

January 2013

Outflow Tract Ablation Using A Conditionally Cytotoxic Feline Immunodeficiency Viral Vector

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**Outflow Tract Ablation using a Conditionally Cytotoxic Feline Immunodeficiency
Viral Vector**

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Ze Zhang
Class of 2013

Under the mentorship of Dr. Nils Loewen

Outflow Tract Ablation using a Conditionally Cytotoxic Feline Immunodeficiency Viral Vector

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Abstract

Purpose: To establish a rat in vivo model of trabecular meshwork (TM) ablation and regeneration. Healthy TM cells are vital to regulate pressure. Ablation may be a useful strategy in glaucoma by reducing outflow resistance and possibly allowing dormant stem cells from adjacent structures to repopulate this structure.

Methods: Inducible, trackable, cytotoxic feline immunodeficiency viral vectors HSVtkiG were produced that expressed herpes simplex virus 1 thymidine kinase (HSVtk) and IRES-mediated eGFP, while the control vector GINSIN expressed cap-dependent eGFP. Filtered vectors were first used to transduce CrFK, GTM3, and NTM5 cells in vitro to confirm that ablation was feasible. Rats then received an intracameral vector into one eye (GINSIN n=10, HSVtkiG n=13), followed by ganciclovir (GCV) administration to trigger ablation. Intraocular pressure (IOP), central corneal thickness (CCT) and slit lamp exams were performed daily. Successful transduction was confirmed by direct gonioscopic visualization of eGFP expression and its disappearance following induction of ablation. Anterior segment histology was obtained at different time points.

Results: Durable and high-grade transgene expression in the TM was achieved both in vitro and in vivo in the rat model, followed by effective removal of the transduced TM cells using ganciclovir. Ablation resulted in an IOP decrease of 25% in HSVtkiG injected eyes 2 days after GCV. No change was observed in the GINSIN controls and non-injected eyes (n=11, P<0.05). The effect persisted for 6 weeks and then regressed. Decreased cellularity was noted at the time of lowest IOP.

Conclusion: We demonstrated successful ablation of TM in vitro and in vivo using a conditionally cytotoxic FIV vector. Selective ablation of TM cells in the rat lead to a statistically significant decrease in IOP without damaging surrounding tissue. This model may be useful to further study the TM, its stem cells and cell migration patterns.

Acknowledgements

I want to thank Dr. Nils Loewen for his incredible mentorship. He is the true definition of an ideal mentor, going above and beyond his duties to guide me. His unparalleled energy and enthusiasm has been and always will be a source of inspiration for me. He has set the example of the clinician scientist I aspire to become, and I truly look forward to continue to learn from him as I begin my training in ophthalmology.

I want to thank the rest of my lab: Harry Tseng, Julius Oatts, and Eberechi Nwogu for their support and camaraderie. Harry was invaluable in helping me with this project and I wish him great luck as he is beginning his journey to becoming a doctor at Albany Medical College. Julius and Eberechi have been incredible classmates as well as lab mates and I look forward to working with them and learning from them as we embark on the next stage of our careers.

I want to thank Dr. Bo Chen for generously offering to review my thesis. I want to thank Dr. Jun Lin for his help and guidance throughout this year. His fund of knowledge was extremely helpful and his advice was always greatly appreciated. I also want to thank Katie McFarland for all her help during my research. Without Katie, this project would not have gone nearly as smoothly. Katie worked tirelessly to ensure we received all our materials in a timely manner.

Finally, I want to thank my family and friends for all their support and encouragement throughout this process.

Funding

This research was supported in part by the American Glaucoma Society Clinical Scientist Award to Dr. Nils Loewen and by a stipend from the Office of Student Affairs and the Office of Student Research at the Yale University School of Medicine to support Ze Zhang.

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INTRODUCTION

Epidemiology of Glaucoma

Glaucoma is a progressive optic neuropathy that is the second leading cause of blindness worldwide and a leading cause of preventable blindness.(1). Glaucoma currently affects 1-in-40 adults who are older than 40 years, or 60 million people. By the year 2020, it will affect almost 80 million people in the world. (2, 3) Because incidence increases with age, prevalence further grows as a result of extending life expectancy. Glaucoma is grossly under-diagnosed even in developed countries. (2, 3) Proper diagnosis requires a thorough ophthalmologic exam of the optic disc and formal visual field testing. (4)

Types of glaucoma

Glaucoma is clinically categorized into open angle glaucomas (OAGs) and angle closure glaucomas (ACGs), both of which are further divided by primary and secondary pathomechanisms. Primarily open angle glaucomas are primary open angle glaucoma (POAG) and low pressure glaucoma (LTG). Pigmentary, exfoliation, steroid-induced, and traumatic glaucomas are classified as secondary open angle glaucomas. ACGs are distinguished from OAGs by the closure of the iridocorneal angle. (2) ACGs include primary angle-closure glaucoma (PACG) and secondary angle closure glaucomas. (2) Secondary angle closure glaucomas result from a known disease pathology, such as neovascularization or uveitis, leading to angle closure. (2) This review will focus on OAGs.

POAG often occurs in the presence of intraocular hypertension and usually progresses over many months to years to cause optic nerve damage and death of retinal ganglion cells (RGC). However, susceptible eyes can also manifest damage from low pressure glaucoma, (5, 6) which

accounts for up to 30% of all OAGs. (7, 8) Characteristic structural loss that is progressive is most commonly seen at the inferior and superior pole of the optic nerve but may be diffuse, leads to corresponding visual field defects. (2)

Pathogenesis of POAG

The pathogenesis of POAG is still poorly understood. The major risk factor is elevated intraocular pressure (IOP). The Baltimore eye Survey (8) and the Barbados eye study (9) showed an association between elevated IOP and glaucoma, and the Ocular Hypertension Treatment Study (OHTS) (10, 11) and the Early Manifest Glaucoma Trial (EMGT) (12, 13) confirmed the causal relationship between lowering IOP and preventing glaucoma progression. However, the level of IOP is not a defining criterion for glaucoma, as low and normal IOPs may lead to glaucoma in individuals with low pressure glaucoma. (2) Prolonged elevation in IOP causes direct damage to the retinal ganglion cell axons and leading to retinal ganglion cell apoptosis that form the optic nerve and worsen vascular compromise that may be present, (14-16) Elevated IOP is generally related to impairment of outflow of aqueous humor from the anterior chamber. (17, 18)

Aqueous humor is produced by the ciliary body, which moves through the pupil into the anterior chamber, flows through the trabecular meshwork (TM), and exits the eye via Schlemm's canal to reach the episcleral veins. (15) The TM is a reticular structure containing highly metabolically active but non-dividing endothelial cells in the angle of the anterior chamber. In humans, the TM generates much of the resistance to aqueous humor outflow. (14, 17, 18) The relationship between aqueous humor production and IOP is summarized by the Goldmann equation: $P_i = F/C + P_v - U$ where P_i is the intraocular pressure in mmHg, F is the rate of aqueous humor

production in microliters per minute, P_e is the episcleral venous pressure in mmHg. C represents the tonographic facility of outflow in microliters per minute per mm Hg, and U is a pressure-independent parameter representing the uveoscleral outflow.(19-21)

Current treatment of glaucoma

No treatment currently exists to reverse the optic neuropathy in glaucoma. Treatment focuses on lowering IOP for all patients with glaucoma. For patients with a diagnosis of POAG, lowering of IOP is always necessary regardless of whether IOP is above 21 mmHg and establishes the basis of the treatment algorithm. (22) The Ocular Hypertension Treatment Study (OHTS) showed that IOP lowering treatment leads to clinical benefit in patients even before initial damage is seen on ophthalmic exam. (10) Current IOP-lowering treatment options for POAG include laser therapy, medical treatment, and surgical interventions.

Laser Therapy

Traditionally, medical therapy has been the first-line regimen for OAGs. However, poor adherence and high cost are among some of the reasons plaguing medical therapy. (23) Laser trabeculoplasty is now recognized as more effective, better, tolerated and considerably more economic than topical medical therapy. In 1995, The Glaucoma Laser Trial (GLT) showed argon laser trabeculoplasty (ALT) is equally effective in the treatment of glaucoma as a single medication. (24, 25) but concerns regarding potential bias and the damaging effect of ALT on the TM lead to poor adoption of ALT as first-line therapy. Ten years later, Francis et al showed that use of selective laser trabeculoplasty (SLT) lead to reduction in the number of medications for 97% of patients at 6 months and 87% at 12 months. (26) Several nonrandomized studies have also shown comparable IOP reduction (up to 30%) using SLT and prostaglandin analogs.(27, 28) Most recently, a study by Katz et al showed similar IOP reduction between 360-

degree-SLT and prostaglandin analogs in a prospective randomized trial of 69 patients with OAG or ocular hypertension (OHT). However, the medication group required greater treatment complexity to maintain the target IOP, supporting the use of SLT as initial therapy in OAG. (29) Despite the advantages, laser trabeculoplasty effects are not permanent (last months to years) with limited options for repetitions and are not effective in all patients. (30)

Medical treatment

Topical medication classes include topical beta blockers, alpha-agonists, prostaglandin analogs, and carbonic anhydrase inhibitors. Topical eye drops function to modulate the aqueous humor production or outflow in order to decrease IOP and fall in three broad categories: decreasing aqueous humor production (alpha agonists, beta blockers and carbonic anhydrase inhibitor), increasing conventional outflow of aqueous humor (pilocarpine), or modulating the uveoscleral outflow pathway (prostaglandin analogs). (31-33) Although medical therapy can be effective, their efficacy is often decreased by high cost, side effects, and poor compliance. Unfortunately, poor adherence to medical therapy is common and very difficult to manage.(23)

Surgical therapy

Surgical therapy remains a mainstay treatment in glaucoma, particularly in patients who have had SLT and are refractory on maximum medical therapy. Surgical interventions range from minimally invasive procedures such as the Trabectome (34) (Neomedix, Tustin, CA), Canaloplasty (35-37) (Science Interventional, Menlo Park, CA), suprachoroidal shunts (Gold Shunt (38) Solx, Waltham MA; CyPass (39), Transcend Medical, Menlo Park, CA; iStent (40) Glaukos, Laguna Hills CA). and endoscopic cyclophotocoagulation (41) (ECP) (EndoOptiks, Little Silver, NJ), to more invasive traditional filtration procedures.

Studies on the Trabectome show up to 42% decrease in IOP after 12 months, and up to 38% mean IOP decrease after 6 years. (42, 43) With Canaloplasty, IOP can be lowered up to 44% when combined with phacoemulsification and 40% when performed alone. (35, 37) The SOLX suprachoroidal gold micro shunt demonstrated a 33% decrease in IOP from baseline in a population of patients who had failed other surgeries. (38) The interim CyPass shunt results recently showed 71.4% reduction in medication use for patients and a low rate of surgical complications. (39) The iStent currently does not have any published results. A recent prospective ECP study with patients with uncontrolled glaucoma with previous tube shunt surgery showed a mean IOP decrease of 30.8% baseline at 12 months with no serious complications. (44)

Traditional surgical therapies involve shunting the aqueous flow into the subtenon space. (45) The most common glaucoma filtering surgery is the trabeculectomy, which involves creating a bleb overlying the sclera for aqueous humor to exit. The procedure can achieve long lasting IOP lowering and adjuvant therapy using topical anti-fibrosis drugs can decrease the risk of bleb failure. (22) Tube shunt surgery is also commonly used and have similar outcomes as trabeculectomy in patients who have a high risk of failure with trabeculectomy. (46) The Tube versus Trabeculectomy (TVT) study showed that tube shunts and trabeculectomy achieved similar IOP lowering: 12.4 mmHg for tube and 12.7 mmHg for trabeculectomy. (45) At 5-year follow-up, the IOP control remained comparable, with 14.4 mm Hg for the tube group and 12.6 mm Hg for the trabeculectomy group ($p=0.12$). (47)

Both tube shunt and trabeculectomy procedures are invasive and carry significant risks, and do not always stop the patients from requiring additional medical therapy. (45) In the TVT study, 21% of tube shunts and 37% of trabeculectomies suffered from early postoperative complications including choroidal effusion, flat anterior chamber, wound leaks, hyphema, corneal edema, etc. (45, 48) At 5-year follow-up, 36% of tube shunts and 38% of trabeculectomies experienced additional complications leading to vision loss of 2 lines or re-operation. Other long term complications include bleb leak and blebitis for trabeculectomy, and endophthalmitis for both. (49)

Comparison of therapies

The effectiveness of the three modalities of IOP lowering treatment have been examined extensively in randomized controlled trials. The Advanced Glaucoma Intervention Study (50) (AGIS), the Collaborative Initial Glaucoma Treatment Study (51) (CIGTS), and the GLT study (25) showed the primary differences lie in the rate of IOP lowering and the side effects associated with treatment. Surgical therapies carry greater risks of vision loss while laser therapy had the lowest rate of side effects. Medical therapy also carries significant side effects, including ocular surface disease and toxicity. (52)

Despite the many treatment options available for POAG, unfortunately, the risk of blindness from glaucoma remains high because of poor efficacy, poor compliance, and high complication rate of existing therapeutic modalities. A permanent therapy with minimal side effects to correct glaucoma pathophysiology would be highly desirable. Thus, a new, simple, lasting, and non-invasive method for IOP control is much needed and currently heavily researched.

Future therapies

Gene therapy

Gene therapy involves replacing mutated gene with a healthy copy of the gene, knocking out aberrant genes or adding or removing genes to change the pathophysiology of disease and change expression of proteins long-term. (52) Gene therapy has been implicated as potential modality for treating human disease for several decades (52) but has been complicated by ethical and safety concerns as well as poor efficacy. The possibility of gene therapy has been greatly increased by the advances in safety and specificity of delivery systems. Gene therapy delivery systems include viral vectors (discussed below), and nonviral systems including liposomes/synthetic polymers, RNA interference, electroporation, and direct DNA injection. (52, 53) Compared to viral vectors, non-viral systems suffer from transient expression and lower production and transfection efficiency. (52)

Advantages of gene therapy as therapeutic modality for glaucoma

The eye allows gene therapy to be used as targeted treatment without the interference of the inflammatory mediators due to lack of vascularization.. The size and accessibility of the eye allow gene therapeutic targeting very effective and the compartmentalized nature of ocular tissues means viral vector doses can remain low and minimize risks of adverse effects. Many genes have been identified related to ocular disease to date, several in glaucoma. (54) After many years of ocular gene therapy research, ocular gene therapy has achieved reality in the treatment of Leber's congenital amaurosis, where replacement of the mutant copy of the RPE65 with the wild type gene in blind patients through subretinal injection lead to significant improvement in vision with no safety effects (55, 56) and other areas of ocular gene therapy are very promising as well. New experimental methods have been developed using both AAV and

lentiviral-mediated vectors for gene transfer in areas of retinitis pigmentosa, age-related macular degeneration, uveitis, and glaucoma. (54)

There has been a significant focus on studying the genetic pathogenesis of glaucoma and the potential for gene replacement therapies and gene silencing as effective treatments. (54)

The nature of glaucoma makes it a good candidate disease for gene therapy. Even though pathogenesis remains poorly understood, the small amount of tissue involved in glaucoma and their accessibility through the anterior eye makes it much easier to achieve adequate levels of transgene expression required to alter aqueous humor dynamics and normalize IOP. A new treatment based on gene therapy would be highly desirable.

Viral vectors in gene therapy

Herpes simplex viral vectors

Herpes simplex virus (HSV) vectors have been previously used for neuronal gene delivery into rat and monkey eyes using a replication-competent HSV type 1 ribonucleotide mutant to express the lacZ gene, which efficiently transduced the TM, ciliary body, and retinal ganglion cells.

However, the limited promoter selection, inflammatory responses to HSV, and possible cytotoxicity limited the duration of the expression of the transgenes. (31)

Adeno-associated viral vectors

Adeno-associated virus (AAV) vectors are commonly used vector systems in both ocular and systemic gene therapy (54) because of the low immunogenicity and lack of association with human disease. AAV is a replication deficient virus that requires adenovirus to function.

Although integration is not required for their life cycle, AAV vectors can integrate into the DNA of both dividing and nondividing cells. (57) The wild type AAV tends to integrate into active

genes, creating concerns for mutagenicity. (57) Although the AAV vector can provide sustained long term expression. (54) it has been unable to transduce the TM in vivo and is limited by low carrying capacity. (58)

Adenovirus vectors

Adenoviruses (Ad) are medium sized non-enveloped DNA viruses that resulted in the earliest vector systems developed. They are non-integrating, able to reach high titers, and infect many dividing and nondividing cells. (54, 59, 60) The recombinant Ad vectors have a high tropism for the outflow tract tissue, including the TM, in many animal species, including human donor eyes, rats, rabbits, mice, and nonhuman primate. (58, 61-64) Expression of transgenes using Ad vectors have lasted up to 1 year. (65) However, Ad vectors are limited by the short transgene carrying capacity and the induction of inflammatory responses at high concentrations. (58) Still, due to the broad tropism Ad vectors remain useful for determining transgene effects and developing elevated IOP models with functional TM.

Lentiviral vectors

HSV and Ad vectors are unable to achieve stable, long-term targeted transgene expression in the outflow tract due to limited duration, induction of inflammation, and lack of successful transduction of the TM. AAV-based vectors are able to achieve long-term expression in the TM but still suffer from small packaging capabilities(58, 66). Lentiviral vectors, on the other hand, have been shown to overcome many of these shortcomings. (1, 67-69)

Lentiviruses are single-stranded RNA retroviruses that integrate into the host genome following reverse transcription.(31, 66) These lentiviral vectors carry the required cellular and molecular components for stable transduction and permanent integration into both mitotically active and

post-mitotic cells in various organs, including the eye. (1, 66, 67, 70) Lentiviral vectors can be concentrated with ultracentrifugation if pseudotyped with envelope proteins that provide high shear force stability (68, 71) to create high titers. Their genomes can be changed to remove almost all potentially deleterious viral components that may cause inflammation or disease in the target host. (66) Additionally, lentiviral vectors are able to provide greater packaging capabilities of 8.7 kilobase pairs (kb) and large polycistronic messages that can contain introns compared to the approximately 4.7 kilobase pairs (Kb) of AAV vectors. (66)

Lentiviral vectors have been derived from various lentiviruses, including primate: HIV-1, HIV-2, SIV, and non-primate: FIV, EIAV, and BIV. (66) The possibility of replication competent lentiviruses created during vector manufacture has been greatly reduced with the use of 3 and 4 plasmid systems.(68, 71) Psychological barriers still remain, especially for non-lethal disorders where other therapies exist. (66) Thus, non-primate lentiviruses such as the FIV and EIAV and BIV can be used to address biosafety concerns, because these viruses are highly species-specific and would be unlikely to replicate or cause any known side effects in human tissue. Moreover, mammalian cells possess the intrinsic ability to restrict retroviral replication using proteins as part of innate immunity. These restriction factors are either constitutively active or inducible and provide further protection against potential retroviral pathogenicity. (72) Additionally, highly deleted and self-inactivating lentiviral vectors have been developed to minimize the concern for oncogenesis. (73, 74) Thus, the rational design of lentiviral vectors, coupled with nature's ability to withstand pathogen threats, render non-primate lentiviral vectors viable and effective means for genetic modification.

Previous use of lentiviral vectors

Lentiviral vectors are pseudotyped through replacement of the lentiviral enveloped protein gp120 with the vesicular stomatitis virus envelope glycoproteins (VSV-G). (71, 75) This provides broad tropisms of host cell entry. Previous studies have used intracameral delivery of VSV-G pseudotyped lentiviral vectors to transduce both the corneal endothelium and the TM in rodent model. (76-80) Both HIV and FIV vectors have been used in many species and successfully delivered to the TM.

FIV vectors' ability to transduce differentiated cells like those in the outflow tract permanently and safely integrate into the genome allowing long-term and stable expression makes FIV vectors a highly desirable model for glaucoma treatment. FIV vectors have successfully targeted the TM in cats and nonhuman primates. (1, 67, 81) In cats, FIV vectors delivered enhanced green fluorescent protein (eGFP) to the TM using both 5' cap translation and internal ribosomal entry site (IRES) translation. In vivo expression was high grade, stable, and sustained in the TM for more than 10 months with no effect on IOP. (70) Similarly, FIV vectors carrying different prostaglandin pathway genes, injected intracamerally, lead to sustained IOP reduction for more than 5 months. (66, 82) In monkeys, FIV transgene expression lasted more than one year after one intracameral injection.

FIV vectors have also transduced the TM after intracameral injection of organ-cultured human eyes with no change in morphology or cellular loss. (14, 67) Thus, FIV vectors enable safe, long-term gene expression that is promising as a gene therapy modality for glaucoma.

Hypotheses regarding POAG outflow resistance

The actual cellular and extracellular mechanisms causing outflow resistance in POAG is still poorly understood. There are conflicting theories regarding where this outflow resistance arises: whether it is due to change in cell density and function of the TM and the Schlemm's canal.(83-85) or due to gradual accumulation and build-up of extracellular matrix materials in the TM. (86-88) There is also evidence to suggest there are TM stem cells located anterior the TM that may be activated after TM injury. (89, 90) Unfortunately, the change in TM cell population is not well understood since there lack tools to selectively remove these cells to study the effect of TM injury on cell repopulation and migration.

HSVtk system to selectively remove the TM

In order to test the cellular hypothesis as a cause for POAG, it is important to be able to selectively remove the TM without damaging the surrounding tissue and to study the effect of TM removal and repopulation on IOP and aqueous humor dynamics. One method to remove the TM is to use the selectively targeting properties of intracameral injections of a FIV vector carrying a suicide gene that would target TM cells for apoptosis without causing surrounding tissue toxicity.

One such system is the herpes simplex virus thymidine kinase (HSVtk) -ganciclovir (GCV) system. (91) HSV-tk containing cells are able to metabolize GCV into a triphosphate form that can be incorporated into the double stranded DNA during replication, which causes base pair mismatches and DNA breakage and fragmentation. (92)

The HSVtk-GCV system is often used in suicide gene therapy in cancer research. (93)

Suicide gene therapy in cancer utilizes the conversion of a non-toxic prodrug into a toxic metabolite by a gene-encoded enzyme system, such as the HSVtk mediated conversion of GCV into a GCV-triphosphate form that is toxic to cells. This was first demonstrated by Moolten et al, when genetic transfer of HSV-tk to tumor cells conferred chemosensitivity to GCV. (94) Subsequently, the HSVtk-GCV system has been used as suicide gene therapy in a wide variety of tumors, including, gliomas, medulloblastomas, prostate tumors, bladder tumors, and lung tumors. (93, 95)

Because of the non-toxic response of GCV in normal cells that do not contain HSVtk, a FIV vector carrying HSVtk, selectively delivered to the TM through an intracameral injection, followed by GCV administration, can remove the TM without damage to surrounding tissue, and enable the study of TM cellular changes and repopulation and their effects on IOP and aqueous humor dynamics.

STATEMENT OF PURPOSE, SPECIFIC AIMS, AND HYPOTHESIS

PURPOSE

To create an inducible cyto-ablative FIV vector that can selectively transduce and remove the TM to study the effect of ablation and repopulation of this structure on IOP in a rodent model.

SPECIFIC AIMS

1. Generate a cyto-ablative (FIV) vector (HSVtkiG) co-expressing a live trackable marker, eGFP, and compare function in vitro to an established FIV vector (GINSIN) expressing eGFP.
2. Establish in vivo transduction of TM and visualization with HSVtkiG and GINSIN in the rat.
3. Evaluate effects of ablation on IOP and the anterior segment over time.

HYPOTHESIS

We hypothesized that selective ablation of TM with a conditionally cytotoxic vector will result in reduced outflow resistance and a lower IOP but that this effect will regress over time indicating regeneration from dormant progenitor cells of adjacent tissue.

METHODS AND MATERIALS

FIV vector construction

Vesicular stomatitis virus glycoprotein G-pseudotyped (VSV-G) feline immunodeficiency viral (FIV) vectors were produced as previously described (68) using a tripartite vector system consisting of envelope plasmid pMD.G (68), packaging plasmid pFP93 (68), and two different FIV transfer vectors, pGINSIN (67, 68) and pHSVtkiG (Figure 1). Control vector GINSIN encoded enhanced green fluorescent protein (eGFP), and neomycin resistance via an internal ribosomal entry site (IRES). Vector pHSVtkiG, expressed herpes-simplex virus 1 thymidine kinase (#12382, Addgene, Cambridge, MA) via a CMV promoter and eGFP via an IRES. HSVtk converts nucleotide analogues to the diphosphorylated form which can be converted by cellular tyrosine kinases to the toxic triphosphate form that competes with normal nucleosides in DNA replication, preventing elongation(92).

Stbl3 E. Coli were transformed with the above plasmids (Invitrogen, Grand Island, NY) at 30°C overnight. Colonies were selected and expanded for DNA extraction using Miniprep kit (Qiagen, Valencia, CA). Plasmids were confirmed as correct as described in the following: pMD.G was restricted using EcoRI resulting in bands with 4153 and 1671 base pairs (bp) and NdeI resulting in bands with 3651, 1556, and 617 bp. pFP93 was restricted using BamHI resulting in bands with 5748, 4352, 990 bp and HindIII resulting in bands with 5869, 2739, 2482 bp. pGINSIN was restricted using EcoRI resulting in 7667 and 6197 bp bands and SphI resulting in bands with 7312 and 4081 bp. pHSVtkiG was restricted using Sph I resulting in bands with 6825 and 1811 bp, and Pst I resulting in bands with 8115, 406, and 15 bp. Correct clones were further expanded

in 100 mL cultures and DNA extracted endotoxin free and confirmed again by restriction digests as above (EndoFree Maxiprep kit, Qiagen, Valencia, CA).

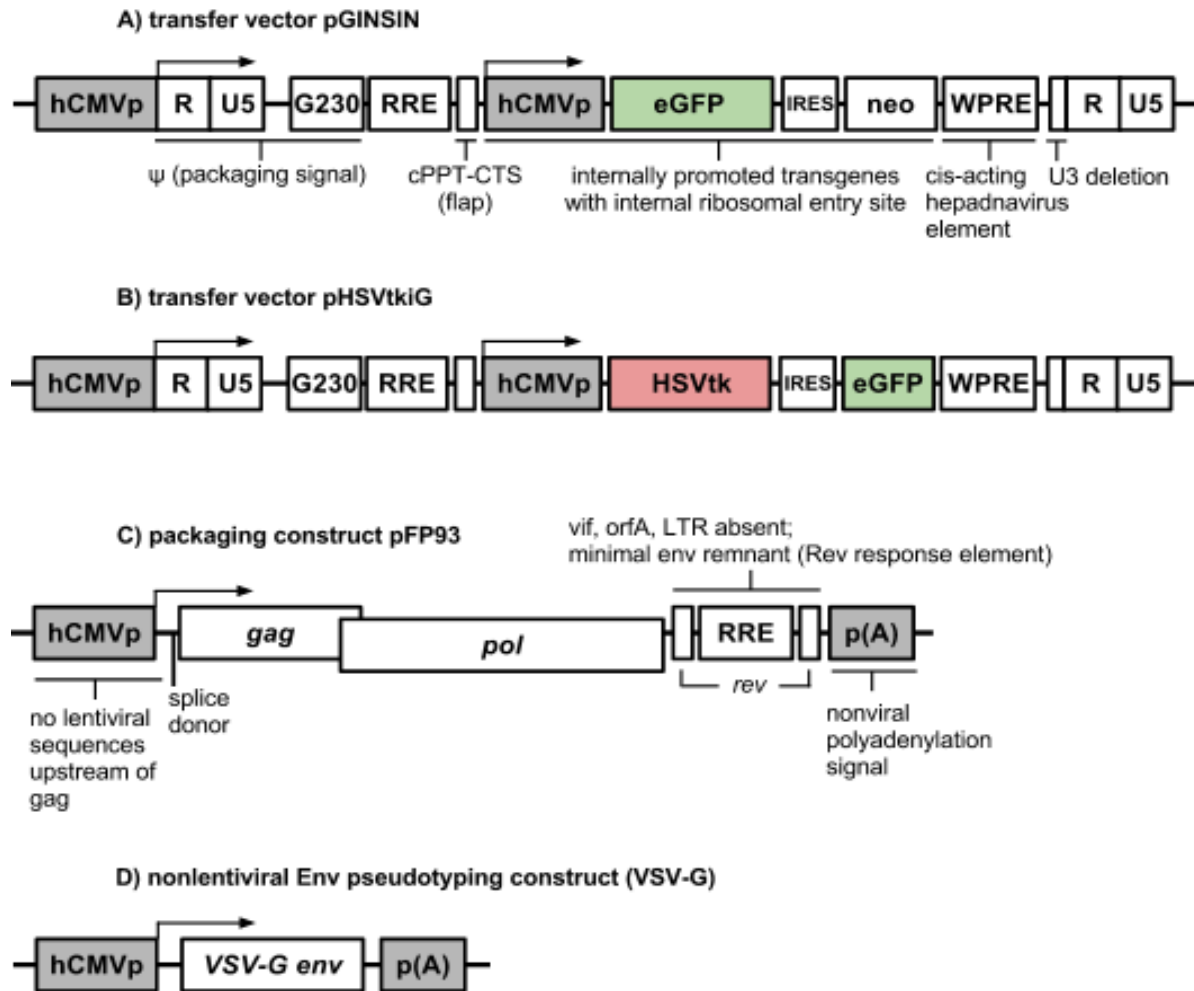


Figure 1. Map of FIV vectors. A) EGFP expressing transfer vector pGINSIN. hCMVp: CMV-promoter; RRE: rev-response element; eGFP: enhanced green fluorescent protein; IRES: internal ribosomal entry site; neo: neomycin-resistance; WPRE: woodchuck hepatitis post-transcriptional regulatory element; cPPT: central polypurine tract; CTS: central termination sequence. B) Cytoablative transfer vector HSVtkiG expressing HSVtk and IRES-mediated eGFP. HSVtk: herpes simplex viral thymidine kinase. C) Packaging construct pFP93 that delivers all . gag: group specific antigen; pol: polymerase; p(A): polypurine tract; VSV-G: vesicular stomatitis virus envelope glycoprotein.

Vector production and titration

FIV vectors were produced by transient transfection of 293T/C17 cells (CRL-11268; ATCC, Manassas, VA) by calcium phosphate precipitation with a 3/3/1 ratio of envelope/packaging/transfer plasmid. (96)

Briefly, 293T/C17 cells were maintained at a high frequency of passage in the logarithmic phase of growth. 500 million 293T/17 cells were seeded onto a Nunc Cell Factory-10 (CF10) (Cell Factories, Nunc, Naperville, IL) with 900 mL of culture medium (high glucose Dulbecco's minimal essential medium with L-glutamine and 10% heat-inactivated FBS and 1% penicillin/streptomycin) and incubated overnight at 37°C in 5% CO₂ environment. To create the calcium phosphate precipitation mix, 250 µg packaging plasmid (pFP93), 250 µg transfer plasmid (pGINSIN or pHSVtkiG), and 83.3 µg envelope plasmid (pMD.G), was added to 0.01 M Tris (pH 8.0) to a volume of 60.75 mL. 6.75 mL 2.5 M CaCl₂ and 6.75 mL 2X HEPES buffered saline (2X HBS, pH 6.95), was then added to the DNA mixture and allowed to precipitate for 3 minutes at room temperature. The media from the CF10 was poured into two 500 mL sterile bottles. Half of the volume of the calcium phosphate-DNA mix was added to each 500 mL bottle containing the culture medium after 3 minutes of precipitation. The medium containing the DNA mixture was then added back to the CF10 and allowed to equilibrate at room temperature for 60 seconds before placed back into the incubator.

Calcium-phosphate-DNA crystals were visualized after addition of transfection mix under low power using a tissue culture microscope. Transfection mix was allowed to settle for 12-16 hours before replacement with fresh culture medium. Vector supernatants were harvested 48 hours

after replacement of transfection mix, filtered through 0.45 µm sterile filter bottle units (Corning, Corning, NY) before storage in -80 °C or further concentration.

900 mL of vector supernatants, separated into 4 separate buckets, were concentrated by double ultracentrifugation using a Type 19 fixed angle rotor (Beckman Coulter, Brea, CA) at 19,000 rpm for 6 hours followed by resuspension in 10 mL of phosphate-buffered saline (PBS) in 4 °C by overnight shaking. Subsequent concentration used a SW-34 swinging bucket rotor (Beckman Coulter, Brea, CA) for 2 hours at 25,000 rpm in a Beckman Coulter Optima L90 ultracentrifuge and again resuspended in 2 mL of PBS by rapid pipetting. (96) Vector titer was determined by fluorescence assisted flow cytometry (FACS) analysis of GFP expression in transduced Crandell feline kidney (CrFK) cells using an LSR-II (BD Biosciences, Sparks, MD) as previously described. (96)

In Vitro Studies

CrFK (CCL-94; ATCC, Manassas, VA), GTM3, and NTM5 cells (gift from Alcon, Fort Worth, TX) were maintained in high glucose Dulbecco's minimal essential medium + L-glutamine (Invitrogen, Carlsbad, CA). The medium was supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin G and 100 µg/mL streptomycin (Invitrogen). Cells were incubated in 5% humidified CO₂ incubator at 37 °C.

CrFK, GTM3, and NTM5 cells were transduced with escalating multiplicity of infection (MOI) using both GINSIN and HSVtkiG vectors. MOIs of 3, 5, 10, and 30 were tested. The medium was replaced twice a week and successful transduction was confirmed by fluorescence at 48 hours. Both GINSIN and HSVtkiG transduced cells were expanded and seeded onto 6-well plates for

ablation using 30 mg/mL of ganciclovir (AK Scientific, Union City, CA). Cell death was assessed by visualization after 24 hours and 48 hours following ganciclovir treatment. All fluorescent images were captured using a digital camera (Coolpix 900, Nikon, Melville, NY) mounted to a fluorescence microscope with an eGFP-optimized filter cube (Axiovert, Zeiss, Oberkochen, Germany) at 24 hours and 48 hours after ganciclovir treatment.

In Vivo Studies

All animal were handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and all protocols were approved and monitored by the Yale University Institutional Animal Care and Use Committee. Eight-week old female Sprague-Dawley rats (Charles River, MA) used in this study were housed up to four in a cage, and maintained in 12-hour light-dark cycles at temperatures of 20-22°C with food and water available ad libitum. All rats were allowed to acclimate for 3 days in the new environment without any procedures performed. After 3 days of acclimation, daily IOP and central corneal thickness measurements were performed for 7 consecutive days prior to injection procedures, as well as on days 1, 3, 7, 10, 14 after injection, and weekly thereafter.

Examination and Measurement

IOP measurement

All IOP measurements were conducted using a rebound tonometer (iCare, Espoo, Finland). Animals were allowed to acclimate to daily IOP measurements for 7 consecutive days prior to injection, after which measurements were performed as described above. For consistency of measurement of IOP without anesthesia, all animals were restrained using the same restraint

device and all measurements were taken at the same time each day for all animals. IOP measurements were taken in triplicate and reported as the mean.

Central Corneal Thickness (CCT) measurement

CCT measurements were made using the DGH Pachmate (DGH technologies, Exton, PA). For consistency of CCT measurements, 1 drop of 0.5% sterile proparacaine was instilled prior to each CCT measurement following IOP measurement. CCT measurements were made in triplicate and reported as the mean.

Animal Injections

At Day 0, prior to injections, the animals were anesthetized with intraperitoneal ketamine (65 mg/kg) and xylazine (5 mg/kg). Appropriate depth of general anesthesia was assessed by paw pinch reflex. Custom-made 32G, 30 ° bevel, 9.52mm long needles attached to a 10 µL Hamilton microsyringe (Hamilton, Reno, NV) was used for all animal injections. A sterile 30-gauge subcutaneous needle was used to puncture the cornea to tap the anterior chamber of the right eye of each animal 10 seconds prior to ocular injection. After the anterior chamber tap, 5µL of 1.5×10^9 TU/mL of FIV vector suspended in PBS was injected intracamerally into the right eye of each animal using the same corneal opening. The needle was left in place for 30 seconds prior to withdrawal to prevent leakage or reflux of injected vector. One drop of 1% prednisolone-acetate and one drop of 0.5% moxifloxacin was applied to the right eye after injection. Eight animals were injected with GINSIN control vector carrying eGFP. 16 animals were injected with HSVtkiG vector carrying HSVTK and eGFP. All animals were monitored in a warm environment until they recovered from anesthesia and were sternally recumbent and ambulatory.

Ablation of the rat TM

Intraperitoneal ganciclovir was administered to each animal in the ablation group for 4 consecutive days at 25 mg/kg twice a day, starting at Day 7 after injection.

Fluorescence biomicroscopy and imaging

Animals were photographed by direct gonioscopy using an inverted fluorescence microscope. Animals were anesthetized with IP ketamine (65 mg/kg) and xylazine (5 mg/kg). Animals were placed on a flat surface on the lateral side, and viscoelastic was applied to the eye before the eye was placed against the flat surface facing the objective of the microscope. All fluorescent images were captured using a digital camera (Coolpix 900, Nikon, Melville, NY) mounted to a fluorescence microscope with an eGFP-optimized filter cube (Axiovert, Zeiss, Oberkochen, Germany).

Quantification of fluorescence

In vivo TM fluorescence was visualized during direct gonioscopy and was graded on a scale from 0 to 4, with sample representative photographs shown in Figure X as previously described (Loewen 2004). Grade 0 was defined as no detectable fluorescence; grade 1 was single fluorescent spots; grade 2 was numerous, nonconfluent fluorescent spots; grade 3 was extensive, mostly confluent transduction; and grade 4 was extensive, high-level and completely confluent transduction of fluorescence.

Histology

At various time points (Table X or Figure X), animals were euthanized with 1 mL of intraperitoneal Euthasol (Virbac Corp, Fort Worth, TX). Eyes were enucleated after confirmation of death. The enucleated eyes were fixed in 10% buffered formalin for 24 hours, before they

were embedded in paraffin wax for sectioning. 5 μ m sections were stained with hematoxylin and eosin reagent for visualization of the ocular structures.

Power calculation

Our power calculation showed we needed n=12 animals to have 80% power to detect an IOP difference of 3 mm Hg with a standard deviation of 1.5 mm Hg using a paired t-test and an alpha error of 0.05.

Statistical analysis

All IOP and CCT data were entered using Microsoft Excel 2010 and transferred to SPSS.

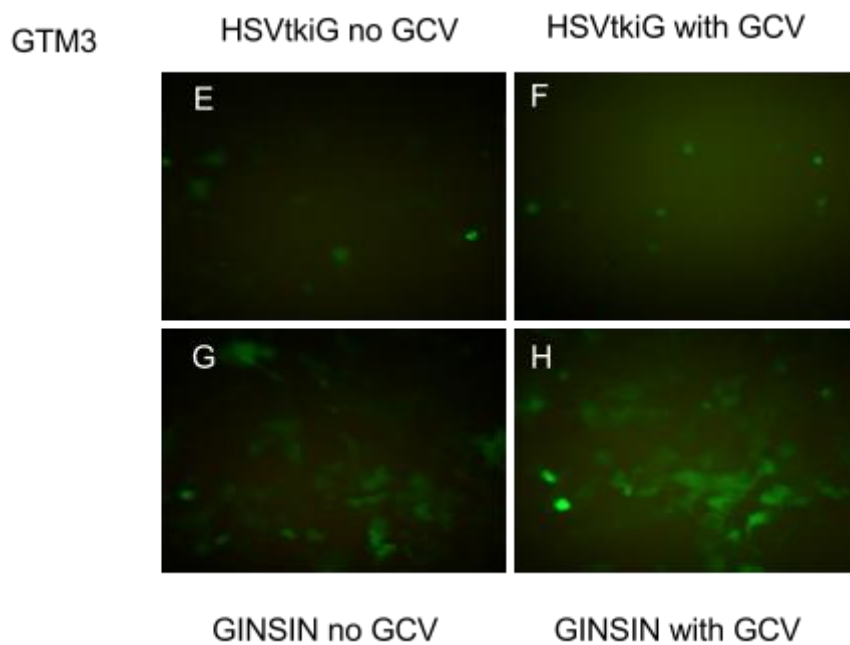
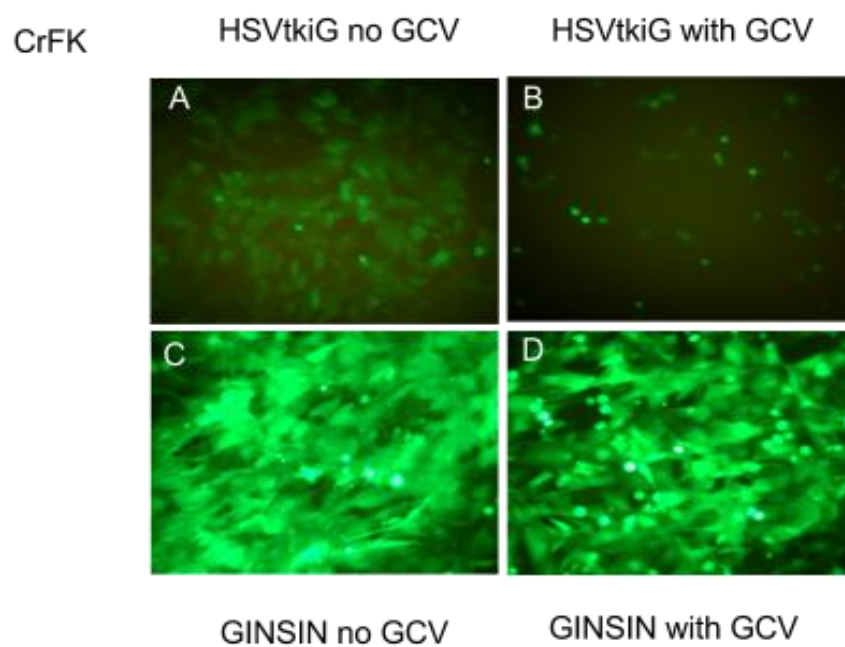
Student's t-test was performed to compare IOP and central corneal thickness between groups.

Differences were considered statistically significant if $p < 0.05$. Statistical significance between groups was determined with statistical analysis software (SPSS version 20, Chicago, IL).

RESULTS

In vitro transduction and ablation

The ablative vector HSVtkiG/eGFP induced apoptosis in transduced CrFK, NTM5, and GTM3 cells within 48 hours of exposure to ganciclovir. The extent of ablation was titrated both by vector dilution and concentration of GCV. A multiplicity of infection (MOI) of 5 transducing units (TU) per cell and treatment with 30 mcg/mL ganciclovir resulted in elimination of virtually all transduced cells and higher GCV concentrations lead to faster cell death in transduced CrFK, GTM3, and NTM5 cells. Cells transduced with HSVtkiG but not exposed to GCV grew to 100% confluence within 48 hours. Cells transduced with the non-ablative control vector GINSIN and non-transduced cells had no response to ganciclovir and grew to full confluence after 48 hours (Figure 2). EGFP was used as the marker to monitor cell proliferation and cell apoptosis. eGFP is dimmer in the cells transduced with HSVtkiG because it is mediated by the internal ribosomal entry site (IRES) (Figure 1, 2).



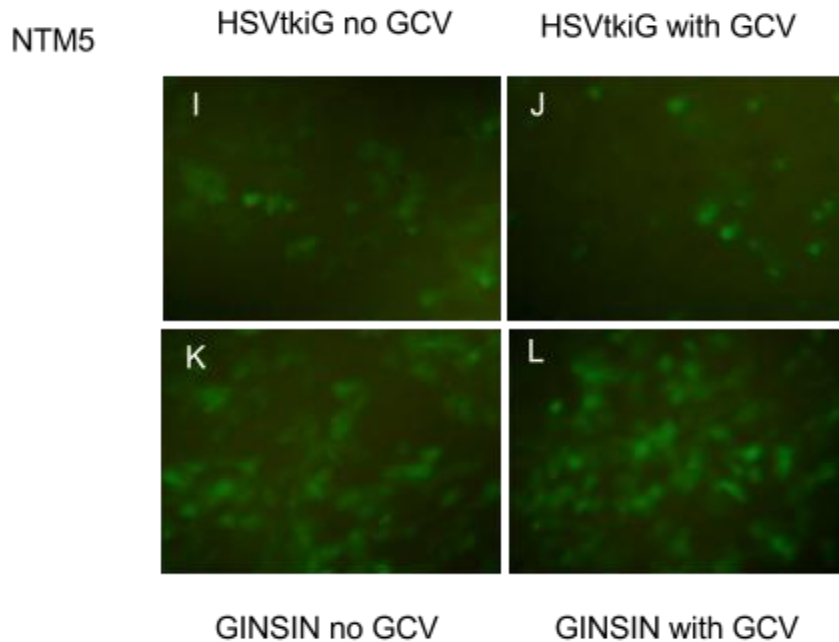


Figure 2. In vitro cytotoxicity of vector HSVtkiG: CrFK, GTM3, and NTM5 cells transduced by HSVtkiG are all ablated by ganciclovir after 48 hours treatment (B,F,J). eGFP serves as live marker that appears dimmer when IRES-mediated (HSVtkiG: A,B,E,F,I, J) compared to 5' cap-dependent (control vector GINSIN: C,D,G,H,K,L). CrFK, GTM3, and NTM5 cells transduced with control vector GINSIN do not respond to ganciclovir treatment (D,H,L).

In vivo results

Intraocular pressure

IOP increased significantly in all injected eyes (both HSVtkiG and GINSIN groups) compared to non-injected control eyes the first day after injection. IOP returned to baseline and did not differ from the uninjected control group starting Day 2 after injection. There was no statistical difference between GINSIN and HSVtkiG group pressures prior to administration of the ablative

drug GCV (16.1 ± 1.1 mmHg, $p=0.66$). Ganciclovir administration started at Day 7 after injection in both GINSIN and HSVtkiG groups. The non-injected control eyes and the eyes in the GINSIN group did not demonstrate any IOP response to GCV administration. The IOP in eyes in the HSVtkiG group decreased. Starting at Day 9 post-injection, after 2 days of ganciclovir administration, the IOP in the HSVtkiG group was significantly lower than the IOP in the control group and the GINSIN group. This difference persisted through Week 5 (Day 35) after GCV administration (Figure 3). Trough IOP was 25% lower than IOP at baseline prior to injection ($n=11$, $p<0.05$). After week 5, the IOP in the HSVtkiG+GCV group rose to baseline, and was no longer statistically lower than IOP in the two control groups and persisted till the end of follow-up.

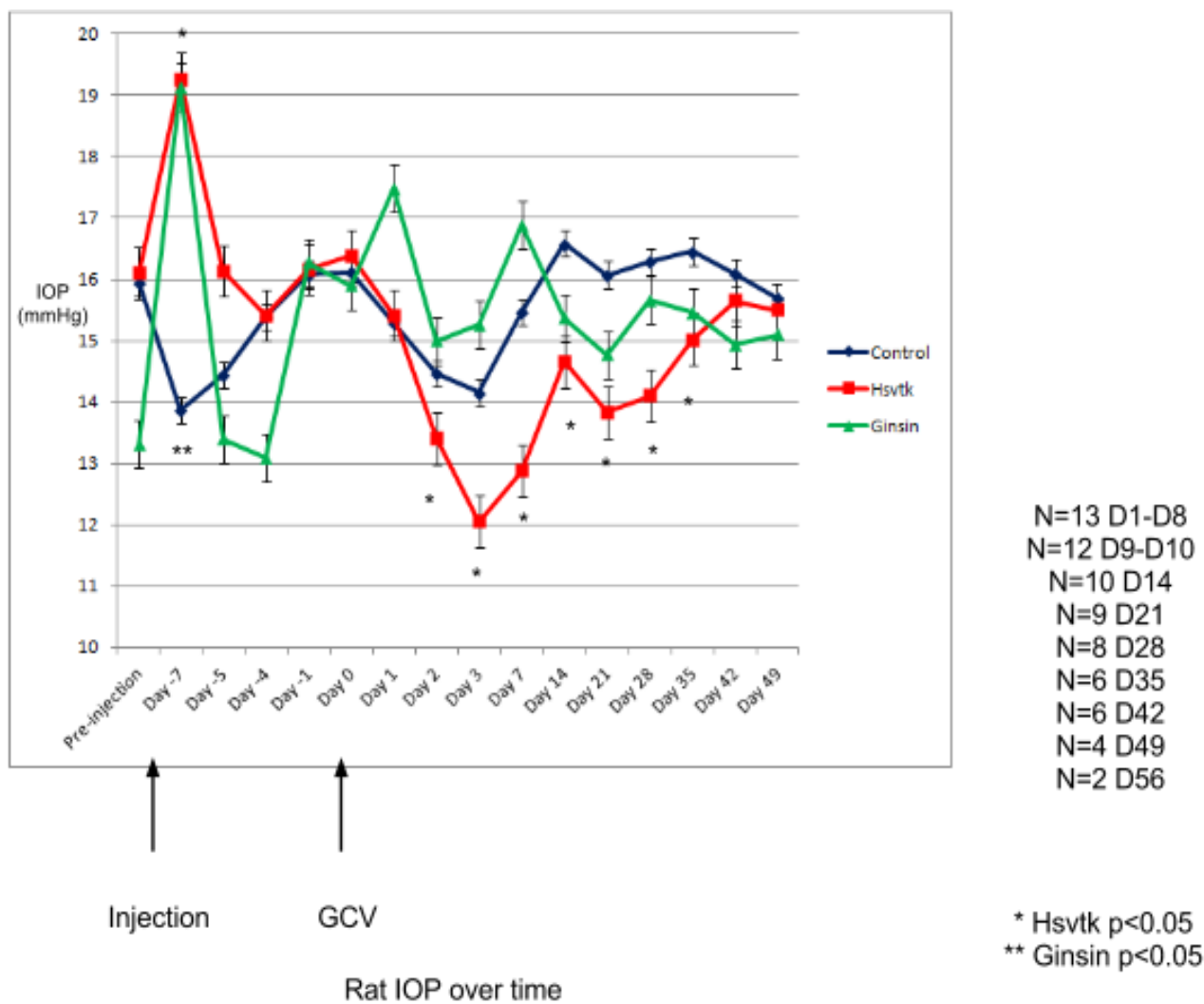


Figure 3. Rat intraocular pressure over time. IOP increases in both GINSIN and HSVtkiG injected eyes compared to uninjected control eyes but returns to baseline after one day. GCV administered 7 days after injection (Day 0). 2 days following GCV administration, IOP declines significantly in HSVtkiG group compared to control and remains decreased throughout day 35 after ablation.

Pachymetry

To monitor changes and potential adverse effects on FIV vector injection on corneal endothelial integrity, CCT was measured prior to injection as well as post-injection for the duration of follow-up. Pre-injection CCT averaged 174 ± 2 μm . The CCT increased post-injection Days 1 through 3 in both HSVtkiG and GINSIN groups compared to the control non-injected group. There was no significant difference in CCT between the two injected groups, GINSIN and HSVtkiG. Both injected groups' CCTs were significantly increased compared to non-injected control. The CCT in the GINSIN group returned to baseline relative to control after Day 2. HSVtk CCT was significantly different through Day 3, after which, it also returned to baseline relative to control. For the remainder of follow-up period, there was not statistically significant difference in CCT among the 3 groups (Figure 4). CCT was significantly thicker only on day 42 onward compared with before transduction ($p < 0.3$).

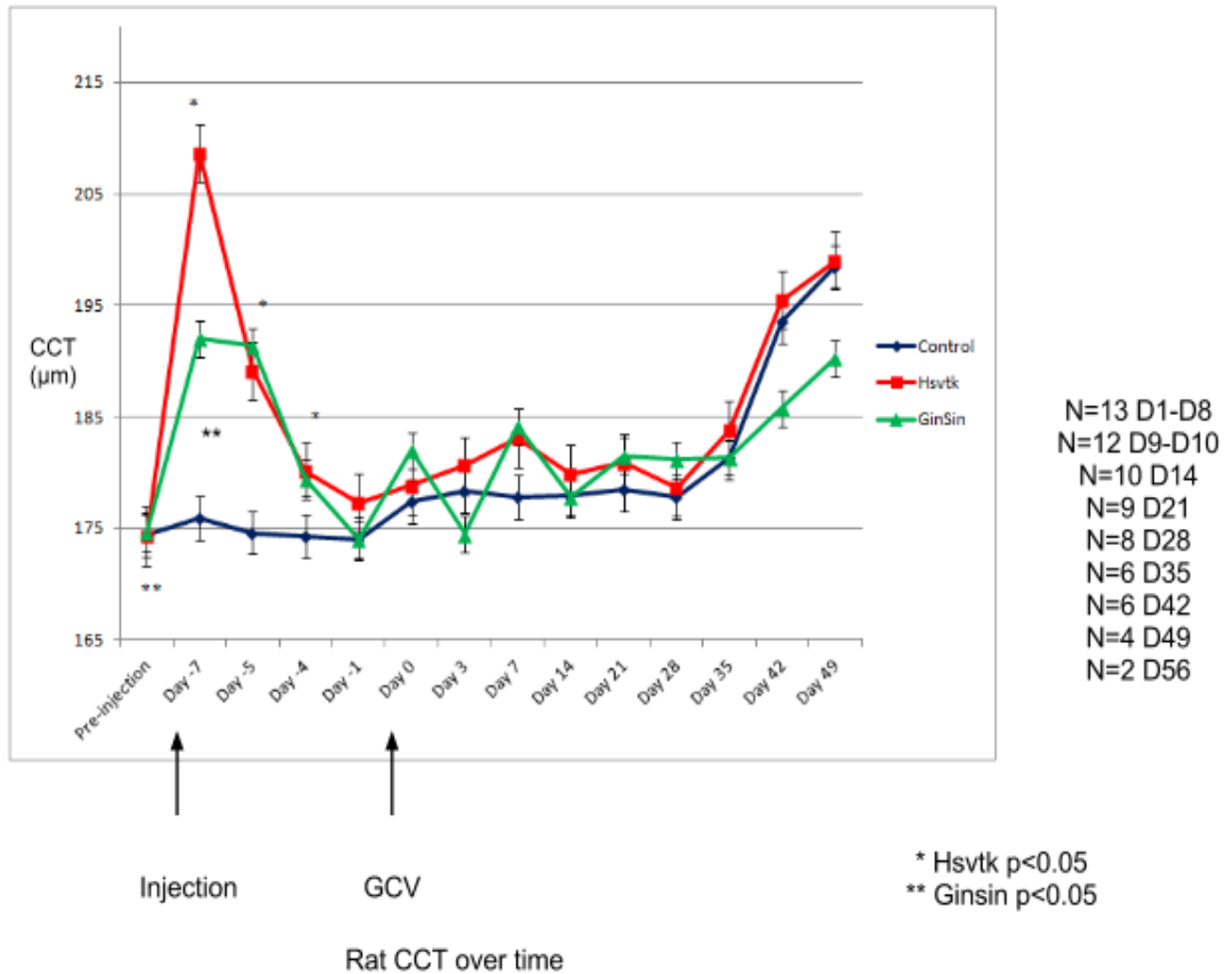


Figure 4. Central Corneal Thickness (CCT). CCT increases after injection in both GINSIN and HSVtkiG eyes but returns to baseline after 2 (GINSIN) and 3 days (HSVtkiG) likely due to inflammatory changes associated with injection. Subsequent CCT remains unchanged even in HSVtkiG transduced and GCV ablated eyes, suggesting normal corneal endothelial function. CCT was significantly thicker only on day 42 onward compared with before transduction ($p < 0.3$).

Direct gonioscopy

We monitored the anterior chamber and eGFP marker expression to confirm successful transduction and ablation. Both GINSIN and HSVtkiG vector injections into the anterior chamber of rats produced efficient and durable TM transduction (Figure 5). Transduction was limited to the TM, with rare eGFP positive cells in the corneal endothelium.

eGFP fluorescence could be visualized by day 7 after intracameral injection of GINSIN or HSVtkiG vector (Figure 5). We confirmed ablation by visualizing decreased fluorescence 2 days after administration of ganciclovir in the HSVtkiG+GCV group. Starting at Day 10 after injection, there was no visualized fluorescence in the HSVtkiG+GCV group, while fluorescence persisted in both the non-ablated HSVtkiG group and the GINSIN group till Week 5 (Day 35). Ex vivo imaging of dissected anterior chamber angles demonstrated that significant eGFP was present in the TM that was not readily apparent by direct gonioscopy (data not shown).

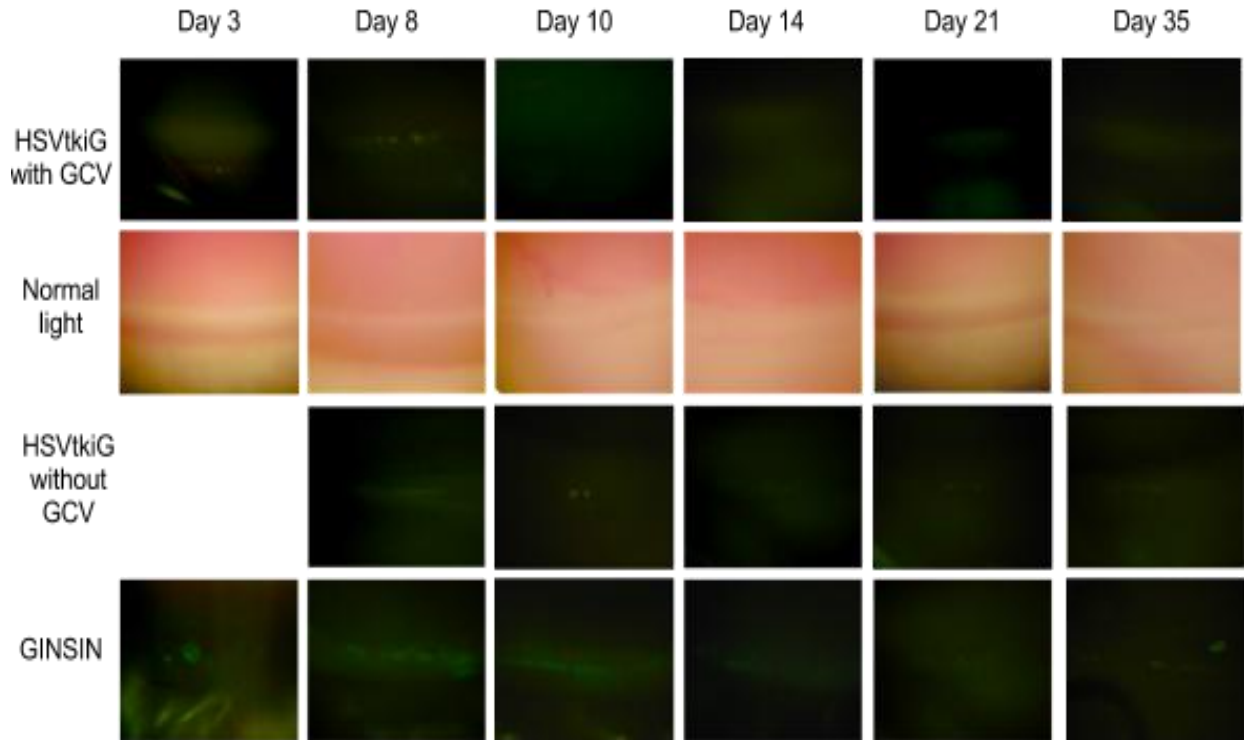


Figure 5. Direct gonioscopy of rat TM. Time is defined as days after injection. HSVtkiG with GCV leads to disappearance of eGFP fluorescence by Day 10 after injection, 3 days after GCV administration. HSVTKiG without GCV ablation showed eGFP persisting through Day 35 after injection, similar to GINSIN group.

Rat Eye Histology

Morphological analysis of rat eyes showed no differences in gross morphology between GINSIN injected eyes, HSVtkiG injected eyes (ablated and non-ablated), or control non-injected eyes.

Hematoxylin and eosin stain of sections of paraffin embedded eyes showed inflammation at injection site in injected eyes. Histology of enucleated rat eyes at various time points showed normal corneal epithelium and endothelium with no signs of corneal edema in all HSVtkiG with and without ganciclovir, GINSIN injected eyes, and non-injected control eyes. The iris and ciliary body appeared intact and normal in all injected eyes in both GINSIN and HSVtkiG group compared to non-injected control eyes. There were no signs of increased inflammation base on slit lamp exam after injection or after ablation compared to control. There were no observed changes in ciliary body architecture after injection or ablation.

Starting at day 9 after injection, Day 2 after GCV administration, HSVtkiG+GCV (ablation) group showed decreased cellularity of the TM and the of endothelium around the Schlemm's canal compared to the HSVtkiG with no GCV (no ablation) group, the GINSIN group, and the non-injected group. Histology also showed a disrupted TM architecture in the HSVtkiG+GCV group compared with the other three groups. There appeared to be a decrease in extracellular matrix (ECM) materials starting at day 2 after GCV administration in the HSVtkiG+GCV group compared to the other groups as well.

The difference in histology was sustained through Day 35 after GCV administration. After Day 35, there appeared to be an increase in both TM cellularity in the HSVtkiG+GCV group that became similar to the control groups (Figure 6).

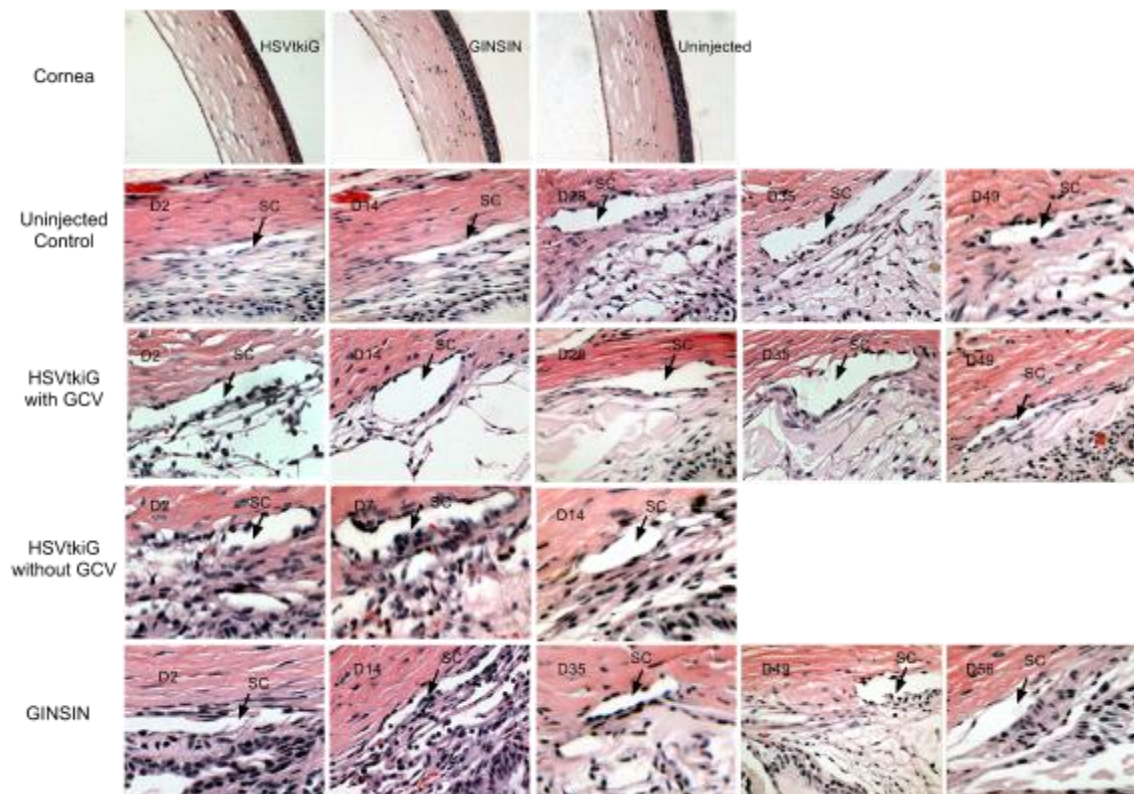


Figure 6. High power view of TM and angle structures in HSVtkiG and control groups. Time is defined as number of days or weeks after GCV administration. There are no differences in corneal histology between the three groups. HSVtkiG with GCV group shows rounding up and cell death of TM cells most evident at Day 2 after GCV administration. TM cellularity is decreased in HSVtkiG with GCV group compared to HSVtkiG without GCV and control groups through Day 35 after GCV administration. SC: Schlemm's canal

DISCUSSION

This is the first study to establish a rat in vivo model of TM ablation and regeneration as a novel method of lowering IOP in glaucoma gene therapy and as a tool to mobilize dormant progenitor cells to migrate into and repopulate this structure. Although lentiviral vectors are typically used to achieve stable long-term expression in nondividing cells, we took advantage of their ability to transduce TM selectively (67) and produce robust marker protein levels to allow in vivo tracking of vector function.(1) Different from lentiviral vectors, standard type C retroviral vectors could not be used to target TM, a terminally differentiated and normally nondividing tissue, since those vectors can only access chromatin during cell division when the nuclear membrane dissolves.(14) We used rats to establish an animal TM ablation model because of their small size but anatomic similarity to the human anterior chamber that has been taken advantage of in other studies of outflow tract transduction.. (61, 63, 97) HSVtk has been used extensively in cyto-ablative cancer gene therapy as an effective means of tumor destruction with a limited bystander effect on non-diseased tissue. (91, 92)

We first determined proper vector function and best amount of GCV in vitro before targeting the TM using one single, small volume intracameral injection. Consistent with prior studies in cat, nonhuman primates, and perfused human eyes, (1, 14, 67, 70) this rat model displayed preferential transduction of TM without apparent toxicity to adjacent structures on slit lamp exam, high power biomicroscopy, gonioscopy and histology. HSVtk-mediated ablation can have a bystander effect on non-transduced, adjacent cells via gap junctions, (98-101) but the effect in TM may be limited from efficient washout and removal of detaching cells via the normal outflow.

EGFP marker expression could be followed through the experimental endpoint at 5 weeks by gonioscopy and was detectable at a higher level by direct visualization in flat mounts.

Diminishing expression of eGFP may indicate that expression levels were initially too high (102-105) and may require reducing the MOI. (1) Alternatively, rats may have evolved more effective mechanisms to silence retro- and lentiviral expression. (106) TM ablation in HSVtkiG transduced eyes did not have a notable effect on CCT or histological appearance of the cornea, consistent with preferential transduction of the outflow tract. The temporary increase immediately following vector injection may indicate trauma from anterior chamber decompression and needle insertion while the moderate increase of CCT in all experimental groups over the entire length of experiments is consistent with the normal growth pattern.

Although the TM of the rat is only 1 to 3 cell layers thick and less able than the human to be able to generate a high flow resistance, we observed a decrease of IOP that became observable within 24 hours after GCV and reached a maximum of 25% on day 3 after induction of the cytotoxic HSVtk + GCV ablation system. This IOP drop does not contradict Bahler et al's finding of reduced outflow when cellular debris was perfused into an intact TM of cultures (107) and correlated well with our findings in vitro experiments when cells started to round up and detach before peaking at day 3. When in vivo eGFP fluorescence started to disappear on serial gonioscopy on day 9, histology demonstrated TM cell changes that reflected in vitro experiments. Around day 10, a decreased cell count was noted when the IOP trough was reached. Despite a rather fast rebound in cellularity, the effect of IOP reduction was sustained requiring approximately 5 weeks to increase to pre-ablation levels. Both the decreased cellularity and the

persistence of the effect after removal of a cellular target in non-glaucomatous eyes with a low and physiological resistance contradict a mere cytoskeletal effect. (108-110)

While this study was sufficiently powered to detect an IOP change it was difficult to establish the rapidly unfolding cellular morphological changes and counts following ablation. This model has a highly confined ocular anatomy that limits the ability to establish the temporal and spatial pattern of transduction with high resolution as in species with larger eyes. (1, 81) It is possible that inflammation may have contributed to a reduced IOP short-term but this effect cannot be expected to persist after transduced and ablated cells are washed out. IOP remained largely unaffected by FIV vectors in past in vivo models and no inflammation was seen. (1, 81) Healthy TM cells are vital to regulate pressure. Ablation may be a useful strategy in glaucoma by reducing outflow resistance and possibly allowing dormant stem cells from adjacent structures to repopulate this structure.

CONCLUSION

In conclusion, we demonstrated successful ablation of TM in vitro and in vivo using a conditionally cytotoxic FIV vector which lead to a statistically significant decrease in IOP without damaging surrounding tissue. This study provides an in vivo model to study the effects of TM cell ablation and turnover that are incompletely understood in glaucoma pathogenesis and therapy.

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AUTHOR CONTRIBUTIONS

Outflow Tract Ablation using a Conditionally Cytotoxic Feline Immunodeficiency Viral Vector

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The purpose of this section is to ensure the proper attribution of each component of the work by section.

ZZ was responsible for the study design, development, execution, and troubleshooting of the protocol, organization and material procurement, data collection, data analysis, performing all statistical analyses, result interpretation and writing the manuscript for this thesis. ZZ also presented this work in part at the Association for Research in Ophthalmology and Visual

Sciences National meeting in May, 2011. HPT assisted with the laboratory experiments, including the in vitro and in vivo data collection. Then entire manuscript was edited by NL, who oversaw and guided the entire study design, execution, and analysis.