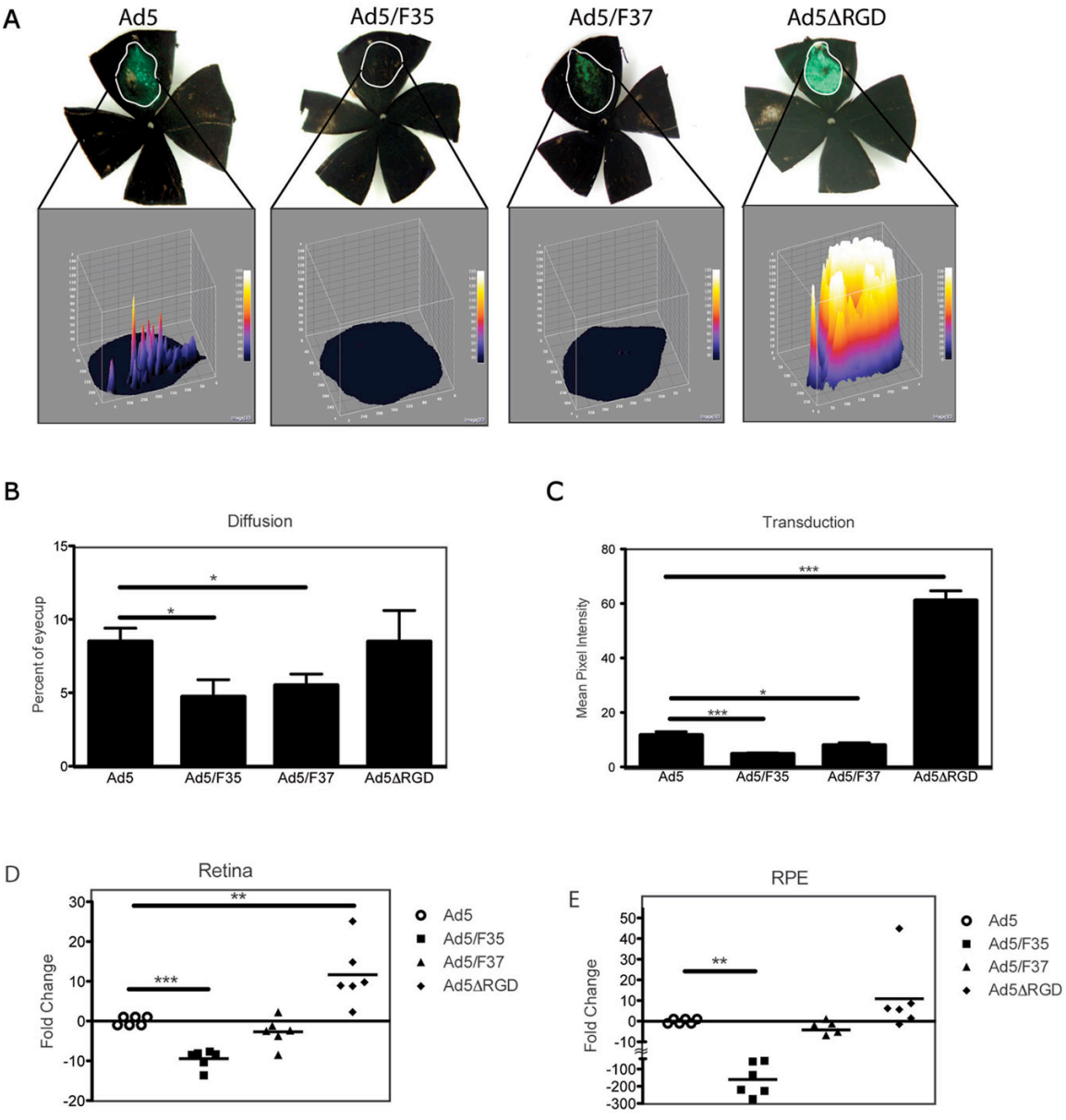


Figure 7: Following a sub-retinal injection Ad5ΔRGD has more robust expression in the retina and RPE compared to Ad5, Ad5/F35, and Ad5/F37.

(A) Flatmounts showing area of transduction. (B) The percent of the eyecup, as measured by image J, transduced by each virus only varies slightly. (C) The intensity of GFP expression, measured as pixel intensity using image J, from Ad5ΔRGD is greatly increased over Ad5, Ad5/F35, and Ad5/F37. (D) qRT-PCR analysis of GFP expression reveals that the Ad5ΔRGD virus is able to express at higher levels in the retina than Ad5, Ad5/F35, or Ad5/F37. (E) qRT-PCR of GFP expression shows that in the RPE, relative to Ad5, both Ad5/F35 and Ad5/F37 are reduced, while Ad5ΔRGD is slightly elevated. (*p≤0.05, **p≤0.01, ***p≤0.001)

In order to identify the specific cell types in the retina infected by each virus, we prepared frozen retinal sections from animals injected in the sub-retinal space. As expected, GFP fluorescence was clearly visible in the RPE of all injected animals. However, transduction of different cell types in the retina was also noted. As described previously, Ad5 infected a small number of photoreceptor cells (Fig. 8A). However, contrary to previous studies, our Ad5/F35 or Ad5/F37 virus did not significantly infect photoreceptors, at least to the extent detectable by observation of direct GFP fluorescence. Whereas Ad5/F35 only infected the RPE, Ad5/F37 infected primarily the müller cells in the retina (in addition to the RPE) (Fig. 8B and C), based on cellular morphology. Müller cell transduction was further confirmed by co-staining with antibodies against glial fibrillary acidic protein (GFAP) and cellular retinaldehyde-binding protein (CRALBP) (Fig. 9). GFP was readily detectable in photoreceptor cell bodies and inner and outer segments in the retina transduced with Ad5ΔRGD with occasional müller cell transduction. However, there was some variability in the GFP expression pattern within the outer nuclear layer (ONL) with Ad5ΔRGD- in some cases sporadic GFP-positive cell bodies had a cytoplasmic expression pattern and in other cases perinuclear GFP was observed in the ONL. In both cases inner and outer segments were also GFP positive (Fig. 8D and E).

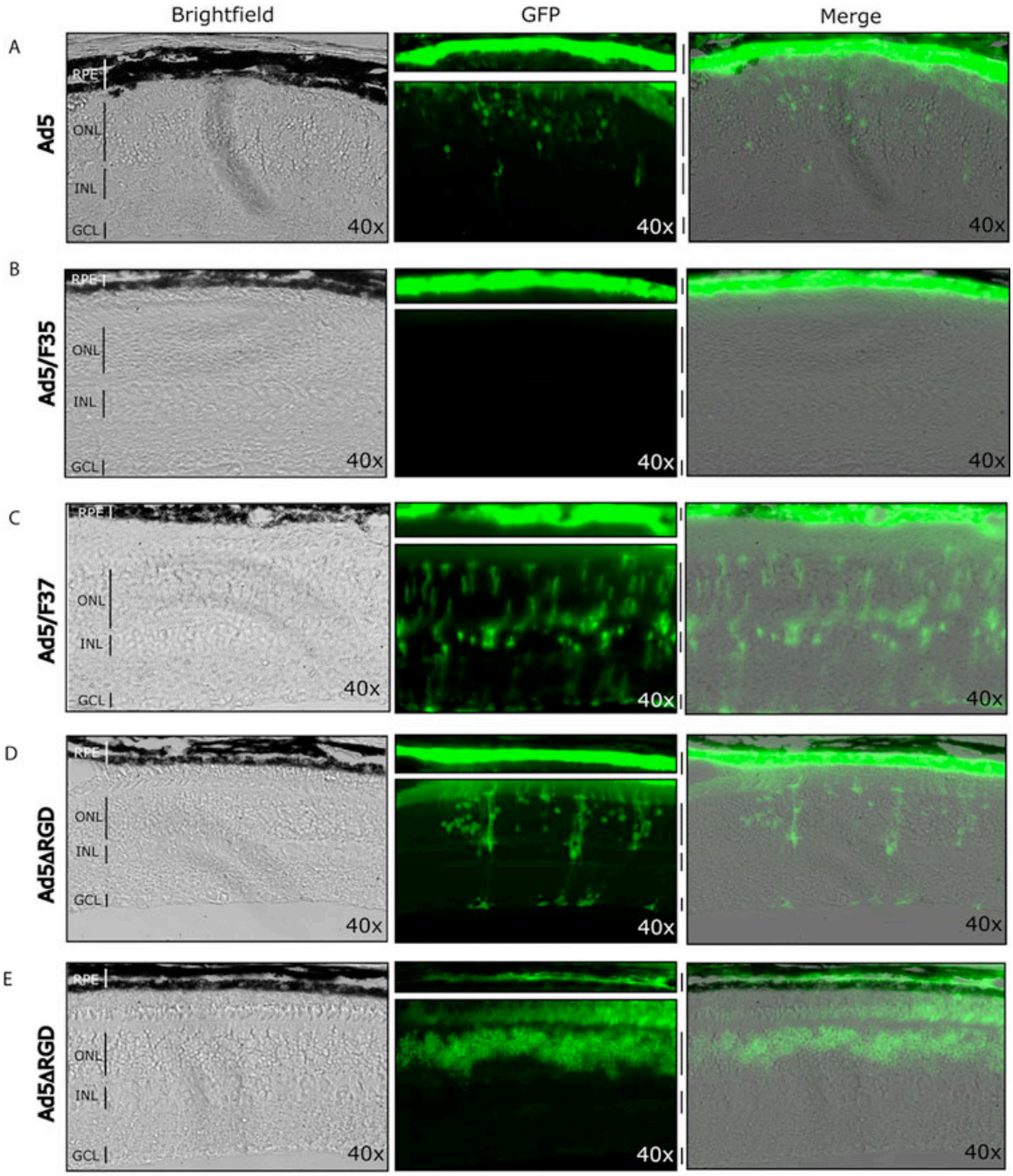


Fig. 8: Fluorescent microscopy of transverse sections of retinas treated with Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD show that while all transduce the RPE, each have a distinct tropism for certain cell types in the retina.

(A) Ad5 transduces a small portion of photoreceptors and scattered müller cells. (B) Ad5/F35 only transduces the RPE with no detectable GFP evident in the retina. (C) Ad5/F37 transduces mainly müller cells in the retina (in addition to RPE). (D) Ad5ΔRGD transduces mostly photoreceptors but also transduces scattered müller cells.

(E) In some regions of the retina Ad5ΔRGD transduced photoreceptors show GFP in the cytoplasm only in the ONL, as well as in inner/outer segments. (ONL=outer nuclear layer; INL=inner nuclear layer; GCL=ganglion cell layer; RPE=retinal pigment epithelium)

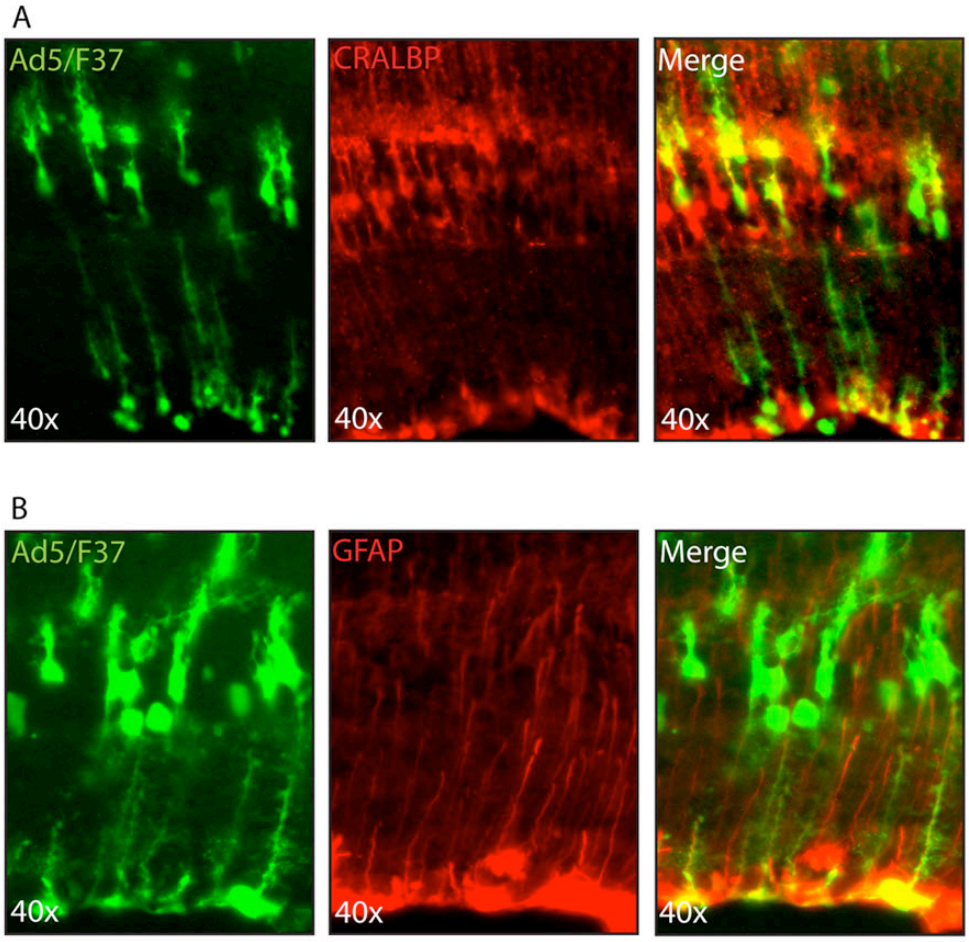


Figure 9: Müller cells are transduced by Ad5/F37.

(A) Ad5/F37 transduced retinal sections labeled with an antibody against CRALBP. A gradient of CRALBP expression, with reduced expression towards the center of the region of injection was observed. Down-regulation of genes involved in the phototransduction cascade has been observed previously[103] following sub-retinal injection. (B) Ad5/F37 transduced retinal sections labeled with GFAP antibody.

The retinal cell layer most efficiently transduced by Ad5ΔRGD is the outer nuclear layer

We found that Ad5ΔRGD has the greatest transgene expression in the retina relative to Ad5, Ad5/F35 or Ad5/F37, and transduces a number of cell types. In order to determine the proportion of transduced cells in the ONL versus the INL, we took a semi-quantitative approach involving a modified stereological technique (microbrightfield). Briefly, serial sections were taken through the entire quadrant of the eye showing GFP expression. A stereological grid was placed over the retina of every tenth section of the region of interest and the total number of GFP positive cells were counted and compared to the total number of cells for both the ONL and INL. We found that of the 52739±0.04 total cells in the ONL, 11507±0.1 were transduced, i.e. 22% (Fig 10A, C). In the INL there were 12816±0.06 total cells and of those 1381±0.3 were transduced, i.e.11% (Fig 10B, C). Transduced cells of the INL tended to occur in discrete patches and likely corresponded to müller cells whereas those in the ONL were evenly distributed (Fig 10B and D).

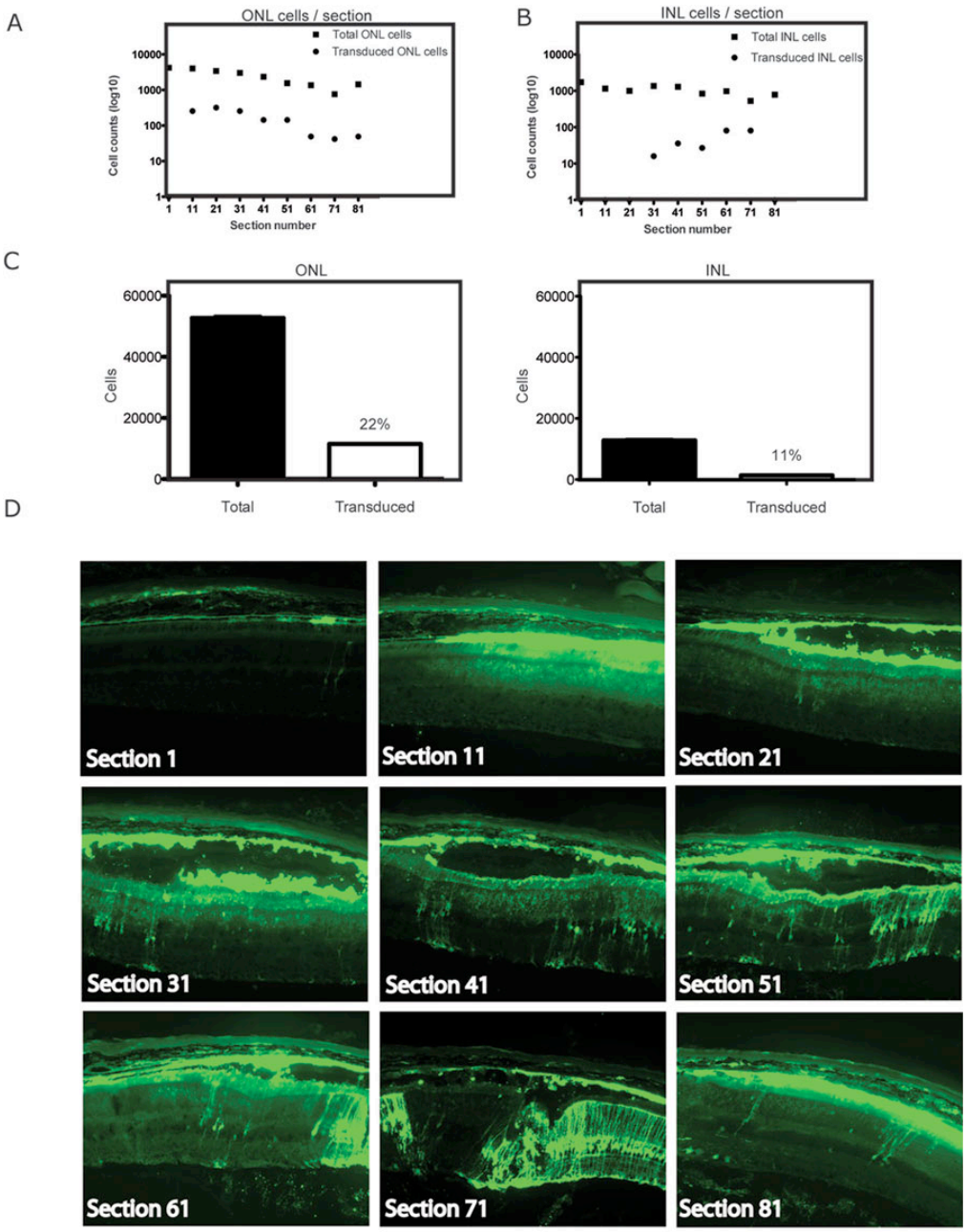


Figure 10: Sections through the region of transduction used for modified stereology to determine portion of cells transduced in the ONL and INL.

(A) Number of transduced cells relative to the total number of cells in the ONL. (B) Number of transduced cells relative to total number of cells in the INL. (C) 22% of the cells in the ONL are transduced while 11% are transduced in the INL. (D) Fluorescent micrographs used to determine cells transduced in ONL/INL. (ONL=outer nuclear layer; INL=inner nuclear layer; RPE=retinal pigment epithelium)

Photoreceptor specific transgene expression from Ad5ΔRGD vectors

Having demonstrated that Ad5ΔRGD vectors can transduce retinal cells more effectively than Ad5, Ad5/F35, or Ad5/F37, we sought to investigate the possibility of achieving photoreceptor specific transgene expression from Ad vectors. Previously, we have demonstrated photoreceptor specific transgene expression *in vivo* using Ad vectors containing a 4.7Kb murine rod opsin promoter[82]. However, transgene expression from such a promoter was achieved only in discrete patches across the retina. Hence, we generated an Ad vector (Ad5sRhoGFPΔRGD) expressing GFP regulated by 257 bp from the 5' conserved enhancer/promoter region of the mouse rod opsin gene (Fig. 11A). This promoter has previously been shown to be photoreceptor-specific in the context of adeno-associated virus (AAV)(21). A total of 10^9 Ad5sRhoGFPΔRGD viral particles were injected into the subretinal space of adult C57BL6J mice and examined 14d post injection in frozen sections. Substantial GFP expression restricted to the ONL that contains primarily the photoreceptor cell bodies was detectable (Fig. 11B). In addition, we observed significant levels of GFP in photoreceptor inner and outer segments.

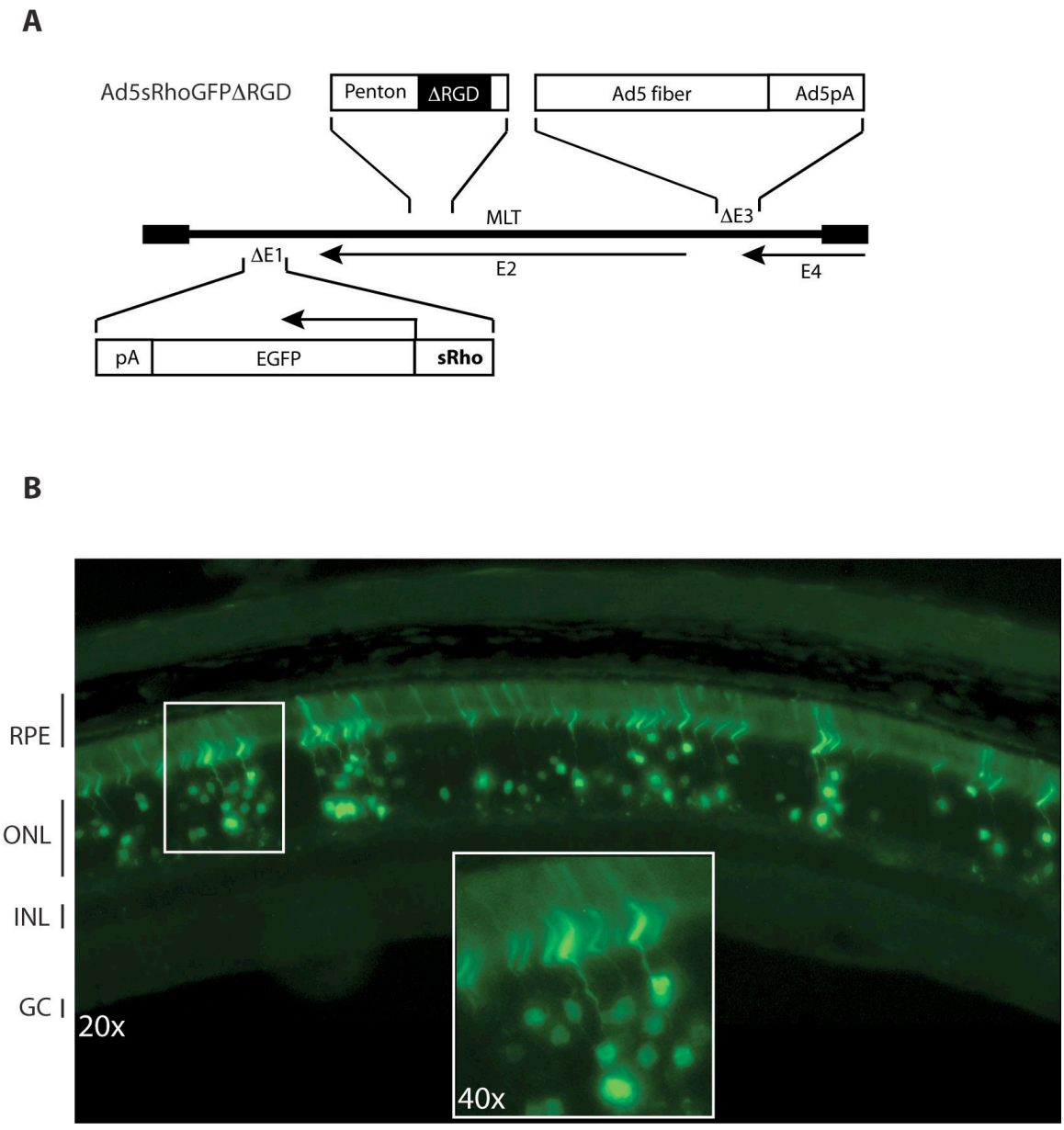


Figure 11: Structure of the Ad5sRhoGFPΔRGD construct.

(A) A 257bp mouse opsin enhancer/promoter (sRho) driving expression of an EGFP cassette was inserted into the Ad5ΔRGD vector in an antisense orientation into the deleted E1 region. (B) Fluorescent micrograph of transverse section through retina 14 days post sub-retinal injection of 10^9 viral particles indicates robust expression restricted to only the photoreceptors.

2.4 Discussion

The viability of adenovirus as a gene therapy vector for ocular disease has recently been demonstrated in two clinical trials, one delivering PEDF for the treatment of age-related macular degeneration and a second delivering thymidine kinase for the treatment of retinoblastoma[56, 58]. Currently, 221 mutations have been implicated in retinal degeneration (http://www.sph.uth.tmc.edu/retnet/disease.htm). Many of these proteins are critical for proper functioning of the phototransduction cascade and expressed exclusively within photoreceptors. One of the major drawbacks to the use of adenovirus in the eye is its inefficient transduction of photoreceptor cells. Three adenoviral vectors containing modification to the Ad5 capsid proteins have been previously described to yield enhanced photoreceptor transduction. Here, for the first time, we directly compare Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD using the same promoter, transgene expression cassette, injection, and detection techniques. Our results indicate that each vector has a distinct tropism for particular cell types of the retina. Our results differ from prior reports that indicated Ad5/F35 and Ad5/F37 have enhanced transduction of photoreceptors relative to Ad5.

It has previously been shown that Ad5/F35 transduces the RPE and photoreceptors[101]. In our study Ad5/F35 transduced exclusively the RPE and with 160 fold less efficiency relative to Ad5. There are however differences between these studies: (1) We utilized the CBA promoter whereas the CMV promoter was used in prior studies. However, we have shown previously that unlike the CMV promoter, the CBA promoter allows us to

observe Ad5 transduction of photoreceptors ; and (2) we did not amplify the GFP signal through the use of an antibody. However, in our studies Ad5 expression is observed without the need for a GFP antibody. Therefore, Ad5/F35 appears to be less efficient at transducing photoreceptors than Ad5.

We have shown that, similar to the Ad5/F35 used in previous studies, Ad5/F35 more efficiently transduces cells that express human CD46 than Ad5[101], Ad5/F37, or Ad5ΔRGD. The need for a higher MOI for Ad5/F35 relative to Ad5 to achieve the same level of transduction in HEPA 1c1c7 cells is further evidence of a re-direction of tropism from the Ad5 receptor, and can possibly be explained further by previously published observations of low expression of CD46 in murine hepatocytes[104]. While mice do carry a CD46 gene, some report that expression is restricted to the testes[105] while others indicate expression by photoreceptors[101]. We were not able to obtain a commercially available antibody for mouse CD46 but did test several tissues from mice for expression by quantitative RT-PCR using primers to mouse CD46. Our data agree with reports that mouse testes have the most CD46 and that levels are much reduced in liver, retina, and RPE/choroid (data not shown). A murine homologue to human CD46, crry, has been shown to have a similar expression pattern and perform a comparable role in the complement system[106]. However, it is not known whether Ad5/F35 is able to bind crry. Expression in the retina of both crry in mice and CD46 in humans is most abundant in the RPE where it is restricted to the basal and lateral surface of the RPE cells[107]. Since Ad5/F35 is injected into the sub-retinal space it would be exposed to

the apical side of the RPE, limiting access to crry. This could be another explanation for less efficient transduction of the RPE by Ad5/F35.

Aside from the RPE, we observed Ad5/F37-mediated GFP expression in müller cell processes at the inner and outer limiting membranes and possibly inner retinal neurons. This is in contrast to what has been found previously[108] in which both the RPE and photoreceptors were transduced. This could be due to several differences among our approach: (1) We use the CBA promoter compared to their use of the CMV promoter; (2) The single amino acid difference between the F37 fiber used in our study and that of prior studies (ours has a threonine at position 10 while in previous studies there is an aspartic acid) and, (3) our mode of injection was sub-retinal versus the previously used intravitreal delivery. However, it would be somewhat surprising that viral diffusion would bypass the photoreceptors to be taken up by müller cells following a sub-retinal injection. We have seen the same pattern of expression following injection with an Ad5/F17 virus[82], both classified as sub-group D adenoviruses. It has been shown that Ad37 binds to sialic acid[67]. While sialic acid is present throughout the retina it does not appear to be preferentially expressed at higher levels on müller cells (data not shown). It has also been shown that Ad37 has a higher binding affinity to integrin than Ad5[64]. This could explain our observation of reduced transduction of Y79 cells by Ad5/F37, which do express sialic acid but very little α_v integrin.

It has been shown that müller cells express 4 integrin subunits (α_1, α_2, α_3, and β_1)[109]. It would be interesting to see if this particular combination of integrin and sialic acid

expression on müller cells is preferentially enhancing transduction for Ad5/F37 over the other vectors tested. Müller cells have been shown to become phagocytic when foreign particles are injected into the eye[110]. Therefore, another possible explanation could be that müller cells are being activated by Ad5/F37, then phagocytosing the virus.

Our Ad5ΔRGD construct was designed to eliminate integrin binding with the intention of reducing viral uptake by the RPE, diverting the virus to the juxtaposed photoreceptors. To this end we do see significantly elevated expression in photoreceptors relative to all the other vectors tested. More surprisingly, we see an increased amount of RPE transduction relative to Ad5. This indicates that, at least in the context of the RPE, integrin binding may not be critical for Ad5 uptake. Aside from increased photoreceptor transduction compared to Ad5 we also see a different pattern of expression within the cells. In some cases the entire photoreceptor cell body along with the inner/outer segments contain GFP. In other regions GFP expression in the cell body appears to be cytoplasmic along with the inner/outer segments expressing GFP. A possible explanation for the two different patterns of expression could be a result of the amount of GFP expressed by the cell. The GFP transgene used in our study does not have a nuclear localization signal and we do not, therefore, have reason to believe that it enters the nucleus with high efficiency. It is possible that nuclear uptake and/or binding of GFP is concentration dependent and that exclusive perinuclear localization occurs in those photoreceptors containing less viral particles per cell. That all 4 vectors have distinct tropism in the retina yet all infect the RPE suggests a possible unknown mechanism for viral uptake by the RPE. One role of the RPE is to phagocytose the outer segments of

photoreceptors[111]. This may be one explanation for how any modified Ad vector enters the RPE independent of its tropism in the retinal layers.

Placing GFP expression under the control of a 257 base pair enhancer/promoter of mouse opsin (sRho) we were able to drive robust expression that was restricted to the photoreceptors. The pattern of expression is consistent with that seen using AAV vectors with the entire cell body and inner/outer segments containing GFP[112]. However, it is not consistent with that reported for non-viral gene delivery vectors where the inner segments lack GFP expression[111]. To our knowledge, this is the first report of robust photoreceptor specific transgene expression from an adenovirus vector.

To summarize, our data suggest that changing either the fiber or deleting the RGD domain in the penton base can be sufficient to enhance Ad transduction of distinct cell types in the retina. We show that Ad5/F35 targets the RPE, Ad5/F37 the RPE and müller cells, and Ad5ΔRGD the RPE, photoreceptors, and some müller cells. Finally, we show that robust expression can be restricted to the photoreceptors by using a 257 base pair mouse opsin promoter in the Ad5ΔRGD construct. This data is relevant for the design of Ad based gene transfer vectors to target specifically those cell populations affected in the various retinopathies.

Chapter 3:

Membrane Cofactor Protein (CD46) Mediated Inhibition of the Alternative Arm of Complement: Therapeutic Implications for Age-related Macular Degeneration

J. Harry Sweigard[1], Siobhan M. Cashman[2], and Rajendra Kumar-Singh[1,2]

Published

Gene Therapy. 2011, Feb 10 (Epub ahead of print)

[1]Program in Cell, Molecular and Developmental Biology

Tufts Sackler School of Graduate Biomedical Sciences, 136 Harrison Avenue, Boston, Massachusetts 02111, USA.

[2]Department of Ophthalmology

Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111, USA.

3.1 Abstract

Activation of the alternative pathway of the complement system has been implicated in the pathogenesis of Age-related Macular Degeneration (AMD). Retinal pigment epithelium (RPE) cells of AMD patients contain membrane attack complex (MAC) on their surface. Membrane cofactor protein (CD46) preferentially regulates the alternative pathway of complement. The aim of this study was to evaluate the potential of increasing CD46 expression on RPE cells as an approach to reduce alternative pathway mediated damage to RPE cells.

3.2 Introduction

Gene transfer approaches to ocular tissues using adenovirus as the gene delivery vector have been found to be safe in a number of clinical trials[56, 58]. Recombinant adenovirus constructs have a significant tropism for retinal pigment epithelium (RPE) cells[53] and can persist in this tissue in rodents for at least one year (latest time point examined)[54] or potentially for the "lifetime" of the animal[51]. In addition, helper-dependent adenovirus vectors can accommodate transgene expression cassettes up to 36 Kb[52, 55], allowing for the expression of multiple transgenes as well as the inclusion of native gene regulatory elements.

Several diseases have been linked to activation of the complement cascade, a component of the innate immune system, including: Atypical Hemolytic Uremic Syndrome (aHUS), arthritis, multiple sclerosis, lupus, and age –related macular degeneration (AMD). AMD is the leading cause of blindness amongst the elderly in the developed world[113, 114]. Currently, the only FDA-approved treatment for AMD targets the less prevalent yet highly debilitating 'wet' form of the disease[115]. While this treatment (ranibizumab/ lucentis) is highly effective, it does not impact the 90% of AMD patients that suffer from the 'dry' form of the disease. A window of opportunity exists for treating 'dry' AMD before it's progression to an advanced stage of 'dry' or the more severe 'wet' form. Early signs of disease can be detected by the formation of extracellular deposits generally referred to as 'drusen' between the retinal pigment epithelium (RPE) and bruch's membrane, abnormalities in pigmentation, or mottling.

While there is considerable debate on the precise mechanism underlying drusen formation, mostly composed of proteins and lipids, there is agreement that they represent a site of chronic inflammation mediated by the complement cascade[116]. The complement cascade is a component of the innate immune system that can be triggered by pathogens (classical pathway) as well as constitutively or spontaneously (alternative pathway). In addition to normal 'tickover', activation of the alternative pathway is triggered by C3 hydrolysis- leading to a cascade of cleavage events that result in the production of anaphylatoxins, opsonins and ultimately the formation of a lytic pore on the cell surface termed membrane attack complex (MAC). MAC has been observed mainly on the bruch's membrane and drusen underlying the RPE in AMD patients[57]. Importantly, proteins of the complement pathway have been identified in drusen[117] and prediction models strongly indicate a higher incidence of AMD in patients with polymorphisms in genes encoding alternative pathway components such as, complement factor H, complement factor B, and complement factor 3[77].

Because activation of the alternative pathway has been implicated in AMD, we wished to identify a complement regulator that will specifically block the alternative arm of the complement system and use adenovirus to locally deliver the regulator to the back of the eye. Membrane cofactor protein (CD46) is a cofactor for factor I which has previously been identified as a key regulator of the alternative pathway[118]. Mutations in CD46 have been identified as a major risk factor for developing diseases associated with chronic alternative pathway activity, such as aHUS[119]. While the importance of CD46

expression has been defined for aHUS, its role in AMD has not been fully evaluated. Analysis of the expression pattern for CD46 in normal human eyes has revealed that it is expressed on the basal and lateral surfaces of RPE cells[93]. Since the basal surface of RPE cells are exposed to drusen, this places CD46 in a prime location to dampen alternative pathway activity. In a state of chronic inflammation, such as AMD, the levels of CD46 may not be sufficient to protect the RPE cells from complement mediated attack. Therefore, increasing the expression of CD46 in RPE cells may be one way to restore the balance between alternative complement activation and regulation in AMD patients.

Both CD46 and factor H negatively regulate the alternative pathway by acting as co-factors for C3b cleavage. Polymorphisms in the *soluble* regulator factor H have been implicated as a major risk factor for developing AMD. Using adenovirus to efficiently deliver CD46, a *membrane* bound complement regulator, to RPE cells may provide localized protection from complement attack by cleaving C3b. This may be a way to compensate for factor H in patients with polymorphisms. To our knowledge this is the first time adenovirus has been used to test the efficacy of CD46 for protecting against a complement mediated disease. While we have focused on AMD our results may suggest the need to extend testing into other disease models, such as aHUS. Currently, kidney transplantation has been the most successful treatment for aHUS patients. Adenoviral delivery of CD46 may be a viable option that bypasses the limited availability of kidney donors for aHUS patients.

Previously, we have described the development of a novel murine model of complement activation to test the capacity for adenoviral delivery of the human complement regulator CD59 to protect against human MAC deposition[120]. This humanized murine model provides a convenient approach to evaluate the role of CD46 in the murine retina. Here, we further adapt this model such that complement activation occurs primarily through the alternative pathway and use this model to evaluate the role of human CD46 in protecting murine RPE cells from alternative pathway mediated complement attack. To our knowledge this is the first time adenoviral delivery of CD46 has been evaluated for its role in protecting RPE cells from alternative complement pathway activity. Our results highlight the need for further investigation of adenoviral delivery of CD46 and its potential use as a therapeutic for AMD.

3.3 Results

Expression of human CD46 from adenovirus in human embryonic retinoblasts and mouse hepatocytes

Human CD46 is a transmembrane protein ranging in size from 48-68kDa[118]. There are 4 isoforms that all contain 4 conserved short consensus sequences (SCR), an O-glycosylated serine/threonine/proline rich area, a hydrophobic transmembrane portion, and an intracellular domain. In order to evaluate the ability of human CD46 (hCD46) to protect tissues against human complement-mediated insult, we first cloned hCD46 into an adenoviral vector under the control of a chicken β actin promoter (CAG) to generate AdCAGCD46. A control adenovirus that does not have a transgene (AdCAGpA) and an adenovirus expressing GFP (AdCAGGFP) were also generated, the GFP-expressing virus

for the purpose of labeling the site of transduction following an *in-vivo* injection (fig 12A). Expression of hCD46 was confirmed by western blot of lysate collected from AdCAGCD46-infected human embryonic retinoblasts (HERs). We observed a band at 63kDa corresponding to the predicted molecular weight of the BC isoform of hCD46[118] (fig 12B). A faint band in the AdCAGpA and uninfected lanes was also detected. This is not surprising since it has been shown that the majority of human cells express CD46[121]. The ability of adenovirus-expressed hCD46 to localize to the cell membrane was confirmed by immunocytochemistry of non-permeabilized mouse hepatocytes (Hepa 1c1c7) infected with either AdCAGCD46 or AdCAGpA . The AdCAGCD46-infected hepatocytes show robust expression on the cell membrane, while no hCD46 is detected on AdCAGpA-infected cells (fig 12C).

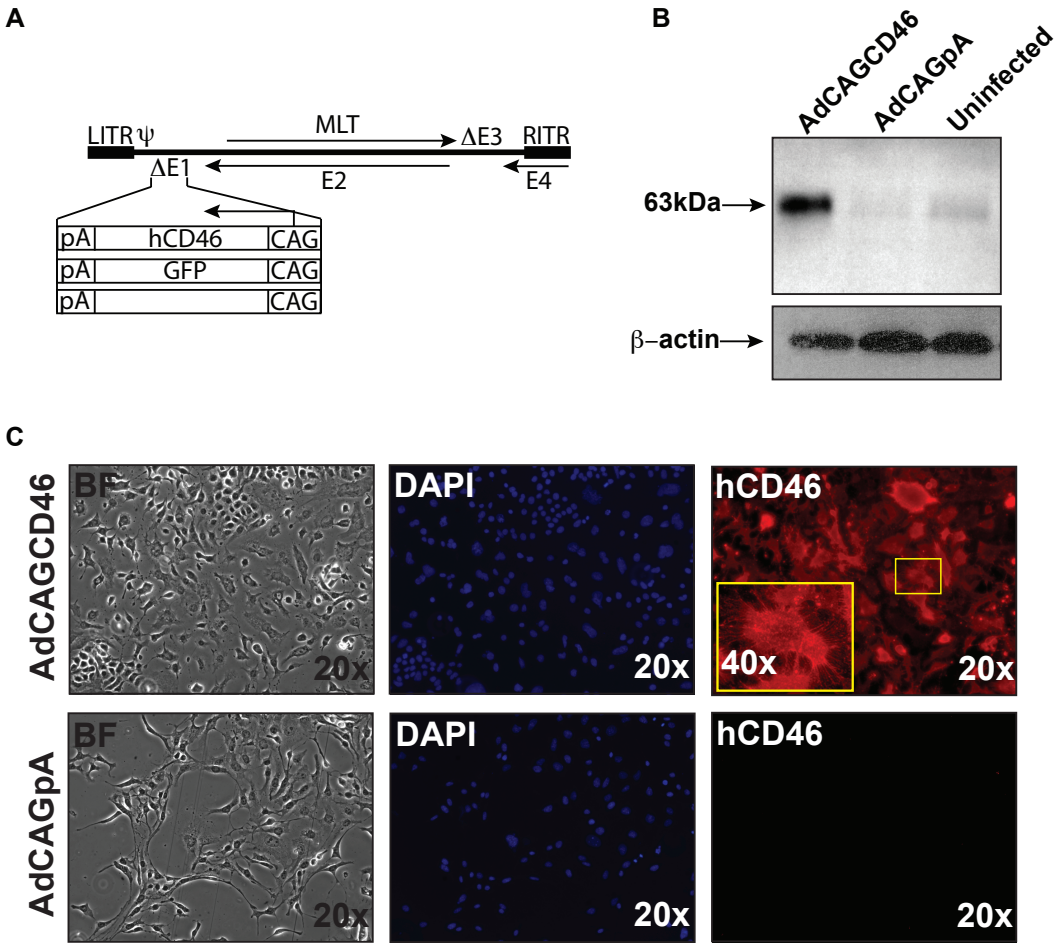


Figure 12: AdCAGCD46 is expressed in HER cells and on the membrane of mouse Hepa 1c1c7 cells.

(A) Adenoviral constructs: expression cassettes for human CD46, GFP, and without transgene were inserted into the E1-deleted region of Ad5. (B) Western blot for hCD46 showing expression in HER cells following infection with AdCAGCD46. (C) Mouse Hepa1c1c7 cells infected with AdCAGCD46 and stained with mouse anti human CD46 (MEM258) showing incorporation of CD46 into the cell membrane. (CAG=chicken b-actin promoter; pA=bovine growth hormone poly A signal; LITR=left inverted terminal repeat; RITR=right inverted terminal repeat; Ψ=packaging signal; MLT=major late transcript; E1-E4=early regions 1-4)

AdCAGCD46 protects mouse hepatocyte (Hepa1c1c7) cells from lysis mediated by the alternative, but not classical, complement pathway

It has previously been shown that hCD46 has a higher affinity for binding C3b relative to C4b, resulting in increased inhibition of convertase formation of the alternative pathway[118]. Therefore, we evaluated the ability of AdCAGCD46 to protect cells from complement mediated cell lysis concomitantly from the classical and alternative complement pathways, or specifically the alternative pathway. Hepa1c1c7 cells pre-incubated with either AdCAGCD46 or AdCAGpA for 3 days were treated with either 25μg/ml emmprin antibody followed by 10% normal human serum (NHS), or MgEGTA-treated NHS (for inhibition of the classical pathway) and cell lysis quantified by FACs analysis of propidium iodide (PI) uptake. When the classical pathway of the complement system was active, we observed no significant protection from lysis in AdCAGCD46-infected cells relative to those infected with AdCAGpA (fig 13A). However, when the classical pathway was inactivated such that cell lysis occurred primarily by the alternative pathway, we observed a 39±0.88% ($p=0.008$) reduction in the amount of PI uptake by AdCAGCD46 relative to AdCAGpA (fig 13B). The difference in PI uptake between AdCAGCD46 and AdCAGpA-infected cells should correlate with the difference in the amount of membrane attack complex (MAC) deposited on the membranes of these cells. To test this hypothesis, we infected Hepa1c1c7 cells with either AdCAGCD46 or AdCAGpA for 3 days then activated the alternative pathway by incubating the cells with 25μg/ml emmprin followed by 10% NHS and MgEGTA. We determined the amount of human MAC deposition using a monoclonal antibody against the C5b9 complex. Cells infected with AdCAGCD46 have less MAC deposited on their surface (fig 13C) than

AdCAGpA-infected cells. Additionally, the morphology of the cells remains largely unaffected by MAC deposition relative to those cells infected with AdCAGpA. Together, these data suggest that AdCAGCD46 confers protection from MAC-mediated cell lysis occurring mainly through the alternative pathway.

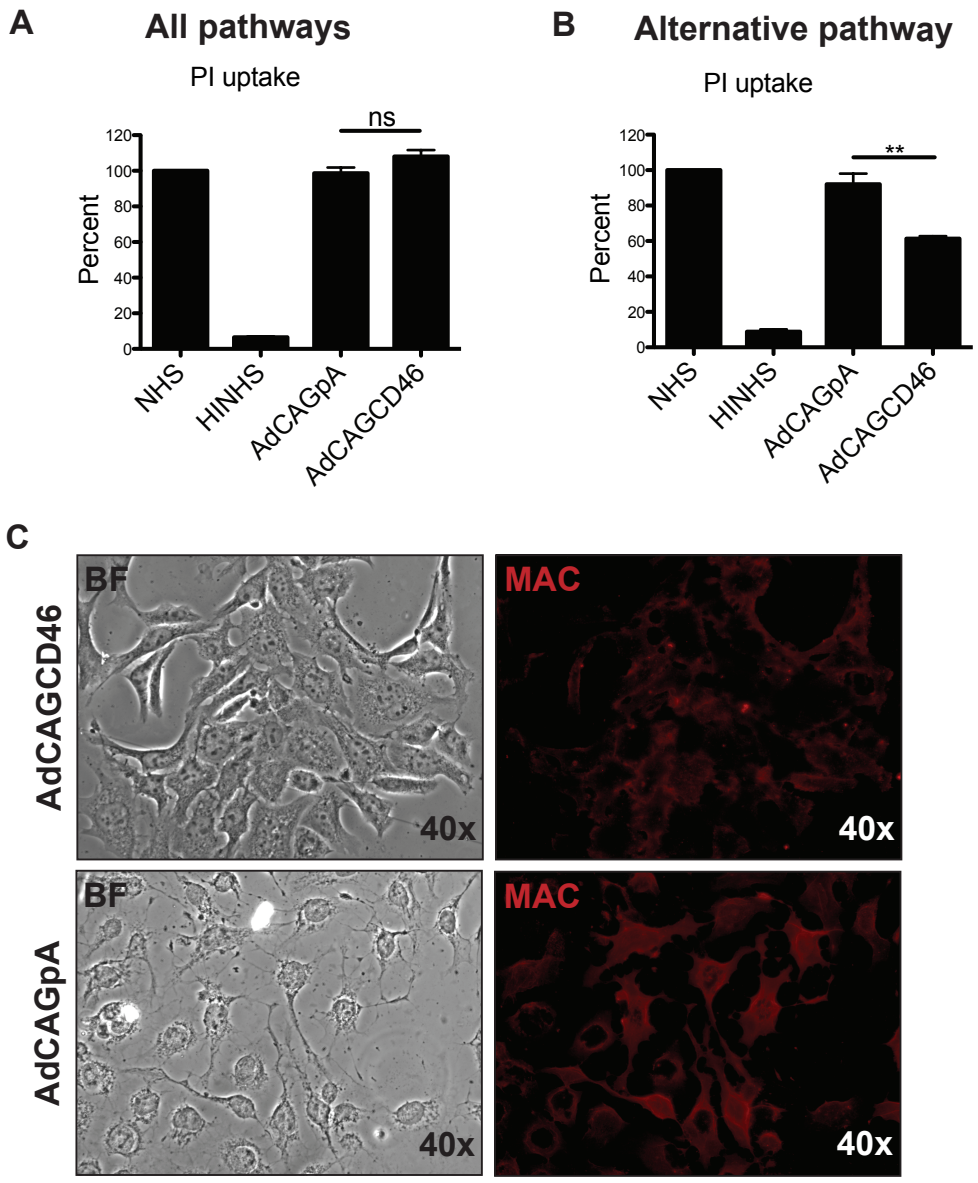


Figure 13: AdCAGCD46 protects mouse Hepa1c1c7 cells from alternative pathway - mediated, but not classical pathway-mediated, MAC deposition.

(A) FACS analysis of PI uptake showing that when all pathways of the complement system are activated hCD46 has no significant effect on MAC deposition on mouse Hepa1c1c7 cells. (B) FACS analysis of PI uptake showing that when the alternative arm of the complement system alone is active, hCD46 protects mouse Hepa1c1c7 cells from MAC deposition by 39±0.88% (p=0.008). (C) Following alternative pathway activation, hCD46 expressing Hepa1c1c7 cells appear healthier and have less MAC deposited on the membrane. (Representative of 3 separate experiments performed in duplicate each time).

(NHS=normal human serum; HINHS=heat-inactivated normal human serum; ns=not significant; PI= propidium iodide; BF=brightfield; MAC=membrane attack complex)

AdCAGCD46 protects mouse primary RPE cells from alternative pathway mediated MAC deposition

Since MAC has been observed on RPE cells in AMD patients and the alternative pathway has been implicated to play a pivotal role in this disease[84, 116], we wished to determine whether AdCAGCD46-infected mouse primary RPE cells are protected from human MAC deposition mediated primarily by the alternative complement pathway. We have previously shown that RPE cells are more resistant to MAC deposition than Hepa1c1c7 cells[120], requiring higher concentrations of antibody and serum. RPE cells were pre-incubated with 50μg/ml emmprin followed by MgEGTA and 50% NHS. MAC was detected by immunohistochemistry (IHC). Quantification of intensity of MAC staining revealed a reduction of 21±1.9% ($p=0.04$) MAC deposition on RPE cells infected with AdCAGCD46 relative to AdCAGpA (fig 14A, B). As the reduction in MAC was less than that of hepatocytes, we considered the possibility that this might be due to a lower infection rate of RPE cells and/or reduced expression of hCD46. To test this, we confirmed the expression of hCD46 from AdCAGCD46 in mouse primary RPE cells by IHC. AdCAGCD46 infected cells showed almost 100% transduction and showed strong expression of hCD46 on the cell membrane, while AdCAGpA infected cells had no detectable expression (Fig. 15A). It may be more likely then that the reduced inhibition of MAC deposition on RPE cells may be due to the increased amount of antibody (and, therefore, increased activation of Classical pathway) required to activate complement on this cell type.

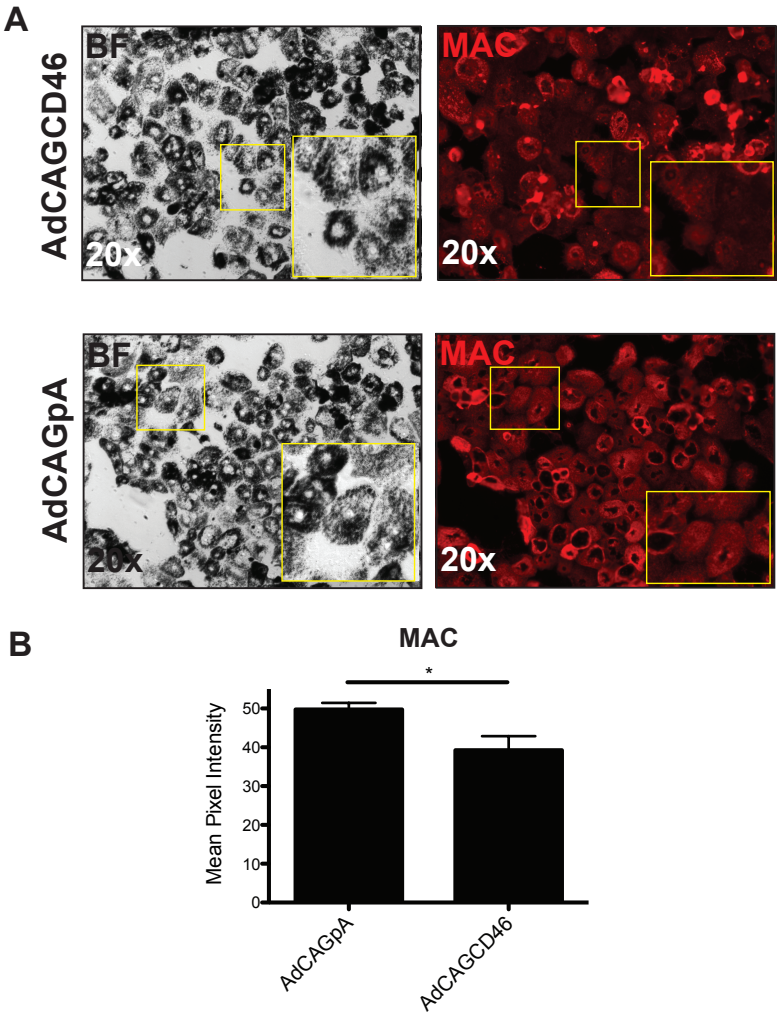


Figure 14: AdCAGCD46 protects mouse primary RPE cells from alternative pathway mediated MAC deposition.

(A) Staining intensity of MAC is not uniform on cells infected with AdCAGCD46 and has areas of reduced MAC deposition. AdCAGpA infected cells display uniform MAC deposition. (B) Quantification of MAC pixel intensity shows an overall reduction in staining intensity of 21±1.9% (p=0.04) on AdCAGCD46 infected RPE cells compared to AdCAGpA. (Representative of 3 separate experiments performed in duplicate each time).

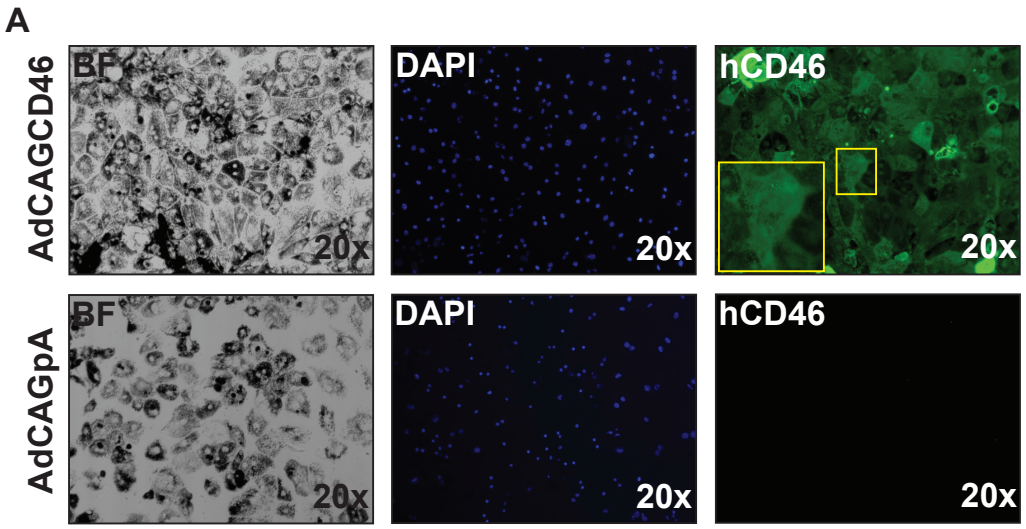


Figure 15: hCD46 is expressed in mouse primary RPE cells following infection with AdCAGCD46.

(A) Mouse primary RPE cells efficiently express hCD46 on their membranes following infection with AdCAGCD46. No hCD46 is detectable in cells infected with AdCAGpA.

hCD46 is expressed on the basal and lateral surface of mouse RPE cells following a sub-retinal injection of AdCAGCD46

In the human eye, CD46 is expressed on both the basal and lateral surfaces of RPE cells[93]. In order to determine if hCD46 can be expressed in a similar pattern in mouse RPE cells *in vivo*, we injected AdCAGCD46 into the sub-retinal space of adult mice. After 8 days the eyes were harvested and examined for CD46 expression. Flat mounts of the eye-cups revealed robust expression in RPE cells with increased intensity of expression observed at the intercellular junctions (Fig. 16A). To determine if expression is also on the basal and lateral surfaces of these cells, cross sections were taken through the injection site and compared with an un-injected region of the eye-cup. The injected region demonstrates expression mainly on the basal and lateral surface of the RPE cells, consistent with that seen in human eyes (Fig. 16B).

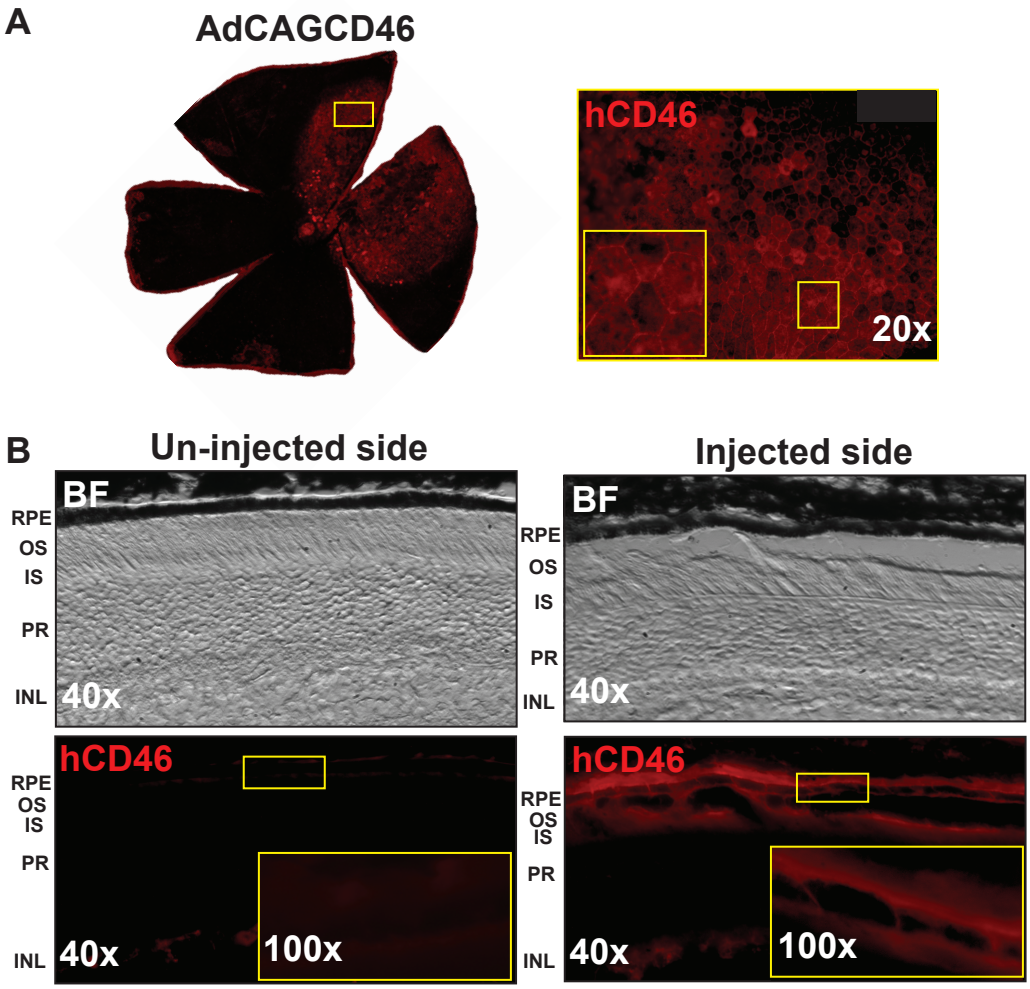


Figure 16: hCD46 is expressed on mouse RPE cells following a sub-retinal injection of AdCAGCD46.

(A) Flat-mount of a mouse eye-cup with the RPE cells exposed reveals a patch of CD46 expression 8 days after a sub-retinal injection of AdCAGCD46. (B) Cross sections through the injection site show that hCD46 expression is strongest on the basal and lateral surface of the RPE cells and that there is no detectable hCD46 expression in the un-injected region of the same eye.

AdCAGCD46 protects mouse RPE cells from alternative pathway mediated MAC deposition following a sub-retinal injection

Since we were able to recapitulate the expression pattern of hCD46 in mouse RPE cells, we then considered whether hCD46 expressed from an adenovirus on mouse RPE cells *in vivo* could offer protection from human MAC deposited via the alternative pathway. Adult mice were injected into the sub-retinal space with either AdCAGCD46 or AdCAGpA. In order to identify the injection site, each virus was mixed with an adenovirus expressing GFP. After 8 days the eyes were enucleated, and the lens and cornea removed to expose the RPE cells. The eyecup was treated with 140μg/ml emmprin followed by 50% NHS and MgEGTA. MAC formation was detected using a monoclonal antibody to the C5b9 complex. Prior to serum treatment, GFP expression was robust. After serum treatment GFP expression was patchy due to damage of RPE cells by the serum. GFP could be seen at the site of injection for both AdCAGCD46 and AdCAGpA (Fig. 17A). Within the region of AdCAGCD46 expression there was a significant reduction in the amount of MAC staining relative to AdCAGpA injected eyes (Fig. 17B, C). We determined a 24±4.5% ($p=0.0001$) reduction in MAC deposition in AdCAGCD46 injected eyes relative to AdCAGpA (Fig. 17D).

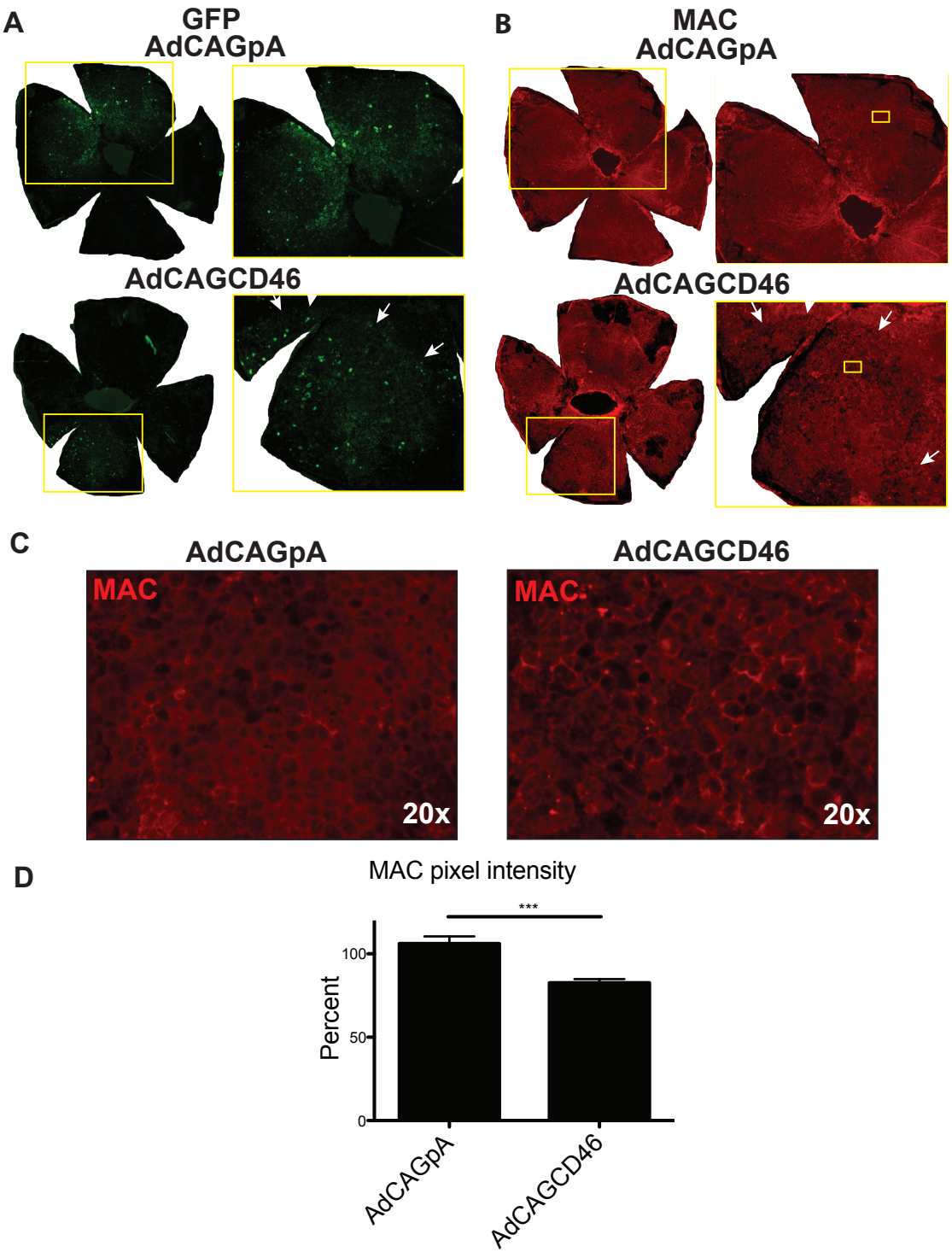


Figure 17: Sub-retinal delivery of AdCAGCD46 reduces the amount of alternative pathway-meditaed MAC deposition on RPE cells.

(A) GFP expression 8 days after a sub-retinal injection of either AdCAGCD46/AdCAGGFP or AdCAGpA/AdCAGGFP. (B) IHC for MAC complex on

eye-cups reveals a visible decrease in MAC pixel intensity within the region of hCD46 expression relative to the uninjected region. No reduction is observed between the injected and uninjected regions for the control (pA) virus. Arrows demarcate the periphery of the injected area. For AdCAGCD46, this coincides with the region of reduction in MAC deposition. (C) Higher power image of the boxed region in B showing that MAC is more uniformly deposited on the RPE cells in the AdCAGpA injected eye compared to AdCAGCD46. (D) Quantification of MAC pixel intensity showing a 24±4.5% (p=0.0001) reduction in MAC staining in AdCAGCD46-transduced eyecups relative to AdCAGpA-injected. (Representative images for AdCAGpA n=7 and AdCAGCD46 n=9).

3.4 Discussion

Recent evidence suggests that increased activity of the complement system plays a significant role in the pathogenesis of AMD. Such evidence includes the presence of fragments of the complement cascade closely associated with drusen[57, 116] or presence of MAC on the surface of RPE and choroidal blood vessels[117]. Genetic studies have identified polymorphisms in complement genes of both alternative and classical pathways, including Factor H, Factor B, C2 and C3[77]. The most significant risk of developing AMD, however, is associated with polymorphisms in the alternative pathway regulator factor H[84]. As the complement cascade is an important first line of defense for the immune system, we considered regulators that would allow it to function locally in ocular tissues and conservatively by dampening the portion of the complement cascade that has been implicated as being over-active in this disease, i.e. the alternative pathway.

We have previously shown localized protection of mouse RPE cells against human MAC deposition in an *ex vivo* humanized mouse model of complement deposition[120]. In that study, human CD59 expressed from an adenovirus specifically on RPE cells, efficiently blocked MAC deposition occurring as a result of all complement pathways. With

evidence accumulating implicating the alternative pathway in AMD, we chose to evaluate the potential of a complement regulator known as membrane cofactor protein (CD46) that has previously been shown to preferentially dampen the alternative pathway[118]. In our study, CD46, despite robust expression on the membrane of mouse hepatocytes transduced with a CD46-expressing adenovirus, displayed no inhibition of complement-mediated cell lysis conferred in the presence of all pathways, but indicated 39±0.88% protection against lysis when examined specifically in the context of the alternative pathway.

One reason for this preference may be that CD46 has a higher binding affinity for the alternative pathway component C3b relative to the classical component C4b. However, another reason may be that it takes longer for CD46 to cleave C3b and C4b than it does for a regulator such as CD59 to disrupt the MAC complex. This could be due to CD46 being a co-factor for cleavage, requiring binding of the serine protease factor I to cleave C3b and C4b. Therefore, limiting the process of MAC deposition to the alternative pathway may allow enough time for CD46 to bind C3b and recruit factor I before the convertase is formed. Furthermore, it has been shown that after the convertase is formed, CD46 can no longer bind to either C3b or C4b[122, 123]. This suggests that once convertase has assembled on the cell membrane, the rate of C3 cleavage may become so rapid that the amount of C3b becomes overwhelming for CD46. It is interesting to speculate whether this may be the case on RPE cells in human AMD.

CD46 in human retinal tissue is localized to the basal and lateral surface of RPE cells[93]. This places CD46 close to the site of complement activation in AMD patients in which MAC deposition has been observed in RPE cells, drusen, and choroidal endothelial cells. It is possible that the levels of CD46 expression in RPE cells could be reduced or insufficient in AMD patients. Of particular interest is the recent observation that CD46 expression is reduced on the RPE of AMD patients[114]. Polymorphisms in CD46 have been linked to the kidney disease aHUS[95], another disease resulting from over-activation in the alternative arm of complement[119]. Renal allographs, which provide wild type expression of CD46, have been a viable therapy for patients suffering from aHUS due to CD46 polymorphisms[124, 125]. Our results show that CD46 expressed from an adenovirus *in vivo* in mice recapitulates the pattern of CD46 expression observed in human RPE, and that this expression is sufficient to provide 24±4.5% protection against an acute insult from human complement attack mediated by the alternative pathway.

The strong linkage observed between polymorphisms in factor H and AMD prompt consideration of CD46 as a viable therapeutic option, as both act at the same step of the complement cascade. Previous studies have shown that CD46 can compensate for the loss or reduction in factor H activity in human serum[118], a situation analogous to that proposed for AMD patients with factor H polymorphisms.

In summary, we have shown that CD46 delivered to murine retina *in vivo* localizes to the basal and lateral membrane of RPE cells and that this expression is sufficient to confer

protection against human complement attack generated by the alternative pathway. Considering the evidence for the role of the alternative pathway in AMD, as well as the specificity of CD46 as a regulator of the alternative pathway, a precision that would allow the rest of the complement system to remain active against invading pathogens, the use of CD46 as a treatment for AMD prompts further investigation.

Chapter 4:

Development of a Soluble Terminator of Activated Complement (STAC) that Contains the Complement Regulatory Function of CD46, CD55, and CD59 as a Potential Therapy for Age-related Macular Degeneration (AMD)

J. Harry Sweigard[1], Siobhan M. Cashman[2], and Rajendra Kumar-Singh[1,2]

[1]Program in Cell, Molecular and Developmental Biology
Tufts Sackler School of Graduate Biomedical Sciences, 136 Harrison Avenue, Boston, Massachusetts 02111, USA.

[2]Department of Ophthalmology
Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111, USA.

4.1 Abstract

Evidence from genetic, proteomic, and IHC studies indicate a correlation between complement activation and the progression of Age-related Macular Degeneration (AMD). Previously, we have generated adenoviral vectors to examine the role of the membrane bound complement regulators CD46, CD55, and CD59 for their ability to protect against complement mediated injury. While each provided protection to RPE cells it was restricted to only the cells expressing the regulator. In the following study we take what we believe to be the smallest functional domain of each regulator and fuse them to make one protein. This is the first description of a novel, chimeric, protein that has 3 regulatory domains theoretically making it capable of blocking complement activation at 3 separate steps of the cascade. Furthermore, we attached a secretory signal to the N-terminus and removed the membrane binding domains creating a protein capable of diffusing away from the initial site of transduction to offer protection throughout the eye.

4.2 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness amongst the elderly in the developed world[113, 114]. Currently, the only FDA-approved treatment for AMD targets the less prevalent yet highly debilitating 'wet' form of the disease[115]. While this treatment (ranibizumab/lucentis) is highly effective, it does not impact the 85% of AMD patients that suffer from the 'dry' form of the disease. Dry AMD can persist for more than a decade with gradual visual loss, severely impacting the quality of life for those living with it and their caretakers. Early signs of disease can be detected by the formation of extracellular deposits generally referred to as 'drusen' between the retinal pigment epithelium (RPE) and Bruchs membrane and loss of RPE cells (geographic atrophy).

Several lines of evidence strongly suggest polymorphisms in complement proteins as a major risk factor for developing AMD. Genetic association studies have revealed that AMD patients are significantly more likely to have mutations in factor H, B, C3, and factor I[75, 87, 92, 126]. Studies analyzing the peripheral blood in AMD patients reveal decreased C3 and elevated levels of C3a, Bb, and C5a, indicating proteolytic cleavage of C3[77]. Immunohistochemical (IHC)[116] and proteomic[127] studies have shown there are cleavage fragments of activated complement in and around drusen. While RPE cells are known to express complement components and regulators[93, 128] it has also been shown that oxidative stress reduces expression levels of the regulators[129]. One hypothesis for AMD disease progression is that the concomitant chronic complement

activity along with a decrease in complement regulators eventually leads to RPE cell stress that culminates in death. Therefore, a potential therapeutic approach is to regulate complement activation on RPE cells.

Gene transfer approaches to ocular tissues using adenovirus as the gene delivery vector has been found to be safe in a number of clinical trials[56, 58]. Recombinant adenovirus constructs have a significant tropism for RPE cells[53], can accommodate transgene expression cassettes up to 36 Kb[52, 55] and can persist in murine ocular tissues for at least one year (latest time point examined)[54] or up to the 'lifetime' of the animal[51]. Furthermore, adenovirus infects a variety of cell types with expression observed within 24 hours of infection. Hence, in the following study we selected adenovirus as a vector for proof of concept to develop a novel regulator of complement.

Previously, we have described independent studies where we examined human complement regulators CD46, CD55, and CD59 for their ability to regulate complement in the membrane bound form on mouse RPE cells[53, 120, 130]. CD46 prevents the formation of C3 convertase by acting as a cofactor for factor I mediated decay of C3b[118]. CD55 acts by dissociating the C3 convertase[131]. CD59 acts furthest in the pathway by preventing the formation of the MAC complex[132]. While each regulator was effective at blocking complement the protection was limited to the site where it was expressed, yielding a 'patch' of protection in the RPE. This prompted us to develop a secreted regulator capable of diffusing through the retina, offering protection to the entire affected region. Here, we build upon our previous findings and on what is currently

known about the complement regulatory sequence of each regulator. We describe a novel protein where we have taken what we believe to be the smallest functional unit of CD46, CD55, and CD59 and combined each via glycine linkers into one protein. Furthermore, we removed the membrane anchors but retained a secretory signal at the N-terminus. This allows us to extend protection from the initial site of transduction to the entire eye. By retaining only the domains of each regulator necessary for complement regulation we were able to maintain a relatively small protein, 130Kd, compared to other known soluble complement regulators such as factor H (150kDa)[133] and sCR1 (200kDa)[134]. Furthermore, there in no known natural regulator that possess the activity of CD46, CD55, and CD59. Therefore, this is the first description of a protein capable of blocking complement at 3 critical steps in the complement pathway. We have termed this new protein soluble terminator of activated complement (STAC) and discuss the therapeutic implications in the context of gene therapy for treating AMD.

4.3 Results

STAC construct and expression vector

STAC is composed of the complement regulatory domains of CD46, CD55, and CD59 while retaining the native secretory signal of CD59 at the N-terminus (figure 18). When designing STAC we modified the sequence of each complement regulator in an attempt to minimize the overall size of the protein by eliminating domains that are not critical for complement regulation. The SCR domains of CD46 and CD55 are essential for complement control while the serine threonine proline (STP) region is heavily glycosylated[135, 136], playing a role in protein stability. With this in mind we removed

the majority of the STR region and retained the full-length SCR domains for each regulator. To separate each domain we added a series of 3 glycines as linkers. We chose glycine because it is the smallest amino acid therefore is the least likely to cause steric hindrance with protein folding.

The STAC transgene was placed into the deleted E1 region of Adenovirus under control of a chicken beta actin promoter (AdCAGSTAC). As a negative control an empty vector was generated that contains only the pA (AdCAGpA) and a GFP negative control vector (AdCAGGFP)(figure 19A). In order to determine if AdCAGSTAC is expressed, secreted, and contains the relevant CD46, CD55, and CD59 domains we infected human RPE cells (ARPE19) then collected the media and probed it by western blot using specific antibodies to the extra-cellular domains of each regulator (figure 18B). A strong signal can be seen with each antibody at 130Kd and a faint band at close to 150Kd. The two bands most likely represent different glycosylation states. This is not surprising since a portion of the STP regions were retained and have been indicated as sites of N and O linked glycosylation.

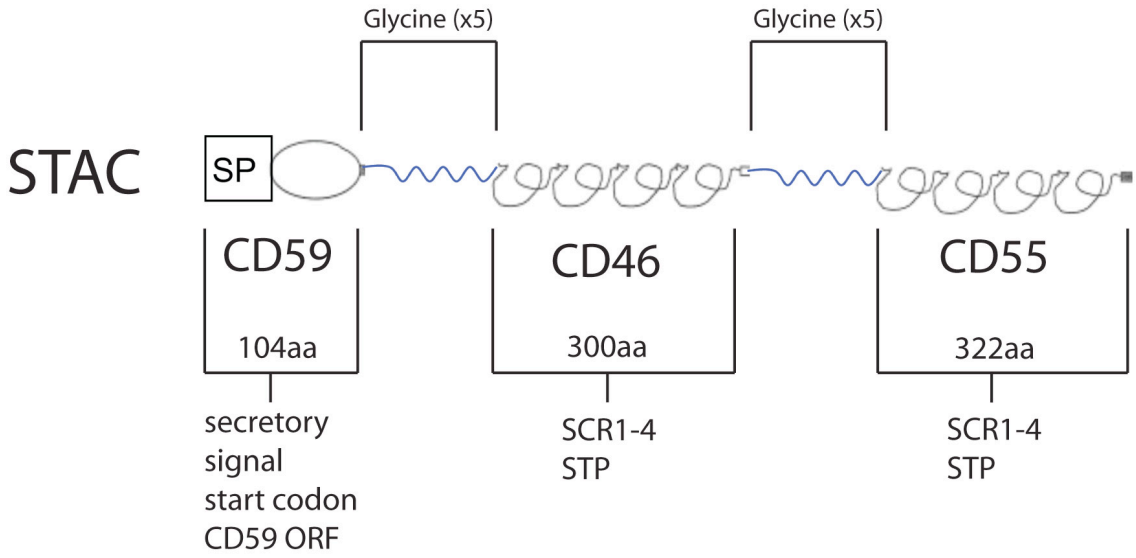


Figure 18: STAC gene sequence.

CD59 contains the start codon, secretory signal and complement regulatory domains. CD46 contains the 4 SCR motifs and STP rich region. CD55 contains the 4 SCR motifs and STP rich region followed by two stop codons (TGA). The membrane anchors were removed and each regulator separated by a series of 5 glycine amino acids.

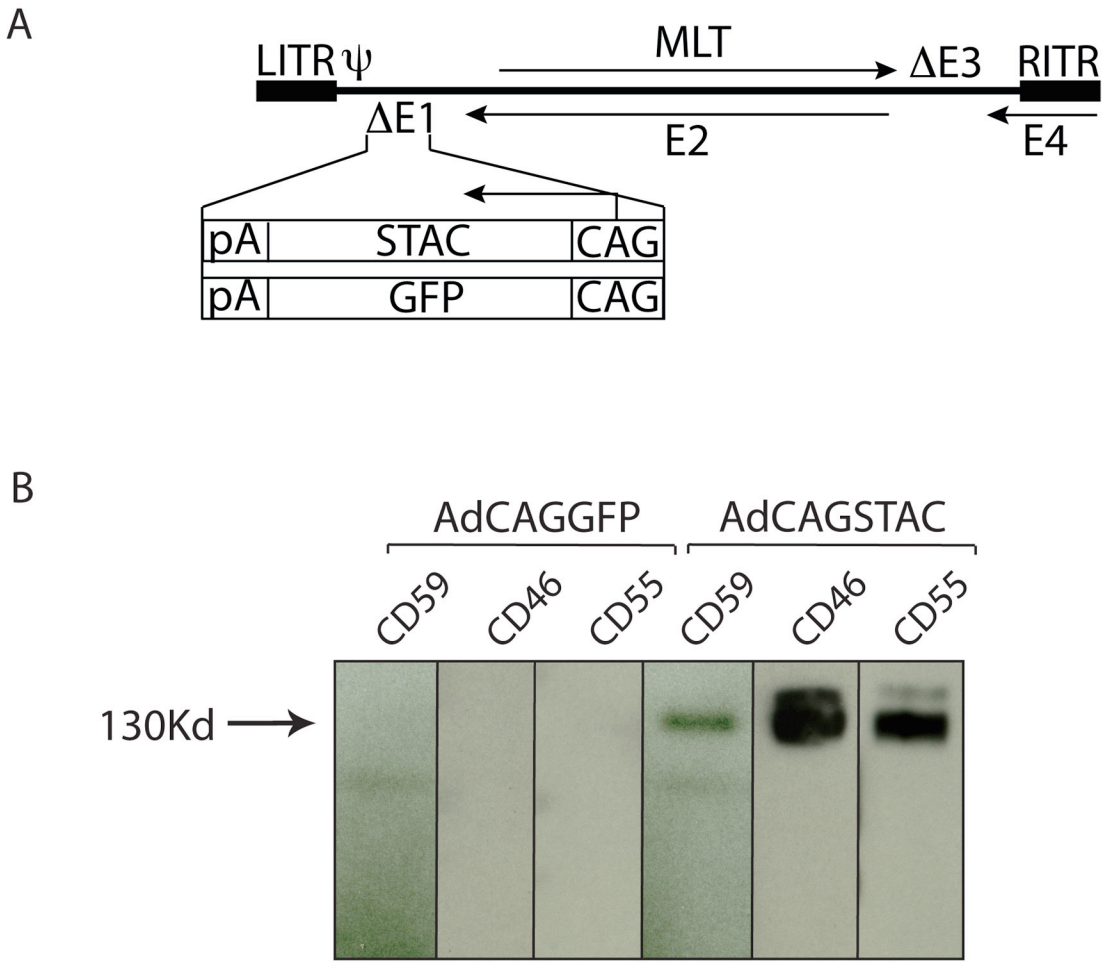


Figure 19: Adenovrial expression vectors.

A) STAC or GFP were placed into the deleted E1 region of first generation adenovirus and expressed using a CMV enhancer/chicken β-actin promoter (CAG). B) Western blot probed with antibodies to detect either CD46, CD55, or CD59 detect a strong band at 130kDa and a faint band at 150kDa. (CAG=chicken β-actin promoter; pA=bovine growth hormone poly A signal; LITR=left inverted terminal repeat; RITR=right inverted terminal repeat; Ψ=packaging signal; MLT=major late transcript; E1-E4=early regions 1-4)

STAC protects Hepa1c1c7 cells from complement activity

In order to test the ability of STAC to prevent MAC deposition we infected ARPE19 cells with either AdCAGSTAC or AdCAGGFP then collected the media. The media was transferred onto plated Hepa1c1c7 (a mouse liver cell line) and complement activated by adding 10% normal human serum (NHS). Hepa cells incubated in STAC media showed a 68% decrease in the amount of MAC formation on the surface of the cell compared to GFP control (figure 20A and C). Furthermore, the morphology of the cells in the control group changed dramatically, consistent with cell lysis, while the morphology of the STAC group remained relatively unchanged (figure 20A inset).

In order to determine physiological relevance of decreased MAC from STAC inhibition we measured the permeability of the Hepa cells following exposure to NHS. We infected ARPE cells with either AdCAGSTAC or AdCAGGFP for 3 days then collected the media and used it to incubate Hepa cells in increasing dilutions of NHS. Afterwards, we measured the amount of propidium iodide (PI) taken up by the cells by FACS. We found that STAC reduces the amount of PI uptake to nearly heat inactivated (Hi) levels up to a concentration of 2% NHS (figure 20C).

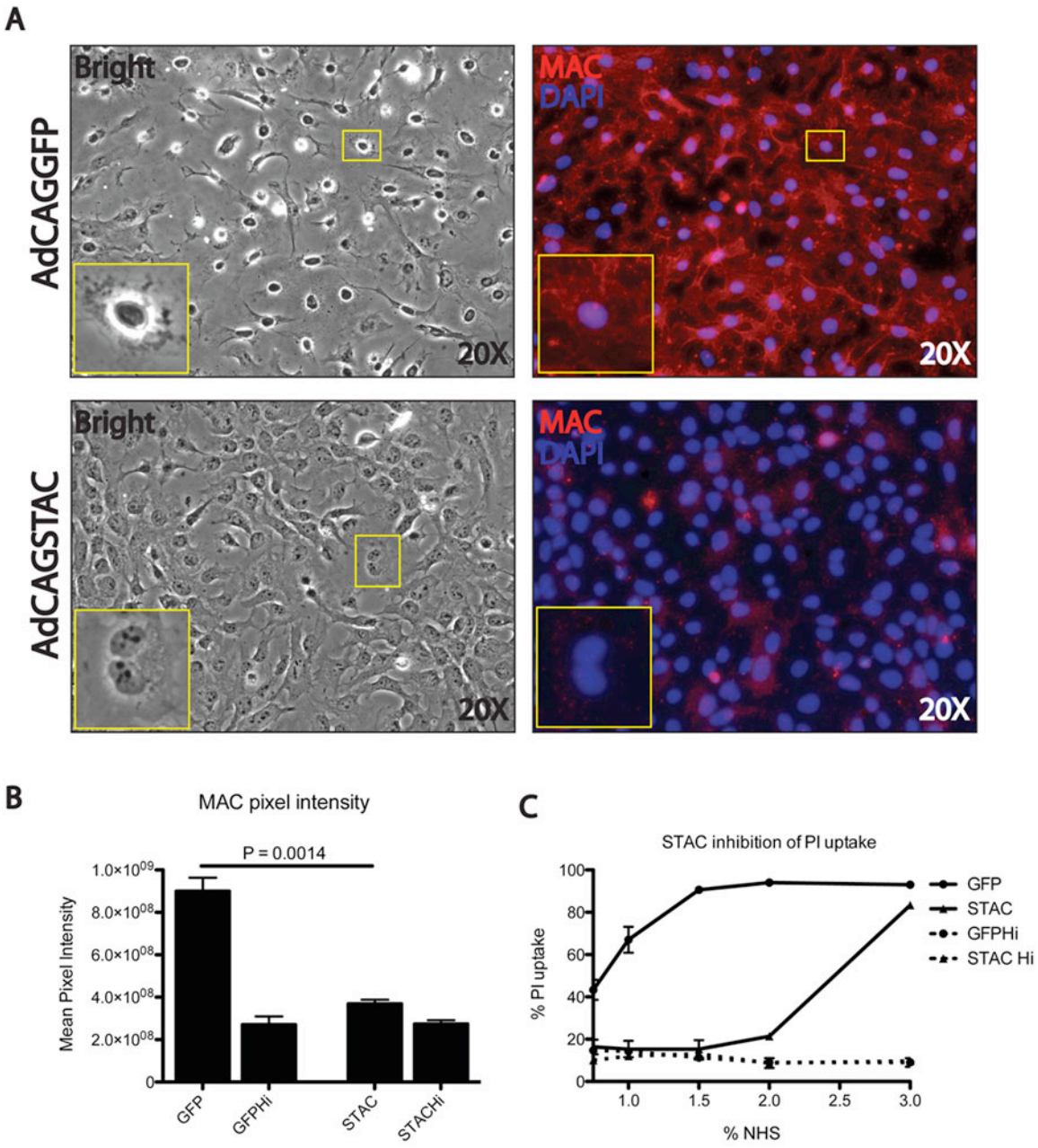


Figure 20: STAC protects Hepa1c1c7 cells from complement-mediated injury.

A) IHC for C5b9 following complement activation show decreased MAC in STAC treated Hepa cells and relatively unchanged cell morphology (inset). B) Quantification of pixel intensity of MAC shows a reduction to nearly Hi levels. C) FACS analysis for PI uptake following exposure to increasing concentrations of NHS. STAC reduces PI uptake to nearly Hi levels up to a concentration of 2% NHS. (Representative of 3 separate experiments performed in duplicate each time). (NHS=normal human serum; HINHS=heat-inactivated normal human serum; ns=not significant; PI= propidium iodide; DAPI= 4',6-diamidino-2-phenylindole; MAC=membrane attack complex)

STAC protects in a humanized *in-vivo* mouse model of complement deposition

To test the ability of STAC for protecting against complement activation in-vivo we developed a humanized mouse model. We injected $5x10^9$ particles of AdCAGSTAC or AdCAGGFP into the intrapeatoneal (IP) space of C57/Bl6J mice. An IP injection of adenovirus results in a high degree of liver transduction, mainly by the Glisson's capsule that surrounds the liver (figure 21A). After 7 days an anti PECAM antibody (250ug in a volume of 200ul) was injected into the left ventricle, causing binding of antibody to the endothelial lining of blood vessels priming them for complement activation. NHS (90% in PBS containing Ca and Mg) was injected into the left ventricle to cause complement deposition on the lining of endothelial cells. Examining cross sections taken from the central region of the left lobe of the liver revealed a 79% decrease in MAC deposition in AdCAGSTAC injected group compared to AdCAGGFP (figure 21B and C).

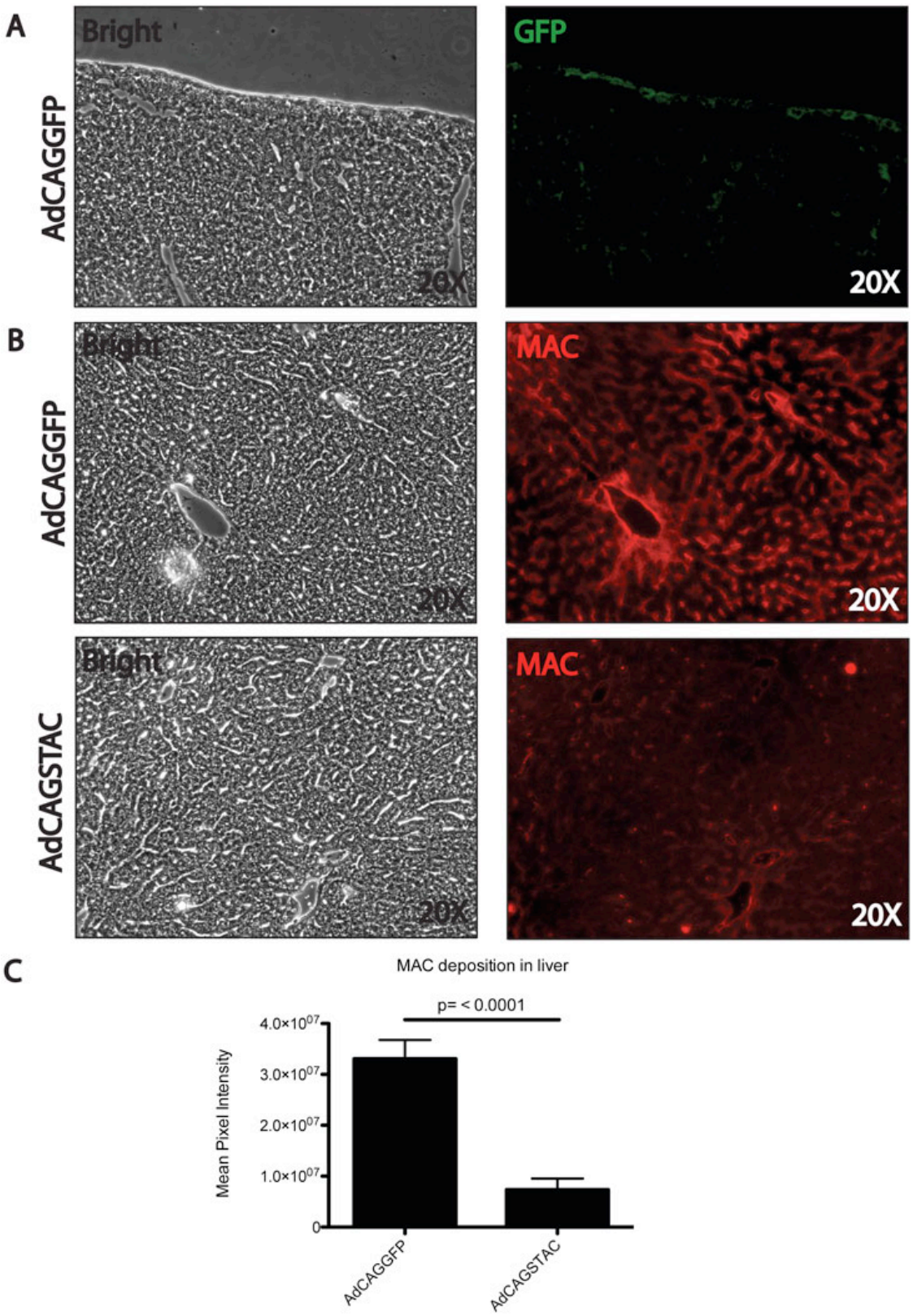

A
Bright
AdCAGGFP
20X
GFP
20X
B
Bright
AdCAGGFP
20X
MAC
20X
Bright
AdCAGSTAC
20X
MAC
20X
C
MAC deposition in liver
p= < 0.0001
Mean Pixel Intensity
4.0×10^07
3.0×10^07
2.0×10^07
1.0×10^07
0
AdCAGGFP
AdCAGSTAC

Figure 21: STAC protection of endothelial cells in a mouse *in-vivo* model of complement activation.

A) GFP expression 7 days after an IP injection of AdCAGGFP showing transduction on the liver capsule. B) Cross-sections taken through the center of the left liver lobe stained for MAC. C) Quantification of MAC pixel intensity showing a reduction in AdCAGSTAC injected group to nearly Hi levels in the center of the left liver lobe. (Data represent 7 mice in AdCAGGFP and 8 mice in AdCAGSTAC) (MAC= membrane attack complex)

4.4 Discussion

We have described a novel chimeric protein, STAC, which contains the relevant complement regulatory domains of CD46, CD55, and CD59. Initial testing of STAC for PI uptake indicates protection of 100% at 1% NHS which prompted us to test increasing serum amounts, as our previous studies using the individual membrane bound regulators did not show complete protection at this concentration. It required a concentration of 2% NHS to see partial protection (66%) using STAC (figure 20C). It is unlikely that removing the membrane anchor would lead to an increase in the ability to protect against complement. One possibility for enhanced protection is that when one portion of STAC binds to complement it becomes sequestered to the membrane. The glycine linkers may allow flexibility between the functional domains so that each unit of STAC can operate to prevent complement activity in the surrounding area. Since STAC contains 3 domains that act on different portions of the cascade when one functional domain has blocked complement another domain may engage. This could allow STAC to remain bound to the membrane for a relatively long period of time, placing it directly at the site where it is needed for complement regulation. Another possibility could be that there is synergistic cooperation amongst the functional domains in STAC. Evidence for this comes from previous studies using CD46 and CD55 where that the presence of both causes an

additive protective effect[137]. The rationale for this is that while CD46 is able to bind and cleave C3b into inactive products it is not able to bind to C3b once it has been incorporated into the C3 convertase. CD55, on the other hand, efficiently disassembles the C3 convertase but does not proteolytically cleave it into inactive components. This means the C3 subunits are able reform and CD55 is required, once again, to disassemble the convertase. Having both CD46 and CD55 present may allow any C3b that escapes CD46 to be regenerated when CD55 disassembles the convertase. Since CD46 is generating inactive C3b cleavage products the amount of convertase formation would be reduced, minimizing the burden on CD55.

While the humanized liver assay demonstrated that STAC protects in an *in vivo* setting it also gave us an opportunity for testing its ability to diffuse through tissue. As demonstrated in figure 21 an IP injection of adenovirus mainly transduces the collagenous Glisson's capsule that surrounds the liver. Therefore, any protection into the central portion of the liver would be due to diffusion of STAC away from the initial site of transduction. There are two scenarios for STAC protection in the center of the liver: 1) STAC secreted from the Glisson's capsule region of the liver diffuses between the densely packed hepatocytes of the liver and enters the blood vessels to protect the endothelial cells. 2) STAC secreted from the Glisson's capsule region of the liver diffuses into the circulatory system then perfuses the vasculature of the liver to offer protection of endothelial cells. Either scenario would indicate that STAC is able to readily diffuse through the liver and endothelial cells.

Currently, there are 4 small molecules in clinical trials targeting the inhibition of complement as a treatment for AMD (www.clinicaltrials.gov), further supporting the convincing connection between complement regulation and AMD. Each is administered through repeat injections either intravenously or into the vitreous. By taking a gene therapy approach we are circumventing the need for frequent repeat administration, substantially benefiting the patient. The small size of STAC should allow diffusion through the retina, offering protection away from the initial site of expression minimizing the number of injection sites. By generating a highly potent inhibitor of complement we have increased the likelihood that complement will be dampened at the furthest site from expression, where the concentration of STAC is the lowest. Future direction may be to consider the best vector for delivery. While we have used adenovirus in our proof of concept studies and it has shown efficacy in clinical trials it may also be desirable to consider AAV vectors, which have achieved recent success in Leber's congenital amaurosis (LCA) patients[138]. Furthermore, AAV injected into the vitreous of patients transduces mainly ganglion cells. This may be a more relevant cell type to target since RPE cells in AMD patients are already compromised due to disease progression. Further stress, due to viral entry, may exacerbate stress to the cells. Whether using adenovirus or AAV STAC is a soluble and potent inhibitor of complement that deserves further investigation as a potential therapy for AMD patients.

Chapter 5:

Summary and Potential Future Direction

Summary

Several polymorphisms have been identified in the retina leading to blindness. While the majority of these mutations occur in photoreceptors (retnet.com) several have also been identified in distinct cell types. Therefore, the ideal approach to correct the gene would be to deliver to only those cells that are affected. Adenovirus as a gene therapy vector for ocular disease has recently been demonstrated in two clinical trials, one delivering PEDF for the treatment of age-related macular degeneration and a second delivering thymidine kinase for the treatment of retinoblastoma[56, 58]. Three adenoviral vectors containing modification to either the Ad5 fiber or penton base have been previously described to yield enhanced photoreceptor transduction, Ad5/F37, Ad5/F35, and Ad5ΔRGD. We directly compared Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD using the same promoter, transgene expression cassette, injection, and detection techniques. Unlike prior reports, our results indicate that each vector has a distinct tropism for particular cell types of the retina. We show that Ad5/F37 mainly transduces müller cells, Ad5/F35 mainly the RPE, and Ad5ΔRGD mainly photoreceptors. Furthermore, we were able to restrict expression to only the photoreceptors by using Ad5ΔRGD and a shortened version of the rhodopsin promoter (figure 14).

The prevailing theory for the pathogenesis of AMD is that chronic activation of the complement system, particularly the alternative arm, places stress on RPE cells leading to cell death. Such evidence includes the presence of alternative pathway fragments closely associated with drusen[57] or presence of MAC on the surface of RPE and choroidal blood vessels[117]. With evidence accumulating implicating the alternative pathway in

AMD, we chose to evaluate the potential of a complement regulator known as membrane cofactor protein (CD46) that has previously been shown to preferentially dampen the alternative pathway[118]. Furthermore, CD46 in human retinal tissue is localized to the basal and lateral surface of RPE cells[93]. This places CD46 close to the site of complement activation in AMD patients in which MAC deposition has been observed in RPE cells, drusen, and choroidal endothelial cells. Importantly, a recent observation has been made that CD46 expression is reduced on the RPE of AMD patients[115]. Therefore, we expressed CD46 from adenovirus and evaluated protection in RPE cells and in an *ex-vivo* mouse model of AMD. We found CD46 to be effective at preventing alternative pathway activation but not classical pathway. Since the alternative pathway has been implicated in AMD this angle of gene therapy would target the damage caused by the alternative pathway leaving the classical pathway to function normally.

In order to extend protection from complement away from the initial site of expression we have described a novel chimeric protein, STAC, which contains the relevant complement regulatory domains of CD46, CD55, and CD59. Initial testing of STAC for PI uptake indicates protection of 100% at 1% NHS which prompted us to test increasing serum amounts, as our previous studies using the individual membrane bound regulators did not show complete protection at this concentration. It required a concentration of 2% NHS to see partial protection using STAC. Using a humanized liver assay we demonstrated that STAC protects in an *in vivo* setting. This model also gave us an opportunity to test the ability of STAC to diffuse through tissue since an IP injection of adenovirus mainly transduces the capsule that surrounds the liver (Fig. 21).

Here for the first time we describe a novel secreted complement inhibitor having the functional domains of CD46, CD55, and CD59 (STAC). No naturally occurring complement regulator is known to have the ability to block complement at 3 separate steps of the complement cascade. We show that STAC is highly effective at blocking complement and, importantly, it is able to diffuse and offer protection at sites distant from initial expression.

While there are currently 4 small molecules in clinical trials aimed at reducing complement activity (www.clinical trials.gov) there are no trials taking a gene therapy approach. Gene therapy would circumvent the need for repeat injections substantially benefiting the patient. By generating a highly potent inhibitor of complement we have increased the likelihood that complement will be dampened at the furthest site from expression so that one injection is sufficient to offer therapeutic benefit.

Potential future direction

We have used the psuedotyped adenoviral vector that best transduces photoreceptors and shown that we are able to then incorporate a cell specific promoter to limit expression to only the photoreceptors. Since the photoreceptors represent the cell type in the retina with the most gene mutations this was the most appropriate cell type to target first. Further experiments should attempt to use specific promoters for the RPE or müller cells. It would then be beneficial to test each in an animal model of photoreceptor, müller cell, or RPE cell dysfunction to assess the efficacy for gene transfer. As the best mode of gene

therapy would be to only treat those cells that are affected this would be the next step towards clinical application.

We have shown that CD46 is capable of modulating the alternative arm of complement but have not extended this finding into an *in-vivo* model. The laser model of CNV is mediated through the alternative arm of complement[139], making it an appropriate model to test CD46. Furthermore, for therapeutic implications it would be pivotal to establish a link between CD46 and AMD pathogenesis. One way to investigate a potential role could be to follow aHUS survival patients, known to develop the disease via a CD46 polymorphism, into later stages of life and determine if they are at an elevated risk Another way to correlate CD46 to AMD would be to partake in a more thorough evaluation of the level of CD46 expression on RPE cells of AMD patients. While some work has been done towards this end more work should be done to corroborate the results.

We have tested and confirmed the ability of STAC to prevent complement-mediated injury to cells and shown that it is highly effective using human serum. As STAC was made using human genetic material human serum was the most appropriate for initial validation. Although, in order to test in an *in-vivo* model of complement mediated injury we must first show that STAC cross-reacts with the species for the animal model being used. Therefore, STAC should be thoroughly evaluated using the serum from various animal species to determine the ability to cross-react. Afterwards, the most appropriate animal could be determined as an AMD model for *in-vivo* testing. While we have shown STAC to be highly functional we have not tested the ability of each domain. At this point it remains unclear how much protection each is offering. Finally, in order to direct STAC

to the site of complement activity it may be beneficial to incorporate a recognition domain for a receptor known to be up-regulated on cells that are undergoing complement mediated cell lysis.

Materials and Methods

Cell lines

HEPA 1c1c7, Y79, and 293 cell lines were obtained from ATCC. Cell culture reagents were purchased from Invitrogen Life Technologies. 293 and 911[140] cells were maintained in DMEM/10%FBS. HEPA 1c1c7 cells in alpha MEM/10%FBS and Y79 cells in RPMI 1640 supplemented with L-Glutamine, D-Glucose, Na pyruvate (1mM), and 15% FBS. Primary mouse RPE cells were obtained from 6-10 week old C57Bl/6 mice. Eyes were enucleated, lens, cornea removed to reveal the RPE cell layer. Eye-cups were incubated in 200µl of 0.25% trypsin/EDTA for 1 hour at 37°C. RPE cells were pulled off in sheets and homogenized in 20µl αMEM/10%FBS. The suspension was placed in the center of an 8 well poly-D-lysine coated chamber slide (Becton Dickenson) for 10 minutes to allow cells to adhere to the plate, and an additional 130µl αMEM/10% FBS was added to each well. Cells were kept in a humidified incubator at 37°C with 5% CO_2 for 3 days prior to use.

Recombinant adenovirus constructs

The hybrid 5/35 fiber was amplified by PCR from an Ad5/F35 plasmid (Obtained from Andre Lieber). Using the primers AdE28403F (5' TATTCAGCAGCACCTCCTTGCC 3') and AdE30702R (5' ATGTAGGCGTGGACTTCTCCTTCG 3') to yield a product of

1.4Kb. An 842bp *Age1/Mfe1* fragment was then cloned into *Age1/Mfe1* digested p6.2FIB[100] to generate p6.2FIBF35. A 4.9kb Pac1/Srf1 fragment of p6.2FIBF35 was then cloned into similarly digested Adeasy1[141] to create pAdEasyF35. This was then recombined with pShCAGGFP[82] to generate AdF35CAGGFP. AdF37CAGGFP was generated by recombination of pShCAGGFP and pAd5F37[100]. Ad5CAGGFPΔRGD has been previously described[82]. The Ad5sRhoGFPΔRGD vector was made using a similar strategy. The 257bp mouse opsin was PCR amplified from SB6.25 (A gift from Wolfgang Baehr) using the primers sRHO-F (5' CCCAGATCTCCCGAATTCCCAGAGGACTCTGG 3') and sRHO-R (5' CCCGTCGACCCCGGCGAGCTCAGCCACTGAC 3'). The PCR product was digested with *BglII/SalI* and cloned into the corresponding sites of pShGFP[82]. The resulting plasmid was named pShsRhoGFP. This was recombined with pAdeasyΔRGD[82] to generate Ad5sRhoGFPΔRGD. Ad5F37 and Ad5ΔRGD were constructed using the AdEasy system[141] and previously described[82, 100]. In all cases adenovirus was recovered in 293 cells as previously described[100]. Viruses were purified using the adenopure purification kit (Puresyn, Inc.), and concentrated using a 100kDa filter (Millipore). Viral titers were determined using a Beckman Coulter DU530 UV/Vis mass spectrophotometer at 260nm.

CD46

E1/E3-deleted adenovirus serotype 5 was used to express human CD46 (hCD46) or no transgene. hCD46, ATCC (MGC-26544), was excised from pBluescriptR using *EcoRI* and *SspI* and inserted into pCAGEN using *EcoRI* and *EcoRV* (gift from C Cepko, Harvard Medical School, Boston MA) between a CMV enhancer/chicken β-actin

promoter (CAG) and a rabbit globin polyadenylation (pA) termination sequence. The sequence containing CAG, hCD46, and pA was excised from pCAGEN using *SpeI* and *HindIII* and inserted into pShuttle[141] using *XbaI* and *HindIII*. The pShuttle was recombined with Adeasy-1 then linearized and transfected into 293 cells. Virus production has been previously described[100]. Following initial transfection virus was amplified in 911 cells. Viral purification was performed using the adenopure purification kit (Puresyn, Inc.) and viral titer determined at OD260 using a spectrophotometer then plaque-purified, previously described[52]. The hCD46 and pA, previously published[100], expressing viruses are subsequently referred to as AdCAGCD46 and AdCAGpA, respectively.

STAC

The N-terminus of STAC contains the human CD59 (ATCC cat.# 10658204) start codon, secretory signal peptide, and SCR domains while lacking the sequence for the C-terminal 26aa, which comprise the signal sequence for attachment of the GPI anchor. Therefore, the CD59 coding region from pOTBCD59 spans nucleotides 165-479. The N-terminus of human CD46 (ATCC cat.# 7491463) was attached via a glycine linker (GGAGGCGGAGGTGGA) to the C-terminus of CD59. The start codon and first 33aa, comprising the secretory signal peptide, were removed from the N-terminus of CD46. The following 300aa were retained, which comprise the SCR domains and the serine/threonine/proline (STP) rich domain. Therefore, the CD46 coding region from pBluescriptRCD46 spans nucleotides 866-1767. The N-terminus of human CD55 (ATCC cat.# 5830488) was attached via a glycine linker (GGAGGCGGAGGTGGA) to the C-terminus of CD46. The start codon and the first 33aa, comprising the secretory

signal peptide, were removed from the N-terminus of CD55. The following 322aa were retained, which comprise the SCR domains and the STP rich region. The C-terminal 26aa have been removed, which comprise the signal sequence for attachment of the GPI anchor. Therefore, the CD55 coding region from pCMVsport6CD55 spans nucleotides 315-1283. Two stop codons (TGA) were added in tandem to the C-terminus. The N-terminus of STAC was flanked by an *XhoI* restriction site and the C-terminus by *SspI* and *SalI* sites. The full length STAC sequence was synthesized by Genscript (Piscataway, NJ) and inserted into the *EcoRV* site of a pUC57 plasmid. STAC was excised from pUC57 using *XhoI* and *SspI* then inserted into pShuttle[141] using *XhoI* and *EcoRV* between a CMV enhancer/chicken β-actin promoter (CAG) and a rabbit globin polyadenylation (pA) termination sequence to generate pShCAGSTAC. Adenovirus production E1/E3 deleted serotype 5 adenovirus was used to express STAC or GFP. The pShuttle[141] containing each insert was recombined with Adeasy-1 then linearized and transfected into 293 cells. Virus production has been previously described[100]. Following initial transfection virus was amplified in 911 cells. Viral purification was performed using the adenopure purification kit (Puresyn, Inc.) and viral titer determined at OD260 using a spectrophotometer.

Western blot

$5x10^9$ viral particles were suspended in 50µl of 100mM Tris-HCL, 0.5M NaCl, 0.1% SDS, and 10% Triton X-100 containing leupeptin (10µg/ml), aprotinin (10µg/ml), and PMSF (1mM). Samples were loaded onto a 10% Tris-Glycine polyacrylamide gel under denaturing conditions. The fiber protein was detected using the monoclonal antibody,

Ab-4 (Clone 4D2, Neomarkers) at a concentration of 1:1000, followed by HRP-conjugated goat anti mouse (Jackson ImmunoResearch) secondary antibody. 911 cells were infected with either AdCAGCD46 or AdCAGpA at a multiplicity of infection (MOI) of 1000. After 24 hours, cells were collected by trypsin treatment and centrifugation, cells lysed in lysis buffer (SDS/triton) containing protease inhibitors (leupeptin, aprotinin, PMSF), and lysate run on a 12.5% tris HCL pre-cast gel (Biorad). Following transfer, the nylon membrane was probed with a mouse anti human CD46 antibody (MEM258, serotec) at a dilution of 1:1000. An HRP conjugated secondary antibody was used followed by detection with luminol (PIERCE chemiluminescent subtrate kit). For Adenoviral constructs 911 cells were infected with AdCAGSTAC, AdCAGsCD46, AdCAGsCD55, AdCAGsCD59, or AdCAGGFP at a multiplicity of infection (MOI) of 1000. After 24 hours, cells were collected by trypsin treatment and centrifugation, cells lysed in lysis buffer (SDS/triton) containing protease inhibitors (leupeptin, aprotinin, PMSF), and lysate run on a 12.5% tris HCL pre-cast gel (Biorad). Following transfer, the nylon membrane was probed with a mouse anti human CD46 antibody (MCA 2113, Serotec, Raleigh NC) at a dilution of 1:10,000, goat anti human CD55 antibody (AF2009, R&D systems, Minneapolis MN) at a dilution of 1:20,000, or a goat anti human CD59 antibody (SC-7077, Santa Cruz, Santa Cruz CA) at a dilution of 1:500 and incubated overnight at 4°C on a shaker plate. An HRP conjugated secondary antibody was used followed by detection with luminol (Cat.#34080, Thermo Scientific, Rockford IL).

In vitro CD46 transfection and blocking

pBluescript containing human CD46 cDNA was obtained from ATCC (MGC-26544). The cDNA was released by digestion with *EcoRI* and *SspI* and cloned into *EcoRI/EcoRV* digested pCAGEN (A gift of Connie Cepko) to generate pCAGCD46. pCAGntLacZ was generated by insertion of a *NotI* fragment of pBntLacZ (pBluescript containing LacZ cDNA) into *NotI* digested pCAGEN. 6μg of either pCAGENCD46 or pCAGntLacZ was transfected into $1x10^5$ HEPA 1c1c7 cells using Lipofectamine 2000 (Invitrogen 11668-027) and maintained in alpha MEM supplemented with 2%FBS at 37°C/5%CO_2 for 48 hours. Cells were then infected with either Ad5CAGGFP (MOI 20) or Ad5F35CAGGFP (MOI 750) for an additional 48 hours.

For blocking experiments

Cells were transfected as described above. After 48hrs the cells were treated with either 5ug/ml of mouse anti human CD46 (Serotec MCA2113) to block the SCR1 domain of CD46 or rabbit anti sheep IgG (Jackson Immuno Research 313-005-006) as a non-specific control for 30 minutes at 37°C 5% CO_2. The cells were then infected with either Ad5CAGGFP (MOI 20) or Ad5F35CAGGFP (MOI 750) as described previously. GFP expression was analyzed using an Olympus IX51 microscope and by flow cytometry using BDFACSCalibur.

Y79 cell transduction

$4.5x10^5$ Y79 cells were infected with either Ad5CAGGFP, Ad5F35CAGGFP, Ad5F37CAGGFP, or Ad5ΔRGDCAGGFP at an MOI of 500. Infected cells were

maintained in RPMI media at 37° 5%CO_2 for 24hrs after which GFP expression was analyzed using BDFACSCalibur.

In vivo transduction

All animal studies were in accordance with the policies set forth by the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and federal and state guidelines. Unless otherwise specified, for each viral construct at least 6 eyes of 6wk old C57BL/6J mice were injected with 1μl containing $1x10^9$ viral particles. After 6 days the eyes were harvested and fixed in 4% PFA.

To analyze surface area and efficiency of viral transduction 6 eyes were harvested for each construct, the cornea and lens removed, and the eyes cut into 4 quadrants and flat-mounted onto a coverglass. An image of each eyecup was captured using a Nikon SMZ800 microscope and Olympus DP20 camera under brightfield and the GFP (2.5 second exposure) filter. To prepare for quantitative analysis all images were sized to 600 x 450 pixels using photoshop. Area of transduction was calculated using Image J version 1.41o (rsbweb.nih.gov/ij/). The percent area of transduction was calculated by dividing the transduced region by the total surface area. The mean fluorescence of the transduced area was calculated by converting the image to grayscale and calculation of the mean pixel intensity using Image J. The surface plots were obtained using the Image J plug-in feature titled 'interactive 3D surface plots'.

To compare viral transduction in the retina versus the RPE/choroid, 6 eyes were harvested for each construct, lens and cornea removed, and the retina peeled away from the RPE/choroid. The retina and RPE/choroid were placed in separate 1.5ml tubes and submerged in liquid nitrogen for 15 seconds. Eyes were not visualized for GFP prior to dissection in order to prevent UV damage and expedite removal of the retina/RPE without protein degradation. An RNeasy kit (Qiagen 74104) was used to extract and purify the RNA, after which 3ug of each sample was DNAse treated using Turbo DNAse (Ambion 2238G). Samples were loaded in triplicate for RT-PCR (Biorad Icycler IQ5) to detect GFP expression using a custom assay to EGFP obtained from Applied Biosystems with the forward primer EGFP-ANYF (5' GAGCGCACCATCTTCTTCAAG 3') the reverse primer EGFP-ANYR (5' TGTCGCCCTCGAACTTCAC 3') and the probe EGFP-ANYM1 (5' ACGACGGCAACTACA 3'). All samples were normalized to mouse beta actin (Applied Biosystems cat. # 4352664).

To analyze the transduction pattern in the retina the quadrant containing the GFP region was dissected from each flatmount used for the transduction study in figure 4 then embedded in OCT. Serial 14μm transverse sections were made through the entire quadrant and images taken using an Olympus BX51 microscope and Retiga 2000R FAST camera with QCapture Pro 5.0 software. Exposure times were adjusted independently for the RPE and retina for each virus to optimize visualization of the cell types transduced.

To determine the proportion of transduced cells in the INL and ONL versus the total number of cells for each layer in Ad5ΔRGD we used a modified stereological technique.

84 serial 14μm sections spanning the transduced area were used for analysis. The fluorescent images were converted to 8 bit and analyzed with stereoinvestigator software version 6.5.5.1. The optical dissector probe was used along with a sampling grid of 86.3μm x 20μm and a sampling periodicity of 10. Prior to sampling, the INL/ONL/RPE were manually traced using the brightfield image to create a region of interest (ROI) for each section. The brightfield image was then used to count the total number of cells and the fluorescent image for GFP positive cells. The raw data, figure 6A, was automatically calculated by the microbrightfield software using CE Gundersen variance. This provides a high, median, and low estimate of cells for each section. Using microbrightfield software for the optical dissector probe the raw data was converted into a 3D reconstruction to estimate the total number of cells/transduced cells, figure 6C. This takes into account the curvature of the tissue, based on the ROI, along with the CE Gundersen variance. The coefficient of error was estimated automatically by the microbrightfield software using the Scheaffer method. The Scheaffer method is designed to calculate the CE for data points collected from a traced ROI taken from randomly sampled areas through a non-uniformly shaped tissue. Therefore, the curvature of the tissue is extrapolated from the traced images.

Immunohistochemistry

Y79 cells were plated at a density of $4.5x10^5$ cells in poly-D-lysine coated chamber slides (BD Biosciences 354632) for 24 hours then fixed in 10% formalin for 15 minutes prior to staining. Tissue sections were fixed in 4%PFA then taken through a graded series of sucrose (15% and 30%) prior to sectioning at 14μm. To detect sialic acid we used

biotinylated Maackia Amurensis Lectin II (Mal II, Vector Laboratories B-1265) (100μg/ml) followed by Cy3 conjugated streptavidin (1μg/ml). To detect integrin we used anti-integrin alpha V mAb (Transduction Laboratories C75120) (1μg/ml) followed by Cy3 conjugated goat anti mouse (1μg/ml). Detection of CD46 was achieved using mouse anti human CD46 (Serotec, clone MEM-258, MCA2113) (20μg/ml) followed by Cy3 conjugated goat anti mouse (1μg/ml). To detect the extracellular region of CAR we used rabbit anti CAR (gift of Jeff Bergelson) (1:2000) followed by Cy3 conjugated goat anti rabbit (1μg/ml). We used two antibodies to detect müller cells. We used rabbit anti GFAP (Novus biologicals NB300-141B2) followed by Cy3 conjugated goat anti rabbit (1μg/ml). We also used mouse anti CRALBP (Abcam Ab15051-50) followed by Cy3 conjugated goat anti mouse (1μg/ml). CRALBP staining was performed on 20μm frozen retinal sections while all other staining was on 14μm sections. All negative controls were obtained by omission of the primary antibody. To detect membrane expression of CD46 on hepa1c1c7 and primary RPE cell cultures, cells were incubated in mouse anti human CD46 (clone MEM258, Serotec) (1:50) in 3% normal goat serum (NGS) (Jackson Immunoresearch) at 4°C for 3 hours prior to fixation. Cells were fixed in 10% NBF overnight at 4°C. Secondary detection was performed with Cy3 goat anti mouse (37.5ng/ml) in 3% NGS for 1 hour. To detect CD46 in fixed 14μm frozen sections and mouse eye-cups, samples were pre-treated with 6% NGS and incubated in mouse anti human CD46 (clone E4.3, BD Pharmingen) (1:100) containing 0.5% triton overnight at 4°C. Secondary detection was performed with Cy3 goat anti mouse (37.5ng/ml) in 0.5% triton for 1 hour. To detect the MAC complex, samples were incubated with mouse anti human C5b-9 (clone aE11, Abcam) (1:100) containing 6% NGS and 0.05% triton for 2.5

hours. Secondary detection was performed with Cy3 goat anti mouse (37.5ng/ml) containing 3% NGS and 0.05% triton for 1.5 hours. For Hepa1c1c7 cells MAC detection was performed using mouse anti human C5b9 (1:50) (ab66768, Abcam, Cambridge MA) in 0.05% triton containing 6% normal goat serum (NGS) for 2.5 hours at room temperature. Secondary detection was achieved using Cy3 conjugated goat anti mouse (1:200) in 0.05% triton containing 3% NGS for 1 hour at room temperature. For MAC detection using mouse liver sections the samples were blocked using 6% NGS in 0.5% triton for 1 hour at room temperature then incubated in rabbit anti human C5b9 (1:400) (Complement Technology, Tyler TX) in 0.5% triton for 2.5 hour at room temperature. Secondary detection was achieved using Cy3 conjugated goat anti rabbit (1:200) for 1 hour at room temperature.

Complement assay on Hepa 1c1c7 cells

Hepa1c1c7 cells were infected (MOI 1000) for 3 days with AdCAGCD46 or AdCAGpA in αMEM/2%FBS. For FACS analysis the cells were collected by trypsinization (0.25%/EDTA), resuspended in 1xPBS containing 0.5% FBS, centrifuged at 1200RPM/4°C, and $5x10^5$ cells re-suspended in 500μl ice-cold Gelatin Veronal Buffer containing Ca^2 and Mg^2 (GVB^{2+}) (Complement Technology). To activate all complement pathways, 25μg/ml rat anti mouse emmprin (Serotec MCA2283) was added for 30 minutes at 4°C followed by 10% Normal Human Serum (NHS) (Sigma), 37°C for 1 hour with constant rotary motion. Cells were treated similarly with 10% Heat Inactivated NHS (HINHS) (56°C for 1 hour) as control. Cell lysis was determined by the propidium iodide (PI) exclusion method. PI was added to each sample (1μl PI into 500μl

GVB^{2+}) and $2.5x10^4$ cells were counted by FACS (FACSCalibur) for PI uptake (CellQuest Pro software, Becton Dickinson).

To preferentially activate the alternative pathway, cells were collected as described above and re-suspended in GVB^{2+}. $5x10^5$ cells were pre-incubated with 25μg/ml rat anti-mouse emmprin antibody and incubated for 30 min. at 4°C. Either 10% NHS or 10% HINHS containing 7mM Mg/10mM EGTA (MgEGTA) was added and incubated at 37°C for 1 hour.

For MAC staining of Hepa1c1c7, cells were plated into an 8 well chamber slide (Becton Dickinson) and infected with either AdCAGCD46 or AdCAGpA for 3 days (MOI 1000). Each well was incubated with 25μg/ml emmprin for 30 min. at 4°C followed by either 10% NHS or 10% HINHS in GVB^2 at 37°C for 5 minutes with MgEGTA to de-activate the classical pathway. Cells were washed twice with cold 1xPBS, fixed for 15 minutes in 10% neutral buffered formalin, and stored in 1xPBS. Cell lysis was determined using the PI exclusion method as described above.

Complement assay on primary mouse RPE cells

Cells were infected with either AdCAGCD46 or AdCAGpA at an MOI of 1000 for 3d in αMEM/10% FBS. The media was removed and 50μg/ml rat anti mouse emmprin added and incubated at room temperature for 1 hour. Immediately following emmprin treatment 50% NHS or 50% HINHS containing MgEGTA was added and cells incubated

at 37°C for 1 hour. Cells were washed 3 times in cold 1xPBS, fixed in 10% NBF for 15 minutes, and stored in 1xPBS at 4°C.

Complement assay on mouse eye-cups

Eight days after subretinal injection of adenovirus mice were euthanized using CO_2 and the lens and cornea removed. The eye-cup was incubated in GVB^{2+} containing 140µg/ml rat anti mouse emmprin for 1 hour at 4°C. Either 50% NHS or 50% HINHS was added directly to the GVB^{2+}/emmprin and incubated for 4 minutes at 37°C. MgEGTA was added to each sample and incubated for an additional 56 minutes at 37°C. Samples were washed 3 times in cold 1xPBS and fixed overnight in 4% paraformaldehyde at 4°C.

Sub-retinal injections

C57Bl/6 mice were purchased (Jackson Laboratories) and maintained in accordance with federal, state, and local regulations. 6-10 week old mice were anesthetized by intraperitoneal injection of xylazine (10mg/ml)/ketamine (1mg/ml). Subretinal injections were performed as previously described[100] using the transcleral-transchoroidal approach with a 32-gauge needle attached to a 5µl glass syringe (Hamilton). Either 1µl of an empty vector control mixture containing 9 parts AdCAGpA (total $5x10^7$ particles) and 1 part AdCAGGFP (total $1x10^6$ particles) or CD46 vector containing 9 parts AdCAGCD6 (total $5x10^7$ particles) and 1 part AdCAGGFP (total $1x10^6$ particles) were injected.

Soluble cell lysis assay

A p100 of ARPE19 cells were infected at an MOI of 1000 using AdCAGsCD46, AdCAGsCD55, AdCAGsCD59, AdCAGSTAC, or AdCAGGFP for 3 days in 6ml of serum free DMEM without phenol red. After 3 days the media was collected, centrifuged at 1200 RPM, then stored on ice. A p100 of Hepa-1c1c7 cells were plated at 50% confluent in αMEM 10% FBS for 1 day then changed to αMEM 2%FBS for 2 days. The αMEM was removed and cells were washed 3x with PBS then trypsinized. $2.5x10^5$ cells were added to a 15ml falcon tube then centrifuged at 1200 RPM for 2 minutes, and resuspended in a total of 500ul of the media collected from the ARPE19 cells, containing STAC or GFP control, plus appropriate concentration of normal human serum or heat inactivated normal human serum. Cells were incubated at 37°C under constant gentle rotary shaking for 1 hour. 2μg of propidium iodide (PI) was added to the cells and PI uptake was measured by counting 25,000 cells using the LSRII (BD biosciences, Rockville MD) flow cytometer.

Assay for MAC deposition in the liver

C57/Bl6 mice ranging in age from 6-10 weeks were injected with $5x10^9$ particles of either AdCAGSTAC or AdCAGpA in a volume of 200μl sterile PBS. After 7 days 200μg of anti mouse PECAM antibody was injected into the left ventricle and kept on heating pad at 37°C for 4 hours. Mice were then perfused with 90% human serum (Complement Technology Inc., Tyler TX) in a total volume of 1.5ml (1.4ml human serum plus 0.1ml PBS containing calcium and magnesium) and incubated in bacterial incubator at 37° for 15 minutes. The median and left lobe of the liver were removed and fixed overnight in 4% paraformaldehyde (PFA). Serial sections were taken at 8μm

through each lobe then used for MAC detection. Images were captured at 10x then MAC pixel intensity, over entire section, was quantified using ImageJ software.

Statistical analysis

Except where otherwise stated, experiments were performed at least 3 times in duplicate. Error bars represent the standard deviation from the mean. Where appropriate, significance was calculated using Student's *t* test with the exception of the stereological data where the Scheaffer's method was employed to determine the coefficient of error.

References

1. Polans A, Baehr W, Palczewski K Turned on by Ca2+! The physiology and pathology of Ca(2+)-binding proteins in the retina. Trends Neurosci 1996; **19**: 547-554. DOI: S0166-2236(96)10059-X [pii]
2. Lambright DG, Sondek J, Bohm A*, et al.* The 2.0 A crystal structure of a heterotrimeric G protein. Nature 1996; **379**: 311-319. DOI: 10.1038/379311a0
3. Lambright DG, Noel JP, Hamm HE*, et al.* Structural determinants for activation of the alpha-subunit of a heterotrimeric G protein. Nature 1994; **369**: 621-628. DOI: 10.1038/369621a0
4. Noel JP, Hamm HE, Sigler PB The 2.2 A crystal structure of transducin-alpha complexed with GTP gamma S. Nature 1993; **366**: 654-663. DOI: 10.1038/366654a0
5. Sondek J, Lambright DG, Noel JP*, et al.* GTPase mechanism of Gproteins from the 1.7-A crystal structure of transducin alpha-GDP-AIF-4. Nature 1994; **372**: 276-279. DOI: 10.1038/372276a0
6. Lolley RN, Racz E Calcium modulation of cyclic GMP synthesis in rat visual cells. Vision Res 1982; **22**: 1481-1486
7. Koch KW, Stryer L Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. Nature 1988; **334**: 64-66. DOI: 10.1038/334064a0
8. Hsu YT, Molday RS Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. Nature 1993; **361**: 76-79. DOI: 10.1038/361076a0
9. Bloomfield SA, Dowling JE Roles of aspartate and glutamate in synaptic transmission in rabbit retina. I. Outer plexiform layer. J Neurophysiol 1985; **53**: 699-713
10. Muller F, Wassle H, Voigt T Pharmacological modulation of the rod pathway in the cat retina. J Neurophysiol 1988; **59**: 1657-1672

11. Slaughter MM, Miller RF 2-amino-4-phosphonobutyric acid: a new pharmacological tool for retina research. Science 1981; **211**: 182-185
12. Yamashita M, Wassle H Responses of rod bipolar cells isolated from the rat retina to the glutamate agonist 2-amino-4-phosphonobutyric acid (APB). J Neurosci 1991; **11**: 2372-2382
13. Baylor DA, Lamb TD, Yau KW Responses of retinal rods to single photons. J Physiol 1979; **288**: 613-634
14. Lagnado L, Baylor D Signal flow in visual transduction. Neuron 1992; **8**: 995-1002. DOI: 0896-6273(92)90122-T [pii]
15. Vuong TM, Chabre M Deactivation kinetics of the transduction cascade of vision. Proc Natl Acad Sci U S A 1991; **88**: 9813-9817
16. Wilden U, Hall SW, Kuhn H Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc Natl Acad Sci U S A 1986; **83**: 1174-1178
17. Kawamura S Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. Nature 1993; **362**: 855-857. DOI: 10.1038/362855a0
18. Schiller PH The central visual system. Vision Res 1986; **26**: 1351-1386. DOI: 0042-6989(86)90162-8 [pii]
19. Franke RR, Konig B, Sakmar TP*, et al.* Rhodopsin mutants that bind but fail to activate transducin. Science 1990; **250**: 123-125
20. Chabre M Trigger and amplification mechanisms in visual phototransduction. Annu Rev Biophys Biophys Chem 1985; **14**: 331-360. DOI: 10.1146/annurev.bb.14.060185.001555
21. Stryer L Visual excitation and recovery. The Journal of biological chemistry 1991; **266**: 10711-10714
22. Wald G Molecular basis of visual excitation. Science 1968; **162**: 230-239
23. McLaughlin ME, Sandberg MA, Berson EL*, et al.* Recessive mutations in the gene encoding the beta-subunit of rod phosphodiesterase in patients with retinitis pigmentosa. Nature genetics 1993; **4**: 130-134. DOI: 10.1038/ng0693-130
24. Danciger M, Blaney J, Gao YQ*, et al.* Mutations in the PDE6B gene in autosomal recessive retinitis pigmentosa. Genomics 1995; **30**: 1-7. DOI: S0888-7543(85)70001-8 [pii]
10.1006/geno.1995.0001
25. McLaughlin ME, Ehrhart TL, Berson EL*, et al.* Mutation spectrum of the gene encoding the beta subunit of rod phosphodiesterase among patients with autosomal recessive retinitis pigmentosa. Proc Natl Acad Sci U S A 1995; **92**: 3249-3253
26. Bayes M, Giordano M, Balcells S*, et al.* Homozygous tandem duplication within the gene encoding the beta-subunit of rod phosphodiesterase as a cause for autosomal recessive retinitis pigmentosa. Hum Mutat 1995; **5**: 228-234. DOI: 10.1002/humu.1380050307
27. Danciger M, Heilbron V, Gao YQ*, et al.* A homozygous PDE6B mutation in a family with autosomal recessive retinitis pigmentosa. Mol Vis 1996; **2**: 10

28. Gal A, Orth U, Baehr W*, et al.* Heterozygous missense mutation in the rod cGMP phosphodiesterase beta-subunit gene in autosomal dominant stationary night blindness. Nature genetics 1994; **7**: 64-68. DOI: 10.1038/ng0594-64
29. Bowes C, Li T, Danciger M*, et al.* Retinal degeneration in the rd mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase. Nature 1990; **347**: 677-680. DOI: 10.1038/347677a0
30. Pittler SJ, Baehr W Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the rd mouse. Proc Natl Acad Sci U S A 1991; **88**: 8322-8326
31. Farber DB, Danciger JS, Aguirre G The beta subunit of cyclic GMP phosphodiesterase mRNA is deficient in canine rod-cone dysplasia 1. Neuron 1992; **9**: 349-356. DOI: 0896-6273(92)90173-B [pii]
32. Suber ML, Pittler SJ, Qin N*, et al.* Irish setter dogs affected with rod/cone dysplasia contain a nonsense mutation in the rod cGMP phosphodiesterase beta-subunit gene. Proc Natl Acad Sci U S A 1993; **90**: 3968-3972
33. Sanyal S, Jansen HG Absence of receptor outer segments in the retina of rds mutant mice. Neurosci Lett 1981; **21**: 23-26. DOI: 0304-3940(81)90051-3 [pii]
34. Hawkins RK, Jansen HG, Sanyal S Development and degeneration of retina in rds mutant mice: photoreceptor abnormalities in the heterozygotes. Experimental eye research 1985; **41**: 701-720. DOI: 0014-4835(85)90179-4 [pii]
35. Travis GH, Brennan MB, Danielson PE*, et al.* Identification of a photoreceptor-specific mRNA encoded by the gene responsible for retinal degeneration slow (rds). Nature 1989; **338**: 70-73. DOI: 10.1038/338070a0
36. Connell G, Bascom R, Molday L*, et al.* Photoreceptor peripherin is the normal product of the gene responsible for retinal degeneration in the rds mouse. Proc Natl Acad Sci U S A 1991; **88**: 723-726
37. Polk TD, Gass JD, Green WR*, et al.* Familial internal limiting membrane dystrophy. A new sheen retinal dystrophy. Arch Ophthalmol 1997; **115**: 878-885
38. Bernardos RL, Barthel LK, Meyers JR*, et al.* Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 2007; **27**: 7028-7040. DOI: 27/26/7028 [pii]
10.1523/JNEUROSCI.1624-07.2007
39. Jacobson SG, Aleman TS, Cideciyan AV*, et al.* Identifying photoreceptors in blind eyes caused by RPE65 mutations: Prerequisite for human gene therapy success. Proc Natl Acad Sci U S A 2005; **102**: 6177-6182. DOI: 0500646102 [pii]
10.1073/pnas.0500646102
40. Jacobson SG, Aleman TS, Cideciyan AV*, et al.* Human cone photoreceptor dependence on RPE65 isomerase. Proc Natl Acad Sci U S A 2007; **104**: 15123-15128. DOI: 0706367104 [pii]
10.1073/pnas.0706367104
41. Kachi S, Oshima Y, Esumi N*, et al.* Nonviral ocular gene transfer. Gene therapy 2005; **12**: 843-851. DOI: 3302475 [pii]
10.1038/sj.gt.3302475
42. Read SP, Cashman SM, Kumar-Singh R POD nanoparticles expressing GDNF provide structural and functional rescue of light-induced retinal degeneration in an adult mouse. Mol Ther; **18**: 1917-1926. DOI: mt2010167 [pii]

10.1038/mt.2010.167
43. Balaggan KS, Binley K, Esapa M*, et al.* Stable and efficient intraocular gene transfer using pseudotyped EIAV lentiviral vectors. The journal of gene medicine 2006; **8**: 275-285. DOI: 10.1002/jgm.845
44. Berkowitz R, Ilves H, Lin WY*, et al.* Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. Journal of virology 2001; **75**: 3371-3382. DOI: 10.1128/JVI.75.7.3371-3382.2001
45. Yanez-Munoz RJ, Balaggan KS, MacNeil A*, et al.* Effective gene therapy with nonintegrating lentiviral vectors. Nature medicine 2006; **12**: 348-353. DOI: nm1365 [pii]
10.1038/nm1365
46. Zaiss AK, Liu Q, Bowen GP*, et al.* Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. Journal of virology 2002; **76**: 4580-4590
47. Afione SA, Conrad CK, Kearns WG*, et al.* In vivo model of adeno-associated virus vector persistence and rescue. Journal of virology 1996; **70**: 3235-3241
48. Acland GM, Aguirre GD, Bennett J*, et al.* Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. Mol Ther 2005; **12**: 1072-1082. DOI: S1525-0016(05)01561-3 [pii]
10.1016/j.ymthe.2005.08.008
49. Auricchio A, Kobinger G, Anand V*, et al.* Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model. Hum Mol Genet 2001; **10**: 3075-3081
50. Maguire AM, Simonelli F, Pierce EA*, et al.* Safety and efficacy of gene transfer for Leber's congenital amaurosis. The New England journal of medicine 2008; **358**: 2240-2248. DOI: NEJMoa0802315 [pii]
10.1056/NEJMoa0802315
51. Kim IH, Jozkowicz A, Piedra PA*, et al.* Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci U S A 2001; **98**: 13282-13287
52. Kumar-Singh R, Yamashita CK, Tran K*, et al.* Construction of encapsidated (gutted) adenovirus minichromosomes and their application to rescue of photoreceptor degeneration. Methods in enzymology 2000; **316**: 724-743
53. Sweigard JH, Cashman SM, Kumar-Singh R Adenovirus-mediated delivery of CD46 attenuates the alternative complement pathway on RPE: implications for age-related macular degeneration. Gene therapy
54. Lamartina S, Cimino M, Roscilli G*, et al.* Helper-dependent adenovirus for the gene therapy of proliferative retinopathies: stable gene transfer, regulated gene expression and therapeutic efficacy. The journal of gene medicine 2007; **9**: 862-874
55. Parks RJ, Chen L, Anton M*, et al.* A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc Natl Acad Sci U S A 1996; **93**: 13565-13570

56. Chevez-Barrios P, Chintagumpala M, Mieler W*, et al.* Response of retinoblastoma with vitreous tumor seeding to adenovirus-mediated delivery of thymidine kinase followed by ganciclovir. J Clin Oncol 2005; **23**: 7927-7935
57. Johnson LV, Leitner WP, Staples MK*, et al.* Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. Exp Eye Res 2001; **73**: 887-896
58. Campochiaro PA, Nguyen QD, Shah SM*, et al.* Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. Human gene therapy 2006; **17**: 167-176. DOI: 10.1089/hum.2006.17.167
59. Ji X, Zhang J, Cheng L*, et al.* Oncolytic adenovirus delivering herpes simplex virus thymidine kinase suicide gene reduces the growth of human retinoblastoma in an in vivo mouse model. Exp Eye Res 2009; **89**: 193-199. DOI: S0014-4835(09)00070-0 [pii]
10.1016/j.exer.2009.03.007
60. Chroboczek J, Ruigrok RW, Cusack S Adenovirus fiber. Curr Top Microbiol Immunol 1995; **199 (Pt 1)**: 163-200
61. Bergelson JM, Cunningham JA, Droguett G*, et al.* Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997; **275**: 1320-1323
62. Stewart PL, Chiu CY, Huang S*, et al.* Cryo-EM visualization of an exposed RGD epitope on adenovirus that escapes antibody neutralization. The EMBO journal 1997; **16**: 1189-1198. DOI: 10.1093/emboj/16.6.1189
63. Wickham TJ, Mathias P, Cheresh DA*, et al.* Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993; **73**: 309-319. DOI: 0092-8674(93)90231-E [pii]
64. Mathias P, Galleno M, Nemerow GR Interactions of soluble recombinant integrin alphav beta5 with human adenoviruses. Journal of virology 1998; **72**: 8669-8675
65. Wang K, Huang S, Kapoor-Munshi A*, et al.* Adenovirus internalization and infection require dynamin. Journal of virology 1998; **72**: 3455-3458
66. Jager L, Ehrhardt A Emerging adenoviral vectors for stable correction of genetic disorders. Curr Gene Ther 2007; **7**: 272-283
67. Arnberg N, Edlund K, Kidd AH*, et al.* Adenovirus type 37 uses sialic acid as a cellular receptor. Journal of virology 2000; **74**: 42-48
68. Gaggar A, Shayakhmetov DM, Lieber A CD46 is a cellular receptor for group B adenoviruses. Nature medicine 2003; **9**: 1408-1412
69. Ting AY, Lee TK, MacDonald IM Genetics of age-related macular degeneration. Curr Opin Ophthalmol 2009; **20**: 369-376. DOI: 10.1097/ICU.0b013e32832f8016
70. Umeda S, Suzuki MT, Okamoto H*, et al.* Molecular composition of drusen and possible involvement of anti-retinal autoimmunity in two different forms of macular degeneration in cynomolgus monkey (Macaca fascicularis). FASEB J 2005; **19**: 1683-1685. DOI: 04-3525fje [pii]
10.1096/fj.04-3525fje
71. Seddon JM, Cote J, Davis N*, et al.* Progression of age-related macular degeneration: association with body mass index, waist circumference, and waist-hip ratio. Arch Ophthalmol 2003; **121**: 785-792. DOI: 10.1001/archopht.121.6.785

121/6/785 [pii]
72. Seddon JM, Willett WC, Speizer FE*, et al.* A prospective study of cigarette smoking and age-related macular degeneration in women. JAMA 1996; **276**: 1141-1146
73. Klein R, Klein BE, Tomany SC*, et al.* The association of cardiovascular disease with the long-term incidence of age-related maculopathy: the Beaver Dam eye study. Ophthalmology 2003; **110**: 636-643. DOI: S0161-6420(02)01448-3 [pii]
10.1016/S0161-6420(02)01448-3
74. Charbel Issa P, Chong NV, Scholl HP The significance of the complement system for the pathogenesis of age-related macular degeneration - current evidence and translation into clinical application. Graefes Arch Clin Exp Ophthalmol; **249**: 163-174. DOI: 10.1007/s00417-010-1568-6
75. Klein RJ, Zeiss C, Chew EY*, et al.* Complement factor H polymorphism in age-related macular degeneration. Science 2005; **308**: 385-389. DOI: 1109557 [pii]
10.1126/science.1109557
76. Sarma JV, Ward PA The complement system. Cell Tissue Res; **343**: 227-235. DOI: 10.1007/s00441-010-1034-0
77. Reynolds R, Hartnett ME, Atkinson JP*, et al.* Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. Invest Ophthalmol Vis Sci 2009; **50**: 5818-5827
78. Miller JW, Schmidt-Erfurth U, Sickenberg M*, et al.* Photodynamic therapy with verteporfin for choroidal neovascularization caused by age-related macular degeneration: results of a single treatment in a phase 1 and 2 study. Arch Ophthalmol 1999; **117**: 1161-1173
79. Gragoudas ES, Adamis AP, Cunningham ET, Jr.*, et al.* Pegaptanib for neovascular age-related macular degeneration. The New England journal of medicine 2004; **351**: 2805-2816. DOI: 351/27/2805 [pii]
10.1056/NEJMoa042760
80. Rich RM, Rosenfeld PJ, Puliafito CA*, et al.* Short-term safety and efficacy of intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Retina 2006; **26**: 495-511. DOI: 10.1097/01.iae.0000225766.75009.3a
00006982-200605000-00002 [pii]
81. Brown DM, Kaiser PK, Michels M*, et al.* Ranibizumab versus verteporfin for neovascular age-related macular degeneration. The New England journal of medicine 2006; **355**: 1432-1444. DOI: 355/14/1432 [pii]
10.1056/NEJMoa062655
82. Cashman SM, McCullough L, Kumar-Singh R Improved retinal transduction in vivo and photoreceptor-specific transgene expression using adenovirus vectors with modified penton base. Mol Ther 2007; **15**: 1640-1646. DOI: 6300203 [pii]
10.1038/sj.mt.6300203
83. Kokotas H, Grigoriadou M, Petersen MB Review: Age-related macular degeneration: genetic and clinical findings. Clin Chem Lab Med. DOI: 10.1515/CCLM.2011.091

84. Edwards AO, Ritter R, 3rd, Abel KJ*, et al.* Complement factor H polymorphism and age-related macular degeneration. Science 2005; **308**: 421-424. DOI: 1110189 [pii]
10.1126/science.1110189
85. Hageman GS, Anderson DH, Johnson LV*, et al.* A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci U S A 2005; **102**: 7227-7232. DOI: 0501536102 [pii]
10.1073/pnas.0501536102
86. Haines JL, Hauser MA, Schmidt S*, et al.* Complement factor H variant increases the risk of age-related macular degeneration. Science 2005; **308**: 419-421. DOI: 1110359 [pii]
10.1126/science.1110359
87. Gold B, Merriam JE, Zernant J*, et al.* Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 2006; **38**: 458-462
88. Jakobsdottir J, Conley YP, Weeks DE*, et al.* C2 and CFB genes in age-related maculopathy and joint action with CFH and LOC387715 genes. PloS one 2008; **3**: e2199. DOI: 10.1371/journal.pone.0002199
89. Despriet DD, van Duijn CM, Oostra BA*, et al.* Complement component C3 and risk of age-related macular degeneration. Ophthalmology 2009; **116**: 474-480 e472. DOI: S0161-6420(08)01299-2 [pii]
10.1016/j.ophtha.2008.09.055
90. Maller JB, Fagerness JA, Reynolds RC*, et al.* Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nat Genet 2007; **39**: 1200-1201. DOI: ng2131 [pii]
10.1038/ng2131
91. Park KH, Fridley BL, Ryu E*, et al.* Complement component 3 (C3) haplotypes and risk of advanced age-related macular degeneration. Invest Ophthalmol Vis Sci 2009; **50**: 3386-3393. DOI: iovs.08-3231 [pii]
10.1167/iovs.08-3231
92. Yates JR, Sepp T, Matharu BK*, et al.* Complement C3 variant and the risk of age-related macular degeneration. The New England journal of medicine 2007; **357**: 553-561
93. Vogt SD, Barnum SR, Curcio CA*, et al.* Distribution of complement anaphylatoxin receptors and membrane-bound regulators in normal human retina. Exp Eye Res 2006; **83**: 834-840. DOI: S0014-4835(06)00211-9 [pii]
10.1016/j.exer.2006.04.002
94. Noris M, Brioschi S, Caprioli J*, et al.* Familial haemolytic uraemic syndrome and an MCP mutation. Lancet 2003; **362**: 1542-1547. DOI: S0140-6736(03)14742-3 [pii]
10.1016/S0140-6736(03)14742-3
95. Richards A, Kemp EJ, Liszewski MK*, et al.* Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. Proc Natl Acad Sci U S A 2003; **100**: 12966-12971. DOI: 10.1073/pnas.2135497100

2135497100 [pii]
96. Hazin R, Freeman PD, Kahook MY Age-related macular degeneration: a guide for the primary care physician. Journal of the National Medical Association 2009; **101**: 134-138
97. Smith AJ, Bainbridge JW, Ali RR Prospects for retinal gene replacement therapy. Trends Genet 2009; **25**: 156-165
98. Kellner U, Kraus H, Heimann H*, et al.* Electrophysiological evaluation of visual loss in Muller cell sheen dystrophy. The British journal of ophthalmology 1998; **82**: 650-654
99. Henry LJ, Xia D, Wilke ME*, et al.* Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in Escherichia coli. Journal of virology 1994; **68**: 5239-5246
100. Cashman SM, Morris DJ, Kumar-Singh R Adenovirus type 5 pseudotyped with adenovirus type 37 fiber uses sialic acid as a cellular receptor. Virology 2004; **324**: 129-139
101. Mallam JN, Hurwitz MY, Mahoney T*, et al.* Efficient gene transfer into retinal cells using adenoviral vectors: dependence on receptor expression. Invest Ophthalmol Vis Sci 2004; **45**: 1680-1687
102. Sakurai F, Murakami S, Kawabata K*, et al.* The short consensus repeats 1 and 2, not the cytoplasmic domain, of human CD46 are crucial for infection of subgroup B adenovirus serotype 35. J Control Release 2006; **113**: 271-278
103. Rattner A, Toulabi L, Williams J*, et al.* The genomic response of the retinal pigment epithelium to light damage and retinal detachment. J Neurosci 2008; **28**: 9880-9889. DOI: 28/39/9880 [pii]
10.1523/JNEUROSCI.2401-08.2008
104. Sakurai F, Kawabata K, Koizumi N*, et al.* Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice. Gene therapy 2006; **13**: 1118-1126
105. Li B, Sallee C, Dehoff M*, et al.* Mouse Crry/p65. Characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. J Immunol 1993; **151**: 4295-4305
106. Yang P, Tyrrell J, Han I*, et al.* Expression and modulation of RPE cell membrane complement regulatory proteins. Invest Ophthalmol Vis Sci 2009; **50**: 3473-3481
107. Von Seggern DJ, Aguilar E, Kinder K*, et al.* In vivo transduction of photoreceptors or ciliary body by intravitreal injection of pseudotyped adenoviral vectors. Mol Ther 2003; **7**: 27-34
108. Guidry C, Bradley KM, King JL Tractional force generation by human muller cells: growth factor responsiveness and integrin receptor involvement. Invest Ophthalmol Vis Sci 2003; **44**: 1355-1363
109. Mano T, Puro DG Phagocytosis by human retinal glial cells in culture. Invest Ophthalmol Vis Sci 1990; **31**: 1047-1055
110. Finnemann SC Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. The EMBO journal 2003; **22**: 4143-4154
111. Farjo R, Skaggs J, Quiambao AB*, et al.* Efficient non-viral ocular gene transfer with compacted DNA nanoparticles. PloS one 2006; **1**: e38

112. Khani SC, Pawlyk BS, Bulgakov OV*, et al.* AAV-mediated expression targeting of rod and cone photoreceptors with a human rhodopsin kinase promoter. Invest Ophthalmol Vis Sci 2007; **48**: 3954-3961
113. Klein R, Klein BE, Knudtson MD*, et al.* Fifteen-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. Ophthalmology 2007; **114**: 253-262
114. van Leeuwen R, Klaver CC, Vingerling JR*, et al.* Epidemiology of age-related maculopathy: a review. European journal of epidemiology 2003; **18**: 845-854
115. Ozkiris A Anti-VEGF agents for age-related macular degeneration. Expert opinion on therapeutic patents; **20**: 103-118
116. Hageman GS, Luthert PJ, Victor Chong NH*, et al.* An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Progress in retinal and eye research 2001; **20**: 705-732
117. Anderson DH, Mullins RF, Hageman GS*, et al.* A role for local inflammation in the formation of drusen in the aging eye. American journal of ophthalmology 2002; **134**: 411-431
118. Barilla-LaBarca ML, Liszewski MK, Lambris JD*, et al.* Role of membrane cofactor protein (CD46) in regulation of C4b and C3b deposited on cells. J Immunol 2002; **168**: 6298-6304
119. Kavanagh D, Richards A, Atkinson J Complement regulatory genes and hemolytic uremic syndromes. Annu Rev Med 2008; **59**: 293-309. DOI: 10.1146/annurev.med.59.060106.185110
120. Ramo K, Cashman SM, Kumar-Singh R Evaluation of adenovirus-delivered human CD59 as a potential therapy for AMD in a model of human membrane attack complex formation on murine RPE. Invest Ophthalmol Vis Sci 2008; **49**: 4126-4136
121. McNearney T, Ballard L, Seya T*, et al.* Membrane cofactor protein of complement is present on human fibroblast, epithelial, and endothelial cells. J Clin Invest 1989; **84**: 538-545. DOI: 10.1172/JCI114196
122. Brodbeck WG, Mold C, Atkinson JP*, et al.* Cooperation between decay-accelerating factor and membrane cofactor protein in protecting cells from autologous complement attack. J Immunol 2000; **165**: 3999-4006
123. Gao LJ, Guo SY, Cai YQ*, et al.* Cooperation of decay-accelerating factor and membrane cofactor protein in regulating survival of human cervical cancer cells. BMC Cancer 2009; **9**: 384. DOI: 1471-2407-9-384 [pii]
10.1186/1471-2407-9-384
124. Caprioli J, Noris M, Brioschi S*, et al.* Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. Blood 2006; **108**: 1267-1279. DOI: blood-2005-10-007252 [pii]
10.1182/blood-2005-10-007252
125. Kavanagh D, Goodship TH Membrane cofactor protein and factor I: mutations and transplantation. Semin Thromb Hemost 2006; **32**: 155-159. DOI: 10.1055/s-2006-939771
126. Fagerness JA, Maller JB, Neale BM*, et al.* Variation near complement factor I is associated with risk of advanced AMD. Eur J Hum Genet 2009; **17**: 100-104

127. Yuan X, Gu X, Crabb JS*, et al.* Quantitative proteomics: comparison of the macular Bruch membrane/choroid complex from age-related macular degeneration and normal eyes. Mol Cell Proteomics; **9**: 1031-1046
128. Wang J, Ohno-Matsui K, Yoshida T*, et al.* Altered function of factor I caused by amyloid beta: implication for pathogenesis of age-related macular degeneration from Drusen. J Immunol 2008; **181**: 712-720
129. Thurman JM, Renner B, Kunchithapautham K*, et al.* Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. The Journal of biological chemistry 2009; **284**: 16939-16947
130. Ma KN, Cashman SM, Sweigard JH*, et al.* Decay accelerating factor (CD55)-mediated attenuation of complement: therapeutic implications for age-related macular degeneration. Invest Ophthalmol Vis Sci; **51**: 6776-6783
131. Lublin DM, Atkinson JP Decay-accelerating factor: biochemistry, molecular biology, and function. Annual review of immunology 1989; **7**: 35-58
132. Kimberley FC, Sivasankar B, Paul Morgan B Alternative roles for CD59. Molecular immunology 2007; **44**: 73-81
133. Ripoche J, Al Salihi A, Rousseaux J*, et al.* Isolation of two molecular populations of human complement factor H by hydrophobic affinity chromatography. The Biochemical journal 1984; **221**: 89-96
134. Yoon SH, Fearon DT Characterization of a soluble form of the C3b/C4b receptor (CR1) in human plasma. J Immunol 1985; **134**: 3332-3338
135. Medof ME, Lublin DM, Holers VM*, et al.* Cloning and characterization of cDNAs encoding the complete sequence of decay-accelerating factor of human complement. Proc Natl Acad Sci U S A 1987; **84**: 2007-2011
136. Xing PX, Russell S, Prenzoska J*, et al.* Discrimination between alternatively spliced STP-A and -B isoforms of CD46. Immunology 1994; **83**: 122-127
137. Xu L, Zhao Z, Sheng J*, et al.* Co-expression of human complement regulatory proteins DAF and MCP with an IRES-mediated dicistronic mammalian vector enhances their cell protective effects. Biochemistry 2008; **73**: 1025-1030
138. Simonelli F, Maguire AM, Testa F*, et al.* Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther; **18**: 643-650
139. Bora PS, Sohn JH, Cruz JM*, et al.* Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. J Immunol 2005; **174**: 491-497. DOI: 174/1/491 [pii]
140. Fallaux FJ, Kranenburg O, Cramer SJ*, et al.* Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Human gene therapy 1996; **7**: 215-222
141. He TC, Zhou S, da Costa LT*, et al.* A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 1998; **95**: 2509-2514

142. Vogt et. al. *ARVO* 2009 Program#/ Poster#: 4180/A436

Acknowledgements

First and foremost I would like to thank my advisor Rajendra for his sincere dedication to my education. After working for several years before entering a Ph.D. program I was vigilant to find a mentor that would create a positive yet discriminating learning environment so that I could grow as a scientist. Rajendra went well beyond what I ever expected to gain from graduate school. He not only critically challenged my data but made sure that my scientific understanding and rational were sound. Furthermore, he was always there when I had questions and took the time to critically analyze the direction of my projects to make sure we could achieve the highest impact with our findings. As I approach graduation his interest in my future remains as he has offered to help in any way that he can to ensure that I secure the perfect post doc. The mentorship I received through Rajendra far exceeded what I had hoped for in graduate school making it the best learning and growing experience I could have imagined.

I would also like to thank Siobhan for her guidance in troubleshooting my many experimental hurdles and for the thought-invoking discussions we've had about my projects. No matter how busy she was her door has always been open for which I am sincerely grateful. Finally, I would like to thank my committee members, Janis Lem, Michele Jacob, and Tom Linsenmayer for their guidance and critical evaluation of my data.

These studies were supported by grants to R.K.-S. from the National Institutes of Health/National Eye Institute, The Foundations for Fighting Blindness, The Ellison

Foundation, the Virginia B Smith Trust; grants to the Department of Ophthalmology at Tufts University from the Lions Eye Foundation and Research to Prevent Blindness.