Published in final edited form as:

Exp Eye Res. 2015 December ; 141: 33–41. doi:10.1016/j.exer.2015.04.003.

Elevation of Intraocular Pressure in Rodents Using Viral Vectors Targeting The Trabecular Meshwork

Iok-Hou Pang^{1,2,3}, J. Cameron Millar^{1,3}, and Abbot F. Clark^{1,3}

¹North Texas Eye Research Institute, University of North Texas Health Science Center, Fort Worth, TX

²Department of Pharmaceutical Sciences, University of North Texas Health Science Center, Fort Worth, TX

³Department of Cell Biology & Immunology, University of North Texas Health Science Center, Fort Worth, TX

Abstract

Rodents are increasingly being used as glaucoma models to study ocular hypertension, optic neuropathy, and retinopathy. A number of different techniques are used to elevate intraocular pressure in rodent eyes by artificially obstructing the aqueous outflow pathway. Another successful technique to induce ocular hypertension is to transduce the trabecular meshwork of rodent eyes with viral vectors expressing glaucoma associated transgenes to provide more relevant models of glaucomatous damage to the trabecular meshwork. This technique has been used to validate newly discovered glaucoma pathogenesis pathways as well as to develop rodent models of primary open angle glaucoma. Ocular hypertension has successfully been induced by adenovirus 5 mediated delivery of mutant MYOC, bioactivated TGF β 2, SFRP1, DKK1, GREM1, and CD44. Advantages of this approach are: selective tropism for the trabecular meshwork, the ability to use numerous mouse strains, and the relatively rapid onset of IOP elevation. Disadvantages include mild-to-moderate ocular inflammation induced by the Ad5 vector and sometimes transient transgene expression. Current efforts are focused at discovering less immunogenic viral vectors that have tropism for the trabecular meshwork and drive sufficient transgene expression to induce ocular hypertension. This viral vector approach allows rapid proof of concept studies to study glaucomatous damage to the trabecular meshwork without the expensive and time-consuming generation of transgenic mouse lines.

Keywords

Animal models; Glaucoma; Viral vectors

Corresponding author: Abbot F. Clark, NTERI/CBH-441, U. North Texas Health Science Center, 3500 Camp Bowie Blvd., Fort Worth, TX 76107, Telephone: 817 735 2094, abe.clark@unthsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Modeling glaucoma in animals has been very challenging as not all animal models mimic all the clinical features associated with glaucoma in humans. One of the most important risk factors for the development and progression of glaucoma in man is elevated intraocular pressure (IOP) (AGIS, 2000; Gordon *et al.*, 2002). Rodents have been increasingly used as glaucoma models. A number of approaches have been used to elevate IOP in rodent models, including: laser-induced outflow pathway photocoagulation (Levkovitch-Verbin *et al.*, 2002; Aihara *et al.*, 2003a), episcleral vein injection of hypertonic saline to sclerose the aqueous outflow pathway (Morrison *et al.*, 1997; Walsh *et al.*, 2009), microbead injection into the anterior segment (Sappington *et al.*, 2010), injection of viscous materials into the anterior segment (Benozzi *et al.*, 2002), extraocular vein cauterization (Shareef *et al.*, 1995), glucocorticoid-induced ocular hypertension (Shinzato *et al.*, 2007; Whitlock *et al.*, 2010; Overby *et al.*, 2014; Zode *et al.*, 2014), among others. IOP elevation in the DBA/2J mouse is due to iris stroma atrophy and pigment dispersion into the anterior segment angle (Anderson *et al.*, 2002), which mimics some features of pigmentary glaucoma. This mouse strain has been extensively used to study the molecular mechanisms of glaucomatous optic neuropathy and retinopathy (Howell *et al.*, 2011) as well as to identify novel therapeutic targets (Anderson *et al.*, 2005; Howell *et al.*, 2011; Howell *et al.*, 2014). In addition, transgenic mouse models have been created that develop elevated IOP including the *Col1a1^{r/r}* mouse (Aihara *et al.*, 2003b), *Tg.hMYOC.Y437H* mouse (Zode *et al.*, 2011), and *Tg.CTGF* (Junglas *et al.*, 2012). However, developing relevant transgenic models of glaucoma takes considerable time and resources.

Another approach to elevate IOP in mice is to use viral transduction of the trabecular meshwork (TM) to overexpress glaucoma associated genes. Several different viral vectors have been used to transduce the TM including: Adenovirus serotypes 5 (Ad5) (Hoffman *et al.*, 1997; Millar *et al.*, 2008), 28 or 35 (Ueyama *et al.*, 2014), self-complementary Adeno-Associated Virus (scAAV) (Buie *et al.*, 2010), Herpes Simplex Virus (HSV) (Spencer *et al.*, 2000), HIV pseudotyped with VEEV-G or VSV-G (Lipinski *et al.*, 2014), and Equine infectious anemia virus (EIAV) pseudotyped with VSV-G (Balaggan *et al.*, 2006).

Ad5 vectors have been used in mice to validate glaucoma associated pathogenic pathways, including: human myocilin mutations (Shepard *et al.*, 2007), secreted frizzled-related protein-1 (sFRP-1) (Wang *et al.*, 2008), transforming growth factor (TGF) β 2 (Shepard *et al.*, 2010; McDowell *et al.*, 2013; Swaminathan *et al.*, 2014), CD44 (Giovingo *et al.*, 2013), and Gremlin (McDowell, submitted for publication). Prolonged transgene expression can lead to optic neuropathy in some mouse strains (McDowell *et al.*, 2012). Aqueous humor dynamics can be measured *in vivo* in the mouse eye (Millar *et al.*, 2011), allowing direct evaluation of transgene expression on the aqueous humor outflow pathway. For example, elevated IOP caused by TGF β 2-induced transgene expression is associated with decreased aqueous humor outflow facility (Shepard *et al.*, 2010). Viral transduction of the TM could also be used to conditionally knockdown the expression of specific genes. This can be done using virus vectors expressing shRNA to knockdown expression of the targeted gene.

However, there are challenges associated with the use of viral transduction of the TM to elevate IOP. Oftentimes, the transgene expression is transient. Ocular inflammation often accompanies injection of viral vectors into the eye (Shepard *et al.*, 2010). Partial suppression of the immune response using antibodies to CD40L can prolong transgene expression and suppress ocular inflammation after Ad5 vector injection (Millar *et al.*, 2008). In addition, mouse strain and age appears to effect long term transgene expression and prolonged IOP elevation (McDowell *et al.*, 2012).

2. Methods

2.1. Viral Vectors

Viruses possess specialized molecular machinery for efficient transduction of their genomes to cells they infect. By replacing viral genes needed for viral replication with foreign (cargo) genes of interest, the resultant recombinant viral vectors can be used to transduce cargo genes, which can then be expressed by the target cell. Goff and Berg (Goff and Berg, 1976) were the first to describe such a process utilizing modified SV40 virus. Since then viruses of several phylogenetic types have been used for the generation of viral vectors. Each type, with their advantages and disadvantages are discussed below. A summary is presented in Table 1.

2.1.1. Herpes Simplex Virus (HSV)—HSV is a large enveloped dsDNA virus, 100 to 150 nm in diameter, Baltimore Classification Scheme (BCS) Group 1. With a capacity of ~150 kb, it is able to accommodate large genes (up to 74–84 kb). HSV is human neurotropic, and HSV vectors have been used to transfect mainly neurons including retinal ganglion cells (RGCs), but also other tissue types including TM and ciliary epithelia (Liu *et al.*, 2005; Wang *et al.*, 2005) in monkey and rat eyes. HSV vectors are helper-independent and can be prepared in titers of 10^9 to 10^{10} transducing units (TU)/mL.

Another HSV vector variant is the amplicon vector, a replication-defective and helper-dependent HSV-1 based vector. Amplicon vectors are plasmids that only require an HSV origin of replication and packaging sequence. They also incorporate a large cargo transgene capacity (> 100 kb), as well as a broad range of cell tropism, and can infect both dividing and non-dividing cells (Epstein, 2005). HSV-1 amplicon vectors have facilitated efficient but transient reporter gene transfer in retinal pigment epithelial (RPE) cells in rats following sub-retinal injection (Fraefel *et al.*, 2005).

However, the use of HSV and amplicon vectors for gene transduction has certain disadvantages, including: cytotoxicity, limited selection of suitable promoters, limited duration of gene expression, and a significant inflammatory response.

2.1.2. Adenovirus (Ad)—Ad is a non-enveloped icosahedral virus, 80 to 100 nm in diameter, incorporating a linear dsDNA genome, classified as BCS Group 1. It has a cargo capacity of 26 to 45 kb. There are 57 known serotypes of Ad. First generation recombinant Ad vectors with E1 and/or E1, E3 gene deletions (genes that assist in evasion of the immune response) have been investigated extensively, especially because they were one of the earliest recombinant DNA viral vector systems developed. They can be prepared to very

high titers, can infect a variety of dividing and post-mitotic or terminally differentiated cells, and lead to expression of high levels of transgene product. A variety of TM cells have been efficiently transduced using recombinant Ad in human perfused anterior segments, as well as living monkey, dog, rat, and mouse eyes. Ad vectors also efficiently transduce iris epithelium and corneal endothelium (Budenz *et al.*, 1995), as well as Muller cells and the RPE. But mature photoreceptors and RGCs have not been successfully transduced using these vectors.

E1, E3-deleted Ad vectors have been used extensively in animal models of experimental glaucoma, but complications have been reported, including immunogenicity which can lead to uveitis and/or corneal complications, and cataract (Shepard *et al.*, 2010). High neutralizing antibody titers limit repeat applications (Yang *et al.*, 1994; Ginsberg, 1996). Gene expression is often transient.

Ad vector developments known variously as: gutless or gutted Ad vectors, high capacity Ad vectors, helper dependent Ad vectors, mini Ad vectors, or encapsidated adenovirus minichromosomes promote less immunogenicity. They have a cargo-carrying capacity of up to 36 kb (Alba *et al.*, 2005). Gutless Ad vectors have been used to successfully transduce liver, muscle, and CNS neurons, as well as retinal photoreceptors and TM cells in mice. Because gutless Ad vectors are devoid of all viral coding genes, they require viral proteins to be supplied *in trans* by a helper Ad. Subsequent removal of the contaminating helper Ad from the final preparation is required. This is usually achieved by excision of the helper packaging signal, most often using the Cre-loxP system. However contamination levels with helper Ad remain at 0.1 to 1%, which, although acceptable for basic research paradigms, render them as yet unsuitable for clinical trials. But better purification techniques are beginning to appear (Alba *et al.*, 2005).

2.1.3. Adeno-Associated Virus (AAV)—AAV is the physically smallest (20 nm diameter) of all known primate viruses. It is a non-enveloped BCS Class II virus, possesses a linear ssDNA genome, and is derived from a replication-deficient, non-pathogenic parvovirus. AAV vectors are capable of transducing both dividing and non-dividing cells, a feature of interest to those seeking to transfect non-dividing TM cells, but they have a very limited cargo gene capacity (4.8 kb), thus they cannot deliver large genes in standard form. Many AAV vectors have no host cell genome integrative capacity due to deletion of packaging (*rep*) and structural (*cap*) genes. Wild-type AAV is a non-pathogenic virus that requires Ad or another helper virus for replication. Recombinant AAV vectors do not possess viral open reading frames and are thus only very mildly immunogenic, although a humoral response can result from exposure to the AAV capsid (Bennett *et al.*, 2000; Acland *et al.*, 2001; Acland *et al.*, 2005). The first AAV recombinants generated contained AAV serotype 2 capsids and genomic sequences (encoding inverted terminal repeats) and are thus termed AAV2. This vector is tropic for skeletal muscle, neurons, vascular smooth muscle, and hepatocytes, and has been used to transduce photoreceptors and RPE in various animal models (Bennett *et al.*, 1997; Hauswirth *et al.*, 2000; Peel and Klein, 2000; Zeng *et al.*, 2004; Min *et al.*, 2005). AAV2 has also been used to transduce RGCs following intravitreal injection (Dudus *et al.*, 1999). However, AAV2 does not transduce the TM, likely because of failure of TM cells to complete second strand synthesis (Borras *et al.*, 2006). Self-

complementary AAV (scAAV) may offer a solution. scAAV packages two shorter complementary strands of DNA, thereby negating the need for second strand synthesis. But scAAV has only half the already very limited cloning capacity of standard AAV. The increasing availability of hybrid and novel engineered serotypes of AAVs with altered transduction properties (Gao *et al.*, 2002) will perhaps improve the potential for using AAV vectors for TM cell transduction, and possibly other cell types of the ocular anterior segment. Examples (and their respective tropisms) include: AAV1 and AAV5 (vascular endothelial cells), AAV6 (airway epithelia cells), AAV7 (murine skeletal muscle cells), and AAV8 (hepatocytes). But previous work from our laboratory using scAAV-2/8.CMV.CNP vectors to transfect TM cells in the living rat showed a transitory response, which lasted no more than 14 days.

2.1.4. Lentivirus—Retroviruses are enveloped ssRNA-RT viruses (BCS Group VI). Following infection, viral reverse transcriptase (RT) reverse-transcribes the ssRNA to dsDNA. The dsDNA then integrates into the host cell genome via the action of viral integrase, facilitating long-term transgene expression. The most commonly used retroviral vectors are lentiviruses, a phylogenetic sub-class of retrovirus, able to infect both dividing and non-dividing cells. Lentiviral vectors based on HIV, FIV, and SIV can deliver genetic information into host cell DNA with high efficiency, and they yield stable, long-term transgene expression, with only transient and low immunogenicity. These vectors lack the genes *tat* and *rev* (required for replication) and *vpr*, *vpu*, and *nef* (required for virulence). They are tropic for CD4+ receptor expressed on the plasma membrane of macrophages, dendritic cells, certain T-lymphocytes, and certain dividing epithelial cells of the gastrointestinal tract. Also, delivery to the TM with either HIV or FIV-based vectors in which the envelope protein gp120 has been replaced with the vesicular stomatitis virus (VSV) glycoprotein (VSV-G) (thus broadening cell tropism) has been tested in several species and found to be highly efficient. Loewen *et al.* (Loewen *et al.*, 2001; Loewen *et al.*, 2004) reported that lentiviral vectors from FIV transduce TM cells after injection into the anterior chamber of the organ cultured human eye, without affecting TM cell morphology or causing significant cellular loss. In the rat, a single intracameral injection of lentiviral vector encoding GFP (10^7 and 10^8 pfu) successfully transduced the TM and corneal endothelial cells, without signs of inflammation or change in IOP (Challa *et al.*, 2005). Similarly, after a single infection, transgene expression persisted stably for 10 months in a cat model, and was highly targeted to the TM. Transient inflammation occurred only at the highest dose (1×10^8 TU). An FIV vector expressing cDNAs for human cyclooxygenase-2 significantly decreased IOP in cats for 5 months after injection into the anterior chamber. Fluorophotometry showed no decrease in aqueous production (Barraza *et al.*, 2010). In non-human primates, FIV transgene expression in the TM was visible for > 1 year after a single transcorneal injection (Barraza *et al.*, 2009).

During manufacture of lentiviral vectors it is challenging to separate the vector from cellular contaminants. Those contaminants may be in part responsible for the (albeit limited) immunogenicity. Other challenges with integrating gene therapy vectors include (1) potential for insertional mutagenesis; and (2) potential for systemic propagation of

replication-competent retroviruses, which could disrupt expression of essential genes, or activate otherwise silent promoter/enhancer regions (Ralph *et al.*, 2006).

2.1.5. Vesicular Stomatitis Virus (VSV)—VSV is an enveloped ssRNA virus (BCS Group V) belonging to the Rhabdoviridae family. It has broad tropism for neurons, ependymal cells, astrocytes, microglia, and endothelial cells. Its envelope glycoprotein (VSV-G) may be altered via molecular and genetic engineering techniques thereby further diversifying its tropism. VSV-G may also be replaced with a glycoprotein from another virus (e.g. non-neurotropic envelope glycoprotein from lymphocytic choriomeningitis virus, Sindbis virus G protein fused to antibody to cell surface receptor of choice, or measles virus envelope glycoprotein fused to antibody to cell surface receptor of choice). Its genome is easily manipulated, but VSV has a limited cloning capacity (4 to 5 kb) and tends to promote encephalitis in experimental animals. Intracameral administration of VSV-G pseudotyped lentivirus vectors resulted in robust transgene expression in the TM (Lipinski *et al.*, 2014).

2.2. Administration of Viral Vectors

Viral vector-based models of primary open angle glaucoma (POAG) have been attempted in several species, including: non-human primates (Barraza *et al.*, 2009), cat (Loewen *et al.*, 2004; Barraza *et al.*, 2010), and rodent. Of these, rodents have shown the greatest promise; hence the following discussions are focused on the rodents.

2.2.1. Intracameral and Intravitreal Injections—Both intracameral and intravitreal injections were successfully used to produce viral transduction of the TM. When the efficacies of two administration routes were compared, it was found that intravitreal injection yielded a better response in mice (Millar *et al.*, 2008). This may be because a high proportion of the relatively small volume injected into the mouse eye via the intracameral route is rapidly washed out through the conventional outflow pathway; there being insufficient left for productive TM cell transduction. In contrast, the vitreous may serve as a slow-releasing depot for the intravitreally injected virus. Intravitreal injection of viral vectors encoding certain myocilin mutant, activated TGF β 2, and sFRP1 has been reported to increase IOP in the mouse (Shepard *et al.*, 2007; Wang *et al.*, 2008; Shepard *et al.*, 2010). The effect takes 4–7 days to fully develop, and endures for at least 11–28 days. It is important to note that, based on our experience as well as that reported by others, the efficiency of transduction, regardless of routes of injection, appears to improve when the vector is delivered slowly over several minutes. It was reported that intracameral infusion over a period of 3 minutes of 5×10^5 particles of Ad.CMV.GFP in CD1 and C57/BL6 mouse eyes yielded better targeting of TM and Schlemm's canal cells, than either bolus injection, or intracameral infusion over a period of 20 to 40 minutes (Li *et al.*, 2013). This was found to be the case regardless of total volume of vector suspension delivered (3 to 10 μ L).

3. Examples

3.1. Green Fluorescent Protein (GFP) Expression

To characterize the expression profile and duration of adenoviral vector transgenes in the mouse eye, we used the jellyfish GFP as a reporter. Various titers of a vector encoding this protein (Ad5.CMV-GFP) were injected intracamerally or intravitreally and GFP expression was examined carefully both *in vivo* and histologically. We found that *in vivo* fluorescence in the BALB/cJ mouse anterior segment was detectable 4 days after intracameral injection, but the intensity was low. In contrast, intravitreal injection produced a significantly stronger signal. And as expected, GFP expression was dependent on the titer administered; a higher titer (1×10^8 pfu) generated more intense fluorescence than a low titer (1×10^7 pfu) (Millar *et al.*, 2008).

When examined by fluorescence microscopy, cross-sections of mouse eyes after Day 5 of intraocular Ad5.CMV-GFP injection showed that GFP expression was typically localized to the TM, with additional expression in the corneal endothelium and occasionally in the iris pigment epithelium, lens epithelium, and ciliary body. No GFP fluorescence was detected in the retina regardless of injection route, suggesting that adenoviral vectors have a lower tropism for cells in the inner retina compared to cells in the anterior segment. Importantly, the observed intensity of GFP expression correlated very well with *in vivo* observations, such that, in a titer-dependent manner, intravitreal injection consistently produced better expression efficacy than intracameral injection (Millar *et al.*, 2008). This is likely due to a relatively quick washout of the vector after intracameral injection, whereas the vitreous may serve as a slow-releasing depot for the intravitreally injected virus and allow a longer exposure of ocular tissues to the virus.

In studies like this and others, adenovirus was shown to exhibit good tropism for the TM with no non-specific effect on aqueous hydrodynamics. However, its transgene expression at times is transient, peaks at one to two weeks, and often returns to a lower level or even to baseline afterward. This is probably due to immune reactions against the viral or encoded proteins, thus limiting its practicality in long-term studies. To overcome this drawback and prolong adenoviral expression, various immunosuppression strategies have been attempted. The anti-CD40L antibody, an immunomodulating agent, is known to suppress both humoral and cellular immune responses and to extend duration of transgene expression in the liver. We tested it and showed that intraperitoneal administration of this antibody (0.5 mg/mouse on Days -1, 0, 1, 2, 5, 9, and 14 relative to adenoviral injection) can significantly increase and maintain GFP expression level over a long period (for at least 6 weeks) in the anterior segment (Millar *et al.*, 2008). Thus, for studies where a prolonged, high, and relatively constant level of transgene expression is required, systemic treatment with the anti-CD40L antibody and perhaps other immunosuppression techniques are viable approaches for an optimized protocol, especially in young mice. In contrast, we found prolonged transgene expression and IOP elevation in older mice (4–6 months old) (McDowell *et al.*, 2012; Giovingo *et al.*, 2013).

3.2. Mutant MYOC

Myocilin (*MYOC*) was the first glaucoma gene identified (Stone *et al.*, 1997). Mutations in *MYOC* account for approximately 4% of POAG (Alward *et al.*, 1998). Mice over-expressing normal mouse or human myocilin do not develop ocular hypertension (Gould *et al.*, 2004; Shepard *et al.*, 2007). IOP also is not affected in *Myoc* null mice (Kim *et al.*, 2001). This suggests that *MYOC* glaucoma is due to a gain-of-phenotype mutation. Myocilin is a glycoprotein normally secreted from the TM (Nguyen *et al.*, 1998; Clark *et al.*, 2001). However, glaucoma associated mutations in *MYOC* inhibit secretion and promote cellular retention of myocilin (Jacobson *et al.*, 2001), which can lead to ER stress in the TM (Joe *et al.*, 2003; Zode *et al.*, 2011).

We used Ad5 *MYOC* vector transduction of mouse eyes to better understand the mechanisms responsible for *MYOC* glaucoma (Shepard *et al.*, 2007). Although over-expression of wild type human *MYOC* did elevate IOP, three glaucoma associated *MYOC* mutations did. In fact, there was a good genotype-phenotype correlation, which closely matched what is seen in human *MYOC* glaucoma (Alward *et al.*, 1998). The Q368X mutation is associated with adult onset glaucoma with milder ocular hypertension, while the juvenile glaucoma Y437H mutation generates early onset juvenile glaucoma with high IOPs. These phenotypes were also apparent in our mouse model. Human myocilin contains a consensus peroxisomal targeting signal-1 (PTS1) on the C-terminus, which normally shuttles PTS1 containing proteins to the peroxisome. As a secreted glycoprotein, myocilin is synthesized in the endoplasmic compartment and is not recognized by PTS1 receptors in the cytosol. However, mutant myocilin is misfolded, which activates the ERAD response and shuttles misfolded myocilin to the cytoplasm for ubiquitination and proteosomal degradation. Mutant myocilin containing the PTS1 signal can now be recognized by the PTS1R within the cytoplasm. We have shown that mutant myocilin is mislocalized to peroxisomes in cultured TM cells, and that there is again a good genotype-phenotype correlation. We therefore hypothesized that the PTS1 signal is required for mutant myocilin-induced ocular hypertension. Interestingly, transgenic mice containing glaucoma mutations in mouse *Myoc* did not develop robust IOP elevation (Senatorov *et al.*, 2006), and mouse *Myoc* lacks this consensus PTS1 signal. We transduced mouse eyes with vectors containing the same human glaucoma *MYOC* mutation that contained or lacked the PTS1 signal and clearly showed that this PTS1 signal was essential for mutant human myocilin to elevate IOP. This may explain the lack of a mutant mouse *Myoc* IOP phenotype and suggests a novel molecular pathogenic mechanism for *MYOC* glaucoma.

In addition, we also performed a mouse strain survey using the Ad5.hMYOC.Y437H vector (McDowell *et al.*, 2012). Mutant *MYOC* expression produced a prolonged (greater than 8 weeks) 2-fold IOP elevation (approximately 25 mm Hg) in BALB/cJ, A/J, and C57BL/6J mice. Interestingly, there was no IOP elevation in the C3H/HeJ strain despite elevated *MYOC* expression in the TM of these mice. In addition, there also appeared to be strain differences in susceptibility to optic neuropathy induced by mutant *MYOC* ocular hypertension. While A/J mice had significant optic nerve damage after 8 weeks of ocular hypertension, neither the BALB/cJ nor the C57BL/cJ mice had optic nerve damage. It

therefore appears that this model could be used to identify potential optic neuropathy susceptibility genes.

3.3. Bioactivated transforming growth factor (TGF) β 2

Many independent studies have demonstrated that glaucoma patients have elevated levels of TGF β 2 in their aqueous humor (for example, Jampel *et al.*, 1990; Tripathi *et al.*, 1994; Inatani *et al.*, 2001). It was proposed that this cytokine plays a causative role in the pathogenesis of the disease. TGF β 2 is produced by the TM cells and modulates a variety of TM functions, such as extracellular matrix metabolism (Fuchshofer *et al.*, 2005; Fleenor *et al.*, 2006; Wordinger and Clark, 2007; Tovar-Vidales *et al.*, 2011; Kang *et al.*, 2013), phagocytosis (Cao *et al.*, 2003), and cell proliferation (Wordinger *et al.*, 1998). Importantly, perfusion of organ cultured human anterior segments with TGF β 2 increases IOP, corresponding to an increased accumulation of extracellular matrix in the TM region (Fleenor *et al.*, 2006; Gottanka *et al.*, 2004).

Our initial attempt at overexpression of human TGF β 2 in rat eyes did not produce any appreciative effect on IOP (Shepard *et al.*, 2010). The reason is that newly synthesized TGF β 2 is a latent, inactive molecule due to dimerization with its latency-associated peptide (LAP). Activation requires proteolytic removal of the LAP-binding region, and this enzymatic activity was apparently not sufficient in the rodent eye. To overcome this hurdle, we mutated the human TGF β 2 coding sequences at positions 226 and 228 in the LAP-binding region from cysteines to serines. The mutant TGF β 2 has a reduced affinity to LAP and is intrinsically bioactive. When adenoviral vector encoding the mutant protein (Ad5.TGF β 2^{226/228}) was injected in the Wistar rat or BALB/cJ mouse eye, IOP was raised. In the rat, intracameral injection of Ad5.TGF β 2^{226/228} (5×10^7 pfu) produced significant ocular hypertension 5 to 12 days after injection, peaking at day 7 at an IOP of 25 mmHg (IOP of null adenoviral vector control = 18 mmHg). In the mouse, intravitreal injection of Ad5.TGF β 2^{226/228} (6×10^7 pfu) significantly elevated IOP from day 4 to day 28 after injection, peaking at day 6 at an IOP of 26 mmHg (IOP of null adenoviral vector control = 12 mmHg) (Shepard *et al.*, 2010). Consistent with the biological effects of TGF β 2 on the TM and its aqueous outflow effects on *ex vivo* perfused human anterior segments, the increase in mouse IOP of the Ad5.TGF β 2^{226/228}-injected eyes correlated with a decrease in aqueous outflow facility by approximately 33% when compared to the non-injected controls (Shepard *et al.*, 2010).

TGF β 2 signals through activation of receptor serine/threonine kinases. The consequent down-stream cellular effects are mediated by Smad proteins or non-Smad signaling pathways such as MAP kinase, GTPase, and Akt pathways (Moustakas and Heldin, 2005; Zhang, 2009). In order to assess whether the Smad pathway plays a predominant role in the ocular hypertensive effect of TGF β 2, we tested Ad5.TGF β 2^{226/228} in the Smad3 knockout mouse (129-Smad3^{tm1Par/J}) and its corresponding wildtype and heterozygous littermates (McDowell *et al.*, 2013). The results showed that intravitreal injection of the Ad5.TGF β 2^{226/228} increased IOP significantly in both the wildtype and, to a less extent, heterozygous mice, but not in the knockout group (McDowell *et al.*, 2013). Postmortem evaluation of these animals showed no gross difference in anterior chamber morphology

among the groups. However, immunohistochemical analyses of their TM and surrounding tissue detected an increased expression of fibronectin, one of the extracellular matrix proteins, in the wildtype mouse treated with Ad5.TGF β 2^{226/228}, but not in the knockout animals, whereas the heterozygous group had an intermediate expression level (McDowell *et al.*, 2013). These data indicate that the canonical Smad pathway is responsible for the pathological IOP effect of TGF β 2. Interestingly, in human TM cells, TGF β 2, via Smad 2/3, JNK, and p38 pathways, can regulate the levels of the Secreted Protein Acidic and Rich in Cysteine (SPARC), one of the extracellular matrix proteins (Kang *et al.*, 2013). In SPARC-null mice, the ocular hypertensive effect of Ad5.TGF β 2^{226/228} was significantly attenuated, suggesting that SPARC is an essential component in the TGF β 2 effect on IOP (Swaminathan *et al.*, 2014).

3.4. Connective tissue growth factor (CTGF)

CTGF is a member of the CCN family of matricellular regulatory proteins and is expressed in the human TM in relatively high amounts (Tomarev *et al.*, 2003). It is involved in various fibroproliferative diseases and induces excessive accumulation of ECM in the affected tissue. The expression of CTGF can be upregulated by a number of factors, among which TGF β 2 is one of the most potent (Leask *et al.*, 2009). In fact, CTGF appears to play an important role in mediating the effect of TGF β 2 on ECM accumulation in the TM (Junglas *et al.*, 2009). Intracameral injection Ad5.CTGF in the mouse produced a significant increase in IOP of approximately 5 mmHg, lasting 63 days, without major structural changes in the chamber angle, TM, or Schlemm's canal (Junglas *et al.*, 2012). Immunohistochemical examination of the anterior segment detected increased level of fibronectin around the Schlemm's canal and in the ciliary process stroma. An increase in immunostaining of α -smooth muscle actin was also observed in the TM, ciliary body, and iris (Junglas *et al.*, 2012). The ocular hypertension in Ad5.CTGF-injected eyes was associated with a loss of optic nerve axons (Junglas *et al.*, 2012). AdV-CTGF has also been used to transduce rat eyes, which was associated with enhanced expression of CTGF, fibronectin, and collagen IV in the aqueous outflow facility as well as an overall decrease in the outflow facility assessed by microbead perfusion (Su *et al.*, 2013).

3.5. Bone morphogenetic proteins (BMPs) and Gremlin

There is significant cross talk between the TGF β 2 and BMP signaling pathways. TM cells and tissues express BMPs 2, 4, 5, & 7 as well as BMP receptors and inhibitors (Wordinger *et al.*, 2002). Both BMP4 (Wordinger and Clark, 2007) and BMP7 (Fuchshofer *et al.*, 2007) suppress TGF β 2 induction of extracellular matrix (ECM) proteins in cultured TM cells. Expression of the BMP antagonist Gremlin 1 (GREM1) is increased in glaucomatous TM cells compared to control cells (Wordinger and Clark, 2007). Gremlin blocks the protective effects of BMPs in TGF β 2 treated TM cells. Gremlin alone increases ECM production as well as stimulates TGF β 2 expression in TM cells and conversely, TGF β 2 increases gremlin expression (Sethi *et al.*, 2011). This sets up a feed-forward profibrotic signaling system, increasing the deposition and cross-linking of ECM proteins in the TM. The addition of recombinant gremlin to perfusion cultured human anterior segments reversibly increases aqueous outflow resistance and elevates IOP (Wordinger and Clark, 2007). We recently showed that transduction of mouse eyes with an Ad5.GREM1 expression vector

significantly elevated IOP (McDowell CM et al, submitted for publication). To determine whether the TGF β 2 Smad signaling pathway was involved in GREM1-induced IOP elevation, we transduced the eyes of wild type as well as *Smad3*^{+/-} and *Smad3*^{-/-} mice. The GREM1 mediated IOP elevation seen in wild type mice was totally abrogated in the *Smad3* knock mice. The *Smad3* heterozygotes had an intermediate ocular hypertensive phenotype. These data provide additional support for the cross talk between the TGF β 2 and BMP signaling pathways in regulating IOP and suggest that perturbations in these pathways (i.e. due to increased GREM1 expression) drive a feed-forward profibrotic pathogenic pathway.

Interestingly, BMP2 has been shown to cause calcification-like phenotype in TM cells (Xue *et al.*, 2006). Intracameral injection of Ad5.BMP2 in Brown Norway and Wistar rats induced IOP elevation starting at approximately 7 days after injection and sustaining for up to 7 weeks. At the 4-week time point, Ad5.BMP2 increased IOP by 25.2 mmHg in the Brown Norway rats, and 3.5 mmHg in the Wistar rats (Buie *et al.*, 2013). The Ad5.BMP2-injected eye showed an intact TM structure, but the region underneath the Schlemm's canal seemed to have less empty spaces between the beams as though there were more accumulation of ECM (Buie *et al.*, 2013). The ocular hypertension in these rats correlated with a loss in RGCs (Buie *et al.*, 2013). Similarly, intracameral injection of Ad.BMP2 in the CD1 mouse decreased aqueous outflow by more 50%, and consequently increased IOP (Li *et al.*, 2014). The IOP elevation lasted for more than 30 days. In these animals, the peripheral cornea and TM were thickened at 7 days after injection. While corneal thickening in the BMP2-transduced eyes returned to normal at 36 days, the TM remained thickened, and the iridocorneal angle was completely closed (Li *et al.*, 2014). At 36 days after Ad.BMP2 injection, there was a 68% decrease in peripheral RGC density (Li *et al.*, 2014).

3.6. Inhibition of WNT signaling

The WNT signaling pathways are responsible in transmitting specific autocrine and paracrine extracellular signals into the cell through cell surface Frizzled protein (FZD) receptors. Based on the signaling molecules involved, they can be classified as canonical and noncanonical pathways. The canonical pathway is activated by the binding of WNT protein ligand to FZD receptor and co-receptor, low-density lipoprotein receptor-related protein (LRP)-5 or -6. Activation of the receptor complex leads to activation of the intracellular Dishevelled (DVL) protein, leading to stabilization and accumulation of β -catenin. β -catenin translocates into the nucleus and affects gene expression by interacting with TCF/LEF transcription factors binding to TCF/LEF cis-elements. When the receptor is not activated, β -catenin is degraded by the β -catenin destruction complex, consisting of proteins Axin, adenomatous polyposis coli (APC), glycogen synthase kinase (GSK) 3 β , and casein kinase 1 (CK1) (MacDonald *et al.*, 2009). The noncanonical pathways also require the activation of DVL. However, downstream of DVL activation, enzymes such as Rho, Rho-associated kinase (ROCK), c-Jun N-terminal kinases (JNK) are activated, resulting in the restructuring of cytoskeleton and gastrulation. Alternatively, phosphodiesterase and phospholipase C may be activated, leading to changes in intracellular calcium mobilization (Logan and Nusse, 2004; Angers and Moon, 2009; Roszko *et al.*, 2009).

In human TM tissues and cultured human TM cells, the canonical pathway has been characterized and demonstrated to be the prevalent WNT signaling pathway in modulating TM functions (Wang *et al.*, 2008; Mao *et al.*, 2012); (Villarreal *et al.*, 2014). We also demonstrated the expression of most of the proteins in this pathway, including WNT2b, WNT5a, WNT5b, FZD1-FZD4, FZD6-FZD8, LRP5, LRP6, DVL1, DVL3, APC, AXIN2, GSK3 β , β -catenin, TCF3, TCF4, TCF7, in the TM (Wang *et al.*, 2008). In addition, the secreted frizzled-related protein-1 (sFRP-1), which is a WNT-binding protein that suppresses WNT signaling, was also found to be present in the TM. Most interestingly, sFRP-1 levels were significantly higher in TM tissues and cells derived from glaucoma patients when compared to non-glaucoma donors (Wang *et al.*, 2008). These findings led to the hypotheses that WNT signaling likely plays a role in the regulation of IOP by modulating TM functions and that abnormal suppression of this pathway, such as by an excessive amount of sFRP-1, may contribute to ocular hypertension and glaucoma. These suppositions were substantiated by subsequent results showing that in human ocular anterior segment organ cultures, perfusion with recombinant sFRP-1 caused a significant degradation of β -catenin in the TM and impeded aqueous outflow facility (Wang *et al.*, 2008).

We found that intravitreal injection of adenoviral vector encoding sFRP-1 (Ad5.sFRP-1) increased sFRP-1 expression in the anterior segments of BALB/cJ mice and raised IOP in a titer-dependent manner. The IOP was doubled from a baseline of approximately 12 mmHg to 25 mmHg four days after injection of Ad5.sFRP-1 (3×10^7 pfu/eye) (Wang *et al.*, 2008). The induced ocular hypertension sustained for more than a month without detectable morphological changes in the eye, making it a practical animal model for glaucoma research. Indeed, we showed that topical ocular administration of a GSK β inhibitor lowered IOP in this model (Wang *et al.*, 2008).

The canonical WNT signaling pathway can also be inhibited by Dickkopf-related protein-1 (DKK1), which interferes with the binding of WNT to LRP-5/6 and thus disrupts the stabilization of β -catenin and its associated downstream effects (Mao *et al.*, 2001). Intravitreal injection of Ad5.DKK1 significantly increased IOP in the mouse. The onset, magnitude, and duration of its effect were comparable to that induced by Ad5.sFRP-1 (Mao *et al.*, 2012). This clearly demonstrates that canonical WNT signaling is responsible for regulating IOP. This therefore is another potential rodent model for glaucoma studies.

3.7. CD44

CD44 is a cell surface glycoprotein that serves as a cell adhesion receptor for a number of ligands, including hyaluronic acid, osteopontin, collagens, and MMPs. Knepper and colleagues have shown significantly increased expression of a soluble form of CD44 (sCD44) in the aqueous humor of POAG patients compared to normal eyes (Knepper *et al.*, 2002). This sCD44 isoform in POAG aqueous humor was also hypophosphorylated compared to sCD44 in normal eyes (Knepper *et al.*, 2005). In addition, aqueous humor concentrations of sCD44 were significantly correlated with visual field loss in POAG subjects (Nolan *et al.*, 2007). Soluble CD44 was toxic to cultured human TM cells (Choi *et al.*, 2005), suggesting that sCD44 could directly compromise TM cell function and lead to elevated IOP. To determine whether CD44 can directly elevate IOP, we used Ad5.CD44 and

Ad5.sCD44 expression vectors to transduce the trabecular meshwork of mouse eyes (Giovingo *et al.*, 2013). Transduction of mouse eyes with either CD44 or sCD44 increased aqueous humor concentrations of sCD44 and caused prolonged IOP elevation (>50 days). Topical administration of a γ -secretase lowered Ad5.sCD44 induced ocular hypertension, and discontinuation of this therapy caused IOP to increase again, implicating that this enzyme may be involved in activating the pathogenic isoform of CD44.

4. Future Applications

Transduction of mouse eyes with viral vectors has been and continues to be an attractive approach for validating pathogenic signaling pathways in rodents. The power of mouse genetics is extremely useful for further dissecting these signaling pathways. Strain differences in induced phenotypes will allow the identification of potential modifier genes.

However, there are still challenges and opportunities in the use of viral transgenes to better understand glaucoma pathogenesis and to establish inducible rodent models of glaucoma using glaucoma associated transgenes. Although the majority of studies have used Ad5 expression vectors because of rapid and high expression of transgenes as well as selective tropism for the TM, there are some deficiencies with this specific vector. Ad5 transgene expression in the TM is often transient, lasting only several weeks, and inflammation/immune response may be responsible (Millar *et al.*, 2008; Shepard *et al.*, 2010). However, sometimes transgene induced IOP elevation can be prolonged in mice (McDowell *et al.*, 2012; McDowell *et al.*, 2013; Giovingo *et al.*, 2013). In addition, there also appears to be mouse strain differences in IOP elevation, despite successful transduction and transgene expression in the TM. There also are strain differences to ocular hypertension induced optic neuropathy, so strain surveys are desirable. Other viral vectors have prolonged transgene expression and fewer propensities to induce inflammation, although these vectors have not been adequately tested to determine whether they can induce ocular hypertension when expressing glaucoma-associated transgenes.

Another exciting development is the use of viral vectors to selectively knock down gene expression in the rodent TM. There continue to be a growing number of conditional knockout mouse strains; however, the lack of TM tissue specific promoter makes it difficult to develop mouse lines expressing the Cre recombinase in the TM with which to cross the floxed mouse strains. Viral vectors with tropism for the TM can be used to deliver the Cre transgene to the TM and selectively knockout the specific floxed gene. Viral vectors can also be used to deliver shRNAs that will induce RNA interference and suppress specific mRNA and protein expression in the TM. Another potentially exciting development would be to use viral vectors to deliver CRISPR/Cas9 for specific genome editing in the TM.

Acknowledgments

We would like to acknowledge grant support from the National Eye Institute (EY016242, EY019977) and the Department of Defense (W81XWH-10-2-003).

Abbreviations

AAV	adeno-associated virus
Ad	adenovirus
APC	adenomatosis polyposis coli
BCS	Baltimore Classification Scheme
BMP	bone morphogenetic protein
CK	casein kinase
CTGF	connective tissue growth factor
DKK1	Dickkopf-related protein-1
ECM	extracellular matrix
EIAV	equine infectious anemia virus
GFP	green fluorescent protein
GSK	glycogen synthase kinase
HSV	herpes simplex virus
IOP	intraocular pressure
JNK	c-Jun N-terminal kinases
LAP	latency-associated peptide
LRP	lipoprotein receptor-related protein
POAG	primary open angle glaucoma
PTS1	peroxisomal targeting signal-1
RGC	retinal ganglion cell
ROCK	rho-associated kinase
scAAV	self-complementary adeno-associated virus
SPARC	Secreted Protein Acidic and Rich in Cysteine
TGF	transforming growth factor
TM	trabecular meshwork
TU	transducing unit
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus glycoprotein

References

- Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J, Dejneka NS, Pearce-Kelling SE, Maguire AM, Palczewski K, Hauswirth WW, Jacobson SG. Long-term restoration of

- rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol Ther*. 2005; 12(6):1072–1082. [PubMed: 16226919]
- Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, Bennett J. Gene therapy restores vision in a canine model of childhood blindness. *Nat Genet*. 2001; 28(1):92–95. [PubMed: 11326284]
- AGIS. The Advanced Glaucoma Intervention Study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration. The AGIS Investigators. *Am J Ophthalmol*. 2000; 130(4):429–440. [PubMed: 11024415]
- Aihara M, Lindsey JD, Weinreb RN. Experimental mouse ocular hypertension: establishment of the model. *Invest Ophthalmol Vis Sci*. 2003a; 44(10):4314–4320. [PubMed: 14507875]
- Aihara M, Lindsey JD, Weinreb RN. Ocular hypertension in mice with a targeted type I collagen mutation. *Invest Ophthalmol Vis Sci*. 2003b; 44(4):1581–1585. [PubMed: 12657595]
- Alba R, Bosch A, Chillon M. Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther*. 2005; 12(Suppl 1):S18–27. [PubMed: 16231052]
- Alward WL, Fingert JH, Coote MA, Johnson AT, Lerner SF, Junqua D, Durcan FJ, McCartney PJ, Mackey DA, Sheffield VC, Stone EM. Clinical features associated with mutations in the chromosome 1 open-angle glaucoma gene (GLC1A). *N Engl J Med*. 1998; 338(15):1022–1027. [PubMed: 9535666]
- Anderson MG, Libby RT, Gould DB, Smith RS, John SW. High-dose radiation with bone marrow transfer prevents neurodegeneration in an inherited glaucoma. *Proc Natl Acad Sci U S A*. 2005; 102(12):4566–4571. [PubMed: 15758074]
- Anderson MG, Smith RS, Hawes NL, Zabaleta A, Chang B, Wiggs JL, John SW. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. *Nat Genet*. 2002; 30(1):81–85. [PubMed: 11743578]
- Angers S, Moon RT. Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol*. 2009; 10(7):468–477. [PubMed: 19536106]
- Balaggan KS, Binley K, Esapa M, Iqbal S, Askham Z, Kan O, Tschernutter M, Bainbridge JW, Naylor S, Ali RR. Stable and efficient intraocular gene transfer using pseudotyped ELAV lentiviral vectors. *J Gene Med*. 2006; 8(3):275–285. [PubMed: 16299834]
- Barraza RA, McLaren JW, Poeschla EM. Prostaglandin pathway gene therapy for sustained reduction of intraocular pressure. *Mol Ther*. 2010; 18(3):491–501. [PubMed: 19953083]
- Barraza RA, Rasmussen CA, Loewen N, Cameron JD, Gabelt BT, Teo WL, Kaufman PL, Poeschla EM. Prolonged transgene expression with lentiviral vectors in the aqueous humor outflow pathway of nonhuman primates. *Hum Gene Ther*. 2009; 20(3):191–200. [PubMed: 19301472]
- Bennett J, Anand V, Acland GM, Maguire AM. Cross-species comparison of in vivo reporter gene expression after recombinant adeno-associated virus-mediated retinal transduction. *Methods Enzymol*. 2000; 316:777–789. [PubMed: 10800714]
- Bennett J, Duan D, Engelhardt JF, Maguire AM. Real-time, noninvasive in vivo assessment of adeno-associated virus-mediated retinal transduction. *Invest Ophthalmol Vis Sci*. 1997; 38(13):2857–2863. [PubMed: 9418740]
- Benozzi J, Nahum LP, Campanelli JL, Rosenstein RE. Effect of hyaluronic acid on intraocular pressure in rats. *Invest Ophthalmol Vis Sci*. 2002; 43(7):2196–2200. [PubMed: 12091416]
- Borras T, Xue W, Choi VW, Bartlett JS, Li G, Samulski RJ, Chisolm SS. Mechanisms of AAV transduction in glaucoma-associated human trabecular meshwork cells. *J Gene Med*. 2006; 8(5):589–602. [PubMed: 16506246]
- Budenz DL, Bennett J, Alonso L, Maguire A. In vivo gene transfer into murine corneal endothelial and trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 1995; 36(11):2211–2215. [PubMed: 7558714]
- Buie LK, Rasmussen CA, Porterfield EC, Ramgolam VS, Choi VW, Markovic-Plese S, Samulski RJ, Kaufman PL, Borras T. Self-complementary AAV virus (scAAV) safe and long-term gene transfer in the trabecular meshwork of living rats and monkeys. *Invest Ophthalmol Vis Sci*. 2010; 51(1):236–248. [PubMed: 19684004]

- Buie LK, Karim MZ, Smith MH, Borrás T. Development of a model of elevated intraocular pressure in rats by gene transfer of bone morphogenetic protein 2. *Invest Ophthalmol Vis Sci.* 2013; 54(8): 5441–5455. [PubMed: 23821199]
- Cao Y, Wei H, Pfaffl MW, Da B. Effect of transforming growth factor beta 2 on phagocytosis in cultured bovine trabecular meshwork cells. *Ophthalmologie.* 2003; 100(7):535–538. [PubMed: 12920553]
- Challa P, Luna C, Liton PB, Chamblin BS, Wakefield JK, Ramabhadran R, Epstein DL, Gonzalez P. Lentiviral mediated gene delivery to the anterior chamber of rodent eyes. *Mol Vis.* 2005; 11:425–430. [PubMed: 15988411]
- Choi J, Miller AM, Nolan MJ, Yue BY, Thotz ST, Clark AF, Agarwal N, Knepper PA. Soluble CD44 is cytotoxic to trabecular meshwork and retinal ganglion cells in vitro. *Invest Ophthalmol Vis Sci.* 2005; 46(1):214–222. [PubMed: 15623776]
- Clark AF, Steely HT, Dickerson JE Jr, English-Wright S, Stropki K, McCartney MD, Jacobson N, Shepard AR, Clark JI, Matsushima H, Peskind ER, Leverenz JB, Wilkinson CW, Swiderski RE, Fingert JH, Sheffield VC, Stone EM. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci.* 2001; 42(8):1769–1780. [PubMed: 11431441]
- Dudus L, Anand V, Acland GM, Chen SJ, Wilson JM, Fisher KJ, Maguire AM, Bennett J. Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. *Vision Res.* 1999; 39(15):2545–2553. [PubMed: 10396623]
- Epstein AL. HSV-1-based amplicon vectors: design and applications. *Gene Ther.* 2005; 12(Suppl 1):S154–158. [PubMed: 16231049]
- Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci.* 2006; 47(1):226–234. [PubMed: 16384967]
- Fraefel C, Mendes-Madeira A, Mabon O, Lefebvre A, Le Meur G, Ackermann M, Moullier P, Rolling F. In vivo gene transfer to the rat retina using herpes simplex virus type 1 (HSV-1)-based amplicon vectors. *Gene Ther.* 2005; 12(16):1283–1288. [PubMed: 15889134]
- Fuchshofer R, Birke M, Welge-Lüssen U, Kook D, Lutjen-Drecoll E. Transforming growth factor-beta 2 modulated extracellular matrix component expression in cultured human optic nerve head astrocytes. *Invest Ophthalmol Vis Sci.* 2005; 46(2):568–578. [PubMed: 15671284]
- Fuchshofer R, Yu AH, Welge-Lüssen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2007; 48(2):715–726. [PubMed: 17251470]
- Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A.* 2002; 99(18): 11854–11859. [PubMed: 12192090]
- Ginsberg HS. The ups and downs of adenovirus vectors. *Bull N Y Acad Med.* 1996; 73(1):53–58. [PubMed: 8804738]
- Giovingo M, Nolan M, McCarty R, Pang IH, Clark AF, Beverley RM, Schwartz S, Stamer WD, Walker L, Grybauskas A, Skuran K, Kuprys PV, Yue BY, Knepper PA. sCD44 overexpression increases intraocular pressure and aqueous outflow resistance. *Mol Vis.* 2013; 19:2151–2164. [PubMed: 24194636]
- Goff SP, Berg P. Construction of hybrid viruses containing SV40 and lambda phage DNA segments and their propagation in cultured monkey cells. *Cell.* 1976; 9(4 PT 2):695–705. [PubMed: 189942]
- Gordon MO, Beiser JA, Brandt JD, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP, Parrish RK 2nd, Wilson MR, Kass MA. The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma. *Arch Ophthalmol.* 2002; 120(6): 714–720. discussion 829–730. [PubMed: 12049575]
- Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci.* 2004; 45(1):153–158. [PubMed: 14691167]
- Gould DB, Miceli-Libby L, Savinova OV, Torrado M, Tomarev SI, Smith RS, John SW. Genetically increasing Myoc expression supports a necessary pathologic role of abnormal proteins in glaucoma. *Mol Cell Biol.* 2004; 24(20):9019–9025. [PubMed: 15456875]

- Hauswirth WW, Lewin AS, Zolotukhin S, Muzyczka N. Production and purification of recombinant adeno-associated virus. *Methods Enzymol.* 2000; 316:743–761. [PubMed: 10800712]
- Hoffman LM, Maguire AM, Bennett J. Cell-mediated immune response and stability of intraocular transgene expression after adenovirus-mediated delivery. *Invest Ophthalmol Vis Sci.* 1997; 38(11):2224–2233. [PubMed: 9344345]
- Howell GR, Macalinao DG, Sousa GL, Walden M, Soto I, Kneeland SC, Barbay JM, King BL, Marchant JK, Hibbs M, Stevens B, Barres BA, Clark AF, Libby RT, John SW. Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. *J Clin Invest.* 2011; 121(4):1429–1444. [PubMed: 21383504]
- Howell GR, MacNicol KH, Braine CE, Soto I, Macalinao DG, Sousa GL, John SW. Combinatorial targeting of early pathways profoundly inhibits neurodegeneration in a mouse model of glaucoma. *Neurobiol Dis.* 2014; 71:44–52. [PubMed: 25132557]
- Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol.* 2001; 239(2):109–113. [PubMed: 11372538]
- Jacobson N, Andrews M, Shepard AR, Nishimura D, Searby C, Fingert JH, Hageman G, Mullins R, Davidson BL, Kwon YH, Alward WL, Stone EM, Clark AF, Sheffield VC. Non-secretion of mutant proteins of the glaucoma gene myocilin in cultured trabecular meshwork cells and in aqueous humor. *Hum Mol Genet.* 2001; 10(2):117–125. [PubMed: 11152659]
- Jampel HD, Roche N, Stark WJ, Roberts AB. Transforming growth factor-beta in human aqueous humor. *Curr Eye Res.* 1990; 9(10):963–969. [PubMed: 2276273]
- Joe MK, Sohn S, Hur W, Moon Y, Choi YR, Kee C. Accumulation of mutant myocilins in ER leads to ER stress and potential cytotoxicity in human trabecular meshwork cells. *Biochem Biophys Res Commun.* 2003; 312(3):592–600. [PubMed: 14680806]
- Junglas B, Yu AH, Welge-Lussen U, Tamm ER, Fuchshofer R. Connective tissue growth factor induces extracellular matrix deposition in human trabecular meshwork cells. *Exp Eye Res.* 2009; 88(6):1065–1075. [PubMed: 19450452]
- Junglas B, Kuespert S, Seleem AA, Struller T, Ullmann S, Bosl M, Bosserhoff A, Kostler J, Wagner R, Tamm ER, Fuchshofer R. Connective tissue growth factor causes glaucoma by modifying the actin cytoskeleton of the trabecular meshwork. *Am J Pathol.* 2012; 180(6):2386–2403. [PubMed: 22542845]
- Kang MH, Oh DJ, Kang JH, Rhee DJ. Regulation of SPARC by transforming growth factor beta2 in human trabecular meshwork. *Invest Ophthalmol Vis Sci.* 2013; 54(4):2523–2532. [PubMed: 23513064]
- Kim BS, Savinova OV, Reedy MV, Martin J, Lun Y, Gan L, Smith RS, Tomarev SI, John SW, Johnson RL. Targeted Disruption of the Myocilin Gene (Myoc) Suggests that Human Glaucoma-Causing Mutations Are Gain of Function. *Mol Cell Biol.* 2001; 21(22):7707–7713. [PubMed: 11604506]
- Knepper PA, Mayanil CS, Goossens W, Wertz RD, Holgren C, Ritch R, Allingham RR. Aqueous humor in primary open-angle glaucoma contains an increased level of CD44S. *Invest Ophthalmol Vis Sci.* 2002; 43(1):133–139. [PubMed: 11773023]
- Knepper PA, Miller AM, Choi J, Wertz RD, Nolan MJ, Goossens W, Whitmer S, Yue BY, Ritch R, Liebmann JM, Allingham RR, Samples JR. Hypophosphorylation of aqueous humor sCD44 and primary open-angle glaucoma. *Invest Ophthalmol Vis Sci.* 2005; 46(8):2829–2837. [PubMed: 16043857]
- Leask A, Parapuram SK, Xu S, Abraham DJ. Connective tissue growth factor (CTGF, CCN2) gene regulation: a potent clinical bio-marker of fibroproliferative disease? *J Cell Commun Signal.* 2009; 3(2):89–94. [PubMed: 19156539]
- Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophthalmol Vis Sci.* 2002; 43(2):402–410. [PubMed: 11818384]
- Li G, Farsiu S, Qiu J, Dixon A, Song Ch, McKinnon SJ, Yuan F, Gonzalez P, Stamer WD. Disease progression in iridocorneal angle tissues of BMP2-induced ocular hypertensive mice with optical coherence tomography. *Mol Vis.* 2014; 20:1695–1709. [PubMed: 25558173]

- Li G, Gonzalez LJ, Camras LJ, Navarro I, Qui J, Challa P, Stamer WD. Optimizing gene transfer to conventional outflow cells in living mouse eyes. *Exp Eye Res.* 2013; 109:8–16. [PubMed: 23337742]
- Lipinski DM, Barnard AR, Charbel Issa P, Singh MS, De Silva SR, Trabalza A, Eleftheriadou I, Ellison SM, Mazarakis ND, MacLaren RE. Vesicular stomatitis virus glycoprotein- and Venezuelan equine encephalitis virus-derived glycoprotein-pseudotyped lentivirus vectors differentially transduce corneal endothelium, trabecular meshwork, and human photoreceptors. *Hum Gene Ther.* 2014; 25(1):50–62. [PubMed: 24125177]
- Liu X, Hu Y, Filla MS, Gabelt BT, Peters DM, Brandt CR, Kaufman PL. The effect of C3 transgene expression on actin and cellular adhesions in cultured human trabecular meshwork cells and on outflow facility in organ cultured monkey eyes. *Mol Vis.* 2005; 11:1112–1121. [PubMed: 16379023]
- Loewen N, Fautsch MP, Peretz M, Bahler CK, Cameron JD, Johnson DH, Poeschla EM. Genetic modification of human trabecular meshwork with lentiviral vectors. *Hum Gene Ther.* 2001; 12(17):2109–2119. [PubMed: 11747600]
- Loewen N, Fautsch MP, Teo WL, Bahler CK, Johnson DH, Poeschla EM. Long-term, targeted genetic modification of the aqueous humor outflow tract coupled with noninvasive imaging of gene expression in vivo. *Invest Ophthalmol Vis Sci.* 2004; 45(9):3091–3098. [PubMed: 15326125]
- Logan CY, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol.* 2004; 20:781–810. [PubMed: 15473860]
- MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell.* 2009; 17(1):9–26. [PubMed: 19619488]
- Mao B, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, Niehrs C. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature.* 2001; 411(6835):321–325. [PubMed: 11357136]
- Mao W, Millar JC, Wang WH, Silverman SM, Liu Y, Wordinger RJ, Rubin JS, Pang IH, Clark AF. Existence of the canonical Wnt signaling pathway in the human trabecular meshwork. *Invest Ophthalmol Vis Sci.* 2012; 53(11):7043–7051. [PubMed: 22956608]
- McDowell CM, Luan T, Zhang Z, Putliwala T, Wordinger RJ, Millar JC, John SW, Pang IH, Clark AF. Mutant human myocilin induces strain specific differences in ocular hypertension and optic nerve damage in mice. *Exp Eye Res.* 2012; 100:65–72. [PubMed: 22575566]
- McDowell CM, Tebow HE, Wordinger RJ, Clark AF. Smad3 is necessary for transforming growth factor-beta2 induced ocular hypertension in mice. *Exp Eye Res.* 2013; 116:419–423. [PubMed: 24184030]
- Millar JC, Clark AF, Pang IH. Assessment of aqueous humor dynamics in the mouse by a novel method of constant-flow infusion. *Invest Ophthalmol Vis Sci.* 2011; 52(2):685–694. [PubMed: 20861483]
- Millar JC, Pang IH, Wang WH, Wang Y, Clark AF. Effect of immunomodulation with anti-CD40L antibody on adenoviral-mediated transgene expression in mouse anterior segment. *Mol Vis.* 2008; 14:10–19. [PubMed: 18246028]
- Min SH, Molday LL, Seeliger MW, Dinculescu A, Timmers AM, Janssen A, Tonagel F, Tanimoto N, Weber BH, Molday RS, Hauswirth WW. Prolonged recovery of retinal structure/function after gene therapy in an *Rsl1h*-deficient mouse model of x-linked juvenile retinoschisis. *Mol Ther.* 2005; 12(4):644–651. [PubMed: 16027044]
- Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC. A rat model of chronic pressure-induced optic nerve damage. *Exp Eye Res.* 1997; 64(1):85–96. [PubMed: 9093024]
- Moustakas A, Heldin CH. Non-Smad TGF-beta signals. *J Cell Sci.* 2005; 118(Pt 16):3573–3584. [PubMed: 16105881]
- Nguyen TD, Chen P, Huang WD, Chen H, Johnson D, Polansky JR. Gene structure and properties of TIGR, an olfactomedin-related glycoprotein cloned from glucocorticoid-induced trabecular meshwork cells. *J Biol Chem.* 1998; 273(11):6341–6350. [PubMed: 9497363]
- Nolan MJ, Giovingo MC, Miller AM, Wertz RD, Ritch R, Liebmann JM, Allingham RR, Herndon LW, Wax MB, Smolyak R, Hasan F, Barnett EM, Samples JR, Knepper PA. Aqueous humor sCD44 concentration and visual field loss in primary open-angle glaucoma. *J Glaucoma.* 2007; 16(5):419–429. [PubMed: 17700283]

- Overby DR, Bertrand J, Tektas OY, Boussommier-Calleja A, Schicht M, Ethier CR, Woodward DF, Stamer WD, Lutjen-Drecoll E. Ultrastructural changes associated with dexamethasone-induced ocular hypertension in mice. *Invest Ophthalmol Vis Sci*. 2014; 55(8):4922–4933. [PubMed: 25028360]
- Peel AL, Klein RL. Adeno-associated virus vectors: activity and applications in the CNS. *J Neurosci Methods*. 2000; 98(2):95–104. [PubMed: 10880823]
- Ralph GS, Binley K, Wong LF, Azzouz M, Mazarakis ND. Gene therapy for neurodegenerative and ocular diseases using lentiviral vectors. *Clin Sci (Lond)*. 2006; 110(1):37–46. [PubMed: 16336203]
- Roszko I, Sawada A, Solnica-Krezel L. Regulation of convergence and extension movements during vertebrate gastrulation by the Wnt/PCP pathway. *Semin Cell Dev Biol*. 2009; 20(8):986–997. [PubMed: 19761865]
- Sappington RM, Carlson BJ, Crish SD, Calkins DJ. The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Invest Ophthalmol Vis Sci*. 2010; 51(1):207–216. [PubMed: 19850836]
- Senatorov V, Malyukova I, Fariss R, Wawrousek EF, Swaminathan S, Sharan SK, Tomarev S. Expression of mutated mouse myocilin induces open-angle glaucoma in transgenic mice. *J Neurosci*. 2006; 26(46):11903–11914. [PubMed: 17108164]
- Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF. Role of TGFbeta/Smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Invest Ophthalmol Vis Sci*. 2011; 52(8):5251–5259. [PubMed: 21642622]
- Shareef SR, Garcia-Valenzuela E, Salierno A, Walsh J, Sharma SC. Chronic ocular hypertension following episcleral venous occlusion in rats. *Exp Eye Res*. 1995; 61(3):379–382. [PubMed: 7556500]
- Shepard AR, Jacobson N, Millar JC, Pang IH, Steely HT, Searby CC, Sheffield VC, Stone EM, Clark AF. Glaucoma-causing myocilin mutants require the Peroxisomal targeting signal-1 receptor (PTS1R) to elevate intraocular pressure. *Hum Mol Genet*. 2007; 16(6):609–617. [PubMed: 17317787]
- Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor- β 2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010; 51(4):2067–2076. [PubMed: 19959644]
- Shinzato M, Yamashiro Y, Miyara N, Iwamatsu A, Takeuchi K, Umikawa M, Bayarjargal M, Kariya K, Sawaguchi S. Proteomic analysis of the trabecular meshwork of rats in a steroid-induced ocular hypertension model: downregulation of type I collagen C-propeptides. *Ophthalmic Res*. 2007; 39(6):330–337. [PubMed: 18046086]
- Spencer B, Agarwala S, Miskulin M, Smith M, Brandt CR. Herpes simplex virus-mediated gene delivery to the rodent visual system. *Invest Ophthalmol Vis Sci*. 2000; 41(6):1392–1401. [PubMed: 10798655]
- Stone EM, Fingert JH, Alward WL, Nguyen TD, Polansky JR, Sunden SL, Nishimura D, Clark AF, Nystuen A, Nichols BE, Mackey DA, Ritch R, Kalenak JW, Craven ER, Sheffield VC. Identification of a gene that causes primary open angle glaucoma. *Science*. 1997; 275(5300):668–670. [PubMed: 9005853]
- Su Y, Cheng J, Liu H, Wang F, Zhao S. Adenovirus conducted connective tissue growth factor on extracellular matrix in trabecular meshwork and its role on aqueous humor outflow facility. *Mol Biol Rep*. 2013; 40(11):6091–6096. [PubMed: 24052232]
- Swaminathan SS, Oh DJ, Kang MH, Shepard AR, Pang IH, Rhee DJ. TGF- β 2-mediated ocular hypertension is attenuated in SPARC-null mice. *Invest Ophthalmol Vis Sci*. 2014; 55(7):4084–4097. [PubMed: 24906856]
- Tomarev SI, Wistow G, Raymond V, Dubois S, Malyukova I. Gene expression profile of the human trabecular meshwork: NEIBank sequence tag analysis. *Invest Ophthalmol Vis Sci*. 2003; 44(6):2588–2596. [PubMed: 12766061]

- Tovar-Vidales T, Clark AF, Wordinger RJ. Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Exp Eye Res.* 2011; 93(4):442–451. [PubMed: 21722634]
- Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res.* 1994; 59(6):723–727. [PubMed: 7698265]
- Ueyama K, Mori K, Shoji T, Omata H, Gehlbach PL, Brough DE, Wei LL, Yoneya S. Ocular localization and transduction by adenoviral vectors are serotype-dependent and can be modified by inclusion of RGD fiber modifications. *PLoS One.* 2014; 9(9):e108071. [PubMed: 25232844]
- Villarreal G Jr, Chatterjee A, Oh SS, Oh DJ, Kang MH, Rhee DJ. Canonical wnt signaling regulates extracellular matrix expression in the trabecular meshwork. *Invest Ophthalmol Vis Sci.* 2014; 55(11):7433–7440. [PubMed: 25352117]
- Walsh MM, Yi H, Friedman J, Cho KI, Tserentsoodol N, McKinnon S, Searle K, Yeh A, Ferreira PA. Gene and protein expression pilot profiling and biomarkers in an experimental mouse model of hypertensive glaucoma. *Exp Biol Med (Maywood).* 2009; 234(8):918–930. [PubMed: 19491369]
- Wang F, Tang W, McGraw HM, Bennett J, Enquist LW, Friedman HM. Herpes simplex virus type 1 glycoprotein e is required for axonal localization of capsid, tegument, and membrane glycoproteins. *J Virol.* 2005; 79(21):13362–13372. [PubMed: 16227258]
- Wang WH, McNatt LG, Pang IH, Millar JC, Hellberg PE, Hellberg MH, Steely HT, Rubin JS, Fingert JH, Sheffield VC, Stone EM, Clark AF. Increased expression of the WNT antagonist sFRP-1 in glaucoma elevates intraocular pressure. *J Clin Invest.* 2008; 118(3):1056–1064. [PubMed: 18274669]
- Whitlock NA, McKnight B, Corcoran KN, Rodriguez LA, Rice DS. Increased intraocular pressure in mice treated with dexamethasone. *Invest Ophthalmol Vis Sci.* 2010; 51(12):6496–6503. [PubMed: 20671272]
- Wordinger RJ, Agarwal R, Talati M, Fuller J, Lambert W, Clark AF. Expression of bone morphogenetic proteins (BMP), BMP receptors, and BMP associated proteins in human trabecular meshwork and optic nerve head cells and tissues. *Mol Vis.* 2002; 8:241–250. [PubMed: 12131877]
- Wordinger RJ, Clark AF. Bone morphogenetic proteins and their receptors in the eye. *Exp Biol Med (Maywood).* 2007; 232(8):979–992. [PubMed: 17720944]
- Wordinger RJ, Clark AF, Agarwal R, Lambert W, McNatt L, Wilson SE, Qu Z, Fung BK. Cultured human trabecular meshwork cells express functional growth factor receptors. *Invest Ophthalmol Vis Sci.* 1998; 39(9):1575–1589. [PubMed: 9699547]
- Xue W, Wallin R, Olmsted-Davis EA, Borrás T. Matrix GLA protein function in human trabecular meshwork cells: inhibition of BMP2-induced calcification process. *Invest Ophthalmol Vis Sci.* 2006; 47(3):997–1007. [PubMed: 16505034]
- Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A.* 1994; 91(10):4407–4411. [PubMed: 8183921]
- Zeng Y, Takada Y, Kjellstrom S, Hiriyanna K, Tanikawa A, Wawrousek E, Smaoui N, Caruso R, Bush RA, Sieving PA. RS-1 Gene Delivery to an Adult Rs1h Knockout Mouse Model Restores ERG b-Wave with Reversal of the Electronegative Waveform of X-Linked Retinoschisis. *Invest Ophthalmol Vis Sci.* 2004; 45(9):3279–3285. [PubMed: 15326152]
- Zhang YE. Non-Smad pathways in TGF-beta signaling. *Cell Res.* 2009; 19(1):128–139. [PubMed: 19114990]
- Zode GS, Kuehn MH, Nishimura DY, Searby CC, Mohan K, Grozdanic SD, Bugge K, Anderson MG, Clark AF, Stone EM, Sheffield VC. Reduction of ER stress via a chemical chaperone prevents disease phenotypes in a mouse model of primary open angle glaucoma. *J Clin Invest.* 2011; 121(9):3542–3553. [PubMed: 21821918]
- Zode GS, Sharma AB, Lin X, Searby CC, Bugge K, Kim GH, Clark AF, Sheffield VC. Ocular-specific ER stress reduction rescues glaucoma in murine glucocorticoid-induced glaucoma. *J Clin Invest.* 2014; 124(5):1956–1965. [PubMed: 24691439]

Highlights

- Viral vectors can be used to express transgenes in the rodent trabecular meshwork
- Over-expression of glaucoma associated transgenes elevates IOP in mice
- These inducible models of ocular hypertension will be useful as models of primary open angle glaucoma

Table 1

Summary of Viral Vectors Used or Potentially Usable in Creation of Animal Models of Glaucoma

Viral Vector	Integrative or Non-Integrative	Cloning Capacity	Advantages	Limitations
Herpes Simplex Virus (HSV)	Integrative	30 kb	Large cloning capacity.	Cytotoxicity; transient transgene expression.
Herpes Simplex Amplicon (Helper-Dependent HSV)	Integrative	>100 kb	Very large cloning capacity; limited immunogenicity; broad tropism.	Transient transgene expression.
Adenovirus (Ad)	Non-Integrative	36 kb	Large cloning capacity; high efficiency of gene delivery to both dividing and non-dividing cells.	Strong immunogenicity.
Helper-Dependent or Gutless Adenovirus (HDAd)	Non-Integrative	Up to 36 kb	Large cloning capacity; high efficiency of gene delivery to both dividing and non-dividing cells.	Transient transgene expression; innate and adaptive host immune response.
Adeno-Associated Virus (AAV)	Non-Integrative	4 to 5 kb	Non-pathogenic; high efficiency of gene delivery to both dividing and non-dividing cells; long lasting transgene expression.	Limited cloning capacity. AAV2 does not transduce TM efficiently. However scAAV8 shows promise.
Lentivirus	Integrative	6 to 8 kb	Stable transgene expression; high efficiency of gene delivery to both dividing and non-dividing cells; low immunogenicity.	Difficult to purify. Potential for insertional mutagenesis.
Vesicular Stomatitis Virus (VSV)	Non-Integrative	4 to 5 kb	Broad tropism with potential for improvement; genome easily manipulated; high efficiency of gene delivery to both dividing and non-dividing cells.	Can cause encephalitis in experimental animals.