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ORIGINAL ARTICLE

Lentivirus-mediated gene transfer to the rat, ovine and human cornea

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Gene therapy of the cornea shows promise for modulating corneal transplant rejection but the most appropriate vector for gene transfer has yet to be determined. We investigated a lentiviral vector (LV) for its ability to transduce corneal endothelium. A lentivector expressing enhanced yellow fluorescent protein (eYFP) under the control of the Simian virus type 40 early promoter (LV-SV40-eYFP) transduced 80–90% of rat, ovine and human corneal endothelial cells as detected by fluorescence microscopy. The kinetics of gene expression varied among species, with ovine corneal endothelium showing a relative delay in detectable reporter gene expression compared with the rat or human corneal endothelium. Vectors containing the myeloproliferative sarcoma virus promoter or the phosphoglycerate kinase promoter were not significantly more effective than LV-SV40-eYFP. The stability of eYFP expression in rat and ovine corneas following ex vivo *transduction of the donor cornea was assessed following orthotopic corneal transplantation. Following transduction* ex vivo, *eYFP expression was maintained in corneal endothelial cells for at least 28 days after corneal transplantation in the sheep and >60 days in the rat. Thus, rat, ovine and human corneal endothelial cells were efficiently transduced by the LV, and gene expression appeared stable over weeks* in vivo.
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Introduction

On a worldwide scale, congenital or acquired corneal disease is the second most common cause of blindness.[1] Corneal transplantation can restore vision in many patients with corneal opacities, but its success is limited by irreversible immunological rejection.[2] *Ex vivo* gene therapy of the donor cornea offers the prospect of improving corneal allograft survival, especially as a number of transgenes that can modulate rejection have already been identified.[3,4] Expressed transgenic proteins capable of prolonging corneal allograft survival in animal models include mammalian interleukin 10,[5] the p40 subunit of interleukin 12,[6] interleukin 4 (albeit not in all studies),[7–9] soluble tumour necrosis factor receptor,[10] endostatin-kringle 5 fusion protein (E-Kr5),[11] soluble CTLA4 or CTLA4-Ig constructs[12,13] and indoleamine 2,3-dioxygenase.[14]

Many studies in which modulation of corneal graft rejection by gene transfer was the experimental objective have utilized non-viral or replication-deficient adenovirus vectors to transduce the cornea, so that long-term expression of the transgene was not achieved and the grafts ultimately failed. Integrative vectors such as recombinant adeno-associated virus (AAV) and lentivirus hold greater promise for achieving long-term transgene expression and corneal graft survival than do non-viral or non-integrative viral vectors. A level of corneal transduction sufficient to inhibit corneal neovascularization has been reported with an adeno-associated vector encoding a soluble vascular endothelial growth factor receptor.[15] Two different lentiviral vectors (LV) have already been reported to transduce corneal endothelium,[16] and a LV encoding E-Kr5 has been shown to prolong rabbit orthotopic corneal allografts significantly, compared with controls.[11]

With the goal of producing safe, long-term gene expression in human corneal endothelial cells transduced *ex vivo*, we investigated the suitability of a vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped LV[17,18] for use in a range of species. This non-replicative, self-inactivating vector, which is based upon human immunodeficiency virus type 1 (HIV-1), utilizes codon-optimized reading frames, and multiple plasmids to extend its safety profile, without compromising its efficiency.[17,18] To assess the efficacy of the vector in transducing corneal endothelial cells, we used a construct encoding the enhanced yellow fluorescence reporter protein (eYFP) and examined gene expression in corneas of the rat (an inbred model), the sheep (an outbred pre-clinical model) and the human (the eventual target species). Expression of eYFP was quantified in rat,

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ovine and human corneas organ cultured *in vitro*, and in rat and ovine corneas following *ex vivo* transduction and orthotopic corneal transplantation. Expression of eYFP from three different promoters in the rat, and from two different promoters in the sheep, was also examined in corneas *in vitro*.

Results

In vitro *transduction of rat, ovine and human corneas with LV-SV40-eYFP*

Rat, ovine and human corneas were transduced with 2.5×10^7 transducing units (TU)/cornea of a VSV-G-pseudotyped lentivector expressing eYFP under the control of the Simian virus type 40 intermediate early promoter (LV-SV40-eYFP), organ cultured *in vitro*, and examined periodically at the fluorescence microscope for reporter gene expression (Figure 1). Approximately 88% of rat and 84% of ovine corneal endothelial cells expressed eYFP after 1 and 2 weeks of organ culture, respectively (Table 1). A few rat corneal epithelial cells also expressed eYFP after 1 week of organ culture (Figure 1a). Few human corneas were available for study, but over 80% of human corneal endothelial cells were transduced by LV-SV40-eYFP after 2 weeks of culture (Table 1). Because of the different sizes of corneas from the different species examined, the multiplicity of infection (MOI) of lentivirus per corneal endothelial cell at a dose of 2.5×10^7 TU/cornea was estimated as being 400 for the rat, 20 for the sheep and 120 for the human.

Kinetics of eYFP expression in rat, ovine and human corneal endothelium

To assess the kinetics of transgene expression in the corneal endothelium of each species, corneas were transduced and examined over a period of *in vitro* organ culture. In our experience, human and ovine corneas can survive for 14 days in organ culture following transduction, whereas rat corneas can tolerate only 8–10 days before losing viability. Therefore, these respective periods were set as limits for assessing transgene expression. Rat corneas demonstrated rapid and efficient transduction by 2.5×10^7 TU/cornea of LV-SV40-eYFP, as measured by the percentage of corneal endothelial cells expressing eYFP, without measurable change in the density of corneal endothelial cells (Figure 2a). In contrast, ovine corneas showed a relative delay in eYFP expression after transduction, but achieved maximal expression by 14 days with no reduction in endothelial cell density (Figure 2b). In human corneas, eYFP expression peaked within 1–4 days of transduction, and persisted for 14 days in organ culture (Figure 3a). Endothelial cell density did not appear to vary significantly with time following transduction (Figure 3b): differences among corneas probably reflected factors such as donor age and time in Eye Bank storage, rather than vector-associated toxicity.

Effect of altering MOI on transgene expression

To investigate whether a lower MOI would be effective in human corneas, transduction with 6.3×10^6 TU LV-SV40-eYFP, equivalent to an MOI of 30, was examined (Figures 3a and b). Transduction at an MOI of 30 produced proportionally lower reporter gene expression compared to transduction at an MOI of 120, but, like the higher dose, maximal expression was achieved after 1 day with no apparent effect on corneal endothelial cell density.

To investigate whether a higher MOI would result in more rapid expression of reporter protein in ovine corneal endothelial cells, transduction with LV-SV40-eYFP at MOIs ranging from 75 to 300 was investigated. Expression of eYFP was still relatively delayed, compared with the rate observed in rat and human corneas. At a vector dose of 4×10^8 TU/cornea (MOI 300), expression of eYFP was achieved in 80% of ovine corneal endothelial cells within 4 days (Figure 4a). However, MOIs of 75 or higher were associated with a reduction in corneal endothelial cell density (Figure 4b).

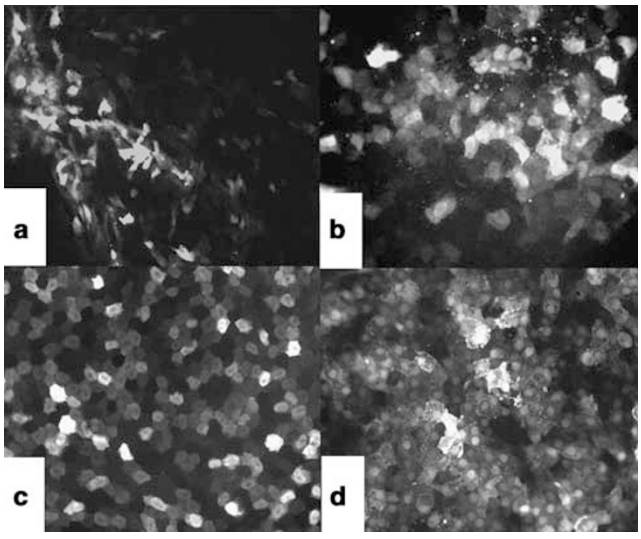

Figure 1 Transduction of corneas with 2.5×10^7 TU LV-SV40-eYFP per cornea. (**a**) Rat corneal epithelium transduced with LV-SV40-eYFP (MOI 400) and examined after 6 days of *in vitro* organ culture. (**b**) Rat corneal endothelium transduced with LV-SV40-eYFP (MOI 400) and examined en-face after 6 days of *in vitro* organ culture. (**c**) Sheep corneal endothelium transduced with LV-SV40-eYFP (MOI 20) and examined en-face after 14 days of *in vitro* organ culture. (**d**) Human corneal endothelium transduced with LV-SV40-eYFP (MOI 120) and examined en-face after 14 days of *in vitro* organ culture. Original magnification $\times 20$.

Table 1 Maximum percentage of corneal endothelial cells expressing eYFP reporter protein after transduction with 2.5×10^7 TU/cornea LV-SV40-eYFP followed by *in vitro* organ-culture

Species	*No. corneas*	*Day post-transduction*	*Percentage eYFP-positive corneal endothelial cells, mean ± s.d.*[a]
Rat	3	8	87.8 ± 18.0%
Sheep	4	14	83.9 ± 5.5%
Human	2	14	83.0 (77–89)%[b]

Abbreviations: eYFP, enhanced yellow fluorescent protein; LV, lentiviral vector ; TU, transducing units.
[a]mean ± s.d. derived from examination of five microscope fields from each cornea.
[b]mean and range.

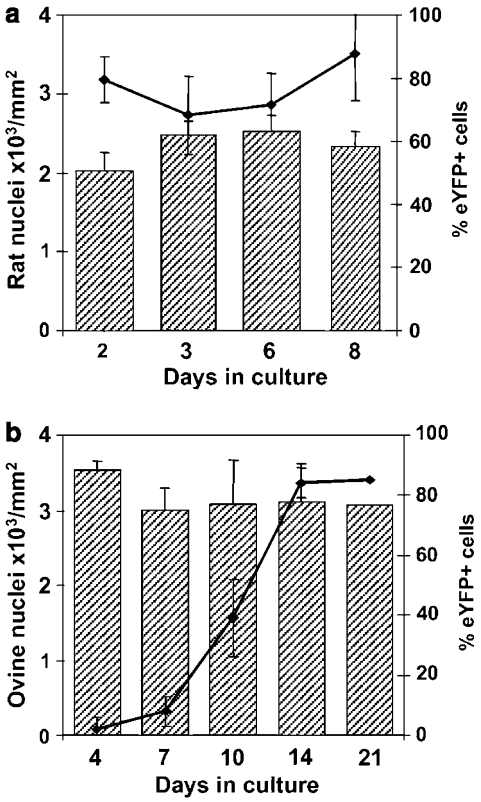

Figure 2 Kinetics of eYFP expression in rat and ovine corneal endothelium following corneal transduction with 2.5×10^7 TU of LV-SV40-eYFP and subsequent *in vitro* organ culture. (**a**) Rat corneal endothelium, MOI 400. (**b**) Ovine corneal endothelium, MOI 20. Hatched columns display the corneal endothelial nuclei density (mean $\pm$ s.d.) after various periods of organ culture. Points represent the percentage corneal endothelial cells (mean $\pm$ s.d.) expressing eYFP. At each time point, 3–7 corneas (five fields/cornea) were examined.

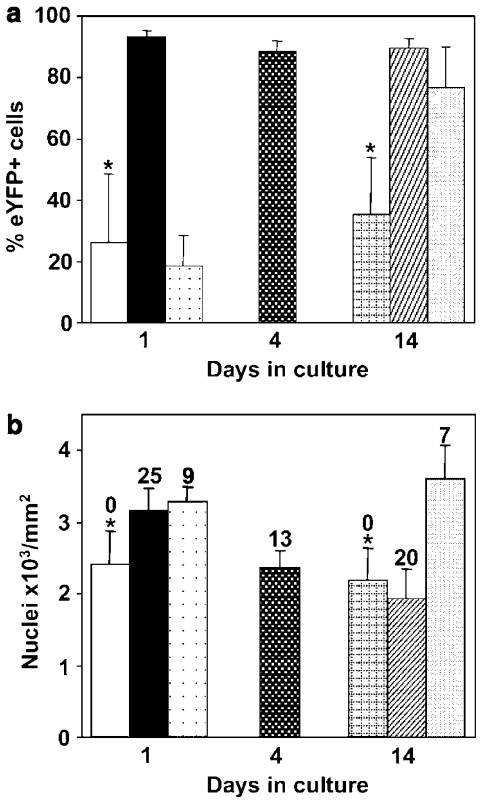

Figure 3 Kinetics of eYFP expression in human corneal endothelium following corneal transduction with two different doses of LV-SV40-eYFP and subsequent *in vitro* organ culture. (**a**) Percentage corneal endothelial cells (mean $\pm$ s.d.) expressing eYFP. (**b**) Corresponding corneal endothelial nuclei density (mean $\pm$ s.d.). A total of seven corneas were examined; each column represents the mean $\pm$ s.d. of five fields for a different cornea at each time point, and an identical hatching pattern is used for each individual cornea. Corneas were transduced with either 6.3×10^6 TU (MOI 30) (asterisked) or 2.5×10^7 TU (MOI 120) of LV-SV40-eYFP. Numbers above the columns represent the number of days the cornea had been stored in the Eye Bank, before being made available for research.

Influence of different internal promoters on reporter gene expression

Plasmids containing the myeloproliferative sarcoma virus promoter (MPSV) or the phosphoglycerate kinase (PGK) promoter, respectively, were used to produce LV-MPSV-eYFP and LV-PGK-eYFP. LV-SV40-eYFP, LV-MPSV-eYFP and LV-PGK-eYFP were used to transduce rat corneas *in vitro* at 5.5×10^5 TU/cornea. A relatively low MOI of 50 and a relatively early time point for examination were selected to maximize potential differences in expression. Expression of eYFP was observed in $6 \pm 4\%$ of rat corneal endothelial cells after 2 days of *in vitro* organ culture in corneas transduced with LV-PGK-eYFP, compared with $13 \pm 2\%$ of cells in corneas transduced with LV-SV40-eYFP and $16 \pm 2\%$ of cells in corneas transduced with LV-MPSV-eYFP (Figures 5a and b). Similarly, ovine corneas were transduced with either LV-PGK-eYFP or LV-SV40-eYFP at an MOI of 20 and examined after 4 days of *in vitro* organ culture. Expression of eYFP was observed in $12 \pm 9\%$ of ovine corneal endothelial cells in corneas transduced with LV-PGK-eYFP, compared with $4 \pm 4\%$ of cells in corneas transduced with LV-SV40-eYFP (Figures 5c and d). No significant differences in gene expression or in endothelial cell density were apparent among the different constructs in either species. As neither of the vectors with alternative promoters resulted in a significantly higher transduction rate, the original vector with the SV40 promoter was chosen for *in vivo* studies in animal models of corneal transplantation.

Stability of reporter gene expression in orthotopic rat and ovine corneal grafts after ex vivo *transduction of donor corneas with a LV*

Rat corneal isografts harvested at 14 and 28 days after *ex vivo* transduction with 2.5×10^7 TU/donor cornea LV-SV40-eYFP showed eYFP in 9.9 ± 3.2 and in $16.7 \pm 6.8\%$, respectively, of donor corneal endothelial cells. Similar isografts harvested at 60–77 days after *ex vivo* transduction with 2.5×10^7 TU/donor cornea LV-SV40-eYFP (MOI 400/endothelial cell) revealed sustained expression of the reporter gene (Figures 6a and b) in the corneal endothelium of four of six grafts. Two ovine corneas transduced *ex vivo* with LV-SV40-eYFP at 5×10^7 TU/donor cornea were transplanted as allografts and harvested post mortem after 20 and 28 post-operative days, respectively, before immunological rejection super-

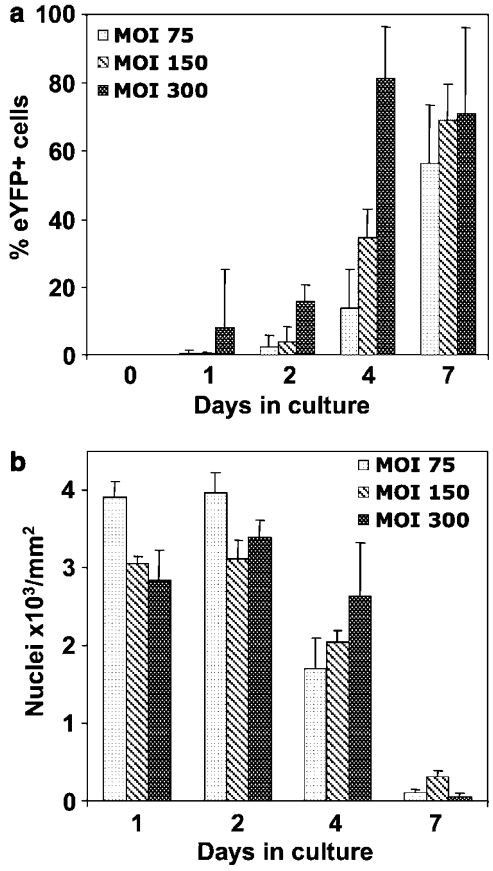

Figure 4 Effect of varying the MOI on (**a**) eYFP expression, and (**b**) corneal endothelial cell density, following 24 h transduction of sheep corneas with LV-SV40-eYFP and *in vitro* organ culture. Each column represents the mean ± s.d. of five fields examined for a single cornea.

vened. Expression of eYFP was widespread within the donor tissue, with approximately 60% of endothelial cells positive for the reporter protein and cell density appearing normal (Figures 6c and d). No endothelial cells in the host peripheral cornea showed reporter protein expression. All rat corneal isografts and both ovine allografts were clear and thin at the time the recipients were killed (Figures 6e and f).

Discussion

We report efficient transduction of rat, ovine and human corneal endothelia with a VSG-G-pseudotyped HIV-1-based LV. For all three species, maximal expression of the transgene in 80–90% of corneal endothelial cells was achieved after *in vitro* transduction and subsequent corneal organ culture. However, unexpected differences in the rate of *in vitro* reporter protein expression were observed among species. Maximal levels of transgene expression were reached within several days in rat and human corneas, but were delayed for up to 2 weeks in ovine corneas. Increasing the lentivirus MOI increased the rate of eYFP expression in ovine corneas to some extent, but was associated with unacceptable endothelial cell loss. The reason for the species difference is unknown. It is possible that the difference reflects suboptimal levels of intracellular deoxynucleotides in ovine corneal endothelial cells, as has been suggested for transduced quiescent fibroblasts *in vitro*.[19] Our data do emphasize the likely importance of testing vectors *in vitro* in the eventual target species (in this case, human), ahead of eventual clinical trials.

Reporter protein expression persisted in organ cultured corneas for as long as they could be maintained *in vitro*. Further, and perhaps of greater interest, stability of transgene expression *in vivo* following *ex vivo* transduction of donor corneas was demonstrated by detection of the eYFP reporter protein after 2 months in orthotopic rat corneal isografts, and 1 month in orthotopic sheep corneal allografts. It was proved to be possible to achieve reporter gene expression in both rat and ovine corneal endothelium with short lentiviral transduction times (3–6 h), and in the absence of any enhancer such as polybrene.

In ovine corneal grafts, the transgene was expressed in at least 60% of donor corneal endothelial cells at 1 month after transplantation, compared with 17% in transplanted rat corneas at the same time. Rat corneal endothelial cells exhibit considerable mitotic potential but repair by division *in vivo* is relatively slow, occurring over weeks.[20] In contrast, ovine corneal endothelial cells are post-mitotic and do not undergo repair by division.[5] In the face of cell repair, rat corneal endothelial cells might be expected to show less overall evidence of vector-mediated toxicity after transduction with an integrative vector. However, if more donor endothelial cells are lost in a small cornea after transplantation because of surgical trauma, then overall transgene expression might conceivably be lower in rat endothelium than in the larger, post-mitotic ovine corneal endothelium, at least in the relatively early stages following corneal transplantation.

The level of transgene expression achieved with a LV in a given cell type depends in part upon the promoter. For example, in a comparison of three different promoters controlling the same transgene, expression in blood-derived vascular endothelial progenitor cells was reported to be greatest with the cytomegalovirus promoter, intermediate with the elongation factor-1 (EF1) promoter and lowest with the PGK promoter.[21] In the mouse retina, the PGK, rhodopsin and EF1 promoters all gave different patterns of expression of the same gene.[22] In terms of reporter gene expression in corneal endothelium, we found no particular advantage in replacing the SV40 early promoter in our original LV with either the MPSV or the PGK promoter.

A wide variety of non-viral vectors (predominantly liposomal agents) and disabled viral vectors (predominantly replication-defective adenovirus, AAV, herpesvirus and retroviruses including lentivirus) have hitherto been used for gene transfer to the cornea.[3,4,23–25] Non-viral vectors, although safe, are generally very inefficient and do not produce long-term gene expression in ocular tissues.[23,26] Adenovirus, the first vector to be systematically explored for gene transfer to the cornea,[5,27] is an efficient vector suitable for both mitotic and post-mitotic cells, but its inherent immunogenicity can limit the duration of transgene expression.[28] Furthermore, because the vector is non-integrative and remains episomal, long-term expression of the transgene is not achieved. There are also concerns over its safety for

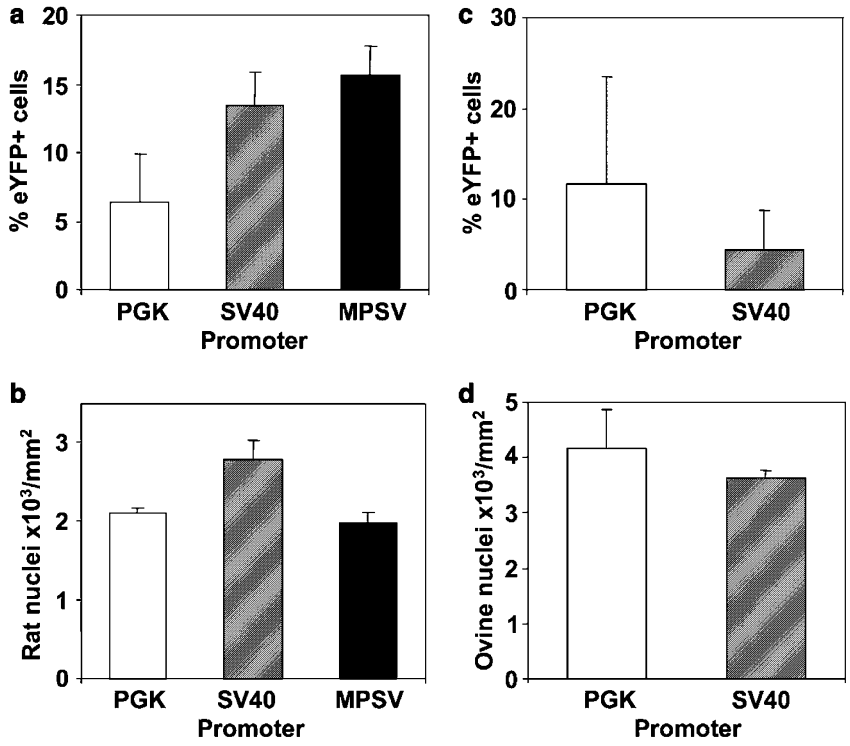

Figure 5 Expression of eYFP driven by different internal promoters after lentiviral transduction of corneas and subsequent *in vitro* organ culture. (**a**, **b**) Rat corneas transduced with 5.5×10^5 TU/cornea (MOI 50) and examined after 2 days of *in vitro* organ culture; (**c**, **d**) sheep corneas transduced with 2.5×10^7 TU/cornea (MOI 20) and examined after 4 days of *in vitro* organ culture. Each column represents the mean $\pm$ s.d. of three corneas examined (five fields/cornea), with the exception of MPSV (**a**, **b**), for which the mean and range of two corneas (five fields/cornea) is shown.

clinical use.[28,29] AAV vectors are less immunogenic and produce persistent expression in target tissues, although the degree to which this is a result of integration is not clear. AAV vectors can also be difficult to grow to high titre and have a limited capacity for foreign DNA.[28,30] Retroviral vectors are integrative but do not transduce non-cycling cells efficiently, and so are not suitable for gene therapy of corneal endothelium, which is post-mitotic.

LV transduce both dividing and non-dividing cells and can produce stable expression of a transgene in both stem cells and in terminally differentiated target tissues.[31] LV encoding reporter genes have been shown to transduce human corneal endothelial cells, keratocytes and epithelial cells for prolonged periods *in vitro*[32] and *in vivo*,[33] and injection of a LV into the anterior chamber of neonatal mice resulted in efficient and stable infection of the endothelium *in vivo*.[34] Corneal epithelial cells[32] and epithelial progenitor cells[35] can also be transduced with LV. Thus, LV show particular promise for the future because of their wide tropism for cells of the anterior segment, including corneal endothelial cells and corneal epithelial progenitor cells, their inherent relative lack of immunogenicity, their failure to induce an immune response after intraocular delivery,[36] and their ability to induce long-term gene expression in ocular cells. Insertional mutagenesis remains a concern,[29] but there is growing evidence that LV are less oncogenic than murine leukaemia virus-based vectors *in vivo*.[37,38] In this respect, the safety profile of lentiviral gene therapy of post-mitotic cells such as the human corneal endothelium might be further improved by the use of a stable integration-deficient vector, such as that recently shown to promote long-term gene expression in ocular tissues.[39]

Our vector is a recombinant HIV-1-based lentivirus[17,18] that has been pseudotyped with VSV-G, is non-replicative and self-inactivating and can produce gene expression for at least 3 months in the murine nasal mucosa.[40] The vector does not contain the post-transcriptional woodchuck hepatitis virus regulatory sequence that improves transgene expression,[41] but that has been associated with liver tumours in mice,[42,43] and which has recently generated some safety concerns.[44] In all three species examined herein, the vector produced stable transgene expression in over 80–90% of corneal endothelial cells *in vitro*. Cationic polymers or detergents were not required to achieve LV transduction of corneal endothelium. The transduction efficiency of our vector compares favourably with that reported by others[16,32] for LV, especially for human corneas. For example, Beutelspacher *et al*.[16] observed transgene expression in approximately 10% of endothelial cells in human corneas transduced with a HIV-1-based vector, and in approximately 25% of corneal endothelial cells transduced with an equine infectious anaemia virus-based vector. Wang *et al*.[32] did not quantify transduction rates in human corneas transduced with an HIV-1-based vector, but achieved good levels of expression in endothelium, as assessed by fluorescence microscopy. Of note, our LV produced sustained expression of donor corneal endothelium in the transplanted ovine cornea *in vivo*, in a model system that bears many similarities to that of

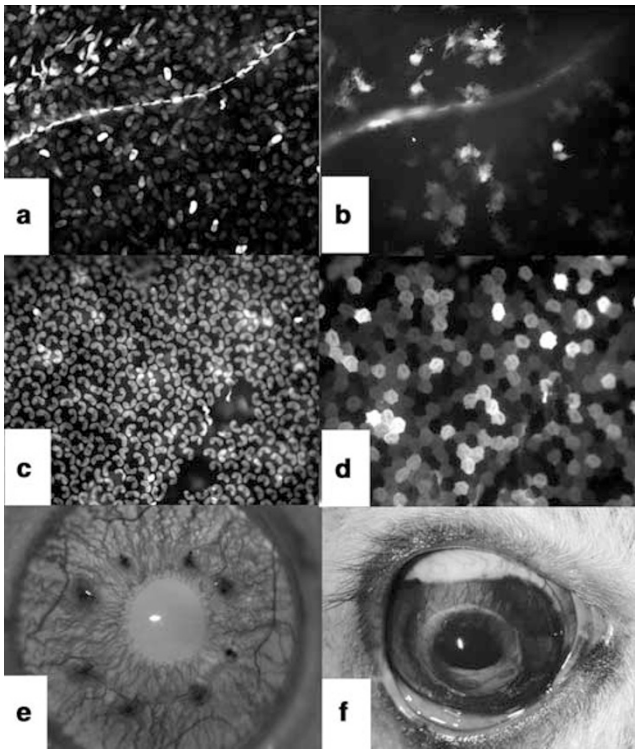

Figure 6 Stability of reporter gene expression after *ex vivo* transduction of the cornea with LV-SV40-eYFP and subsequent orthotopic corneal transplantation. (**a**, **b**) Rat corneal isograft harvested 60 days after transplantation; (**c**, **d**) sheep corneal allograft harvested 20 days after transplantation. (**a**) and (**c**) Show staining with Hoechst 33258 dye; (**b**) and (**d**) show eYFP expression in identical fields of the same corneas. Original magnification $\times 20$. (**e**) Long-surviving lentivirus-transduced rat corneal isograft at 62 days post-operatively; (**f**) lentivirus-transduced ovine corneal allograft at 20 days post-operatively.

orthotopic corneal transplantation in humans. The performance of the vector prompts its exploration as a means of achieving sustained expression of therapeutic transgenes in human corneal transplantation.

Materials and methods

Production, purification and titre determination of lentiviral vectors

Human embryonic kidney 293T cells (SD3515, American Type Culture Collection, Rockville, MO, USA) were co-transfected with five different plasmids comprising pHIV-1SDmSV-eYFP-ΔLTR (shuttle plasmid), pHCMV-G (VSV-G), pcDNA3tat101 ml (HIV-1 Tat), pHCMV whvrevml (HIV-1 Rev), and pHCMVwhvgagpolml (HIV-1 GagPol) to produce LV-SV40-eYFP.[45] Plasmids were prepared using commercially available kits (Endo-free Maxi kit; Qiagen, Valencia, CA, USA) to minimize bacterial endotoxin contamination. In the original construct, the eYFP reporter gene was controlled by the SV40 promoter. Analogous plasmids pHIV-1SDmMPSV-eYFP-ΔLTR and pHIV-1SDmPGK-eYFP-ΔLTR containing the MPSV or the PGK promoter, respectively, were used to produce LV-MPSV-eYFP and LV-PGK-eYFP. Viral vectors were concentrated as described previously.[45] Concentrated LV (LV-SV40-eYFP, LV-MPSV-eYFP or LV-PGK-eYFP) was filtered through a 0.8 μm filter, further concentrated by ultracentrifugation at 50 000 *g* for 90 min at 4°C, resuspended in ophthalmic balanced salt solution (Cytosol Ophthalmics, Lenoir, NC, USA), aliquotted and stored at −80°C. All vector stocks were tested for replication-competent lentivirus by assaying expression of HIV-1 p24 (HIV-1 p24 ELISA kit, PerkinElmer Inc, Boston, MA, USA) in transduced cells over 3 weeks and found to be negative. Titres of vectors carrying the eYFP reporter gene were determined by flow cytometric analysis of transduced human lung carcinoma A549 cells (CCL-185, American Type Culture Collection, Rockville, MO, USA) as described previously.[45] Titres of $>10^9$ A549 cell TU/ml were routinely obtained.

Lentivirus-mediated transduction of the cornea

Inbred Fischer 344 (F344) rats were bred within the institution. Outbred Merino cross-breed sheep were obtained from local farmers, and enucleated ovine eyes were obtained within 3 h of donor death from a local abattoir (Normanville Meatworks, Normanville, SA, Australia). Experimentation in rats and sheep was performed with approval from the institutional Animal Welfare Committee. Human corneas considered unsuitable for clinical transplantation were obtained from the Eye Bank of South Australia (Adelaide, SA, Australia) with permission from the next-of-kin and with approval from the institutional Committee on Clinical Investigations. Human corneas had been stored for variable periods at 4°C in Optisol GS corneal storage medium (Bausch and Lomb, Rochester, NY, USA) before being made available for research.

Rat corneas: Eyes removed from rats killed by an overdose of inhalation anaesthetic were decontaminated in 10% (w/v) povidone-iodine (Faulding Pharmaceuticals, Salisbury, SA, Australia) for 2 min, and rinsed twice in ophthalmic balanced salt solution. Corneas dissected with a 1–2 mm scleral rim were placed in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered RPMI 1640 medium (ICN, Costa Mesa, CA, USA) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin sulphate, 2 mM L-glutamine and 2% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (FCS) (all from Invitrogen, Rockville, MD, USA), hereafter described as HEPES-RPMI-2% FCS, in a sterile 96-well round-bottomed plate (Nalge Nunc International, Rochester, NY, USA). Corneas were transduced with LV-SV40-eYFP (5.5×10^5–2.5×10^7 TU/cornea), LV-MPSV-eYFP (5.5×10^5 TU/cornea) or LV-PGK-eYFP (5.5×10^5 TU/cornea) diluted in 100 μl HEPES–RPMI–2% FCS for 3–6 h at 37°C, rinsed twice in medium, and were then either organ cultured *in vitro* in 2 ml HEPES–RPMI–10% FCS at 37°C in air, or used within 1 h for transplantation.

Ovine corneas: Sheep eyes were decontaminated for 3 min in 10% (w/v) povidone-iodine and washed twice in sterile 0.9% (w/v) NaCl. Corneas were removed with a 2 mm scleral rim, suspended in 2 ml HEPES–RPMI–10% FCS and transduced by application of LV-SV40-eYFP (2.5×10^7–4.0×10^8 TU/cornea) or LV-PGK-eYFP (2.5×10^7 TU/cornea) to the corneal endothelium in 300 μl HEPES–RPMI–2% FCS, for 24 h at 37°C. Then a further 8 ml of the same medium was added and corneas were organ cultured. For transplantation studies, the transduction time of the donor cornea was reduced to 4 h.

Human corneas: Corneas were transduced with 2.5×10^7 TU/cornea LV-SV40-eYFP at 37°C for 24 h in a total volume of 200 μl HEPES–RPMI–2% FCS. They were then organ cultured in 10 ml HEPES–RPMI–10% FCS at 37°C.

Orthotopic corneal transplantation

Corneal (3 mm in diameter) isografts from F344 donor rats were transplanted as isografts to the eyes of F344 recipients, as described previously.[46] For sheep, 12-mm-diameter corneal allografts were transplanted to 11-mm-diameter graft beds of adult, outbred recipients, as described previously.[47] Topical immunosuppression was not administered to either rat or ovine corneal grafts. Rat isografts were examined three times weekly for at least 60 days at the operating microscope for clarity and oedema;[46] sheep allografts were examined daily at the hand-held slit-lamp until rejection was considered imminent.[47]

Quantification of eYFP reporter gene expression

Corneas were fixed in buffered formalin and counter-stained with 10 mg/ml Hoechst 33258 dye (Sigma Chemical Co. St Louis, MO, USA) for 30 min. Rat corneas were flat-mounted and examined under a fluorescence microscope (Olympus BX50) equipped with a digital camera (Photometrics CoolSNAP high resolution cooled CCD, 1.0 × tube). Different layers of the flat-mounts were observed by traversing through different planes within the same optical field. Corneal epithelial cells, stromal cells and endothelial cells were clearly distinguishable by nuclear morphology and size.[48] For the thicker ovine and human corneas, the endothelium and Descemet's membrane were stripped from the underlying stroma with a scalpel blade before flat-mounting. The number of Hoechst 33258-positive nuclei and the number of eYFP-positive cells were counted in five microscope fields (each 0.15 mm^2) from each cornea and expressed as a mean $\pm$ s.d. Transduction efficiency was defined as the percentage of corneal endothelial cells expressing eYFP.[48]

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