

**BRIEF COMMUNICATION**

# Activity analysis of housekeeping promoters using self-inactivating lentiviral vector delivery into the mouse retina

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For most retinal degeneration disorders, no efficient treatment exists to preserve photoreceptors (PRs) and, consequently, to maintain vision. Gene transfer appears to be a promising approach to prevent PR loss. In order to design adequate vectors to target specific retinal cell types, we have analyzed the expression pattern of three different promoters (mouse phosphoglycerate kinase 1 (PGK), elongation factor-1 (EFS), rhodopsin (Rho)) in newborn and adult DBA/2 mice retinas using self-inactivating lentiviral vectors. At 7 days after intraocular injection and in optimal conditions, cell transduction was observed up to 1.5 mm from the injection site. PGK promoter expression was predominant in the retinal pigment epithelium (RPE), especially in adult mice, whereas the EFS promoter allowed a broad expression in the

retina. Finally, as expected, the Rho promoter was specifically expressed in PRs. Differences in the cell types transduced and in transduction efficiency were observed between newborn and adult injected eyes emphasizing the importance of such basic studies for further gene therapy approaches as well as for understanding the transcriptional changes during retinal maturation. Thus, for future attempts to slow or rescue retinal degeneration by lentiviral delivery, PGK and EFS are more suitable to control the expression of a supporting secreted factor, PGK being mainly expressed in RPE and EFS in different cell types throughout the entire retina, whereas Rho should allow to specifically deliver the therapeutic gene to PRs.

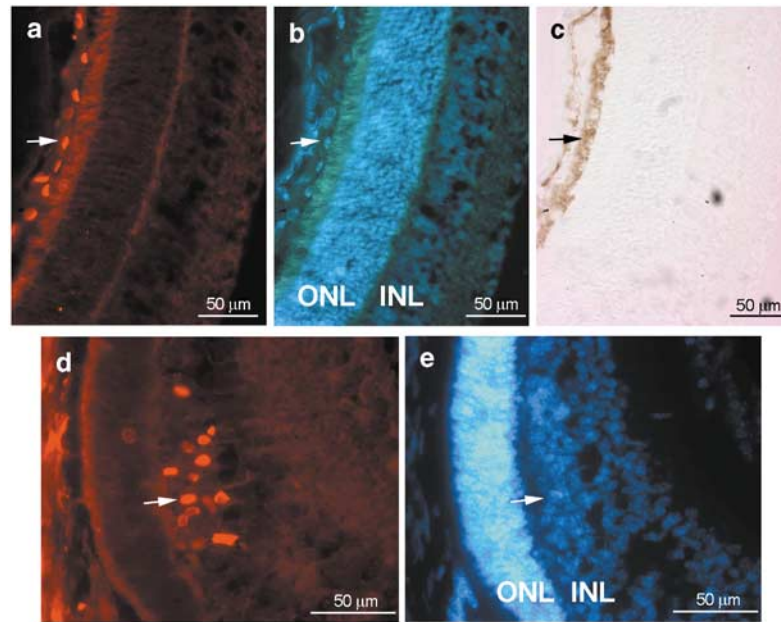
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Gene transfer approaches have been evaluated in the retina for less than 10 years using both adenovirus and adeno-associated virus (AAV) as vectors.<sup>1–4</sup> However, only a few studies describe the potential use of lentiviral vectors in the retina. The effect of the injection site on cell transduction was explored by Bainbridge *et al*<sup>5</sup> and Takahashi *et al*,<sup>6</sup> whereas the tropism of different pseudotyped lentiviruses was determined by Auricchio *et al*.<sup>7</sup> Since retinal degeneration can be due to dysfunctions of different retinal cell types such as retinal pigment epithelium (RPE) cells or photoreceptors (PRs), it is of prime importance to specifically target each cell population. We propose here an alternative approach to target specific retinal cell subtypes by studying the expression patterns of different promoters in the retina.

Although encouraging preliminary results were obtained concerning the use of HIV-derived lentiviral vectors in the retina,<sup>8,9</sup> long-term transgene expression and safety studies as well as the optimization of specific gene targeting still need to be performed before envisaging lentiviral-based clinical trials on patients suffering from eye disorders. In the optic of increasing our

knowledge concerning this vector in the ocular context, Bainbridge *et al*<sup>5</sup> described the consequence of lentiviral delivery sites on cell transduction. According to these authors, anterior chamber injections promote corneal endothelial cell transduction, subretinal injections promote RPE and PR transduction, whereas intravitreal injections are inefficient. Recently, the efficiency of the bovine immunodeficiency virus (BIV) pseudotyped with the VSV-G envelope was also analyzed following intravitreal or subretinal injections in adult mice.<sup>6</sup> RPE transduction efficiency was demonstrated using subretinal injections, whereas other retinal cells were rarely transduced. Alternatively, Auricchio *et al*<sup>7</sup> studied the tropism of different pseudotyped HIV-derived lentiviral vectors expressing green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter in murine retinas. After subretinal injection, VSV-G-pseudotyped lentiviral vectors allowed transduction of both RPE and PR, whereas Mokola-pseudotype lentiviral vectors are specific for RPE. This observation is of prime importance for targeted lentiviral applications, such as specific RPE delivery of a therapeutic gene. Among the different characteristics of viral vector used for gene therapy, specific cell-type targeting is an important consideration to limit undesirable effects and to ensure safe expression of the therapeutic gene. To



**Figure 1** PGK activity in PN11 retina of DBA/2 mice. The SIN-PGK-lacZ-WPRE self-inactivating lentiviral construct is described by Deglon et al.<sup>11</sup> and high titer preparation was performed as described by Naldini et al.<sup>12</sup> We determined p24 antigen titer by ELISA and injected, respectively, the equivalent of 240 and 400 ng of p24 per newborn eye and adult eye. As determined in Deglon et al.,<sup>11</sup> this amount of p24 antigen corresponds, respectively, to around  $5 \times 10^5$  and  $9 \times 10^5$  infectious units for this vector. The volume of viral vector suspension injected was adapted to eye dimensions, which are different between newborn and adult mice. Intraocular injections of 0.8 and 1.5  $\mu$ l of concentrated preparation in newborn and adult eyes ( $n=8-10$ ) were performed by the temporal transchoroidal approach using a 5  $\mu$ l syringe (Hamilton, Bonaduz, Switzerland) with a 34G beveled needle. PGK promoter drives the expression of  $\beta$ -Gal frequently not only in RPE cells (a–c arrow), but also in some cells of the INL (d, e arrow). a, d,  $\beta$ -Gal labeling; b, e, Dapi staining; c, bright light.

achieve this goal, either viral entry or transgene expression should be cell type-dependent. Complementary to the study of Auricchio *et al.*,<sup>7</sup> which described different cell-type targeting in function of the lentiviral envelope, we chose to study the pattern of expression of different promoters in the retina. The initial work done by Miyoshi *et al.*<sup>8</sup> demonstrated PR transduction following subretinal injection, in rats, of a lentivirus expressing the GFP under the control of the rhodopsin (Rho) or the CMV promoter. Following this study, Takahashi *et al.*<sup>9</sup> provided the proof of principle for gene replacement therapy using an HIV-derived lentiviral vector in the *rd* mouse retinal degeneration model. We propose here the study of two additional promoters whose pattern of expression was unknown in the retina.

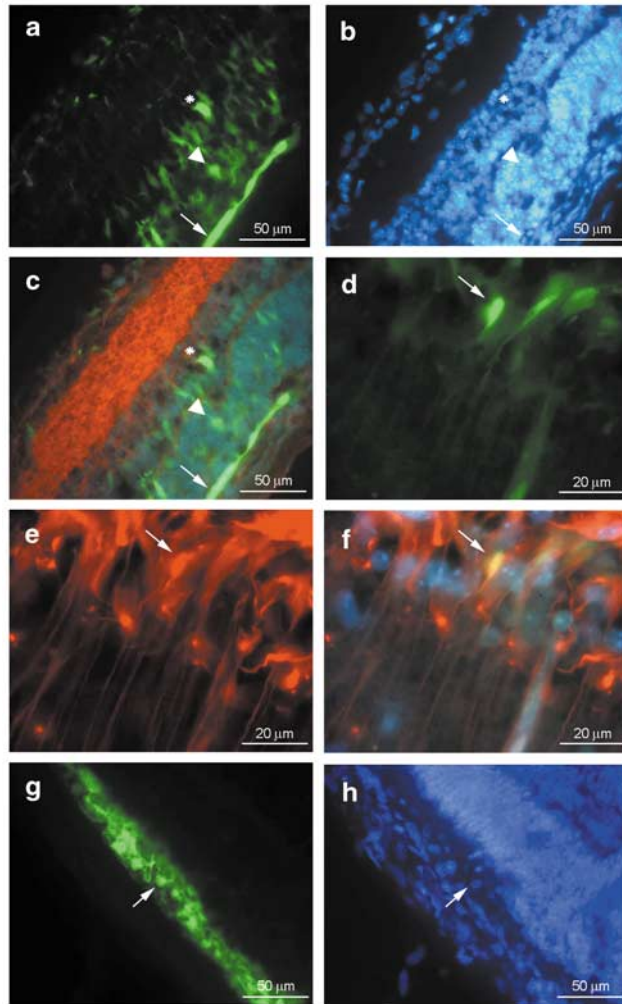
In the present work, we analyze the expression pattern of phosphoglycerate kinase 1 (PGK), elongation factor-1 short (EFS) and Rho<sup>10</sup> promoters following intraocular injections in the eye posterior chamber of HIV-1-derived self-inactivating lentiviral vectors in newborn and adult mice. LacZ or GFP reporter genes were used to follow the pattern of expression 7 days postinjection.

Consistent with the study of Bainbridge *et al.*<sup>5</sup>, injection in the posterior chamber does not lead to cell transduction in the anterior chamber but uniquely in the retina and/or in the RPE depending on the promoters. Moreover, no transgene expression, neither LacZ nor GFP, was observed in eyes injected with the vehicle only (PBS+ 3% BSA). No obvious immune response was observed and the absence of infiltrating cells was confirmed.

The lentiviral vector SIN-PGK-lacZ-WPRE was shown to drive *in vivo* expression in the CNS, mostly in

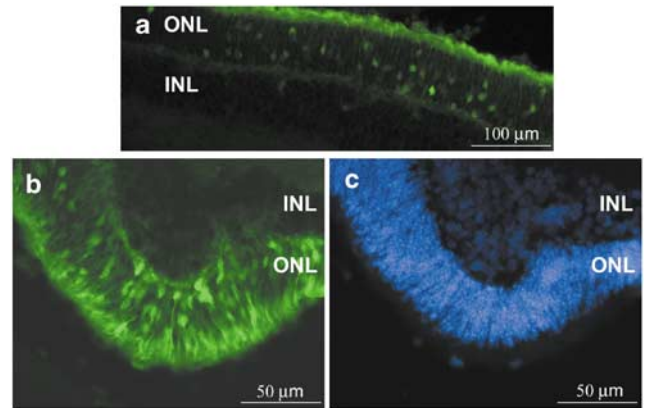
neurons,<sup>11</sup> we thus expected that it would also be expressed in retinal neurons because they have the same developmental origin. However, in newborn-PGK-lacZ-injected retina,  $\beta$ -galactosidase-positive cells were mainly detected in the RPE and only rarely in the inner nuclear layer (INL; Figures 1 and 4). Under optimal conditions, we estimated up to 18 000 RPE and 19 400 INL cells to be positive in newborn injected mice. In adult injected eyes, very few transduced INL cells were observed, but specific expression in the RPE was detected (data not shown). In the best injection cases, we estimated 9000 RPE and 1200 INL cells to be positive in injected adult eyes. Transduced RPE were observed up to 1.5 mm from the injection site. Moreover, in the region where transduced cells are observed, more than 95% of RPE cells express the LacZ gene, whereas less than 5% of INL cells express the transgene.

The EFS promoter is broadly active in different retinal cell types in both newborn and adult animals (Figures 2 and 4). Indeed, the EFS promoter allows GFP expression in RPE as well as in neurons and PR (Figure 2a–c, 2g–h). Additionally, double staining with GFAP demonstrated expression in glial cells (Figure 2d–f), an effect not seen using the PGK promoter. A higher number of GFP-positive cells was observed in newborn (14 000 RPE and 12 700 other retinal cells) compared to adults (5800 RPE and 2400 other retinal cells). As expected, the Rho promoter is specific for PRs in newborn injected mice but, surprisingly, has a very weak activity in injected adult eyes (Figures 3 and 4). In newborn injected eyes, the proportion of GFP-expressing cells varies from 2 to 10% of the PRs in the region of the injection site, which



**Figure 2** EFS activity in PN11 retina DBA/2 mice. The Hlox-EFS-GFP is derived from the pLOX-GFP described by Salmon et al<sup>13</sup> by replacement of the CMV promoter with the EFS promoter and deletion of the second cistron. The EFS promoter is derived from a pEF-Bos plasmid<sup>14</sup> and corresponds to nucleotides 378–610 of the EF1 $\alpha$  gene (GeneBank Accession no. J04617). Concentrated viral preparations were performed as described by Naldini et al.<sup>12</sup> The viral titer was determined as previously described<sup>15</sup> by Hela cell transduction in six-well dishes followed by the counting of GFP-positive cells by FACS analysis. The same amount of physical particles, as measured by p24 antigen dosage, as for SIN-PGK-LacZ-WPRE was injected per eye. Thus, intraocular injections of  $4 \times 10^6$  and  $7 \times 10^6$  infectious units were performed, respectively, per newborn and adult mouse eye. The viral titer variations observed between the concentrated preparations of the SIN-PGK-LacZ-WPRE vector and the Hlox-EFS-GFP vector probably reflect the difference in the backbone construction, the first one being a third-generation vector and the second one being a second-generation vector. The EFS promoter is ubiquitously active in the mouse retina. The GFP transgene (green) is expressed in the INL (a–c, star), ONL (a–c, arrowhead), RPE cells (a–h, arrow) and in glial cells as shown by double labeling with GFAP antibody (d–f; arrow). (a, d, g): GFP fluorescence; (b, h): Dapi staining; (e), anti-GFAP staining (red); (c), merge of (a, b) and staining with anti-syntaxin (red); (f): merge of (d, e) and Dapi staining (blue).

extends to around 250  $\mu$ m. Notably, best scores, up to 20%, were obtained in regions where the retina was detached, or disturbed by the surgical act, suggesting that the retinal structure itself may decrease the viral transduction efficiency by limiting viral diffusion, or that an injured retina is more prone to Rho promoter activity.



**Figure 3** Rho activity in PN11 retina DBA/2 mice. The 2.5 kb human Rho promoter defined by the Spel-XbaI fragment of the original 5.3HRPlacZ clone from Bennett et al<sup>16</sup> was cloned in place of the EFS in the pLOX-EFS-GFP plasmid using BamHI and XhoI blunted restriction sites. High titer preparation was performed as described by Naldini et al<sup>12</sup> and the p24 antigen titer was determined by ELISA. A similar number of Hlox-Rho-GFP physical particles to the precedent lentiviral vectors described in this study was injected into newborn and adult eyes. Owing to the specificity of the Rho promoter, we cannot define the infective titer for this lentiviral vector, but we can estimate that the transforming units/ng ratio is similar to Hlox-EFS-GFP ratio and that the same amount of infective particles as with Hlox-EFS-GFP were injected in mice eyes for this vector. The Rho promoter drives the expression exclusively in the ONL (a, b). The percentage of PRs GFP-positive varies from 2 to 10% (a) to 20% in region where the surgical act injured the retina (b, c). (a, b) GFP fluorescence; (c), Dapi staining.

	Rho		PGK		EFS	
	postnatal	adult	postnatal	adult	postnatal	adult
choroid						
RPE	-	-	++++	++	+++	++
ONL	+++	+/-	-	-	} +++++	+
INL	-	-	++	+/-		
GCL	-	-	-	+		
vitreous						

**Figure 4** Schematic and summarized representations of PGK, EFS and Rho patterns of expression in the retina. The retina cell layers are schematically represented and named adjacent to a table describing the pattern of expression of PGK, EFS and Rho in those layers. Cell layers consist of the retinal pigmented epithelium (RPE), the outer nuclear cell layer (ONL: photoreceptors), the inner nuclear layer (INL: mainly interneurons, but also glial cells) and the ganglion cell layer (GCL: ganglion cells and astrocytes). For each promoter, results are given for both postnatal injected mice (days 4–5) and adult injected mice (8 weeks). The number of transduced cells is proportional to the number of +, and absence of transduced cells is mentioned as -. Rho: rhodopsin promoter, PGK: mouse phosphoglycerate kinase 1 promoter, EFS: elongation factor-1 short promoter.

This study indicates that although more infectious particles were injected into adult eyes than into newborn eyes, PGK-driven expression was more efficient in newborn than in adult injected mice, suggesting that the developing mouse retina is more favorable for

expression of this promoter in the INL. This may be because of the metabolic state of differentiating neurons to a different pool of transcription factors or to a different chromosomal context, which could influence the expression and integration of the transgene cassette. Higher permissibility of the developing retina could also allow better diffusion through the different layers.<sup>2</sup> However, additionally, we cannot exclude that infection of RPE is due to the presence of viral particles in the subretinal space at the time of needle withdrawal and not to the diffusion of the lentiviral preparation from the vitreous cavity to the RPE cell layer. Nonetheless, PGK-derived expression is predominant in RPE during adulthood, a selectivity not seen with the EFS promoter. However, as the different lentiviral vectors doses were adjusted by their physical particle amount (p24 content), 10-fold more infectious particles (as titered on Hela cells) were administered with the EFS-driven lentiviral vector in comparison to the PGK-driven lentiviral vector. This higher level of infectious-injected particles could account for the broader expression pattern of EFS, which comprises both RPE cells and other retinal cells. However, when we injected 10-fold less Hlox-EFS-GFP to reach the same infective titer as with the SIN-PGK-LacZ-WPRE, the same broad EFS expression pattern was observed, but with less transduction efficiency (data not shown). This later observation confirms that PGK is preferentially active in RPE of adult injected mice, while EFS is also active in some other retinal cells.

These results suggest that the PGK promoter should be useful for targeting specific expression in the RPE either to restore an RPE function or to express a secreting factor, which may diffuse and promote the survival of the adjacent PRs. Furthermore, the EFS promoter should be used to express transgenes throughout the entire retina, whereas the Rho promoter is suitable for expressing a transgene specifically in PRs either to restore a wild-type function in these cells or to deliver a ribozyme in order to inhibit a dominant negative function.

These preliminary data obtained using intraocular injections reveal that targeted expression can be achieved using specific promoters and that the rapid expression of a transgene delivered by a lentiviral vector is a convenient tool for such an investigation. Moreover, the study of promoter activities is essential for elaborating gene therapy strategies as those activities may be different with the age of injection.

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