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Alexis-Pierre Bemelmans, Sébastien Bonnel, Leila Houhou, Noëlle Dufour, Emeline Nandrot, et al.. Retinal cell type expression specificity of HIV-1-derived gene transfer vectors upon subretinal injection in the adult rat: influence of pseudotyping and promoter.. *The Journal of Gene Medicine*, 2005, 7 (10), pp.1367-1374. 10.1002/jgm.788 . hal-00187439

**HAL Id: hal-00187439**

**<https://hal.science/hal-00187439>**

Submitted on 6 Nov 2020

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Full Title:

Retinal cell type expression specificity of HIV-1-derived gene transfer vectors upon subretinal injections in the adult rat: influence of pseudotyping and promoter.

Short Title:

Pseudotyped lentivector for retinal gene transfer

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Key Words: gene transfer, lentiviral vector, pseudotyping, promoter, retinal pigment epithelium, photoreceptors.

## ABSTRACT

**Background:** Gene therapy, and particularly gene restoration, is currently a great hope for non-curable hereditary retinal degeneration. Clinical applications require a gene transfer vector capable of accurately targeting particular cell types in the retina. To develop such a vector, we compared the expression of a reporter gene after subretinal injections of lentiviral constructs of various pseudotypes and with the transgene expression driven by various promoters.

**Methods:** Lentiviral vectors expressing green fluorescent protein (GFP) under the transcriptional control of cytomegalovirus (CMV), mouse phosphoglycerate kinase (PGK), human elongation factor 1- $\alpha$  (EF1 $\alpha$ ), or human rhodopsin (RHO) promoters were pseudotyped by vesicular stomatitis virus or Mokola virus envelope proteins. These constructs were injected into the subretinal space of adult *rdy* rats. GFP expression was analysed *in vivo* one and four weeks after injection by fundus examination. The precise location of transgene expression was then determined by immunohistochemistry and *in situ* hybridization.

**Results:** Constructs of both vesicular stomatitis virus and Mokola pseudotypes with ubiquitous promoters led to a strong expression of GFP *in vivo*. Histological studies confirmed the production of GFP in the retinal pigment epithelium in most cases. However, only the combination of the VSV pseudotype with the RHO promoter led to GFP production in photoreceptors, and did so in a sporadic manner.

**Conclusions:** Mokola-pseudotyped lentiviral vectors are effective for specific gene transfer to the retinal pigment epithelium. Neither vesicular stomatitis virus nor Mokola-pseudotyped lentiviral vectors are adequate for efficient gene transfer to photoreceptors of adult rats.

## INTRODUCTION

Eye diseases are becoming one of the most promising targets for gene therapy. Various eye diseases, and in particular those involving a neurodegeneration of the retina, fulfill numerous conditions required for “proof of concept” testing for therapeutic gene transfer: (i) animal models of retinal disease allow validation of therapeutic strategies; (ii) several therapeutic genes have been identified and understanding of retinal physiopathology is continuing to improve; (iii) ocular gene transfer needs only local delivery; (iv) existing vitreal-retinal surgical techniques are available to deliver gene transfer vectors to a precise location in the human eye.

For many diseases, particular therapeutic genes will have to be targeted to specific cell types in the retina. In the case of a retinitis pigmentosa caused by a mutation in a gene expressed in photoreceptor cells (PRs), a restorative therapy, in which a fully functional copy of the gene is expressed in PRs, may be possible. Alternatively, protective therapy could be developed based on a factor secreted by the retinal pigment epithelial cells. In both cases a vector that is able to drive transgene expression in specific cell types in the retina is required.

Adenovirus, adeno-associated virus (AAV) and lentivirus-derived vectors have been widely used to transfer genes to retinal cells. Subretinal injections of adenoviral vector lead to efficient transduction of the retinal pigment epithelium (RPE) and only limited transduction of the PRs [1-3]. However, adenoviral vectors trigger an immune response which leads to the rejection of the transduced cells [4, 5]. Even the last generation of adenoviral vectors, totally devoid of viral coding sequences, do not escape from a transient immune response on administration [6]. This last generation of adenoviral vectors allows transgene expression for longer times than the previous generations, but expression remains transient [7]. Studies in animals suggest that lentiviral and AAV vectors are more likely to fulfill the criteria for future

clinical trials [8-10]. AAV have been extensively used to transduce retinal cells after subretinal injections. According to the serotype used, they transduce PRs only or PRs and the RPE with a high efficiency [8, 11, 12]. AAV-mediated gene transfer to the eye appears stable, although one study reported latency in transgene expression that can last between one and several months in primates [10]. This delay might be a serious hurdle for clinical applications requiring rapid treatment. Once their effectiveness in transducing CNS cells *in vivo* was demonstrated, lentiviral vectors were used for the eye [9], and they transduce the RPE with a high efficiency. Nevertheless, their ability to transfer genes to PRs remains unclear, with some studies demonstrating efficient transduction and others reporting poor or no transduction [13-16].

There are two main strategies currently under investigation for targeting transgene expression to specific cell types. One consists in using an expression cassette containing a cellular specific promoter. This approach has been used with success in various viral vectors, including adenoviruses and lentiviruses [9, 17]. The second strategy is to modify the viral vector to transduce cells not naturally infected by the virus. In the case of lentiviruses the possibility of pseudotyping the virus by an envelope other than the widely used vesicular stomatitis virus G protein (VSV-G) protein has recently emerged as a powerful tool to expand the vector tropism to diverse cell types (reviewed in [18]).

We report here an investigation of whether HIV-1 derived lentiviral vectors can be engineered to target different cell types of the rat retina, using various combinations of specific promoters and pseudotypes. Recombinant lentiviruses encoding green fluorescent protein (GFP) under the transcriptional control of various ubiquitous and cellular specific promoters were constructed and produced. They were pseudotyped with the classical VSV-G or the lyssavirus Mokola G protein (Mok-G). After subretinal injection into adult *rdy* rats, GFP expression was analysed to assess the influence of promoters and pseudotyping on lentiviral gene transfer to

rat retinal cells. We found that only the combination of the VSV-G pseudotype with the cellular specific rhodopsin promoter allowed efficient transgene expression in few PRs. Contrarily, RPE cells were very efficiently transduced with both VSV-G and Mok-G.

## **EXPERIMENTAL METHODS**

### ***Lentiviral vectors construction and production.***

Plasmids containing the HIV-1 derived vector genome were all constructed from pTrip-CMV-GFP (described in [19]) by replacing the CMV promoter by either the mouse phosphoglycerate kinase 1 promoter (PGK, described in [20]), a 234-bp fragment (ranging from -203 to +31) of the elongation factor 1 $\alpha$  promoter (EF1 $\alpha$ , described in [21]) or a 2.2-kb fragment of the human rhodopsin promoter (RHO, amplified from clone pJHN7, kindly provided by Dr J. Bennett, [22]). In all cases, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was added 3' to the GFP gene to increase transgene expression [23]. Vector stocks were produced by transient transfection of 293T cells with the vector genome plasmid, a packaging plasmid and an envelope-coding plasmid as described previously [19]. Briefly, the cell supernatant was harvested 48 hours after transfection, treated with DNase, filtered through 0.45  $\mu$ m pore-sized filters, and subjected to ultracentrifugation at 64'000 g for 90 minutes. The virus pellet was resuspended in PBS. Physical titers of the viral stocks were determined by ELISA quantification of the p24 capsid protein concentration using Beckman Coulter kit (Villepinte, France) according to the manufacturer's instructions. All the stocks used in this study had a p24 concentration of the same order of magnitude, ranging from 130 to 250 ng. $\mu$ l<sup>-1</sup>.

### ***Animal care and treatment***

Animals were handled in strict accordance with the Helsinki Declaration and with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research. The animals were kept at 21°C with a 12-h light:12-h dark cycle and fed *ad libitum*. All the experiments were performed on young adult (2 to 3 months old) *rdy* rats (kindly provided by Dr M.M. La Vail, University of California, San Francisco, CA). Subretinal injections were performed under general anaesthesia induced by intra-muscular injection of ketamine/xylazine. Eyes were treated by consecutive local delivery of oxybuprocaine, neosynephrin and tropicamid to inhibit the eyelid reflex and induce pupil dilation. Subretinal injections were given through a trans-scleral approach with a 34G modified Hamilton syringe. The infectious titres of vectors consisting of various promoters and pseudotypes being not comparable, we thus used the amount of p24 protein to define the dose of vector: 100 ng of vector was delivered to each eye in 3 µl of saline containing a small amount of fluorescein. The pressure of injection was set to allow serous detachment without retinal breaks. The injection site was immediately checked by visualisation of the fluorescein position in the fundus under the operating microscope: this allowed differentiating correct subretinal injection from choroidal or intra vitreous injections. Treated and untreated eyes were covered with sterdex paste (Ciba Vision, Basel, Switzerland) containing dexamethasone and oxytetracycline.

### ***In vivo imaging of GFP expression: Fundus Photography and Fluorescein Angiography***

*In vivo* GFP fluorescence was monitored by fundus photography one week and one month after subretinal injection. Rats were anaesthetized by intra-muscular injection of ketamine/xylazine and eyes were treated with neosynephrin and tropicamid to induce pupil dilation. A Canon CF-60ZA human retinal camera modified as previously described was used

for fundus photography and fluorescein angiography [24]. Blue light was used for excitation to detect GFP fluorescence. Then the rats were intraperitoneally injected with 0.15 g/kg of 10% sodium fluorescein (Ciba Vision, Basel, Switzerland) and serial photographs were taken during the 10 min following the injection [25] to detect any alteration of retinal tissues induced by the gene transfer and transduction.

### ***Histological procedures***

Rats were deeply anesthetized by intra-peritoneal injection of chloralhydrate (500 mg.kg<sup>-1</sup>) and perfused transcardially by ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Eyes were then enucleated, and the anterior chamber, cornea, lens and vitreous were discarded. Enucleated eyes were immersed for 2 hours in the same fixative, overnight in PBS containing 30% sucrose, and then in OCT compound immediately before being frozen in dry ice. Serial sections (16 µm) were cut on a cryostat, collected on Superfrost slides, and stored at -80°C until further processing. Alternatively, flat-mounted retinas (after perfusion, dissection and post-fixation) were prepared: neural retina was separated from RPE/choroid/sclera layers and both parts were mounted under coverslips with glycergel. For immunohistochemical staining of GFP, sections were consecutively incubated in a solution of 5% deionised formamide, 5% 20x saline sodium citrate (20x SSC) and 5% H<sub>2</sub>O<sub>2</sub> in water until depigmentation, in PBS containing 10% normal goat serum (NGS) and 0.2% Triton X-100 for two hours, and overnight in PBS containing 1% NGS, 0.2% Triton X-100 and anti-GFP rabbit polyclonal antibody (ab290, Abcam, Cambridge, UK) diluted 1:5000. Bound primary antibody was then detected according to the manufacturer's instructions with Vectastain ABCkit (Vector laboratories, Burlingame, CA, USA) and using diaminobenzidine (DAB) as the chromogen. Sections were then dehydrated, lightly counterstained with haematoxylin and mounted under coverslips in non-aqueous medium. For



immunofluorescence labelling, sections were incubated in PBS containing 10% NGS, 1% gelatine and 0.2% Triton-X100 for two hours, and then overnight in the same solution containing anti-GFP rabbit polyclonal antibody (1:1000 dilution, ab290, Abcam, Cambridge, UK) and anti-GFAP mouse monoclonal antibody (1:300 dilution, GA5, Novocastra Laboratories, Newcastle, UK). Sections were then rinsed three times in PBS, incubated for one hour with secondary antibodies (1:100 FITC-conjugated polyclonal Goat anti-rabbit IgG (H+L), TebuBio, Le Perray en Yvelines, France and 1:300 Cy3-conjugated affininpure goat anti mouse IgG (H+L), Jackson Immunoresearch Laboratories, West Grove, PA, USA), rinsed three times in PBS and mounted under coverslips in Mowiol.

For *in situ* hybridization, the EGFP coding sequence was inserted into pGEMT-easy (Promega, Charbonnières, France), which was then linearized with NcoI. An antisense riboprobe labelled with digoxigenin (DIG)-UTP was synthesized by the SP6 polymerase and used to probe on the cryostat sections as described in [26]. 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (both from Roche, Meylan, France) were used to obtain a blue staining.

As negative controls, all the histological procedures aiming at detecting the GFP expression have been tested on non-injected eyes and failed to give any signal. Similarly, no direct fluorescence could be detected on flat-mounted retinas of non-injected eyes.

## **RESULTS**

### **GFP fluorescence was detected *in vivo* by fundus photography.**

We studied GFP expression after subretinal administration of HIV-CMV-GFP pseudotyped with VSV-G or Mok-G. The subretinal location of the injection was assessed by fundus

examination. When subretinal injection was successful, subretinal fluids appeared as a precisely delimited bleb with the retinal vessels clearly visible, whereas inadequate intravitreal injection of the vector resulted in a deposit with blurred outlines and masking the retinal vessels (data not shown). For each vector type a total of at least eight correct subretinal injections were performed. One week after injection fundus examination revealed a strong GFP fluorescence in all animals given HIV-CMV-GFP vectors (Figure 1a and b) pseudotyped with either VSV-G or Mok-G. In every case, the area of GFP expression was strictly delimited, and this area was entirely within the subretinal bleb. In some cases, the centre of the transduced area showed no GFP expression, with the most intense fluorescence at the margin of the bleb; this pattern may reflect toxicity associated with the surgery or the vector. We then tested vectors encoding GFP under the control of house keeping gene promoters (PGK and EF1 $\alpha$ ) or a cellular specific promoter (RHO). GFP was detected in rats treated with HIV-PGK-GFP and HIV-EF1 $\alpha$ -GFP although the signal was lower than with CMV (data not shown). In HIV-RHO-GFP-treated rats, no GFP was detected with either the VSV-G pseudotype or with the Mok-G pseudotype. After testing for GFP, all the animals were injected intra-peritoneally with fluorescein to examine retinal blood vessels and retinal layers (Figure 1c and d). Blood vessels and retinal layers appeared normal in all animals, indicating that subretinal injection of 100 ng of lentiviral vector is not harmful and does not induce a major toxicity event in the retina with reorganization of the various cell layers. Fundus examination one month after vector injection gave the same findings than the examination one week post injection. Thus, irrespective of the pseudotype, vectors with ubiquitous promoters drive strong transgene expression in the retina, whereas GFP was not produced in detectable amounts from the GFP gene under the RHO promoter.

**VSV-G and Mok-G pseudotypes transduce the RPE with high efficiency.**

We searched for the site of transgene expression. Animals injected with the various vectors were sacrificed one month post injection, after the second fundus examination, and GFP was detected histologically. Firstly, flat-mounted retinas were prepared from two animals of each group to examine direct GFP fluorescence (Figure 2). There was no fluorescence in the neural retina layers with none of the vectors tested as assessed by microscopy. In the RPE/choroid/sclera layers, GFP was detected in eyes that had received HIV-CMV-GFP, HIV-PGK-GFP, or HIV-EF1 $\alpha$ -GFP pseudotyped with VSV-G or Mok-G. Higher magnification revealed that the GFP was in cells with typical morphology of the RPE: bi-nucleated and hexagonally shaped (Figure 2c). No fluorescence was detected in flat-mounted retinas of eyes injected with the HIV-RHO-GFP vector stocks. GFP fluorescence was also detected on cryostat tangential sections in an eye injected with a VSV-G pseudotype HIV-CMV-GFP vector (Figure 2d).

### **VSV-G pseudotype and RHO promoter are necessary to obtain transgene expression in PRs.**

Tangential cryostat sections were prepared from all the remaining eyes and used for immunohistochemistry to determine the site of GFP expression in the retina. In all cases, one month following the transient retinal detachment associated with the subretinal injection, the neural retina had re-attached to the RPE (Figure 3f). We observed a strong staining of the RPE layer in all the eyes injected with ubiquitous promoters containing vectors (HIV-CMV-GFP (n=12), HIV-PGK-GFP (n=11), or HIV-EF1 $\alpha$ -GFP (n=8)) (Figure 3). This pattern was independent of the pseudotype, consistent with the findings from flat-mounted retinas (Figure 2). We did not detect any staining in the other retinal layers except in one eye of the Mok-G pseudotyped HIV-PGK-GFP group (n=6, Figure 5c) and one eye of the VSV-G pseudotyped HIV-CMV-GFP group (n=6, Figure 5a). In these two cases, in the middle of the transduced

area, GFP immunostaining was evident in the PRs segments, but surprisingly no staining appeared in the corresponding outer nuclear layer (ONL) (Figure 5a and c). In eyes injected with HIV-RHO-GFP vectors, there was a discrete staining of the RPE layer with both VSV-G and Mok-G pseudotypes (Figure 4a and c respectively). In all eyes given VSV-G pseudotyped HIV-RHO-GFP vector (n=8), there were a few PRs that were intensely stained from the nucleus to the segment (Figure 4a). No GFP was found in PRs in eyes given Mok-G pseudotype (n=8). The observations done on retinal sections after immunohistochemical staining of GFP are summarized in table 1.

### **The RHO promoter displays a weak and ectopic activity in the RPE.**

To confirm localization of the transduced cells, we looked for GFP mRNA by *in situ* hybridization. Consistent with the immunohistochemical staining of GFP, PRs containing GFP mRNA were only found in eyes injected with VSV-G pseudotyped HIV-RHO-GFP (Figure 4b). In these eyes, the colorimetric reaction had to be extended to eight hours to detect GFP mRNA in the RPE layer, confirming the weak activity of the RHO promoter in the RPE (Figure 4b and d). In contrast, an intense staining appeared in the RPE layer after a colorimetric reaction of one hour in eyes injected with HIV-CMV- or HIV-PGK-GFP vectors. No GFP mRNA was detected in the PRs in these groups even after prolonged reactions (Figure 5b and d).

In the VSV-G pseudotyped HIV-RHO-GFP injected eyes, the cells expressing GFP in the neural retina displayed the characteristic morphology of photoreceptors, with the segments, the nuclei and the synaptic endings clearly visible (Figure 6a and d). No GFP labelling was detected in other retinal layers, except in RPE where some cells were positive, presumably due to an ectopic activity of the RHO promoter (data not shown). To assess for potential inflammation, we performed a labelling of glial fibrillary acidic protein (GFAP), a glial

marker up-regulated during inflammation. Close to the injection site, GFAP was up-regulated, as demonstrated by the labelling throughout the cell body of Müller cells, from the ganglion cell layer (GCL) to the ONL (Figure 6b). Further from the injection site, GFAP was detected only close to the GCL (Figure 6e), indicating that GFAP up-regulation was probably due to the surgical act than to transduction of neighbouring cells by the lentiviral vector. No double-labelled cells were observed, excluding the possibility of Müller cells transduction (Figure 6c and f).

## DISCUSSION

We report a study of the tropism of HIV-1 derived lentiviral vectors pseudotyped with the VSV-G envelope or the Mok-G envelope and expressing GFP reporter gene under the transcriptional control of various ubiquitous or cell-specific promoters after injection into the subretinal space of adult *rdy* rats. Both VSV-G and Mok-G pseudotyped vectors transduced RPE efficiently. In contrast, only VSV-G vectors transduced PRs but the efficiency was poor. Furthermore, PRs were only transduced by constructs carrying the cell-specific rhodopsin promoter, suggesting that the ubiquitous promoters used in this study are not efficient for transgene expression after lentiviral gene transfer in PRs of adult *rdy* rats.

In our experimental model, the adult *rdy* rat, VSV-G pseudotyped lentiviral vectors transduced cells of the ONL with a very low efficiency. Few cells were transduced by constructs with the RHO promoter (Figure 4 and 6). Substantial GFP immunoreactivity was found in the PR segment layer in two of the thirty-one eyes injected with CMV or PGK vectors but *in situ* hybridization on adjacent sections indicated that the PRs had not been transduced (Figure 5). The GFP immunostaining observed in the PRs segment layer in these

two cases might be the consequence of GFP overproduction in the RPE leading to the presence of GFP in the microvilli of RPE cells or to the release of GFP in the extracellular matrix due to abnormal function of RPE cells.

All studies describing subretinal injections of VSV-G pseudotyped lentiviral vectors encoding reporter genes in rodents or non-human primates report efficient transduction of the RPE, but few demonstrate efficient transduction of the PRs. The discrepancies between studies may be due to the age of the injected animals and the dose of vector used. Miyoshi *et al.* described GFP expression in RPE and PRs by VSV-G pseudotyped HIV-derived vectors with a CMV and a RHO promoter, and found transduction to be more efficient when rats were injected at 2 to 5 days old than at 4 weeks old, suggesting that maturation of PRs makes them resistant to transduction by VSV-G pseudotyped HIV-derived vectors [9]. Similarly, Auricchio *et al.* described an efficient transduction of the PRs with the same type of vector, but the work was done on 5-7 weeks old mice given large doses of vector [13]. Using a VSV-G pseudotyped FIV vector injected in the subretinal space of cynomolgus monkeys, Lottery *et al.* showed efficient transduction of the RPE and sporadic transduction of PRs [15], similar to our findings here. Our results are also in accordance with a number of studies showing that in adult rodents, subretinal administration of VSV-G pseudotyped lentiviral vectors preferentially transduce the RPE layer of the retina [14, 16, 27, 28]. Thus, it appears that VSV-G-pseudotyped lentiviral vectors are only suitable for transducing PRs in particular conditions, each of the species, the age, and the dose of vector used might as well be important.

Results obtained by different teams with Mok-G pseudotyped lentiviral vectors are less divergent. Like previous studies [13, 16], we found that this type of lentiviral vector transduces RPE with an efficiency similar to the one obtained with VSV-G pseudotyped vectors; however these vectors are unable to transduce neuronal cells of the retina. This result

is consistent with recent studies showing that after injection into the brain parenchyma, Mok-G pseudotype vectors transduce astroglial cells more efficiently than neuronal cells (Sarkis *et al.* submitted, [29]).

Specific promoters could possibly be used instead of pseudotyping to obtain transgene expression restricted to a particular cell type. We compared the activity of ubiquitous promoters with that of the rhodopsin promoter. The rhodopsin promoter drove GFP expression in PRs, as demonstrated by immunostaining and *in situ* hybridization (Figure 4), but surprisingly, led also to a weak but detectable expression in the RPE. This ectopic expression from the rhodopsin promoter is probably a consequence of a lack of specificity of the fragment used for the genetic construction and of an overload of RPE cells by the vectors, with both the VSV-G and the Mok-G pseudotypes. Kostic *et al.* described the expression of GFP as being more efficient following transduction of PRs using a VSV-G pseudotyped HIV vector when the subretinal injections were given to newborn mice in comparison to adults [17]. Moreover, they report that PRs transduction was favoured by the surgical trauma suggesting that the retinal structure itself may present a physical barrier to transduction of neuronal cells. Thus, it appears that for efficient transduction of rodent PRs with a VSV-G pseudotyped lentivirus, the vector has to be administered very early in life.

The absence of PRs transduction with Mok-G pseudotyped lentiviruses, even, as demonstrated here, with the RHO promoter which is strongly expressed in these cells, demonstrates the value of this pseudotype for prolonged expression of transgenes specifically in RPE cells. An alternate strategy to obtain a transgene expression restricted to RPE cells could be the use of a promoter specific to the RPE, such as the one from the RPE65 gene. Nevertheless, RPE65 promoter has not yet proven to have an expression strictly restricted in the RPE [30]. This kind of promoter might be useful for RPE65 gene replacement but Mokola pseudotyping appears currently to be the best strategy for obtaining a transgene expression

strictly restricted to RPE cells after lentiviral-mediated gene transfer. This type of vector could be use to correct a genetic defect of the RPE by providing a fully functional gene, or to make these cells function as biological pumps for long-term secretion of a survival factor for the nearby PRs.

## ACKNOWLEDGMENTS

SB is the recipient of a PhD Grant from "Association Française des Amblyopes Unilatéraux". This work was supported by "Association Rétina-France", "Association Française contre les Myopathies", the French "Ministère de la Recherche Programmes Bio-Ingénierie 2000 et 2001", and "Agence Nationale pour la Valorisation de la Recherche" (ANVAR).

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## LEGENDS

### Figure 1:

GFP direct fluorescence *in vivo*. Fundus photographs of living rats seven days after subretinal injection of HIV-CMV-GFP, VSV-G pseudotyped (A-C) or Mok-G pseudotyped (B-D). C and D show views of the eyes illustrated in A and B respectively 5 minutes after an intraperitoneal bolus injection of fluorescein to evidence the blood vessels.

### Figure 2:

GFP direct fluorescence on flat-mounted retinas and cryostat sections. (A) Bright field photograph of a flat mounted-RPE: after fixation, the lens and the anterior chamber were removed, the posterior chamber was cut into four quadrants and flattened on a glass slide. The RPE was then parted from the neural retina and both layers were mounted separately. (B) GFP direct fluorescence in a flat-mounted RPE from an eye administered VSV-G pseudotyped HIV-CMV-GFP, showing the whole transduced area. (C) Higher magnification of transduced RPE after subretinal injection of VSV-G pseudotyped. The hexagonal shape and the binuclear aspect is characteristic of RPE cells. (D) GFP direct fluorescence visualized on a cryostat tangential section of a VSV-G pseudotyped HIV-CMV-GFP injected eye. (E) phase contrast view of D. CHO: choroid layer; RPE: retinal pigment epithelium; ONL: outer nuclear layer. Scale bar represents 150  $\mu\text{m}$  in B, 20  $\mu\text{m}$  in C, D and E.

### Figure 3:

GFP immunohistochemical staining of rat retinal cryostat sections one month after subretinal injection of vectors containing various ubiquitous promoters. A,D: CMV promoter; B,E: PGK promoter; C: EF1 $\alpha$  promoter. A-C: VSV-G pseudotyped vectors; D,E: Mok-G pseudotyped

vectors. Strong GFP staining was evidenced in every RPE, whereas no GFP was detected in the PRs layer. A: all the neural retina layers are totally devoid of staining. F: photomontage of a retinal section taken at the centre of the eyeball after injection of Mok-G pseudotyped HIV-PGK-GFP. The transduced area, delineated by arrows, covered more than half of the RPE, and, one month after injection, no retinal detachment could be seen. ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 20  $\mu\text{m}$  in (A-E), 300  $\mu\text{m}$  in F.

**Figure 4:**

GFP expression one month after subretinal injection of HIV-RHO-GFP vector pseudotyped with either VSV-G (A, B) or Mok-G (C, D). GFP expression was evidenced by immunohistochemical staining (A, C) or by *in situ* hybridization (B, D). Both detection methods revealed weak GFP expression in the RPE layer independently of the pseudotype used (arrowheads in B, C and D). PRs expressing GFP were detected only after injection of the VSV-G pseudotyped vector, and, in this case, the immunohistochemical staining showed that the GFP protein was located both in the nucleus (arrows in A) and in the segment (arrowheads in A) of PRs. Transgene expression in the PRs was confirmed by detection of GFP mRNA in the PRs layer by *in situ* hybridization (arrows in B). Scale bar: 20  $\mu\text{m}$ .

**Figure 5:**

Transgene expression revealed by immunostaining (A, C) and *in situ* hybridization (B, D) on adjacent sections of the two eyes displaying GFP protein in the PRs segment after injection of HIV-CMV-GFP VSV-G pseudotyped (A, B) or HIV-PGK-GFP Mok-G pseudotyped (C, D). Although GFP protein is clearly revealed in the segment layer by immunostaining in both cases (A, C), the *in situ* hybridization showed that there is no GFP mRNA in the corresponding cells (B, D). Scale bar: 40  $\mu\text{m}$ .

**Figure 6:**

Double labelling for GFP and GFAP after injection of VSV-G pseudotyped HIV-RHO-GFP. GFP was detected in the nuclei, the segments (arrows) and the synaptic endings (arrowheads) of PRs (A, D). GFAP immunoreactivity was more intense close to the injection site (B) than at distance (E) where GFAP expression was restricted to the part of Müller cells apposed to the GCL. C (A and B merged) and F (D and E merged): GFAP (in Müller cells) and GFP were not co-localized. CHO: choroid; GCL: ganglion cell layer. Scale bar: 25  $\mu$ m.

**Table 1:**

Summary of immunohistochemical results. GFP expression was estimated on both the quantity of expressing cells and the quality of expression in these cells. ++++: strong expression in numerous cells; ++: strong expression in few cells; +/-: weak expression; -: no expression.

Figure 1

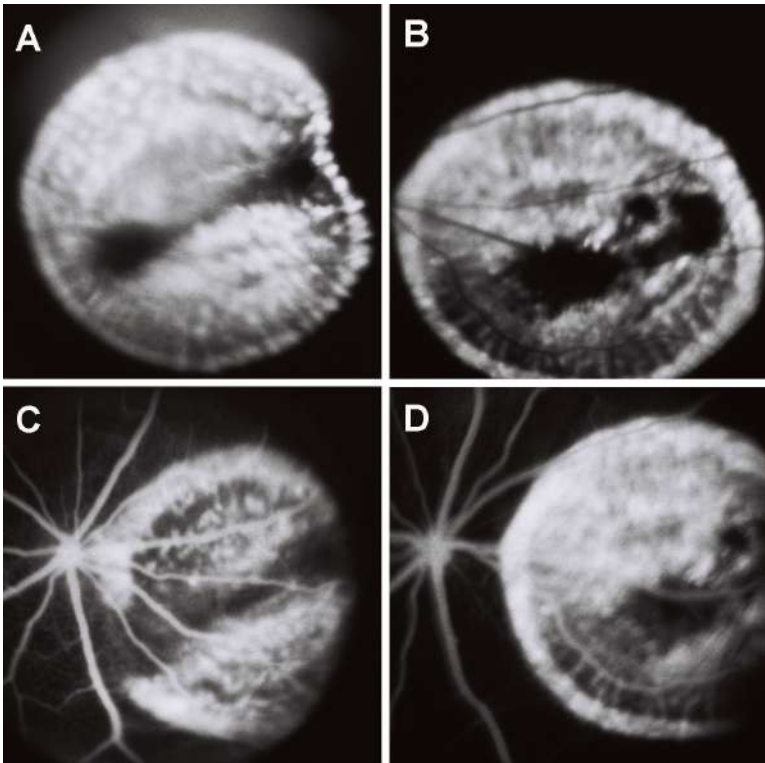


Figure 2

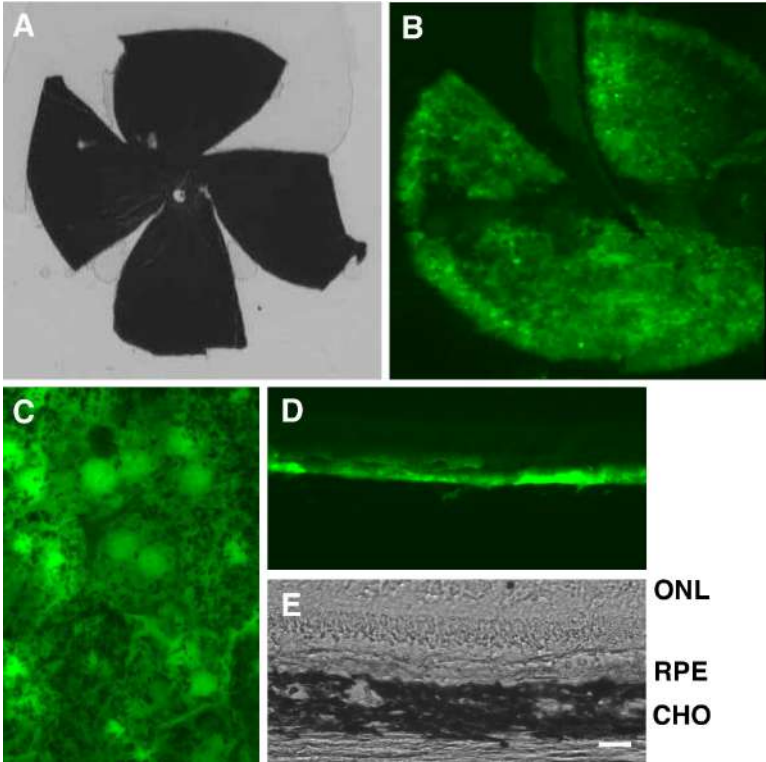




Figure 3

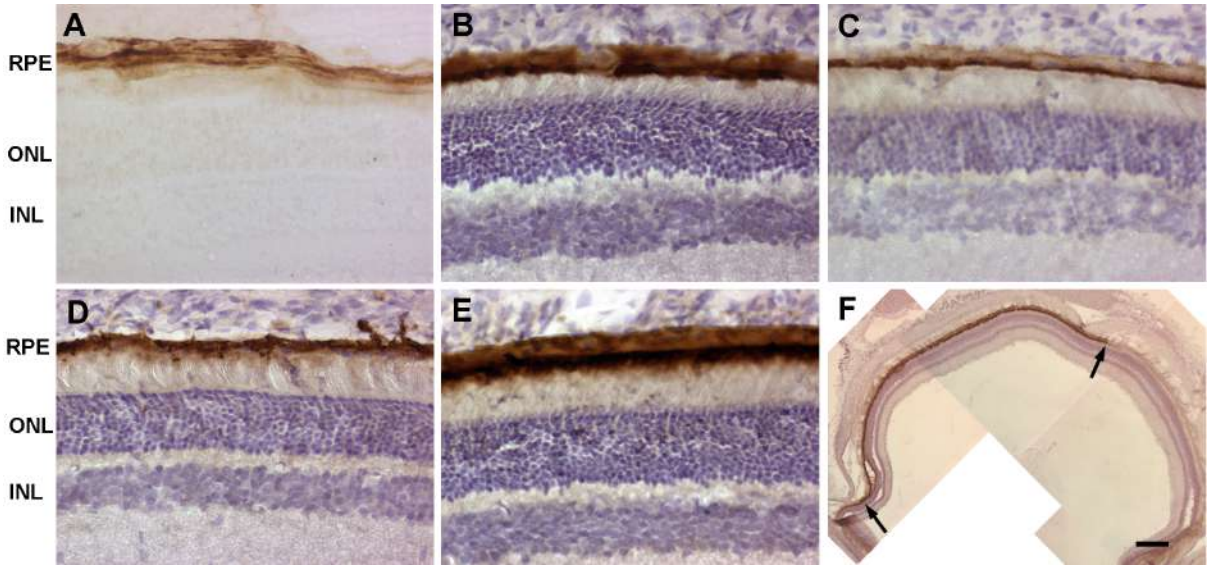


Figure 4

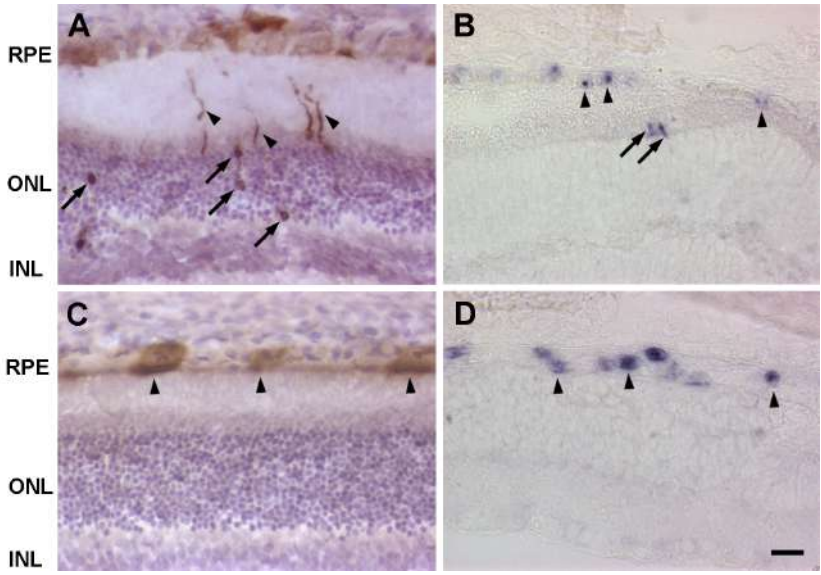


Figure 5

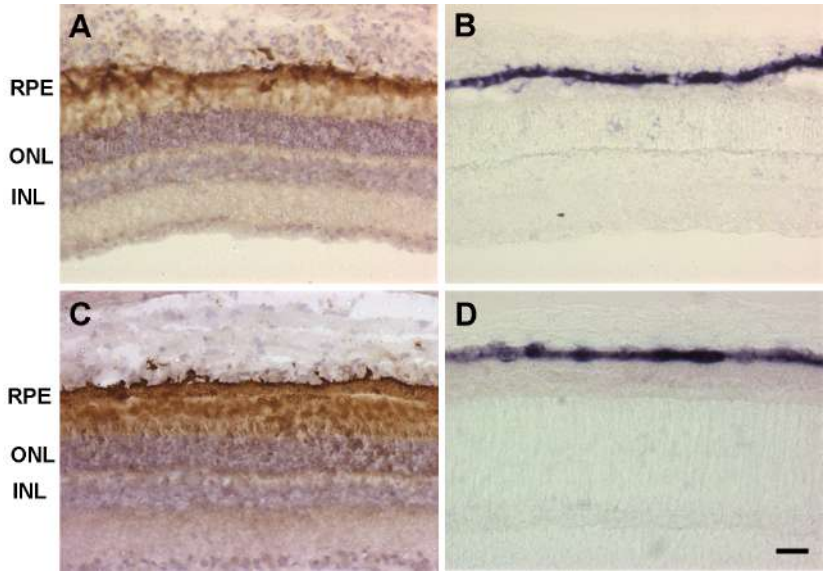


Figure 6

