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Prospects for Lentiviral Vector Mediated Prostaglandin F Synthase Gene Delivery in Monkey Eyes *In vivo*

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Abstract

Currently, the most effective outflow drugs approved for clinical use are prostaglandin F_{2α} analogues, but these require daily topical self-dosing and have various intraocular, ocular surface and extraocular side effects. Lentiviral vector-mediated delivery of the prostaglandin F synthase (PGFS) gene, resulting in long-term reduction of IOP, may eliminate off-target tissue effects and the need for daily topical PGF_{2α} self-administration. Lentiviral vector-mediated delivery of the PGFS gene to the anterior segment has been achieved in cats and non-human primates. Although these results are encouraging, our studies have identified a number of challenges that need to be overcome for prostaglandin gene therapy to be translated into the clinic. Using examples from our work in non-human primates, where we were able to achieve a significant reduction in IOP (2 mm Hg) for 5 months after delivery of the cDNA for bovine PGF synthase, we identify and discuss these issues and consider several possible solutions.

Keywords

Intraocular pressure; Lentiviral vector; Prostaglandin F Synthase; Restriction; Glaucoma

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Glaucoma

The glaucomas are a heterogeneous group of optic neuropathies that affect an estimated 70 million people worldwide (1). The major causal risk factor in this disease is chronic stress to the optic nerve, often, but not always, associated with increased intraocular pressure (IOP) which, in turn, results from increased resistance to the outflow of aqueous humor from the eye.[reviewed in (2)] In both elevated and normal tension glaucoma, lowering IOP pharmacologically or surgically remains the mainstay of glaucoma therapy, and can be effective in reducing optic nerve damage and visual field loss (3, 4).

Prostaglandins and the Monkey Eye

Currently, the prostaglandin (PG) $F_{2\alpha}$ analogues are the most effective outflow drugs approved for clinical use.[reviewed in (5)] In the ocular normotensive monkey $PGF_{2\alpha}$ -isopropyl ester ($PGF_{2\alpha}$ -ie) and various analogues given at μ g topical doses for several days are capable of reducing IOP by <5 mm Hg (6). This is accomplished by a several-fold enhancement of uveoscleral outflow (7, 8) with little or no effect on aqueous humor formation (9), trabecular outflow facility (pressure dependent trabecular outflow)(10) or episcleral venous pressure (11). Interestingly, pressure-independent trabecular outflow is decreased by 75% compared to controls in monkey eyes following $PGF_{2\alpha}$ -ie treatment, suggesting a redirection from trabecular to uveoscleral outflow (10).

Prostaglandin F (FP) receptors are abundant in ciliary muscle (12). The $PGF_{2\alpha}$ -induced enhancement of flow from the anterior chamber through the ciliary muscle and into the suprachoroid appears to result in part from relaxation of the muscle (13, 14). However, the majority of the outflow effect is likely due to extracellular matrix remodeling in the anterior segment, characterized by an increase in matrix metalloproteinases (MMP)-1, 2, and 3 and reduction in collagen types I, II and IV, within the ciliary muscle, iris root and peri-ciliary body sclera (15-17).

Prostaglandin-induced changes in the sclera also are important in the regulation of uveoscleral outflow and might be used to enhance trans-scleral delivery of peptides and other high-molecular-weight substances to the posterior segment of the eye (18). Human scleral permeability to dextrans, measured in an Ussing chamber following exposure to $PGF_{2\alpha}$ and latanoprost acid for 1-3 days, increased in a dose- and time-dependent manner. This was accompanied by an increase in MMP concentration in the media, with greater increases in MMP-2 and -3 compared to MMP-1 (19). $PGF_{2\alpha}$ and latanoprost acid also induce increases in mRNA for MMPs and tissue inhibitors of matrix metalloproteinases in human scleral organ cultures (20).

Immunohistochemistry studies show that EP2 receptors are the most abundantly expressed EP subtype in human ocular tissues (21). EP2 selective agonists are effective in lowering IOP and increasing uveoscleral outflow in monkeys (22, 23). Sagittal semithin sections through the anterior ciliary body of a butaprost treated eye revealed enlarged intermuscular spaces between bundles of the longitudinal and reticular portion of the muscle that extended farther posteriorly. Electron micrographs revealed enlarged intermuscular spaces lined by endothelial-like cells forming contacts with the vessel wall of the capillary (22). Long-term

treatment with various subtype selective prostaglandins resulted in intermuscular spaces becoming more organized and lined with an incomplete layer of endothelial cells resembling lymphatic pathways (24).

Markers indicating lymphatic channels are found in human circular, radial and longitudinal portions of the ciliary muscle, stroma, and ciliary processes as well as surrounding many blood vessels in the ciliary body (25). Further evidence of a uveolymphatic outflow pathway was demonstrated *in vivo* following intracameral injection of ¹²⁵I human serum albumin into living sheep and subsequent detection of tracer in peripheral lymph nodes (26).

The uveoscleral outflow system likely evolved to protect the eye in several ways during inflammation. In the normal monkey eye, in the absence of inflammation or other treatment, particles and spheres up to 1.0 μm in diameter can pass through the ciliary muscle bundles into the suprachoroid to the posterior portion of the eye, reaching the macular and optic nerve head regions in 3 hours (27). In the presence of inflammation the trabecular meshwork may be compromised or obstructed by inflammatory debris, and the choroid is overloaded with debris and extravasated proteins that must be removed from the eye (28). In this situation, prostaglandins are released and, as autoids or hormones that are synthesized, released, and locally acting, could induce the changes described. Redirection of aqueous outflow from the trabecular to the uveoscleral pathway via mechanisms similar to those described above following topical PG treatment including elevated levels of MMPs and extracellular matrix turnover(29), would both rid the eye of excess proteins and maintain physiologic IOP. This could also explain the very low IOP that often accompanies uveitis; during experimental iridocyclitis in monkeys, uveoscleral outflow increases approximately four-fold (30).

In vivo, prostaglandins are synthesized from arachidonic acid via the rate-limiting step involving the cyclooxygenase enzyme to an intermediate which is then converted by various PG synthases to the different PGs (31). The genes for the various steps of the cascade are known, making it possible to consider over-expressing these genes to cause the same IOP-lowering response as with pharmacotherapy. Advances in genetic technology may allow genetic “resetting” of the outflow or inflow tissues to a desired “performance” level as an alternative to classical repetitive exogenous small molecule pharmacotherapy, which requires the patient to be the delivery system, with all the compliance issues that this engenders (32).

Viral Vectors for Glaucoma Gene Therapy

Several viral based vector systems can be used to deliver genes to tissues relevant for glaucoma therapy including Adeno-Associated virus (AAV)(33, 34), self-complementary AAV (scAAV)(35), Adenovirus (Adv)(36), Herpes simplex virus (HSV)(37) and lentiviruses such as Feline Immunodeficiency virus (FIV) (38). Each of these vector systems have advantages and disadvantages related to ease of production, size of the transgene that can be delivered, and whether the transgene remains as an episomal element or integrates into the target cell genome. Standard AAV vectors do not efficiently transduce trabecular meshwork cells because the cells are not efficient at completing the second strand DNA

synthesis that is required to convert the single-stranded DNA vector genome to the double-stranded form (39, 40). This has been addressed through the use of self-complementing (scAAV) vectors where the single-stranded vector genome forms a double-stranded DNA hairpin (35). However, AAV vectors can only deliver approximately 4.5 Kb of DNA and in the scAAV vectors this is reduced by half. Several genes that might be useful for glaucoma therapy are large and there is usually no extra room to co-deliver a marker gene. Adv vectors have a larger carrying capacity but the transgene does not integrate. Lentiviral vectors can deliver approximately 7 to 8 Kb of DNA and they have the advantage that they can deliver genes to non-dividing cells (41). In addition, lentiviral vectors also integrate the transgene into the target cell genome. For these reasons lentiviral vectors have several advantages for glaucoma gene therapy.

Lentiviral vector design and evaluation

FIV vectors were first used to transduce non-dividing human cells in 1998 (42). FIV normally does not productively infect human cells, so for use as a vector, it was necessary to identify the restriction points. These had been attributed to inefficient promoter activity of the LTR(43, 44) and problems with *rev* activity (45). Poeschla et al., replaced the U3 element in the 5'LTR with the CMV promoter (CT5 vector) and subsequently showed that the 5' U3 element was the most important determinant of restriction in human cells (42). Subsequently, a slightly modified vector expressing lacZ, where all but the first 311 bases of the gag gene were deleted (this improves packaging efficiency), was used to efficiently transduce human trabecular meshwork in an eye organ culture system, opening the way for the use of FIV-based vectors for treating glaucoma (46).

Additional studies showed that bicistronic expression vectors (eGFP and neomycin resistance) could efficiently transduce the TM (47). In this bicistronic vector (GiNMF), the CMV promoter drove expression of a hybrid mRNA where eGFP was expressed as the 5' open reading frame and the neomycin resistance gene was translated from an internal ribosome entry site (IRES) element. Loewen et al. (47) also introduced improved production methods for large scale packaging of FIV-based vectors. In terms of delivery, most studies have used anterior chamber injection, but delivery to Schlemm's canal via a viscocanalastomy procedure in human eye organ culture has also been demonstrated (48).

FIV delivery in animal models

Up to this point, FIV vectors had been used in cell culture and in human eye organ cultures, but had not been tested in animals. In a study designed to determine an optimal vector dose in cats, Loewen et al. (49) found that 10^7 transducing units (TU) of a GFP vector were optimal, whereas 10^8 TU of the corresponding lacZ vector was optimal. This difference was due to GFP-induced toxicity at higher doses. Khare et al. (50) constructed a series of dual vectors using an IRES element that expressed GFP, neoR, and myocilin in various positions (5' or 3' to the IRES) and injected them into the anterior chamber of cats. Expression of GFP was monitored non-invasively and was detected for up to 2.3 years, establishing that safe, long-term dual expression could be achieved. Similar studies were then done in non-human primates, where expression of GFP was evident non-invasively for up to 15 months (51).

Having demonstrated successful gene delivery in two animal species, the next step was to test a potential therapeutic strategy for glaucoma. As noted elsewhere, prostaglandins have become a mainstay of glaucoma therapy, but frequent dosing and patient compliance issues are problems. A next logical step would be to propose that increasing long-term levels of prostaglandins in the TM and/or CM would circumvent some of the problems. Based on the observation that cyclooxygenase 1 or 2 (COX1 or COX2) carries out the rate limiting step in prostaglandin synthesis (52), Barraza et al. (38) constructed FIV vectors expressing either the human COX2 enzyme, the prostaglandin F2 synthase gene, or the FP receptor, and delivered them to the TM of cats. Initially, expression of human COX2 was poor and this was subsequently attributed to the high AU-rich sequence of the human mRNA. Barraza et al. (38) subsequently changed 382 of the 1,815 nucleotides to reduce the AU content and this codon optimization resulted in the substantially improved expression of total COX2 and GFP and subsequently improved levels of PGF2 α . They also carried out codon optimization on the FP receptor. When cat TM was transduced with the codon optimized COX2 and FPR vectors, a sustained 32% reduction IOP was obtained. These results suggest further development of prostaglandin gene therapy for glaucoma is warranted.

Prostaglandin synthase gene delivery lowers intraocular pressure in non-human primates

To determine if delivery of the gene for Prostaglandin F Synthase (PGFS) could lower IOP in non-human primates we cloned the cDNA for bovine PGFS (BOVPGFS)(53) with a myc tag into a FIV-based dual expression vector, FIV.BOVPGFS-myc.GFP (System Biosciences; SBI, Mountain View, CA). As a control we used the same vector expressing only GFP (Figure 1). After verifying that the vector expressed BOVPGFS in cell culture, we delivered the control and BOVPGFS vectors to the anterior chamber of 5 monkey eyes and measured IOP for 400 to 550 days. Figure 2 shows the schedule of procedures for each animal in the study. Not all monkeys were injected at the same time (Figure 2). K691 was injected first and when an IOP lowering response was observed, we opted to re-inject the vector to see if IOP would be reduced further, which was not observed. At the same time, K681 and K685 were injected and when the IOP for all three returned to baseline, we re-injected to see if the response could be restored, which it was not. We then injected the last two monkeys, K674 and K679 which were not re-injected, as re-injection failed to generate a response in the first three animals. Approximately 1×10^8 transducing units were delivered to each eye. As shown in Figure 3, when data from all 5 monkeys were considered, ANOVA analysis indicated IOP was significantly decreased (-2.05 mm Hg) in FIV.BOVPGFS-myc.GFP treated eyes compared to control FIV.GFP treated eyes after correction for baseline during the 0-5 month interval ($p=0.003$), and was significantly decreased (-1.14 mm Hg) when all data were considered ($p=0.012$). These results were encouraging and raised the possibility that delivery of a prostaglandin synthase gene could result in a long-term therapeutic effect in humans. Testing gene therapy approaches in non-human primate eyes will be essential for advancing glaucoma gene therapy to the clinic.

Nonetheless, a number of problems remain to be solved for lentiviral mediated prostaglandin gene delivery to be successful and we will use selected results from our studies in non-human primates to illustrate these problems and discuss potential solutions for them.

Weak IOP Responses

The IOP decrease was not as much as seen with topical prostaglandin therapy in monkey eyes (23). For successful gene delivery in humans, a stronger effect will be needed. One factor that could affect the efficacy is efficiency of gene delivery. The percentage of cells that express the transgene will affect the amount of prostaglandin synthesized and secreted, thus it is desirable to transduce as many cells as possible. Scoring systems for grading the efficiency of delivery have been used previously for both gonioscopic images and tissue sections (47, 49-51) and have indicated FIV transduction efficiencies in human trabecular meshwork ranging from 70 to 95% (47). The vector we used expressed both PGF synthase and copepod GFP that allowed for live imaging of the GFP expression. Figure 4 shows the imaging results for one of the animals in our study where single GFP positive cells are visible and Table 1 summarizes the quantitative analysis of the gonioscopic images (49, 51). GFP was detectable at 48 weeks in all animals and all but one animal at 52 weeks.

One way to increase transduction efficiency would be to deliver more virus. This is problematic in the eye because of the small volume of the anterior chamber. Typically we can deliver 100 μ l by removing some aqueous and replacing it with the vector solution but larger volumes are not advisable. Increasing the titer of the vector could result in more efficient delivery but increasing titers beyond what is currently achievable (approximately 10^9 /ml) would be difficult. In addition, increasing titers also can result in increased aggregation in the vector preparations and other problems so we are probably at or near the limit in terms of delivering more transducing particles.

The FIV vector we use is pseudotyped with the Vesicular Stomatitis G protein which is responsible for attachment and entry of the virus. The receptor for the G protein is ubiquitous allowing one to transduce numerous cell types. This makes it unlikely that the lack of receptors on the target cells is responsible for reducing the efficiency of transduction.

Another factor that might significantly affect transduction efficiency is the existence of species-specific innate resistance mechanisms that block lentiviral infection (54-56). Cells express several proteins that specifically target lentiviruses such that FIV infection of monkey or human cells can be blocked. Some of these include APOBEC3G, TRIM5 α , and tetherin (57, 58). APOBEC3G is incorporated into virus particles in an infected cell and when a new cell is infected it deaminates cytosines in the viral genome and inhibits reverse transcriptase (59). This results in the generation of mutations during reverse transcription essentially mutating the virus out of existence. The HEK293 cells commonly used for packaging vectors do not express APOBEC3G so this is probably not a significant factor. TRIM5 α in the target cell causes capsids to be destroyed by the proteasome system. It also induces premature disintegration of capsids, which blocks reverse transcription. Therefore, TRIM5 α would be an important factor (60). Tetherin prevents viral particles from being released from cells (61, 62). Because tetherin acts at the release step and the vectors are

replication deficient, it is probably not affecting the efficiency of transduction. Countering these innate resistance systems could increase the efficiency of gene delivery.

In studies to date (38, 51), the delivery efficiency has been reported to be in the range of 70 to 95% suggesting that species specific restriction may not be a significant problem (47). This is probably due to the delivery of large amounts of transducing particles that saturate the TRIM5 α and other factors in the cell. Interfering with restriction systems through delivery of genome deficient viral particles to packaging cells has been shown for another retrovirus (63). Strategies such as transient inhibition of proteasomes, interfering with other innate antiviral defenses, pre-delivery of a dominant negative TRIM5 α or pre-delivery of genome deficient viral particles could result in more efficient transduction of the TM and/or CM (64-66). One could argue that the 70-90% transduction efficiency reported in studies should be sufficient, but this is probably due to saturation of the innate defenses with large amounts of transducing particles. Countering these systems could increase delivery even more or result in vector sparing, where highly efficient transduction could be achieved with fewer viral particles. Increasing the efficiency of transduction or vector sparing would result in the need to use fewer transducing particles thereby affecting production issues and the costs associate with producing very high titer stocks of transducing viruses.

Species-specific restriction of lentiviral vectors creates an additional problem. Because of the species specificity of the blockage it may be necessary to use SIV vectors for testing in monkey eyes, FIV vectors for cat studies, and ultimately HIV vectors for use in humans. Barraza et al. (51) have discussed this and suggested that species specific restriction is not a significant problem. However, this requires the production of very high titer stocks of vector which is labor intensive and expensive. Typically the FDA requires safety studies in multiple species. If the lentiviral vectors are most efficient in their species of origin then the FDA may need to accept data using FIV in cats and SIV in monkeys as a surrogate for using HIV in humans. It is not clear that this would be acceptable. Engineering the vectors to avoid the species-specific restriction would enable a single vector to be used in multiple animal species.

The decrease in IOP lasted for only 5 months

The 2 mm Hg decrease in IOP in the monkeys was lost at 5 months post-transduction, yet we were still able to visualize GFP expression in the monkeys (Table 1). This strongly suggested that expression of the BOVPGFS was shutting off at some point, most likely around 5 months. To determine if transgene expression had shut off we collected the eyes at 18 months post-transduction, isolated RNA and used PCR to test for expression. The results are summarized in Table 2. Note that for some tissues, RNA yields were low, which may account for the lack of signal. As predicted, we were able to detect GFP and control gene expression but we were not able to detect any expression of the BOVPGFS transgene. We were also able to detect expression of the endogenous monkey PGFS. This supports the hypothesis that the loss of the IOP effect was due to transgene shutoff.

There are several potential explanations for why BOVPGFS expression was lost and GFP was retained. The GFP transgene is expressed from a housekeeping promoter, Elongation

Factor 1 (EF1). Housekeeping promoters typically are active constitutively in all cells and at lower levels than induced genes. This could explain why GFP was expressed for 18 months in our animals. In contrast, BOVPGFS is expressed from the Human Cytomegalovirus (CMV) major immediate early gene promoter. This promoter expresses at high levels but the level of expression varies substantially. Expression from the CMV promoter has been found to last from a few weeks to approximately 2 years (49-51). One possible explanation for these variable results is that the CMV promoter is a NF κ B responsive promoter and loss of NF κ B activity could terminate expression. In one study where an Adenovirus vector was used to deliver a gene to liver cells, administration of a known activator of NF κ B reactivated expression of the transgene after it had shut off (67). During the delivery of the viral particles it is likely that innate inflammatory responses are activated, including NF κ B, and this would drive expression from the CMV promoter. After time, however, the inflammation would subside, NF κ B would be deactivated and this could terminate expression. In addition to the availability of transcription factors, other mechanisms might be causing the termination of expression. The host cells could be methylating the CMV promoter or histone modifications could be converting the transgene to a heterochromatin state. The host cells may be reducing gene expression through siRNA or shRNA mechanisms. If so, potential solutions could include using species-specific PGFSynthase or codon optimization as was done previously for COX-2 cDNA expression in cats (38). Clearly further studies are needed to determine why gene expression is terminating. It would also be advantageous if more trabecular meshwork and/or ciliary muscle specific promoters were available.

Differences in Vector Structure

Our study and that of Barraza et al. (51) used slightly different FIV-based vectors raising the question as to whether the early shutoff is due to differences in the vectors? Barraza et al. found that GFP expression driven by the CMV promoter lasted for at least 15 months. In our work, we also detected GFP expression driven from the EF1 promoter for a similar period of time. A more detailed comparison of the structures of the two vectors is shown in Figure 5. The vectors are very similar making it unlikely that the shutoff of PGF synthase expression is due to vector sequences. Potentially significant differences include a neomycin resistance gene in pGINSIN vs. PGF synthase in FIV.BOVPGFS-myc.GFP, eGFP vs. copGFP, the placement of GFP either before or after the second gene expressed from the vector, and the presence or absence of an SV40 virus polyadenylation sequence in FIV.BOVPGFS-myc.GFP. Since both vectors expressed GFP for up to 15 months it is unlikely that the different GFPs are involved in the shutoff of PGFSynthase expression. Although we cannot rule it out, the SV40 sequence has been used extensively in vectors making it unlikely that it is involved. This makes it likely that the critical difference is the presence of the bFGFSynthase gene in FIV.BOVPGFS-myc.GFP vs. the neomycin gene in pGINSIN. Whether it is the CMVp-pFGFs combination or the bPGFS itself is not clear and requires further work.

Gene Delivery Vectors Induce Uveitis

Previous work from our laboratories has shown that viral vectors can be injected into rodent eyes without inducing uveitis (65). However, in non-human primate eyes a single injection

of Adv, HSV, or lentiviral vectors into either the anterior or posterior chambers induces a transient uveitis (36, 37, 51). For example in our prostaglandin study, following transcorneal injection of vector into the living monkey eyes, biomicroscopic examination initially revealed cells (up to 3+) and flare (up to 2+) in the anterior chamber and some corneal haze that was largely resolved by 3 weeks post injection. This was not due to bacterial endotoxin contamination of our vector preparations and is likely the result of activation of innate viral sensors in the eye. These would include Toll-like receptors (TLRs), RIG-I, MDA-5 and others (66, 67). Since the vectors are all replication deficient, the inflammation is being triggered in the absence of viral replication. Since humans are more likely to resemble non-human primates, it is critical that we identify the triggering mechanism so that we can develop strategies to counter the response.

Inflammatory responses can be induced by prostaglandins and inflammation can result in increased prostaglandin production (28, 68-70). This raises the possibility that at least part of the decrease in IOP we measured could have been due to vector-induced prostaglandin release. It is unlikely that viral vector induction of the transient inflammatory response was responsible for the decrease in IOP for at least two reasons. First, the degree of inflammation was the same in the eyes injected with the GFP-expressing vector and the BOVPGFS-myc expressing vector. Second, the uveitis was transient and cleared by 7-10 days post-transduction, whereas the effect on IOP in the BOVPGFS-myc transduced eyes was evident out to 5 months post-transduction. Any inflammation-induced effect would be diminished as the inflammation cleared and would not be expected to last for 5 months.

Conclusions

The delivery of genes for prostaglandin synthase using lentiviral vectors has now been achieved in cats and non-human primates, resulting in significant reductions in IOP. These results are encouraging and suggest that prostaglandin gene therapy would be feasible in humans. However, there are a number of challenges that must be overcome to translate these findings to the clinic. The effect on IOP needs to be stronger, possibly through more efficient delivery of the transgene by engineering the vector to resist species-specific restriction factors or the use of a different promoter. We need to determine the mechanism whereby transgene expression is lost after a few months and identify other promoters to drive gene expression. Finally, we need to identify the trigger for the induction of uveitis to develop preventive strategies.

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Figure 1.

Schematic diagram of the FIV vector maps for control and BOVPGFSmyc vectors. The BOVPGFS cDNA in the pUCPF1 vector was obtained from K. Watanabe (Kyoto, Japan) (53) and PCR amplified with primers that added a 5' XbaI restriction site and replaced the 3' stop codon with a myc epitope tag, stop codon, and EcoRI restriction site. The PCR amplicon was then gel purified and subcloned into the MCS of pZero using the XbaI and EcoRI restriction sites. Once the myc-tagged BOVPGFS construct sequence was verified, the construct was subcloned into the FIV expression vector FIV.BOVPGFS-myc.GFP using the XbaI and EcoRI restriction sites down stream of the CMV promoter. The packaging vector (pFIV-34N) contains the structural, regulatory, and replication genes that code for the proteins required for packaging the vector genome. The pVSV-G vector expresses the envelope glycoprotein of the vesicular stomatitis virus (VSV, replacing the lentiviral *env* gene). Structural features are indicated. LTR, Long Terminal Repeat; CMV promoter, Human Cytomegalovirus major immediate early gene promoter; MCS, multi-cloning site; copGFP, copepod GFP gene; WPRE, Woodchuck post-transcriptional regulatory element; SV40 Poly A, SV40 virus polyadenylation signal; BOVPGFS, bovine prostaglandin F synthase gene; EF-1, Eukaryotic Initiation Factor-1 promoter. Vectors were packaged according to the supplier's instructions.

Figure 2.

Schedule of procedures for individual animals. Six procedures are shown for each animal. 0) FIV injections; 1) IOP/SLE - intraocular pressure and slit lamp examination (biomicroscopy); 2) GFP photos; 3) AHF - aqueous humor formation determination by fluorophotometry; 4) OF - outflow facility determination; 5) AC taps - anterior chamber aqueous humor sampling.

Figure 3.

IOP difference compared to baseline in all monkeys after vector injection. IOP after correction for baseline was significantly decreased in all FIV.BOVPGFS-myc.GFP injected eyes compared to contralateral control FIV.GFP injected eyes by ANOVA analysis for the following time periods: for all data until euthanasia (black bars), $p=0.012$; during the period 0-5 months (red bars), $p=0.003$. After 5 months (white bars) until euthanasia, the IOP was not significantly different between the eyes.

Figure 4.

Detection of GFP expression *in vivo* after FIV.BOVPGFS-myc.GFP or FIV.GFP transduction. GFP expression was monitored serially and noninvasively as previously described (36). Briefly, after installation of 0.5% proparacaine hydrochloride, a Swan-Jacob gonioscopy lens was placed on the corneal surface of anesthetized, supine-positioned monkeys to view the iridocorneal angle and trabecular meshwork tissue. A customized Nikon microscope (Nikon Instruments) with a 12-bit, monochromatic, cooled- CCD (charge-coupled device) camera (Retiga-2000RV; Q-Imaging, Surrey, BC, Canada) and a specific GFP filter set was also used to collect images at all time points (71). Representative images are shown for monkey K691. GFP was detected with the research microscope system until shortly before euthanasia. Top row, first three panels: there is clearly expression overlying and perhaps within the ciliary muscle. Expression in the trabecular meshwork is more visible, but the images do not prove that ciliary muscle expression is negligible compared to trabecular meshwork since the depth of expression (outward from the inner aspect) could not be assessed *in vivo*. Presumably, deeper expression is masked by pigment in the ciliary muscle. Far right panels are from a different monkey and are shown for orientation purposes only. Little or no GFP expression was present in the 626-day post-1st injection images (both of these panels are the same magnification). The text at the bottom of figure indicates the number of days post (d p) 1st, 2nd, and 3rd injections for both eyes.

Figure 5.

Detailed structure of the FIV-based FIV.BOVPGFS-myc.GFP and pGINSIN vectors. hCMVp, Human Cytomegalovirus Immediate Early Promoter; 5' LTR, 5' Long Terminal Repeat; gag, the 5' 230 nucleotides of the gag gene; RRE, Rev Response Element; central polypurine tract important for nuclear translocation and integration of the vector genome; PGF synthase, the bovine prostaglandin F2 α synthase gene; copGFP, copepod Green Fluorescent Protein; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element which increases mRNA stability; 3' LTR, the 3' Long Terminal Repeat containing a 167 nucleotide deletion in the U3 region important for self-inactivation, SV40 Poly-A, SV40 virus polyadenylation signal; eGFP, enhanced Green Fluorescent Protein, IRES, internal ribosome entry site that allows for internal initiation of translation of the eGFP/Neo mRNA; neo, neomycin resistance gene for selection of transduced cells. The structure of pGINSIN is from Saenz et al. (72).

Table 1

GFP *in vivo* grading

Animal#	K674)			K679			K681			K685			K691		
Time post 1 st injection	OD	FIV.GFP	OS	OD	FIV.GFP	OS	OD	FIV.GFP	OS	OD	FIV.GFP	OS	OD	FIV.GFP	OS
	1 × 10 ⁷ TU	1 × 10 ⁷ TU	FIV.PGFS, GFP	1 × 10 ⁷ TU	1 × 10 ⁷ TU	FIV.PGFS, GFP	2 × 10 ⁷ TU	2 × 10 ⁷ TU	FIV.PGFS, GFP	2 × 10 ⁷ TU	2 × 10 ⁷ TU	FIV.PGFS, GFP	2 × 10 ⁷ TU	2 × 10 ⁷ TU	FIV.PGFS, GFP
1 wk							0	0	0	1.5	0.5				
2 wk	1.5	0.5	#	#	#	#							1.0	1.0	1.0
4±1 wk							1.0	1.0	1.0	2.0	1.0	1.0	1.5	1.0	1.0
8±1 wk	1.0	0	0	0.5	0	0	0.5	0	0	2.5	1.0	1.0	1.0	2.5	2.5
12±2 wk	1.5	1.0	1.5	1.5	1.5	1.5	1.0	1.0	1.0	2.0	1.0	1.0	1.0	2.0	2.0
16±2 wk	1.5	1.0	1.0	1.5	1.5	1.5	1.0	0.5	0.5	2.0	0.5	0.5	2.5	1.5	1.5
20±2 wk				1.5	1.0	1.0	0.5	1.5	1.5	1.5	0.5	0.5	2.0	1.5	1.5
24±2 wk	1.5	1.0	#	#	#	#	1.0	1.0	1.0	2.0	1.0	1.0	2.0	2.5	2.5
28±2 wk							0	§	§				1.0	2.0	2.0
32±2 wk							0.5	0.5	0.5	0.5	0.5	0.5	2.0	1.5	1.5
36±2 wk							0.5	1.0	1.0	2.0	1.0	1.0			
40±2 wk										2.0	1.0	1.0	2.0	#	#
44±2 wk	*	1.0	1.0	1.0	1.0	1.0	0	0.5	0.5	1.0	0.5	0.5	2.0 §	1.0 §	1.0 §
48±2 wk	*	1.0					0.5	0.5	0.5	1.5	0.5	0.5	2.0	1.5 §	1.5 §
52±2 wk or final				1.0 §	1.0 §	1.0 §	1.0 § (75 wk)	1.0 § (75 wk)	1.0 § (75 wk)	2.0 (77 wk)	1.5 (77 wk)	1.5 (77 wk)	0 (91 wk)	0.5 (91 wk)	0.5 (91 wk)

* cornea defect—poor view, unable to detect GFP

expression detected with fundus camera but no photos from GFP research microscope available for grading

§_{bad} bad view

italics – results after 2nd injection at the same dose

bold – results after 3rd injection of 2 × 10⁷ TU

Note: detection of GFP is dependent on ability to obtain a suitable image which may not always have been possible

Table 2

Determination of BOVPGFS expression by RT-PCR

		RNA detected by RT-PCR							
		Cornea		Limbus/TM		Iris		Ciliary Body	
Monkey	Gene	OS	OD	OS	OD	OS	OD	OS	OD
K679	bovPGF								
	GFP	X	X	X	X	X	X		X
	endoPGF			X	X				X
K674	bovPGF		X						
	GFP	X	X	X	X				
	endoPGF		X	X	X				
K681	bovPGF								
	GFP	X	X	X	X	X	X		
	endoPGF			X	X				
K685	bovPGF								
	GFP	X	X	X	X	X	X		
	endoPGF				X	X	X	X	X
K681	bovPGF								
	GFP			X	X	X		X	
	endoPGF	X	X			X	X	X	X