Delivery of Helper-Dependent Adenoviral Vectors
to the Subretinal Space of Mice

By

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Graduate Department of Laboratory Medicine and Pathobiology,
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Abstract

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Laboratory Medicine and Pathobiology, University of Toronto

The helper-dependent adenoviral (HD-Ad) vector is the latest generation of Ad vectors. It ameliorates the vector-associated immunogenic problems with increased capacity for carrying DNA because all viral coding genes are removed. I hypothesize that HD-Ad vectors can be effective vehicles for retinal gene delivery. The objectives of this study are to determine if HD-Ad vectors can deliver reporter genes, GFP or lacZ, driven by a CMV or a MOPS promoter, into specific retinal layers. Subretinal injections were performed and eyes removed at time points from 1 week to 3 months, processed for fluorescent microscopy, X-gal staining, and H&E staining. Transgene expression was detected for at least 3 months. A dose dependent relationship was revealed between the level of transgene expression and viral vector dose. Distinctively, the MOPS promoter drove photoreceptor cell specific expression. Notably, no sign of inflammation or tissue toxicity was detected, demonstrating the benefits of the HD-Ad vector.
Acknowledgements

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List of Abbreviations

A2E - pyridinium bisretinoid
AAV - adeno-associated virus
ABCA4 - adenosine triphosphate binding cassette 4
Ad - adenovirus
AMD - age-related macular degeneration
ATP - adenosine triphosphate
β-gal - β-galactosidase
BDNF - brain derived neurotrophic factor
bFGF - basic fibroblast growth factor
bp - base pair
CAR - coxsackievirus and adenovirus receptor
cDNA - complementary DNA
cGMP - cyclic guanosine 3’-5’ monophosphate
CMV - cytomegalovirus immediate-early promoter
CNTF - ciliary neurotrophic factor
CTD - C terminus domain
CTL - cytotoxic T lymphocyte
DAPI - 4’,6-Diamidino-2-Phenylindole
dd - double-stranded
DTT - dithiothreitol
EGTA - ethylene glycol tetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ERG</td>
<td>electro retinogram</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FG-Ad</td>
<td>first generation adenovirus</td>
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<td>FIX</td>
<td>factor IX</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GTF</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>HD-Ad</td>
<td>helper-dependent adenovirus</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparin sulphate proteoglycan</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>IFT</td>
<td>intraflagellar transport</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IRBP</td>
<td>interphotoreceptor retinoid-binding protein</td>
</tr>
<tr>
<td>IS</td>
<td>inner segment</td>
</tr>
<tr>
<td>ITR</td>
<td>inverted terminal repeats</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>LacZ</td>
<td><em>E. coli</em> β-galactosidase gene</td>
</tr>
<tr>
<td>LCA</td>
<td>leber's congenital amaurosis</td>
</tr>
<tr>
<td>LRAT</td>
<td>lecithin:retinal acetyltransferase</td>
</tr>
<tr>
<td>LV</td>
<td>lentivirus</td>
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</table>
MHC - major histocompatibility complex
mRNA - messenger RNA
MOI - Multiplicity of infection
MOPS - murine opsin promoter
MSHα - Melanocyte stimulating hormone alpha
N-PE - N-retinylidene phosphatidylethanolamine
OD - Optical density
OH - hydroxyl group
ONL - outer nuclear layer
OS - outer segment
PBS - phosphate buffered saline
PDL - programmed death ligand
PE - phosphatidylethanolamine
pfu - Plaque forming unit
PIC - pre-initiation complex
polyA - polyadenylation
PR - photoreceptor
PSF - penicillin-streptomycin-fungizone
RDH - retinol dehydrogenase
RNA pol II - RNA polymerase II
RP - retinitis pigmentosa
RPE - retinal pigment epithelium
ss - single-stranded
TGFβ - transforming growth factor beta
Treg - regulatory T cells
tRNA - transfer RNA
UAS - upstream activating site
URS - upstream repressing site
vp - viral particle
VSV-G - vesicular stomatitis virus G
X-gal - 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
INTRODUCTION

THE EYE

Overview: the retina

Light enters through the cornea, and then passes through the anterior chamber, pupil, lens and vitreous humour to reach the outermost layer that lines the back of the eye. It is this retinal layer of terminally differentiated neuronal cells that initiates a cascade of chemical and electrical events that ultimately trigger nerve impulses delivered by the optic nerve to the visual centers of the brain.

The retina is a complex structure. It measures no more than 0.5 mm thick with three layers of neurons interconnected with two layers of synapses (figure 1). The sensory modality that we call vision is initiated in the outermost layer of the retina, a region that lies at the interface between the vascularised choroid and the inner retina. Cellular components of the outer retina include a monolayer of cells called the retinal pigment epithelium (RPE) and the light-sensing rod and cone photoreceptor (PR) cells. The specialized interaction of these two cell types is intimately linked to the vision cycle. The inner retina is divided into two neuronal layers: cells of the inner nuclear layer (INL), which serve as secondary neurons to transmit signals sent from the PRs, and the retinal ganglion layer, which extend their axons to the optic nerve.
**Photoreceptor cells**

There are 2 specialized types of PRs in the vertebrate retina that function in phototransduction: cones and rods perform under normal lighting conditions and dim lighting conditions, respectively. The human retina consists of roughly 6 million cones and 120 million rods with cones being highly concentrated near the foveal pit and rods dispersed throughout the peripheral retina. The large number of rods are highly sensitive to light and contain the visual pigment rhodopsin necessary to induce the vision cascade. Cones, being less sensitive to light, contain 3 different photopsins used to detect red (long $\lambda$), green (medium $\lambda$), and blue (short $\lambda$) light and are responsible for colour vision and high visual acuity.

PRs (figure 2) are highly compartmentalized in their structure and organization and consist of five principle regions: 1) the outer segment (OS) that captures light and undergoes phototransduction processing; 2) the inner segment (IS) that houses the biosynthetic and metabolic machinery of the cell necessary to synthesize proteins; 3) the connecting cilium that joins the inner and outer segments together and allows selective passage of certain proteins; 4) the cell body that holds the nucleus; and 5) the synaptic region that allows for signal transduction to occur between bipolar cells and other secondary neurons of the inner retina.

**Photoreceptor outer segments**

The OS of PR cells are of particular interest because their unique structure and highly specialized organization allows for the initial stages of phototransduction. Cone OS differ from those of rods in a couple respects. First, cones are shorter in length with a wider base
and tapered top compared to the rectangular shape of rods. Second, rod cells consist of ordered stacks of closed disks surrounded by a separate plasma membrane, whereas in cones, the disk and plasma membrane are continuous [1]. It is within these disk membranes that the visual pigments opsins are exclusively found.

Interestingly, these stacks are constantly and consistently undergoing a renewal process whereby newly formed disks are added to the proximal region of the OS and old disks are displaced and phagocytosed in the distal end by the RPE [2]. There, they are broken down by lysis, with some parts recycled back to the PR for new disc assembly. Typically, a rod disc has a lifetime of about 10 days and studies have shown that disc shedding occurs in the morning as judged by an increase in phagosomes detected in the epithelium shortly thereafter [3]. Cones, on the other hand, undergo phagocytosis during light offset [3, 4].

**Protein trafficking in photoreceptor cells**

The PR is a highly polarized cell type containing many photosensitive proteins concentrated in the OS. For example, rhodopsin makes up ~85% of all protein components on the disk membranes; they are sparsely located elsewhere in the PR [5]. A fundamental question concerning PR cell biology is: “How do proteins traffic from the IS (where protein synthesis occurs) to the photosensitive OS compartment?” One possibility is intraflagellar transport (IFT) which has been proposed to facilitate this transport of proteins through the connecting cilium [6, 7]. IFT uses cilia and flagella to mobilize delivery vesicles along microtubules throughout the PR, bidirectionally from the ciliated OS.
This process has been divided into 4 phases [8]. Briefly, phase one is a connection step in which the IFT complex assembly at the base of the connecting cilium binds to both microtubule motors and to cargo vesicle (in this case, components of the phototransduction cascade like opsin). Phase two uses the motor actions of kinesin II to anterograde traffick the IFT super-complex towards the OS. Phase three and four consists of cargo delivery and the subsequent switching of motors from microtubules to cytoplasmic dynein to power the retrograde trafficking of the IFT complex back to the basal body.

Although it is suggested that PRs use the IFT as one mechanism of protein delivery, very little is known about this process in regards to the retina. The role of the IFT in promoting polarized protein localization will continue to be an area of fascinating research as more information is required to fully understand the mechanisms that regulate PR cell protein polarization.

**Retinal pigment epithelium**

Between the choriocapillary vessels and the OS of the PR, lie a monolayer of cells that closely interacts with the neural retina playing a role in the maintenance of visual function. This layer of cubical cells is collectively known as the RPE. These cells contain an apical membrane that interacts with the PR and a basolateral membrane that faces the Bruch’s membrane in order to ensure retinal separation from the fenestrated endothelium of the capillaries. The epithelium functions in a variety of complex roles that are essential for vision, including phagocytosis of PR outer segments, isomerisation of molecules in the visual cycle, and maintaining a homeostatic ionic environment to nourish the retina.
Interactions of RPE and PR cells

The close juxtaposition of the RPE to the PR cells allows a specialized interaction that is intimately linked to vision. The RPE is made up of pigmented cells and therefore has the ability to absorb excess light energy that has been focused onto the retina to protect PRs against photo-oxidation [9]. The apical membrane operates to induce voltage-dependent ion conductance that stabilizes ion composition in the subretinal space to ensure PR excitability [10]. Another way the RPE interacts with PRs is to participate in the visual cycle by re-isomerizing retinal to 11-cis retinal and transporting it back to the PR [11]. Importantly, the RPE participates in the daily phagocytosis of rod and cone OS [2, 9, 12], that recycle essential substances such as retinal back to the PRs to rebuild new disk membranes. Furthermore, tight junctions between RPE cells restrict passive passage of virtually all proteins and metabolites allowing only a subset of molecules (those that are absolutely essential for the survival of the retina) to selectively pass. The RPE also exports water, ions, and metabolic end products from the subretinal space to the blood while delivering nutrients such as glucose, retinol, and fatty acids from the blood to the PRs [13-16]. Additionally, the RPE can secret growth factors that maintain the structural integrity of both the choriocapillaris endothelium and the PRs [17]. Finally, the immune privileged status of the eye is partly attributed to the physical presence of the RPE acting to provide a blood-retina barrier while secreting immunosuppressive factors [18-20].

In summary, the RPE provides a significant regulatory role in the maintenance of the retinal environment. A failure of any of the aforementioned functions can lead to retinal degeneration and blindness. For example, improper phagocytosis of rod OS can result in an autosomal form of retinitis pigmentosa [21-23]. Diabetic retinopathy can be induced by the
formation of macular edema which is caused by damage to the blood-retina barrier created by the RPE [24, 25]. The pathogenesis of age-related macular degeneration can be a result of alterations in transepithelial transport of ions and nutrients from the RPE [25]. An RPE exclusive isomerase enzyme, RPE65, is involved in a critical step of the retinoid cycle and without it, accumulation of retinyl esters in the RPE will fester and result in PR cell apoptosis and vision impairment [11, 26, 27]. This is the classic symptom of Leber’s congenital amaurosis, a childhood blindness disease. Another juvenile inherited retinal disorder is Stargardt disease. In this disorder the protein deficiency is located in the disk membrane of the OS, although the initial degeneration occurs in the RPE, which inevitably leads to PR cell death and loss of vision [28].

The anatomical relationship between the PRs and the RPE is critical to the visual cycle. While not a part of the neural retina itself, the RPE is essential for the normal function and survival of PRs. Due to the specialized and close interaction of the PR cells with the RPE, any defect in the cellular processes of these two cell types can lead to their concurrent cell death, as loss of one cell type eventually affects survival of the adjacent.

**Cells of the inner retina**

Once the PR cell has been excited by light, a signal must be transmitted to the optic nerve for processing by the brain. This occurs via two synaptic regions: one connecting the PRs with the cells of the INL (bipolar, horizontal, and amacrine), and the other connecting the INL to the ganglion cells (figure 1). Bipolar cells send their processes into the outer and inner plexiform layers allowing the travel of both a direct and indirect path of action potential to ganglion cells. Specifically, bipolar cells receive synaptic input from either
cones or rods, but not both, and are designated cone bipolar and rod bipolar cells. Cones release glutamate to control the polarization state of the bipolar cell, which in turn transmits the signal to ganglion cells [29]. Rod bipolar cells on the other hand do not synapse directly with ganglion cells but instead, require the presence of amacrine cells to transmit information to neural ganglions.

Laterally oriented horizontal cells are far less numerous than bipolar cells and indirectly transmit information by receiving input from multiple PRs and communicating them to bipolar cells. HI, HII, and HIII are the three types of horizontal cells but their selectivity to the three cone types are still undergoing heavy debate [30]. Nevertheless, they all summate inputs from cones and inversely hyperpolarize by releasing controlled amounts of the neurotransmitter γ-Aminobutyric acid (GABA), constituting a form of lateral inhibition [31]. In so doing, they increase spatial resolution and thus making the eye more sensitive to contrast and differences in intensity.

Amacrine cells synapse with bipolar cells and serve to signal ganglion cells. In fact, amacrine cells directly input 70% of the information sent to ganglion cells as well as regulate the bipolar cells that send the remaining 30% [32]. Surprisingly, most of the 40 different types of amacrine cells lack axons and, like horizontal cells, work in a lateral manner. They are specialized because each cell connects to a particular bipolar cell and utilizes a particular neurotransmitter special to that connection. Despite their importance, relatively little is known about their functional roles except that they are thought to provide inhibitory feedback mechanisms to bipolar and ganglion cells.
Retinal ganglion cells are significantly different from one another in terms of cell size, connections, and responses to visual stimuli, and can be classified into 5 main groups based on their function. Midget, bistratified, parasol, photosensitive, and other retinal ganglion cells, make up the roughly 1.5 million retinal ganglion cells active in the human retina [32]. Each ganglion cell synapses with either a bipolar cell or amacrine cell so that the neurotransmitter, glutamate, GABA, or glycine, sent out by these retinal interneurons can bind to specialized receptor proteins on the ganglion cell [33, 34]. This neurotransmission initiates a change in membrane potential to convey visual signals to be delivered to several regions in the midbrain, mesencephalon, thalamus, or hypothalamus for vision interpretation [35].

Müller cells

Of the three basic glial cell types in the retina, Müller cells are principal. They span across the thickness of the retina providing structural support and defining the inner and outer limiting membranes. Their cell bodies sit in the INL, but their processes project in every direction including both the inner and outer plexiform layers as well as throughout the INL. It is thought that through two phases, a single progenitor cell gives rise to both Müller cells as well as retinal neurons. Phase one produces cones, horizontal cells, and ganglion cells whereas the second phase gives rise to rods, bipolar cells, and amacrine cells [36].

Müller cells possess a variety of different roles, all contributing to the vitality and health of retinal cells, forming in a symbiotic relationship with the neurons. Waste products such as CO$_2$ and ammonia, which may accumulate in the retina, are collected and disposed of by the Müller cells [36]. They also play regulatory roles by recycling excess glutamate so as
to protect surrounding neurons from over-exposure to these neurotransmitters [36]. Correspondingly, they take up and re-distribute deleterious extracellular potassium ions which may cause an imbalance in homeostasis by disrupting the ionic environment [36]. Synthesis of retinoic acid, release of GABA and phagocytosis of neuronal debris also contribute to their many functions [36, 37].

Recently, it has been published that Müller cells can serve to direct light towards the rods and cones by acting somewhat analogous to a fiber optic plate [38]. Their roles in neural regeneration are also being tested, but results in this field has been limited [39-42]. Interestingly, in the past several years, it has been suggested that upon retinal injury, these glial cells can undergo de-differentiation into multipotent progenitor cells of the retina which can then differentiate into PRs [43]. However, a complete understanding of these cells requires further investigation.

**The classic visual cycle**

The classic visual (retinoid) cycle follows a series of steps involving specialized enzymes and retinoid-binding proteins cycled between PR and RPE cells. Our understanding of this cycle is based largely on studies looking at rod cells; cones are believed to rely on the same system, but it is proposed that they require an additional pathway.

The cycle begins with the absorption of a photon of light by rhodopsin (figure 3). This initiates a photochemical reaction to change the conformation of the opsin molecule from 11-cis retinal to its all-trans isomer [44, 45]. All-trans retinal is then released into the inner leaflet of the OS disk bi-layer to complex with phosphatidylethanolamine (PE) resulting in an equilibrium mixture of N-retinylidene-phosphatidylethanolamine (N-
retinylidene-PE) and all-trans retinal [44, 45]. Most of the all-trans retinal is reduced to all-trans retinol (vitamin A) by retinol dehydrogenase (RDH) on the cytoplasmic surface of disc membranes [45, 46]. The remaining N-retinylidene-PE then gets flipped from the lumen to the cytoplasmic side of the disk membrane via the ATP-binding cassette transporter, ABCA4 [47-49]. Once there, N-retinylidene-PE dissociates into all-trans retinal and PE [28]. RDH then reduces all-trans retinal to all-trans retinol for further processing in the visual cycle [45, 46]. Vitamin A exits the PR by way of an interphotoreceptor retinoid binding protein (IRBP) to be shuffled to the RPE [50, 51]. There, it is converted to all-trans retinylester by the enzyme lecithin:retinol acetyltransferase (LRAT) and subsequently isomerized to 11-cis retinol by the RPE65 isomerase [11, 26, 27, 52-54]. Additionally, vitamin A from systemic circulation can pass through the basal surface of the RPE cells for esterification by LRAT [55]. The visual cycle is completed when 11-cis retinol is oxidized to 11-cis retinal by 11-cis RDH and transported back to the rod cell by IRBP [55]. Once there, it can recombine with opsin in the disk membranes to regenerate the photosensitive rhodopsin visual pigment, completing the cycle.

**The cone visual cycle**

Cones follow the visual cycle as proposed for rods, but are also theorized to have their own unique vision cycle pathway. They function in particular during the day where constant light increases the demand for 11-cis retinal and they interact with Müller glia cells for phototransduction [56]. The proposed model asserts that all-trans retinol generated in photoreceptors are transported to Müller cells where an unidentified isomerase changes it to 11-cis retinol [56]. This molecule is then oxidized to 11-cis retinal in the IS of cones [57]. Although a functional relationship has not been established between Müller cells and cone
IS, the ability of cones to efficiently regenerate visual pigment in perpetual light may rely on more than just the RPE interaction as proposed by the classic visual cycle.

**The Phototransduction Cascade**

Visual phototransduction is a process whereby photons of light in the surrounding environment is converted into an electrical stimulus as generated by PR cells, which can then be sent to the visual centers of the brain for vision interpretation. Contrary to many other neuronal types, in their resting state (dark), PRs are said to be depolarized [58]. An important second molecule responsible for maintaining the depolarized state in the PR is the nucleotide cyclic guanosine 3′-5′ monophosphate (cGMP). High levels of this second messenger keep cGMP-gated ion channels in an open state, allowing inwards positive cations to enter into the cell [59]. During this influx of positive cations, glutamate is signalled to be released from the synaptic vesicle of cones and rods to their respective bipolar cell [58].

When a photon of light strikes rhodopsin, a G-protein coupled receptor, 11-cis retinal is converted to 11-trans retinal, causing a conformational change in the rhodopsin molecule [44, 45]. This subsequently leads to an inactive GDP-bound transducin, a G-protein, to exchange GDP for GTP [60]. The now GTP-bound transducin will increase the activity of cGMP phosphodiesterase, which in turn breaks down cGMP in the cytoplasm causing the closing of cGMP-gated ion channels [60]. The PR is now hyperpolarized and restricts glutamate from being released [58]. In order to terminate the light response and depolarize PRs again, rhodopsin kinase phosphorylates rhodopsin, followed by the binding of arrestin to the phosphorylated rhodopsin [61]. This effectively prevents the activation of transducin, terminating the phototransduction cascade.
GENE THERAPY

General overview

Gene therapy is a form of molecular medicine with the potential to significantly change the way modern medicine is practiced. It holds promises to remedy inherited and acquired diseases originally thought to have no form of cure or treatment. The basic concept of gene therapy is very simple: introduce genetic material into target cells that will restore protein production originally absent or deficient, in the hopes that it will slow down or stop the progression of disease. Although the notion seems straightforward, safe and effective vehicles capable of delivering functional genes into target cells has been shown to be one of the biggest stumbling blocks to a successful outcome.

The vector used to ferry genes into host cells requires a number of features before successful transduction can occur. First, it needs to be determined whether or not the vector used can target the specific cell types, those being either dividing or non-dividing. Subsequently, receptors present on host cells should recognize the vector to be internalized. Upon entry, the vector must then migrate towards the nucleus of the cell all the while evading endosome and lysosome degradation. Once nuclear uptake is completed, stable and sustained retention of the therapeutic DNA must ensue, which requires either integration of the vector DNA with host genome or the maintenance of an episome. Following stable gene retention, the expression of the therapeutic gene will need to be monitored whereby transcription is regulated either by host cell machinery or induced by manipulations of its regulatory elements. Importantly, vector transduction should elicit minimal immune responses so as to reduce pathogenic or adverse effects to host tissue as well as prevent
immune clearance of the vector. Finally, the ideal vector should be produced in a highly concentrated form so that the volume of vector suspension injected into host cells is minimal. Along with that, a convenient and reproducible schematic on vector production should be available.

**Gene Expression**

Once viral genome is stably introduced into host cells, viral gene transcription is regulated by host RNA polymerase II (pol II). This polymerase requires the help of accessory proteins and general transcription factors (GTFs) to help direct site-specific initiation for the commencement of transcription as dictated by the core promoter elements. GTFs assemble at the promoter in a step-wise manner to create a stable pre-initiation complex (PIC) consisting of the RNA pol II and various GTFs. The core RNA pol II is the key catalytic enzyme in the PIC that is responsible for transcription. This polymerase contains a large subunit, RPB1, that has a C-terminus domain (CTD), which is the target of kinases and phosphatases. The CTD participates in several critical phases of RNA synthesis and processing [62].

Upon PIC attachment to the DNA, regulatory elements are then required to bind to regulatory sequences for transcription initiation. These *cis*-acting sequences fall into three categories: promoters, enhancers and silencers. *Trans*-acting factors then bind to the *cis*-acting sequences to elicit specific and regulated gene expression in response to environmental, developmental or cellular signals.

As transcription proceeds, phosphorylation of the CTD of RNA pol II serves to copy the DNA into mRNA by complementary base pair matching of RNA nucleotides to the DNA
template [63]. In many cases, newly transcribed pre-mRNA is not ready for duty. Instead, it must undergo post-transcriptional RNA processing to become functional as well as be protected from degradation by endo and exonucleases. This is accomplished by processes that take place co-transcriptionally and involves the addition of a 5’-cap, the addition of a 3’-poly(A) tail, and the excision of introns by splicing factors.

A fully processed mRNA now exits the nucleus of the host cell and enters the cytoplasm where it can be translated into a polypeptide of amino acids facilitated by ribosomes. When released from the ribosome, newly synthesized proteins are usually unable to function, instead, they must fold into the correct conformation and undergo chemical modifications. Modification of amino acid side chains usually takes place in the golgi apparatus and undergo a plethora of common processes including phosphorylation, methylation, acetylation, glycosylation, somulation, and disulfide bond formations, all of which serve as a reversible switch to control protein activity. Once fully processed, a signal sequence on the C terminus end of the protein dictates where the protein will be delivered to for further cellular use.

**Progress and challenges – Vector design**

Over the years, gene transfer vehicles capable of overcoming the above mentioned obstacles have been developed and can be categorized as synthetic or viral vectors. Synthetic vectors, also known as non-viral vectors, directly deliver genetic material via injection of DNA, liposomes, and nanoparticles. The benefits of using non-viral vectors include the ability to produce large amounts of vector that cause minimal toxicity or have little immunogenic properties. However, gene transfer by this method seems to be largely
inefficient and transgene expression is often transient in nature, thereby excluding their application to diseases that require long term, high level gene expression [64]. Perhaps in the future, when improvements can be made to the efficiency of the non-viral vector system, it will be the vector of choice. Presently, however, the use of viral vectors predominates in gene therapy studies.

Viruses are highly evolved biological machines that efficiently gain access to host cells and exploit the cellular machinery to facilitate their replication. Their main goal is to produce as much progeny as possible in as little time as possible. Unfortunately, the process of viral infections leads to many deleterious effects in the host including destruction of infected cells and surmounting a harmful immune reaction. Therefore, the ideal virus-based vectors for most gene therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to replication and cell toxicity.

When designing viral-based vectors, the viral genome should be characterized and separated into necessary cis-acting sequences required for packaging and integration into host cells and dispensable viral coding sequences which include structural and regulatory elements required for propagation of infectious progeny. The viral genes which promote pathogenicity and immunogenicity are excised out and replaced with the therapeutic gene of interest. The vector is then introduced into producer cell lines that provide the necessary structural elements in trans needed for the proper production of replication defective viral particles, which can now be used as delivery vehicles into host cells.

There is a steady increase in the number of viral vectors currently under development for gene therapy trials. Presently, four main classes of viruses (table 1) are heavily favoured
for clinical trials and can be divided into 2 groups; those that can integrate into host sequences and those that cannot. Retroviruses, including the lentivirus (LV), and some adeno-associated virus (AAV) have the ability to insert their genome into chromosomal DNA, giving them the potential to supply lifelong gene expression in dividing cells. Integration however does not guarantee lasting transgene expression because vector genomes can be silenced over time [65]. Adenovirus (Ad), herpes simplex virus (HSV), and most AAV on the other hand remain in the nucleus as an episome which eliminates the chance of insertional mutagenesis and has been shown to mediate persistent transgene expression in non-dividing cells.
EYE GENE THERAPY

Ocular diseases

Many forms of untreatable eye diseases stem from single gene defects in the retina (table 2) and affect millions of North Americans, leading to either severe vision impairment or complete blindness. Retinitis pigmentosa (RP) is a term given to a family of inherited retinal generation with initial rod degeneration followed by cone death and subsequent degeneration of the RPE, gradually leading to permanent loss of vision [66-69]. It is the most prevalent form of hereditary retinal degenerative disease and over 100 different loci have been identified to cause different forms of RP with an estimated 1.5 million people worldwide being affected [70]. Another leading group of retinal diseases called age-related macular degeneration (AMD), combines both genetic susceptibility with environmental exposures [68, 71]. Patients in this group tend to be over the age of 50 and suffer from atrophy of PRs and RPE cells and have an enhanced risk of choroidal neovascularisation, resulting in severe vision loss [71-74]. Of concern with AMD is that prevalence will greatly rise with a projected increase in the elderly population [71].

Leber’s congenital amaruosis (LCA) is a group of severe inherited retinal diseases affecting 3 in 100,000 children; patients typically progress to complete blindness by the third decade of life [26]. So far, 14 different genes have been shown to cause the various forms of LCA [27]. Perhaps one of the most studied forms is that of a mutation in RPE65, an isomerase enzyme found in the RPE involved in a critical step of the retinoid cycle [11, 26, 27]. Patients with a defective RPE65 gene suffer from accumulation of retinyl esters in the RPE, which in turn leads to death of PR cells [27, 75]. Positive results have been shown in
human gene therapy trials involving the delivery of a functional RPE65 gene to the retina [76-79] and a promising outlook on gene therapy for this disease is in sight.

Another disease occurring from an abnormality in the visual cycle is Stargardt disease, which is the most common hereditary recessive macular dystrophy having an estimated incidence rate of 1 in 10,000 [28, 80]. It is characterized by loss of central vision, progressive bilateral atrophy of PR cells and RPE and frequent appearance of orange-yellow flecks distributed around the macula [28, 81, 82]. A mutation in the PR specific ABCA4 gene, encoding an ATP-dependent binding cassette transporter is thought to be the cause in this recessive dystrophy [47, 81]. In short, a dysfunctional or absent ABCA4 transporter leads to excessive accumulation of pyridinium bisretinoid (A2E), a toxic byproduct in the RPE, negatively affecting their function and survival [28]. Since the RPE provide many nutrients for PR cell survival, the death of epithelial cells will result in subsequent PR cell death and overall vision compromisation.

It is estimated that without improvements in prevention and treatment opportunities for people with eye diseases, the number of blind or visually impaired patients will grow to 5.5 million in North America by the year 2020 [83]. Not only are eye diseases detrimental to patient’s quality of health, but it also burdens the economy of a nation. The National Eye Institute estimates that in the United States alone, the annual cost of vision impairment is $68 billion. Because treatment options for many ocular diseases are currently limited, it is extremely beneficial to explore the ability of gene therapy to permanently resolve the genetic malignancies in these patients.
**Advantages of eye gene therapy**

The eye has several unique features that make it a well suited target organ for gene therapy. It has a highly compartmentalised structure which allows for the accurate delivery of a small volume of vector suspension to a specific subset of cells. The precise targeting of a particular cell type minimises viral dissemination and unwanted systemic effects. Additionally, immune responses resulting from intraocular administration are attenuated compared to those following systemic administration because the eye has both physical barriers as well as an internal environment that promote tissue preservation and protects against harmful inflammatory responses which can limit transgene expression. Non-invasive imaging of gene expression can be applied *in vivo* due to the unique optical transparency of the eye. As well, visual function can be readily measured under psychophysical and electrophysiological parameters. Importantly, since most retinal diseases are bilaterally symmetrical, the contra lateral untreated eye can be used as an invaluable experimental control. Lastly, a wide range of well characterized animal models are available to be used that parallel human disease progression [84].

**Immune privilege**

The eye possesses a number of unique features which qualifies it as being an immune privileged organ. In fact, perhaps the oldest and most successful form of organ transplantation takes place in the eye. Corneal transplantation has been successfully performed on human patients for over 90 years and over 40,000 annual operations were performed last year in America with a low rejection rate [85]. This extraordinary success can be attributed to the ability of the eye to enjoy acceptance and not reject the foreign antigens
present on the graft. Tolerance is not only induced in the cornea, but in all areas of the eye, including the anterior chamber, vitreous or subretinal space [86-88].

Multiple overlapping mechanisms in the eye contribute to a state in which foreign antigens can successfully survive without immune clearance. First, the RPE physically prevents inflammatory cells from entering the retina by helping create the blood-retina barrier, which acts as a physical obstacle that prevents a mass infiltration of immune mediators. If T cells do manage to pass through this barrier, the naturally reduced expression of MHC II in the eye decreases its ability to present antigens [89]. Also, stromal cells can induce immune cell apoptosis without tissue damage or inflammation by strategically expressing death inducing molecules like PDL-1 and FasL [90, 91] located throughout the cornea, retina, iris, and ciliary body [92-96]. They also have the ability to convert activated T cells into regulatory T cells (Tregs), further inducing tolerance [97-99]. Recently, it has also been established that the RPE also retains the ability to change primed T cells into Tregs [18]. Next, immunosuppressive factors including neuropeptides, αMSH, somatostatin, anti-inflammatory cytokines like TGFβ-2, and complement inhibitors circulate in the eye to create a suitable immune suppressing environment [100-102]. Combined, the above mentioned mechanisms of reducing inflammation and inducing tolerance explain why the eye is an excellent organ for sustained transgene expression.

**Routes of vector delivery**

There are two main routes of vector delivery when introducing genes into the ocular space: subretinal and intravitreal (figure 4). Preference of one method over another can be due to the type of cells that are targeted. When studying PR cells and the RPE, it would be
logical to directly inject the viral vector to the subretinal space. This way, the vector particles are in direct contact with the two cell layers. Alternatively, if the inner retinal cells were to be targeted, then perhaps intravitreal injection may be more beneficial because vector particles have direct accessibility to that cell layer. In fact, it has been shown that subretinal injections lead to expression predominantly in the RPE and the PR cell layer [103-109] where as expression after intravitreal injection is located in the retinal ganglion cells, Müllera cells, and amacrine cells [105, 110-113].

Interestingly, studies have shown that transgene expression in the retina after subretinal injection is stronger and more intense than that after intravitreal injections [105, 114]. The vitreous body has been shown to inhibit retroviral vector mediated gene transduction of the retina by hindering the vector from direct contact with viral entry receptors [115]. Ad and AAV vectors also need to establish this physicality with retinal cells and since the vitreous body can potentially pose a big obstacle by preventing contact between vector and target cells, intravitreal injections may not be as efficient in transduction as subretinal injections.

One concern with any gene delivery method is inflammation. Although the eye is an immune privileged organ, there can still be factors which can bring about an immune response, leading to vector clearance and/or tissue damage. When comparing injection methods, it has been shown that inflammation and electro-retinogram (ERG) changes were stronger with intravitreal injections [114]. This may in part be due to more mechanical damage to the lens capsule after intravitreal injection, which breaks down the blood-retina barrier, leading to a stronger inflammatory response [105, 114]. Overall, when targeting
cells of the outer retina, the best suited method of delivery to induce maximum transduction and minimum immune response is via the subretinal route.

**RPE and photoreceptor cell transduction**

The majority of inherited retinal diseases stem from gene defects expressed in the PR cell (figure 5) (table 2). However, targeting of PRs has been difficult and the majority of transgene expression in the retina stems from the RPE. Transduction of the RPE is simplistic as compared to PR cells due to their morphology, accessibility, and receptors. Retinal cells located in various sub-cellular compartments express different receptors necessary for viral vector entry. This difference in expression will lead to variable viral transduction efficiency and gene expression levels. During subretinal injections, the majority of viral vectors come in contact with the PR outer segment. However, CAR, HSPG, and αβ5 receptors, those that facilitate entry of Ad, AAV, and LV are distinctively absent from the OS and solely located on the IS and cell body [116, 117]. Hence, steric hindrance of the tightly packed PR layer prevents efficient attachment of viral particles to their respective viral receptors. On the other hand, the RPE is a monolayer of cells with viral receptors located all along the apical side where they are in constant contact with viral particles upon injection into the subretinal space [116]. Thus, the uptake of viral vectors is more efficient as compared to PR cells and gene expression levels in the RPE will be greater than that of rods and cones.

It has also been proposed that the tropism of viral vectors for retinal cell types are dictated by the stage of retinal development such that PR transduction is efficient in mice pups but rarely in adult mice [107, 118]. Complete retinal differentiation occurs by post natal day (p) 21 and it has been suggested that there is a purported shift in viral tropism from
PR cells to RPE cells by p10 [119]. Consequently, by using adult mice, viral vectors will have a natural preference to target RPE cells over PR cells. On a different note, daily phagocytosis may also play a role in more transgene expression detected in the RPE. Any transgene product expressed in the PR outer segment will be engulfed by the RPE and hence an increased build-up of protein expression will be detected in the RPE.

Since targeting of PR cells is critical in many retinal diseases, strategies have been developed to circumvent RPE only targeted expression, by cell specific promoters. The ubiquitous cytomegalovirus immediate-early (CMV) promoter is highly expressed in RPE cells, and theoretically should have the same activity in PRs. However, this is not the case as the majority of studies using the CMV promoter yields results predominantly, if not exclusively, in the RPE layer [107, 108, 120, 121]. The development of PR specific promoters: rhodopsin kinase, cone opsin, and murine opsin promoter (MOPS), have allowed cell specific expression in rods and cones and have been heavily utilized in AAV gene therapy trials [106, 122].
VECTORS USED IN EYE GENE THERAPY

Substantial progress in understanding the molecular pathology of ocular diseases has been made resulting in more than 120 different disease causing genes to be identified, mapped and cloned [123]. Correcting gene defects in inherited ocular disorders is highly plausible by way of gene-based therapies to replace absent genes or silence defective ones. In recent years, there has been an influx of studies demonstrating proof-of-principle that gene based therapies can significantly improve ocular morphology and visual function that have otherwise been impossible to achieve [122, 124-129]. These advancements have been made possible by the development of suitable viral vectors for ferrying therapeutic genes into the ocular compartment in a safe and stable manner.

Many viral vectors have been developed for the delivery of transgene into the retina, of which the most widely used are LV, AAV, and Ad. Although they all have very different capacities, the common characteristic of these viral vectors is their ability to transduce non-dividing cells, of which the retina is comprised. Here, we review some of the most commonly used viral vectors, discussing the biological infection pathway as well as their current status in eye gene therapy.

Lentivirus

Currently, retroviral vectors are the most commonly used vectors in the delivery of genes into higher animals and human cells both \textit{in vitro} and \textit{in vivo} [130, 131]. However, a major drawback in gene transfer with a simple retroviral vector is the innate inability to invade quiescent cells without nuclear membrane breakdown. LV vectors, also known as
complex retroviral vectors, have been the major focus of development in this class of virus to resolve the limitation of nuclear targeting in post-mitotic cells.

The genome is comprised of two identical single-stranded (ss) RNAs packaged into a viral capsid encoded by gag, pol, and virus specific reverse transcriptase. These viral proteins synthesize the double-stranded (ds) DNA provirus, protease and integrase. Upon reverse transcription of the viral genome in the cytoplasm, this class of viral vector has the ability to efficiently pass through the nuclear pore of non-dividing cells via the interactions of the pre-integration complex containing a nuclear localization sequence and karyopherins or importins located in nuclear pore complex [132-134]. Once inside the nucleus, the provirus can integrate into the cell genome via non-homologous end joining [135]. LVs have a natural tropism for haematopoietic cells, the central nervous system, muscle cells and liver cells and have been proven to exhibit long term gene expression in the absence of inflammation [136-140].

The vector forms of this virus are mainly derived from the human immunodeficiency virus (HIV) and are often pseudotyped with vesicular stomatitis virus G (VSV-G). They have a carrying capacity of 7 kb and contain no viral accessory proteins other than the minimal viral sequences necessary for virus propagation, such as the inverted terminal repeats (ITR) found on the ends of the genome. Hence, low immunogenicity with this vector results due to the absence of viral coding elements.

**Lentiviral vectors in eye gene therapy**

LV vectors have been implemented in studies to correct genetic diseases in rodent models of retinal dystrophies such as those with phenotypes similar to the human LCA
condition, RP, and Stargardt disease. However, although LV vectors efficiently transduce the PR cells of neonatal mice or rat pups, they predominantly transduce the RPE in the adult counterpart [107, 141]. In a study examining retinal degeneration (rd1) mice, it was shown that vector administration slowed rod degeneration in neonates but no effect on the retinal degeneration of adults [142]. Even under the direction of a rhodopsin promoter, adult rats were shown to have poor gene expression in PR cells [118]. Another study using a non-human primate retinal degeneration model, used gene transfer of a neurotrophic factor with the simian immunodeficiency virus (SIV)-based vector [103]. They reported that transgene expression was maintained for 4 years without significant decline but expression was predominantly localized in the RPE [103].

Indeed, it is possible to transduce PR cells with the LV vector, but pseudotyping is necessary. Studies have shown that a LV vector pseudotyped with equine immunodeficiency virus (EIAV) or VSV-G can transduce PR cells, but the level of transgene expression is reported to be variable and sporadic [118, 143]. No doubt, RPE cells are easily targeted by LV. For example, when RPE65−/− mice were given a subretinal injection of a functional human RPE65 gene, they were found to successfully express that protein in the RPE [127]. These mice also exhibited functional rescue, as assessed by ERGs showing significant improvements in ERG waveform [127].

Taken together, this vector type does not efficiently transduce adult PRs, which renders them unsuitable for human gene therapy trials unless administrated in utero, when the fetal retina is not fully developed [144]; a feat that is not yet possible. Nonetheless, despite evidence suggesting that LV based methods are unsuitable for efficient PR targeting, correction of Stargardt disease phenotype was recently published. The study used a mouse
model of Stargardt disease, and reported efficient delivery of the wild type human ABCA4 gene via an EIAV-derived vector into subretinal space of ABCA4−/− mice. Five to 20% of PR cells were successfully transduced and A2E accumulation was reduced to levels comparable with mock treated control eyes [145].

**Adeno-associated virus**

AAV is a member of the non-pathogenic human parvovirus family and requires co-infection with helper viruses such as Ad or HSV for viral propagation. AAV virions are small non-enveloped particles that carry a linear ssDNA genome measuring 4.7 kb in size. Upon receptor-mediated endocytosis of the virus into host cells, the ssDNA is uncoated and converted to a double-stranded form. This rate-limiting step occurs either by annealing with a complementary DNA strand from another virus or by the host cell machinery [146]. Once converted, the dsDNA may retain in episomal form by forming concatamers via head to tail recombination of the ITRs or it may randomly integrate into the host genome at a low frequency (<10%) [147].

The vector form of this virus is derived from numerous serotypes of AAV, each utilizing a different approach for cell entry via different receptors and co-receptors, resulting in a variety of different cellular tropisms. Perhaps the best characterized and most studied serotype is AAV serotype 2 (AAV2). This vector displays a broad host-range by using the ubiquitous heparin sulphate proteoglycan for primary attachment and fibroblast growth factor receptor 1 and integrins $\alpha_v\beta_5$ as co-receptors [148, 149]. Like AAV2, AAV1 and AAV3 share homology in their cap proteins and use heparin sulphate for internalisation. AAV1 transduces muscle and liver cells efficiently [150, 151], whereas AAV3 shows strong tropism
for hematopoietic cells [152]. Alternatively, AAV4 and the AAV5 require the presence of sialic acid and platelet derived growth factor (PDGF) receptors for internalisation [153]. AAV5 displays a strong preference for retinal cell transduction as well as airway epithelia [154, 155]. These observations suggest that altering the capsid proteins present on the surface of the AAV vector allows for tissue specific targeting.

The development of hybrid pseudotyped AAV vectors have considerably expanded the repertoire of available AAV vectors that can potentially target specific tissue subsets with high efficiency. To do so, an AAV plasmid is packaged into the capsid of an AAV vector of a different serotype. For instance, an AAV2 plasmid packaged into an AAV2 capsid (designated AAV2/2) has been shown to target both PR cells and RPE cells, but AAV2/4 and AAV4/4 deliver expression restricted to the RPE. The different combinations of capsid and genome can be used to generate a large range of vectors each with specific tissue tropisms and expression kinetics.

**Adeno-associated viral vectors in eye gene therapy**

AAV remains the most commonly used vector in retinal gene therapy and possesses many benefits for the targeting of ocular tissue. Most importantly, the vector is safe for human administration as the wild type virus does not cause human disease [156]. Its small particle size and low immunogenicity allows for a high administration of vector dose without eliciting an acute inflammatory response or toxic side effects. Also, it has a strong tropism and transduction pattern that leads to stable and efficient transfer to RPE and PR cells. As a result, a wide range of neovascular and retinal degenerative diseases have been studied using AAV mediated gene therapy.
Unlike the LV, AAV is the currently the only vector consistently capable of efficiently transducing both the RPE and PR cells [106, 157-159]. Whereas larger viruses such as HIV (120 nm in size) are prohibited from easily accessing the inter-photoreceptor matrix and the outer limiting membrane due to the sheer size of the viral particle, AAV virions measure 20 nm in size [160, 161]. Different serotypes of AAV vectors have also been engineered to better target PR cells. Under the direction of PR-specific promoters, mainly those of rhodopsin, mops, and rhodopsin kinase, AAV 2/5 can efficiently transduce PR cells specifically [106]. More recently, it was shown that driven by PR-specific promoters, AAV2/7 and AAV2/8 are the most efficient serotypes for PR targeting [157]. Even using the ubiquitous CMV promoter, a canine retina was shown to exhibit significant transgene expression in PR cells [162].

AAV vectors have also mediated efficient gene replacement therapy in mice models of inherited retinal degeneration. In a mouse model of RP, Prph2Rd2/Rd2 mice (lacking a membrane protein essential for the formation and stability of PR outer segments) were given a single injection of AAV2 with the peripherin-2 gene under a rhodopsin promoter and found to have restored formation of OS discs and improved retinal function by ERG for up to 14 weeks [126]. In a model of LCA, localization of a GTPase regulator interacting protein is abnormal, leading to PR death. When mice were injected with a functional replacement gene under the guidance of a rhodopsin promoter, PR cells survived and retained retinal function for at least 5 months post injection [122]. In another study, using Merkt in an AAV2 vector, PR degeneration was slowed in the Royal College of Surgeons rat [124].

Pre-clinical studies investigating gene transfer using AAV in large animal models have been extremely successful. These models are useful in the development of clinical
applications because their anatomy is much more similar to that of humans. The Swedish Briard dog is a popular model used to study the effects of a null mutation in RPE65 [128]. These dogs are phenotypically similar to humans with the same mutation and several groups have demonstrated “proof of concept” for gene replacement therapy in this model. Following subretinal injections of RPE65, animals showed significant improvement in visual function as evidenced by behavioural assessments and ERGs. These improvements were present up to 3 years post-injection [129, 163]. In another canine model, subretinal injection of AAV2/2 resulted in long term expression of transgene in the RPE and PR cells [162].

Immunological and biological similarities between primates and humans are essential for determining safety and tolerance of vectors. One study has shown that rod PR cells had higher transduction efficiencies than cone PR cells when injected with an AAV2 vector carrying a CMV promoter [164]. Other instances show that AAV5/5 has preferential tropism for rod PR cells in non-human primates [165]. Interestingly, one study compared the results of a long term experiment looking at transgene levels in rat, dog, and non-human primate and found that the level of transgene expression was strongest at 2 months post-injection for all species [166]. Collectively, these experiments demonstrate that diseases caused by loss-of-function mutations can be overcome by gene replacement strategies with the AAV vector. The positive results from long term functional improvements in vision with large animals as well as small scale clinical studies, have made it possible for additional human trials of AAV mediated gene therapy for patients with LCA [26, 76, 77].
**Adenovirus**

Ads are non-enveloped dsDNA viruses that in nature cause relatively mild upper respiratory tract infections, gastroenteritis or conjunctivis but have not been associated with any neoplastic diseases in humans [167]. This 60-90 nm virus has a natural tropism for epithelial cells of the respiratory and GI tracts. The uptake of the viral particle occurs with initial interaction between the fiber protein on the virus and the coxsackievirus-adenovirus receptor (CAR) on host cells [168]. Receptor-mediated endocytosis follows by the binding of the viral penton base protein to the $\alpha_v\beta_3$ integrins [169]. Upon entry into the cell, the virus uncoats and migrates into the nucleus through the nuclear pore, where it remains in episomal form for the remainder of the cell’s life-span [170]. Usually, a rapid immune response occurs after Ad infection including both cytotoxic and humoral responses. These result from neutrophil and macrophage infiltration as well as the activation of T helper lymphocytes of both Th1 and Th2 phenotypes leading to specific immunoclearance of the infected cells by cytotoxic T lymphocytes (CTL) [167, 171]. In the process, low levels of IgG immunoglobulins are sustained in the blood; these act to enhance the immune response upon subsequent infections [172]. In addition, a large proportion of the human population has been exposed to the virus and ~80% of adults have pre-existing antibodies [173].

The Ad genome is 30-38 kb of DNA and can be divided into 4 functional units, E1 to E4 [167] (figure 6). The first-generation vector of this virus (FG-Ad) was created by substituting a transgene for E1 (the region responsible for viral gene expression and DNA replication) resulting in a replication defective vector with a cloning capacity of 4kb. Since E3 products are also not essential for Ad replication, deletions in both the E1 and E3 regions increased the vector carrying size to 7kb [174]. Although 51 different serotypes exists, Ad5
and Ad2 are most commonly used for viral vector production [175]. Compared to all other vector systems, Ad vectors most efficiently deliver genes into a broad range of hosts [170]. Furthermore, they have the ability to transduce both dividing and non-dividing cells of nearly all types of human tissue including muscle, blood, bone, nerve, liver, and skin at high efficiency [167, 170, 176].

**Adenoviral vectors in eye gene therapy**

Surprisingly, despite its strong immunogenicity, the first two published clinical ocular gene therapy trials employed the Ad vector as the delivery tool [177, 178]. The first trial used a FG-Ad carrying the herpes simplex thymidine kinase that was delivered to children with retinoblastoma. No dose limiting toxicities were reported even at a high concentration of viral particles, but mild inflammation was documented [177, 178]. The other trial utilized patients with advanced AMD and intravitreally injected a second-generation Ad vector with genome deletions in the E1, E3 and E4 regions carrying a pigment epithelium-derived factor. Again, no dose-limiting toxicities were documented in any of the patients, but mild and transient intraocular inflammation was observed in 25% of the patients [177, 178]. Both trial groups claimed that the inflammation was clinically manageable. Based on these results, it may warrant discussion on how gene transfer in the murine retina is not a worthy predictor of results expected in humans since immunogenicity was greatly reduced in humans compared to rodents. Nonetheless, to alleviate some of the immunogenicity problems associated with the FG-Ad vector, researchers have focused on developing a vector devoid of all viral coding genes.
**Helper-dependent adenovirus**

After many generations of modified Ad vectors, significant improvements in the safety and efficacy of these vectors came with the development of the HD-Ad vector, also known as the gutless, gutted, and high capacity Ad vector. Many attractive features about this vector render it a prime choice of gene delivery as it addresses the issues of capacity, toxicity, and immunogenicity brought upon by LV, AAV and FG-Ad vectors.

The main difference in genome composition between the HD-Ad vector and its parental FG-Ad vector is that the HD-Ad vector is fully eradicated of all its viral coding genes, leaving only the ITR and ψ sequences necessary for vector replication and packaging, respectively (figure 6). This strategy prevents the production of all viral proteins which in turn reduces or eliminates the CTL response brought upon by viral delivery [179-181]. A negligible immune response reduces toxicity to host cells, and with no vector clearance, long-term gene expression can be achieved. In fact, upon HD-Ad injection, gene expression has been shown to have life-long persistence [182]. Along with these viral gene deletions, a large carrying cassette of ~37 kb is now available [183]. Such a large carrying capacity allows the packaging of larger transgenes complete with cis-acting control elements necessary for proper transgene expression.

Other worthwhile features of this vector include its ability to transduce both dividing and terminally differentiated resting cells. As well, they have a broad tropism for a wide variety of cell types. In addition, since HD-Ad vectors remain in an episomal state, there is no risk of insertional mutagenesis. The episomal state also provides prolonged transgene expression when transduced into resting or slowly replicating cells.
**Production of HD-Ad vectors**

The HD-Ad vector, because all of its viral coding sequences have been deleted, requires a complementing helper virus to provide all of the necessary proteins *in trans* for its packaging [184, 185]. Originally, one of the major setbacks in preparing large quantities of this vector was the inability to remove high levels of contaminating helper viruses. The first efficient system for producing high titres of viable HD-Ad vectors with low volumes of helper virus was developed by Graham and coworkers in 1996 using the Cre-loxP system (figure 7) [186].

In the developing stages of this method, production of only modest amounts of vector was attainable, limiting their use to small animal experiments that required low doses of virus. The technical issue of large preparations of high quality vector production was addressed by Palmer and Ng [187]. To produce large quantities of this vector, a system was developed that used suspension-adapted producer cell lines with high Cre expression and a mutation resistant helper virus. This method reduced the length of time required (2 weeks) to produce the virus. In addition it increased the yield of viral particles (upwards of $1 \times 10^{13}$ viral particles) with very little helper virus contamination (0.02% to 0.01%) [187].

**Benefits of HD-Ad in eye gene therapy over other vectors**

Currently, Ad, AAV, and LV vectors have been successfully used for the delivery of transgenes into the ocular space of different animal models. However, little information is known about the effects of employing HD-Ad vectors in ocular gene therapy trials. Many characteristics of this vector render it an excellent choice when pursuing the delivery of transgenes into the retina compared to the other vectors currently being employed.
Low immunogenicity and long term transgene expression

The major setback to using the FG-Ad vector is its immunogenetic effect. FG-Ad vectors activate both innate and adaptive immune responses, which are directed against viral capsids and the transduced cells [179, 188]. Upon initial delivery of the viral vector, transgene expression is highly expressed. However, expression typically decreases to low or undetectable levels several weeks post injection [170]. This can in part be explained by the understanding that despite deletion of the E1 and E3 regions, vectors of this type still contain residual viral genes that synthesize viral proteins [189, 190]. As a result, CTLs are activated, transduced cells are attacked and destroyed, and transgene expression is eradicated [170].

The HD-Ad vector retains many of the advantages of the FG-Ad vector including high transduction efficiency and high level transgene expression, but owing to the absence of all viral coding regions, the vector possesses the ability to evade host immune responses. This in turn mediates not only high level but also long term transgene expression, without the risk of chronic toxicity and transient transgene expression. Although this is of less concern for ocular gene therapy given the immune privilege status of the eye, studies have demonstrated that FG-Ad vectors promote only short-lived ocular expression [191-193]. Not surprisingly, several studies have demonstrated a CTL mediated immune response after the introduction of FG-Ad vectors into an ocular environment in mice resulting in only transient transgene expression [194, 195].

Distinctly different in genome composition, the HD-Ad vector evades much of the CTL response because no viral genes are present. Recently, two reports have found HD-Ad
gene expression to last more than 1 year post injection in the eye [120, 196]. In contrast, it has been found that the majority of the population possesses Ad-neutralizing antibodies as a result of natural infection [197], and even a single injection of the FG-Ad vector can elicit a strong humoral immune response against the viral capsid [198]. This response makes FG-Ad vectors unusable for re-administration. Since the majority of retinal gene therapy requires long term transgene expression, it is required that the vector be both stable and possess low immunogenicity. For these reasons the HD-Ad is the vector of choice.

**No risk of insertional mutagenesis**

The retina is composed of primarily post-mitotic cells, therefore, dilutional loss of an episomal transgene due to cell division is of little concern. In addition, since the HD-Ad genome exists in an episomal state, there is no risk of insertional mutagenesis and oncogenic transformation which may result from LV or AAV vector integration. Historically, one of the most remarkable clinical gene therapy trials involved the successful treatment of 11 children with severe combined immunodeficiency disease using an integrating Moloney retroviral vector. Unfortunately, insertional mutagenesis resulted in leukemia in 4 patients and death in another [199, 200]. Wild type AAV vectors naturally integrate into human chromosome 19 and rAAV vectors show random integration at a frequency of ~10%, making insertional mutagenesis a concern when using this vector type [147, 201-204]. The need for persistent gene expression via an integrating vector is not required in the post-mitotic retina, which raises the question of whether or not AAV or LV vectors serve as a good platform for retinal gene therapy if efficient long term transgene expression can be achieved by way of episomal vectors.
Fast onset of transgene expression

Another disadvantage in using the AAV vector is its initial slow rise in gene expression levels. Transgene levels were shown to be absent for the first few weeks after administration. In addition, transgene levels when expressed in these vectors require a significantly longer time period to reach a stable plateau as compared to other vector types [106, 157]. Conversely, HD-Ad vectors elicit transgene expression almost immediately after vector administration. From as little as three days to one week, strong GFP expression was detected in mouse retina upon intraocular injections [120, 196]. The AAV lag phase, whereby the genome is converted from a single strand to a double strand, greatly retards the rate of gene expression. This long lag phase may impose hurdles when it is necessary to rescue a rapidly deteriorating retina.

Large cloning capacity

The HD-Ad vector proves to be superior to the AAV, FG-Ad, and LV vector because of its large cloning capacity of 38 kb. With such a considerable amount of space, the vector can house whole genomic loci, multiple transgenes, and large *cis*-acting elements to enhance, prolong, and regulate transgene expression. Transgenes expressed in PR cells should be tightly regulated in terms of gene expression and cell specificity because it has been shown that even with a 5-fold increase in expression of normal opsin in rod cells can lead to retinal degeneration [205]. Similarly, a 23% over-expression of rhodopsin also leads to retinal degeneration [206]. Alternatively, complete PR degeneration results after 3 months in a model of rhodopsin−/− mice [207]. Taken together, it seems that there is a narrow range of gene expression that is tolerable by PR cells in order to remain functional.
In order to control the amount of gene expression, native gene regulatory elements can be used; these are generally too large for the limited cloning capacity of the AAV vector (4.7kb), FG-Ad vector (7 kb), and LV vector (7 kb) because once the cDNA is cloned into the cassette, there is little room left for large regulatory sequences. For example, the cDNAs of ABCA4, CEP290, and myosin VIIa measure to be 6.8 kb, 7.4 kb, and 6.6 kb respectively; all of which already exceed the capacity allowed by the AAV vector. Additionally, evidence suggests that tissue-specific promoters suitable for targeting selective cells are typically larger than can be accommodated by these vectors [208]. For instance, while short rhodopsin kinase promoters can promote transgene expression specifically in rods, it has been shown to be more beneficial to use the larger 4.7 kb rod opsin promoter to enhance transgene expression [106, 108].

To circumvent the obstacle of a small packaging capacity, the exploitation of AAVs unique ability to form concatemers by head to tail recombination of its ITRs has been explored, increasing its delivery size up to 10 kb. This can be accomplished by splitting up the transgene sequences between 2 different AAV vectors which can be later fused together after host infection to form a functional gene [209, 210]. This approach, “trans-splicing”, although theoretically sound, is relatively inefficient and requires gene sequence-specific modifications [211]. In addition, positive results of this method are not readily reproducible. All in all, it seems that specific and regulated transgene expression in larger retinal genes requires a vector with a large carrying capacity, such as that of the HD-Ad.
RATIONAL, HYPOTHESIS AND OBJECTIVES

Currently, several vectors have been used in retinal gene therapy trials including those derived from AAV and LV. However, limitations do exist that prevent their widespread and consistent use for all forms of retinal dystrophies. Little information is known about the HD-Ad vector but many characteristics of this vector render it an excellent choice for transgene delivery.

The ideal vector for transducing retinal cells should: 1) have a large cloning capacity, 2) be a non-integrating genome that can exist in the episomal form in the nucleus, 3) possess a high tropism for non-dividing cells in the post mitotic retina, 4) be able to sustain long term transgene expression, 5) lack immunogenicity, 6) and allow for re-administration if necessary. The HD-Ad vector presents all these characteristics which make it an ideal candidate for retinal gene therapy. I hypothesize that the HD-Ad system can be used to deliver transgenes into PRs and RPE cells. To that end, I conducted this study and asked the following questions:

- What is the expression of CMV driven reporter genes upon subretinal injections?
- What is the dose response of viral vector mediated reporter gene expression?
- Can I target PR cells using the mops promoter?
Figure 1 – Structure of the retina. Simplified schematic diagram of the retina showing the major cell layers. Perception of light by rods and cones initiates a phototransduction cascade to second order (bipolar cells, amacrine cells and horizontal cells) and third order (ganglion cells) neurons that project the signal to the brain via the optic nerve. Sandwiched between the two nuclear layers are two plexiform layers where synaptic interactions are made. RPE – retinal pigment epithelium; INL - inner nuclear layer; IPL – outer plexiform layer; OPL - outer plexiform layer; ONL - outer nuclear layer. Modified from Matlin and Foley (2009).
**Figure 2 – General organization of a rod PR cell.** Rod cells are structurally made up of 5 distinct segments: the outer segments allows for phototransduction to occur; the connecting cilium holds the outer and inner segments together; the inner segment houses the machinery for protein synthesis; the cell body holds the nucleus; and the synapse signals to secondary neurons. RPE – retinal pigment epithelium; OS - outer segment; CC - connecting cilium; IS - inner segment; CB - cell body; N - nucleus; S – synapse; ER – endoplasmic reticulum; ROS – rod outer segment. Modified from Molday (2007).
**Figure 3 – The classic visual cycle.** The visual cycle begins when light hits rhodopsin (yellow oval), changing its conformation from $11$-cis retinal to all-trans retinal, which then gets released into the cytoplasmic side of the disc membrane. Alternatively, all-trans retinal combines with phosphatidylethanolamine (PE) to form N-retinylidene-PE (N-PE). ATP binding cassette 4 (ABCA4) transports N-PE to the cytoplasmic side of the disc membrane where it dissociates into all-trans retinal and PE. The free floating all-trans retinal is then reduced to all-trans retinol by retinol dehydrogenase (RDH) and subsequently released into the subretinal space where interphotoreceptor retinoid binding protein (IRBP) then carries it to the RPE. There, through a series of enzymatic steps, $11$-cis retinal is regenerated and exported back out into the subretinal space. IRBP reshuffles it back to the rod cell where $11$-cis retinal can now bind with an opsin molecule to be reused. RPE65 – retinal pigment epithelium 65; LRAT – lecithin:retinol acetyltransferase.
**Figure 4 – Routes of vector administration.** The 2 main methods of vector suspension delivery are injection into the vitreous cavity or the subretinal space. Subretinal administration involves vector particles to be delivered into the potential space between the PR outer segment and the underlying RPE, which results in a temporary separation of these layers that re-appose spontaneously after a few days. Histology of the retina shows the nuclear layers to stain purple sandwiched between the plexiform layers. IPL - inner plexiform layer; INL - inner nuclear layer; OPL - outer plexiform layer; ONL - outer nuclear layer; IS - inner segments; OS - outer segments. Modified from Bainbridge, Tan and Ali (2006)
Figure 5 – Localization of proteins involved in retinal dystrophies. Some of the prominent proteins involved in retinal dystrophies are localized in the RPE and different compartments of the PR, including the outer segments, connecting cilium, and cell body. For the diseases associated with these gene mutations, refer to table 2. RPE – retinal pigment epithelium; ROS – rod outer segment; RIS – rod inner segment; COS – cone outer segment; CIS – cone inner segment
**Adenovirus**

**First Generation Adenoviral vector**

**Helper Dependent Adenoviral vector**

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**Figure 6** – Simple schematic of the adenovirus genome, first generation genome, and helper dependent genomes. The adenovirus genome is composed of 4 viral coding regions: E1 to E4 (grey). ITRs (red) flank the ends of the genome for viral propagation and the ψ (yellow) sequence codes for the packing sequence. First generation vectors replace the E1 region with a transgene (blue), which prevents the recombinant adenoviral vector from replication and cell lysis. Helper dependent adenoviral vectors are deleted of all viral coding regions and contain only the transgene of interest, the ITRs, and the ψ. Non-coding stuffer DNA (purple) builds the genome to a size of 27 kb to 38 kb for efficient packaging into Ad capsid.
**Figure 7 – HD-Ad production.** The expression cassette is cloned into the gutted HD-Ad vector backbone along with some stuffer DNA, the ITR and ψ. Upon digestion with PmeI to eliminate bacterial sequences, the linear genome is transfected in Cre-expressing 293 cells and subsequently infected with helper virus bearing a packaging signal flanking loxP sites. The helper virus *trans* complements the replication and encapsidation of the HD-Ad genome and the packaging signal is excised rendering itself unpackagable. Serial co-infections of 293 Cre cells amplify the HD-Ad genome to a desired titre and CsCl ultracentrifugation separates the vector from the helper virus. Modified from Ng et al. (2002).
<table>
<thead>
<tr>
<th>Vector</th>
<th>Genetic material</th>
<th>Packing capacity</th>
<th>Immune response</th>
<th>Vector genome</th>
<th>Main limitation</th>
<th>Main advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>ssDNA</td>
<td>4.7kb</td>
<td>Low</td>
<td>Episomal (90%)</td>
<td>Small packing capacity, lag-phase expression delay</td>
<td>Non inflammatory, non-pathogenic</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>dsDNA</td>
<td>7 kb</td>
<td>High</td>
<td>Episomal</td>
<td>High Inflammatory response</td>
<td>Extremely efficient transduction of most tissues</td>
</tr>
<tr>
<td>HD-Ad</td>
<td>dsDNA</td>
<td>38 kb</td>
<td>High</td>
<td>Episomal</td>
<td>Production,</td>
<td>Non-pathogenic, efficient transduction of most tissue, large carrying capacity</td>
</tr>
<tr>
<td>HSV-1</td>
<td>dsDNA</td>
<td>40 kb, 150 kb</td>
<td>High</td>
<td>Episomal</td>
<td>Inflammation, transient transgene expression in non neuronal cells</td>
<td>Large carrying capacity, strong tropism for neurons</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>RNA</td>
<td>7 kb</td>
<td>Low</td>
<td>Integrated</td>
<td>Insertional mutagenesis, human pathogenicity</td>
<td>Long term gene expression in non-dividing cells</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>RNA</td>
<td>7 kb</td>
<td>Low</td>
<td>Integrated</td>
<td>Limited to dividing cells, insertional mutagenesis</td>
<td>Persistent gene expression in dividing cells</td>
</tr>
</tbody>
</table>

**Table 1 – Major viral vectors in gene therapy.** Compiled characteristics of the major viral vectors currently employed in gene therapy trials.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell</th>
<th>Cellular function</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA4</td>
<td>PR (OS)</td>
<td>Flippase; visual cycle</td>
<td>Recessive Stargardt</td>
</tr>
<tr>
<td>CEP290</td>
<td>PR (CC)</td>
<td>Vesicle transport to OS</td>
<td>PR degeneration</td>
</tr>
<tr>
<td>CNG</td>
<td>PR(OS)</td>
<td>Phototransduction cascade</td>
<td>PR degeneration</td>
</tr>
<tr>
<td>CRB1</td>
<td>PR (IS)</td>
<td>Retinal morphogenesis</td>
<td>Recessive RP, LCA</td>
</tr>
<tr>
<td>CRX</td>
<td>PR (N)</td>
<td>Transcription factor, photoreceptor differentiation</td>
<td>PR degeneration, LCA III, Cone-Rod dystrophy 2</td>
</tr>
<tr>
<td>GNAT</td>
<td>PR(OS)</td>
<td>Phototransduction cascade</td>
<td>PR degeneration</td>
</tr>
<tr>
<td>LRAT</td>
<td>RPE</td>
<td>Phototransduction cascade</td>
<td>LCA</td>
</tr>
<tr>
<td>MYO7A</td>
<td>PR (CC)</td>
<td>Myosin motor, transport in PR and RPE</td>
<td>Usher Syndrome 1B</td>
</tr>
<tr>
<td>RDH</td>
<td>RPE, PR</td>
<td>Reduces and oxidizes retinoids</td>
<td>Autosomal recessive fundus albipunctatus</td>
</tr>
<tr>
<td>RDS</td>
<td>PR (OS)</td>
<td>Disc rim protein, disk membrane morphogenesis and structure</td>
<td>Dominant RP, MD</td>
</tr>
<tr>
<td>RHO</td>
<td>PR (OS)</td>
<td>Light receptor for visual cycle, disk membrane targeting, morphogenesis and structure</td>
<td>Dominant or recessive RP</td>
</tr>
<tr>
<td>RIMS1</td>
<td>PR (OS)</td>
<td>Vesicle exocytosis, PR synapse</td>
<td>Cone-rod dystrophy</td>
</tr>
<tr>
<td>RP1</td>
<td>PR (OS)</td>
<td>Photoreceptor differentiation, disk membrane stacking</td>
<td>Dominant RP</td>
</tr>
<tr>
<td>RPE65</td>
<td>RPE</td>
<td>Visual cycle</td>
<td>Recessive RP, LCA</td>
</tr>
<tr>
<td>βPDE</td>
<td>PR (OS)</td>
<td>Effector molecules of visual cycle</td>
<td>RP, congenital night blindness</td>
</tr>
</tbody>
</table>

**TABLE 2 – Retinal disease gene mutations.** RP-Retinitis Pigmentosa; LCA-Leber’s Congenital Amaurosis; MD- Macular Degeneration; OS-Outer segment; IS-Inner segment; N-Nucleus.
MATERIALS AND METHODS

HELPER-DEPENDENT ADENOVIRAL VECTOR

Vector constructs

Three viral constructs (figure 8): HD-Ad-CMV-GFP, HD-Ad-CMV-LacZ, and HD-Ad-MOPS GFP were used in this study. The HD-Ad-CMV-GFP vector contains a green fluorescent protein (GFP) under the control of the cytomegalovirus immediate-early promoter (CMV). The HD-Ad-CMV-LacZ vector comprises of the E coli β-galactosidase (LacZ) cDNA under the control of the CMV promoter. The HD-Ad-MOPS-GFP vector contains a GFP under the control of a 500 b.p. murine opsin promoter (MOPS).

HD-Ad-MOPS-GFP was made by a previous lab member Richard Xiang from an AAV2 plasmid given to us by Dr. Robert Molday’s lab. The HD-Ad-CMV-LacZ and HD-Ad-CMV-GFP vectors were made in our lab by Cathleen Duan. Constructs (promoter and reporter gene) were cloned into bacterial plasmid (pBSIISK) backbones and placed into HD-Ad vectors (pC4HSU) devoid of all viral coding sequences except for the ITR and ψ (these minimal cis-acting elements are necessary for DNA replication and encapsidation). Since a genome size of 27 kb to 38 kb is required for the efficient packaging into the Ad capsid, stuffer DNA, usually that of non-coding mammalian DNA with minimal repeating sequences, is included in the viral backbone to increase stability [212, 213].
**HD-Ad vector preparation**

For the production of the HD-Ads vectors, producer cell line 116 and helper virus AdNG163R-2 were used. 116 producer cells were generated from 293N3S cells by transfection of pNG159 which contain Cre and the hygromycin resistance gene. Cells were grown in Dulbecco’s Modification of Eagle’s Medium (DMEM) with 10% Fetal bovine serum (FBS) (Sigma), 1% antibiotics, and 100ug/ml hygromycin. Cells were kept in an incubator set at 37°C and passed at 90% confluency. The helper virus contains a E1 deleted Ad genome with a ψ flanked with loxP sites. These sites are the target sequences for Cre, and thus after infection of cells expressing cre recombinase, the packaging signal is excised from the helper viral genome by Cre-mediated site-specific recombination between the loxP sites. The helper virus is now rendered unpackagable but is still able to undergo DNA replication and so can *trans* complement the replication and encapsidation of the Hd-Ad genome.

6 cm plates of 116 cells were transfected with the PmeI digested linearized viral genomes vectors when the confluency of cells were at 90%. Helper virus was then added at a multiplicity of infection (MOI) of 5 pfu/cell. Viral mixture was incubated in 37°C for 3 days and then frozen at -80°C until virus was ready to be harvested for the next passage. Serial co-infections of 116 cells at 90% confluency with 20% of the crude lysate from previous passages were carried out for the next 2 passages with an MOI of 3 pfu/cell and 1 pfu/cell of helper virus for P1 and P2 respectively. For the third passage, P3, a larger 15 cm dish of 116 cells at 80% confluency was co-infected with 30% of the crude lysate from P2 and 1 pfu/cell of helper virus. Viral mixture was incubated at 37°C for 3 days and frozen at -80°C.
The large scale production of the HD-Ad starts with 116 cells cultured in a spinner flask with 1L of growth media (Jolkik’s MEM with 10% FBS, 1% PSF, and 100ug/ml hygromycin). Virus was gently agitated in a magnetic stir plate spinning at 60-75 rpm at 37°C overnight. Over the next 3 days, an additional 2 L of growth media was added to the flask. The following day, cells were harvested and re-suspended in 5% volume of conditioned media and co-infected with the entire P3 lysate and helper virus at an MOI of 1 pfu/cell. Virus was gently agitated in a magnetic stir plate (60-75 rpm) at 37°C for 2 hours consisting of 25% conditioned media and 75% fresh viral media and cells were harvested after 72 hours and stored in 10 mM Tris buffer (pH 8.0) until viral purification.

Viral purification requires that cells be lysed with 5% sodium deoxycholate and 10 µl of benzonase to be incubated at room temperature for 1 hour. Cells were then spun at 5500 rpm for 15 minutes at 4°C and purified by CsCl gradient. The HD-Ad viral band was collected and dialyzed at 4°C for 24 hours, against 500ml buffer of 10mM Tris-HCl, pH 8.0. The buffer was changed three times at 8 hour intervals, the volume of dialyzate measured and glycerol added to a final concentration of 10%. The final virus concentration was 8.5x10⁹ vp/ml by OD measurement and the viral vector was stored at -80°C until further use.

ANIMAL CARE AND SUBRETINAL INJECTIONS

One month old female CD-1 mice (Charles River) were housed in cages with 12 hour light/dark cycles. Mice were treated humanely in strict compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Animals were anaesthetized via intraperitoneal injections of a mixture of Ketamine (100mg/ml), Rompun
(20mg/ml), and Saline. Pupils were dilated with a topical application of a mixture of 1% cyclogyl, 25% mydfrin, and 0.5% tropicamide in ddH₂O for 30 seconds.

Under a dissection microscope, a small incision was made through the cornea, adjacent to the limbus with a 30½-gauge needle. A blunt needle fitted to a 5 µl Hamilton syringe was then inserted through the incision, with special care to avoid the lens, and was pushed through the retina to the subretinal space (potential area between the RPE and the PR cells) where the virus was injected at a rate of 1 µl/min. Each animal received 1 µl of virus in the right eye, leaving the left eye as a negative control. Animals were sacrificed by cervical dislocation at 1 week, 2 weeks, 1 month, 2 months, and 3 months post injection and eyes were enucleated for further tissue processing.

TISSUE PROCESSING

GFP detection

Eyeballs were harvested and a small cornea puncture was made with a 30½-gauge needle. Eyes were fixed with 4% paraformaldehyde in 0.1 M Phosphate buffer for 6-8 hours at 4°C then submerged in 30% sucrose overnight at 4°C for cryopreservation. Eyes were embedded with tissue-freezing medium (O.C.T. Kaltek tissue) in dry ice and stored at -80°C for further use. 30 serial sections (18 µm thick) and 30 serial sections (6 µm thick) were cut and distributed on slides to represent the whole eye at different levels. Sections were mounted with mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vectashield-Vector Laboratories) and stored at -20°C. Sections were observed directly with
confocal microscopy or processed for immunohistochemistry. Images from confocal microscopy were processed with Velocity 5.

**X-gal staining of whole eyeball**

Eyes were enucleated and fixed in fixative solution (1% formaldehyde, 0.1% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA in 0.1 M sodium phosphate buffer, pH 7.8) for 30 minutes at 4°C rocking. Fixed tissues were washed with wash buffer (2 mM MgCl₂, 0.01% Deoxycholate, 0.02% NP-40 in 0.1 M sodium phosphate buffer) at 4°C rocking and stained with X-gal solution (5 mM K₄Fe(CN)₆.3H₂O, 5 mM K₃Fe(CN)₆ in wash solution with 40mg/ml of dimethyl formaide) at 37°C shaking for 3 hours. After staining, samples were washed 3 times with 70% alcohol and post-fixed with 10% formaldehyde at 4°C for 4 hours. Samples were then sent to the Pathology department of The Hospital for Sick Children where they were embedded in paraffin blocks. Upon receiving the paraffin blocks back, 60 serial sections (6 μm thick), cut at the horizontal meridian, were distributed on 10 slides representative of the whole eye at different levels to be counterstained with H&E or neutral red.

**H&E staining**

Briefly, tissues were deparaffinised and rehydrated in a series of alcohol rehydration steps with xylene (3 min x 3), 100% EtOH (3 mins x 3), 95% EtOH (3 mins), 70% EtOH (3 mins), 50% EtOH (3 mins) and running cold tap water to rinse. Oxidized particles were removed on the slide with a Kimwipe before moving on. Slides were stained with hematoxalin (Poly Scientific) for 3 minutes and rinse with deionized water. Tissues were dipped briefly in acid ethanol to de-stain and rinse with deionized water. Excess water was
blotted from the slide before staining tissue with eosin (Poly Scientific) for 1 minute.

Tissues were dehydrated in a series of alcohol dehydration steps starting with 95% EtOH (5 mins x 3), 100% EtOH (5 mins x 3), and xylene (15 mins x 3). Tissues were mounted with xylene-based mounting media, Permount (Fisher Scientific) and covered with a coverslip.

**Neutral red staining**

Briefly, tissues were deparaffinised and rehydrated in a series of alcohol rehydration steps with xylene (3 min x 3), 100% EtOH (3 mins x 3), 95% EtOH (3 mins), 70% EtOH (3 mins), 50% EtOH (3 mins) and running cold tap water to rinse. Oxidized particles were removed on the slide with a Kimwipe before moving on. Slides were stained with neutral red staining solution (0.1% Neutral red in ddH2O with acetate buffer, filtered) for 2 minutes and rinsed in running tap water until dye has been removed from slides. Tissues were dehydrated in a series of alcohol dehydration steps by dipping briefly in 70% EtOH, 95% EtOH (2 mins), 100% EtOH (2 mins x 2), and xylene (5 mins x 2). Tissues were mounted with xylene-based mounting media, Permount (Fisher Scientific) and covered with a coverslip.

**β-Galactosidase reporter assay**

Eyes were enucleated and the lens and vitreous was removed under a dissection microscope, leaving only the eyecup. Tissue was homogenized in lysis buffer (potassium phosphate buffer [pH 8.0] with 0.5mM DTT, 10% Triton X-100, and proteinase inhibitor). Samples were centrifuged for 15 minutes at 12,000rpm in 4ºC. Supernatant was collected and either processed immediately or stored at -80ºC. Lysate was heat inactivated at 48ºC for 50 minutes and allowed to cool to room temperature. Incubate 20 μl of tissue sample in 50
µl of β-Galactosidase reaction buffer (1:100 Dilute Galacton Substrate with Reaction Buffer Dilutent) for one hour at room temperature in 96 well plates. Luminescence was measured by a luminometer (EG+G Berthold, Microplate Luminometer LB 96V MicroLumatPlus)

**IMMUNOHISTOCHEMISTRY**

**Antibodies**

The following antibodies (dilutions) were used for immunohistochemical analysis after dilution in blocking buffer: primary rabbit polyclonal Anti-GFP antibody (1:1000) from ABCam (category# Ab290); secondary Anti-rabbit antibody conjugated to Alexa 594 fluorophore (1:200) from Invitrogen (category# A-10239); primary rabbit polyclonal Anti-rhodopsin antibody (1:300) from Imgenex (category# img-71474); secondary Anti-rabbit IgG conjugated to Cy3 antibody (1:500) from Invitrogen (category# A10520).

**Immunostaining**

Six µm tissue sample slides (cut from frozen sections) were air dried in room temperature and immersed in ice-cold acetone for 10 minutes. Boundaries were drawn around tissues with ImmEdge Hydrophobic Barrier Pen (Vector Laboratories). Samples were then place in a humidifier chamber protected from light for the rest of the immunostaining to avoid drying out of the tissue and photobleaching of the fluorophore. For GFP detection, samples were rehydrated with 1x PBS and blocked with 10% goat serum for 30 minutes. Samples are then rinsed in PBS and incubated with the primary GFP antibody for 30 minutes. After a brief washing in PBS, the secondary antibody conjugated to Alexa
594 was incubated on the slides for 30 minutes. Slides are then rinsed in PBS and samples were mounted with mounting medium with DAPI (Vectashield-Vector Laboratories).

For rhodopsin detection, sections were rehydrated with PBS and blocked with 10% rabbit serum for 1 hour. Then samples were incubated with anti-rhodopsin for 1 hour, rinsed with PBS and incubated with the secondary antibody conjugated to Cy3 for 1 hour. Slides were then briefly rinsed in PBS and samples were mounted with mounting medium with DAPI. Slides were observed by a confocal microscope immediately or stored in -20°C.

**MICROSCOPY**

Three types of microscopy were performed in the analysis of retinal tissues. Confocal images were taken from a ZEISS Axiovert 100M microscope and processed with Velocity 5. Light microscope images were taken on a LEICA DM IRB microscope and fluorescent images were obtained from the same light microscope, but with the addition of the arc lamp (mbq52ac) and a green filter.

**STATISTICS**

All statistical analysis were done in Graphpad Prism 4.03.
Figure 8 – Schematic diagram of HD-Ad vector constructs. 3 viral constructs (promoter and reporter gene), MOPS-GFP, CMV-GFP, and CMV-LacZ were each cloned into a bacterial bluescript II SK phagemid (pBSIISK) vector, then linearly digested with BshII and cloned into an HD-Ad vector (pC4HSU) digested at the AscI site.
RESULTS

AIM 1 - To examine the general expression of CMV-driven reporter genes

Detection of the presence transgene expression

Mice were given subretinal injections with 3 different viral vectors: CMV LacZ, CMV GFP, and MOPS GFP. At 4 different time points, detection of transgene was determined via X-gal staining of the CMV LacZ injected eyeballs and by fluorescence microscopy for the GFP- tagged vectors and marked as either present or absent. The percentage of mice that showed expression with the CMV LacZ vector (figure 9) was not statistically different at 1 week, 2 weeks, 1 month and 2 months (chi-square, P>0.05). Similarly, mice injected with the CMV GFP and MOPS GFP vector also demonstrated no difference in the 4 time points tested, with respect to the percentage of mice that showed transgene expression (chi-square, P>0.05). Additionally, we found no statistically significant difference between the 3 different viral vectors used and percentage of transgene expression (chi-square, P>0.05).

Histological detection of LacZ for 2 months

The HD-Ad vector has never been successfully shown to deliver a lacZ gene into the retinal layer. We have cloned the CMV-LacZ construct into a Hd-Ad vector and subretinally delivered 1 μl of the virus at 1x10^8 vp/μl. Mice were sacrificed at 4 time points: 1 week, 2 weeks, 1 month, and 2 months. Whole eyeballs were X-gal stained and the serial sections of the
Figure 9 – Detection of transgene expression. Mice were injected with 3 different viral vectors: CMV LacZ, CMV GFP, and MOPS GFP and transgene expression was recorded as either being detectable or undetectable over 4 different time points. Crosstabulations comparing time points within each vector and comparing vectors within each time point did not reveal a statistically significant difference (chi-square, P>0.05). (n=48-68 per vector construct).
eyes were counterstained with neutral red to reveal the layers of the retina. We saw that expression of the β-galactosidase is prominent starting at 1 week post injection with consistent staining along the majority of the retina (figure 10A). The X-gal positive areas were seen in the RPE predominantly, with sporadic expression found in the proximal regions of the OS. At 2 weeks (figure 10B) and 1 month (figure 10C), similar results were observed: X-gal staining was present all along the retinal layer with expression predominantly in the RPE layer and occasional expression in the OS of the PR cells.

Likewise at 2 months (figure 10D), the furthest time point tested, trends in expression were comparable to the other time points. X-gal staining was present along the retinal layer with the majority of expression in the RPE layer and little expression in the OS of the PR cells. As well, there seemed to be no marked change in the level of expression or distribution pattern seen along the retina from earlier time points. In general, starting at 1 week and lasting to 2 months, strong expression is seen along the retina with no perceptible changes at any time. Control eyes (figure 10E), those not injected with a viral vector, were processed under the same X-gal staining conditions but no expression was recorded.

At all time points, no sign of tissue damage or cell death was apparent as the morphology of the injected mice resembled that of the negative control. Nuclear layers in all groups seemed intact with straight, densely packed orientation of 9 to 10 rows of nuclei bodies indicating no retinal degeneration. As well, no retinal haemorrhage or retinal detachment was observed in any of the treated mice starting at 1 week.
Figure 10 – Histological detection of LacZ expression at 2 months post-delivery. Eyes were processed for X-gal staining and cut at serial sections of 6 μm and counterstained with neutral red. Expression was detected at 1 week (A), 2 weeks (B), 1 month (C), and 2 months (D) in the RPE layer of the retina. Some expression was also detected in the proximal OS. Control eyeball (E) with no vector injection reveals no β-galactosidase activity. Original magnification 40x. RPE retinal pigment epithelium; OS outer segments; ONL outer nuclear layer; INL inner nuclear layer.
H&E staining reveal no sign of inflammation

H&E is a popular staining procedure that give a general overview of whether or not an immune response was generated in most tissues. Mice were injected with the CMV LacZ vector at 3 different viral vector doses, $1 \times 10^7$ vp/µl, $1 \times 10^8$ vp/µl, $1 \times 10^9$ vp/µl, and sacrificed at 1 week and 2 weeks. Tissues were processed for X-gal staining, to verify that vector delivery was successful and transgene expression was present, and counterstained with H&E to detect for immune cell infiltration. Control eyes (figure 11A) were processed under the same conditions and revealed normal physiology of mouse retina.

At the lowest vector dose (figure 11B), X-gal staining revealed that transgene expression was present after 1 week, but H&E staining revealed no sign of inflammation as tissue morphology was comparable to that of the control eyes. The integrity of the ONL was preserved with no difference in the thickness of the ONL, indicating no retinal degeneration. As well, no folding or out pouching in the retinal layer were observed, a common characteristic of inflammation which occurs when granules creating invaginations in the ONL leads to folding of the layer.

Mice injected with higher vector doses, $1 \times 10^8$ vp/µl (figure 11C) and $1 \times 10^9$ vp/µl (figure 11D), also reveal no sign of inflammation. Likewise, tissue morphology and integrity seemed to be preserved with no tissue damage or necrosis. Results detected at 2 weeks were comparable to that of the 1 week mice. These results show that the HD-Ad vector, can be subretinally injected into mice, without noticeable signs of damage or danger to the eye.
Figure 11 – H&E staining reveals no sign of inflammation. Eyes were processed for X-gal staining at 3 different concentrations of viral vector dose at 1 week post injection and counterstained with H&E. Control mouse (A) with no injection revealed no β-galactosidase activity and normal physiology. CMV LacZ injection at 1x10^7 vp/μl (B), 1x10^8 vp/μl (C), and 1x10^9 vp/μl (D) all show signs of transgene expression in the RPE layer, without visible signs of inflammation or cell toxicity. Original magnification 40x. RPE retinal pigment epithelium; OS outer segments; ONL outer nuclear layer; INL inner nuclear layer.
Detection of GFP 3 months post injection

A GFP reporter gene driven by a CMV promoter was cloned into the HD-Ad vector backbone and 1μl of it was delivered to the subretinal space of mice at a concentration of 5x10⁹ vp/μl. Serial sections were cut all along the eyeball and tissue was counterstained with DAPI to reveal the nuclear layers of the retina. Four different time points were looked at ranging from 1 week to 3 months. Direct GFP detection on the green channel was observed on a confocal microscope.

Observation at 1 week (figure 12D) revealed that GFP was present and bright. Expression was located around the site of injection and was not dispersed throughout the entire retinal layer, covering about 25% of the retina. DAPI staining shows that the GFP expression is localized in the RPE layer. Tissue morphology demonstrated no sign of damaged ONL and nuclei were arranged in a manner comparable to the control eye (figure 12I). At 2 weeks (figure 12E) and 1 month (figure 12F), GFP was still strong and expression was comparable with those of the mice at 1 week. As well, no sign of tissue damage was observed and PR cells seem to be healthy. At 3 months (figure 12G), the last time point tested, expression was still present, however the intensity of the GFP signal seemed to diminish slightly. As well, inconsistent expression along the retina was observed with some adjacent RPE cells lacking transgene expression.

To verify that the GFP observed in the tissue samples above were indeed true GFP and not autofluorescence or artifact, control staining with GFP antibodies were performed. Green channel reveals native GFP expression (figure 12A) and red channel reveals the GFP antibody staining (figure 12B). When the two channels are merged (figure 12C), native GFP
Figure 12 – GFP expression in mice at 3 months post-delivery. Frozen serial sections of 18 μm were counterstained with DAPI. Native GFP was detected in the green channel (A) and GFP antibody was detected on the red channel (B). Merged together (C), GFP and GFP antibody directly overlapped one another. CMV GFP at 5x10⁹ vp/μl were injected into mice eyes and GFP fluorescence was detected strongly at 1 week (D), 2 weeks (E) and 1 month (F) post injection. Detection at 3 months (G) revealed transgene expression was still present but slightly inconsistent along adjacent RPE cells around the retina. Expression in all cases seemed to be localized to the RPE layer of the retina with little or no expression in PR cells. Control eyes (I) had no sign of GFP or GFP antibody expression (H). Original magnification 40x. RPE retinal pigment epithelium; OS outer segments; ONL outer nuclear layer; INL inner nuclear layer.
overlapped the areas where the GFP antibodies are found, confirming that the native GFP detected is in fact true GFP expression and not an artifact. Control mice (figure 12I) had no detection on the green or red channel at any time points using the same microscope settings.

AIM 2 - To determine the dose response of viral vector mediated reporter gene expression

**Correlation between LacZ expression and viral vector concentration**

To determine whether a dose dependent relationship exists between viral vector dose and transgene expression levels, we injected mice with 4 different viral vector concentrations of CMV LacZ and detected for β-galactosidase expression by both histological (X-gal staining) and quantitative (protein activity assay) means.

At the lowest vector dose, 1x10^6 vp/µl, the eyeball (figure 13B) show negligible LacZ activity and was comparable to the negative control (figure 13A). Upon cutting serial sections (figure 13G), only a few slides showed positive staining for transgene activity in limited cells.

At a higher viral particle number, 1x10^7 vp/µl, transgene expression was detected (figure 13C), but consistency of expression around the entire retina was not observed as only several areas around the retina showed X-gal staining (figure 13H). Serial sectioning of tissues at this dose revealed that expression was present, but many cells had not been transduced (figure 14A) and expression was absent from a large proportion of the retina. Also, expression of the transgene was confined to the RPE layer of the retina (figure 14D) and absent from PR cells.
Figure 13 – X-gal staining reveals a correlation in transgene expression with viral vector concentration. Mouse eyes were injected with 1μl of a CMV LacZ vector and enucleated at 2 weeks post injection and processed for X-gal staining (A-E). Serial sections were cut at 6 μm and tissues were counterstained with neutral red to reveal retinal layers (F-J). Control eye with no vector injection (A, F) reveal no sign of β-galactosidase activity. Mice injected at 1x10⁶ vp/μl (B, G) showed minimum X-gal staining. Eyes injected at 1x10⁷ vp/μl (C, H) showed transgene expression to be present, but sporadic and inconsistent around the retina. At higher doses of 1x10⁸ vp/μl (D, I) and 1x10⁹ vp/μl (E, J), expression was detected to cover the entire retinal layer.
Figure 14 – Correlation between LacZ transgene expression and viral vector concentration. Mouse eyes were injected with 1μl of a CMV LacZ vector and enucleated at 2 weeks and processed for X-gal staining. Serial sections were cut at 6 μm and tissue counterstained with neutral red. Eyes injected at 1x10^7 vp/μl (A, D) showed β-galactosidase expression to be inconsistent and sporadic and confined to the RPE. At 1x10^8 vp/μl (B, E), expression was seen around the entire retinal layer and detected in the RPE predominantly. At 1x10^9 vp/μl (C, F), significant expression was detected around the entire retinal layer with staining in both the RPE layer as well as PR segments. Control eye (G) reveal normal retinal morphology and no sign of transgene expression. Original magnification (A-C) 20x. Original magnification (D-F) 100x. Original magnification (G) 40x.
At 1x10^8 vp/μl, significant LacZ expression was observed all around the eyeball (figure 13D). Reporter expression was not localized to area of viral injection, instead, widespread lacZ staining means that the viral particles were able to spread all across the retina and transduce all the cells (figure 13I). Serial sectioning of tissues in this group reveals that there were no areas of the retina in which we did not observe transgene expression (figure 13I and 14B). Expression was consistent in the RPE layer of the retina with some mice showing little expression in the OS of the PR cell (figure 14E).

At the highest vector concentration, 1x10^9 vp/μl, histological examinations of the whole eye (figure 13E) rendered fairly similar to the 1x10^8 vp/μl group, with constant and widespread expression along the entire retina (figure 13J). However, the expression of the LacZ seemed to be stronger in this group (figure 14C). Expression at this dose revealed robust staining in the RPE as well as in the OS and some IS of PRs (figure 14F), suggesting that perhaps at higher concentrations, viral vectors can target multiple vertical cell layers of the retina.

Control eyes with no injections were processed under the same conditions as the above mentioned groups and revealed no expression of β-galactosidase (figure 13A, 13F, 14G). Again, morphology of the retinas seems to be preserved in all groups of mice as compared to the control eye. No damage or inflammation was evident from the injection of the viral vector.
**Dose dependent transgene expression: quantitative analysis**

Next, we wanted to quantify the amount of transgene expression at various vector doses over the time span of 2 months. Mice eyes were injected with vector doses of $1 \times 10^6$ vp/μl, $1 \times 10^7$ vp/μl, $1 \times 10^8$ vp/μl and $1 \times 10^9$ vp/μl, processed for X-gal staining, and assayed for β-galactosidase activity. Transgene expression was determined by luminescence and reported in relative luminescence units (RLU).

At the lowest dose $1 \times 10^6$ vp/μl, no significant difference in transgene activity was detected and results were comparable to that of the control group mice (figure 15). With an increase in vector concentration in the remaining groups, a significant correlation of an increase in transgene expression levels seemed to exist. At $1 \times 10^7$ vp/μl, the level of transgene activity was measured to be 4 times that of the control group. A 6-fold increase in amount of transgene activity was detected in the $1 \times 10^8$ vp/μl group and a 7-fold increase in β-galactosidase activity was recorded for the highest group $1 \times 10^9$ vp/μl. Correspondingly, each vector dose group had a statistically significant difference in transgene activity from all subsequent vector dose groups (1-way ANOVA, P<0.05). This assay demonstrated a dose dependent relationship between vector concentration and amount of reporter gene expression.

Additionally, at each of the vector dose groups, β-galactosidase activity levels were also measured over different time points: 1 week, 2 weeks, 1 month, and 2 months (figure 15). At $1 \times 10^6$ vp/μl, mice were only tested up to 2 weeks post injection because there was no statistically significant difference with the control group detected at either time points nor was there evidence to suggest a difference would arise (1-way ANOVA, P>0.05). Mice
injected with $1 \times 10^7$ vp/μl had no statistical difference in the amount of transgene activity reported at any time points in that group. Similarly, mice injected with $1 \times 10^8$ vp/μl showed no significant difference in the amount of transgene activity at the four time points either (1-way ANOVA, P>0.05). Likewise, results in the $1 \times 10^9$ vp/μl group also showed no statistical difference in the level of β-galactosidase activity at any of the time points tested (1-way ANOVA, P>0.05). In general, no sign of reduced expression at any viral vector concentration was evident over the course of 2 months, the latest time point tested.

Overall, using a 2-way ANOVA, there was no significant association between time points and transgene activity levels. However, a statistically significant effect was found between the effect of dose and transgene activity levels (2-way ANOVA, dose P<0.05, time P>0.05, interaction P>0.05). With time eliminated as a factor, we examined the effect of vector concentration with all time points grouped together and with a 1-way ANOVA, we found there was a significant difference between the vector concentration groups (1-way ANOVA, P<0.05). Using Bonferroni corrected pair wise tests, we found a significant difference between all vector dose groups (P<0.05) except for the negative control group and the $1 \times 10^6$ vp/μl group, signifying that a change in transgene activity level is solely based on the vector dose having no correlation with time over the period tested.
Figure 15 – Quantitative analysis of transgene expression with vector concentration. Mouse eyes were injected with 1 μl of a CMV LacZ vector at increasing concentrations of 1x10$^6$ vp/μl, 1x10$^7$ vp/μl, 1x10$^8$ vp/μl and 1x10$^9$ vp/μl. Eyes were enucleated, processed for X-gal staining and measured for β-galactosidase activity levels. Transgene expression was determined by luminescence and reported in relative luminescence units (RLU). Using a two way ANOVA, no significant association between the 4 time points and their associated activity levels exists. However, activity levels were determined to be significantly increasing with the increase in viral vector dose suggesting that a dose dependent relationship exists with time being of no effect, atleast among the time points selected here (2-way ANOVA, dose P<0.05, time P>0.05, interaction P>0.05). Only 1x10$^6$ vp/μl activity levels were comparable to that of the control group (P>0.05). Error bars indicate standard error of the mean (SEM).
Correlation of GFP expression with viral vector concentration

To verify that the results obtained with the CMV LacZ vector are reproducible with another reporter gene, we injected mice with a CMV GFP vector at 3 different viral particle concentrations. Indeed, a dose dependent relationship was observed in terms of GFP fluorescence intensity and viral vector dose.

At lowest dose, $5 \times 10^8$ vp/μl, GFP expression was present but intensity of the fluorescence was not strong and a higher laser power was used in order to obtain a signal (figure 17B). Expression was inconsistent around the retina and GFP was localized to only a small area around the injection site (~5% of retina). DAPI staining of the nuclei layers reveals that only a few cells of the RPE layer seemed to exhibit positive expression.

At a 10-fold increase in viral particle number, $5 \times 10^9$ vp/μl, there was significantly more expression seen along the RPE with no absence of fluorescence between adjacent epithelial cells (figure 16C). GFP was more consistently detected around 30% of the retina and not restricted to the immediate injection site. Intensity of the GFP was also enhanced compared to the lower vector dose and required reduced laser power for adequate visualization. No expression in the ONL was detected. However, expression in the outer segments of the PR cells was present (figure 18E).

At the highest viral vector concentration, $1 \times 10^{10}$ vp/μl, significant expression was detected around 40% of the retina and spanned multiple vertical layers (figure 16D). The RPE was fully transduced as no adjacent cells were absent in fluorescence. Inner and outer segments of the PR cells also produced a strong GFP signal as well as some cell bodies of the ONL. Fluorescence in the INL were also detected, although it was not explored further.
Figure 16 – GFP expression increases with increasing viral vector dose. Mouse eyes were injected with 1μl of a CMV GFP vector at increasing concentrations of 5x10⁸ vp/μl, 5x10⁹ vp/μl and 1x10¹⁰ vp/μl. Eyes were enucleated and serial sections of 18 μm were counterstained with DAPI. At 5x10⁸ vp/μl (B), very little GFP was detected. Expression was localized to few RPE cells and was inconsistent around the entire retina. At 5x10⁹ vp/μl (C), significant expression was detected in the RPE cells. The highest dose of 1x10¹⁰ vp/μl (D), revealed extensive GFP expression spanning multiple vertical layers of the retina including the RPE, PRs, and some INL expression. No GFP was detected in control mice (A). Original magnification 40x. RPE retinal pigment epithelium; OS outer segments; ONL outer nuclear layer; INL inner nuclear layer.
which cell types were transduced (horizontal, amacrine or bipolar cells). Intensity of the fluorescence was strong and a low laser power was able to pick up significant expression. Tissue morphologies of all vector doses are comparable to that of the negative control (figure 16A) and no sign of retinal degeneration was observed. PR cell bodies were aligned in rows and columns similar to the control mice and no retinal folding was detected for the most part, indicating a lack of inflammatory responses and tissue damage.

**AIM 3 - To target PR cell expression driven by the mops promoter**

**Mops drives photoreceptor cell specific expression**

Rhodopsin is an OS specific protein and can be used as a marker to determine transgene expression location. GFP is detected in the green channel (figure 17A), and rhodopsin, as detected by antibody, is seen under the red channel (figure 17B). Areas of overlay represent expression of GFP in the OS of the PR cells. Control mice showed no evidence of GFP expression in the retinal layer but rhodopsin antibody staining clearly indicated the presence of the visual pigment in the OS of the PR cell (figure 17F).

GFP driven by a mops promoter was cloned into an HD-Ad backbone and mice were injected with 3 different doses. At the lowest dose, $8 \times 10^8$ vp/μl, GFP expression is present (figure 17D), although faint, and is located in areas where there is rhodopsin antibody staining, suggesting GFP expression is located specifically in photoreceptor OS. No expression was detected in the RPE layer of the retina. GFP was hard to detect and a high laser power was necessary in order to pick up some fluorescence. Expression was present only around the injection site (~5% of retina).
At a higher dose, \(4 \times 10^9\) vp/\(\mu\)l, GFP intensity increased and the majority of expression was confined to the outer segments of the PR cells (figure 17C). Some GFP is also detected below the area of rhodopsin antibody staining, suggesting that it is present in the IS of the PR cells as well. In some mice, both rhodopsin and GFP were found to be present on the apical side of the RPE cells, which can be explained by the phagocytic properties of the RPE.

At the highest vector dose, \(8 \times 10^9\) vp/\(\mu\)l, GFP signal was intense in ~ 25% of the retina. Again, the expression of the transgene seems to mostly be located in the OS of the PR cells (figure 17E). However, at the highest dose, more GFP expression was evident in the IS of the PR cells. No ONL layer showed expression.

At 3 different doses, expression is mostly seen to be confined to the areas where rhodopsin antibody staining was found. Similar to the CMV GFP results, the level of GFP intensity and presence seems to increase with increasing viral vector particle number, again suggesting that there is a dose-dependent relationship. No significant GFP is detected in the RPE layer as well as any other layers apart from the PR cells suggesting that with the MOPS promoter, expression is localized to the PR cells.

**CMV drives both RPE and PR expression**

It is generally accepted that the CMV promoter is highly active and should be expressed ubiquitously throughout the retina. However, no studies have shown the use of this promoter to drive GFP transgene expression in the HD-Ad vector. Here, we show that under the control of a CMV promoter, transgene expression can be detected in both the RPE and the PR cell layer. GFP is detected in the green channel (figure 18A), and rhodopsin antibody is seen under the red channel (figure 18B). Areas of overlay represent expression
Figure 17 – GFP is expressed exclusively in the PR cell layer under the control of a MOPS promoter. Mouse eyes were injected with 1μl of a MOPS GFP vector. Eyes were enucleated and serial sections of 18 μm were immunostained with rhodopsin antibody (B) and counterstained with DAPI. Native GFP detected after injection with $4 \times 10^9$ vp/μl (A) revealed that GFP expression was localized to areas where rhodopsin is expressed (C). Injections with $8 \times 10^8$ vp/μl (D) revealed GFP intensity was reduced but still confined to the OS. At the highest vector dose $8 \times 10^9$ vp/μl (E), GFP had greater intensity and was predominantly expressed in the OS, with some expression also found in the IS. Control eyes (F) revealed no GFP expression in or around the area of the rhodopsin antibody staining. Original magnification 40x. RPE retinal pigment epithelium; OS outer segments; IS inner segments; ONL outer nuclear layer; INL inner nuclear layer.
of GFP in the OS of the PR cells. Control immunostaining reveals that rhodopsin is present, but not GFP (figure 18F), even at the highest laser power.

Mice were injected with 3 different CMV GFP vector doses. At the lowest dose, 5x10^8 vp/μl, GFP expression was faint and inconsistent around the retina (figure 18B). Upon DAPI and rhodopsin antibody staining, GFP expression was detected in both the RPE as well as some OS of the PR cells (figure 18D). No GFP was expressed in the IS or the ONL at this vector dose.

At a medium dose, 5x10^9 vp/μl, GFP expression is more abundant and seemed to be significantly present in the RPE. Some GFP is also detected in the PR cells (figure 18E), but expression remains mainly localized to the OS. A few cells showed expression in the IS, and no PR cell body produced fluorescence.

At the highest dose, 1x10^10 vp/μl, significant GFP expression was detected in multiple cell types of the retina (figure 16D). GFP was seen in the RPE and throughout the PR cells (figure 18C). However, not all cells in the PR layer were transduced. Cell bodies of the INL also show GFP expression, but this observation was not further explored. This suggests that an increase in vector particles promotes multiple retinal layer transduction.
Figure 18 – GFP expression is detected in the RPE and PRs when under the control of the CMV promoter. Mouse eyes were injected with 1 μl of a CMV GFP vector. Eyes were enucleated and serial sections of 18 μm were stained with rhodopsin antibody (A) and counterstained with DAPI. Detected after injection with 1x10^10 vp/μl (B) and overlapped with rhodopsin antibody staining (C) reveals GFP expression was present in both the RPE as well as in PRs. Some expression was also detected in the INL. Injections with 5x10^8 vp/μl (D) revealed GFP intensity was greatly reduced and expression predominantly located in the RPE with sporadic expression in the PR cells. At 5x10^9 vp/μl (E), GFP expression was expressed along the RPE layer with some overlap in areas of rhodopsin, as well as a few cells expressing GFP in the IS. Control eyes (F) with no injection revealed no GFP expression anywhere in the retina. Original magnification 40x. RPE retinal pigment epithelium; OS outer segments; IS inner segments; ONL outer nuclear layer; INL inner nuclear layer.
DISCUSSION

Currently, the majority of retinal gene therapy trials are carried out by AAV vectors, and little is known about the effects of the HD-Ad vector as a vehicle for gene delivery. This vector however, has many features which render it suitable for ocular gene therapy trials and I have shown that it is possible to obtain transgene expression for up to 3 months post injection with minimal toxic effects. I have also demonstrated a correlation between the level of transgene expression and viral vector particle concentration.

Subretinal injection techniques affect transgene expression

Mice injected with three different viral vectors and sacrificed at 4 different time points all showed similar results in terms of having reporter gene expression or having no expression (figure 9). The slight variations in presence of transgene expression can be potentially explained by injection techniques. The delicate puncturing of the cornea should be carried out with no larger than a 30½-gauge needle so as to avoid too much breakdown of ocular integrity. Importantly, avoidance of blood vessel rupture is crucial for the nutrition and survival of retinal cells. Delivery of the virus into the subretinal layer needs to occur at an extremely slow rate, exceeding no more than 1 μl injected per minute at constant flow rate. Failure to follow this rate can induce irreversible retinal detachment and damage. As well, viral particles may escape into other areas of the eye, leading to unwanted gene expression or inflammation. Due to ocular pressures already present in the eye, there is a tendency for the vector suspension to exit the eye following the path of the Hamilton syringe. When pulling the needle out, it is very important to do so very slowly so as not pull out any
of the viral vector particles along with it by back pressure and capillary pressure. Sterility may also play a factor as contamination of any surgical instrument used may bring on a potent inflammatory event that promote vector clearance and prevents proper vector transduction. A steady hand is required for mastering subretinal injections and failure to follow precise instructions and timings may prevent proper, if any, gene expression. In general, for the results obtained in this study, the lack of expression in mice can be mainly attributed to problems in vector delivery as opposed to vector transduction.

**Transgene expression is detected up to 3 months post injection**

HD-Ad vectors have attributes that make them desirable in gene therapy trials where prolonged gene expression is sought after. Due to their genome being devoid of all viral coding genes, little or no CTL response arises, and vector can persist in host for a very long time where they stay in episomal form. Since retinal cells are terminally differentiated, dilutional loss of episome is unlikely, and an integrating vector is not necessary for long term gene expression. Furthermore, since the genome is non-integrating, unlike AAV or LV, there is no risk of insertional mutagenesis. As seen in figure 10, injection of the CMV LacZ viral vector results in detectable transgene expression in mice from week 1 to 2 months, with no sign of decreased expression. Injection with the CMV GFP vector resulted in expression being detected up to 3 months, with a slight decrease in transgene expression at that time, although the decrease was minimal (figure 12). Overall, I predict that reporter gene expression would continually persist had further time points been examined with no evidence suggesting otherwise.
**HD-Ad vectors have low immunogenicity**

The eye is immune privileged, rendering it an organ which can greatly benefit from gene therapy. However, studies have shown that inflammation can indeed persist in the ocular compartment to clear viral vectors and eliminate transgene expression [191-193]; such is the concern when using FG-Ad vectors upon vector delivery. Studies have shown that the inflammatory response brought about by the Ad vector can lead to vector clearance, tissue damage and transient transgene expression lasting for only 3 weeks post injection [114, 167, 171]. Consequently, low levels of IgG immunoglobulin will be left circulating the system which will enhance the immune response upon consequent infections [172]. However, because HD-Ad vectors are devoid of all viral coding genes, the immune response elicited on the host is attenuated as compared to first generation vectors [179-181].

My results show that upon injection, morphology of the retina remained normal with little or no sign of tissue damage or inflammation which may have arisen if a strong immune response persisted (figure 11). My data also demonstrate lengthy transgene expression lasting for at least 3 months post injection (figure 12). Hence, our HD-Ad vector seems to be superior in terms of not eliciting a strong immune response and vector clearance as compared to the first generation parental vector. This lack of immunogenicity will lead to prolonged transgene expression in the host, which is extremely valuable because most retinal diseases require lifelong genotypic correction. Additionally, although this was not tested in my study, the ability to re-administer the viral vectors with no harmful side effects or immediate vector clearance is feasible with the HD-Ad vector.
PR cell death can be detected by measuring the thickness of the ONL. Normal ONL possess 9 to 10 rows of neatly stacked PR nuclei. During an inflammatory response, granules creating invaginations in the ONL occurs and a disturbance in the nuclear layer organization manifests. Data from my results show no folding or out-pouching in the retina at any time points or at any viral vector dose in the mice that were injected. Also, the integrity of the ONL in the injected mice appeared to be comparable to those of control mice, suggesting that our vector does not elicit a strong immune response that leads to tissue damage or PR cell death.

Overall, my results reaffirm previous findings that the HD-Ad vector elicits minimum immunogenicity [120, 196]. However, thorough studies over a protracted period of time are required to evaluate the immunogenetic response of the eye after treatment with HD-Ad vectors. It is said that an immune response can initially appear hours after injection and can subside in a few days. Since our earliest evaluation was at 1 week, perhaps future studies will involve sacrificing mice at days 1, 2, 4 and 6 to detect for possible inflammation.

**Transgene expression controlled by viral vector concentration**

The potential for tissue damage and inflammation increases with higher viral load. Hence, it is beneficial to determine the lowest amount of vector required for maximum gene expression. Alternatively, if only limited amounts of transgene expression are required, it would be beneficial to determine how much expression can be achieved with a given viral vector dose. My results demonstrate that the concentrations of virus delivered in these experiments follow a simple dose response trend in expression levels, as determined by histological and quantitative assays.
Mice injected with CMV LacZ and processed for X-gal staining revealed that transgene expression was barely detectable with the lowest (1x10^6 vp/μl) viral particle concentration. However, at higher concentrations of 1x10^8 and 1x10^9 vp/μl, LacZ was consistently detected throughout the entire retina (figure 13 and 14). At a higher magnification, in all vector doses, expression was located in the RPE layer of the retina. However, at the highest dose, 1x10^9 vp/μl, reproducible transgene expression was also observed in the OS and some IS of the PR cell layer as well, suggesting that perhaps at higher vector concentrations, there is an increased ability for the vectors to target multiple retinal cell layers.

I used another reporter gene, GFP, under the direction of the CMV promoter to verify the results obtained with the LacZ construct. Indeed, a dose response correlation was again observed in terms of viral vector dose and output fluorescence and (figure 16). Using 3 concentrations of viral particles, I found that at the highest vector dose, 1x10^10 vp/μl, not only was there robust expression in the RPE, but also significant GFP in PR cells. Moreover, some expression was detected in the cells of the INL, although this was not explored further. At the lower vector doses, a proportional reduction in GFP presence was noted, signifying again that an increase in vector concentration leads to an increase in gene expression.

My β-galactosidase activity assays showed quantitatively that protein expression remained constant throughout the length of the study period. However, as suggested above, with an increase in vector dose, there was a significant difference in protein activity (figure 15). These results suggest that it is possible to control the level of therapeutic transgene expression; this is an important finding because some retinal diseases require robust transgene expression and others require a more controlled level of transgene expression. It is
important to regulate the amount of transgene activity as over-expression or under-expression of key proteins leads to deleterious effects. For example, a 5 fold expression increase of normal opsin in rod PR cells can lead to permanent retinal degeneration [205] and a 23% over-expression of rhodopsin can also lead to PR cell apoptosis [206]. In contrast, complete PR degeneration results after 3 months in a model of rhodopsin⁺ mice [207].

Another way to regulate the amount of transgene activity is under the guidance of cis-acting elements and promoters. AAV vectors (4.7 kb) are generally unable to carry these large regulatory sequences, but HD-Ad vectors with their large 38 kb cloning capacity can house whole genomic loci, multiple transgenes and native regulatory elements that promote desirable gene expression. LV and FG-Ad, both with a carrying capacity of 7 kb, are also hindered by a limited space because the cDNA alone of some ocular diseases require a significant amount of space. For example, the cDNAs of ABCA4, CEP290, and myosin VIIa measure to be 6.8 kb, 7.4 kb, and 6.6 kb respectively. Of all the vectors discussed here, only the HD-Ad vector has the carrying capacity to package these therapeutic cDNAs as well as beneficial native regulatory elements.

**Fast onset of transgene expression**

Since there were no difference in transgene activity at any of the time points tested, onset of maximum gene expression started no later than 1 week post injection (figure 15) a significant improvement over the AAV vector which requires 4 to 8 weeks to achieve strong transgene expression [106, 157]. The AAV lag phase results in a delayed onset of transgene expression which makes the HD-Ad vector superior if immediate transgene expression is desired. For example, acute damage by physical trauma to the eye resulting in fast retinal
cell deterioration will require a vector that quickly delivers survival factors to rescue rapidly
dying cells. The delivery of various neurotrophic factors, growth factors, and cytokines
protect neurons from cell death in these instances including BDNF, CNTF, neurotrophin-3
and -4, and bFGF [214-217]. Additionally, the damaging effects of constant light exposure
were also found to be dampened with the introduction of these survival factors into the rat
retina [218]. Therefore, in certain instances where immediate rescue of retinal cells is
necessary, AAV vectors are handicapped due to their slow onset, making the HD-Ad vector a
better choice.

**RPE and photoreceptor cell transduction**

Upon subretinal injection, the majority of transgene expression is localized in the
RPE (figure 12 and 14). There are several reasons as to why the PR layer is a more difficult
target compared to the RPE. First, reduced transduction efficiency can be explained by steric
hindrance caused by the tight packing of PR cells that can interfere with effective binding of
the large Ad fibre to the PR inner segments where the CAR receptors are located [116, 117].
Second, subretinal injection places the vector particles in direct contact with the OS, but viral
particles must travel and migrate through the densely packed OS to reach the IS. Therefore,
the sheer increase in particle number alone will increase the odds that the virus will contact a
host cell receptor. Indeed, my results show that with an increase in vector particle numbers,
there is an increase in the amount of PR cell expression (figure 14 and 17 and 18).

In contrast, the RPE alternatively, is a monolayer of cells with CAR receptors located
throughout its apical side [116]; here, they are in constant contact with the viral particles
upon subretinal injections. Uptake of virus in the RPE is therefore extremely efficient as
compared to PR cells. Due to its organization, the simplicity in vector entry to the RPE heavily favours higher transgene expression as compared to PR cells. Indeed, my results show that RPE expression is present at all vector concentrations, suggesting that transgene expression is limited primarily at the very first level of viral transduction and not gene expression.

Studies have shown that PR cell transduction is efficient in mice pups but rarely in their adult counterparts, suggesting that the tropism of viral vectors is dictated by the stage of retinal development [107, 118]. Complete retinal differentiation occurs by p21 and there is a purported gradual shift in tropism from PR cells to RPE cells after p10 [119]. In my study, I injected adult mice ranging from 1 month to 2 months of age, and results show that despite fully differentiated retinas, our vector can deliver genes into PR cells, albeit at a lower frequency than RPE cells (figure 17 and 19). Although some studies employ mice between p3 and p21, gene delivery into adult mice is important because the symptoms associated with retinal diseases in humans are not apparent until after birth when the retina is already fully differentiated. Although mice eyes may not parallel exact morphology and structure of human eyes, the mouse model of retinal development forms a good starting foundation on which to test delivery methods and therapeutic agents.

Daily phagocytosis of rod and cone OS may also play a role in the quantitative differences in transgene expression detected in the RPE. Since OS disk membranes undergo regular shedding, proteins originally localized in the OS may become engulfed into the RPE, resulting in the experimental detection of more transgene product in the epithelial layer. The primary location of these transgenes may well have been in the PR cells; this hypothesis requires detection of localization of transgene product at earlier time points than were used in
these studies. Figure 17 shows that rhodopsin, which is normally exclusive to the rod OS, was unexpectedly found to localize, albeit in small quantities, in some RPE cells. This is consistent with my hypothesis that the phagocytic properties of the RPE on the OS accounts for some of the localization results, e.g., the markedly strong GFP and LacZ detection in the epithelium.

**MOPS promoter drives PR cell specific expression**

Inherited retinal degenerative disease result from a mutation in any one of more than 120 different retinal genes [123], and many of these diseases may be amenable to direct gene transfer therapies. The ability for cell specific targeting is extremely valuable because PR cells are the predominant and often only cell type expressing the mutant proteins (table 2).

In previous studies, it has been shown that CMV promoters primarily drive expression in RPE as opposed to PR cells [107, 108, 120, 121]. Therefore, one objective of my study was to test the effects of using a PR specific promoter to direct cell specific expression. Recently, a number of studies have utilized the MOPS promoter to guide PR specific expression in AAV vectors [106, 122, 157]. However, little is known about how the transgene functions when driven by this promoter in a HD-Ad vector. I tested the hypothesis that the MOPS promoter would drive GFP expression specifically in PR cells, and I found that when delivered in the HD-Ad vector via subretinal injection, GFP fluorescence was localized in the OS (figure 17).

Consistent with our other GFP-tagged vector, I saw that the level of GFP intensity seemed to be correlated with increasing viral vector particle number under the direction of the mops promoter, again verifying a dose dependent relationship. The total number of PR
cells transduced is a key parameter in evaluating the potential of a regimen for correcting or ameliorating genetic defects. In retinal gene therapy, a substantial fraction of PR cells need to be targeted while keeping the injection volume to the least possible in order to minimize tissue damage. In my study, a single 1μl injection transduced approximately 25% of PR cells, which is enough to improve or delay retinal degeneration [219].

In general, when using the MOPS promoter, I found little to no significant GFP in the RPE. The low level of expression that was occasionally detected can be a secondary effect attributed possibly as the result of the RPE phagocytosing the OS of the PR cells [2, 9, 12]. My data also reveals that the MOPS GFP expression is localized almost exclusively in the photoreceptor OS (figure 17). However, it is still unclear why protein expression is absent in the rest of the PR. One possible explanation is the highly polarized state of the PR cell has a tendency to shift proteins to the distal end of the outer segments. Likewise, GFP and rhodopsin may have been carried to the OS via the same vesicle and hence, expression of GFP will only localize to areas where rhodopsin is found. Subcellular trafficking of many PR outer segment proteins is an area of great mystery and much is to be learned.

**CMV promoter drives expression in both RPE and PR cells**

Unlike previous studies using the CMV promoter in which transgene expression was predominantly expressed in RPE cells [107, 108, 120, 121], using our vector system I was able to transduce both RPE and PR cells. My results showed that transduction with the CMV LacZ vector, resulted in strong β-galactosidase expression in the RPE layer and as well as some expression seen in the OS of the PR cells (figure 15). Biologically, the majority of proteins made in the PR are transported to the OS after protein synthesis occurs [1].
Therefore, transgene expression should have been found, at least in small quantities, in the IS as well. Strangely, my results showed expression of the transgene was only detected in the proximal regions of the OS; it was virtually non-existent in the IS. A potential explanation for this finding is that this vector did not actually transduce PR cells, but instead the observed β-galactosidase staining was so strong in the RPE that expression in the OS was an artifact due from the intense staining in the RPE. A potential experiment that could address this question would be to detect the lacZ by in situ hybridization to probe for the location of the vector genome.

Unlike the LacZ results, I found significant GFP expression throughout the entire PR cell; both inner and outer segments (figure 16 and 18). At the lower vector doses, cell morphology, accessibility to CAR receptors, and phagocytosis of OS can again be applied to explain why the expression was limited to the RPE layer. However, at the highest vector dose, GFP was detected to be present in all segments of the PR cells. These results were obtained with a confocal microscope, so no bleeding effect can be attributed to PR cell expression. Therefore, our CMV GFP vector, at high concentration can transduce PR cells that previous studies have not been able to achieve. Currently, the GFP results have only been confirmed by confocal microscopy. Further investigation into quantifying the level of GFP expression will better determine a dose and effect relationship numerically and with statistical significance. To do so, we need to quantify the intensity of fluorescence as detected from the confocal microscope and standardize these values using the same microscope settings for each vector dose group.

A potential explanation as to why there is stronger PR transgene expression at the highest dose of CMV GFP (1x10^{10} vp/μl) vector compared to the highest dose of CMV LacZ
(1x10^9 vp/μl) vector, is the difference in vector concentrations. Perhaps, if mice were injected with a higher concentration of CMV LacZ, the sheer increase in the number of viral particles will increase the likelihood of the vector attaching to the confined IS region where the viral receptors are located [116, 117]. Future studies may involve standardizing vector concentrations between the 2 reporter constructs to examine PR transduction efficiencies. However, a problem arises when trying to quantify two different viral batches because at present there is no easy way to determine the amount of active virus in a given preparation. Therefore, each viral batch has minor differences in contaminating helper virus and active particles. Using this reasoning it is possible that the batch of the CMV GFP had a higher titer of active virus than the CMV LacZ preparation used in my study. This alone can account for the difference in expression levels between the CMV-GFP and CMV-LacZ findings.

Although many inherited retinal disease genes are expressed in the PR, there are still many diseases that would benefit from rescue under the direction of the CMV promoter. For instance, in a form of Usher syndrome, the MYO7A gene expressed both in the RPE and PRs, will greatly benefit from gene therapy trials utilizing a ubiquitous promoter [220]. Moreover, diseases with a predominant gene defect in the RPE, such as certain forms of LCA, will also benefit from CMV promoters because this promoter drives high-level transgene expression in the epithelial cells. Therefore, the ability of our CMV vectors to target both cell layers is highly valued.

**Conclusions**

An ideal vector for ocular gene therapy should be one that has the ability to evade biological barriers to reach only the desired cells, a natural tropism for retinal cells, be able to
circumvent the immune system from clearance of the vector as well as prevent an immune reaction that may damage ocular tissue, retain a relatively large carrying capacity for long transgene sequences, and maintain long term transgene expression. The results of this study demonstrate that our HD-Ad vector is a useful tool in retinal gene therapy trials when long term transgene expression, low immunogenicity, high transduction efficiency and PR specific targeting is sought after. Moreover, its large carrying capacity triumphs over any other viral vectors currently employed in retinal gene therapy trials.

The results and knowledge attained in my study will be expanded upon by cloning the ABCA4 gene driven by a human rhodopsin promoter into the HD-Ad vector with the hopes of protein detection in a knockout ABCA4\(^{-/-}\) mouse model. This potentially can further elucidate the therapeutic benefits of this vector to patients with Stargardt disease.
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