LENTIVIRUS-MEDIATED GENE EXPRESSION IN CORNEAL ENDOTHELIUM

Douglas George Anthony Parker

BMedSc GradDipEx&SpSc MBBS (Hons)

Department of Ophthalmology

School of Medicine

Faculty of Health Sciences

Flinders University of South Australia

Thesis submitted for the degree of

Doctor of Philosophy

December 2007

To the memory of my father, R.W.R. Parker QC, who always encouraged me to have a love of learning.

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Summary

Modulation of corneal transplant rejection using gene therapy shows promise in experimental models but the most appropriate vector for gene transfer is yet to be determined. The overarching aim of the thesis was to evaluate the potential of a lentiviral vector for use in human corneal transplantation. Specific aims were: (i) to assess the ability of an HIV-1-based lentiviral vector to mediate expression of the enhanced yellow fluorescent protein (eYFP), and a model secreted protein interleukin-10 (IL10), in ovine and human corneal endothelium; and (ii) to examine the influence of lentivirus-mediated IL10 expression on the survival of ovine corneal allografts.

Four lentiviral vectors expressing eYFP under the control of different promoters, were tested: the simian virus type-40 (SV40) early promoter, the phosphoglycerate kinase (PGK) promoter, the elongation factor-1α (EF) promoter, and the cytomegalovirus (CMV) promoter. Two lentiviral vectors expressing IL10 were tested: one containing the SV40 promoter and another containing a steroid-inducible promoter (GRE5). Lentivirus-mediated expression in transduced ovine and human corneal endothelium was assessed by fluorescence microscopy, real-time quantitative RT-PCR and ELISA, following alterations of transduction period duration (2–24 hr) and vector dose, as well as in the presence or absence of polybrene or dexamethasone (GRE5 vector). It was also compared to expression mediated by adenoviral vectors. Orthotopic transplantation of *ex vivo* transduced donor corneas was performed in outbred sheep. Allografts were reviewed daily for vascularisation and signs of immunological rejection.

Lentivirus-mediated eYFP expression was delayed in ovine corneal endothelium compared to human. However, in both species the final transduction

rate was >80% and expression was stable for at least 14 d *in vitro*. Lentivirus-mediated expression in ovine and human corneal endothelium was higher with the viral promoters in comparison to the mammalian promoters. A 24 h transduction of ovine corneal endothelium with the lentiviral vector encoding IL10 resulted in expression levels which were increasing after 15 d of organ culture but logarithmically lower than those achieved by adenovirus. Shortening the lentiviral transduction period to 2 h led to a reduction in expression, but the addition of polybrene (40 µg / ml) to the transduction mixture restored expression to levels comparable to those attained after a 24 h transduction period. Lentivirus-mediated IL10 expression was higher and more rapid in human corneal endothelium compared to ovine corneas. Dexamethasone-responsive transgene expression was observed in both ovine and human corneal endothelium using the lentiviral vector containing the GRE5 promoter. Lentivirus-mediated expression in ovine corneal endothelium was stable for 28 d *in vivo*. A modest prolongation of ovine corneal allograft survival (median of 7 d) was achieved by transduction of donor corneas for 2–3 h with the lentivirus expressing IL10. Attempts to increase the expression of IL10 by the addition of polybrene (40 µg / ml) to the transduction mixture, resulted in a toxic effect on corneal allografts which abrogated the beneficial effect of IL10.

The lentiviral vector shows potential for the stable expression of therapeutic transgenes in human corneal transplantation. However, the mechanisms underlying the species-specific differences in HIV-1-mediated transgene expression will need to be elucidated and overcome if the ovine preclinical model is to provide justification for a clinical trial.

Declaration

I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Douglas G.A. Parker

Acknowledgements

I first thank Professor Doug Coster for the privilege of studying in such a distinguished department. Despite heavy demands on his time, Doug performed over fifty corneal grafts in the sheep and always retained his trademark good humour. He has been wonderfully encouraging both personally and professionally. To my mentor Professor Keryn Williams, I am deeply grateful. Keryn did her utmost to make a scientist out of me, and I dare say she might just have succeeded. Her scientific vision and rigour were invaluable, and she also encouraged me to maintain a healthy balance in life. Associate Professor Don Anson designed the lentiviral vector and supplied the expertise and resources to produce it; I thank him for all his help and for the opportunity to apply his vector to the eye. Dr Helen Brereton taught me all I know about laboratory science. Her input was a deciding factor in my success and enjoyment of the project. Dr Claude Kaufmann was my companion for the first two years of the project, and helped with much of the vector preparation; we have forged a lasting friendship. I acknowledge Dr Sonja Klebe for her pioneering work in gene therapy of the ovine cornea, and thank her for her expert input regarding histopathology.

I thank Professor Prue Hart and Dr Cecilia Prele from the Telethon Institute for Child Health Research, Subiaco, WA, for conducting an assay which presented technical difficulties. I also thank Stanley Tan, Kirsty Marshall, Dr Claire Jessup, Dr Paul Badenoch, Dr Shiwani Sharma, Angela Chappell, Margaret Philpott, Dr George Mayne, Dr Damian Hussey, Dr Peter Van Wijngaarden, Associate Professor Pamela Sykes, Dr Thu-Lan Kelly, Dr Michael Michael, and Dr Rachel Koldej—all of whom contributed to the project in important ways. I am also grateful to other members of the Department of Ophthalmology who assisted me during the project: Melinda Tea,

David Dimasi, Dr Jamie Craig, Dr Kathryn Burdon, Lauren Mortimer and Sarah Brice. I also thank Ray Yates, Stuart Lisk and the staff of the animal house, who were so dedicated in handling and caring for the sheep. I thank Bruce Wedd, the proprietor of Normanville Meatworks, who provided ovine eyes throughout the project at no charge, and Joyce Moore and Lyn Harding, who were so helpful to me in the office.

On a personal note, I thank Dr Dan Peet for his friendship and for showing me how an excellent scientist can also be a humble man of great faith. I also pay tribute to my dear father- and mother-in-law, Professor Doug Stace and Nitia Stace. Together, they have taught me the value of maintaining a strong family life whilst making a contribution to the lives of others. My mother Merrilee Parker has been a constant source of encouragement and I am so grateful for her example of unconditional love. I thank my brother Will for his friendship and his technical expertise in producing a thesis of such high print quality. This thesis is dedicated to the memory of my father, whose sudden illness and passing during my final year was so devastating for us all. He always impressed on me the importance of having what he called a ‘relish for learning’. His dignity, integrity and devotion to his profession have shaped me enormously. Without his example I could not have seen the race to its finish; I am only sorry that he was not here to see me break the tape.

Finally, to my darling wife Heidi, I offer my utmost gratitude. The journey has not been easy, but her dedication to the success of the project has never wavered. During my candidature we were blessed with two beautiful children, Jackson William and Allegra Jade. I am so thankful to Heidi for her devotion to them through long hours of my absence. I know she agrees that it has been a great adventure for us all. With Heidi by my side, I anticipate the next challenge with confidence.

Publication arising from this thesis

Parker DG, Kaufmann C, Brereton HM, Anson DS, Francis-Staite L, Jessup CF, Marshall K, Tan C, Koldej R, Coster DJ, Williams KA. Lentivirus-mediated gene transfer to the rat, ovine and human cornea. *Gene Ther* 2007; **14:** 760-767.

Abbreviations and symbols

<	less than
>	greater than
µg	microgram
µl	microlitre
µm	micrometre
AAV	adeno-associated virus
ACAID	anterior chamber-associated immune deviation
Ad	adenoviral vector
Amp	ampicillin
APC	antigen-presenting cell
BIV	bovine immunodeficiency virus
bp	base pair
BSS	balanced salt solution (balanced for intraocular use)
CD	cluster defined antigen
cDNA	complementary DNA
cm	centimetre
CMV	cytomegalovirus
CPE	cytopathic effect
cPPT	central polypurine tract
CsCl	caesium chloride
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated protein-4 (CD152)
Da	dalton
DDH20	double distilled water
DEPC	diethylpyrocarbonate

DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	deoxynucleotide triphosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
EF	elongation factor 1 alpha
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anaemia virus
ELISA	enzyme linked immunosorbent assay
eYFP	enhanced yellow fluorescent protein
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
g	gram; unit of gravity
G	gauge
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hour
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
his	histidine
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HRP	horseradish peroxidase
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IU	international unit

iu	infectious unit
Kan	kanamycin
kb	kilobase
kDa	kilodalton
L	litre
LPS	lipopolysaccharide
LTR	long terminal repeat
LV	lentiviral vector
M	molar
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MW	molecular weight
n	sample size
ng	nanogram
NK	natural killer
nm	nanometre
°C	degree Celsius
OD_X	optical density at wavelength X (nanometres)
ori	origin of replication, part of adenoviral genome
PBS	phosphate buffered saline
PCR	polymerase chain reaction

pfu	plaque forming unit
PGK	phosphoglycerate kinase
polyA	polyadenylation site
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rAAV	recombinant adeno-associated virus vector
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev response element
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s	second
SA	streptavidin
SD	standard deviation of the mean
SV40	simian virus type-40
TCR	T cell receptor
TGF-β	transforming growth factor beta
T_m	melting temperature
TU	transducing unit
TNF-α	tumour necrosis factor alpha
UV	ultraviolet light
v/v	volume per volume
VEGF	vascular endothelial growth factor
w/v	weight per volume

CHAPTER 1

INTRODUCTION AND AIMS

1.1 Overview

Blindness due to acquired disease of the cornea is second only to cataract as a cause of visual loss worldwide.[1] In many patients, corneal transplantation is the only means of restoring vision and improving quality of life. Corneal transplantation is regarded as very successful but overall survival rates have not improved over the past 20 years.[2] Improvements in the survival of renal allografts over the same period may be ascribed to improvements in immunosuppression, HLA matching and the use of organs from living related donors. These advancements have not had an impact on corneal transplantation, either because evidence for their benefit is scarce or they are not practical or feasible. Therefore, there is a need for novel strategies if corneal transplantation is to realise its full therapeutic potential. Gene therapy has shown promise in animal models but to date no clinical trials of gene therapy for corneal transplantation have been performed. Reasons for this include a shortage of robust preclinical models of corneal graft rejection, and debate over which vector and transgene will be best for clinical use. With access to a unique preclinical model in the outbred sheep, and an HIV-1-based lentiviral vector with potential advantages over other vectors for achieving safe and stable expression in the clinical setting, I characterised the performance of the vector in the ovine model and tested it in human corneas *in vitro* as a prelude to clinical studies. The overarching aim of the project was to move gene transfer technology for corneal transplantation one step closer to the clinic, where thousands of patients worldwide stand to benefit.

1.2 Normal function of the cornea

The cornea is the transparent tissue at the front of the eye (Figure 1.1). It functions as a powerful lens, contributing two thirds of the overall focussing power of the eye, whilst also providing a barrier to external insults. The human cornea comprises five layers (Figure 1.2). Most anteriorly is a nonkeratinised, nonsecretory epithelium, overlying Bowman's layer. Underneath this is the corneal stroma, which accounts for over 90% of corneal thickness. It contains multiple lamellae of precisely arranged collagen fibrils suspended in a matrix of proteoglycans, and interspersed with keratocytes. On the posterior surface of the cornea, adjacent to the anterior chamber of the eye, is the corneal endothelium, a monolayer of cells resting on Descemet's membrane.

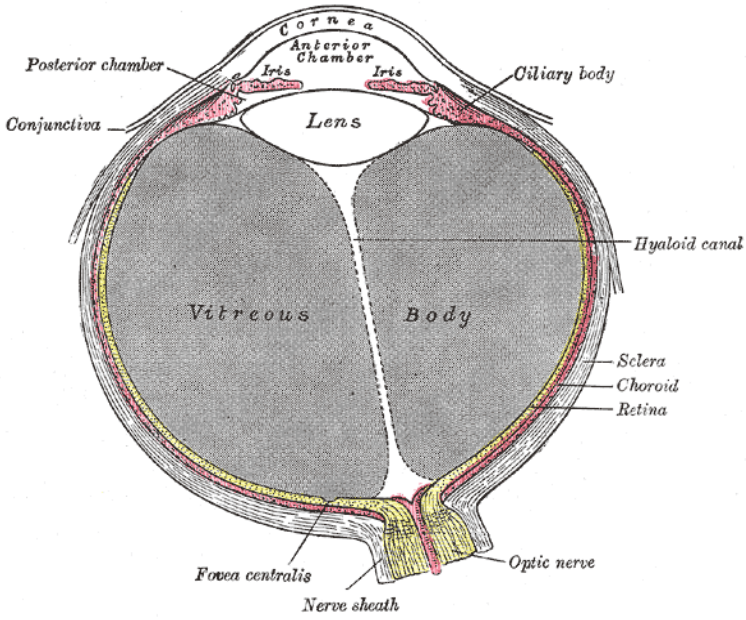


Figure 1.1: Schematic cross-sectional diagram of the human eye

The cornea constitutes the anterior boundary of the anterior chamber. It protects the eye from external insults and focuses incoming light on the retina. [Adapted from Gray's Anatomy (1942)[3]]

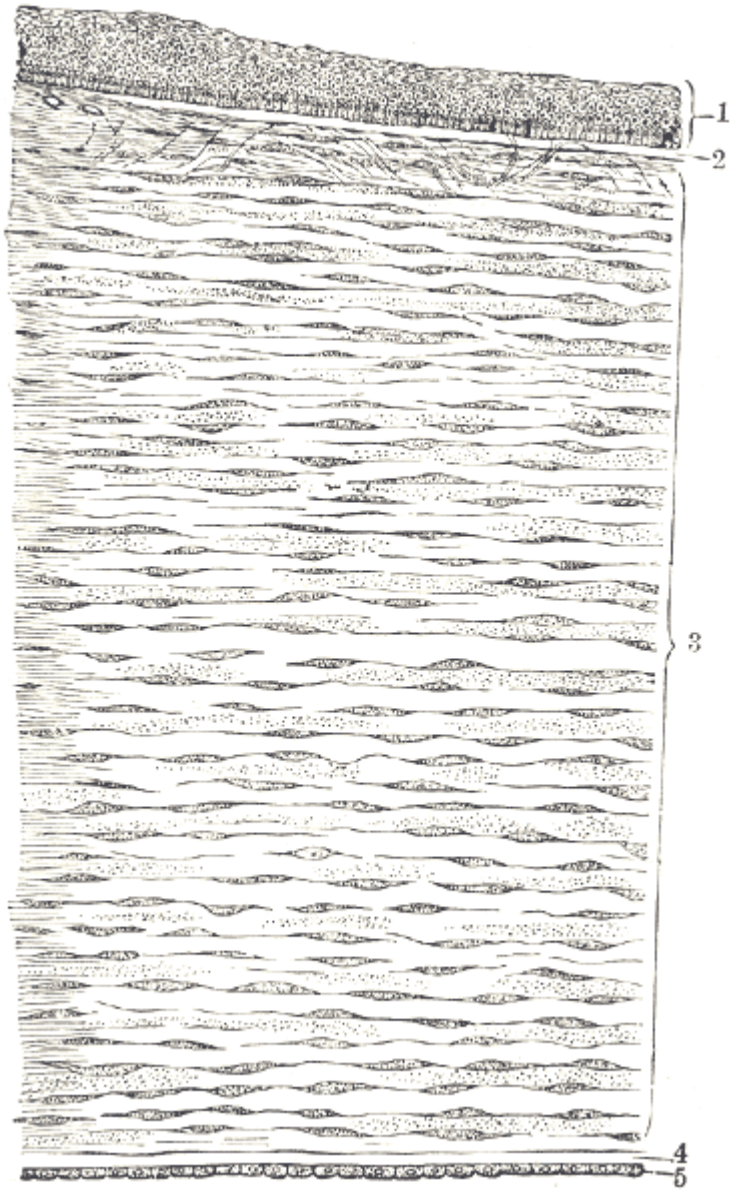


Figure 1.2: Transverse section of human cornea

The five labelled layers of the cornea are: (1) the epithelium, (2) Bowman's layer, (3) the stroma, (4) Descemet's membrane, and (5) the endothelium. The cells of the endothelial monolayer are adjacent to the anterior chamber and bathed by aqueous humour. [Adapted from Gray's Anatomy (1942)[3]]

The optical properties of the cornea are dependent on maintenance of the precise spatial arrangement of stromal collagen fibrils.[4] The stroma has a high water content and an inherent tendency to swell. This reflects the water-binding properties of the proteoglycans in the extracellular matrix. If the spatial relationship of collagen fibrils in the stroma is disrupted the cornea becomes cloudy, resulting in impaired vision.

Normal stromal deturgescence, corneal thickness, and consequent corneal clarity, are maintained by the corneal endothelium.[5] Corneal endothelial cells are highly metabolically active, and have both a barrier and a pump function. Their active transport of ions preserves an osmotic gradient favouring the flow of water from the stroma into the anterior chamber. Leaky tight junctions between adjacent cells are the result of an incomplete zonula occludens, allowing nutrients and other molecules to enter the stroma from the aqueous humour. Processes which result in a loss of endothelial cells and depletion of the endothelial monolayer eventually result in decompensation of the endothelial pump and swelling of the stroma.[6] In humans, endothelial cells are arrested in early G1-phase of the cell cycle.[7,8] It has been suggested that this may be due to cellular contact inhibition. Whilst they can be induced to divide in cultured corneas, human corneal endothelial cells do not divide under normal physiological conditions. Thus, the human corneal endothelium has no effective replicative capacity and, as a consequence, maintaining a healthy endothelial monolayer is critical to normal vision.

1.3 Corneal transplantation

A range of diseases may lead to disruption of normal corneal architecture and function. In severe cases, corneal transplantation using healthy cadaveric donor tissue may be the only means of restoring useful vision. Corneal transplantation is

the oldest form of solid tissue engraftment. Bigger's report of a graft performed in a pet gazelle in 1837 and Von Hippel's lamellar graft in 1888 are two of the earliest descriptions of corneal graft surgery.[9,10] The first successful penetrating allokeratoplasty in a human was carried out by Eduard Zirm in 1905 (Figure 1.3);[11] the first successful human kidney transplant was not achieved until fifty years later.[12] Today, more corneal transplants are carried out annually than any other solid tissue allograft. In the United States alone there are more than 45,000 grafts performed each year.[13] In Australia over 18,000 grafts have been performed since the inception of the Australian Corneal Graft Registry (ACGR) in 1985[2] (and personal communication), and over 1,000 are now performed each year.

Corneal diseases warranting transplantation may be primary or acquired. The majority of primary corneal diseases comprise keratoconus and the corneal dystrophies. The acquired diseases include bullous keratopathy—a blinding condition due to endothelial cell failure—as well as microbial infection, trauma and scarring, and failure of a previous graft.[14]

There is a common misconception that corneal grafts are almost invariably successful.[15] Data from the ACGR show overall survival of penetrating grafts is 86% at one year and 62% at 10 years.[2] The median survival of a penetrating graft is almost 16 years. However, these figures do not provide the full picture. Firstly, graft survival varies enormously depending on the indication for the graft and the condition of the recipient graft bed. Graft survival at 10 years is 90% for keratoconus but only 40% for bullous keratopathy, and for patients receiving a second graft this figure drops to 36%. Furthermore, whilst the survival of other solid organ transplants has significantly improved over the last 30 years,[16] data from the Australian Corneal

Figure 1.3: Eduard Konrad Zirm

The Austrian ophthalmologist carried out the first successful full-thickness human corneal transplant on 7 December, 1905, in the town of Olomouc, Moravia.[11] The recipient was a labourer, Alois Glogar, who was suffering from bilateral corneal scarring following lime burns sustained a year earlier. Both corneas were grafted, and one enjoyed sustained survival, allowing Glogar to return to his occupation. The donor was an 11 year old boy, Karl Bräuer, who had been irreversibly blinded by metallic foreign bodies. Zirm performed the operation without the aid of the operating microscope,[17] which did not come into being for another 50 years.

Graft Registry show that corneal graft survival rates have remained static since the Registry's inception in 1985 (Figure 1.4).[2]

The leading cause of failure in human corneal allografts is irreversible immunological rejection, accounting for a third of all failures.[2] The actual number may indeed be higher than that reported. It is possible that a proportion of cases of graft failure secondary to rejection are misclassified because the classical signs are obscured by the underlying condition or a concomitant process such as infection. Grafts that fail to clear in the early postoperative period are regarded as primary nonfunctioning grafts; these occur in less than 1% of cases.[14] Other less common causes of corneal allograft failure include endothelial cell failure, infection and glaucoma. Immunological rejection occurs as a result of a breakdown in corneal immune privilege. This is demonstrated by the clear association of the indication for the graft, the degree of host prevascularisation, and the number of previous ipsilateral grafts, with the probability of graft survival. Patients grafted for keratoconus—a primary disease leading to a misshapen cornea—have the best survival, but patients with any form of pregraft inflammation have significantly lower success rates.[14]

1.4 Corneal immune privilege

The high rate of acceptance among first-time corneal allografts for primary conditions such as keratoconus without the need for systemic immunosuppression or HLA matching, is attributable to the immune privilege of the cornea. The key features underpinning the immune privilege of the cornea have been elucidated in rodent studies: the existence of a blood-eye barrier; an absence of blood vessels and lymphatics; low numbers of mature antigen presenting cells (APCs) in the central cornea; low expression of major histocompatibility complex (MHC) class I and class II determinants; the presence of immunomodulatory substances in the aqueous

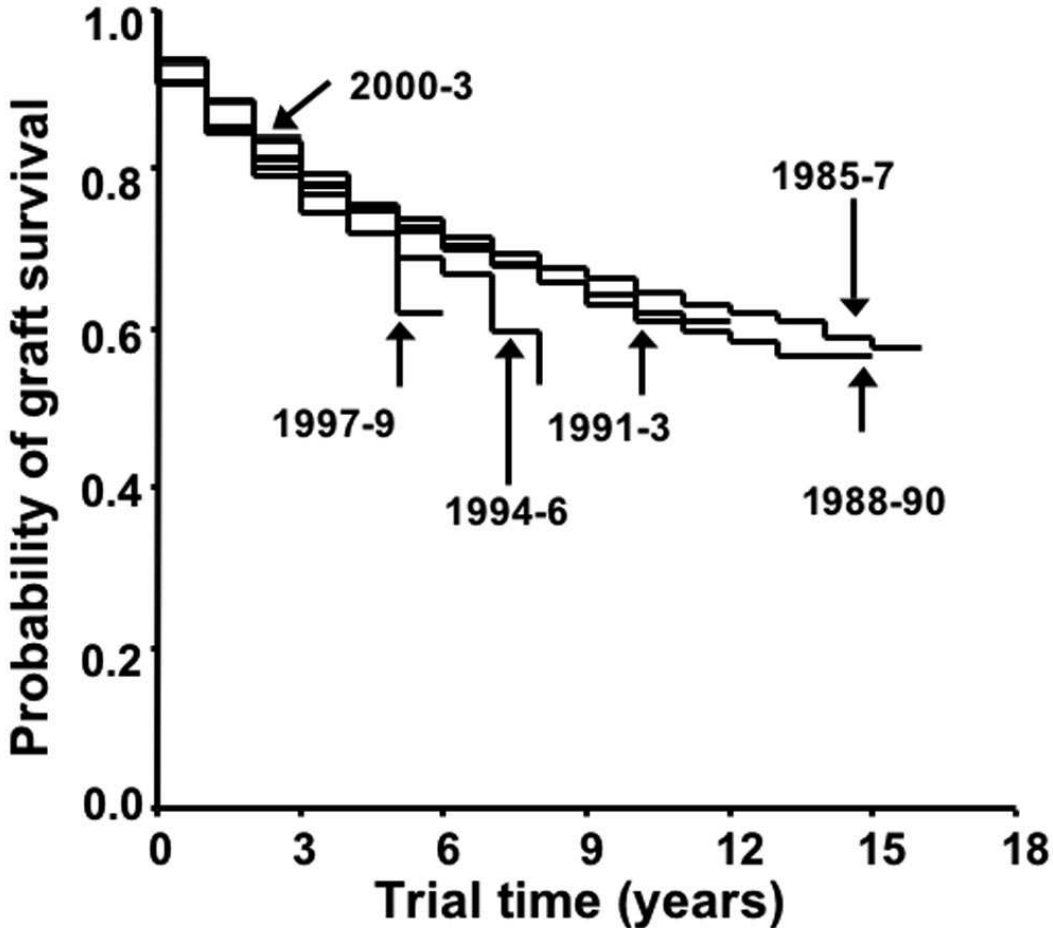


Figure 1.4: Corneal graft survival by era

The Kaplan-Meier plot shows probability of corneal graft survival divided into six eras since the inception of the Australian Corneal Graft Registry. There has been no demonstrable improvement in survival since 1985. [From Williams et al. (2006)[2]]

humour (including transforming growth factor-β [TGF-β], α-melanocyte-stimulating-hormone and vasoactive intestinal peptide); constitutive expression of Fas-ligand; and the phenomenon of anterior chamber-associated immune deviation (ACAID).[18] Despite the effectiveness of these mechanisms in promoting immunologic unresponsiveness, the immune privilege of the cornea is readily compromised by inflammation.[19] Acquired corneal diseases such as those due to infection and trauma may result in significant vascularisation and inflammation of the graft bed, damaging all the mechanisms contributing to privilege. The development of any strategy to improve the survival of corneal grafts is dependent on an appreciation that immunological rejection is fundamentally linked to the erosion of immune privilege.

1.5 Corneal allograft rejection

1.5.1 Clinical presentation of corneal allograft rejection

The clinical appearance of corneal graft failure as a result of immunological rejection was defined by Maumenee in 1962.[20] A typical episode begins with symptoms of irritation and a slight clouding of vision in the grafted eye, accompanied by anterior segment inflammation and reduced graft transparency. The eye displays circumcorneal injection and, as the episode progresses, the stroma becomes progressively more oedematous. Opacification of the graft may be accompanied by the growth of limbal blood vessels into the graft tissue (Figure 1.5).

The corneal epithelium, stroma and endothelium may each undergo rejection separately or together.[21] Endothelial rejection may be characterised by keratic precipitates—collections of leucocytes on the endothelium. These may be arranged in a line, commonly referred to as a rejection line or Khodadoust line, which does not involve the recipient corneal endothelium. A linear defect may also be seen

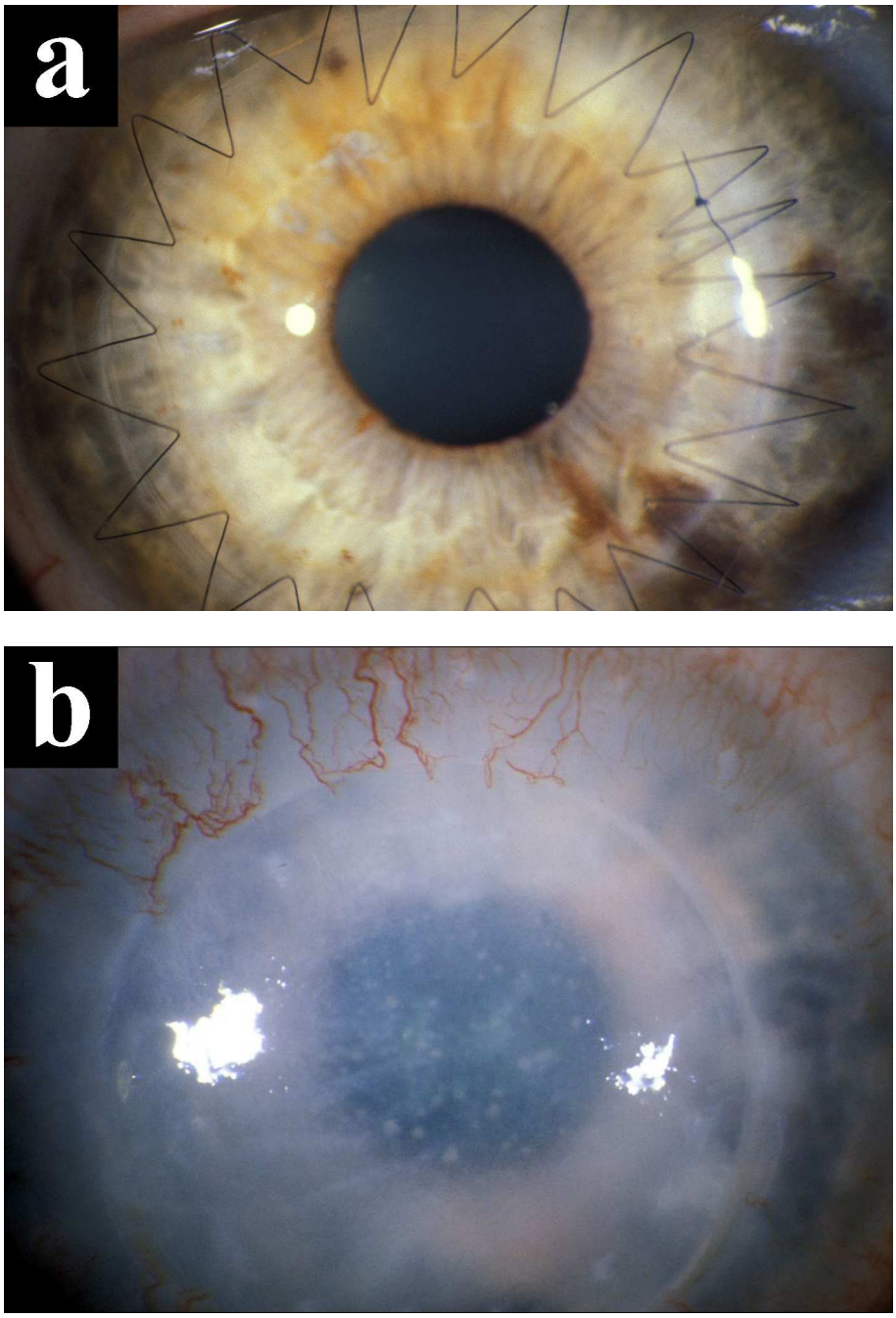


Figure 1.5: Appearance of human corneal allograft rejection

Photographs show: (a) a healthy corneal allograft, with an intact continuous suture; and (b) a corneal allograft undergoing immunological rejection—note loss of clarity, keratic precipitates and growth of limbal blood vessels into the graft (seen here superiorly). The continuous suture has been previously removed. [Pictures provided by D.J. Coster and A. Chappell]

progressing across the epithelium of the donor tissue in the case of rejection of this layer. Rejection of the stroma is accompanied by circumcorneal injection and stromal haze, followed by endothelial rejection and clouding of the graft.

The rejection process may also be appreciated clinically, by detection of an anterior chamber reaction and the appearance of corneal infiltrates, followed by corneal oedema, in a previously thin, clear graft.

1.5.2 Corneal allograft rejection at the cellular and molecular level

Our understanding of the corneal allograft rejection process at the cellular and molecular levels has aided in the identification of potential targets for immuno-modulation. The rejecting corneal allograft contains an infiltrate of T cells (both CD4+ and CD8+), macrophages, natural killer (NK) cells and a smaller proportion of neutrophils.[22-25] These different cell types are involved in the efferent or effector arm of the allograft response which is directed at all layers of the graft. However, the endothelium with its limited capacity to replicate, is the major target of the rejection process.[6]

Role of cell-mediated immunity

It is widely accepted that CD4+ T cells play a crucial role in the rejection of corneal allografts,[26-30] and a close correlation has been established between the development of delayed-type hypersensitivity (DTH) to donor histocompatibility antigens and corneal allograft rejection.[31] However, the mechanisms by which CD4+ T cells mediate the corneal allograft rejection process remain unclear. Recent studies suggest that CD4+ T cells act directly as effector cells rather than purely as helper cells,[32] and that macrophages, which have been shown to play a critical role in corneal allograft rejection,[33] may be largely restricted to a role in antigen presentation.

Classical delayed-type hypersensitivity (DTH) is mediated by a subset of CD4+ T cells, known as Th1 cells. The Th1/Th2 model proposed by Mossman et al. is founded on observations in mice that there are two distinct populations of CD4+ T cells which can be characterised on the basis of cytokine production.[34] Th1 cells produce interleukin-2 (IL2) and the pro-inflammatory cytokine interferon-γ (IFN-γ). Lesions of the Th1 type display a characteristic mononuclear infiltrate and a conspicuous absence of granulocytes. Th2 cells produce a variety of cytokines including IL4, IL5 and IL10. The development of IgE antibodies, an eosinophilic reaction and suppression of IFN-γ production are evident in Th2-type inflammatory states. A cross-regulation occurs between the two subsets of cells, as the production of subset-specific cytokines tends to inhibit the proliferation of the other. This apparent dichotomy of T cells has been challenged; individual T cells and clones display remarkable diversity in their cytokine profiles, collectively forming a continuous spectrum in which Th1 and Th2 cells may be only two extremes of the possible range of phenotypes.[35] However, it does provide a useful framework within which to investigate and classify immune reactions. Studies of corneal graft rejection have established the process as typical of a DTH immune response, in which CD4+ Th1 cells play the pivotal role. The Th1/Th2 paradigm underpins the selection of several Th2 cytokines as therapeutic transgenes in attempts to prolong corneal allograft survival using gene therapy.

Cytotoxic T lymphocytes (CTLs) are allospecific CD8+ cells which recognise target cells bearing MHC class I determinants and cause cytolysis by the release of perforins. Clinicians have proposed that the rejection lines which may be seen in corneal epithelium or endothelium during acute rejection represent an interface between advancing CTLs and the progressively depleted graft cell layer. Such

lesions are indeed consistent with the piecemeal necrosis brought about by CTLs. However, whilst donor-specific CTLs are acquired during corneal graft rejection their contribution has been uncertain. Studies in CD8 knockout (KO) and perforin KO mice demonstrate that corneal graft rejection continues unabated, in spite of the fact that neither mouse strain is capable of mounting an allospecific CTL response.[36] Further work in mice by Boisgerault et al. has provided evidence that although alloreactive CD8+ T cells are activated via the direct pathway, they are not fully competent and are unable to contribute to the rejection process unless they receive an additional signal provided by professional APCs in the periphery.[37] It appears that whilst CD8+ T cells are capable of contributing to the rejection process, their contribution is not obligatory and their exact role remains to be elucidated.

Like the CTL, the role of the NK cell in corneal graft rejection is not yet fully appreciated. However, there are reports implicating NK cells as possible mediators of the rejection process, and certainly the absence of constitutive expression of MHC class I molecules on corneal endothelial cells makes them potential targets for NK cell-mediated cytolysis.[38,39]

Role of alloantibody

There is evidence that alloantibody is involved in corneal graft rejection but its precise role is not yet clear. Studies in mice have shown that hosts deficient in either antibody or complement reject corneal allografts as effectively as their wild-type counterparts, suggesting that the primary default pathway for graft rejection is independent of alloantibody.[40] Nonetheless, alloantibody can kill corneal endothelial cells in a complement-dependent or complement-independent manner.[41,42] Components of the intraocular microenvironment may play a role in modulating the contribution of antibody to an immune response. Studies suggest that the

complement system is constantly active at a low level in the normal eye and is strictly regulated by intraocular complement regulatory proteins.[43,44] It is not known whether a humoral response is responsible for the gradual but supranormal decrease in endothelial cell density observed following corneal transplantation, as a form of chronic rejection.

Antigen presentation

The effector response to the corneal allograft is dependent on successful antigen presentation to naive T cells, and results in proliferation and clonal expansion of allospecific T cells.[45] The capture and presentation of antigen by APCs constitutes the afferent arm of the allograft response. Antigen presenting cells may be present as passengers on the donor tissue, participating in so called direct presentation. Alternatively, they may move into the graft from the surrounding host cornea triggered by inflammation, internalise histocompatibility antigens from the graft, and present them to naive host T cells. This is referred to as indirect antigen presentation.[46] Both pathways have been found to operate after corneal transplantation, and despite the fact that the indirect pathway is less efficient, it is believed to be far more important than the direct pathway in the rejection of corneal allografts.[13] Corneal grafts confronting the host with only minor histocompatibility disparities are still rejected, even though minor histocompatibility antigens cannot induce alloimmune responses via the direct pathway.[47]

Activation of T cells requires the interaction of the T cell receptor/CD3 complex with the MHC present on APCs. Moreover, costimulatory signals involving the interaction of B7 antigens (CD80 and 86) of APCs with the CD28 molecule of T cells are also essential for full activation of this immune reaction.[48,70] The site of T cell sensitisation and proliferation in corneal allograft rejection remains uncertain,

but there is mounting evidence that it takes place in draining lymph nodes, rather than inside the eye itself.[49,262]

1.5.3 Current interventions to prevent corneal allograft rejection

The mainstay of treatment to prevent and reverse corneal allograft rejection is topical glucocorticosteroids.[50] These have been in use for over 40 years, and there is anecdotal evidence that their introduction resulted in an appreciable improvement in graft survival.[51] Corticosteroids have an array of actions, but the most important may be their ability to inhibit migration of effector leucocytes into the graft in the efferent arm of the allograft rejection response.

Conventional interventions which have contributed to improvements in survival of renal allografts have not had the same impact on corneal allografts. Systemic immunosuppression is not widely used in corneal transplantation for two reasons. First, there is limited evidence for benefit, with studies yielding inconsistent results.[52-54] Second, the clinical context differs from that of other solid tissue transplants. The majority of corneal transplant recipients are otherwise well, and few patients are prepared to accept the risks associated with the use of systemic immunosuppressive agents.

Tissue matching is the other strategy which has had profound benefits in renal transplantation but is not routinely performed for corneal transplantation. Perhaps the primary reason that histocompatibility matching for MHC determinants has not become widely used for corneal grafts is that the largest study to date, the American Collaborative Corneal Transplant Study (CCTS) reported no benefit.[55] Those results stand in conflict with benefits from MHC matching seen in a number of other studies conducted in parts of Europe[56-58] and Canada.[59] There may indeed be a benefit to be gained by tissue matching for corneal transplantation, but the issue

remains unresolved and practical challenges further reduce the likelihood of such an approach being implemented in Australia.

1.5.4 The need for novel approaches to improving corneal allograft survival

The lack of improvement in corneal allograft survival evident in data collected over the past 20 years should not be surprising. The introduction of topical corticosteroids took place approximately 40 years ago and minor changes in surgical technique have not significantly affected outcomes.[60] Now, more than one hundred years after the first successful corneal transplant, what was once the leading light of transplantation has become a muted glow. However, the advent of recombinant DNA technology offers new prospects for modulating corneal allograft rejection. Gene therapy has achieved successes in experimental models of corneal transplantation, opening up new opportunities to exploit its full therapeutic potential.

1.5.5 Experimental models of corneal allograft rejection

In order to test gene therapy strategies with a view to improving human corneal allograft survival, useful animal models are required. Much of the current understanding of the mechanisms underlying corneal allograft rejection has been derived from experiments in rodents, based on the penetrating keratoplasty model in rats established by Williams and Coster[61] and the mouse model developed by She and Moticka.[62] Whilst, as in humans, the inflammatory infiltrate in rejecting rat allografts contains macrophages, T cells (more CD4+ than CD8+), NK cells and neutrophils,[25,63] the rejection process in the rat differs from that seen in humans at a clinical level; rejection lines are rarely observed. Importantly, the corneal endothelium of rodents has replicative capacity, in contrast to the amitotic human

endothelium.[64,65] Episodes of rejection may be short and healing of the endothelium may occur, restoring corneal clarity. Whilst the rodent model is valuable as a means of investigating the mechanisms underlying the graft rejection process, it does not constitute a preclinical model of corneal transplantation.

Alternative models to the rodent include the rabbit and the cat. Corneal allografts in rabbits are generally well accepted. Studies have shown that retained graft-host sutures significantly increase the rate of rejection, making this a useful model for assessing mechanisms of graft rejection.[66] Unlike the rodent, and as is the case in humans, the corneal endothelium of the rabbit is essentially nondividing. However, its replicative capacity may be age-dependent, with a higher mitotic rate in younger animals.[67] Cats have virtually no corneal endothelial mitotic potential,[68] but present unique handling challenges.

There is a scarcity of large mammal models of corneal allograft rejection. Monkeys are known to have an essentially amitotic endothelium, but have seldom been used for studies of corneal allotransplantation, probably on account of their cost and ethical issues associated with their use. A miniature pig model has been described, but the model is inherently low-risk, as the cornea is avascular.[69]

A model of orthotopic corneal transplantation in the outbred sheep has been established by Williams et al.[70] The ovine cornea has a degree of vascularisation under normal conditions, rendering the model high-risk. The graft rejection process is macroscopically and histologically similar to that observed in humans (Figure 1.6). In the absence of immunosuppression, rejection has been observed at a median of 20 days following orthotopic corneal allotransplantation in the sheep. Both endothelial and epithelial rejection lines are observed. At a histological level, the inflammatory infiltrate in rejecting grafts has been found to contain both CD4+ and

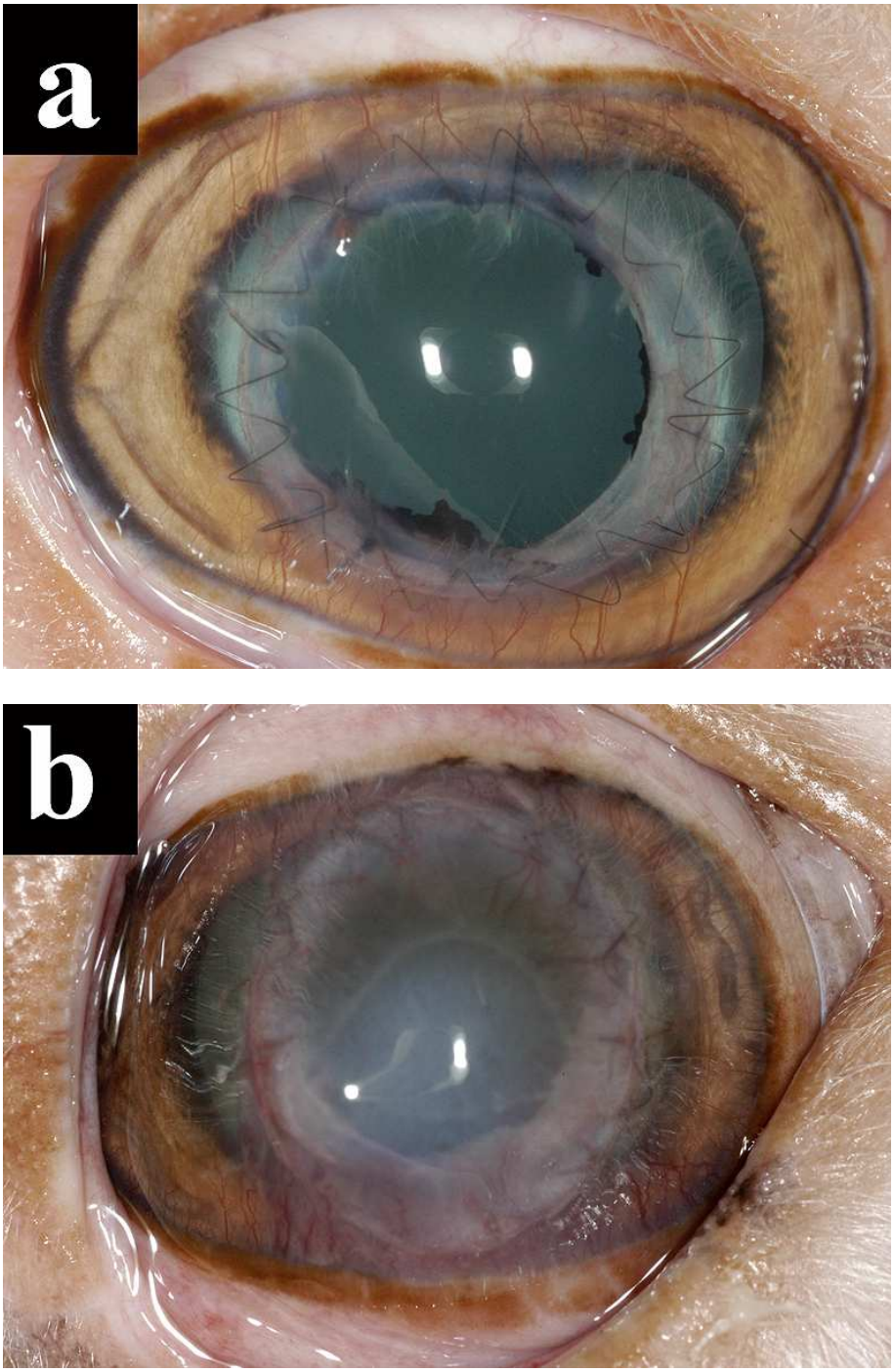


Figure 1.6: Appearance of corneal allograft rejection in the sheep

Photographs show: (a) a healthy ovine corneal allograft (the line apparent in the lower left quadrant of the graft is a reflection); and (b) an ovine corneal allograft undergoing immunological rejection—note loss of clarity, ingrowth of blood vessels into the graft, and epithelial rejection line. [Photographs taken by A. Chappell]

CD8+ T cells, and is accompanied by the local production of IL2 and the pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and IFN-γ.[70] In view of the tempo and rate of rejection of ovine allografts in the absence of immunosuppression, and the clinical similarity to the process seen in humans, the ovine model is an ideal preclinical model of high risk corneal allograft rejection. Studies of gene transfer methods for corneal transplantation in the ovine model have previously been undertaken using nonviral and adenoviral vectors.[71] For these reasons, the outbred sheep was chosen as the preclinical model in which to characterise the lentiviral vector under investigation in this project.

1.6 Gene therapy as a strategy to prolong corneal graft survival

At its most basic level, gene therapy entails the use of a vector to deliver modified DNA into the nuclei of target cells, whereupon that DNA is transcribed and translated to produce a protein with a therapeutic effect. The most commonly used vectors are engineered virus particles, but nonviral methods, such as liposomes, are also used. The field of gene therapy has received considerable media attention since its beginnings in the early 1990s. This attention has often been misdirected, either overstating its potential and capabilities, or focussing on adverse events and failures. In truth, there have been some successes in gene therapy trials, most notably the treatment of children suffering from X-linked severe combined immunodeficiency disease (SCID-X1) who might otherwise have died.[72] But there have also been difficulties and setbacks. Understanding the biology of the individual condition to be treated presents certain challenges, but tuning the gene transfer technology to address it can prove particularly problematic. The biological processes involved in the

rejection of corneal allografts are complicated but a number of immunomodulatory strategies have been devised based on the mechanisms described above. A range of transgenic molecules have been trialled in experimental models and show great therapeutic potential.[73] A greater challenge for gene therapists of the cornea is developing a vector that has an acceptable biosafety profile, and can achieve efficient, stable transgene expression in both a preclinical model and the human cornea itself.

The corneal endothelium as a target for gene transfer

The cornea is regarded as an ideal target for gene therapy.[48,74] It has a well-defined anatomy and is easily accessible—facilitating rapid, noninvasive examination. Furthermore, donor corneas may be stored for up to 28 days in eye bank culture media prior to transplantation. They are therefore uniquely available for *ex vivo* manipulation, given that the corneal endothelium is an exposed surface and highly accessible to vectors. This approach also obviates the need for systemic administration of the vector, minimising systemic dissemination and its associated risks, and facilitating more precise titration of dose.

The choice of transgene to prolong corneal allograft survival

A number of different transgenes have been shown to prolong corneal allograft survival in experimental models (Table 1.1). These include the immunomodulatory cytokine interleukin-10 (IL10),[75] inhibitors of T cell activation and proliferation,[76-81] an anti-angiogenic factor,[82] and an anti-apoptotic factor.[83]

Table 1.1: Transgenes shown to confer a prolongation of corneal allograft survival in experimental models

Transgene	Vector	Route	Model	Outcome	Reference
IL10	Adenovirus	*Ex vivo* transduction of cornea	Sheep	Median survival extended from 20 d to 55 d	75
Soluble TNFR-Ig	Adenovirus	*Ex vivo* transduction of cornea	Rabbit	Treatment with Ad-control significantly shortened graft survival (p=0.0185); marginal prolongation of Ad-TNFR-treated grafts compared to negative controls (p=0.0832)	84
CTLA4-Ig	Adenovirus	*Ex vivo* transduction of cornea; or intravenous injection	Brown-Norway rats to Lewis rats	*Ex vivo* transduction of cornea: extended median survival time from 9 d to 10 d; systemic injection extended survival from 9 d to 18 d	76
IL4 with CTLA4	MIDGE vectors	Gene gun to corneal epithelium 1 d pre-transplant	C3H mice to BALB/c mice	Survival extended from 27±19 d to 64±28 d after treatment of recipient cornea	77
IL4 with CTLA4	MIDGE vectors	Gene gun to lower eyelid 1 d pre-transplant	C3H mice to BALB/c mice	Survival extended from 26.6±5.7 d to 61.7±25.6 d; no extension after treatment of leg	78
E-Kr5	HIV-based lentivirus	*Ex vivo* transduction of cornea	Rabbit	2 negative controls failed at 14 d and 18 d; none of 10 LV-E-Kr5-treated rabbits failed by 39 d	82
p40-IL12	Adenovirus	*Ex vivo* transduction of cornea	Sheep	Median survival extended from 20 d to 45 d	85
CTLA4-Ig vIL10	Adenovirus	*Ex vivo* transduction of cornea; or intra-peritoneal injection (2 different doses)	Dark Agouti rats to Lewis rats	*Ex vivo* transduction of cornea: mean survival extended from 13.1 d to 15.8 d (Ad-CTLA4-Ig only) or 15.3 d (Ad-CTLA4-Ig and Ad-vIL10); intra-peritoneal Ad-CTLA4-Ig: mean survival extended to 22.7 d (low dose) and 40.4 d (high dose)	79
IDO	EIAV-based lentivirus	*Ex vivo* transduction of cornea	C3H/He mice to BALB/c mice	Median survival extended from 11 d to 21 d	80
vIL10	Liposomes Adenovirus	*Ex vivo* transduction of cornea; or intra-peritoneal injection (2 different doses)	Wistar-Furth rats to Lewis rats	*Ex vivo* transduction: no extension with liposomes or adenovirus; intra-peritoneal Ad-vIL10: mean survival extended from 10.6 d to14.6 d at lower dose	86

Continued below

Table 1.1 continued: Transgenes shown to confer a prolongation of corneal allograft survival in experimental models

Transgene	Vector	Route	Model	Outcome	Reference
NGF, CTLA4-Ig	Adenovirus	*Ex vivo* transduction of cornea; or intra-peritoneal injection	Dark Agouti rats to Lewis rats	Ad-NGF to cornea only: mean survival extended from 13.1 d to 16.8 d; Ad-NGF intra-peritoneal only: no extension; Ad-NGF to cornea plus intra-peritoneal Ad-CTLA4-Ig: 6 of 7 animals surviving at 70 d	81
Bcl-xL	HIV-based lentivirus	*Ex vivo* transduction of cornea	C57/Bl6 mice to BALB/c mice	90% survival rate at 8 wk (LV-Bcl-xL-treated corneas) compared to 40% (negative control) and 30% (LV-control)	83

***Footnotes:
IL10 cellular interleukin-10, d days, wk weeks, TNFR-Ig tumour necrosis factor receptor immunoglobulin fusion protein, CTLA4-Ig cytotoxic lymphocyte antigen-4 immunoglobulin fusion protein, IL4 interleukin-4, MIDGE minimalistic immunologically defined gene expression, HIV human immunodeficiency virus, E-Kr5 endostatin-kringle 5 fusion protein, LV lentivirus, p40-IL12 p40 subunit of interleukin-12, vIL10 viral interleukin-10, IDO indoleamine 2,3-dioxygenase, EIAV equine infectious anaemia virus, NGF nerve growth factor

1.6.1 The choice of vector for gene transfer to corneal endothelium

Characteristics of the ideal vector for gene transfer to human corneal endothelium are: the ability to transduce nondividing cells, efficient transduction, low immunogenicity, stability of transgene expression, large transgene carrying capability, capacity for inducibility, and an acceptable biosafety profile. A range of methods, including viral and nonviral vectors, have been hitherto trialled for achieving gene delivery to the corneal endothelium. An outline of these approaches is provided below, culminating in a discussion of the proposed advantages of the lentiviral vector which was investigated for this thesis.

Nonviral gene transfer

Physical methods of gene transfer include naked DNA, liposomes, and ballistic gene delivery. Considerable effort has been invested in devising such vectors as noninflammatory alternatives to viral vectors; this has yielded some success, notably the targeted liposomes and dendrimers of the George and Larkin group.[87-89] However, their main drawback has been relatively low efficiency of gene transfer and transient gene expression.[74,90] Ballistic delivery of minimalistic immunologically defined gene expression (MIDGE) vectors expressing IL4 and CTLA4, to the corneal epithelium, has been shown to prolong corneal graft survival in a mouse model.[91] However, ballistic gene transfer requires careful optimisation to avoid causing damage, particularly in the case of the corneal endothelium. Previous studies using a gene gun to deliver genes to the ovine corneal endothelium resulted in an unacceptable degree of cell damage.[92] A range of other nonviral vectors were trialled in the ovine corneal endothelium and were found to be highly inefficient compared to

an adenoviral vector.[71] One remarkably efficient nonviral vector was a polylysine-based vector incorporating the integrin-binding domain of American pit viper venom; it transduced 100% of rabbit corneal endothelial cells by *ex vivo* gene delivery.[93] However, gene expression waned after seven days.

Adenovirus-mediated gene transfer

Adenovirus is a 35 kb double-stranded DNA virus that causes mild self-limiting respiratory tract infection and keratoconjunctivitis in humans. Recombinant adenoviral vectors are the most commonly tested vectors for gene transfer to the corneal endothelium, and have been studied in a variety of species including rodents, rabbits, sheep and humans (Table 1.2). By and large, adenoviral vectors have been found to be more efficient than nonviral methods of gene delivery to the corneal endothelium.[94] Significant advantages of adenoviral vectors include their large transgene capacity and high efficiency of transduction. In addition, adenoviral vectors have the ability to infect nondividing cells such as the corneal endothelium, and they can be produced to high titres.

Disadvantages of recombinant adenoviral vectors include their inherent immunogenicity, and the fact that they remain episomal rather than integrating into the host cell genome. These factors may contribute to short-lived expression, counteracting any benefit conferred by the transgene.[84] Immunogenicity has been addressed by the production of so-called “gutless” vectors which lack nearly all viral coding sequences not required for production of useful vector or transgene expression.[95] Despite these improvements, transgene expression can still be short-lived.[96] *Ex vivo* adenovirus-mediated transgene expression in human corneas has been reported to be maintained at high levels for only seven days, with a diminution of expression to zero by 28 days.[97] Studies of adenoviral gene transfer to rabbit and

Table 1.2: Transduction of corneal endothelium by adenoviral vectors

Species	Vector/Transgene	Transduction conditions	Transgene expression	Reference
Mouse	Ad-lacZ	AC injection: 2–3 µl of 10^{11} particles/ml	LacZ expression present at 14 d post-injection	98
Mouse	Ad-GFP	6×10^{6}–6×10^{7} pfu for 2 h at 37°C or 4°C, followed by syngeneic or allogeneic graft	37°C /syngeneic: GFP expression lasted < 2 wk 4°C /syngeneic: GFP expression for up to 12 wk, and peaked at 5 d to 5 wk 4°C /allogeneic: GFP expression undetectable after 4 wk	99
Mouse	Ad-GFP		99% transduction efficiency	100
Rat Rabbit	Ad-lacZ	5×10^{6} pfu for 3 h	90% at 3–7 d 70% at 14 d Undetectable at 21 d	101
Rat	Ad-rIL4	2×10^{7}–2×10^{8} pfu for 3 h at 37°C	rIL4 detectable in culture supernatants at 24 h, 72 h and 6 d	102
Rat	Ad-β-Gal	AC injection: 2 µl of 10^{11} pfu/ml	Strong β-Gal expression at 4 d and 7 d Reduced intensity of expression at 10 d Not detectable after 10 d	103
Rat	Ad-CTLA4-Ig	5×10^{7} pfu for 3 h at 37°C	CTLA4-Ig production maximal in first 7 d and continued to 27 d	76
Rat	Ad-CD4-GFP	5×10^{7} pfu for 2 h at 37°C	69% positive for GFP at 3 d Anti-CD4 scFv production peaked at 4–5 d	49
Rat	Ad-CTLA4-Ig Ad-vIL-10 Ad-β-Gal	0.5–1×10^{8} pfu for 3 h at 37°C	CTLA4-Ig detected after 48 h	79
Rat	Ad-vIL10 Ad-β-Gal	1×10^{8} pfu for 3 h at 37°C	Not reported	86
Rat	Ad-NGF Ad-β-Gal	1×10^{8} pfu for 3 h at 37°C	NGF detected in supernatants collected at 3 d	81
Rabbit	Ad-lacZ	(a) *Ex vivo* transduction and organ culture: 1.5×10^{7} pfu for 3 h (b) *Ex vivo* transduction and transplantation: 1.5×10^{7} pfu for 3 h, 36–40 h culture, grafted	(a) 75–100% cells positive at 1 d; <5% by 21 d (b) 90% cells positive at 1 d; <5% at 7 d	104
Rabbit	Ad-TNFR-Ig	Corneal segments: 1×10^{6} pfu for 1 h	TNFR-Ig detected by ELISA in supernatants for up to 28 d	84

Continued below

Table 1.2 continued: Transduction of corneal endothelium by adenoviral vectors

Species	Vector/Transgene	Transduction conditions	Transgene expression	Reference
Sheep	Ad-lacZ	6.6×10^{7} pfu for 2 h at room temperature	Expression detectable at 1 d, 4 d, and reached 70% at 7 d	71
Sheep	Ad-lacZ Ad-IL10	6.6×10^{6}–6.6×10^{7} pfu for 0.5–2 h at room temperature	30% positive for lacZ at 24 h 70% positive at 6d, and persisted until 28 d IL10 mRNA detected within 24 h and up to 21 d	75
Sheep	Ad-GFP Ad-p40 IL12 Ad-IL4	$0.8–1\times10^{7}$ pfu for 2 h at room temperature	80–100% positive for GFP at 3–4 d IL4 and p40 IL12 mRNA detected at 2 h and up to 21 d	85
Pig	Ad-GFP	MOI 100	27% positive for GFP after 2 d, and 31% positive for GFP at 12 d	105
Human	Ad-GFP	MOI 100	6% positive for GFP after 2 d, and 45% positive for GFP at 12 d	105
Human	Ad-lacZ Ad-CTLA4-Ig	$1–1.5\times10^{8}$ pfu for 3 h	lacZ high until 7 d and undetectable by 28 d Adenoviral DNA persisted for at least 56 d High levels of active CTLA-4 Ig protein detected in supernatants for up to 28 d	97
Human	Ad-vIL10	2×10^{8} pfu/ml for 3 h at 37°C	Transgenic protein (vIL10) detected in supernatant up to 22 d	106
Human	Ad-lacZ	10^{6} pfu (MOI 100) applied to cultured endothelial cells for 24 h	80% positive for lacZ at 24 h	107
Human	Ad-CD4-GFP	5×10^{7}–3×10^{8} pfu for 2.5 h at room temperature	12–100% positive for GFP at 72–96 h Anti-CD4 scFv production peaked at 2 wk and declined to half that level at 1 month	108

***Footnotes:
Ad adenovirus, lacZ β-galactosidase, AC anterior chamber, d days, h hours, wk weeks, GFP green fluorescent protein, pfu plaque forming units, rIL4 recombinant interleukin-4, β-Gal β-galactosidase, IL10 interleukin-10, vIL10 viral IL10, CTLA4-Ig cytotoxic lymphocyte antigen-4 immunoglobulin fusion protein, NGF nerve growth factor, TNFR-Ig tumour necrosis factor receptor immunoglobulin fusion protein, MOI multiplicity of infection

rat corneas *ex vivo* demonstrated greatest transgene expression at three to seven days post-transduction, falling to undetectable levels by 21 days.[101] Importantly, the safety of adenoviral vectors has been brought into question by the death of a patient involved in a clinical trial of gene therapy in which the vector was administered systemically.[109,110]

Despite their deficiencies, adenoviral vectors remain the most efficient means of achieving rapid transgene expression in the corneal endothelium. In our own laboratory, the use of an adenoviral vector expressing a reporter gene has resulted in transduction of up to 70% of endothelial cells in cultured ovine corneas.[71] Adenoviral vectors also constitute the method of gene transfer utilised in achieving a prolongation of allograft survival in several animal models, including the rat and the sheep.[71,76,79,81,85]

Adeno-associated virus-mediated gene transfer

Recombinant adeno-associated virus vectors (rAAV) are based on the 4.7 kb single-stranded DNA virus which is not associated with any human disease. Given its very simple genomic structure AAV requires a helper virus, usually adenovirus, to replicate.[111] The genome contains only two open reading frames (ORFs) flanked by inverted terminal repeats (ITRs). The wild-type viral DNA integrates into the host cell genome via site-specific recombination directed by the viral rep function.[112,113] Recombinant AAV vectors have a broad host range, infect both dividing and nondividing cells, and integrate randomly into the host genome at sites of double-stranded DNA breaks.[114] However, it appears that the greater part of rAAV vectors persist episomally as concatemers formed by head-to-tail recombination, switching between linear and circular forms.[115]

Like adenovirus, rAAV vectors can be grown to high titre. There are now more than 10 different known AAV serotypes,[116] with AAV-2 most commonly used for gene therapy, and AAV-4 and -5 packaged vectors showing tropism for retina.[117,118] Relative disadvantages of rAAV include limited transgene capacity and the need for a helper virus in production. The limited transgene capacity has been addressed by taking advantage of the AAV genome's ability to undergo concatemerisation after transduction. Transgenes larger than 4.9 kb are packaged into two different rAAV vectors and delivered concomitantly. However, this approach significantly reduced transduction efficiency.[119,120] To minimise the risk of contamination with wild-type adenovirus, some groups have developed helper-virus-free methods of rAAV production.[121]

A significant issue in the use of rAAV vectors for clinical use is the presence of neutralising antibodies. Most people have neutralising antibodies to AAV due to prior infection.[122] Using vectors developed from different serotypes, such as AAV-7 and AAV-8 isolated from Rhesus monkeys, has been proposed as a means of circumventing this problem, to allow effective long-term treatment with rAAV vectors.[74]

A number of studies have been performed examining rAAV-mediated gene transfer to the corneal endothelium. Studies using rAAV to transduce rabbit and human corneal endothelial cells resulted in low efficiency, and were limited by technical difficulties in achieving high titres.[123] However, when injected into the anterior chamber of rabbits, rAAV containing the cytomegalovirus (CMV) promoter driving a reporter gene transduced almost 90% of corneal endothelial cells.[124] Expression was shown to be inducible by an injection of lipopolysaccharide, given up to 60 days after transduction. In another study, a level of corneal transduction

sufficient to inhibit corneal neovascularisation was reported with rAAV encoding a soluble vascular endothelial growth factor (VEGF) receptor.[125] More recently, over 90% of corneal endothelial cells in human corneoscleral rims were transduced with rAAV encoding a reporter gene, and expression remained stable for 14 days in organ culture.[126]

Given their lack of pathogenicity and capacity for long-term expression, rAAV vectors are an attractive choice for achieving stable expression of immunomodulatory transgenes after corneal transplantation. However, their limited transgene capacity and the possibility of neutralising antibodies abrogating expression are relative shortcomings.

Herpes simplex virus-mediated gene transfer

Herpes simplex virus (HSV) is a nonintegrative, pathogenic double-stranded DNA virus with a broad host range, and the ability to infect both dividing and nondividing cells. It has a very large capacity and can be grown to high titre but is cytotoxic. Trials of HSV vector transduction in corneal endothelial cells have been disappointing, resulting in a low efficiency of transduction, transient expression, and cytotoxicity.[71,123] In their current form, such vectors are not a good prospect for clinical use in corneal transplantation.

Gammaretrovirus-mediated gene transfer

Gammaretrovirus is a genus of the retroviridae family of enveloped RNA viruses. Gammaretroviral vectors commonly derived from murine leukaemia virus have been widely used in gene therapy experiments and as general purpose gene expression vectors. They are vectors which integrate into the host cell genome and thus can

achieve sustained transgene expression. However, their inability to infect nondividing cells is a significant drawback.

Greatest success using retroviruses to transduce corneal cells has been achieved in keratocytes,[127,128] and one group has reported transduction of epithelial progenitor cells using a retroviral vector.[129] Whilst keratocytes and epithelial progenitor cells are cycling cell populations, the human corneal endothelium is nondividing and therefore not an appropriate target for transduction with gammaretroviral vectors.

Lentivirus-mediated gene transfer

The vector chosen for study in this project was an HIV-1-based lentiviral vector. Background information concerning lentiviral vectors is presented below, followed by a description of the specific vector used, and a rationale for its selection over other lentiviral vector systems.

1.6.2 Lentiviral vectors

Structure and genome

The lentiviruses are a genus of retroviridae that have the ability to transduce both dividing and nondividing cells.[130] They include nonprimate viruses—such as equine infectious anaemia virus (EIAV), bovine immunodeficiency virus (BIV), and feline immunodeficiency virus (FIV)—as well as the primate viruses, simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV). Lentiviruses have a cylindrical core structure composed of Gag proteins, and a viral genome comprising two identical copies of RNA. The genome encodes three classical retrovirus genes—*gag*, *pol* and *env*—as well as a varying number of ORFs (Figure 1.7). The *gag* sequence encodes four proteins which form the viral core,

including the p24 capsid protein and the p17 matrix protein. The *pol* sequence also encodes four proteins, including the viral reverse transcriptase and integrase enzymes. The *env* gene codes for the viral envelope protein gp160, which is transported to the cell membrane and cleaved into two subunits, gp120 and gp41. These form spikes on the surface of enveloped virus particles as they bud off from the cell membrane. The ORFs are most numerous in HIV-1, in which they are designated *vif*, *vpu*, *vpr*, *nef*, *tat* and *rev*. The first four are termed accessory genes, coding for proteins which determine the pathogenic features of the virus. The *tat* and *rev* genes code for virus regulatory proteins, which control HIV-1 expression at transcriptional and post-transcriptional levels.

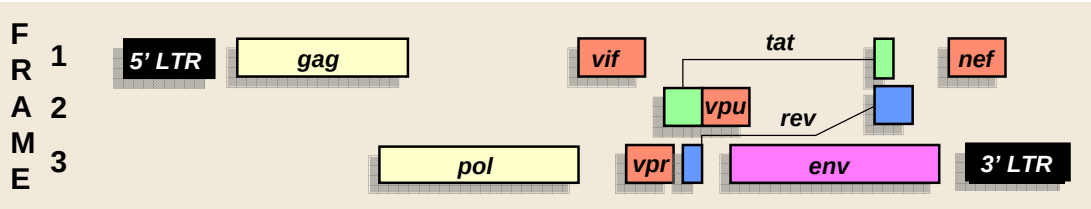


Figure 1.7: Schematic genome map of HIV-1

The genome encodes the protein and enzyme components of the lentiviral particle in the *gag*, *pol* and *env* genes. The *tat* and *rev* spliced exons code for regulatory proteins which modulate transcriptional and post-transcriptional steps of viral gene expression. The *vif*, *vpr*, *vpu*, and *nef* genes are encoded by a number of open reading frames (marked), and code for accessory proteins believed to be involved in virus pathogenicity. Note that lengths of genes are not to scale. LTR: long terminal repeat. [Adapted from HIV-1 gene map at http://www.hiv.lanl.gov/content/hiv-db/MAP/landmark.html in November 2007]

Like other retroviruses, lentiviruses infect cells through an interaction between viral envelope proteins and receptors on the target cell. In the case of wild-type HIV, this interaction takes place between viral gp120, and CD4 on the surface of T cells. Following attachment and receptor-mediated entry in host cells, viral reverse transcriptase and integrase enzymes mediate reverse transcription and integration of the virus genome into the host cell chromatin. This facilitates stable gene expression—a key attribute of a prospective vector for modulation of corneal graft rejection. To confer the capacity for HIV-based vectors to transduce a wide range of cell types including the corneal endothelium, the parental HIV-1 envelope is commonly substituted with the vesicular stomatitis virus G protein (VSV-G). This process—referred to as pseudotyping—also stabilises vector particles, allowing concentration by ultracentrifugation and the generation of higher titres.[131]

In addition to their ability to transduce nondividing cells and capacity for long-term expression, lentiviral vectors are appealing because of their low immunogenicity. In one study, subretinal administration of lentivirus appeared to induce an immune-deviant response, showing significant induction of the Th2 cytokines IL4, IL10, and TGF-β.[132] No neutralising antibodies were identified and inflammatory cells were not apparent at the site of subretinal injection upon immunohistochemical examination. Another study reported injection of lentiviral particles into the anterior chamber of live rat eyes, and no evidence of inflammation was detected.[133]

Generation of HIV-1-based vectors

In view of the pathogenic nature of HIV-1 in humans, a great emphasis has been placed on safety in the development of HIV-1-based vectors.[134] A number of strategies have sought to minimise the likelihood of generating replication-competent

virus. These include minimising the homology between constituent sequences, and segregation of the cis-acting elements in the genome required for vector RNA synthesis, packaging, reverse transcription and integration, from protein-encoding trans-acting sequences. Envelope-encoding sequences are also separated from the rest of the HIV-1 packaging cassette as an additional safety measure.[131]

The earliest HIV-based vectors contained the first-generation packaging cassette developed by Naldini et al.,[130] which included all of the HIV-1 accessory genes (Figure 1.8). Given that these vectors retained a large portion of the HIV genome, there was a significant risk of recombination-mediated generation of wild-type virus. Subsequent studies determined that only the regulatory genes *rev* and *tat* are required for *in vitro* viral propagation. The second-generation packaging cassette was developed, in which all accessory genes were deleted with the exception of *rev* and *tat.*[135] Yet another significant modification was introduced when the third-generation of packaging cassette separated the *gagpol* and *rev* genes between two different plasmids and the *tat* gene was dispensed with altogether.[136] This was made possible by replacing the 5' LTR with the CMV promoter, thus rendering the system Tat-independent.[137] Vectors made using this cassette had a reduced likelihood of generating replication-competent retroviruses, but were still able to be generated at high titres.

Further advancements in the design of HIV-based vectors have included the use of synthetic (codon-optimised) reading frames,[138] and the development of self-inactivating (SIN) vectors.[137] During reverse transcription in the wild-type virus the 3' U3 region, containing the HIV-1 enhancer/promoter sequences, is transferred to the 5' LTR. Making deletions in the 3' U3 region deprives the vector of its parental enhancer/promoter sequences, thus rendering it incapable of transcribing full length

vector RNA that may be packaged. This reduces the likelihood of generating replication-competent virus and the risk of inadvertently activating silent host cell promoters.

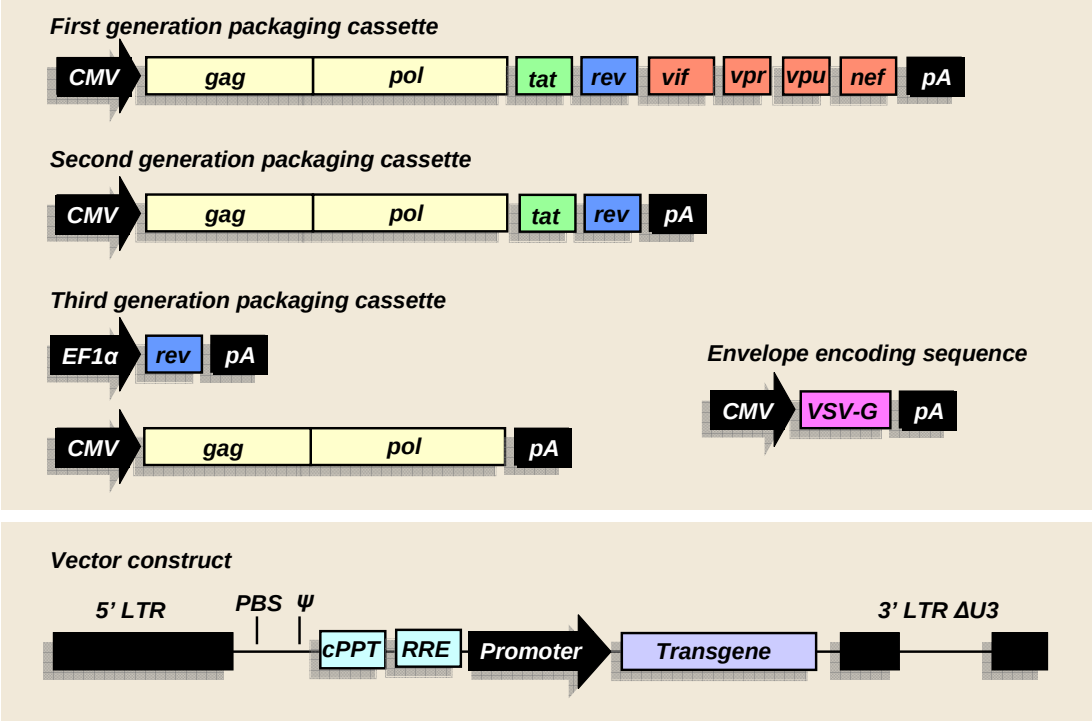


Figure 1.8: Progressive development of HIV-based lentiviral packaging cassettes

The first generation packaging cassette contained all the accessory genes, which were subsequently excluded in the second generation packaging cassette. The third generation packaging cassette divided the *gagpol* and *rev* genes between two separate plasmids, and dispensed with the *tat* gene altogether. A further development was the introduction of deletions in the U3 region of the 3' LTR in the vector construct resulting in a self-inactivating (SIN) vector, incapable of transcribing the full-length vector RNA. CMV: cytomegalovirus promoter, EF1α: elongation factor-1α promoter, pA: polyadenylation signal, VSV-G: vesicular stomatitis virus G protein, LTR: long terminal repeat, PBS: primer binding site, ψ: retroviral packaging signal, cPPT: central polypurine tract, RRE: Rev response element, ΔU3: deletion in U3 region of 3'LTR.

Nonhuman lentiviral vectors

A range of vectors based on nonhuman lentiviruses such as EIAV, BIV and FIV are being developed. The proposed rationale is that such vectors, derived from viruses which cannot replicate in human cells, pose fewer risks and might improve safety in clinical trials. However, there is little known about the risks involved in introducing nonhuman viruses into the human genome. Indeed, there is evidence that FIV and SIV full-length RNA can be cross-packaged by HIV-1 particles, raising the concern that such an approach may lead to the generation of a chimeric virus capable of infecting both animals and humans.[131] No lentivirus has been the subject of greater scrutiny than HIV-1, and HIV-based vectors retain the most rigorously tested safety profile of any lentiviral vectors yet developed.

Transduction of the corneal endothelium with lentiviral vectors

Many studies have demonstrated expression of transgenes by corneal endothelium following transduction with lentiviral vectors (Table 1.3). Long-term expression has been demonstrated in several species; reporter gene expression has been detected 10 months after anterior chamber injection of mice with an EIAV vector,[139] and endothelial cells on pieces of human cornea transduced with an HIV-based vector, have been reported to express a reporter gene for 60 days in organ culture.[140] A prolongation of corneal allograft survival has been demonstrated in three studies using lentiviral vectors;[80,82,83] recently, an HIV-based vector encoding the anti-apoptotic factor Bcl-xL improved corneal allograft survival in a murine model.[83]

Table 1.3: Transduction of corneal endothelium by lentiviral vectors

Species	Vector/Transgene	Transduction conditions	Transgene expression	Reference
Mouse	HIV-1-based CMV promoter eGFP	AC injection: 2 µl of 2×10^8 TU/ml	eGFP detected by 7 d Unchanged at 6 and 12 wk Not quantified	141
Mouse	FIV VSV-G CMV promoter β-Gal	Intravitreous injection: 2 µl containing 1×10^8 TU	β-Gal expression detected at 1 and 3 wk	142
Mouse	BIV-based eGFP	Intravitreous injection: $5x10^4$–$5x10^5$ TU	eGFP detected at post-mortem (20 wk)	143
Mouse	EIAV-based HIV-1-based eGFP		EIAV 95% transduction efficiency HIV-1 75% transduction efficiency	100
Mouse	EIAV-based CMV promoter Pseudotyped with either VSV-G or rabies-G eGFP	AC injection: 2 µl of $1.5x10^8$–$1.4x10^{10}$ TU/ml	EIAV-VSV-G: eGFP detected at 1 wk and maintained at 10 months; not quantified EIAV-rabies-G: undetectable	139
Mouse	HIV-based CMV promoter VSV-G eGFP, ZsGreen, bcl-xL	Corneas incubated for 18 h in Optisol® GS containing 6 µl/ml polybrene and 1×10^7 iu/ml lenti-IZsGreen or lenti-IZsGreen-xL, or 5.5×10^6 iu/ml lenti-eGFP	eGFP detected on day 3 post-transplantation and persisted for at least 56 d in syngeneic graft ZsGreen expressed in 15.8% at 1 wk and persisted at 8 wk	83
Rat	HIV-based VSV-G EF1α promoter eGFP	AC injection: 10 µl (10^7 pfu)	eGFP detected; not quantified No information on time course of expression	133
Rabbit	HIV-based pHR' vector CMV promoter E-Kr5 fusion protein	Whole buttons: 50 µl of 10^8 particles/ml Incubated for 18 h at 37°C Transplanted	E-Kr5 transcripts detected by RT-PCR at 30 d and 40 d	82

Continued below

Table 1.3 continued: Transduction of corneal endothelium by lentiviral vectors

Species	Vector/Transgene	Transduction conditions	Transgene expression	Reference
Human Rabbit Mouse	3 plasmid system EIAV envelope EIAV-based HIV-based CMV promoter lacZ, eGFP	Full thickness corneal segments: 5×10^5 particles for 3 h Murine corneal endothelial cell line: MOI 300 for 3 h Temperature not stated	lacZ: expression at 0.5 wk and 8 wk, in human and rabbit segments for both EIAV- and HIV-based vectors; maximum of 5–40% expression EIAV-eGFP: 83% at 7 d in murine cell line	144
Human	HIV-based pHR' vector 3 plasmid system VSV-G CMV promoter eGFP	Pieces of corneal tissue: 10^6–10^7 particles for 24 h at 37°C	eGFP detected at 3 d and still present at 60 d Not quantified	140
Human	3 plasmid system EIAV-based CMV promoter Pseudotyped with either VSV-G or rabies-G eGFP	Primary human cultured corneal endothelial cells: 12 h transduction with 5×10^8–1×10^9 TU/ml to achieve MOI of 100 in volume of 200 µl	eGFP expression in 30% cells after 2–3 d; still present at 12 d post-transduction	145

***Footnotes:

HIV human immunodeficiency virus, CMV cytomegalovirus, eGFP enhanced green fluorescent protein, AC anterior chamber, TU transducing units, d days, h hours, wk weeks, FIV feline immunodeficiency virus, β-Gal β-galactosidase, EIAV equine infectious anaemia virus, VSV-G vesicular stomatitis virus G, BIV bovine immunodeficiency virus, ZsGreen green fluorescent protein, IZsGreen ZsGreen in vector containing internal ribosomal entry site, iu infectious units, EF1α elongation factor-1α, pHR' first generation HIV-based vector (Naldini), E-Kr5 endostatin-kringle 5 fusion protein, lacZ β-galactosidase, MOI multiplicity of infection

Risk of insertional mutagenesis

Given that lentiviral vectors integrate into host cell chromosomes, like gammaretroviruses they possess the risk of insertional mutagenesis. However, unlike the clinical cases of *LMO2* proto-oncogene activation in the French SCID-X1 trial in which an oncogenic retrovirus was used,[146] to date there have been no known instances of HIV-1 activating oncogenes via proviral integration.[147] The use of SIN vectors reduces the likelihood of insertional mutagenesis by depriving the proviral vector of its parental promoter/enhancer sequences.[148] However, it should be noted that SIN vectors consequently rely on an internal promoter which itself may have trans-activating potential.[149] A promising development has been the design of nonintegrating lentiviral vectors (NILVs) which may circumvent the risk of insertional mutagenesis altogether by creating mutations in the viral integrase protein.[150] So far such vectors have shown similar transduction efficiencies *in vivo* to their integrating counterparts in the rat retina,[151] but further work is needed to determine residual rates of integration, and as far as I am aware, their capacity for stable expression in corneal endothelial cells is unknown.

The Anson lentiviral vector as a prospect for human transplantation

This project was concerned with the development of a safe, efficient and stable lentiviral vector for ultimate application in a clinical trial of gene therapy to modulate corneal allograft rejection. A range of lentiviral vector systems are privately and commercially available but not all of them have been developed with a view to clinical application. The lentiviral vector under study in this project was specifically selected on the basis of features facilitating clinical use. It is an HIV-1-based SIN vector which is pseudotyped with the VSV-G protein. It has been designed and

developed by Associate Professor D.S. Anson of the Department of Genetic Medicine, Women's and Children's Hospital, Adelaide, South Australia.[152] It contains none of the HIV accessory genes, but retains both the regulatory genes *rev* and *tat* (Figure 1.9). All sequences have been codon-optimised to enable the generation of high titres without increasing the risk of generating replication-competent viruses. Homology between constituent sequences has been minimised.

Like traditional third-generation lentiviral packaging cassettes, the Anson lentiviral system expresses all proteins from different constructs.[153] However, unlike other third-generation systems, the 5' HIV-1 LTR U3 sequence is not replaced with a constitutive promoter, but retains a third packaging plasmid coding for Tat. Whilst it has been argued that SIN vectors utilising a heterologous constitutive promoter in place of the HIV-1 U3 region are safer as they do not contain the sequences necessary to reconstitute a functional HIV-1 LTR, such vectors remain Tat-inducible.[137] In the interests of safety, the Anson SIN vector has been kept Tat-dependent, on the basis that generation of a functional replication-competent virus would require not only repair of the LTR, but also acquisition of the ability to express Tat.[154]

A further important aspect of a lentiviral vector with potential for clinical use is the ability to scale-up production whilst still achieving high titres of purified virus under biosafety conditions. The Anson approach utilises a hollow-fibre ultrafiltration system which is readily adapted to large-scale processing, and results in acceptable recoveries with rapid processing times. In combination with ultracentrifugation, the method allows multi-litre volumes of virus to be concentrated up to 1,000-fold with relative ease.[154]

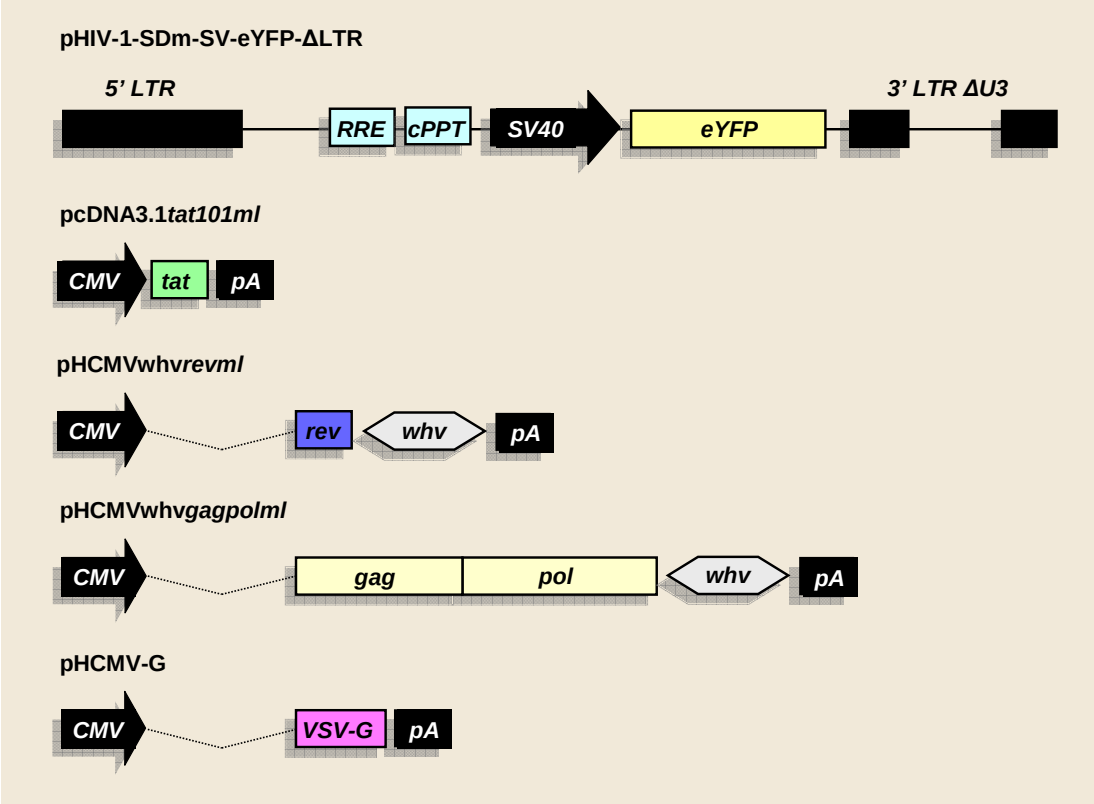


Figure 1.9: Anson lentiviral vector expression constructs

Schematic diagrams of the vector and four helper plasmids are shown. In the vector pHIV-1-SDm-SV-eYFP-ΔLTR, RRE represents the Rev response element, cPPT the central polypurine tract, SV40 the simian virus type-40 early promoter, and eYFP the enhanced yellow fluorescent protein coding sequence. For pcDNA3.1*tat101ml*, CMV represents the simian cytomegalovirus immediate early promoter. The *tat101ml* coding sequence is followed by the bovine growth hormone gene polyadenylation signal. For the pHCMV constructs, hCMV represents the human cytomegalovirus immediate early promoter, the dashed lines splicing signals from the rabbit β-globin gene, whv the woodchuck hepatitis virus post-transcriptional regulatory element followed by the rabbit β-globin polyadenylation signal, and VSV-G the vesicular stomatitis virus G protein coding sequence, followed by the rabbit β-globin polyadenylation signal. [Adapted from Koldej et al. (2005)[154]]

1.7 Summary and plan for the thesis

Selection of the Anson lentiviral vector and the ovine preclinical model

There is now an impetus to develop gene therapy vectors which meet the demands of a clinical trial for human corneal transplantation. Of the viral vectors currently available, adenoviral vectors remain the most efficient but rAAV vectors and lentiviral vectors appear to have the greatest potential for long-term transgene expression (Table 1.4).

Table 1.4: Features of viral vectors for gene transfer to corneal endothelium

Feature	Adenovirus	rAAV	Lentivirus
Genome	dsDNA	ssDNA	ssRNA
Genome size (kb)	35	4.7	7-11
Transgene capacity (kb)	~30	~5	~9
Integration into chromosomes	No, remains episomal	Yes, but inefficient	Yes
Transduction efficiency in human corneal endothelium	Consistently high	Reports vary from very low to very high	Reports vary from low to moderate
Stability of expression[†]	Poor	Potentially high	High
Immunogenicity	May be high	Moderate to low	Low
Biosafety issues	Systemic toxicity	Theoretical risk of insertional mutagenesis	Theoretical risk of insertional mutagenesis

***Footnotes: rAAV recombinant adeno-associated virus vector, ds double-stranded, ss single-stranded, [†] also dependent on internal promoter

Lentiviral vectors have been shown to produce stable expression in human corneal endothelium[140,144] and have low immunogenicity in the eye.[132,133] The Anson lentiviral vector possesses safety and production features which make it a realistic prospect for use in clinical trials of gene therapy to prevent corneal allograft rejection. In the existing range of established preclinical models of corneal allograft rejection, the outbred sheep has advantages, primarily its high-risk nature and amitotic endothelium. For these reasons, I investigated the ability of the Anson lentiviral vector to deliver transgenes to the ovine and human corneal endothelium, and evaluated the vector's potential for use in human corneal transplantation.

The choice of a reporter gene and interleukin-10 as transgenes

The focus of the project was to characterise the ability of the HIV-1-based Anson lentiviral vector to achieve transgene expression in ovine and human corneal endothelium, not to explore the merits of a novel therapeutic transgene. To this end, a vector encoding the enhanced yellow fluorescent protein (eYFP) was chosen to allow qualitative and quantitative assessment of transduction rates and expression stability. However, since many of the potential therapeutic transgenes for use in corneal transplantation are secreted proteins, it was decided to investigate the expression of one of these also. Given that the immunomodulatory cytokine IL10 has been shown to prolong corneal graft survival in the outbred sheep following adenovirus-mediated gene transfer, it was chosen as a secreted protein with which to characterise the performance of the lentiviral vector both *in vitro* and *in vivo*.

Comparison of lentiviral vectors with different constitutive promoters

Many of the early studies using retroviral vectors were conducted using the simian virus type-40 (SV40) early promoter, whilst the elongation factor-1α promoter (EF1)

and phosphoglycerate kinase (PGK) promoters are constitutive mammalian promoters which have also been widely used. The human CMV immediate early promoter is known to be a strong viral promoter and has been shown to drive robust and stable expression in corneal endothelium of several different species, including sheep and human. Therefore, performance of vectors encoding these different internal promoters was assessed in both the ovine and the human corneal endothelium, to determine which promoters held most promise for use in the preclinical ovine model and for ultimate clinical application in humans.

Regulation of lentiviral-mediated expression by a steroid-inducible promoter

Regulation of transgene expression by an external agent such as an eye drop is a highly desirable attribute of a vector for the modulation of corneal allograft rejection. Topical corticosteroids are routine prophylaxis and treatment for corneal allograft rejection.[155] Therefore, in addition to assessing constitutive promoters, I investigated the incorporation of a steroid-inducible promoter based on five glucocorticoid response elements (GRE5) upstream of the adenovirus 2 major late promoter TATA box (Figure 1.10). This promoter construct has been shown to drive marked glucocorticoid-induced upregulation of transgene expression in a number of systems both *in vitro* and *in vivo.*[156-159]

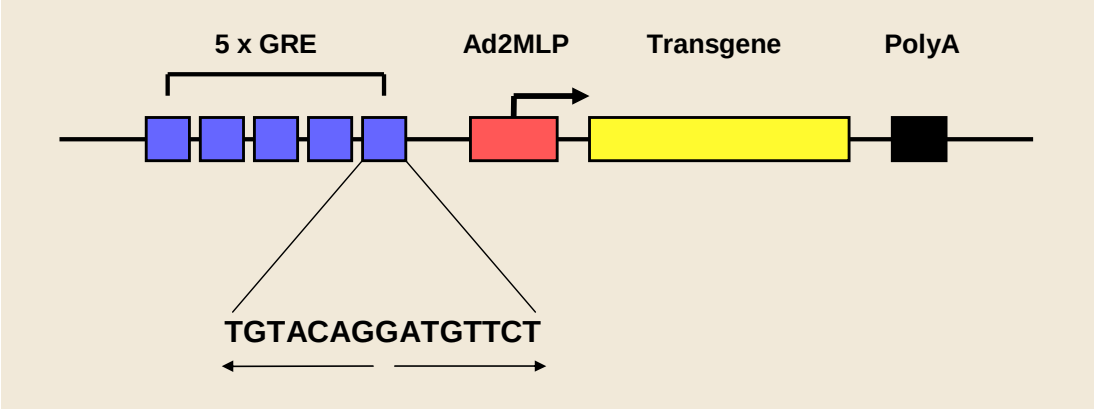


Figure 1.10: Schematic representation of the GRE5 steroid-inducible promoter

The chimeric promoter represents five glucocorticoid response elements (GREs) from the rat tyrosine aminotransferase gene, inserted upstream of the adenovirus 2 major late promoter (Ad2MLP).

Analysis of parameters influencing lentivirus-mediated expression

In addition to the influence of the internal promoter on lentivirus-mediated transgene expression, a range of other factors were also assessed, including: the duration of the transduction period (the time during which the concentrated virus is incubated in close proximity with the corneal endothelium), the multiplicity of infection (MOI), and use of the polycation polybrene, which is commonly used to improve retroviral transduction rates in tissue culture. Stability of expression was examined both *in vitro* and *in vivo* in ovine corneal endothelium, and in human corneas *in vitro*. Given the extensive use of adenoviral vectors to transduce corneal endothelium and their success in mediating a prolongation of corneal allograft survival in sheep, adenoviral vectors were used as a benchmark for the performance of the lentiviral vectors.

In vivo trial of lentivirus-mediated expression of interleukin-10

Finally, the potential therapeutic effect of the Anson lentiviral vector was investigated by conducting a trial of lentivirus-driven IL10 expression in the ovine

model of corneal allograft rejection. Studies were also conducted to determine the viability of ovine donor corneas following periods in organ culture.

1.8 Aims of the thesis

1.8.1 Overarching aim

To study the ability of the HIV-1-based Anson lentiviral vector to achieve efficient and stable gene expression in corneal endothelium, thereby evaluating its potential for use in human corneal transplantation.

1.8.2 Specific aims

In vitro

- To construct lentiviral vectors encoding a reporter gene, and the model secreted protein IL10, under the control of different internal promoters;
- To characterise the kinetics and stability of lentivirus-mediated expression of a reporter gene and IL10, in ovine and human corneal endothelium;
- To investigate the influence of transduction period duration, multiplicity of infection, and the use of polybrene, on lentivirus-mediated transgene expression in ovine and human corneal endothelium;

In vivo

- To investigate the stability of lentivirus-mediated transgene expression, and the effects of organ culture and transfer of IL10, in the ovine model of corneal transplantation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

General chemicals

Unless otherwise stated, all chemicals were of analytical reagent grade, and obtained from: Sigma (St Louis, MO, USA), AJAX chemicals (Auburn, NSW), or BDH chemicals (Kilsyth, VIC). All water used was double glass distilled (DDH_2O), unless otherwise specified. Recipes for buffers and solutions are in Section 2.2.

Molecular biology reagents and PCR primers

Reagents used in molecular biology are listed in Table 2.1. Primers used in endpoint polymerase chain reaction (PCR) and real-time PCR were synthesised by GeneWorks (Thebarton, SA) at sequencing-grade purity. These are listed in Table 2.2 and Table 2.3 respectively.

Materials used in bacterial cell culture

Bacterial culture plates were purchased from Techno-Plas (St. Mary's, SA). Antibiotics used in bacterial cell culture were: ampicillin (50 mg / ml; Boehringer Mannheim, Germany) and kanamycin (10 mg / ml; Sigma, St Louis, MO, USA), which were prepared in sterile water, and chloramphenicol (Sigma, St Louis, MO, USA) which was prepared in ethanol (35 mg / ml). Working concentrations of antibiotics were 100 µg / ml for ampicillin, 10 mg / ml for kanamycin, and 35 µg / ml for chloramphenicol. All stocks were stored at 4°C until use. Bacterial cell lines and plasmids used during the course of the project are listed in Table 2.4 and Table 2.5 respectively.

Table 2.1: Molecular biology reagents

Reagent	Manufacturer
Ethidium bromide (10 mg / ml)	Sigma (St Louis, MO, USA)
6× Loading dye	Promega (Madison, WI, USA)
20 bp DNA ladder (20 bp–1 kb)	GeneWorks (Thebarton, SA)
2 log DNA ladder (100 bp–10 kb)	New England Biolabs (Beverly, MA, USA)
Restriction endonucleases	New England Biolabs (Beverly, MA, USA)
Restriction endonuclease reaction buffers (NEBuffers 1,2,3,4)	New England Biolabs (Beverly, MA, USA)
One-Phor-All® restriction endonuclease buffer	Amersham Biosciences Corporation, (Piscataway, NJ, USA)
T4 DNA Ligase (400,000 cohesive end units / ml)	New England Biolabs (Beverly, MA, USA)
10× T4 DNA Ligase Reaction Buffer (containing 10 mM ATP)	New England Biolabs (Beverly, MA, USA)
Elongase®	Invitrogen (Carlsbad, CA, USA)
Platinum® Taq DNA polymerase	Invitrogen (Carlsbad, CA, USA)
10× PCR buffer (specific for either Platinum® Taq or Elongase®)	Invitrogen (Carlsbad, CA, USA)
dNTPs (10 mM for each: dATP, dCTP, dGTP and dTTP)	Amersham Biosciences Corporation, (Piscataway, NJ, USA)
DNA-*free*™ DNase I	Ambion (Austin, TX, USA)
SuperScript III® First-Strand Synthesis System	Invitrogen (Carlsbad, CA, USA)
QuantiTect™ SYBR Green Master Mix	Qiagen (Valencia, CA, USA)

Table 2.2: Endpoint PCR primer sequences

Target gene/application	Primer	Length (bp)	Sequence	T_m (°C)	Amplicon Size (bp)
Confirmation of IL10 sequence by PCR and sequencing	IL10for	20	5' GCA GCT GTA CCC ACT TCC CA 3'	56	300
	IL10rev	17	5' AGA AAA CGA TGA CAG CG 3'	44	
Amplification of IL10 sequence from pRC-CMV-IL10, introducing a 5' *Kpn*I site and 3' his6-tag and *Hind*III restriction site	IL10kpnfor	36	5' CGC AGG TAC CTC CGC CAT GCC CAG CAG CTC AGC CGT 3'	75	580
	IL10hindrev	55	5' ATC GCA AGC TTA GAT TAG TGG TGG TGG TGG TGG TGC ATC TTC GTT GTC ATG TAG G 3'	73	
Amplification of GRE5 promoter sequence from pGRE5-luc, introducing a 5' *Bam*HI site and 3' *Kpn*I site	GREbamfor	20	5' CGC GGA TCC TTA ATT CGG GG 3'	56	320
	GREkpnrev	24	5' CCG GTA CCT GGC CCT CGC AGA CAG 3'	66	
Amplification of CMV sequence with 5' *Bam*HI site and 3' *Cla*I site	CMVbamfor	27	5' CGC GGA TCC AAT AGT AAT CAA TTA CGG 3'	58	580
	CMVclarev	26	5' CCA TCG ATG GAT CTG ACG GTT CAC TA 3'	59	

***Footnotes: PCR polymerase chain reaction, bp base pairs, T_m melting temperature, IL10 interleukin-10, for forward, rev reverse, his6-tag six histidine residues, GRE5 five glucocorticoid response elements, CMV cytomegalovirus promoter

Table 2.3: Real-time PCR primer and probe sequences

Gene (Accession Number†)	Primer/Probe	Length (bp)	Sequence	T_m (°C)	Amplicon Size (bp)
Ovine IL10	IL10RTfor	20	5’ CAA GCC TTG TCG GAA ATG AT 3’	60	89
(U11421)	IL10RTrev	20	5’ TTC ACG TGC TCC TTG ATG TC 3’	60	
Ovine β-actin	oBAfor	19	5’ CAC CCA GCA CGA TGA AGA T 3’	60	105
(U39357)	oBArev	20	5’ ACA TCT GCT GGA AGG TGG AC 3’	60	
Ovine GAPDH	oGAP1for	20	5’ ATC AAT GGA AAG GCC ATC AC 3’	60	81
(AF030943)	oGAP1rev	20	5’ CAC GTA CTC AGC ACC AGC AT 3’	60	
HIV-1 *gag*	Forward	23	5' AGC TAG AAC GAT TCG CAG TTG AT 3'	53	
	Reverse	25	5' CCA GTA TTT GTC TAC AGC CTT CTG A 3'	56	65
	Probe (FAM/NFQ)	17	5' CCT GGC CTG TTA GAA AC 3'	47	
Mouse transferrin	Forward	24	5' AAG CAG CCA AAT TAG CAT GTT GAC 3’	54	
	Reverse	25	5' GGT CTG ATT CTC TGT TTA GCT GAC A 3’	56	76
	Probe (FAM/NFQ)	15	5' CTG GCC TGA GCT CCT 3’	47	

***Footnotes: PCR polymerase chain reaction, † NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez), bp base pairs, T_m melting temperature, IL10 interleukin-10, for forward, rev reverse, GAPDH glyceraldehyde 3-phosphate dehydrogenase, HIV human immunodeficiency virus

Table 2.4: Bacterial strains of *E. Coli*

Strain of *E. coli*	Genotype	Details/Application
BJ5183	endA sbcBC recBC galK met thi-1 bioT hsdR (Strr)	High levels of homologous recombination; intermediate transformation efficiency; used for cotransformation of adenoviral backbone with shuttle vector in production of adenoviral vectors.
DH5α	SupE44 ΔlacU169 Φ80 lacZΔM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Recombination-deficient suppressing strain used for amplification of recombinant plasmids.
DH10β	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG	Recombination-deficient suppressing strain; allows efficient cloning of both prokaryotic and eukaryotic genomic DNA and efficient plasmid rescue from eukaryotic genomes; used for amplification of recombinant plasmids.

***Footnotes: *E. coli* Escherichia coli

Table 2.5: Plasmids

Designation	Description	Sequence of interest	Resistance	Source
pRC-CMV-IL10	Mammalian expression vector containing the ovine IL10 gene sequence driven by a CMV promoter, followed by a polyadenylation site	Ovine IL10 gene	Ampicillin	pRC-CMV from Promega (Madison, WI, USA); ovine IL10 from Professor M. Pericaudet, Institut Gustave Roussy, Villejuif, France
pAdEasy-1 (pAdEasy)	E1-, E3-deleted adenovirus serotype-5 backbone	Adenoviral sequences	Ampicillin	Professor B. Vogelstein, Johns Hopkins University, Baltimore, MD, USA
pAdTrack-CMV	Shuttle vector with polylinker under control of CMV promoter, and co-expressing eGFP on separate CMV promoter	CMV promoter, eGFP gene	Kanamycin	Professor B. Vogelstein, Johns Hopkins University, Baltimore, MD, USA
pGRE5-luc	Plasmid containing five glucocorticoid response elements upstream of the adenovirus major late promoter TATA box, driving expression of luciferase	GRE5 promoter region	Ampicillin	Professor J. White, McGill University, Montreal Canada
pHIV-1-SDm-SV-eYFP-ΔLTR	HIV-1 lentiviral vector plasmid	SV40 early promoter eYFP gene	Chloramphenicol	A/Professor D. Anson, Women's and Children's Hospital, Adelaide, SA
pcDNA3.1*tat101ml*	Helper plasmid encoding HIV-1 Tat	HIV-1 Tat	Chloramphenicol	A/Professor D. Anson
pHCMVwhv*revml*	Helper plasmid encoding HIV-1 Rev	HIV-1 Rev	Chloramphenicol	A/Professor D. Anson
pHCMVwhv*gagpolml*	Helper plasmid encoding HIV-1 GagPol	HIV-1 GagPol	Chloramphenicol	A/Professor D. Anson
pHCMV-G	Helper plasmid encoding VSV-G protein	VSV-G protein	Chloramphenicol	A/Professor D. Anson

Continued below

Table 2.5 continued

Designation	Description	Sequence of interest	Resistance	Source
pHIV-1-SDm-PGK-eYFP-ΔLTR	HIV-1 lentiviral vector plasmid	PGK promoter eYFP gene	Chloramphenicol	A/Professor D. Anson
pHIV-1-SDm-EF-eYFP-ΔLTR	HIV-1 lentiviral vector plasmid	EF promoter eYFP gene	Chloramphenicol	A/Professor D. Anson
pHIV-1-SDm-SV-empty-ΔLTR	HIV-1 lentiviral vector plasmid	SV40 early promoter	Chloramphenicol	A/Professor D. Anson
pHIV-1-SDm-CMV-eYFP-ΔLTR	HIV-1 lentiviral vector plasmid	CMV promoter eYFP gene	Chloramphenicol	CMV promoter cloned into pHIV-1-SDm-SV-eYFP-ΔLTR
pHIV-1-SDm-SV-IL10-ΔLTR	HIV-1 lentiviral vector plasmid	SV40 early promoter Ovine IL10 gene	Chloramphenicol	Ovine IL10 gene cloned into pHIV-1-SDm-SV-empty-ΔLTR
pHIV-1-SDm-GRE-IL10-ΔLTR	HIV-1 lentiviral vector plasmid	GRE5 promoter Ovine IL10 gene	Chloramphenicol	GRE5 promoter cloned into pHIV-1-SDm-SV-IL10-ΔLTR

***Footnotes: CMV cytomegalovirus, IL10 interleukin-10, eGFP enhanced green fluorescent protein, GRE5 five glucocorticoid response elements, SV40 simian virus type-40, eYFP enhanced yellow fluorescent protein, VSV vesicular stomatitis virus, PGK phosphoglycerate kinase, EF elongation factor-1α

Materials used in mammalian cell culture

Tissue culture flasks, disposable pipettes and disposable graduated tubes were obtained from Nunclon (Copenhagen, Denmark), or Falcon (Franklin Lakes, NJ, USA). Powdered media for mammalian cell culture were obtained from Thermo Electron (Melbourne, VIC) and GIBCO BRL (Gaithersburg, MD, USA); details are provided in Section 2.2. All media were prepared in endotoxin-low glassware treated with E-Toxa-Clean™ according to the manufacturer's instructions (Sigma, St Louis, MO, USA), and 0.22 µm filter-sterilised prior to use. The HEPES-buffered RPMI medium 1640 and bicarbonate-buffered DMEM medium, will hereafter be referred to as RPMI and DMEM respectively. Unless otherwise stated, tissue culture media were supplemented with penicillin (100 IU / ml), streptomycin (100 µg / ml) and L-glutamine (2 mM) (all from GIBCO BRL, Gaithersburg, MD, USA). These were replenished every two weeks. Foetal calf serum (FCS; GIBCO BRL, Gaithersburg, MD, USA) was heat-inactivated at 56°C for 30 min and stored at -20°C. Media were supplemented with 2–10% v/v FCS. A medium will be referred to by the % v/v of FCS present followed by its name (e.g. 10% RPMI refers to HEPES-buffered RPMI 1640 containing 10 % v/v FCS and supplemented with penicillin (100 IU / ml), streptomycin (100 µg / ml) and L-glutamine (2 mM)). Mammalian cell lines used during the course of the project are listed in Table 2.6.

Miscellaneous reagents

These are listed in Table 2.7.

Table 2.6: Mammalian cell lines

Designation	Characteristics	Applications	Catalogue Number/Source
HEK-293A	Human embryonic kidney cells; adherent; expressing adenovirus serotype-5 E1 sequence	Production of adenovirus serotype-5 vector	ATCC CRL-1573 Obtained from: QBiogene Inc (Carlsbad, CA, USA)
HEK-293T	Human embryonic kidney cells; adherent; highly susceptible to transfection	Production of lentiviral vector; testing lentiviral vector stocks for replication competence	ATCC SD3515
A549	Human alveolar epithelial cells; adherent; high transduction efficiency for lentivirus	Titre determination of lentiviral vector stocks expressing reporter gene	ATCC CCL-185
NIH3T3	Mouse fibroblasts; adherent; high transduction efficiency for lentivirus	Titre determination of lentiviral vector stocks not expressing reporter gene	ATCC CRL-1658

***Footnotes: HEK human embryonic kidney, ATCC American Type Culture Collection (Manassas, VA, USA)

Table 2.7: Miscellaneous reagents

Reagent	Description	Source
Water for irrigation	Sterile, nonpyrogenic	Baxter (Old Toongabbie, NSW)
Sodium chloride for irrigation	0.9% w/v isotonic, nonpyrogenic, sterile	Baxter (Old Toongabbie, NSW)
Glycogen	20 mg / ml in distilled water	Roche (Mannheim, Germany)
Tween-20	Polyethylene sorbitan monolaurate	Sigma (St Louis, MO, USA)
Streptavidin-HRP	Purified strepavidin horseradish peroxidise conjugate (0.7 g / L)	DakoCytomation (Glostrup, Denmark)
Dextran T500	Used as an osmotic agent in corneal organ culture	Pharmacia Bitoech AB (Uppsala, Sweden)
Hoechst-33258 dye	Nuclear stain	Sigma (St Louis, MO, USA)
2-Mercaptoethanol	β-mercaptoethanol ≥98% (M-7154)	Sigma (St Louis, MO, USA)
Polybrene	Hexadimethrine bromide ≥94% (52495)	Sigma (St Louis, MO, USA)
DEPC	Diethylpyrocarbonate ≥97% (D-5758)	Sigma (St Louis, MO, USA)
Dexamethasone sodium phosphate	8 mg / 2 ml Used as an induction agent for GRE5 promoter	Mayne Pharma (Mulgrave, VIC)

Sheep

All experiments involving sheep were approved by the Animal Welfare Committee of Flinders University of South Australia. Outbred adult Merino-cross ewes and wethers were obtained from various properties in South Australia. Animals weighed between 40 and 60 kg and were housed in individual crates in groups of two to four. They had unlimited access to lucerne chaff and water, and were housed so that at

least one other sheep was visible at all times. Only the right eye of any animal was operated on. Animals were acclimatised for one week prior to surgery. Materials used in orthotopic corneal transplantation are listed in Table 2.8.

Ovine eyes

Whole ovine eyes were obtained from a local abattoir (Normanville Meatworks, Normanville, SA) within 1 h of slaughter and had not been heat-treated in any way.

Table 2.8: Materials used in orthotopic corneal transplantation

Material	Manufacturer
1% Atropine sulphate (Minims™)	Chauvin Pharmaceuticals Ltd. (Kingston-Upon-Thames, England, UK)
1% Tropicamide (Mydriacyl™)	Alcon Laboratories (Frenchs Forest, NSW)
1% Cyclopentolate hydrochloride (Minims™)	Chauvin Pharmaceuticals Ltd. (Kingston-Upon-Thames, England, UK)
10% Phenylephrine hydrochloride (Minims™)	Chauvin Pharmaceuticals Ltd. (Kingston-Upon-Thames, England, UK)
Chloramphenicol ointment (10 mg chloramphenicol per g) (Chloromycetin™)	Pfizer Australia Pty. Ltd. (West Ryde, NSW)
0.5% Chloramphenicol drops (5 mg / ml) (Chlorsig™)	Sigma Pharmaceuticals (Clayton, VIC)
Balanced salt solution	Cytosol Laboratories (Braintree, MA, USA)
Methylprednisolone acetate (40 mg / ml) (Depo-Medrol™)	Pharmacia (Kalamazoo, MI, USA)
Corneal trephines (11 mm and 12 mm)	Medtronic Ophthalmics (Jacksonville, FL, USA); Tecfen Corporation (Santa Barbara, CA, USA)
4.0 braided silk suture	Syneture (Norwalk, CT, USA)
9.0 monofilament nylon suture	Alcon Laboratories (Fort Worth, TX, USA)

2.2 Buffers and Solutions

Buffered formalin

Compound	*Amount*
Formalin (40% w/v)	50 ml
$NaH_2PO_4.H_2O$	2 g
Na_2HPO_4	3.25 g
DDH_2O	made up to 500 ml

0.1% v/v Diethylpyrocarbonate (DEPC)-treated water/saline

Compound	*Amount*
DEPC	1 ml
DDH_2O or 0.9% w/v NaCl	1000 ml

Placed at 37°C overnight, autoclaved to inactivate DEPC.

DMEM Medium

Compound	*Amount*
Powdered medium	1 sachet (MultiCel™ Cat No: 50-125-PA; Thermo Electron, Melbourne, VIC)
$NaHCO_3$	3.7 g
D-glucose (anhydrous)	3 g
Sterile, nonpyrogenic H_2O	made up to 1000 ml

Adjusted pH to 7.2, filter sterilised through a 0.22 μm filter, stored at 4°C.

RPMI Medium 1640 (with 25 mM HEPES buffer)

Compound	*Amount*
Powdered medium	1 sachet (GIBCO™ Cat No: 23400-021; Invitrogen, Gaithersburg, MD, USA)
$NaHCO_3$	2 g
Sterile, nonpyrogenic H_2O	1000 ml

Adjusted pH to 7.2, filter sterilised through a 0.22 μm filter, stored at 4°C.

2× HEPES-buffered saline (HeBS)

Compound	*Amount*
NaCl	1.6 g
Na_2HPO_4	0.021 g
HEPES	1.2 g
Sterile, nonpyrogenic H_2O	made up to 100 ml

Adjusted to pH 7.4, filter-sterilised through a 0.22 μm filter, stored at 4°C.

Luria Bertani (LB) bacterial medium

Compound	*Amount*
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
DDH_2O	made up to 1000 ml

Adjusted to pH 7.0, autoclaved.
To make LB agar plates, 15 g / L agar was added.
To make low salt LB, the quantity of NaCl added was halved.

Phosphate-buffered saline (PBS) 10× stock

Compound	*Amount*
$Na_2HPO_4.2H_2O$	28.55 g
$NaH_2PO_4.2H_2O$	6.25 g
NaCl	70 g
DDH_2O or sterile, nonpyrogenic H_2O	made up to 1000 ml

Adjusted to pH 7.4, autoclaved.

SOC medium

Compound	*Amount*
Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
0.25M KCl	10 ml
NaOH	100 µl
DDH_2O	made up to 1000 ml

Autoclaved before addition of sterile $MgSO_4$ and glucose:

2M $MgSO_4$	5 ml
1M glucose	20 ml

Tris borate EDTA (TBE) 5× stock

Compound	*Amount*
Trizma base	54 g
Boric acid	27.5 g
0.5M EDTA	20 ml
DDH_2O	made up to 1000 ml

Used at 0.5× for agarose gel electrophoresis.

Trypsin-EDTA

Compound	*Amount*
Trypsin powder (1:250)	0.5 g
$EDTA.Na_4.4H_2O$	0.2 g
10× PBS	100 ml
Sterile, nonpyrogenic H_2O	made up to 1000 ml

Filter-sterilised through a 0.22 µm filter, and stored at –20°C in 20 ml aliquots.

2.3 Methods

2.3.1 Molecular biology

Agarose gel electrophoresis

Products of plasmid preparations, digests, ligations, polymerase chain reaction (PCR) and RNA extraction were analysed by agarose gel electrophoresis. Gels were prepared by adding 0.8–2% w/v agarose (Promega, Madison, WI, USA) to 100 ml of 0.5× TBE buffer, with the addition of 5 µl ethidium bromide (10 mg / ml) (Sigma, St Louis, MO, USA). Electrophoresis was performed in 0.5× TBE buffer. Samples were loaded in 6× loading dye (Promega, Madison, WI, USA). Gels were examined and photographed under ultraviolet (UV) light using a commercially available gel documentation and analysis system (GeneSnap, Syngene; Synoptics Ltd., Frederick, MD, USA). The size of products was determined by comparison with DNA ladders: 2 log ladder (New England Biolabs, Beverly, MA, USA) and 20 bp ladder (GeneWorks, Thebarton, SA).

Plasmid preparation

Plasmid DNA was generated in *E. coli* bacteria (Table 2.5), and purified using commercially available plasmid purification kits, according to the supplied protocols (Mini-, Midi-, Maxi- and Mega Kits; Qiagen, Valencia, CA, USA). For some applications, such as purification of transfection grade vector plasmid DNA for virus production, endotoxin-low kits were used (EndoFree Maxi- and Mega Kits; Qiagen, Valencia, CA, USA). Quantification of purified plasmid DNA was carried out using spectrophotometry. Absorbance at 260 nm was used to determine concentration, and the 260:280 nm ratio was used to determine purity. Preparations of DNA at low

concentrations were quantified using ethidium bromide plates. In these cases, 1 µl of each sample at various dilutions was dispensed onto an agarose gel plate, containing 1% w/v agarose in DDH_2O and 0.005% v/v ethidium bromide (10 mg / ml). Each plate included a series of DNA standards, using equal volumes of serial dilutions of herring sperm DNA (Sigma, St Louis, MO, USA). Samples were quantified by visual comparison with standards under UV light.

Preparation of electrocompetent bacteria

A single colony was picked from a freshly-streaked plate of DH5-α or DH10-β *E. coli* bacteria, used to inoculate 20 ml of Luria Bertani (LB) medium, and incubated at 37°C for 16 h. This culture was then added to 1,000 ml of low salt LB medium and incubated until an optical density (OD) at 600 nm of 0.6–0.8 was attained. Cells were then repeatedly pelleted and washed in chilled sterile water by three successive centrifugations at 2,000 g for 15 min at 2°C. Following the third centrifugation, pelleted cells were resuspended in 10 ml of 10% v/v glycerol, divided into 60 µl aliquots, snap-frozen in liquid nitrogen and stored at -80°C.

Restriction enzyme digestion

Restriction enzyme digests were carried out in 20–125 µl volumes, containing the appropriate buffer (NEBuffer 1, 2, 3 or 4, New England Biolabs, Beverly, MA, USA; or One-Phor-All®, Amersham Biosciences Corporation, Piscataway, NJ, USA), restriction enzyme at 10 U / µg of DNA (New England Biolabs, Beverly, MA, USA), the DNA itself, and distilled water. Reactions were carried out at 37°C for 60 min. In vector digests, shrimp alkaline phosphatase (USB, Cleveland, OH, USA) was added to the reaction mixture for 30 min, to prevent self-religation of the cut vector. The reaction was terminated by incubation at 65°C for 15 min. Purification of the

digested DNA was carried out by gel extraction (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA, USA) or a QIAquick spin column, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Agarose gel electrophoresis was performed to confirm digestion of the vector DNA.

Cloning methods

In general, cloning of insert DNA sequences into plasmid shuttle vectors was carried out as follows. A double-digestion was performed on the vector using the appropriate restriction enzymes, and the insert DNA was amplified by PCR, using the proof-reading enzyme Elongase® (Invitrogen, Carlsbad, CA, USA) and primers incorporating the respective restriction sites. Both double-cut vector and amplified insert were purified and quantified following gel extraction (QIAquick Gel Extraction Kit; Qiagen, Valencia, CA, USA). The insert DNA was subjected to double-digestion using the specific enzymes to create sticky ends, followed by purification and quantification. Ligation reactions were then performed.

Ligation reactions

Each ligation reaction contained: 2 µl of 10× ligation buffer, 1 µl T4 DNA ligase (New England Biolabs, Beverly, MA, USA), and vector DNA and insert DNA (at either a 1:1 or 1:3 vector:insert molar ratio), made up to a total volume of 20 µl with DDH_2O. Reactions were placed at 4°C overnight in 0.5 ml tubes.

Cleanup of ligation reactions by ethanol precipitation

The volume of each ligation reaction mixture was made up to 200 µl with DDH_2O. Then 1 µl glycogen (10 mg / ml in water) and 20 µl of 3 M sodium acetate, pH 5.2, were added. Next, 2.5× volumes of ice-cold ethanol were added and the mixture

placed at -20°C for 30 min to allow the DNA to precipitate. The mixture was then centrifuged in a microcentrifuge at 10,000 g for 15 min at 4°C. The supernatant was removed, being careful not to disturb the pellet, which was then rinsed with 1 ml of 70% ethanol. A 5 min centrifugation was carried out at 10,000 g at 4°C. This step was repeated. Finally as much supernatant as possible was removed without disturbing the pellet. The tube was placed in a SpeediVac high vacuum centrifuge (Edwards High Vacuum Ltd., Crawley, England, UK) for 5 min to ensure drying before being redissolved in 10 µl of DDH_2O.

Electroporation

One to 2 µl of plasmid DNA or cleaned-up ligation mixture was added to 60 µl aliquots of electro-competent DH5-α or DH10-β *E. coli*, mixed and incubated on ice for 5 min. Cells were then transferred to electroporation cuvettes (1 mm gap), electroporated using an electroporator (Gene Pulser II; BioRad, Hercules, CA, USA), set at 1.8 kV, 25 µF and 200 Ohms, and then added to 1 ml of SOC medium. Following incubation at 37°C for 1 h, cells were pelleted, resuspended in 200 µl and plated on LB agar plates supplemented with the appropriate antibiotic. Plates were incubated at 37°C for 16 h.

Endpoint PCR

Individual endpoint PCR was carried out in a total volume of 25 µl per tube, containing: 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$, 100 µM dNTPs, 0.5 U Platinum® Taq (Invitrogen, Carlsbad, CA,USA), 0.4 µM forward and reverse primers, DDH_2O, and 2 µl DNA template. A bulk reaction mixture was made up excluding the DNA template, which was then added to individual reactions. All reactions were carried out in duplicate. Positive controls containing a DNA sequence

of interest, and negative controls containing 2 μl of DDH_2O substituted for the DNA template, were included in all assays. The PCR was performed in the OmniGene Thermal Cycler (Hybaid Ltd, Middlesex, England, UK). Parameters of PCR varied depending on the application. Typical parameters were: (i) an initial denaturation stage at 94°C for 5 min; (ii) 20–40 cycles of three steps: a denaturation step at 94°C for 30 s, an annealing step dependent on the melting temperature of primers, for 30 s, and an extension step at 72°C for 1 min; and (iii) a final stage at 72°C for 4 min. Products of PCR were analysed by electrophoresis using 0.8–2% w/v agarose in 0.5× TBE buffer (depending on the expected size of the product).

2.3.2 Tissue Culture

Propagation of cell lines

The adherent cell lines, HEK-293A, HEK-293T, A549, and NIH3T3 were propagated in 5–10% DMEM. On reaching confluence, cells were split by washing once with 1× PBS and incubation with 10% v/v trypsin-EDTA in PBS (undiluted trypsin-EDTA was used for A549 cells) at 37°C for 5 min. After detachment, cells were washed once in growth medium, centrifuged at 800 g for 5 min, and resuspended in growth medium for replating. Cells were replated at 1:10 to 1:4 depending on the incubation time until the next split.

TNF-α synthesis inhibition assay to confirm function of transgenic IL10

An assay was kindly carried out by Professor Prue Hart and Dr Cecilia Prele at the Telethon Institute for Child Health Research, Subiaco, WA, to confirm the function of secreted transgenic ovine interleukin-10 (IL10) in supernatants from transduced cells. Primary human monocytes were isolated from peripheral blood using Lymphoprep (Axis-Shield, Oslo, Norway) and enriched by centrifugal elutriation.

Cells were cultured at 0.5×10^6 cells per 500 µl culture in 48-well plates, and stimulated by the addition of lipopolysaccharide (LPS; Sigma, St Louis, MO, USA) (500 ng / ml). Experimental supernatants were added at a dilution of 1:5. At 24 h after stimulation, monocyte culture supernatants were assayed for the concentration of tumour necrosis factor-α (TNF-α), using a commercially available ELISA kit (BD Biosciences, Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. All samples were assayed in duplicate. Controls comprised untreated cells (negative controls), and cells cotreated with LPS and recombinant human IL10 (10 ng / ml) (positive controls).

2.3.3 Production of adenoviral vectors

Introduction

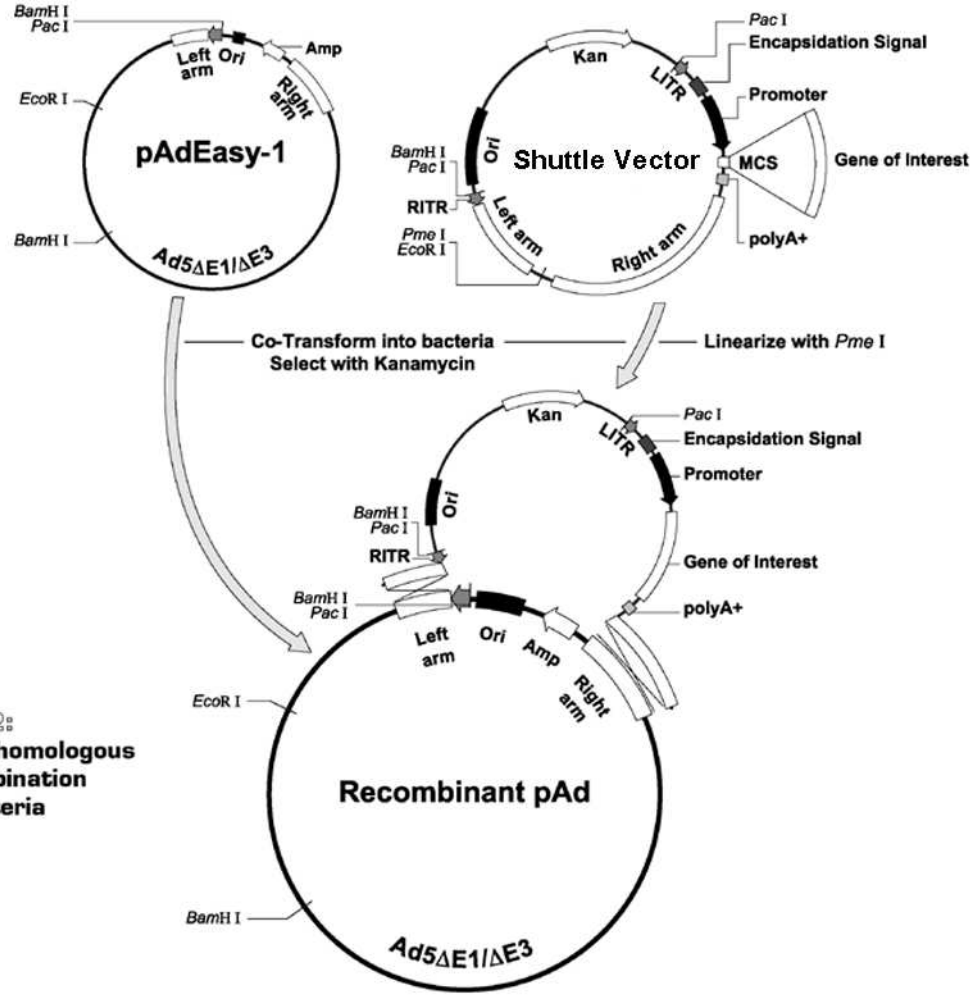
The adenoviral vectors used in the studies were E1-, E3-deleted adenoviruses of serotype-5, produced using the AdEasy system (Dr. B. Vogelstein, Baltimore, MD)[160] (Figure 2.1). All procedures were carried out in a Class II biosafety cabinet and under Level 2 physical containment (PC2) conditions. Two adenoviral vectors were produced: one encoding the enhanced green fluorescent protein (eGFP) under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter (Ad-GFP), and a second encoding both eGFP and ovine IL10, each under the control of individual CMV promoters (Ad-GFP-IL10). The methods used for the construction and production of both adenoviral vectors are described below. Details of the cloning of the IL10 gene into the adenoviral shuttle vector, and cotransformation with the adenoviral backbone pAdEasy to produce the recombinant adenoviral vector plasmid Ad-GFP-IL10, are provided in Chapter 4 (page 143-144).

Figure 2.1: Schematic representation of the AdEasy approach to generating recombinant adenoviral vector stocks

The gene of interest was first cloned into a shuttle vector, and the resultant plasmid linearised by digesting with restriction endonuclease *Pme*I (Step 1). The plasmid was then cotransformed with the adenoviral backbone plasmid, pAdEasy-1, in *E. coli* BJ5183. Recombinants were selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analysis (Step 2). The linearised recombinant plasmid was transfected into an adenovirus packaging cell line, HEK-293A, expressing the E1 gene of the adenovirus genome (Step 3). Purified adenoviral vector stocks were obtained by passing infected cell lysates through caesium chloride density gradients. MCS: multicloning site, LITR: left inverted terminal repeat, RITR: right ITR, Ori: origin of replication; Kan: kanamycin resistance sequence [diagram adapted from AdEasy Vector System Application Manual[161]].

Step 1:

cDNA cloning in Shuttle Vector



Step 2:

In vivo **homologous recombination in bacteria**

Step 3:

Virus production in 293A cells

Ready to be amplified recombinant adenovirus

Transfection of HEK-293A cells with Ad-GFP-IL10 vector plasmid

A transfection with the Ad-GFP-IL10 vector plasmid was carried out in a 25 cm^2 tissue culture flask containing 2×10^6 HEK-293A cells in 10% DMEM. Twenty µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was added to 4 µg of *Pac*I-linearised, endotoxin-low Ad-GFP-IL10 vector DNA, diluted in 500 µl of serum-free OptiMEM medium (GIBCO BRL, Gaithersburg, MD, USA). The mixture was incubated for 20 min at room temperature, whilst the cells were washed once with 4 ml serum-free OptiMEM medium, covered with 2.5 ml serum-free OptiMEM medium and incubated at 37°C. The transfection mixture was added dropwise to the cells and incubated at 37°C with 5% CO_2 in air. After 6 h, the Lipofectamine-DNA mix was replaced by 6 ml of 10% DMEM. Cells were reviewed daily for evidence of cytopathic effect and eGFP expression, using an inverted fluorescence microscope (Leitz Fluovert microscope with Osram HBO 50W lamp and blue light filter block). When evidence of maximal expression was observed (2–3 d), cells were harvested using a cell-scraper and collected by centrifugation at 800 g for 5 min at 4°C. Cells were then resuspended in 3 ml of supernatant and lysed by sonication (Sonicator W0375, Heat Systems, Ultrasonics, Adelaide), using setting 2, 40% duty cycle, continuous pulse, for 10 s. The cell lysate was considered Passage 0 virus stock and stored at -80°C.

Production of unpurified Ad-GFP-IL10 virus

In order to produce large quantities of Ad-GFP-IL10 virus for purification, HEK-293A cells were subjected to repeated rounds of infection with crude lysate from infected cells. Passage 0 crude lysate was thawed in a waterbath at 37°C, cell debris pelleted by centrifugation at 1,200 g for 10 min at 4°C, and the supernatant passed

through a 0.2 µm filter (Minisart; Sartorius AG, Goettingen, Germany). Two 25 cm^2 tissue culture flasks containing HEK-293A cells at 90% confluence were each infected with 1 ml of sterilised Passage 0 supernatant, in a total volume of 3 ml 5% DMEM for 90 min at 37°C. Cells were observed daily for evidence of cytopathic effect and eGFP expression (usually achieved within 3 d). Collection and lysis of these cells by sonication resulted in Passage 1. This lysate was stored at -80°C and subsequently used for infection of two 75 cm^2 tissue culture flasks of HEK-293A cells at 90% confluence. The 4 ml infection mix contained 1 ml of Passage 1 and 3 ml 5% DMEM, with each flask of cells receiving 2 ml for 90 min at 37°C. As before, the cells were observed, collected and lysed, resulting in Passage 2. Three 175 cm^2 tissue culture flasks were each infected with 4 ml of Passage 2 infection mix, containing 1 ml Passage 2 and 11 ml 5% DMEM, in order to produce Passage 3. For production of a purified batch of adenoviral vector, twenty 75 cm^2 tissue culture flasks were each infected with 2 ml of a Passage 3 mixture containing 0.5 ml of Passage 3 and 39.5 ml of 5% DMEM. This stage was carried out a total of four times to produce sufficient Passage 4 stock for caesium chloride (CsCl) purification.

Caesium chloride purification and dialysis of adenoviral vectors

Passage 4 infected cell lysates were thawed at 37°C, pooled and cellular debris excluded by centrifugation at 1,400 g for 10 min at 4°C. The viscosity of the resultant supernatant was reduced by passing it through a 22G needle three times. The supernatant was made up to 17 ml with 10 mM HEPES buffer pH 7.4, and divided into two volumes of 8.5 ml. Two Quickseal Ultraclear tubes (Beckman Instruments, Palo Alto, CA, USA) were each filled with a discontinuous gradient of CsCl in 10 mM HEPES buffer pH 7.4, consisting of: 2.5 ml of 2.2 mM CsCl, on top of 2.5 ml of 4M CsCl. A long Pasteur pipette was then used to dispense each volume

of supernatant carefully onto each CsCl gradient. The two tubes were heat-sealed and centrifuged at 100,000 g for 2 h at 4°C in a Beckman L8-M preparative ultracentrifuge and fixed angle rotor (Ti 80) (Beckman Instruments, Palo Alto, CA, USA). Each tube was then punctured at the top using a 19G needle. The band containing the virus particles was collected in approximately 3 ml, by puncturing the tube using a 22G needle attached to a 5 ml syringe. This solution was made up to 13 ml with saturated CsCl in order to make two more gradients. The solution was divided into two 6.5 ml volumes and each was overlayed with 3 ml of 4M CsCl followed by 4 ml of 2.2M CsCl, in a new Quickseal Ultraclear tube. The tubes were heat-sealed and centrifuged at 120,000 g for 3 h at 4°C in the Beckman L8-M preparative ultracentrifuge and fixed angle rotor (Ti 80). Once again, tubes were punctured at the top using a 19G needle. The band containing the virus particles was collected by puncturing the tube with a 22G needle attached to a 5 ml syringe, and injected directly into a Slide-a-lyzer dialysis cassette with a 10,000 Da molecular weight cut-off (Pierce Chemical Company, Rockford, IL, USA). The solution was dialysed against a total volume of 2 L sterile PBS 10% v/v glycerol at 4°C over a 24 h period. Finally the virus solution was collected and passed through a 0.2 µm filter (Minisart, Sartorius AG, Goettingen, Germany), before being divided into aliquots and stored at -80°C.

Production of adenoviral vector expressing eGFP

Concentrated stock of the adenoviral vector expressing eGFP under the control of the human CMV immediate early promoter (Ad-GFP) was produced by four rounds of infections using a Passage 3 of unpurified virus stock, previously generated in the laboratory by Dr C. Jessup, as described above. Lysates were stored at -80°C until being pooled for CsCl purification.

Titration of adenoviral vectors

The titre of concentrated adenoviral vector stock was determined using the $TCID_{50}$ method (tissue culture infectious dose 50%) and then converting to pfu per ml. Briefly, serial 10-fold dilutions of the virus from 10^{-2} to 10^{-13} were each added to 10 wells each containing 1×10^4 HEK-293A cells, in 96-well plates. Each dilution of virus was accompanied by two negative control wells of HEK-293A cells to which only medium was added. Following 10 d in culture at 37°C, wells were examined by fluorescence microscopy for the presence of cells expressing the eGFP reporter gene, indicating successful gene transfer. Wells were deemed positive even if there was only a single patch of fluorescent cells present. The proportion of the 10 wells at each dilution which were positive was expressed as a decimal ratio, assuming the 10^{-1} dilution (not performed) to be 1. These were then summed and termed 'S' as the sum of ratios. Titre was calculated as: Titre = $10^{S+0.8}$ pfu per ml, according to the Karber statistical method, and correcting for the difference between $TCID_{50}$ and standard plaque assay, according to the AdEasy system protocol.[161]

2.3.4 Production of lentiviral vectors

Introduction

Lentiviral vectors were produced at the Gene Technology Unit, Department of Genetic Medicine, Women's and Children's Hospital, Adelaide, SA. All procedures were conducted under PC2 conditions, including use of a Class II biosafety cabinet. A four-plasmid packaging system was used, in which all proteins required for production of the virus were expressed on different constructs (Figure 1.9, page 41). Four vector plasmids—three encoding the enhanced yellow fluorescence protein (eYFP) under the control of the simian virus type-40 (SV40) early promoter (pHIV-

1-SDm-SV-eYFP-ΔLTR), the murine phosphoglycerate kinase promoter (pHIV-1-SDm-PGK-eYFP-ΔLTR) and the human elongation factor-1α promoter (pHIV-1-SDm-EF-eYFP-ΔLTR) respectively, and one containing the SV40 promoter driving an empty transgene cassette (pHIV-1-SDm-SV-empty-ΔLTR)—as well as the four helper plasmids, were kindly provided by Associate Professor D.S. Anson of the Gene Technology Unit, Department of Genetic Medicine, Women's and Children's Hospital, Adelaide, SA. All lentiviral expression plasmids are listed in Table 2.5.

The vector sequences were situated between the *Not*I and *Apa*I sites of the 3.4 kb pBC KS+ phagemid (Stratagene, La Jolla, CA, USA). The helper plasmids encoding the viral proteins were: pcDNA3.1*tat101ml* (HIV-1 Tat), pHCMVwhv*revml* (HIV-1 Rev), and pHCMVwhv*gagpolml* (HIV-1 GagPol). The pHCMV-G plasmid was used for expression of the vesicular stomatitis virus (VSV) G envelope glycoprotein. The eYFP coding sequence had previously been isolated from pEYFP (Clontech, Mountain View, CA, USA). The SV40 promoter had previously been isolated from pLNSX.[162] Plasmids were prepared using commercially-available endotoxin-low kits as described above, (Endo-free Mega/Giga kit; Qiagen, Valencia, CA, USA) to minimise bacterial endotoxin contamination.

Concentrated, purified lentiviral vector preparations were produced on a large-scale, medium-scale or small-scale. In each case, HEK-293T cells (SD3515, American Type Culture Collection, Manassas, VA, USA) were transfected by calcium-phosphate coprecipitation using a preprepared mixture of the five plasmids made in 250 mM calcium chloride ($CaCl_2$) according to strict ratios (Table 2.9), combined with HEPES-buffered saline (HeBS). For large and medium-scale preparations, the supernatant collected from transfected cells was concentrated by

filtration and ultrafiltration using a benchtop system with hollow-fibre cartridges, followed by ultracentrifugation. In the case of small-scale preparations, supernatant was collected from transfected cells and stored without further purification or concentration. A table listing the lentiviral vector preparations used during the project is presented in Appendix 1.

Table 2.9: Ratios of lentiviral plasmids used for transfection of HEK-293T cells

Plasmid	Amount of DNA per plate† (µg)
Vector	158
pcDNA3.1*tat101ml*	3.16
pHCMVwhv*revml*	3.16
pHCMVwhv*gagpolml*	15.8
pHCMV-G	7.9

† Calculated for a 245 mm square plate seeded with 4×10^7 cells.

Cloning of internal promoters and transgenes into lentiviral vectors

Preparations of six different lentiviral vectors were produced during the project. The cloning methods described above (pages 63–64) were used to construct vector plasmids containing the human CMV immediate early promoter driving expression of eYFP (pHIV-1-SDm-CMV-eYFP-ΔLTR), and the SV40 and GRE5 promoter sequences driving the ovine IL10 coding sequence (pHIV-1-SDm-SV-IL10-ΔLTR and pHIV-1-SDm-GRE-IL10-ΔLTR). The specific details of cloning for these vectors are presented in Chapter 3 (CMV vector driving eYFP expression) and Chapter 4 (vectors driving ovine IL10 expression).

Large-scale production of lentiviral vectors

To produce approximately 3 ml of concentrated lentiviral vector stock, HEK-293T cells were seeded on twenty 245 mm square plates (Corning Incorporated, Corning,

NY, USA) at 3.75×10^5 cells per ml in 105 ml of 5% DMEM per plate. After 20–24 h, transfection was performed by calcium phosphate coprecipitation. All solutions were brought to room temperature. The transfection was carried out on two square plates at a time: one volume (6.5 ml) of the DNA/$CaCl_2$ solution was added to one volume of 2× HeBS over 5–10 s, while mixing on a vortex; the mixture was then vortexed for a further 20 s, and left to sit for a final 90 s, before 6.4 ml was carefully dripped onto the medium of each of the two plates in turn. This procedure was repeated nine times to transfect the remaining 18 plates of cells. After an 8 h incubation at 37°C in 5% CO_2 in air, the medium was replaced with 170 ml of Opti-Pro serum free medium (SFM) (Invitrogen, Carlsbad, CA, USA) per plate, being careful not to dislodge any cells.

After a further 40 h incubation at 37°C in 5% CO_2 in air, the 3,400 ml of supernatant was harvested into 450 ml centrifuge buckets and centrifuged at 2,000 g for 10 min at 20°C. The supernatant was then filtered through a 0.45 μm hollow-fibre cartridge (CFP-4-E-4MA, Amersham Biosciences Corporation, Piscataway, NJ, USA) at room temperature, using the QuixStand™ benchtop system (Amersham Biosciences Corporation, Piscataway, NJ, USA) (Figure 2.2). Next, the filtrate was concentrated down to a volume of 150 ml with a 750 kDa cut-off hollow-fibre ultrafiltration cartridge (UFP-750-E-4x2MA), again using the QuixStand™ benchtop system, at room temperature. The retentate was then passed through a 0.8 μm syringe filter unit (Millex-AA, Millipore, Carrigtwohill, Co. Cork, Ireland) before ultracentrifugation at 50,000 g for 90 min (Beckman SW40) at 4°C. The resulting pellet was gently resuspended in 3 ml of ophthalmic balanced salt solution (Cytosol Ophthalmics, Lenoir, NC, USA). A final filtration through a 0.45 μm filter (Minisart;

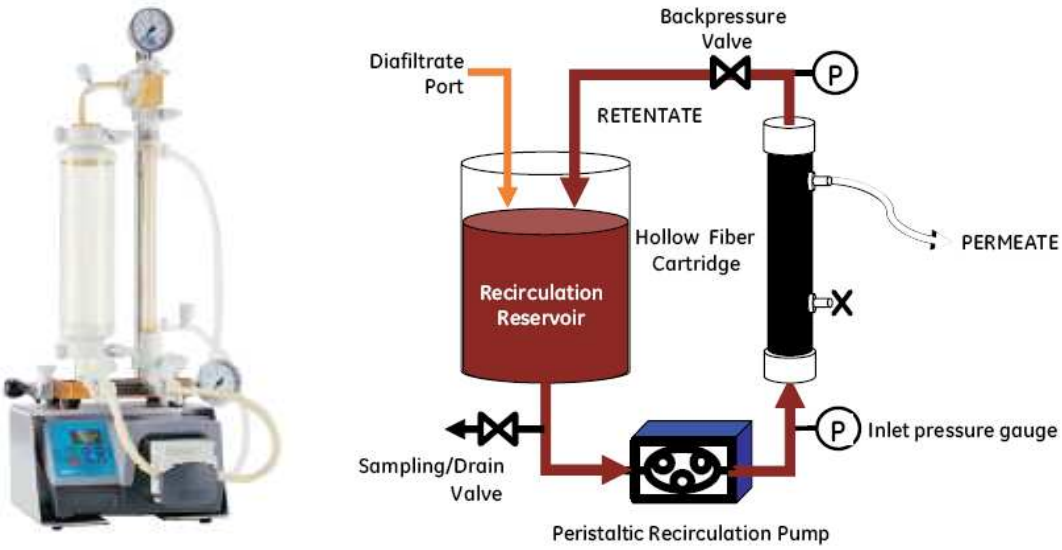


Figure 2.2: The QuixStand™ benchtop system used for concentrating large and medium-scale lentivirus preparations

The system is a compact, laboratory scale cross flow hollow fibre system designed for fast, efficient concentration and/or diafiltration of a wide range of biological solutions. It has a peristaltic pump, for recirculating the supernatant or filtrate through a reservoir and a hollow fibre cartridge. Initially, 3.5 L of supernatant from transfection of HEK-293T cells with the lentiviral plasmids was introduced into the recirculation reservoir with the 0.45 μm cut-off hollow fibre cartridge in position. The filtered supernatant containing virus was collected as the 'permeate'. In the second step, the freshly filtered virus-containing supernatant was recirculated through the 750 kDa cut-off hollow fibre cartridge and concentrated down to 150 ml in the reservoir as the 'retentate'. In this step, the permeate constituted waste. [Pictures from QuixStand Benchtop System User Manual (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)].

Sartorius AG, Goettingen, Germany) was followed by distribution of concentrated virus into 25–100 µl aliquots and storage at -80°C.

Medium-scale production of lentiviral vectors

To produce approximately 500 µl of concentrated lentiviral vector stock, HEK-293T cells were seeded on three 245 mm square plates at 3.75×10^5 cells per ml in 105 ml 5% DMEM. After 20–24 h, transfection was performed by calcium phosphate coprecipitation as described above. After an 8 h incubation at 37°C in 5% CO_2 in air, the medium was replaced with 170 ml of Opti-Pro SFM per plate.

After a 40 h incubation at 37°C in 5% CO_2 in air, the 510 ml of supernatant was harvested into 450 ml centrifuge buckets and centrifuged at 2,000 g for 10 min at 20°C. The supernatant was then filtered through a 0.45 µm Polydisc™ TF filter device (Whatman, Kent, England, UK) at room temperature. The filtrate was concentrated down to a volume of 50 ml using the 750 kDa hollow fibre cartridge, again at room temperature. The retentate was passed through a series of filters of pore size 5 µm, 1.2 µm, and 0.8 µm to further filter the solution (Millex-AA; Millipore, Carrigtwohill, Co. Cork, Ireland) before ultracentrifugation at 50,000 g for 90 min (Beckman SW40) at 4°C. The resulting pellet was gently resuspended in 500 µl of balanced salt solution. A final filtration through a 0.45 µm filter was followed by distribution of concentrated virus into 10–25 µl aliquots and storage at -80°C.

Small-scale production of lentiviral vectors

For small-scale preparations of unconcentrated virus, HEK-293T cells were seeded on 6-well plates (Corning Incorporated, Corning, NY, USA) at $3.75\text{x}10^5$ cells per ml in 2 ml 5% DMEM. The transfection was performed 20–24 h later by calcium phosphate coprecipitation as described. After an 8 h incubation at 37°C in 5% CO_2 in

air, the medium was replaced with 2 ml Opti-Pro SFM per well. After a further 40 h incubation at 37°C in 5% CO_2 in air, supernatants were collected and stored at -80°C until use.

Titration of lentiviral vector preparations by flow cytometry

In order to determine the titre of lentiviral vector stocks expressing eYFP, A549 cells (CCL-185, American Type Culture Collection, Rockville, MD, USA) were seeded on a 24-well plate at 2.5×10^5 cells per well in 5% DMEM, and left to adhere for 3 h. After attachment of cells, medium was replaced with 0.5 ml per well of 5% DMEM supplemented with 4 µg / ml polybrene (Sigma, St Louis, MO, USA) and 50 µg / ml gentamicin. Serial 10-fold dilutions were performed on concentrated vector stock using 5% DMEM, and a range of three different doses (1 in 100, 1 in 200, and 1 in 500) were added to wells in triplicate. Nontransduced control wells were also included in triplicate on every plate. The cells were incubated at 37°C in 5% CO_2 in air. After 24 h, the medium was replaced by 5% DMEM supplemented with 50 µg / ml gentamicin and incubated. After a further 24 h, cells were split 1 in 4 and incubated for another 48 h. At that time, cells were detached using undiluted trypsin-EDTA and pipetted to produce a single cell suspension in 1% PBS. Cells were then centrifuged at 800 g for 5 min, and the supernatant aspirated and discarded. The cell pellet was resuspended in 200 µl of 1% w/v paraformaldehyde in DDH_2O and transferred to tubes for flow cytometry. The cell population was gated by side and forward scatter and eYFP expression analysed on a FACScan machine (Becton Dickinson, Franklin Lakes, NJ, USA). The results were analysed using Cell-Quest software v3.0.1f (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry of nontransduced cells was used to provide a background control. The corrected percentage of positive cells in the experimental sample was calculated as

the percentage of eYFP positive cells in the transduced samples minus the average percentage of positives in the control samples. Viral titre was calculated by multiplying the corrected percentage of positive cells by the number of cells plated and corrected for the amount of virus assayed:

A549 transducing units per ml

= ((250,000 cells × (transduced % gated – control % gated) / 100) x 1000)

per µl of virus added to well

Titration of lentiviral vector preparations using real-time PCR

The titres of lentiviral vector stocks not expressing eYFP were determined by a real-time PCR assay detecting the beginning of the *gag* sequence in the vector. Concentrated virus was diluted 1:1000 and 20 µl added to 2.5×10^5 NIH3T3 cells (CRL-1658, American Type Culture Collection, Rockville, MO, USA) per well, in a 24-well plate. Control wells were included to which no virus was added. Experiments and controls were performed in triplicate. Cells were harvested after 5 d incubation, and genomic DNA isolated using a commercially available kit (Wizard SV Genomic DNA Purification System; Promega, Madison, WI, USA), in which DNA is bound to and eluted from spin columns. Real-time PCR assays were carried out using an ABI 7300 cycler (Applied Biosystems, Foster City, CA, USA). Primers and fluorescent probes were designed to detect the *gag* sequence and mouse transferrin sequence in isolated genomic DNA (Table 2.3). Reaction mixtures of 20 µl were made using 10 µl of 2× TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µl of 20× assay mix (containing specific primers and probes), water, and genomic DNA template. During each run, a standard made from genomic DNA isolated from NIH3T3 cells containing one copy of the virus genome, and two copies of the transferrin gene per cell, was used as a calibrator

sample. In addition, no-template-controls (NTCs) were included in every run for both primer and probe sets, in order to detect contamination. All standards and NTCs were run in triplicate. Each run consisted of 40 cycles.

At the completion of a run, the cycle threshold bar was positioned to ensure that it intersected all amplified samples in the linear portion of the amplification curve. Cycle threshold (Ct) values were determined for each sample using Sequence Detection Software v1.2.2 (Applied Biosystems, Foster City, CA, USA) and the ΔΔ-Ct method used to calculate the copy number of *gag* per cell. Viral titre was derived by multiplying the number of cells (2.5×10^5) per sample, by the dilution factor of the virus (1000), and the *gag* copy number per cell, and dividing this figure by the volume of virus added to cells (20 µl). The result was then multiplied by 1000 to represent the number of NIH3T3 infectious units (iu) per ml.

Assay for the detection of replication-competent lentivirus

To ensure that no replication-competent lentivirus was present in concentrated vector stock, a sample from each vector preparation was subjected to a p24 protein disappearance assay. To set up the assay, HEK-293T cells were seeded at 5×10^5 cells per well in 5% DMEM, on a 12-well plate, and left for 3 h to adhere. After the cells had attached, the medium was replaced with 1 ml per well of 5% DMEM supplemented with 4 µg / ml polybrene (Sigma, St Louis, MO, USA), and 50 µg / ml gentamicin. Then, 1 µl of concentrated vector was applied to three wells, and the cells incubated at 37°C in 5% CO_2 in air. Three wells of nontransduced cells were included as controls. After 24 h, medium was replaced with 5% DMEM supplemented with 50 µg / ml gentamicin. After a further 24 h had elapsed, cells were split 1 in 10. On days 6, 9, 12 and 15, cells were split 1 in 10. At 6, 12 and 18 d, 450 µl of supernatant was harvested form each well and stored at -20°C. Finally,

supernatants were tested in a commercially available ELISA for the HIV p24 protein (Perkin-Elmer, Boston, MA, USA). A stock of vector was deemed to be free of replication-competent lentivirus if p24 was readily detectable in the supernatant immediately following transduction but declined to control levels over the 18 d period of the assay.

2.3.5 *In vitro* studies

Processing of ovine corneas

Experimentation in sheep was performed with approval from the institutional Animal Welfare Committee. Enucleated ovine eyes were obtained within 1 h of donor death from a local abattoir (Normanville Meatworks, Normanville, SA, Australia) and decontaminated for 3 min in 10% w/v povidone-iodine. They were then washed twice in sterile 0.9% w/v NaCl. Dissections were performed to remove the corneas with a 2 mm scleral rim, whereupon they were suspended in 7 ml of 2% RPMI until transduction with viral vectors.

Processing of human eye bank corneas

Human corneas considered unsuitable for clinical transplantation were obtained from the Eye Bank of South Australia (Flinders Medical Centre, Adelaide, SA) with permission from the next-of-kin and 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.

Transduction and organ culture of corneas

Corneas undergoing transduction with viral vectors underwent a 2–24 h transduction period in the presence of concentrated vector. Corneas were placed onto sterilised plastic eye cups (The Dodge Company, Cambridge, MA, USA) in the bottom of 50 ml pots with the endothelium facing upwards. For transduction of ovine corneas, a predetermined dose of virus was applied topically to the endothelium in a total volume of 300 μl of 2% RPMI. Human corneas were transduced in a total volume of 200 μl of 2% RPMI. These respective volumes were established on the basis that the entire corneal endothelium would be exposed to the transduction mixture. After the completion of the transduction period—the duration of which varied according to the experiment—a volume of 10–20 ml of 10% RPMI supplemented with 0.25 μg / ml amphotericin B (Amphostat; Thermo Electron, Melbourne, VIC) was added to each pot, and corneas were then cultured for a period of 1–21 d. Medium was replenished every 3–4 d.

Calculation of multiplicity of infection

The doses of vectors tested throughout the project were in the order of 10^6–10^8 plaque forming units (adenovirus), transducing units or infectious units (lentiviral vectors) per cornea. Multiplicity of infection (MOI) was used as a means of simplifying the description of doses, and for comparing transduction efficiencies between ovine and human corneas. Multiplicity of infection denotes the ratio of plaque forming, transducing or infectious units to the number of target cells exposed to the vector during the transduction period. To calculate the number of target endothelial cells per cornea in each species, the surface area of the corneal endothelium was multiplied by the respective endothelial cell density.

The approximate surface area of the corneal endothelium was derived from calculations of the surface area of an ellipsoidal dome for the sheep, and a spheroidal dome for the human. According to a Cartesian coordinate system, the radii along the *x*, *y*, and *z*-axes were 10.7 mm, 8.8 mm, and 4.6 mm respectively, for the ovine cornea, and 5.8 mm, 5.8 mm, and 2.1 mm for the human cornea. Endothelial cell density was assumed to be 4,000 cells per mm^2 for the ovine cornea, and 2,500 cells per mm^2 in the case of the human cornea. On this basis, MOIs were based on a total of 1.4×10^6 and 2×10^5 endothelial cells being exposed to the vector, in ovine and human corneas respectively. The calculation of MOI was considered an approximation, as it was recognised that true anatomical dimensions and endothelial cell densities would vary between individual corneas; MOIs were rounded out to the closest five or ten.

Detection and quantification of reporter gene expression in corneal endothelium

To quantify the number of corneal endothelial cells expressing eGFP or eYFP, corneas were removed from culture and fixed in ice-cold buffered formalin for 10 min, prior to counterstaining with 10 μg / ml Hoechst-33258 dye (Sigma, St Louis, MO, USA) for 30 min. The corneal endothelium and underlying Descemet's membrane were stripped away from the posterior stroma using a scalpel blade (No. 15) and flat mounted (endothelium upwards) in buffered glycerol (Bartel's buffered glycerol mounting medium; Trinity Biotech, Bray, Co. Wicklow, Ireland) under a cover slip on a glass slide. The edges of the cover slip were sealed with nail polish. Mounted corneal endothelial specimens were examined under a fluorescence microscope (BX50; Olympus Corporation, Tokyo, Japan) and photographed using a digital camera (Photometrics CoolSNAP high resolution cooled CCD, 1.0 x tube)

and RS Image imaging software (Photometrics, Tucson, AZ, USA). Images were acquired using two filter combinations; one to detect the nuclear stain (Olympus U-MNUA) and one to detect the reporting fluorescent chromophores eGFP or eYFP (Chroma, Rockingham, VT, USA) (Table 2.10). Five representative fields were acquired per cornea, using the 20× objective (each 0.15 mm^2) and cell counts were made using image analysis software to determine the percentage of endothelial cells expressing the reporter gene (Adobe Photoshop 7.0, Adobe Systems Incorporated, San Jose, CA, USA). For each field, the image of the nuclear staining and the image of reporter fluorescence were superimposed. The transduction rate of each cornea after a period of organ culture was calculated by determining the mean percentage reporter gene expression of five fields examined.

Table 2.10: Filters used in fluorescence microscopy

Fluorophore	Filter	Wavelength (nm)		
		Excite	Dichroic	Emit
Hoechst-33258	Olympus U-MNUA	360-370	400	420-460
eGFP, eYFP	Chroma 31001	465-495	505	515-555

***Footnotes: eGFP enhanced green fluorescent protein, eYFP enhanced yellow fluorescent protein

Harvesting of corneal endothelial tissue for mRNA extraction

In corneas intended for real-time quantitative RT-PCR, two different methods were trialled in order to optimise the yield and purity of RNA. Initial attempts were made to extract RNA from whole ovine corneas by trephination, snap-freezing and pulverisation. This approach resulted in yields below 100 ng / µl—the level required for accurate quantification using ultraviolet spectrophotometry.[163] This was partly attributed to difficulty homogenising the ovine cornea, as a result of its high collagen content. An alternative method was adopted, in which the endothelium (including Descemet's membrane) was stripped away from the underlying stroma of each

cornea, and then subjected to RNA extraction. Briefly, transduced and/or cultured corneas were rinsed and transferred from culture medium into 2% RPMI. Immediately before dissection, corneas were rinsed in chilled DEPC-treated normal saline (4°C). After fixation to a piece of polystyrene foam using four 25G needles, the corneal endothelium with Descemet's membrane was incised at its perimeter, and dissected away from the underlying stroma, using a scalpel blade (no. 15). The tissue was immediately transferred into a 1.5 ml nuclease-free tube containing 600 µl of lysis buffer (Buffer RLT, Qiagen, Valencia, CA, USA), with 1% v/v 2-mercaptoethanol (Sigma, St Louis, MO, USA). The sample was then vortexed for 2 min without delay, and finally stored at -20°C until RNA extraction. To minimise RNA degradation, gloves were worn at all times, and the dissection was carried out in less than 5 min per sample.

Extraction of endothelial RNA

Total RNA was extracted from each sample using the RNeasy Mini-Kit (Qiagen, Valencia, CA, USA), in accordance with the supplied protocol. Nuclease-free tubes, and pipette tips were used at all times. The thawed lysate was transferred onto a QIAshredder spin column (Qiagen, Valencia, CA, USA), for the purpose of homogenisation of the sample. Unlysed bulky tissue was not transferred onto the Qiashredder. Following recovery of the homogenised lysate, and the addition of 1× volume of 70% ethanol, the sample was placed onto the RNeasy spin column, and the subsequent washes carried out according to the supplied protocol for animal tissues. Finally, the bound RNA was eluted in 20 µl of RNase-free water. Residual genomic DNA was removed by treatment with commercially available DNase I (DNA-*free*™, Ambion, Austin, TX, USA), according to the supplied protocol for

rigorous DNase treatment. The RNA was transferred to a clean tube, and stored on ice until quantification.

Quantification of endothelial RNA

Following extraction of RNA, samples were assessed for quality and DNA contamination by electrophoresis in 1% w/v agarose gel. Samples yielding bands corresponding to the 28S and 18S ribosomal RNA subunits at a ratio of approximately 2:1, and free of evidence of DNA contamination, were subjected to spectrophotometry to determine yield. Absorbance at 260 nm and 280 nm was measured at a dilution of 1 in 25, in a 50 µl disposable cuvette on a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). Samples with an absorbance at 260 nm of greater than 0.1, and a 260:280 nm ratio of ≥1.9 were deemed satisfactory for reverse transcription. The method of dissecting the endothelium resulted in samples consistently yielding 1–4 mg per cornea, with satisfactory 260:280 nm absorbance ratios. Such samples were used for reverse transcription, including RNA pooled from adenovirus and lentivirus-transduced corneas, which was used for the synthesis of a cDNA standard. Equal quantities of input RNA were used in cDNA synthesis reactions.

Synthesis of cDNA

Synthesis of complementary DNA (cDNA) was achieved using a commercially available kit (SuperScript III® First-Strand Synthesis System; Invitrogen, Carlsbad, CA, USA) according to the supplied protocol. For each reaction, 0.5 µg of input RNA was added to 1 µl of random hexamers (50 ng / µl) and 1 µl of dNTP mix (10 mM). The volume was made up to 10 µl with nuclease-free water. This RNA/primer mix was incubated at 65°C for 5 min and then cooled on ice for at least 1 min. A

10 µl cDNA synthesis mix containing 2 µl of 10× RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, and 1 µl of SuperScript III® reverse transcriptase (Invitrogen, Carlsbad, CA, USA), was added to each RNA/primer mix, and mixed thoroughly. A 10 min incubation at 25°C was followed by 50 min at 50°C. The reactions were terminated by a 5 min incubation at 85°C and transferred to ice. Finally, 1 µl of *E. coli* RNaseH (2U / µl) was added to each reaction, followed by a 20 min incubation at 37°C. For each RT-positive reaction performed, a corresponding RT-negative control sample was synthesised by the substitution of reverse transcriptase enzyme with nuclease-free water. Upon completion of the synthesis reactions, the neat cDNA samples were diluted to 1 in 20, and stored at 4°C for use in real-time PCR. The remaining undiluted cDNA was stored at -20°C. In addition, corneal RNA from adenovirus- and lentivirus-transduced corneas was pooled and used for the synthesis of a cDNA standard.

Primers for real-time quantitative PCR

Primers for the detection of ovine IL10, ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ovine β-actin—by real-time quantitative PCR—were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) with the recommended parameters (Table 2.11). Primers were synthesised by GeneWorks (Thebarton, SA) at sequencing grade (Table 2.3).

Table 2.11: Parameters for design of real-time PCR primers using Primer3

Parameter	Parameter range		
	Minimum	Optimum	Maximum
Product size (bp)	80		110
Primer size (bp)	15	20	22
Primer T_m (°C)	54	60	62
Primer G/C%	20		70
Hybridisation probe size (bp)	25	30	35
Probe T_m (°C)	65	70	72
Probe G/C%	20	50	70

***Footnotes: PCR polymerase chain reaction, bp base pairs, T_m melting temperature
All other parameters were set to default.

Real-time quantitative PCR

Real-time quantitative polymerase chain reaction (qPCR) was performed on a Rotor-Gene 3000 real time thermal cycler (Corbett Research, Mortlake, NSW). Each 20 µl reaction mixture included 10 µl of QuantiTect™ SYBR Green PCR Master Mix containing hot-start Taq DNA polymerase, SYBR Green I, dNTPs and PCR buffer (5 mM $MgCl_2$, Tris-Cl, KCl, $(NH_4)_2SO_4$ pH 8.7), 2 µl each forward and reverse primers (0.5 µM final concentration), and 6 µl cDNA sample diluted 1/100 v/v with Ultra Pure water (Fisher Biotech, West Perth, WA, Australia). Reaction conditions were: initial denaturation (95°C, 10 min), 40 cycles of denaturation (95°C, 10 s), annealing (52°C, 15 s), and extension (72°C, 20 s). Test cDNA samples were generated from corneas subjected to a range of treatments. Every sample was tested in triplicate. The standard cDNA pool was included in triplicate in each PCR run. A single RT negative control for each sample and two water controls (no template control) were included in each experiment.

The "Takeoff" point of a given sample was calculated automatically by Rotor-Gene analysis software v6.0 (Corbett Research, Mortlake, NSW), using the

feature “Comparative Quantitation”. This is used to compare the relative expressions of samples to a control sample in a run when a standard curve is not available. The second derivative of the amplification plot produces peaks corresponding to the maximum rate of fluorescence increase in the reaction. The Takeoff point is defined as the cycle at which the second derivative is at 20% of the maximum level, and indicates the end of the noise and the transition into the exponential phase. A negative control sample was confirmed to be negative if the Takeoff point for amplification was more than five cycles greater than the corresponding test sample.

Analysis of real-time PCR products

Real-time PCR products were separated by agarose gel electrophoresis. The DNA was extracted from agarose gels using the QIAquick column purification system (Qiagen, Valencia, CA, USA) and eluted in 10 mM Tris.Cl pH 8.5 for quantification and sequencing. Purified DNA was labelled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and resolved using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Melt-curve analysis was used to confirm amplicon specificity for each test sample for each gene of interest. Melting of PCR products was performed with 1°C steps every 5 s, from 47–95°C.[164] Results of analysis of real-time PCR products are described in Chapter 4 (page 153).

Analysis of gene expression data

Relative gene expression levels were calculated by importing Takeoff point values into qBase v1.3.5, a free program for the management and automated analysis of qPCR data.[165] The software calculates amplification efficiencies of primer sets from serial dilutions of the cDNA standard, and employs a modification of the ΔΔ-Ct

method, adjusting for differences in amplification efficiency, and allowing the use of multiple reference genes. In the analysis of each treatment, raw relative reference gene expression levels were analysed for variability between replicates, or trends indicating that the treatment was influencing expression. The levels of expression of each gene were then normalised to the normalisation factor, defined as the geometric mean of the relative expression levels of both reference genes, GAPDH and β-actin, in a given sample.[166]

Quantification of IL10 in supernatants by ELISA

To quantify levels of secreted transgenic IL10 from transduced corneas in culture, supernatant was collected at three-day intervals. At each collection point (3, 6, 9, 12 and 15 d post-transduction), all of the culture medium (10 mL) was replaced. The removed medium was filtered through a 0.2 µm membrane (Minisart; Sartorius AG, Goettingen, Germany), and stored at -20°C until processing. The relative quantity of ovine IL10 present in corneal culture supernatants was measured by sandwich ELISA. A mouse anti-bovine IL10 monoclonal antibody (Serotec, Kidlington, UK) was used as a capture antibody by dilution at 1 in 167 in coating buffer comprising 0.1 M sodium carbonate, pH 9.5. The coated ELISA plate (Nunc, Rosklide, Denmark) was stored at 4°C overnight. The plate was washed three times with 0.05% v/v Tween-20 in 1× PBS before blocking using 200 µl 10% FCS in PBS per well for 1 h at room temperature. The plate was washed three times. Supernatant samples were diluted in 10% v/v FCS in 1× PBS, added at 100 µl per well and the plate incubated at room temperature for 2 h. The plate was washed five times before the addition of a biotinylated mouse anti-bovine IL10 monoclonal detector antibody (Serotec, Kidlington, UK), diluted 1 in 500 in 10% v/v FCS in 1× PBS, followed by a 1 h incubation at room temperature. The plate was then washed five times before

each well received 100 µl of streptavidin-HRP (DAKO, Denmark), diluted 1 in 1000 in 10% v/v FCS in 1× PBS. The plate was incubated for 1 h at room temperature and washed five times before the addition of TMB substrate reagent (BD Biosciences, Pharmingen, San Diego, CA, USA). The TMB reagent was made up by the combination of reagents A and B at 1:1, and applied at 100 µl per well. The plate was incubated in the dark at room temperature for 30 min. The reaction was terminated by the addition of 50 µl 1M H_2SO_4. Optical density at 450 nm (OD_{450}) was read using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA). For each assay, serial dilutions of an arbitrarily assigned sample were included to create a standard curve. Experimental samples were tested in duplicate at various dilutions to ensure the OD_{450} fell on the linear part of the standard curve.

2.3.6 *In vivo* studies

Ex vivo transduction of ovine donor corneal allografts

In the majority of experiments, ovine donor corneas were transduced for 2–3 h in the manner described on page 82, before being transplanted to the right eye of outbred recipient sheep, on the same day. In studies of organ culture following *ex vivo* transduction, donor allografts were transduced for 24 h and stored at 37°C for 1–14 d in sealed 50 ml plastic pots containing 10–40 ml of 10% RPMI, with supplementary amphotericin B (0.25 µg / ml). In a limited number of cases, donor corneas were transferred into sealed 50 ml plastic pots containing 40 ml of 10% RPMI with 5% w/v Dextran T500 (termed deswelling medium) for the final 24–48 h prior to transplantation.

Orthotopic corneal transplantation in sheep

Animals were fasted for 18–20 h prior to surgery.[70] Mydriasis and cycloplegia were achieved by topical application of 1% atropine sulphate 12 h before surgery, and then 1% atropine sulphate, 1% cyclopentolate hydrochloride, 1% tropicamide and 10% phenylephrine hydrochloride on the morning of surgery (Table 2.8). Anaesthesia was induced by intravenous injection of 25 mg / kg sodium thiopentone, followed by intubation, and maintenance by 1.5–2% halothane in 2:1 air/oxygen. A blood sample was taken at the time of anaesthetic induction. Surgery was performed by a highly practiced ophthalmic surgeon, with prior experience of corneal transplantation in the sheep (Professor D.J. Coster, Department of Ophthalmology, Flinders Medical Centre, Bedford Park, SA). The globe was stabilised with 4.0 silk subconjunctival sutures. A trephined donor cornea 12 mm in diameter was transplanted into a graft bed 11 mm in diameter, in the right eye. The graft was secured by insertion of four to seven 9.0 monofilament nylon cardinal sutures and one continuous 9.0 monofilament nylon suture. When necessary, the anterior chamber was reformed with an injection of balanced salt solution (Cytosol Laboratories, Braintree, MA, USA); no viscoelastic was used. At the end of each procedure chloramphenicol ointment was applied to the eye (Table 2.8). A record was maintained of each surgical case (Appendix 2).

Postoperative treatment and assessment of sheep

On the day of surgery, animals were observed postoperatively until capable of standing and feeding independently. Daily examinations were conducted using a handheld slit lamp, and markers of ocular inflammation were graded on a scale of zero to four with respect to: (i) graft clarity, (ii) graft oedema, (iii) presence of cells

in the anterior chamber, (iv) presence of fibrin in the anterior chamber, (v) and the degree of generalised ocular inflammation (Appendix 2). The extent of corneal vascularisation was also measured in four quadrants. Preoperative vascularisation of the host was noted, and the day on which the blood vessels breached the graft-host junction was recorded. Comparison was made with the untreated eye. The onset of corneal graft rejection was defined as spreading oedema or loss of clarity in a previously thin, clear graft (equivalent to a score of ≥2 centrally), or the appearance of a corneal epithelial or endothelial rejection line. Animals were euthanased after the onset of immunological rejection, by overdose of intravenously injected veterinary sodium phenobarbitone (6.5 g per sheep). Blood samples were taken preoperatively, and at the time of death, in collection tubes containing clot activator. Serum was extracted by centrifugation at 1,500 g for 5 min and stored at -20°C.

Endpoint histology of ovine corneal allografts

Corneal tissue was harvested and fixed in buffered formalin, embedded in paraffin wax, cut at 8 μm and stained with haematoxylin and eosin.

2.3.7 Statistical analysis

Statistical tests were performed using the software package SPSS v14.0.0 (SPSS Inc., Chicago, IL, USA). Differences in expression between corneas transduced with different vectors and subjected to *in vitro* organ culture were analysed using the 2-tailed, unpaired t-test. Corneal graft survival data were analysed using the 2-tailed Mann-Whitney U-test, corrected for ties. The level of significance was set to $p=0.05$.

CHAPTER 3

LENTIVIRUS-MEDIATED

REPORTER GENE EXPRESSION *IN VITRO*

3.1 Abstract

Aims

To construct lentiviral vectors encoding a fluorescent reporter protein under the control of different internal promoters, and to characterise transgene expression in ovine and human corneal endothelium.

Methods

Four lentiviral vectors were produced, expressing the enhanced yellow fluorescent protein (eYFP) under the control of: the simian virus type-40 early promoter (LV-SV40-eYFP), the human cytomegalovirus immediate early promoter (LV-CMV-eYFP), the murine phosphoglycerate kinase promoter (LV-PGK-eYFP), and the human elongation factor-1α promoter (LV-EF-eYFP). Transduction rates and kinetics of reporter gene expression in transduced ovine and human corneas were measured by fluorescence microscopy following organ culture. The influence of the cationic polymer polybrene on lentiviral transduction efficiency was investigated. An adenoviral vector expressing the enhanced green fluorescent protein (Ad-GFP) under the control of the CMV promoter was tested as a positive control in ovine corneas.

Results

The lentiviral vector LV-SV40-eYFP achieved >80% transduction of ovine and human corneal endothelial cells after a 24 h transduction period in the absence of polybrene. However, evidence of maximal transgene expression was not observed in ovine corneal endothelium until 14 d post-transduction. The kinetics of lentivirus-mediated transgene expression varied between the species, with ovine corneal endothelium showing a relative delay in detectable reporter gene expression compared with human corneal endothelium. Lentivirus-mediated expression in ovine and human corneal endothelium was stable for up to 21 d and 14 d, respectively.

LV-PGK-eYFP and LV-EF-eYFP were not significantly more effective in transducing ovine corneas than LV-SV40-eYFP. However, LV-CMV-eYFP achieved a significantly higher level of expression than LV-SV40-eYFP at 4 d post-transduction ($p<0.05$), and also appeared to be the strongest promoter in human corneas. Reducing the transduction period in ovine corneas from 24 h to 2 h resulted in a significant decrease in final transduction rate. Addition of polybrene to the transduction mixture increased the final transduction rate achieved following the shorter transduction period. High doses of polybrene (40 µg/ ml) did not appear to cause toxicity *in vitro*, as judged by cell count and nuclear morphology. The adenoviral vector transduced >80% of ovine corneal endothelial cells, with expression evident within 4 d of a 2 h transduction period.

Conclusions

Lentivirus-mediated reporter gene expression was delayed in ovine corneal endothelium compared to human, but in both species the final transduction rate was >80% and expression was stable for at least 14 d *in vitro*. Adenovirus-mediated reporter gene expression in ovine corneal endothelial cells was more efficient than that achieved by the lentiviral vector. Lentivirus-mediated expression in ovine and human corneal endothelium was higher with the viral SV40 and CMV promoters in comparison to the mammalian PGK and EF promoters.

3.2 Introduction

Expression of a fluorescent intracellular reporter protein is a valuable means of analysing the ability of a gene delivery method to transfer genes to a target tissue. It allows detection of the onset of expression, measurement of the kinetics of expression, and an indication of stability of expression. Reporter genes are particularly useful when comparing the performance of different internal promoters, although if expression is generally high, low doses of vector may be required to detect differences between constructs. Adenoviral vectors expressing a reporter gene under the control of the human cytomegalovirus (CMV) immediate early promoter have previously been shown to achieve efficient transduction and high levels of expression, in both ovine[71] and human corneal endothelial cells.[97,108] I am not aware of any reports of lentivirus-mediated gene transfer to ovine corneal endothelium, and such studies in human corneal endothelium have either not quantified reporter gene expression,[140] or reported relatively low transduction rates.[144,145]

As the first step in determining the ability of the Anson lentiviral vector to produce efficient, long-term transgene expression in corneal endothelial cells, I produced vectors encoding the enhanced yellow fluorescent reporter protein (eYFP) under the control of different constitutive internal promoters. Two strong viral promoters—the simian virus type-40 (SV40) early promoter and the human CMV immediate early promoter—and two mammalian promoters, the murine phosphoglycerate kinase (PGK) and the human elongation factor-1α (EF) promoters, were assessed. The vectors were tested in both ovine and human corneal endothelium. Ovine corneas transduced with an adenoviral vector encoding enhanced green fluorescent protein (eGFP) under the control of the CMV promoter were also

examined, to provide data against which to evaluate the performance of the lentiviral vector.

With a view to conducting studies of orthotopic corneal transplantation in sheep using donor corneas transduced *ex vivo*, aspects of the transduction process were assessed for their effect on final transduction rate; it was considered that a short transduction period might facilitate transduction and transplantation on the same day. The cationic polymer polybrene is routinely used for improving retrovirus transduction in cell culture assays, and has also been used in lentiviral transduction of mouse corneal endothelium.[83] Therefore, transduction period duration and the addition of polybrene to the transduction mixture were investigated to determine their influence on final transduction rate.

3.3 Specific aims

- To construct and produce lentiviral vectors encoding a reporter gene under the control of different internal promoters;
- To assess the ability of the HIV-1-based Anson lentiviral vector encoding a reporter gene, to transduce the ovine and human corneal endothelium;
- To investigate the influence of constitutive viral and mammalian promoters on lentivirus-mediated transgene expression in corneal endothelium;
- To study the effect of transduction period duration and the use of polybrene, on lentivirus-mediated transgene expression in ovine corneal endothelium;
- To determine the *in vitro* stability of reporter gene expression following transduction of ovine and human corneal endothelium with the Anson vector.

3.4 Results

3.4.1 Production of lentiviral vectors containing different internal promoters

Lentiviral vectors expressing eYFP under the control of the SV40, EF, PGK, and CMV promoters were produced as described in Section 2.3.4 (pages 72–81), using the corresponding vector plasmids: pHIV-1-SDm-SV-eYFP-ΔLTR, pHIV-1-SDm-EF-eYFP-ΔLTR, pHIV-1-SDm-PGK-eYFP-ΔLTR and pHIV-1-SDm-CMV-eYFP-ΔLTR. The first three plasmids were kindly provided by Associate Professor D.S. Anson, Department of Genetic Medicine, Women's and Children's Hospital, Adelaide, SA. To produce pHIV-1-SDm-CMV-eYFP-ΔLTR, the 580 bp sequence encoding the human CMV immediate early promoter was first amplified from the adenoviral shuttle vector pAdTrack-CMV by PCR using the primers CMVbamfor and CMVclarev (Table 2.2). This insert was then cloned into the plasmid pHIV-1-SDm-SV-eYFP-ΔLTR, in place of the SV40 promoter sequence, between the unique restriction sites *Bam*HI and *Cla*I (Figure 3.1). A PCR of potential clones, using the primers CMVbamfor and CMVclarev, confirmed successful ligation (Figure 3.2(a)). Plasmid mini-preps were carried out on four of the clones and subsequent *Bam*HI/*Cla*I and *Eco*RI/*Pst*I restriction enzyme digestions demonstrated the expected liberation of a 580 bp fragment and a 969 bp fragment respectively, from the vector DNA, in three cases (Figure 3.2(b)). The DNA from one of these clones was used for production of LV-CMV-eYFP. The vectors containing the other promoters were referred to as: LV-SV40-eYFP, LV-PGK-eYFP and LV-EF-eYFP. All virus preparations achieved final titres of 10^8–10^9 A549 transducing units (TU) per ml.

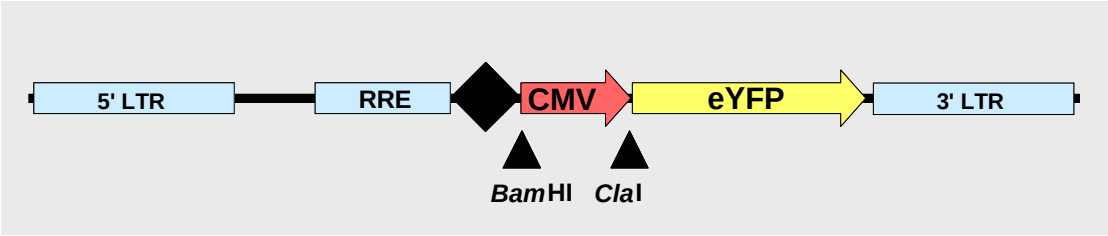
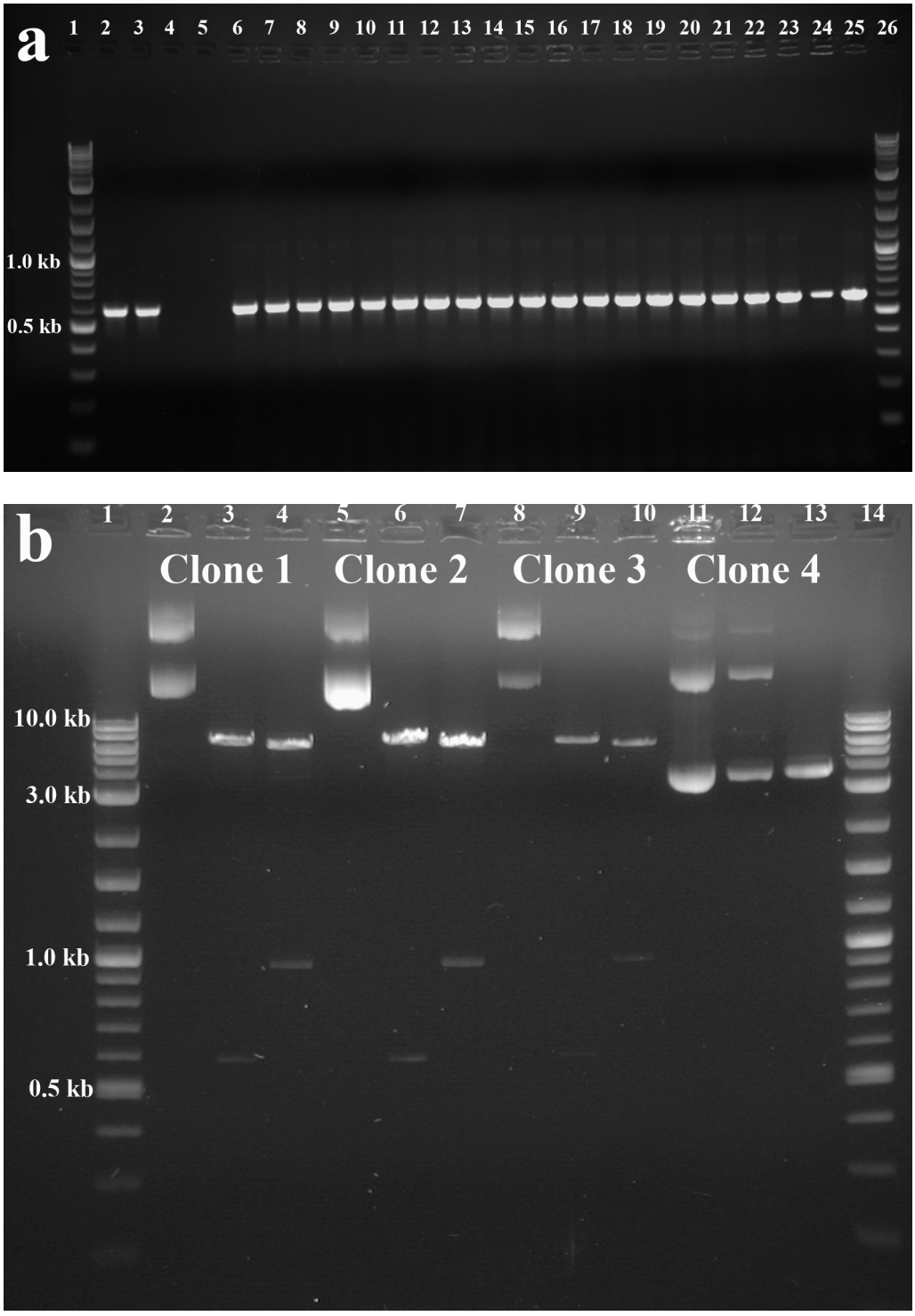


Figure 3.1: Schematic representation showing cloning of the CMV promoter sequence into the lentiviral vector plasmid

The 580 bp sequence containing the CMV promoter was ligated into the vector pHIV-1-SDm-SV40-eYFP, after cutting the vector with the restriction enzymes *Bam*HI and *Cla*I to excise the simian virus type-40 (SV40) promoter sequence. LTR: long terminal repeat, RRE: rev response element, diamond symbol: central polypurine tract, CMV: cytomegalovirus, eYFP: enhanced yellow fluorescent protein.

Figure 3.2: Confirmation of cloning the CMV promoter sequence into the lentiviral vector plasmid

(a) A photograph of an agarose gel shows products of polymerase chain reaction (PCR) carried out on 20 potential clones using the primers CMVbamfor and CMVclarev to amplify the cytomegalovirus (CMV) promoter sequence, and reveals a fragment of expected size (580 bp) in all cases; lane 1 contains 2 log ladder DNA marker; lanes 2–3 contain positive control DNA for CMV promoter; lanes 4–5 contain PCR negative controls; lanes 6–25 contain clones 1–20; lane 26 contains 2 log ladder DNA marker. (b) A photograph of an agarose gel, following restriction digestion of four potential clones identified by the PCR, using *Bam*HI and *Cla*I (lanes 3, 6, 9, 12), and *Eco*RI and *Pst*I (lanes (4, 7, 10, 13): lanes 1 and 14 contain 2 log ladder DNA marker; lanes 2, 5, 8 and 11 contain uncut clone DNA; clone DNA is labelled 1 to 4. *Bam*HI/*Cla*I digestion liberated a 580 bp fragment, and *Eco*RI/*Pst*I digestion liberated a 969 bp fragment, indicating successful ligation in clones 1, 2 and 3.


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
1.0 kb
0.5 kb
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Clone 1
Clone 2
Clone 3
Clone 4
10.0 kb
3.0 kb
1.0 kb
0.5 kb

3.4.2 Transduction of ovine corneas with an adenoviral vector

Adenoviral vectors have previously been shown to achieve efficient transduction of corneal endothelium. Therefore an initial experiment using adenovirus to transduce ovine corneal endothelium was conducted as a positive control with which to compare further studies of lentivirus-mediated reporter gene expression. An adenoviral vector expressing the enhanced green fluorescent protein (eGFP) under the control of the human CMV immediate early promoter (Ad-GFP) was produced as described in Chapter 2 (pages 66–72). The transduction conditions for Ad-GFP were based on previous studies in the laboratory.[71] A 2 h transduction period was used and, in view of the variation in titre inherent in adenoviral vector preparations, doses were tested over a range from 1×10^6–1×10^7 pfu per cornea.

The titre of purified Ad-GFP vector, as determined by the $TCID_{50}$ method and converted into pfu per ml, was 2.5×10^9. Intense expression of eGFP was achieved by the vector within 4 d of transduction (Figure 3.3). Doses of 5×10^6–1×10^7 pfu per cornea (MOIs of 5–10) resulted in expression by >80% of endothelial cells after a 2 h transduction period (Figure 3.4). Although there was a reduction in endothelial cell density compared with nontransduced tissue as doses of adenoviral vector increased, the variation was not statistically significant (2-tailed, unpaired t-test: $p>0.05$).

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

Having established that adenoviral transduction of corneal endothelium resulted in expected rapid and efficient expression, initial transduction experiments were carried out in the same system using the lentiviral vector LV-SV40-eYFP. Ovine and human

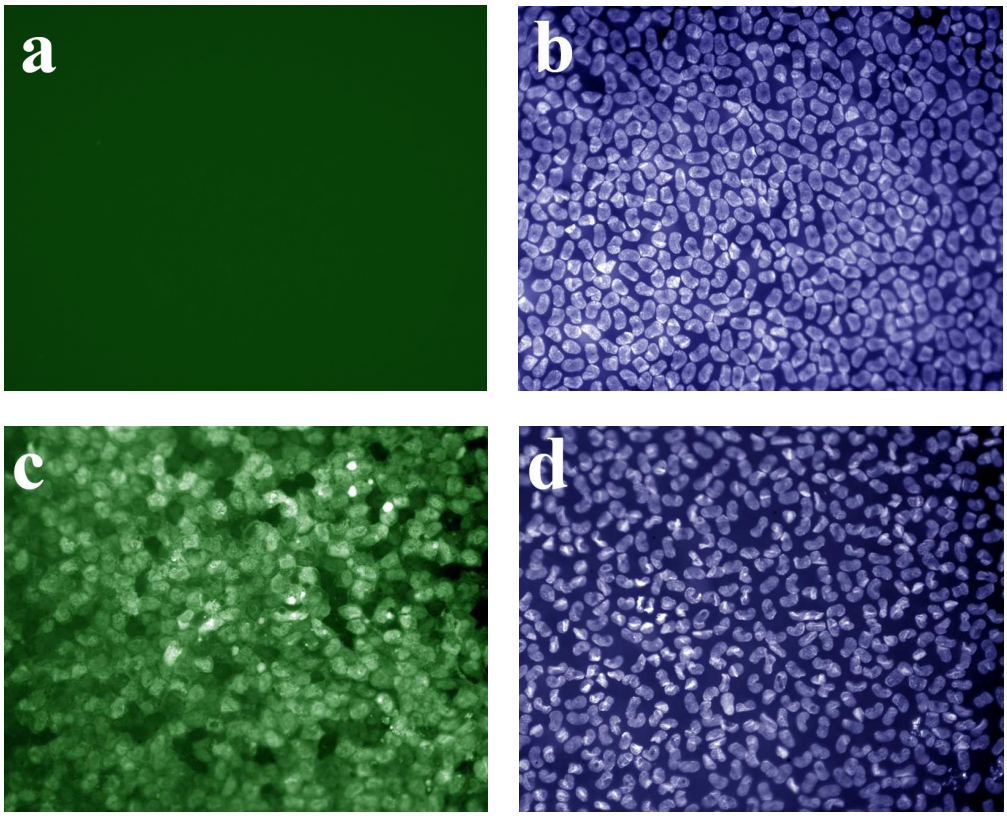
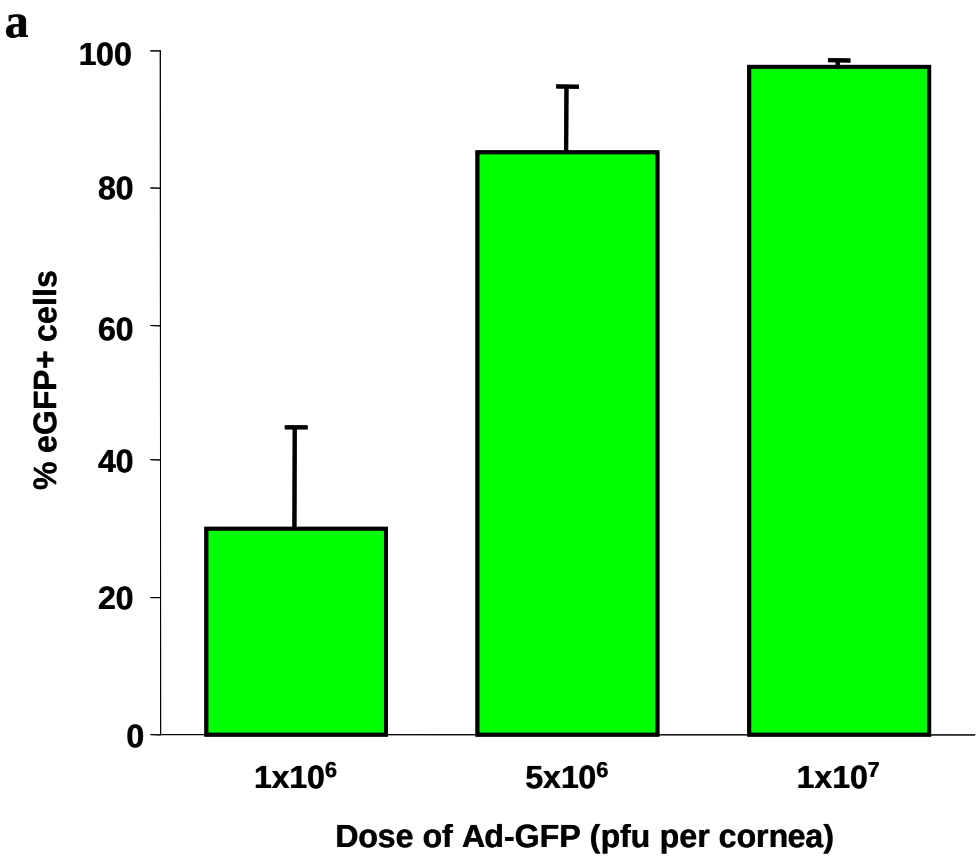


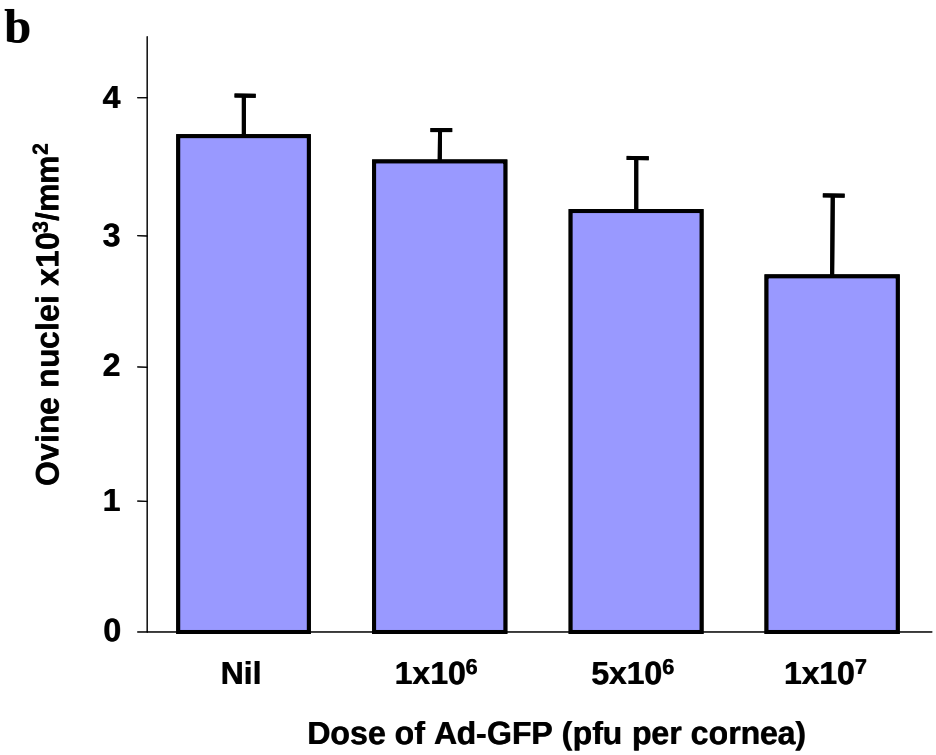
Figure 3.3: Expression of eGFP by ovine corneal endothelium transduced with Ad-GFP

Ovine corneas were transduced for 2 h with (a, b) medium only, or (c, d) 5×10^6 pfu per cornea of Ad-GFP (MOI 5), and subjected to 4 d of *in vitro* organ culture. Fluorescence micrographs of representative central fields show expression of: (a, c) enhanced green fluorescent protein (eGFP) with blue light excitation, and (b, d) endothelial cell nuclei stained with Hoechst-33258 under ultraviolet illumination of each respective field. Original magnification 20×.

Figure 3.4: Transduction of ovine corneal endothelium with increasing doses of Ad-GFP

Corneas were transduced for 2 h with increasing doses of Ad-GFP and subjected to *in vitro* organ culture. After 4 d the corneas were fixed, and the corneal endothelia were stained with Hoechst-33258 and flat-mounted for fluorescence microscopy. Columns show: (a) the percentage of endothelial cells expressing enhanced green fluorescent protein (eGFP) (mean±SD) as determined by fluorescence microscopy, and (b) the respective endothelial cell density (mean±SD) as determined by counting Hoechst-33258-stained nuclei under ultraviolet illumination. For each dose, three corneas were examined. No eGFP expression was observed in nontransduced control corneas (data not shown). SD: standard deviation of the mean.


a
100
80
60
40
20
0
% eGFP+ cells
1x10⁶
5x10⁶
1x10⁷
Dose of Ad-GFP (pfu per cornea)


b
4
3
2
1
0
Ovine nuclei x10³/mm²
Nil
1x10⁶
5x10⁶
1x10⁷
Dose of Ad-GFP (pfu per cornea)

corneal endothelia were transduced for 24 h with 2.5×10^7 TU per cornea of LV-SV40-eYFP, and harvested for fluorescence microscopy of reporter gene expression after *in vitro* organ culture (Figure 3.5). A 24 h transduction period was tested initially, since it has been reported that maximal retroviral infection requires prolonged exposure of target cells to the virus.[167] Approximately 84% of ovine corneal endothelial cells expressed eYFP after 14 d of organ culture, and this level persisted at 21 d. Over 80% of human corneal endothelial cells were transduced by LV-SV40-eYFP after 14 d of culture. According to the method described in Chapter 2 (pages 82–83), the multiplicity of infection (MOI) of lentivirus transducing units per corneal endothelial cell at a dose of 2.5×10^7 TU per cornea, was estimated as being approximately 20 for the sheep and 120 for the human.

3.4.4 Kinetics of eYFP expression in ovine and human corneal endothelium

Having established a final transduction rate of >80% in both ovine and human corneas transduced with LV-SV40-eYFP, the kinetics of transgene expression in the corneal endothelium of each species was assessed. Corneas were transduced for 24 h with 2.5×10^7 TU per cornea LV-SV40-eYFP, and harvested for fluorescence microscopy to demonstrate reporter gene expression after increasing periods of *in vitro* organ culture. Human and ovine corneas can survive for between 14–21 d in organ culture following transduction. Therefore, this period was set as a limit for assessing transgene expression. Ovine corneal endothelium showed a relative delay in eYFP expression after transduction, but expression in >80% of endothelial cells was achieved by 14 d with no reduction in endothelial cell density (Figure 3.6).

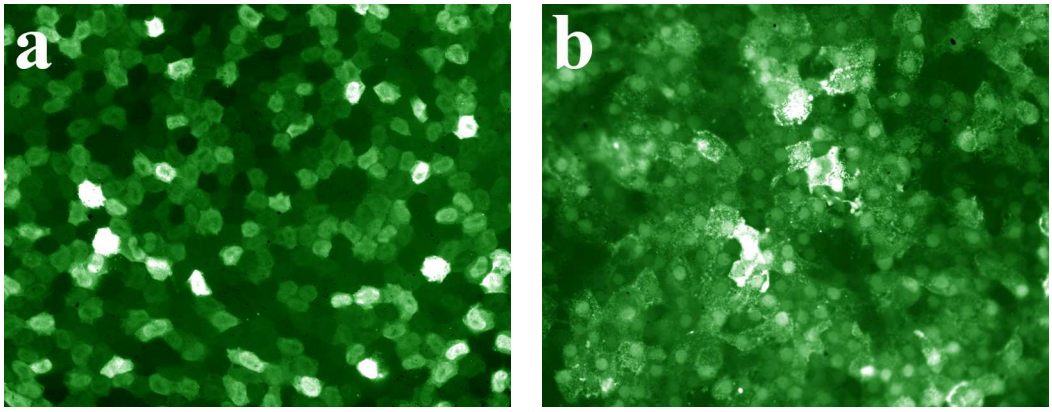


Figure 3.5: Expression of eYFP by ovine and human corneal endothelium following transduction with LV-SV40-eYFP

Corneas were transduced for 24 h with 2.5×10^7 TU per cornea of LV-SV40-eYFP. Fluorescence micrographs show representative central fields of (a) ovine corneal endothelium (MOI 20), and (b) human corneal endothelium (MOI 120), examined by fluorescence microscopy after 14 d of *in vitro* organ culture. eYFP: enhanced yellow fluorescent protein. Original magnification 20×.

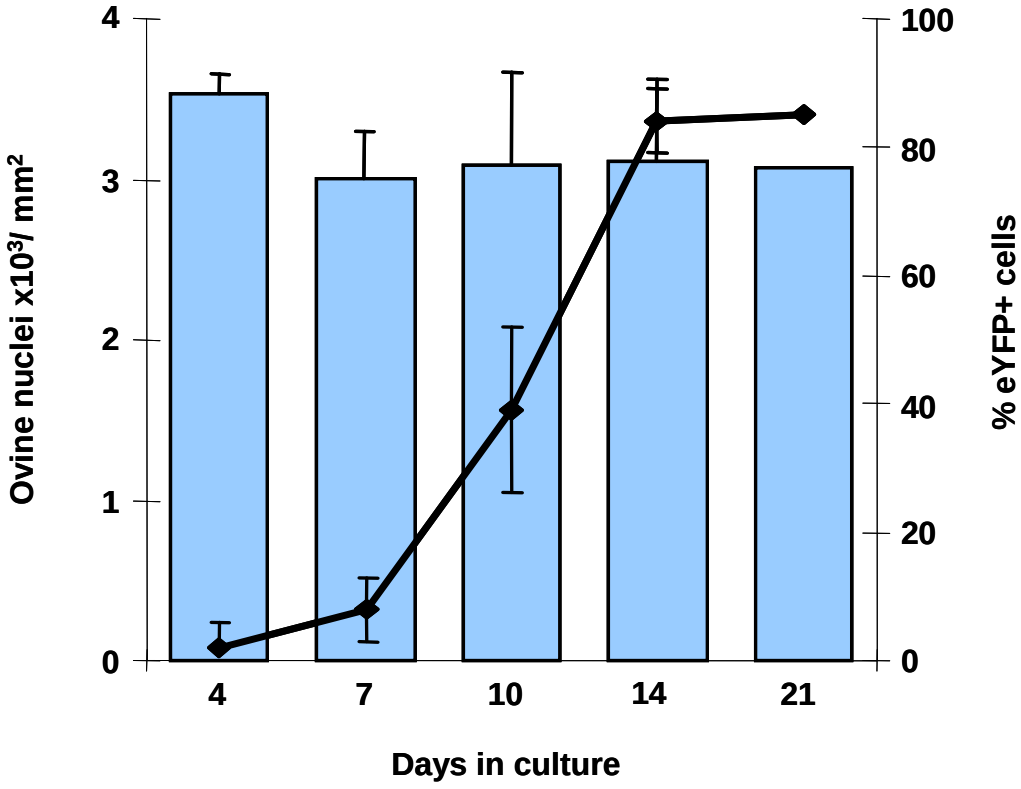


Figure 3.6: Kinetics of eYFP expression in ovine corneal endothelium transduced with LV-SV40-eYFP

Ovine corneas were transduced for 24 h with 2.5×10^7 TU per cornea of LV-SV40-eYFP (MOI 20), and harvested for fluorescence microscopy after increasing periods of *in vitro* organ culture. Columns show the corneal endothelial nuclei density (mean±SD) as determined by counting Hoechst-33258-stained nuclei under ultraviolet illumination. Points represent the percentage of corneal endothelial cells (mean±SD) expressing enhanced yellow fluorescent protein (eYFP) as determined by fluorescence microscopy. At each time point, three corneas were examined (except at 21 d, where n=1). SD: standard deviation of the mean.

In human corneas, eYFP expression peaked within 1–4 d of transduction, and persisted for 14 d in organ culture (Figure 3.7(a)). Endothelial cell density did not vary according to time following transduction (Figure 3.7(b)). Differences were evident between individual human corneas, probably reflecting factors such as donor age and time in Eye Bank storage, rather than vector-associated toxicity; all human corneas had been discarded from the Eye Bank as being unsuitable for transplantation. Intense expression of eYFP was observed in Eye Bank corneas which had been subjected to hypothermic storage in Optisol® GS for up to 25 d.

3.4.5 Effect of multiplicity of infection on transgene expression

Higher multiplicities of infection in ovine cornea

To investigate whether higher MOIs would result in more rapid expression of the reporter gene in ovine corneal endothelial cells, transduction with LV-SV40-eYFP at MOIs of 75, 150 and 300 was investigated. Some increase in the rate of expression of eYFP was seen but expression was still relatively delayed compared with the rate observed in human corneas. At a dose of 4×10^8 TU per cornea (MOI 300), expression of eYFP was achieved in 80% of ovine corneal endothelial cells within 4 d (Figure 3.8(a)). However, at 7 d post-transduction, all MOIs tested (75, 150 and 300) were associated with a reduction in corneal endothelial cell density (Figure 3.8(b)).

Lower multiplicities of infection in human cornea

To investigate whether a dose of lentivirus lower than that used previously (MOI 120) would be effective in human corneas, transduction with 6.3×10^6 TU of LV-SV40-eYFP, equivalent to an MOI of 30, was examined (Figure 3.7). Transduction at an MOI of 30 produced proportionally lower reporter gene expression compared to

Figure 3.7: Kinetics of eYFP expression in human corneal endothelium following transduction with LV-SV40-eYFP

Eye Bank 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, and harvested after 1 d, 4 d or 14 d of *in vitro* organ culture. (a) The percentage of corneal endothelial cells (mean±SD) expressing enhanced yellow fluorescent protein (eYFP) was determined by fluorescence microscopy, and (b) the corresponding corneal endothelial nuclei density (mean±SD) was determined by counting Hoechst-33258-stained nuclei under ultraviolet illumination. A total of seven corneas were examined; each column represents the mean±SD of five fields examined for a different cornea at each time point, and a unique colour is used for each individual cornea. Numbers above the columns represent the number of days the cornea had been stored in the Eye Bank, before being transduced. SD: standard deviation of the mean.

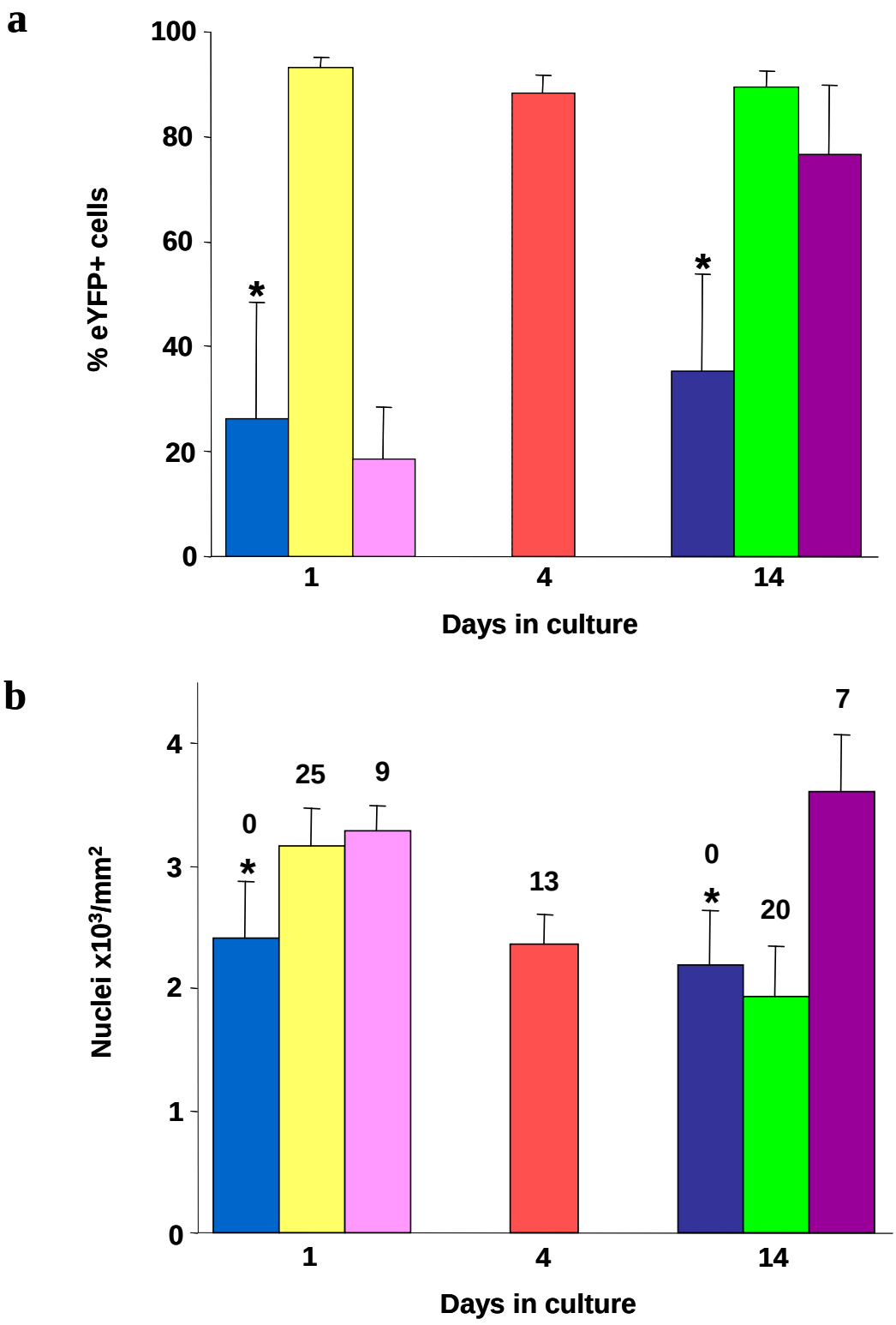
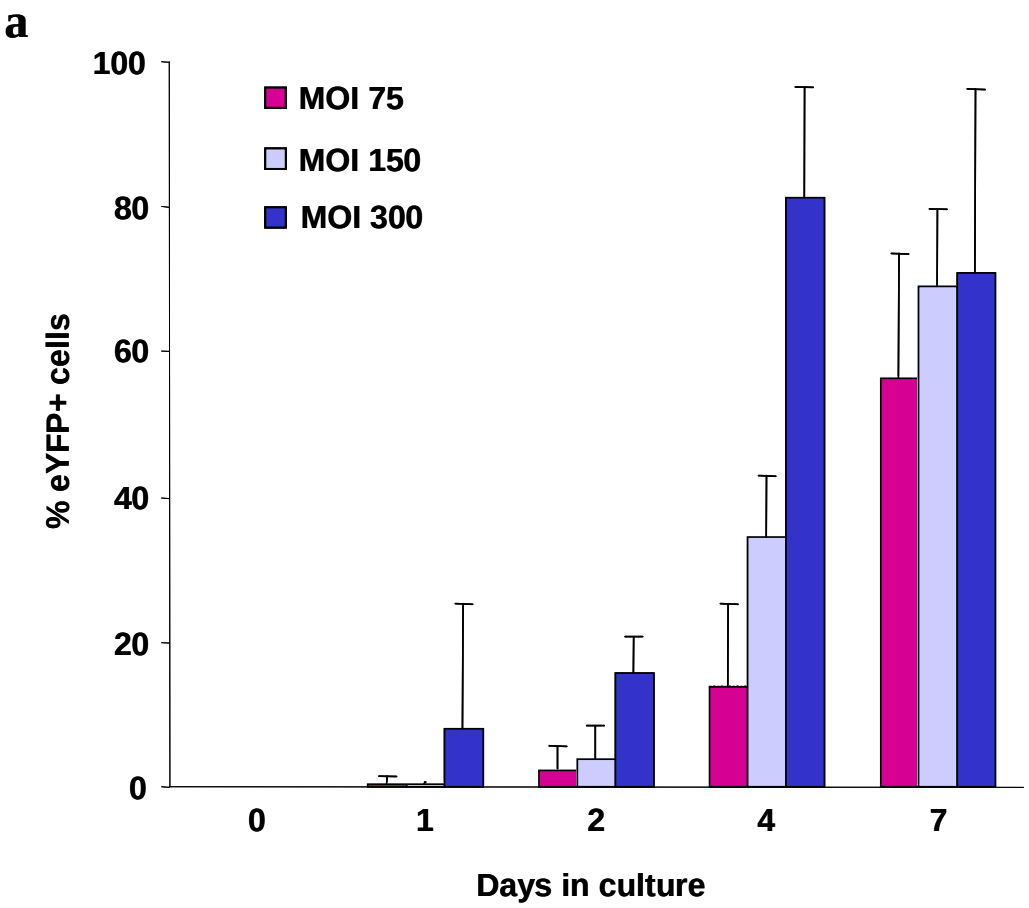
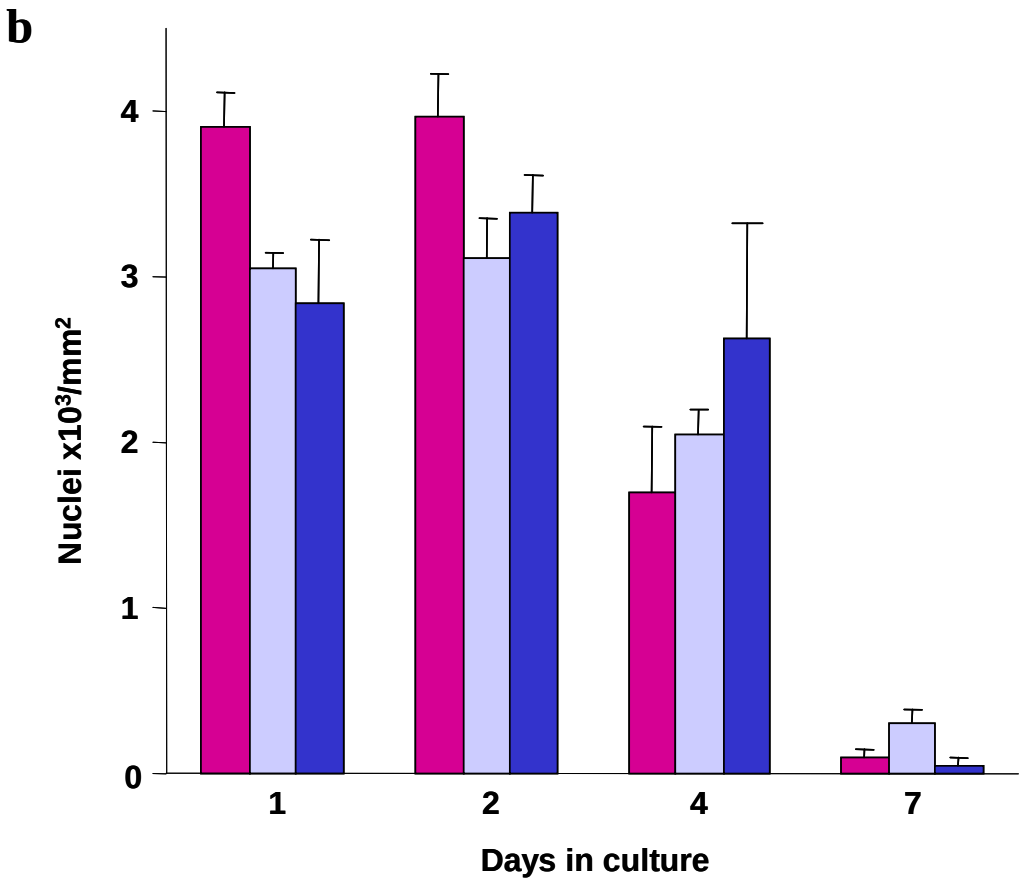

a
100
80
60
40
20
0
% eYFP+ cells
*
*
1
4
14
Days in culture
b
4
3
2
1
0
Nuclei x10³/mm²
0
25
9
13
0
20
7
*
*
1
4
14
Days in culture

Figure 3.8: Effect of high multiplicities of infection (MOIs) on eYFP expression and corneal endothelial cell density, following transduction of ovine corneal endothelium with LV-SV40-eYFP

Ovine corneas were transduced for 24 h with increasing MOIs of LV-SV40-eYFP and harvested at various time points following transduction. (a) The percentage of corneal endothelial cells expressing eYFP was determined by fluorescence microscopy, and (b) the corneal endothelial cell density in each sample was determined by counting Hoechst-33258-stained nuclei under ultraviolet illumination. Each column represents the mean±SD of five fields examined for a single cornea. eYFP: enhanced yellow fluorescent protein, SD: standard deviation of the mean.


a
100
80
60
40
20
0
MOI 75
MOI 150
MOI 300
% eYFP+ cells
0
1
2
4
7
Days in culture


b
4
3
2
1
0
Nuclei x10³/mm²
1
2
4
7
Days in culture

transduction at an MOI of 120, but maximal expression was achieved after 1 d with no apparent effect on corneal endothelial cell density.

3.4.6 Effect of transduction period on maximal expression in ovine corneas

To investigate the feasibility of carrying out *ex vivo* transduction and transplantation of ovine corneas on the same day, the duration of the transduction period was reduced from 24 h to 2 h. Ovine corneas transduced with LV-SV40-eYFP at an MOI of 20 were harvested for fluorescence microscopy after 14 d of *in vitro* organ culture. The transduction rate for the 2 h transduction period was lower than that achieved following a 24 h period: 10.2% ± 5.3% (mean±SD) versus 83.9% ± 4.5% respectively. The difference was statistically significant (2-tailed, unpaired t-test: $p<0.05$) (Figure 3.9).

3.4.7 Effect of polybrene on transduction rate in ovine corneas

In view of the striking difference in transduction rate following a 2 h transduction period compared to a 24 h period, the polycation polybrene was explored as a means of improving transduction rate achieved during the shorter transduction period. An initial study was performed to identify any direct toxic effect of polybrene on the corneal endothelium over a range of doses. Polybrene is generally used in cell culture transductions at 4–8 µg / ml. Corneas were incubated for 3 h in 2% RPMI, with either no polybrene, or polybrene at a concentration of 4 µg / ml or 40 µg / ml. After a further 4 d of *in vitro* organ culture, corneas were fixed, stained with Hoechst-33258 and examined by fluorescence microscopy. No evidence of a direct toxic effect was seen, either in the form of an increase in apoptotic nuclei, or a gross

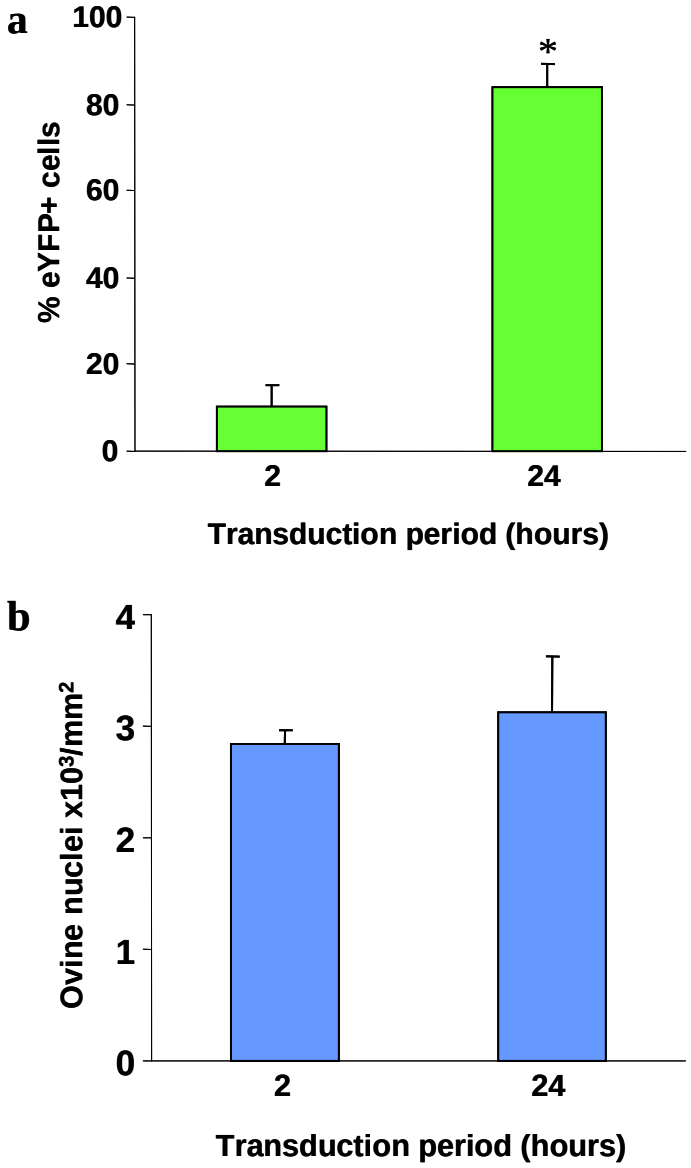


Figure 3.9: Influence of the duration of transduction on eYFP expression in ovine corneal endothelium following transduction with LV-SV40-eYFP

Ovine corneal endothelia were transduced with 2.5×10^7 TU per cornea of LV-SV40-eYFP (MOI 20) for either 2 h or 24 h, and harvested after 14 d of *in vitro* organ culture. Each column represents (a) the percentage of corneal endothelial cells expressing enhanced yellow fluorescent protein (eYFP) (mean±SD) as determined by fluorescence microscopy, and (b) the respective endothelial cell density (mean±SD) determined by counting Hoechst-33258-stained nuclei under ultraviolet illumination. At least three corneas were examined in each group. * 2-tailed, unpaired t-test: $p<0.05$, SD: standard deviation of the mean.

reduction in endothelial cell density (Figure 3.10). Therefore, the higher dose was used in transduction studies. Next, polybrene was added to a 2% RPMI transduction mixture at a concentration of 40 µg / ml, this time with 2.5×10^7 TU per cornea of LV-SV40-eYFP (MOI 20). Corneas were incubated for 3 h before the addition of 10 ml 10% RPMI and a further 14 d of *in vitro* organ culture. Corneas transduced in the presence of polybrene showed a higher transduction rate compared to those without polybrene (approximately 80% versus 10% respectively) (Figure 3.11).

3.4.8 Influence of internal promoter on reporter gene expression

Ovine cornea

To investigate the influence of the internal promoter on lentivirus-mediated reporter gene expression, ovine corneas were transduced for 24 h with either LV-PGK-eYFP, LV-SV40-eYFP, LV-EF-eYFP or LV-CMV-eYFP at 1×10^7 TU per cornea (MOI 8) and examined after 4 d of *in vitro* organ culture. This time point was chosen in view of the relative delay in expression observed in earlier experiments in which LV-SV40-eYFP was used. Low levels of expression of eYFP were observed in corneas transduced with LV-PGK-eYFP and LV-SV40-eYFP, whilst expression was virtually undetectable in corneas transduced with LV-EF-eYFP (Figure 3.12). Expression appeared strongest in corneas transduced with LV-CMV-eYFP. To investigate further, corneas were transduced for 24 h with either LV-SV40-eYFP or LV-CMV-eYFP at 2.5×10^7 TU per cornea (MOI 20; that is, at 2.5× the earlier dose) and again examined after 4 d of *in vitro* organ culture. Expression of eYFP was observed in 21±2% (mean±SD) of ovine corneal endothelial cells in corneas transduced with LV-CMV-eYFP, compared with 4±4% of cells in corneas

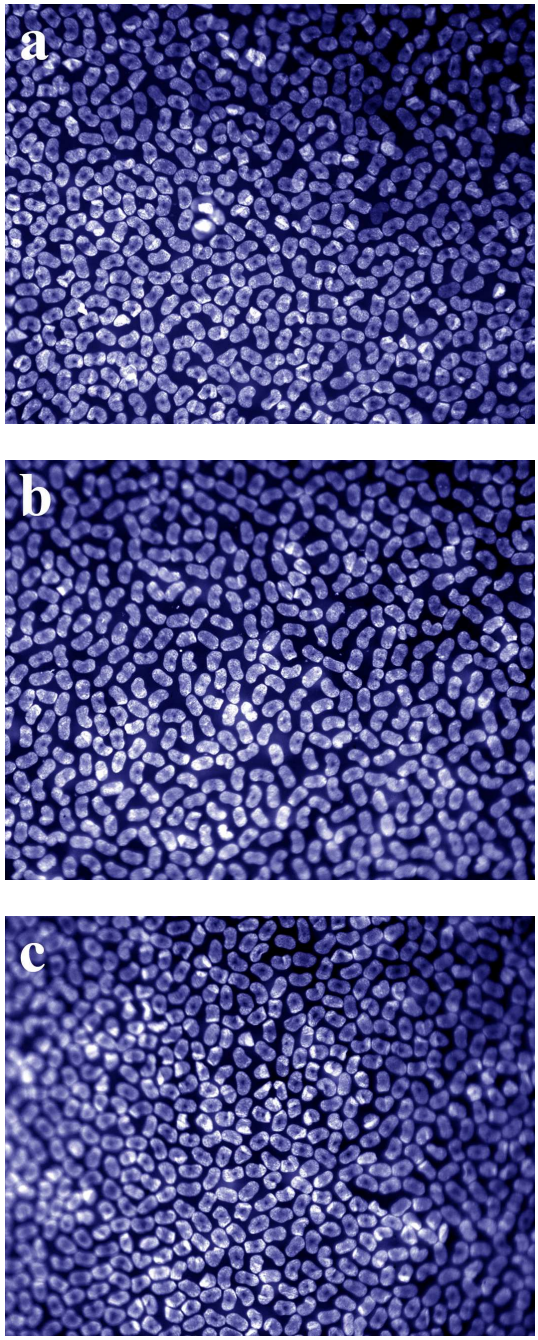


Figure 3.10: Density and nuclear morphology of ovine corneal endothelium following an incubation with polybrene

Ovine corneas were incubated for 3 h with various concentrations of polybrene: (a) nil, (b) 4 μg / ml and (c) 40 μg / ml. They were then harvested after 4 d of *in vitro* organ culture. Fluorescence micrographs of representative central fields show Hoechst-33258-stained endothelial cell nuclei taken under ultraviolet illumination. Original magnification 20×.

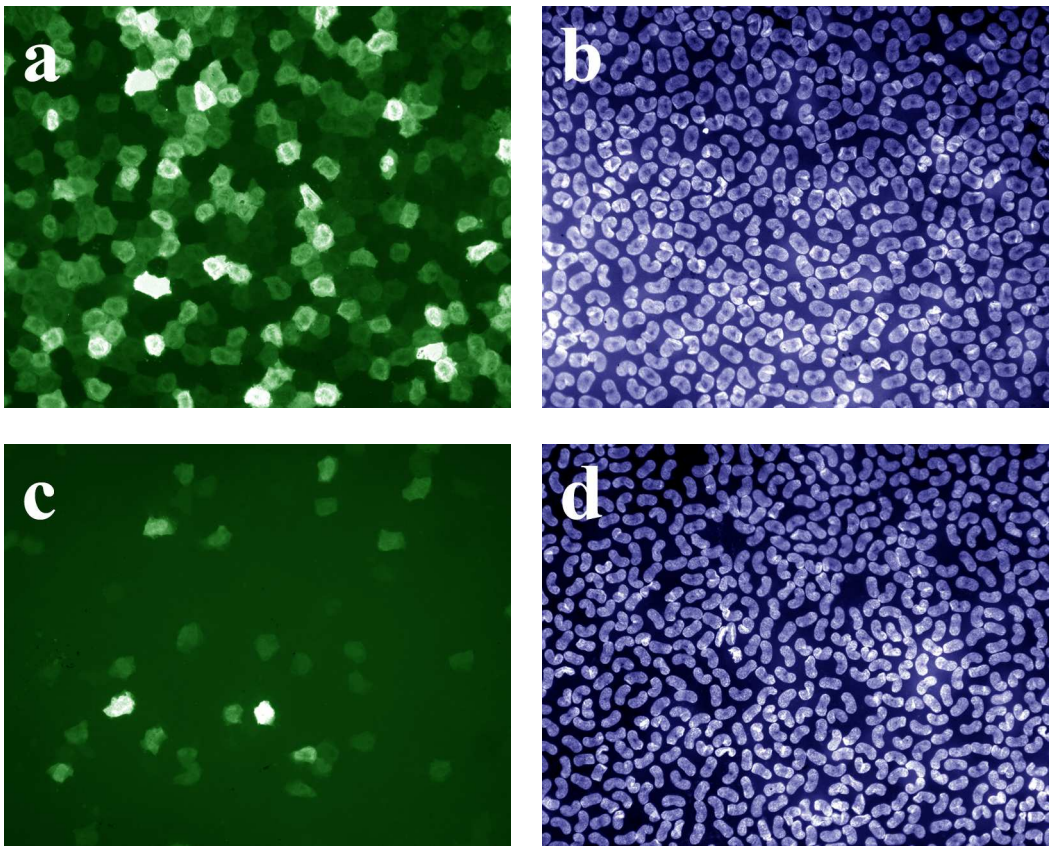
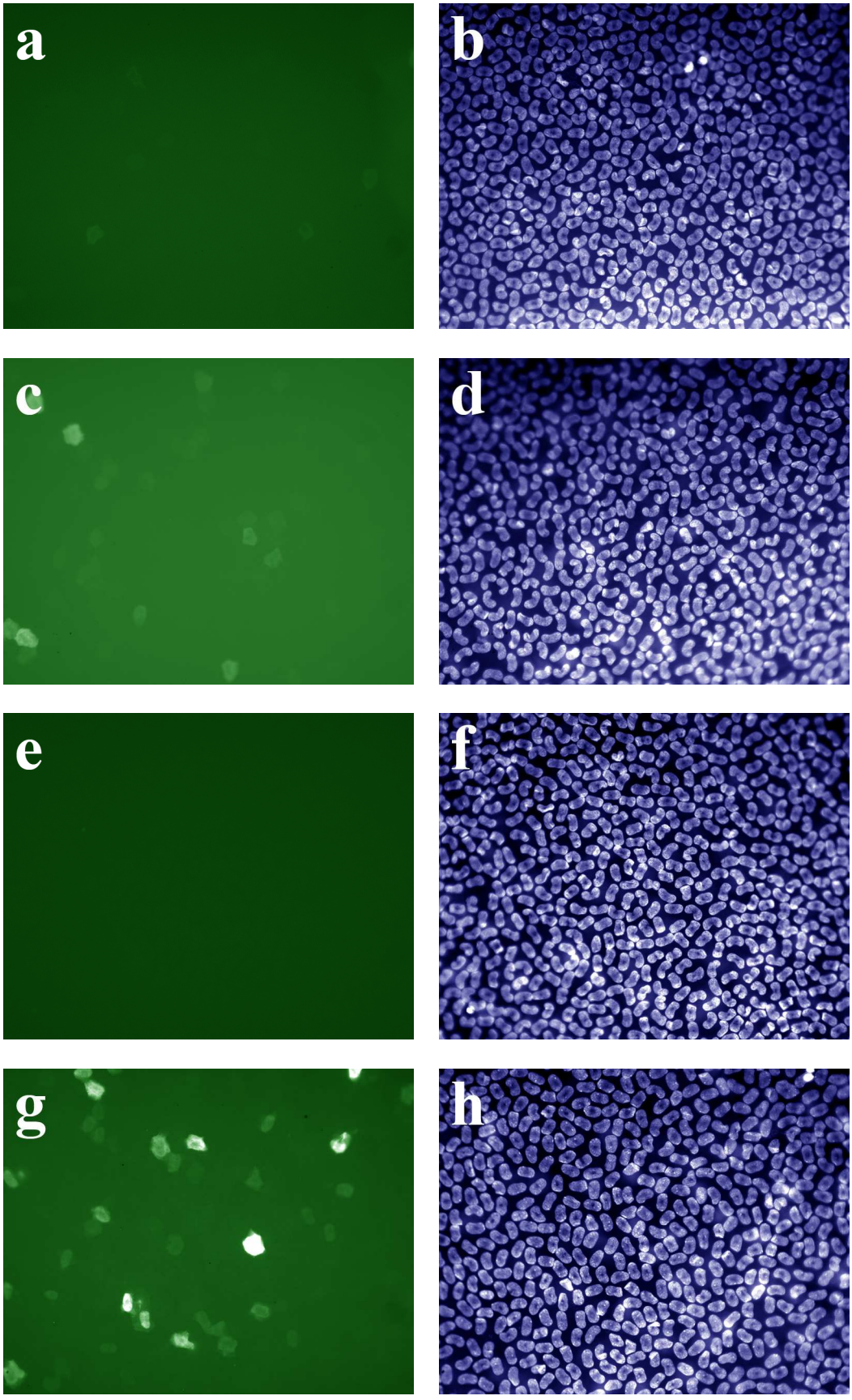


Figure 3.11: Effect of polybrene on transduction rate in ovine corneal endothelium following transduction with LV-SV40-eYFP

Corneas were transduced for 3 h with 2.5×10^7 TU per cornea of LV-SV40-eYFP (MOI 20) in (a, b) the presence or (c, d) the absence, of polybrene 40 μg / ml, and harvested after 14 d of *in vitro* organ culture. Fluorescence micrographs of representative central fields show (a, c) expression of enhanced yellow fluorescent protein (eYFP) under blue light excitation, and (b, d) endothelial cell nuclei stained with Hoechst-33258 under UV illumination, of each respective field. Original magnification 20×.

Figure 3.12: Expression of eYFP in ovine corneal endothelium following transduction with four lentiviral vectors containing different internal promoters

Ovine corneas were transduced for 24 h with 1×10^7 TU per cornea (MOI 8) of vectors containing either (a, b) the phosphoglycerate kinase promoter (LV-PGK-eYFP), (c, d) the simian virus type-40 early promoter (LV-SV40-eYFP), (e, f) the elongation factor-1α promoter (LV-EF-eYFP), or (g, h) the human cytomegalovirus immediate early promoter (LV-CMV-eYFP), and harvested 4 d after transduction. Fluorescence micrographs of representative central fields show expression of (a, c, e, g) enhanced yellow fluorescent protein (eYFP) under blue light excitation, and (b, d, f, h) Hoechst-33258-stained endothelial cell nuclei under ultraviolet illumination of each respective field. Original magnification 20×.


a
b
c
d
e
f
g
h

transduced with LV-SV40-eYFP (Figure 3.13). The difference between these expression levels reached statistical significance (2-tailed, unpaired t-test: $p<0.05$). There were no differences in endothelial cell density between the different constructs and nontransduced control corneas (2-tailed, unpaired t-test: $p>0.05$).

Human cornea

To investigate the influence of the internal promoter on lentivirus-mediated reporter gene expression in human corneas, Eye Bank corneas were transduced for 24 h with either LV-PGK-eYFP, LV-SV40-eYFP, LV-EF-eYFP or LV-CMV-eYFP at 6.3×10^6 TU per cornea (MOI 30) and examined after 1 d of *in vitro* organ culture. The earlier time point was chosen in view of the relatively rapid and intense expression observed in human corneal endothelium in earlier experiments. The LV-CMV-eYFP vector achieved expression in >80% of cells, and LV-SV40-eYFP in approximately 40% of cells. In the cases of LV-PGK-eYFP and LV-EF-eYFP, reporter gene expression was undetectable after 1 d of culture (Figure 3.14).

3.4.9 Kinetics of expression following LV-CMV-eYFP transduction

Ovine cornea

To assess the kinetics and stability of eYFP expression following transduction with the vector containing the CMV promoter, corneas were transduced with LV-CMV-eYFP at 2.5×10^7 TU per cornea (MOI 20) and cultured for up to 14 d. As observed in ovine corneas transduced with LV-SV40-eYFP, there appeared to be a delay in expression. However, a transduction rate of >80% was evident after 14 d of *in vitro* organ culture (Figure 3.15).

Figure 3.13: Comparison of eYFP expression in ovine corneal endothelium following transduction by lentiviral vectors containing either the simian virus type-40 (SV40) or cytomegalovirus (CMV) promoters

Ovine corneas were transduced for 24 h with 2.5×10^7 TU per cornea of LV-SV40-eYFP or LV-CMV-eYFP (MOI 20) and harvested 4 d after transduction. Control corneas were transduced with medium only. Each column represents the mean±SD for: (a) the percentage of endothelial cells expressing enhanced yellow fluorescent protein (eYFP) as determined by fluorescence microscopy, and (b) respective endothelial cell density as determined by counting of Hoechst-33258-stained nuclei under ultraviolet illumination. Three corneas were examined for each group. * 2-tailed, unpaired t-test: $p<0.05$ compared to SV40, SD: standard deviation of the mean.

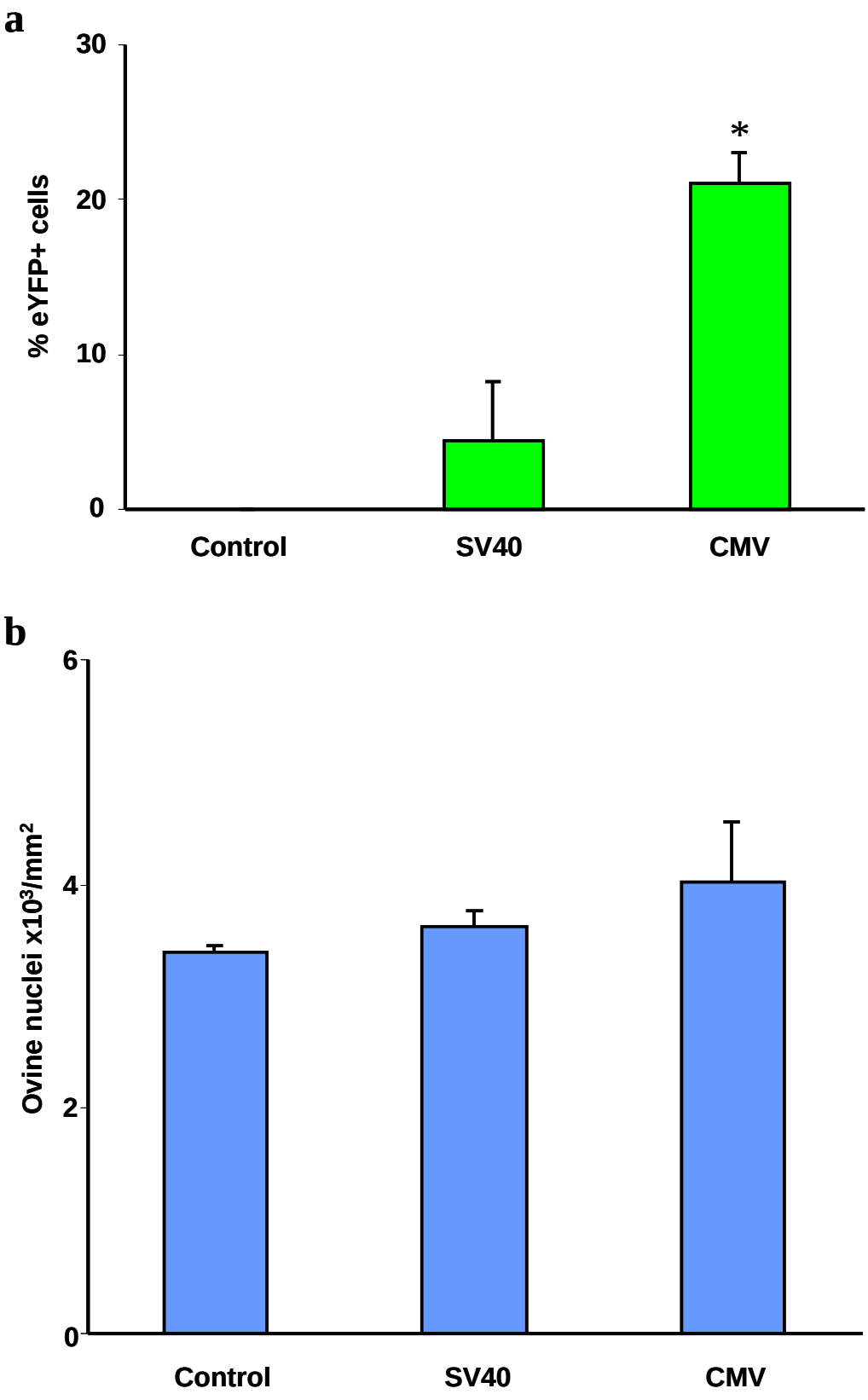
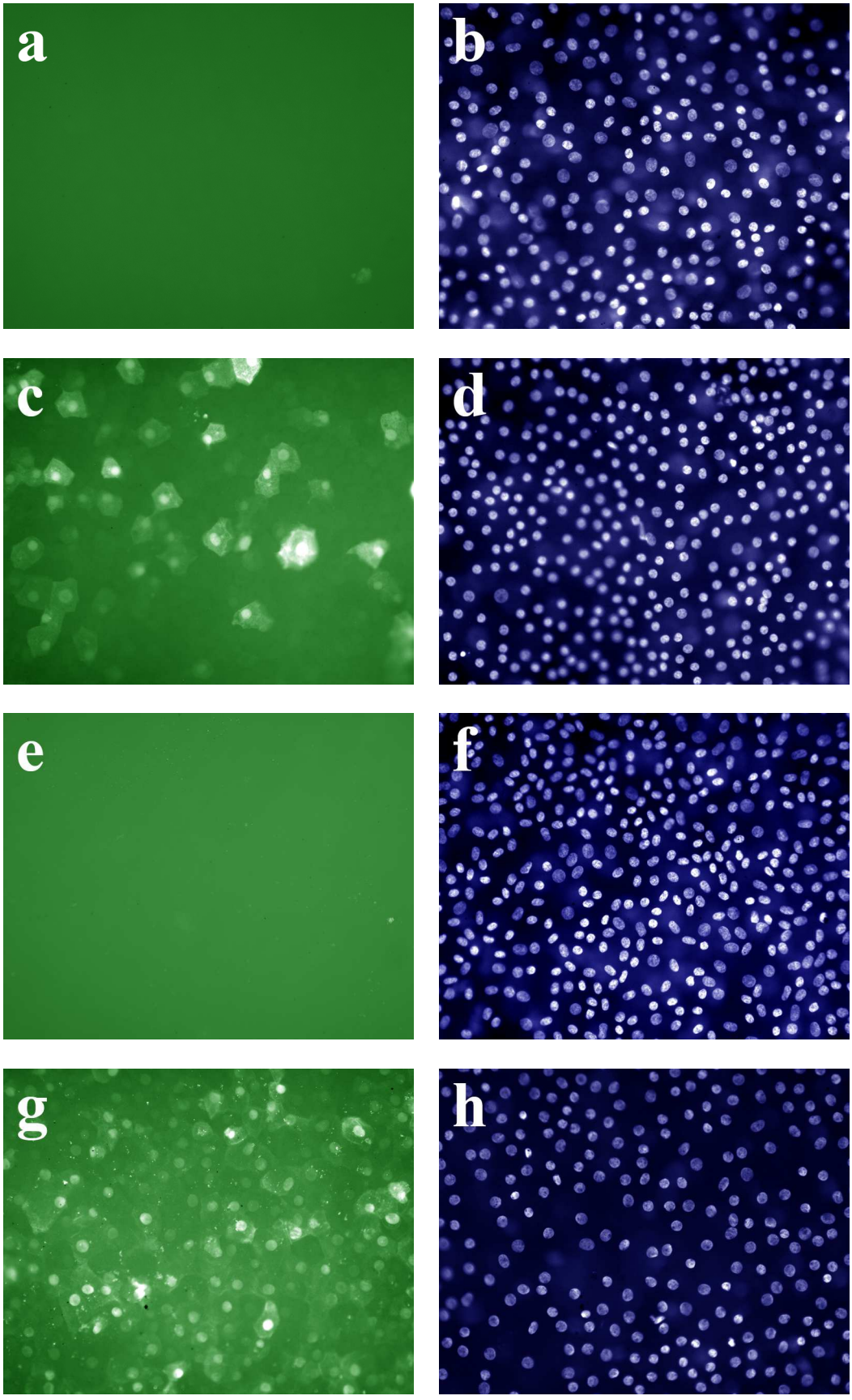

a
30
20
10
0
% eYFP+ cells
*
Control
SV40
CMV
b
6
4
2
0
Ovine nuclei x10³/mm²
Control
SV40
CMV

Figure 3.14: Expression of eYFP in human corneal endothelium following transduction with four lentiviral vectors containing different internal promoters

Human corneas were transduced for 24 h with 6.3×10^6 TU per cornea (MOI 30) of vectors containing either (a, b) the phosphoglycerate kinase promoter (LV-PGK-eYFP), (c, d) the simian virus type-40 early promoter (LV-SV40-eYFP), (e, f) the elongation factor-1α promoter (LV-EF-eYFP), or (g, h) the human cytomegalovirus immediate early promoter (LV-CMV-eYFP), and harvested 1 d after transduction. Fluorescence micrographs of representative central fields show (a, c, e, g) expression of enhanced yellow fluorescent protein (eYFP) under blue light excitation, and (b, d, f, h) Hoechst-33258-stained endothelial cell nuclei under ultraviolet illumination of each respective field. Original magnification 20×.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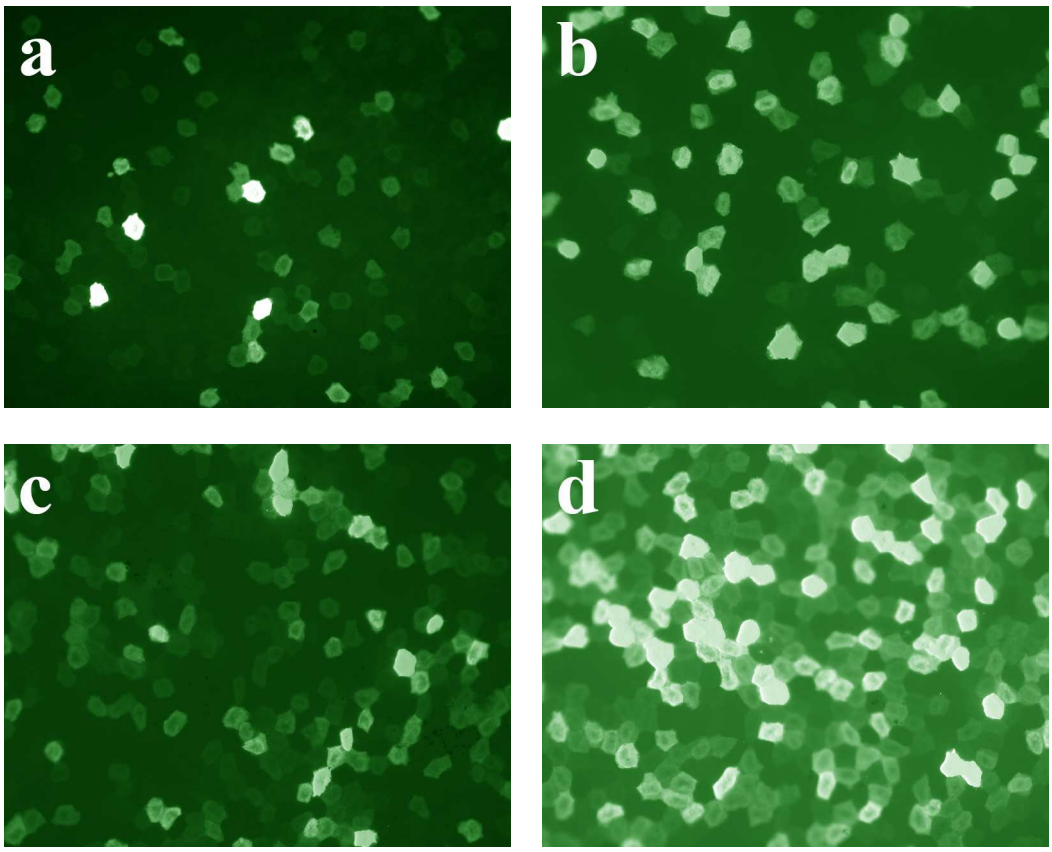


Figure 3.15: Expression of eYFP by ovine corneal endothelium following transduction with LV-CMV-eYFP

Ovine corneas were transduced for 24 h with 2.5×10^{7} TU per cornea (MOI 20). Fluorescence micrographs of representative central fields show enhanced yellow fluorescent protein (eYFP) expression in corneas harvested after (a) 4 d, (b) 7 d, (c) 10 d, and (d) 14 d of *in vitro* organ culture. Original magnification 20×.

Human cornea

A human cornea transduced with LV-CMV-eYFP at 6.3×10^6 TU per cornea (MOI 30) was harvested for examination after 15 d of *in vitro* organ culture. As previously seen at 1 d post-transduction with the same vector, reporter protein expression was evident in >80% cells, and the endothelium was intact (Figure 3.16).

3.5 Discussion

The major findings of these studies were that the Anson lentiviral vector transduced a high proportion of both ovine and human corneal endothelial cells, and demonstrated stable expression for up to two weeks *in vitro* using the SV40 and CMV promoters. The duration of the transduction period and the addition of polybrene to the transduction mixture, were both found to influence the final transduction rate in ovine corneal endothelium.

High transduction rates were achieved by lentiviral vectors

Lentiviral vectors pseudotyped with the VSV-G protein are capable of transducing a wide range of tissues including brain,[168] liver,[169] skeletal muscle,[169] retina,[170] haematopoietic cells,[171] and myocardium.[172] The capacity of lentiviral vectors to transduce nondividing cells is of particular interest with respect to the amitotic human corneal endothelium. However, I am aware of no more than three reports of lentiviral transduction of human corneal endothelial cells,[140,144,145] and the transduction rates achieved using the HIV-1-based Anson vector (>80%) compare favourably with all of them. Suh and colleagues transduced primary cultured endothelial cells with an Equine Infectious Anaemia Virus-based (EIAV) vector and reported eGFP expression in 30% of cells two to three days after transduction.[145]

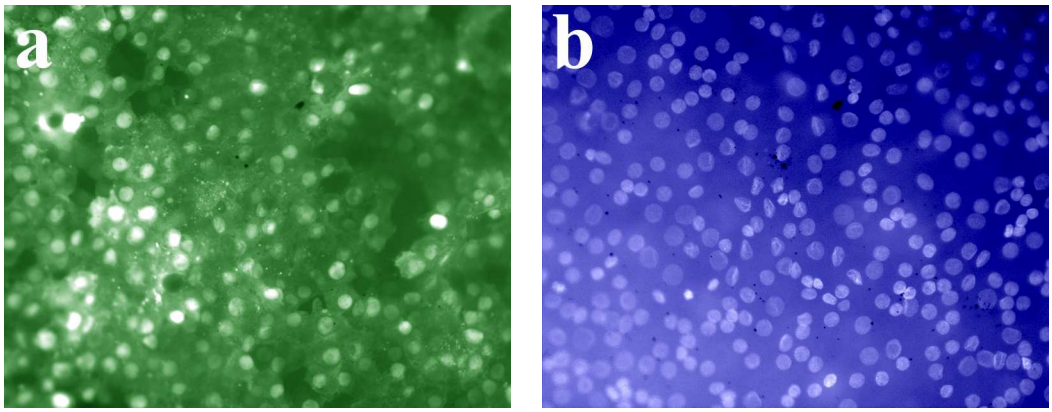


Figure 3.16: Expression of eYFP by human corneal endothelium following transduction with LV-CMV-eYFP and *in vitro* organ culture

A human cornea was transduced for 24 h with 6.3×10^6 TU of LV-CMV-eYFP (MOI 30) and subjected to 15 d of *in vitro* organ culture. Fluorescence micrographs of a representative central field show (a) expression of enhanced yellow fluorescent protein (eYFP) with blue light excitation, and (b) endothelial cell nuclei stained with Hoechst-33258 under ultraviolet illumination of the same field. Original magnification 20×.

Beutelspacher and co-workers observed transgene expression in approximately 10% of endothelial cells in human corneas transduced with an HIV-1-based vector, and in approximately 25% of those transduced with an EIAV vector.[144] Wang et al. detected reporter gene expression in endothelial cells of human corneas transduced with an HIV-1-based vector but did not quantify transduction rates.[140] The current studies demonstrated expression in >80% of endothelial cells in whole human corneas for up to two weeks of *in vitro* organ culture. Importantly, this expression was observed in some human corneas which had been in hypothermic storage in the Eye Bank for up to 25 days prior to transduction.

The higher transduction rates observed in the present studies, compared to previous reports, may be due to differences in vector design and transduction protocols. The vectors used by Beutelspacher and colleagues contained an EIAV envelope protein, rather than the VSV-G protein used in the current studies and in those of Suh and co-workers. Both the Beutelspacher and Suh groups used a three-plasmid transfection system, in contrast to the four-plasmid system used to produce the Anson vector. Furthermore, whereas a 24 hour transduction period was used in the current studies, Suh et al. used a transduction period of 12 hours while Beutelspacher and colleagues reported using a transduction period of just 3 hours. I did not assess a transduction period of less than 24 hours in human corneas. However, a relationship between duration of transduction and final transduction rate was observed for ovine corneal endothelium, and it seems reasonable that this may have contributed to the discrepancy in transduction rates achieved in human corneas in the current studies compared to those reported previously. Finally, whilst it is impossible to make an accurate comparison of the MOIs used in each system, the dose of vector reportedly used by Beutelspacher and co-workers (5×10^5 infectious

particles per cornea) was considerably lower than the dose used here (2.5×10^7 TU per cornea). The highest titre of virus produced in their system was reportedly 10^8 infectious particles per ml—10-fold lower than those attained in the current studies. Use of the VSV-G protein, as in the Anson system, which stabilises vector particles during ultracentrifugation and allows concentration to higher titres,[131] may be partly responsible for this difference.

Kinetics of expression varied between vectors and species

The time course of transgene expression is important in the selection of a vector for the prevention of corneal allograft rejection. Early-onset expression of an immunomodulatory transgene may be required to subvert the afferent arm of the immune response. The majority of studies demonstrating a prolongation of graft survival with gene therapy have used adenoviral vectors (Table 1.1). In the present studies, an adenoviral vector attained a high transduction rate (>80%) within four days of transduction. This is consistent with previous reports of adenoviral transduction of ovine corneal endothelia,[71,85] and of other animals, including the rabbit[104] and rat.[101]

Compared to the adenovirus, lentivirus-mediated expression in ovine corneal endothelium was delayed, with similar transduction rates (>80%) not evident until 14 days of *in vitro* organ culture had elapsed. This may be partly related to the distinct biology of each virus. Adenovirus enters cells by receptor-mediated endocytosis, binding to the cell surface Coxsackie-adenovirus receptor (CAR) and being internalised via clathrin-coated pits.[173] Once inside the cell, the virus is disassembled and released by endosomal lysis—the genome being transported into the nucleus, where it remains episomal and transcription takes place. The entire process may occur in a matter of hours.[174] Conversely, retroviral transduction is known to be a

relatively inefficient process,[175] and delays in expression have been noted previously.[172] In transducing rat cardiomyocytes *in vitro,* Fleury and co-workers detected eGFP expression in a majority of cells treated with adenovectors at three days, but not those treated with lentivectors. By day four, transduction rates were comparable, although mean fluorescence values remained lower with lentivectors. The slower kinetics of lentiviral gene transfer have been attributed to several factors—including reverse transcription, second-strand DNA synthesis, nuclear translocation of the preintegration complex, and proviral integration.[172] The differences in the life-cycle of the parent viruses may partly explain the difference in kinetics of expression between adenoviral and lentiviral vectors in the ovine corneal endothelium.

More surprising than the difference in kinetics observed between adenoviral and lentiviral vectors, was the finding that the delay in lentivirus-mediated expression observed in the ovine corneal endothelium was not seen in the case of the human corneal endothelium. In the human, maximal levels (>80%) of transgene expression were evident as early as one day after transduction. Increasing the multiplicity of infection in ovine corneas increased the rate of eYFP expression, but was associated with toxicity—probably due to the VSV-G protein, which is known to be cytotoxic in many mammalian cell lines.[176] The precise reason for the delay in expression observed in the ovine corneal endothelium is unknown. It did not appear to be due solely to the promoter, as a delay was evident following transduction with vectors containing both the SV40 and CMV promoters—albeit less pronounced in the case of CMV vector. It has been reported that in certain cell types—such as primary T lymphocytes—reverse transcription is the rate limiting step in retroviral transduction, and that preincubation of intact HIV-1 virions with deoxynucleotide

triphosphates (dNTPs) increases transduction efficiency.[130] Perhaps the ovine corneal endothelium, which has been shown to be effectively nonproliferating,[75] has low cytoplasmic pools of dNTPs. However, if this were the case, one might expect to see a delay in expression in the human corneal endothelium also, since it is known to be arrested in the G1-phase of the cell cycle.[8] The difference in expression rate appears to be species-specific and may have important ramifications for *ex vivo* expression of therapeutic transgenes in the ovine model of orthotopic corneal transplantation.

Viral promoters produced higher expression than mammalian promoters

Lentivirus-mediated transgene expression is known to vary depending on the target cell type and the choice of internal promoter. In a comparison of three promoters driving expression of a reporter gene, expression in blood-derived vascular endothelial progenitor cells was found to be highest with the CMV promoter, intermediate with the EF promoter and lowest with the PGK promoter.[177] In delivering a reporter gene to adult cardiomyocytes, HIV-1-based lentiviral vectors achieved higher expression with the CMV promoter than the EF or PGK promoters.[172] Almost all studies of lentivirus-mediated gene transfer to corneal endothelium to date have utilised vectors containing the CMV promoter. The one exception utilised an HIV vector containing the EF promoter, but did not quantify expression nor directly compare it with the CMV promoter.[133]

In the present studies, lentivirus-mediated expression in both ovine and human corneal endothelium was found to be highest with the human CMV immediate early promoter, high with the SV40 early promoter, and lowest with the PGK and EF promoters. The CMV promoter sequence was shared by the adenoviral vector, which achieved rapid and strong expression in ovine corneal endothelium. Expression was observed in both ovine and human corneal endothelium for at least

two weeks following transduction with the SV40 and CMV promoter lentiviral vectors. Despite these promising results, the prospect of promoter silencing must be considered. Transcriptional silencing of the CMV promoter by methylation in adenoviral vectors has been reported previously.[178] Given the half-life of the reporter protein is measured in days, longer observation would be required to ensure that lentivirus-mediated expression driven by the CMV promoter in corneal endothelium is not subject to transcriptional silencing. However, it is worth noting that Wang and colleagues used the CMV promoter in their lentiviral vector and reported no diminution of eGFP expression in human corneal endothelial cells 60 days after transduction.[140]

In choosing a promoter for use in an integrative vector, it must be recognised that promoter elements have the potential to trans-activate host genes. This was epitomised by the development of leukaemia-like proliferations in three patients undergoing retroviral gene therapy for X-linked severe combined immune deficiency (SCID-X1). It is believed that overexpression of the *LMO2* gene, as a result of trans-activation by the retroviral LTR, was responsible for tumorigenesis in these patients.[146] Self-inactivating (SIN) vectors such as the Anson vector reduce the risk of such an event occurring by deletion of the viral enhancer/promoter sequences in the U3 region of the LTR. Such vectors require an internal promoter to drive transgene expression, and thus the potential for unwanted trans-activation events is not eliminated completely. Interestingly, the trans-activation potential of the SV40, CMV, EF, and PGK promoters has recently been investigated in three different human cell lines and compared with retroviral and SIN LTRs.[149] The results suggested that trans-activation was cell-type dependent and often correlated with the inherent transcriptional strength of the promoter in that cell line. I am not aware of

any adverse events in gene therapy applications arising from the use of one of the four promoters tested in the current studies.

The SV40 virus is known to be oncogenic in animal models and it has been implicated in the development of human malignancies.[179] However, the oncogenic capacity of the virus is understood to be due to sequestration by the large and small T-antigens of important tumour suppressor proteins, such as p53 and pRb. I am aware of no evidence that the SV40 early promoter sequence is, in and of itself, pathogenic. Given that superior expression was achieved in both ovine and human corneal endothelium using the viral promoters compared to the mammalian promoters, and that the SV40 promoter construct was readily available for cloning purposes in the early stages of the project, further experiments were performed with vectors containing the SV40 promoter.

Final transduction rate was influenced by the duration of the transduction period and the use of polybrene

The final transduction rate achieved in ovine corneal endothelia, after a two hour transduction period with the lentiviral vector, was significantly lower than that observed after a 24 hour period. This was not surprising, given a report by Morgan and colleagues that, after exposing murine fibroblasts to a retrovirus for two hours, the majority of infectious virus was not bound to the cells but free in solution.[167] The use of the cationic polymer polybrene led to an increase in final transduction rate achieved during the shorter transduction period. These findings are consistent with the known inefficiency of retroviral particles in adsorbing to target cells. Retroviral particles reach the surface of target cells by diffusion and bind to receptors whilst subject to the electrostatic repulsion between their own negative charge and that of the cell membrane.[175,180,181] Cationic polymers are believed to enhance

adsorption by neutralising this electrostatic repulsive force,[182,183] and are commonly used to improve retroviral transduction in tissue culture (Figure 3.17). However, high doses can have antiproliferative and cytotoxic effects.[184] In the current studies, the use of polybrene at up to 40 µg / ml was not associated with any evidence of toxicity in ovine corneal endothelium, as judged by cell density and nuclear morphology following Hoeschst-33258 staining. However, this method of assessment may have failed to detect dead or injured endothelial cells which had not detached from Descemet's membrane. That might have been achieved using a vital stain such as trypan blue, or the viability stains calcein acetoxymethyl ester and ethidium homodimer, which measure intracellular esterase activity and plasma membrane activity respectively.[185] A potential toxic effect on the viability of treated allografts could not be ruled out until *in vivo* testing was performed (Chapter 5).

Further studies

These experiments established that the transduction efficiency of the Anson lentiviral vector was superior to previous reports of lentiviral transduction of human corneal endothelium. It was also shown that the SV40 and CMV promoters produced higher expression than the EF or PGK promoters, in both ovine and human corneal endothelium. The results showed promise for sustained expression of therapeutic transgenes in corneal endothelium using the Anson vector. Further studies were undertaken to characterise expression of the immunomodulatory cytokine interleukin-10, under the control of the SV40 promoter, prior to testing in the ovine preclinical model of corneal transplantation. These are described in Chapter 4.

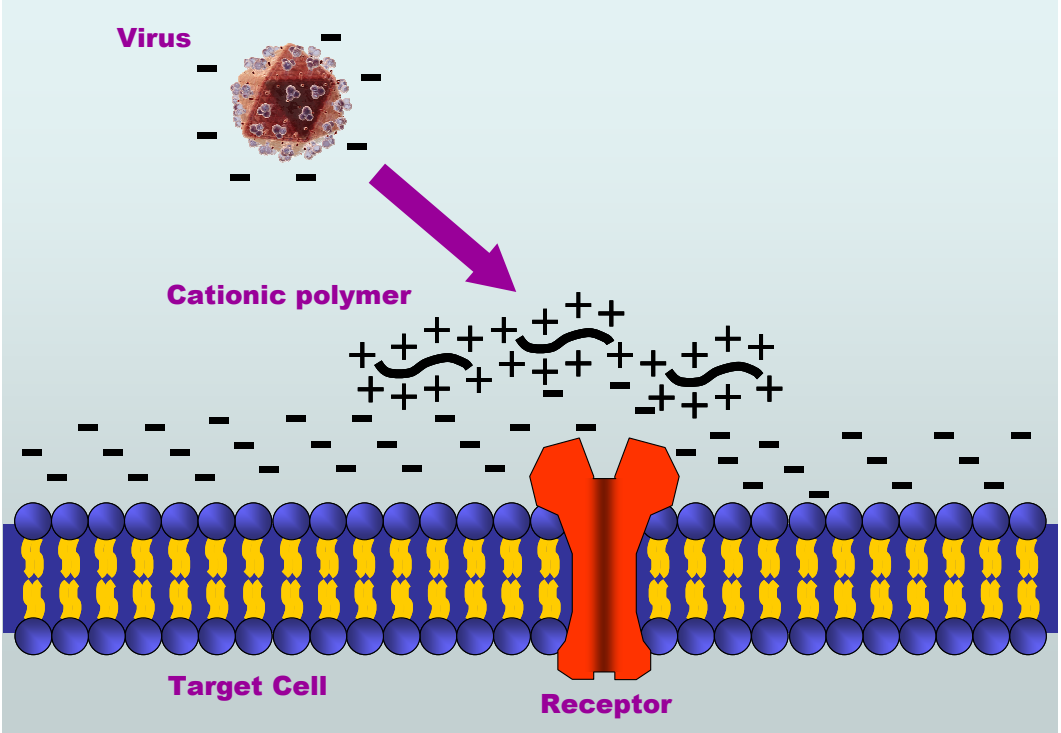


Figure 3.17: Proposed mechanism of action of cationic polymers in improving retroviral transduction efficiency

Cationic polymers such as polybrene are believed to enhance adsorption by a form of charge shielding, whereby the electrostatic repulsive force between the negatively charged cell membrane and virus particles is neutralised. As a result, a greater proportion of virus particles are able to interact with cell surface receptors, and gain entry to target cells.

CHAPTER 4

LENTIVIRUS-MEDIATED

EXPRESSION OF INTERLEUKIN-10 *IN VITRO*

4.1 Abstract

Aims

To construct lentiviral vectors and an adenoviral vector, all encoding the model secreted protein interleukin-10 (IL10), and to quantify relative transgene expression in ovine and human corneal endothelium *in vitro*.

Methods

Three vectors were produced and tested: (i) a lentiviral vector expressing ovine IL10 under the control of the simian virus type-40 early promoter (LV-SV40-IL10); (ii) a lentiviral vector expressing IL10 under the control of a promoter consisting of five glucocorticoid response elements (GRE5) upstream of the adenovirus 2 major late promoter TATA box/initiation site (LV-GRE-IL10); and (iii) an adenoviral vector, co-expressing enhanced green fluorescent protein (eGFP) and IL10 (Ad-GFP-IL10), under the control of separate human cytomegalovirus (CMV) immediate early promoters. Transgene expression in transduced ovine (all vectors) and human (LV-SV40-IL10 and LV-GRE-IL10) corneas was measured using real-time qRT-PCR to detect ovine IL10 mRNA, and an ELISA for ovine IL10 protein in corneal organ culture supernatants. For LV-SV40-IL10, rates of transcription and secretion were analysed following alterations in the dose of vector and the duration of the transduction period, as well as after transduction in the presence or absence of polybrene (40 µg / ml). The performance of the steroid-inducible vector LV-GRE-IL10 was assessed by an ELISA for ovine IL10 in organ culture supernatants of transduced ovine and human corneas.

Results

Real-time qRT-PCR of ovine corneal endothelium demonstrated a 10^4-fold increase in IL10 mRNA, 4 d after transduction with LV-SV40-IL10, compared to control

cornea. Secretion of IL10 by LV-SV40-IL10-transduced ovine corneas was detected within 6 d of transduction, with the rate of secretion increasing to approximately 20-fold higher than control levels over 15 d in culture. Lentivirus-mediated IL10 expression increased with higher multiplicities of infection (MOIs), and reducing the duration of the transduction period from 24 h to 2 h led to a reduction in expression at both the mRNA and secreted protein levels. The addition of polybrene (40 μg / ml) to the transduction mixture restored the expression rate in ovine corneas undergoing a 2 h transduction period, to levels comparable to those achieved using a 24 h transduction period. Transduction of ovine corneal endothelium with Ad-GFP-IL10 achieved a level of IL10 mRNA expression which was 10^3-fold higher than that achieved in corneas transduced with LV-SV40-IL10 at 4 d post-transduction. Secretion of IL10 from Ad-GFP-IL10-transduced ovine corneas demonstrated a rapid increase over the first 6 d in culture, reaching a peak 10^4-fold higher than control levels after 10–12 d. Expression of IL10 in LV-SV40-IL10-transduced human cornea, as measured by ELISA, was dose-responsive, and more rapid than in ovine cornea; levels of expression approximately 10-fold higher were achieved at 15 d post-transduction. LV-GRE-IL10-mediated expression of IL10, as measured by ELISA, showed an approximately 10-fold increase in the presence of 50 nM dexamethasone in ovine and human corneal endothelium. In both species, expression levels were comparable to, or greater than, those produced by LV-SV40-IL10.

Conclusions

Transduction of ovine corneal endothelium for 24 h with the Anson lentiviral vector resulted in expression levels which were increasing after 15 days of organ culture but logarithmically lower than those achieved by an adenoviral vector. Shortening the lentiviral transduction period to 2 h led to a reduction in expression, but the addition

of polybrene (40 μg / ml) to the transduction mixture restored expression to levels comparable to those attained after a 24 h transduction period. Transduction of human corneal endothelium with the Anson lentiviral vector resulted in higher and more rapid IL10 secretion than was observed in ovine corneas. Dexamethasone-responsive transgene expression was observed in both ovine and human corneal endothelium using a lentiviral vector containing the GRE5 steroid-inducible promoter.

4.2 Introduction

In Chapter 3, a high proportion of ovine and human corneal endothelial cells (>80%) were shown to be transduced with the Anson lentiviral vector containing the SV40 or CMV promoter, over 14 days *in vitro*. Those studies identified the influence of transduction period duration on transgene expression, and the beneficial effect of polybrene (40 μg / ml) in improving expression after a short transduction period. They also identified a hitherto unexplained delay in HIV-1-lentivirus-mediated expression kinetics in the sheep versus the human corneal endothelium. In this chapter, I describe experiments establishing the kinetics of transgenic IL10 expression in lentivirus-transduced corneas; the cytokine was chosen as a prototypical secreted therapeutic protein which could be quantified at the level of both mRNA and protein. As a benchmark for the performance of the Anson vector, the expression kinetics of an adenoviral vector expressing IL10 were also assessed.

Lentivirus-mediated *ex vivo* delivery of the gene encoding IL10 to the corneal endothelium is aimed at achieving sustained expression of the immunomodulatory cytokine in the anterior segment for weeks to years following corneal transplantation. Interleukin-10 exerts a range of effects on antigen presenting cells. These include downregulation of MHC Class II and costimulatory molecule expression,[186,187] and inhibition of Langerhans cell migration by the generation of functional decoy

receptors which act as molecular sinks and scavengers for inflammatory chemokines.[188,189] Furthermore, IL10 inhibits production of monokines such as IL1, IL6, IL8 and TNF-α. Thus, gene transfer leading to sustained expression of IL10 by a corneal allograft is a strategy for impairing effective antigen presentation, reducing graft immunogenicity, and inhibiting inflammation. Adenovirus-mediated transfer of the gene encoding IL10 has been shown to prolong allograft survival in rodent models of cardiac transplantation,[190,191] and after corneal transplantation in the outbred sheep model.[75] The putative advantage of a lentiviral vector over an adenoviral vector in expressing transgenic IL10 after corneal transplantation is the generation of stable expression. These experiments sought to establish the optimal transduction conditions for testing lentivirus-mediated IL10 expression in the ovine model of corneal transplantation.

In addition to measuring the expression of lentivirus-mediated IL10 secretion under the control of a constitutive promoter, I also transduced ovine and human corneas with a lentiviral vector containing an inducible promoter. The ability to regulate transgene expression is a key feature of the ideal gene therapy vector for corneal transplantation. The corneal endothelium is a tissue readily amenable to regulation of transgene expression by virtue of its accessibility. Inducible expression systems include those utilising a tetracycline-responsive promoter,[192] a promoter responsive to changes in intracellular cyclic adenosine monophosphate (cAMP),[193] and the use of glucocorticoid response elements (GREs) to allow induction of gene expression by steroids,[156] amongst many others. Since topical corticosteroids are the mainstay of the prophylaxis and treatment of corneal graft rejection, it is inconceivable that any gene transfer strategy to pretreat human corneal donor allografts would preclude their use. A steroid-responsive promoter might confer the

ability to limit expression of a therapeutic transgene to clinically evident episodes of acute rejection, reducing the risk of adverse effects associated with sustained expression. Given the Anson vector is intended for clinical application, these experiments included the construction and testing of a vector expressing IL10 under the control of the GRE5 promoter designed by Mader and White.[156]

4.3 Specific aims

- To construct a lentiviral vector and an adenoviral vector, both encoding IL10, and compare levels of transgene expression;
- To measure the kinetics and relative levels of lentivirus-mediated IL10 expression in ovine and human corneal endothelium;
- To determine the influence of multiplicity of infection, duration of transduction, and the use of polybrene, on lentivirus-mediated IL10 expression in ovine corneal endothelium;
- To construct a lentiviral vector containing a steroid-inducible promoter, and measure the inducibility and expression levels of transgenic IL10 in transduced ovine and human corneal endothelium.

4.4 Results

4.4.1 Production of an adenoviral vector encoding IL10

Cloning of IL10 gene into adenoviral shuttle vector

As a benchmark for the performance of the lentiviral vector, the adenoviral vector Ad-GFP-IL10, expressing ovine IL10 and the enhanced green fluorescent protein (eGFP)—both under the control of individual human CMV immediate early promoters—was produced and tested. The 580 bp cDNA sequence for ovine IL10

was amplified from the plasmid pRC-CMV-IL10 by PCR, using the primers IL10kpnfor and IL10hindrev (Table 2.2), and cloned into the polylinker of the shuttle vector pAdTrack-CMV using the restriction sites *Kpn*I and *Hind*III (Figure 4.1(a)). A tag of six histidine residues was incorporated at the 3' end of the IL10 coding sequence to facilitate purification of the protein, but was not subsequently used. Successful ligation, resulting in the formation of the shuttle vector pAdTrack-CMV-IL10, was confirmed by PCR using the primers IL10kpnfor and IL10hindrev, and restriction enzyme digestion (Figure 4.1(b)). The sequence and orientation of the ovine IL10 insert were confirmed by sequencing using the primers IL10for and IL10rev (Table 2.2) and the ABI BigDye Terminator sequencing kit on an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Cotransformation of IL10-shuttle vector with adenoviral backbone

The shuttle vector pAdTrack-CMV-IL10 was linearised with the restriction enzyme *Pme*I, and cotransformation with the adenoviral backbone pAdEasy was performed by electroporation of BJ5183 *E.coli* with 400 ng of the shuttle and 100 ng of pAdEasy. Plasmid mini-preps of potential recombinants were digested with *Pac*I, and confirmed the expected 30 kb and 4.5 kb fragments (Figure 4.2). An endotoxin-free plasmid maxi-prep was made of one such recombinant, which was then linearised with the restriction enzyme *Pac*I.

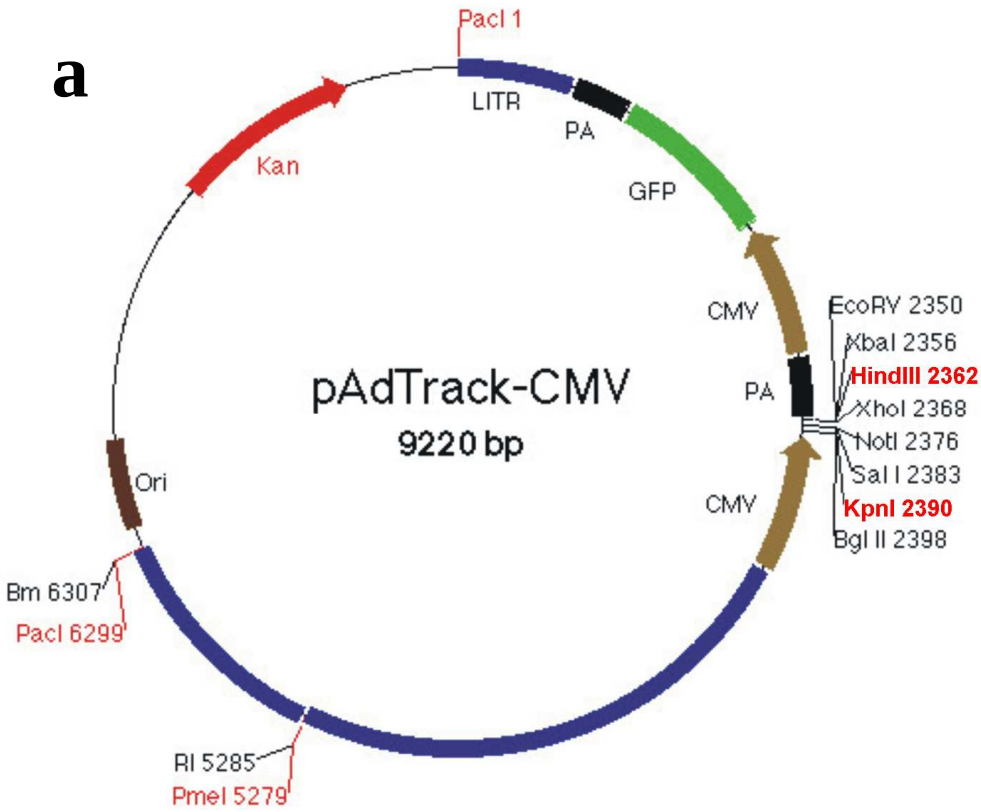
Transfection of HEK-293A cells with Ad-GFP-IL10 vector plasmid

A transfection was carried out in HEK-293A cells using the linearised Ad-GFP-IL10 vector plasmid, as described in Section 2.3.3 (page 69). Reverse transcription-PCR (RT-PCR) conducted on transfected cells using the primers IL10kpnfor and IL10hindrev (Table 2.2), confirmed expression of the IL10 transgene (Figure 4.3).

Figure 4.1: Cloning of the IL10 sequence into the pAdTrack-CMV shuttle vector

(a) A map of the pAdTrack-CMV shuttle vector shows the multicloning region (MCS), including *Kpn*I and *Hind*III restriction sites. Note the presence of two cytomegalovirus (CMV) promoters, one upstream of the MCS, and a second driving expression of the enhanced green fluorescent protein (GFP). PA: polyadenylation signal, LITR: left inverted terminal repeat, Ori: origin of replication, Kan: kanamycin resistance sequence [diagram adapted from AdEasy Vector System Application Manual[161]] (b) A photograph of an agarose gel, following restriction digest of DNA purified from the clone, demonstrates the liberation of a 580 bp fragment (arrow) representing the size of the interleukin-10 (IL10) insert sequence. Lane 1 contains 2 log ladder DNA marker; lane 2 contains uncut clone DNA; lane 3 contains clone DNA single-cut by *Hind*III only; lane 4 contains clone DNA double-cut by *Kpn*I and *Hind*III.

a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LITR
PA
GFP
CMV
PA
CMV
Kan
Ori
pAdTrack-CMV
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XbaI 2356
HindIII 2362
XhoI 2368
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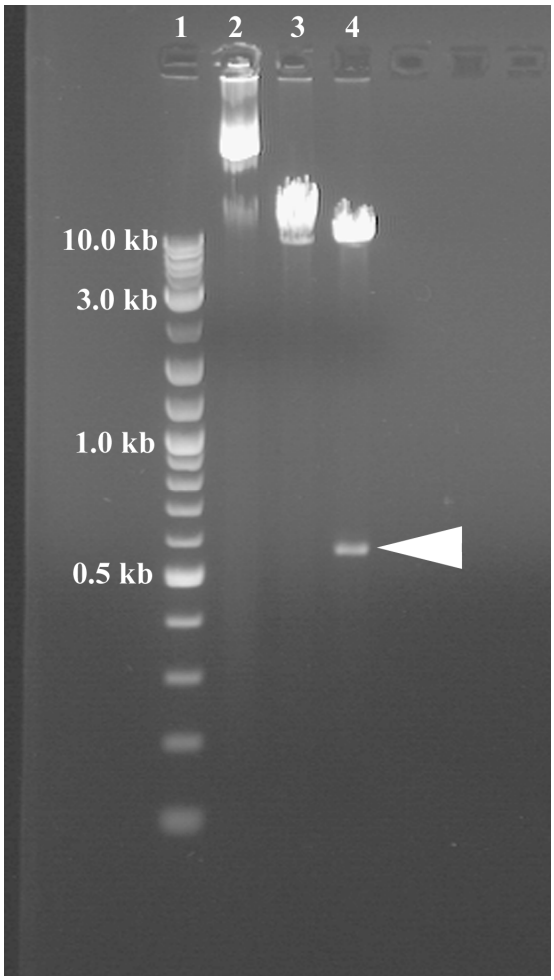
b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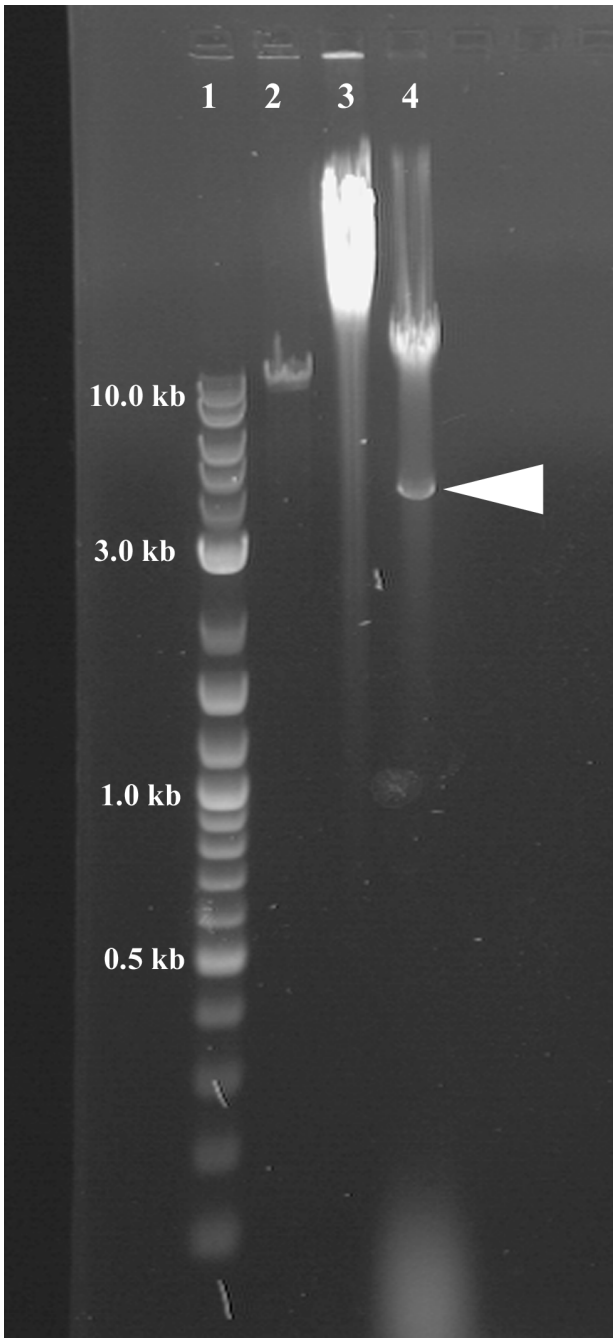


Figure 4.2: Restriction digest confirming cotransformation of the pAdTrack-CMV-IL10 shuttle vector with the pAdEasy viral backbone

A photograph shows agarose gel electrophoresis following *Pac*I digest of DNA purified from a potential recombinant. Lane 1 contains 2 log ladder DNA marker; lane 2 contains *Pme*I-cut pAdTrack-CMV-IL10; lane 3 contains *Pac*I-cut pAdEasy; lane 4 contains *Pac*I-cut recombinant and shows bands of expected size—one band at >10 kb and one band at approximately 4.5 kb (arrow).

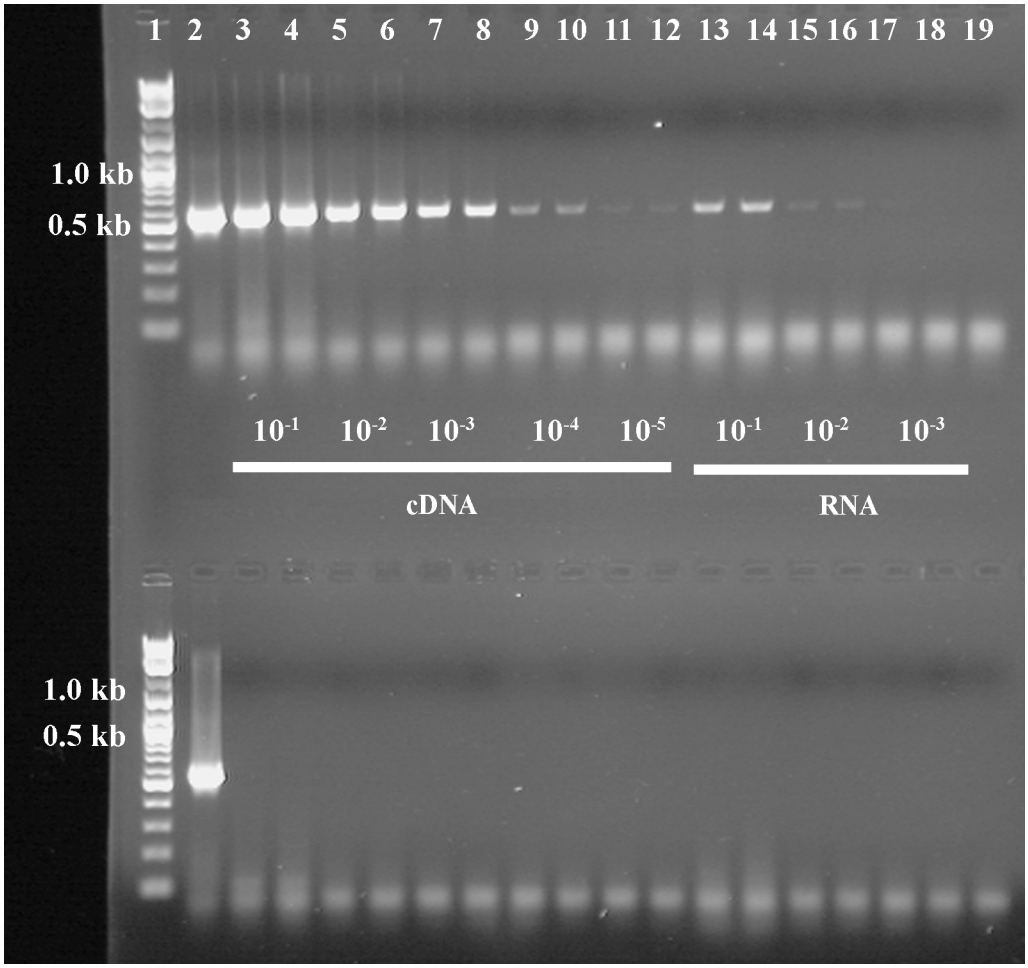


Figure 4.3: Confirmation of IL10 expression in HEK-293A cells transfected with Ad-GFP-IL10 vector DNA

A photograph of an agarose gel shows reverse transcription-PCR products from Ad-GFP-IL10-transfected cells (upper row), and mock transfected cells (lower row). After RNA extraction, cDNA was synthesised and used as template for a PCR with the primers IL10kpnfor and IL10hindrev (Table 2.2). In each row, lane 1 contains 2 log ladder DNA marker; lane 2 contains positive control PCR product for interleukin-10 (IL10) DNA (580 bp); lanes 3–12 contain duplicates of products from PCR of cDNA (serial dilutions as marked); lanes 13–18 contain duplicates of products from PCR in which extracted RNA was used as template (serial dilutions as marked); lane 19 contains a PCR negative control.

Production of purified adenoviral vector stock

In order to produce large quantities of Ad-GFP-IL10 for purification, HEK-293A cells were subjected to repeated rounds of infection with subsequent passages of crude lysate from infected cells. Purified stocks of Ad-GFP-IL10 were obtained by passing infected cell lysates through caesium chloride density gradients. These procedures are described in detail in Section 2.3.3 (pages 69–71).

4.4.2 Production of lentiviral vectors encoding IL10

Cloning of IL10 gene into lentiviral vector plasmid

With the aim of producing the lentiviral vector LV-SV40-IL10, the gene encoding ovine IL10 was cloned into the plasmid pHIV-1-SDm-SV-empty-ΔLTR (Figure 4.4(a)). The plasmid was linearised with a *Kpn*I restriction site at one end and a blunt cloning site at the other. The IL10 coding sequence was isolated from the pAdTrack-CMV-IL10 adenoviral shuttle plasmid by restriction digestion. Successful ligation resulting in the shuttle plasmid pHIV-1-SDm-SV-IL10-ΔLTR was confirmed by PCR (Figure 4.4(b)), and the sequence and orientation of the ovine IL10 insert confirmed by sequencing, using the primers IL10for and IL10rev (Table 2.2) and the ABI BigDye Terminator sequencing kit on an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Cloning of GRE5 promoter sequence into lentiviral vector plasmid

The 320 bp sequence encoding five glucocorticoid response elements from the rat tyrosine aminotransferase gene, upstream of the adenovirus 2 major late promoter (AdMLP) TATA box/initiation site, was obtained in the shuttle plasmid pGRE5-luc, as the generous gift of Dr John White, Department of Physiology, McGill University,

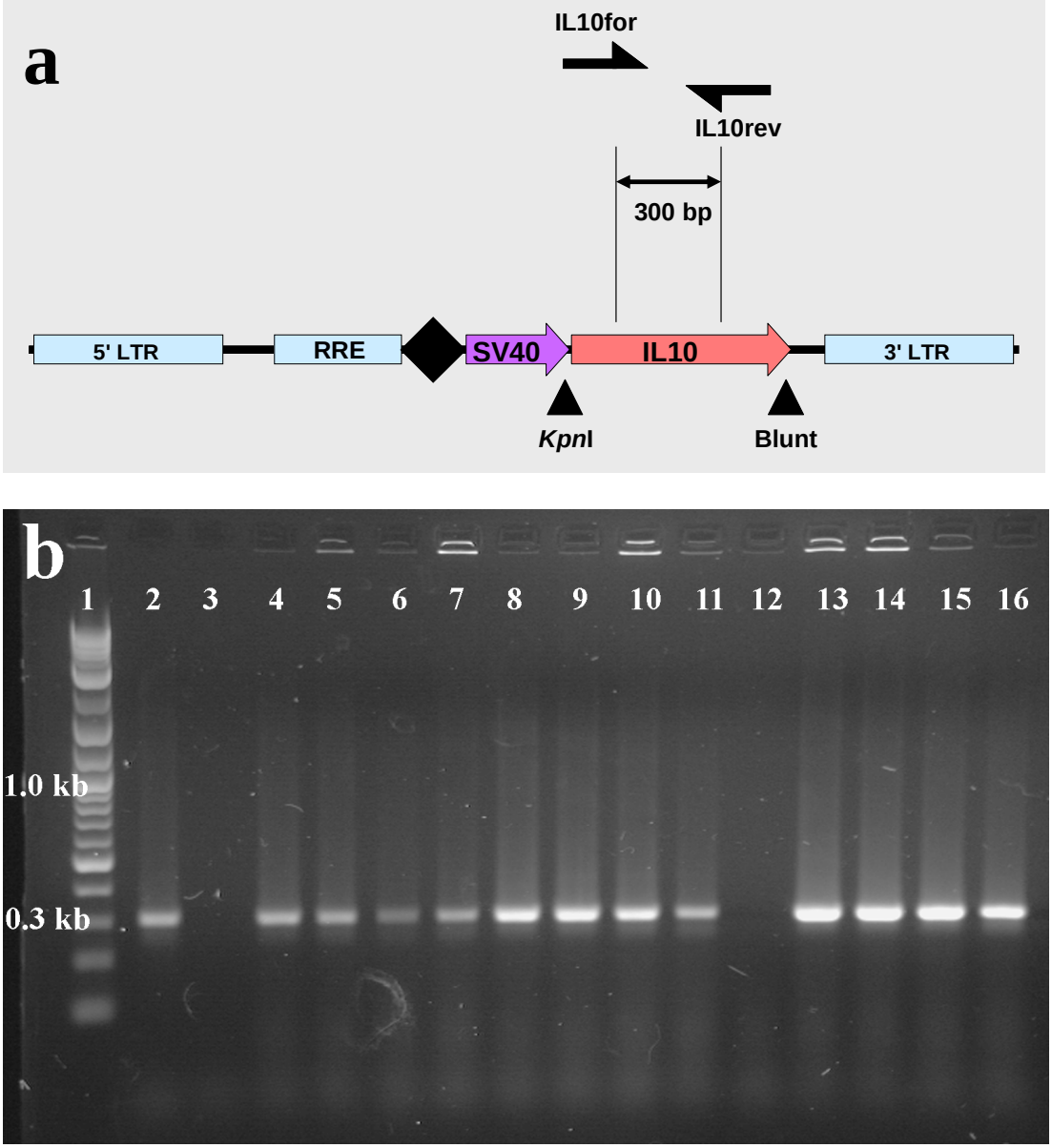


Figure 4.4: Cloning of the IL10 coding sequence into the lentiviral vector plasmid

(a) A schematic representation of the vector pHIV-1-SDm-SV-IL10-ΔLTR shows the *Kpn*I and blunt restriction sites, between which the IL10 sequence was cloned. LTR: long terminal repeat, RRE: rev response element, diamond represents central polypurine tract, SV40: simian virus type-40 early promoter (b) A photograph of an agarose gel shows PCR products amplified from potential clones after ligation, using primers IL10for and IL10rev (Table 2.2). Lane 1 contains 2 log ladder DNA marker: lane 2 contains IL10 DNA positive control (300 bp); lane 3 contains a PCR negative control; lanes 4–16 contain DNA from clones 1–13, showing the expected 300 bp fragment in 12 out of 13 clones.

Montreal, Quebec, Canada. The GRE5 promoter sequence was amplified by PCR and cloned into the lentiviral vector plasmid pHIV-1-SDm-SV-IL10-ΔLTR, by substitution of the sequence encoding the SV40 promoter at the restriction sites *Bam*HI and *Kpn*I (Figure 4.5(a)). Successful ligation was confirmed by PCR (Figure 4.5(b)), and resultant plasmid DNA sequenced, using the primers GREbamfor and GREkpnrev (Table 2.2) and the ABI BigDye Terminator sequencing kit on an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Production, purification and titre determination of lentiviral vectors

The lentiviral vectors LV-SV40-IL10 and LV-GRE-IL10 were produced by transfection of HEK-293T cells with the corresponding vector plasmid and the four helper plasmids, before being purified according to the protocol for large-scale (LV-SV40-IL10) or medium scale (LV-GRE-IL10) lentiviral vector preparations described in Section 2.3.4 (pages 74–77). Vector titres were determined by real-time PCR on transduced NIH3T3 cells, as described on page 79, and were consistently 10^8–10^9 NIH3T3 iu per ml. The vector stocks were found to be free of replication-competent lentivirus by assaying expression of HIV-1 p24 (HIV-1 p24 ELISA kit, PerkinElmer Inc, Boston, MA, USA) in transduced cells over three weeks, using the method described on page 80.

Difference in titre measurement between IL10 and reporter gene vectors

Titres determined using real-time PCR for *gag* in transduced NIH3T3 cells were found to be 5–10-fold higher than those determined using flow cytometry of A549 cells transduced with vectors encoding the reporter gene (data not shown). This was probably due to detection of both transcribed and nontranscribed proviral copies by real-time PCR, whereas the flow cytometric method provided a more accurate

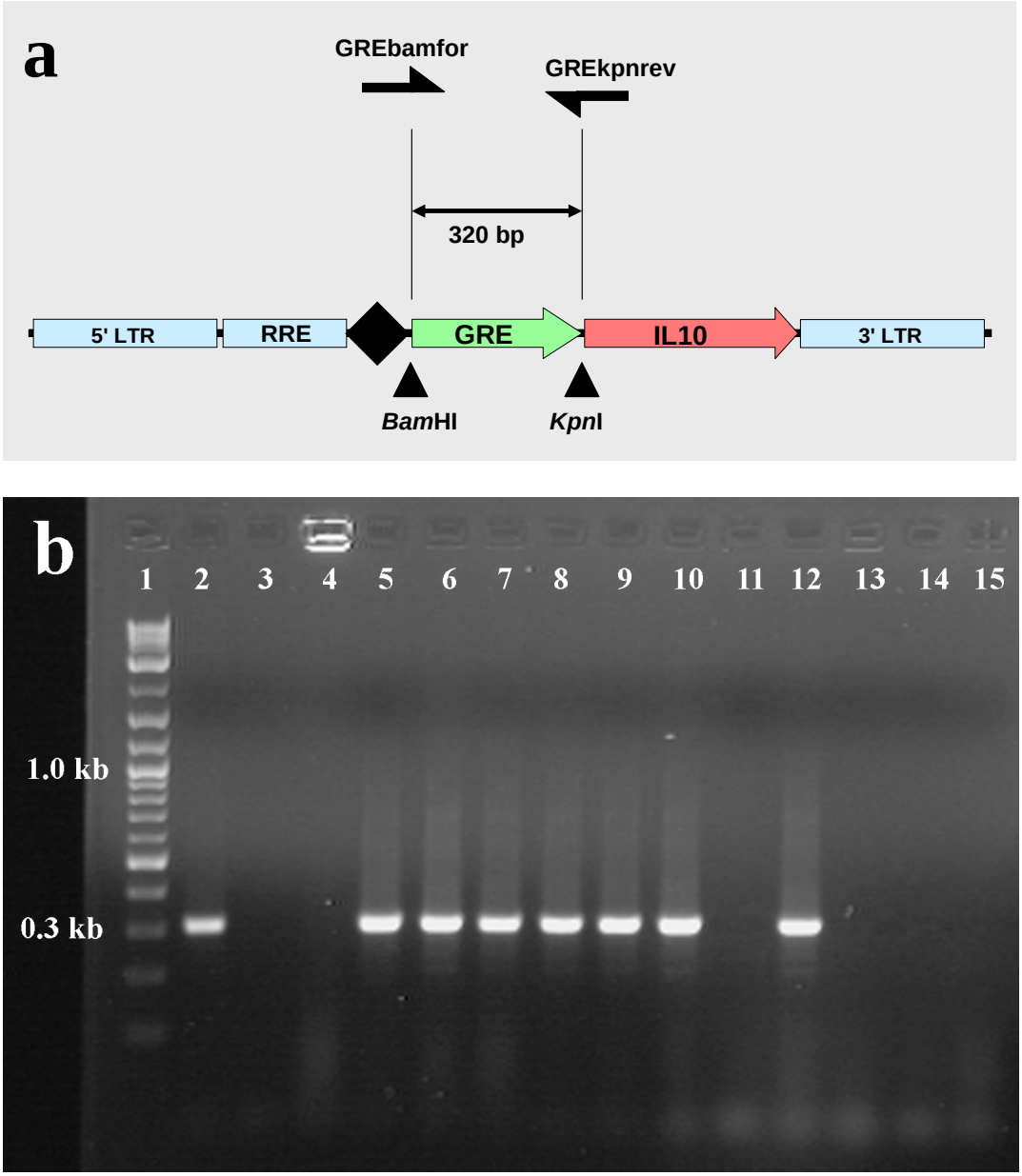


Figure 4.5: Cloning of the GRE5 promoter sequence into the lentiviral shuttle vector

(a) A diagram of the vector pHIV-1-SDm-GRE-IL10-ΔLTR shows the restriction sites *Bam*HI and *Kpn*I, between which the GRE5 (glucocorticoid response element) promoter sequence was cloned. LTR: long terminal repeat, RRE: rev response element, diamond symbol: central polypurine tract, IL10: interleukin-10 (b) A photograph of an agarose gel shows PCR products amplified from potential clones after ligation, using primers GREbamfor and GREkpnrev (Table 2.2): lane 1 contains 2 log ladder DNA marker; lane 2 contains GRE5 positive control (320 bp); lane 3 contains a PCR negative control; lanes 4–15 contain DNA from clones 1–12, showing the expected 320 bp fragment in seven out of 12 clones.

reflection of transcribed proviral copies alone. For this reason, multiplicities of infection (MOIs) of the IL10-expressing lentiviral vectors are not directly comparable to MOIs of the reporter gene vectors described in Chapter 3.

4.4.3 Quantification of IL10 expression in transduced ovine corneas by real-time qRT-PCR

In order to measure transgenic IL10 expression in transduced ovine corneas at the mRNA level, a real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay was set up. Selection of reference genes was limited by the relative paucity of fully-sequenced ovine genes. The sequences for ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ovine β-actin were both available and have been used previously in qRT-PCR experiments in ovine tissues.[194,195] I was not aware of any previous studies of their expression in ovine corneal endothelium. Therefore, the transcriptional stability of both genes in ovine corneal endothelium was assessed under various conditions, including lentiviral and adenoviral transduction, and *in vitro* organ culture.

Analysis of primer sets for real-time PCR

Real-time quantitative polymerase chain reaction was performed on cDNA samples from experimental corneal endothelia using primer sets for the gene sequences encoding ovine IL10, ovine GAPDH and ovine β-actin (Table 2.3). Primers were designed as described on page 87. Real-time PCR products were separated by agarose gel electrophoresis (Figure 4.6). A solitary band indicating a single product was identified for the ovine IL10 and ovine GAPDH primer sets. An intense band of expected size was observed for ovine β-actin (105 bp), but was accompanied by a very faint band at around 180 bp. The existence of β-actin pseudogenes has been

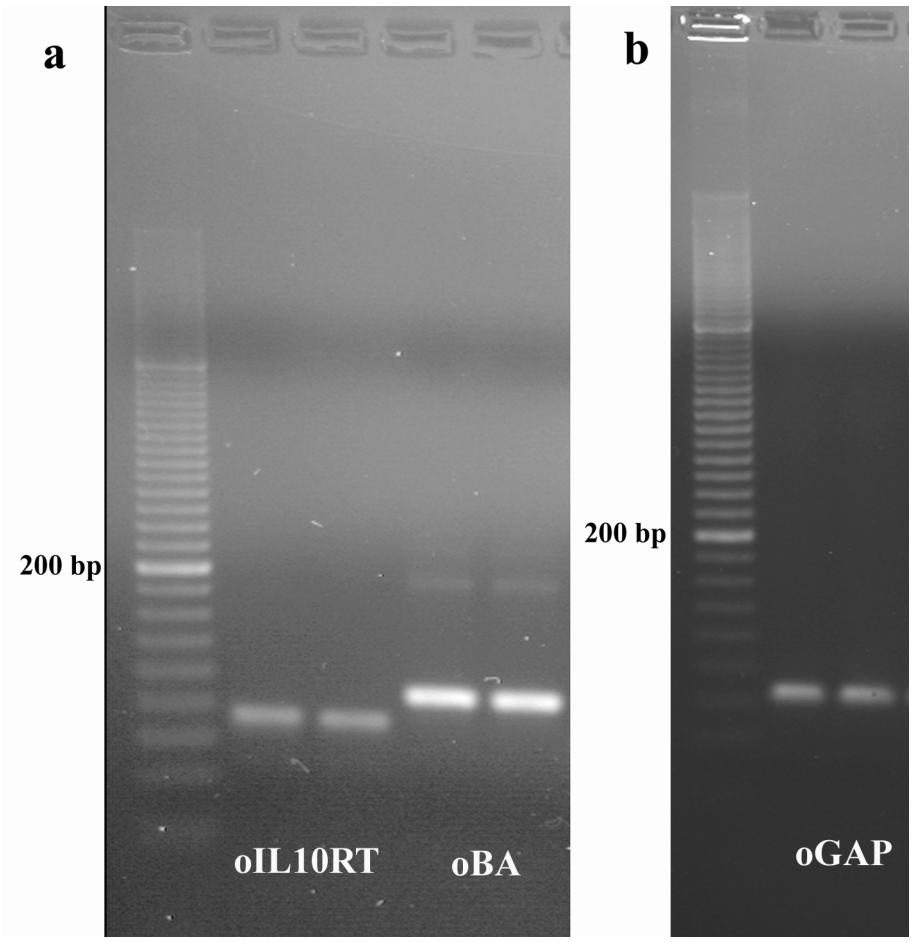


Figure 4.6: Agarose gel electrophoresis of real-time PCR products

Photographs show real-time PCR primer set products following agarose gel electrophoresis. The first lane of each gel contains a 20 bp DNA ladder and PCR products have been run in duplicate in adjoining lanes. The sizes of the principal products match the expected amplicon lengths for (a) the oIL10RT (89 bp) and oBA (105 bp) primer sets, and (b) the oGAP (81 bp) primer set. oIL10RT: ovine interleukin-10, oBA: ovine β-action, oGAP: ovine glyceraldehyde 3-phosphate dehydrogenase.

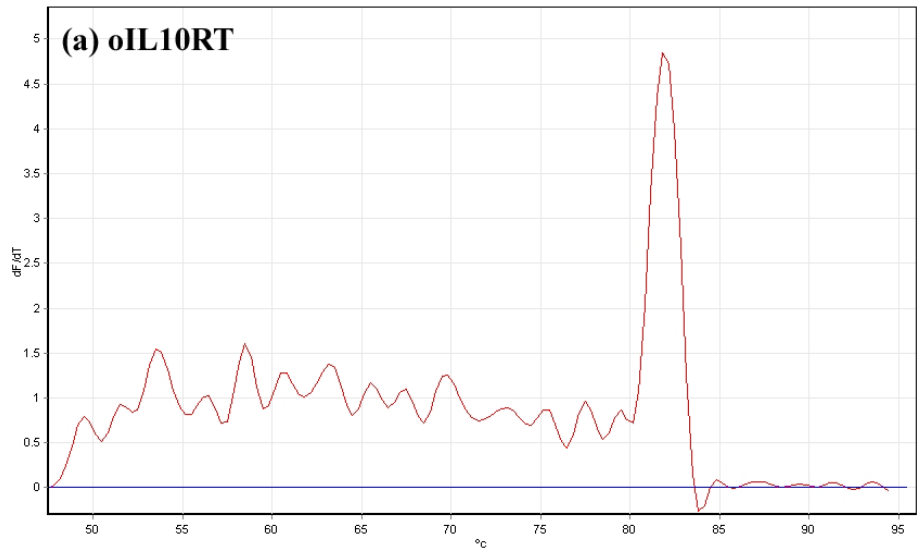
reported,[196] and this band may have represented amplification from a pseudogene or genomic DNA despite the DNase treatment. The DNA from the intense bands was extracted from agarose gels using the QIAquick column purification system (Qiagen, Valencia, CA, USA) and eluted in 10 mM Tris.Cl pH 8.5 for quantification and sequencing. Purified DNA was labelled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and resolved using corresponding primer sets (Table 2.3) and the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences confirmed those predicted for all primer sets. Melt-curve analysis was used to confirm amplicon specificity for each test sample for each set of primers (Figure 4.7). Melt-curves for all primer sets demonstrated a single tall peak consistent with a single PCR product. This indicated that the product represented by the faint band on the agarose gel was not interfering with amplification of the ovine β-actin primer set amplicon.

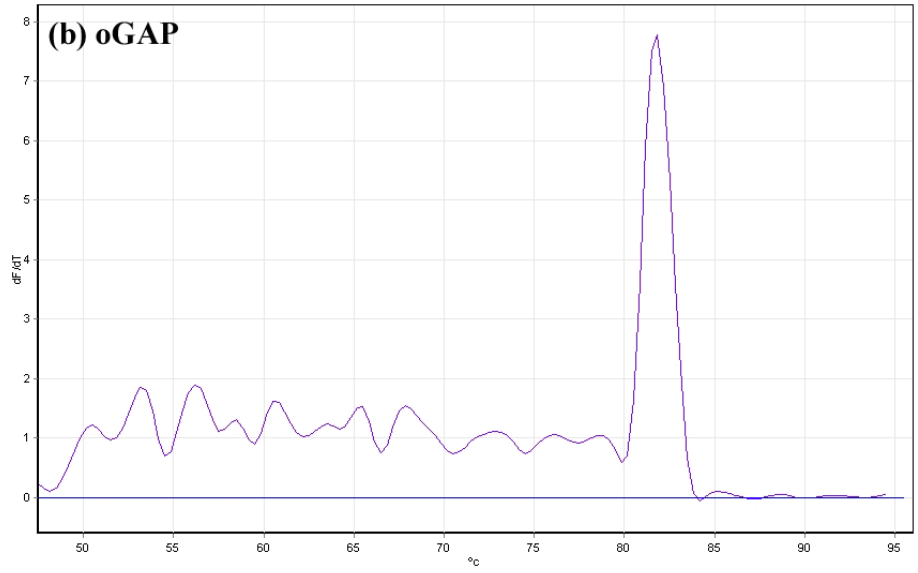
Amplification efficiencies for real-time PCR primer sets

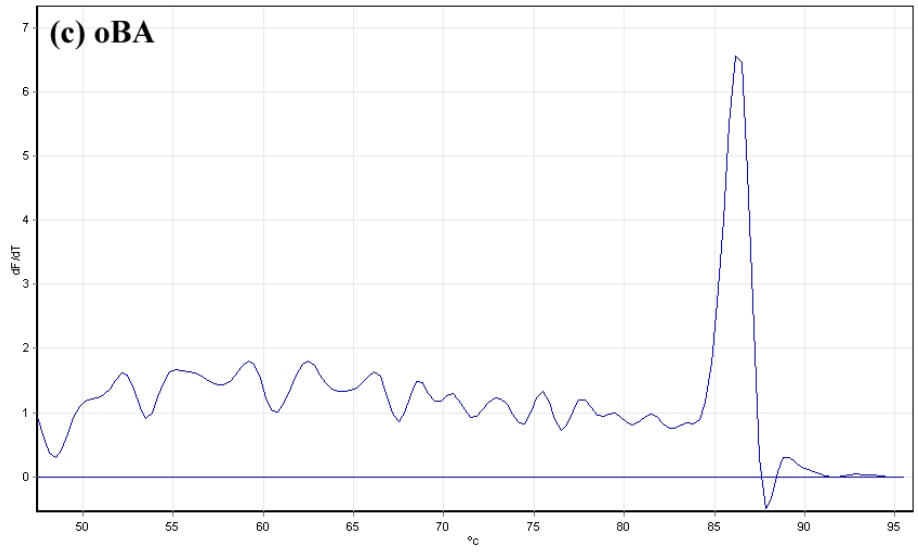
Amplification efficiencies for the real-time PCR primer sets were calculated by importing Takeoff points for serial dilutions of the cDNA standard into qBase v1.3.5, a free program for the management and automated analysis of qPCR data.[165] Amplification efficiencies for the ovine IL10, ovine GAPDH and ovine β-actin primer sets were 104%, 105%, and 103% respectively.

Figure 4.7: Melt-curve analysis of real-time PCR products

At the end of each run, products of real-time PCR were subjected to progressively increasing temperatures to generate melt-curves. These curves demonstrate peaks at the points of maximal change in fluorescence (dF/dT), representing melting of the amplicon or any other products present. Melt-curves run for the three sets of primers each display a single well-defined peak at a reproducible temperature, indicative of a single PCR product: (a) ovine interleukin-10 (oIL10RT), (b) ovine glyceraldehyde 3-phosphate dehydrogenase (oGAP), and (c) ovine β-actin (oBA).


(a) oIL10RT
dF/dT
°c


(b) oGAP
dF/dT
°c


(c) oBA
dF/dT
°c

Assessment of reference gene stability by real-time PCR

An analysis of relative reference gene expression, using qBase v1.3.5, was performed on samples subjected to a range of treatments, including *in vitro* organ culture, transduction with adenovirus or lentivirus, and exposure to polybrene (Table 4.1). Raw expression levels of β-actin resulted in a coefficient of variation of 51% across all samples, and those for GAPDH resulted in a coefficient of variation of 67%. Given the variability of expression for each gene, rather than use one or the other it was decided that a normalisation factor based on the expression of both genes should be calculated. This was done by determining the geometric mean of relative expression levels for each reference gene according to the method of Vandesompele.[166] After normalisation of the raw expression of each gene to the normalisation factor, the coefficients of variation for β-actin and GAPDH improved to 28% and 35%, respectively (Figure 4.8). These were deemed acceptable. For further analysis of relative IL10 expression, raw IL10 expression was normalised to the normalisation factor calculated for each sample.

Expression of IL10 mRNA in transduced ovine corneal endothelium

The first experiment carried out using the vectors expressing IL10 was an analysis of IL10 mRNA expression levels in lentivirus- and adenovirus-transduced ovine corneas, 4 d after transduction. Corneas were transduced for 24 h with 5×10^7 iu per cornea of LV-SV40-IL10 (MOI 40) or for 2 h with 5×10^7 pfu per cornea of Ad-GFP-IL10, and subjected to 4 d of *in vitro* organ culture, before harvesting of endothelial RNA for qRT-PCR. Normalised expression of IL10 mRNA in corneas transduced with LV-SV40-IL10 was approximately 10^4-fold higher than in nontransduced,

Table 4.1: Ovine corneal cDNA samples subjected to qRT-PCR

No.	Sample	Vector	MOI	TP (h)	OC time (d)	Polybrene (µg/ ml)
1	Pooled standard	NA	NA	NA	NA	NA
2	NTNC 1	Nil	Nil	0	0	0
3	NTNC 2	Nil	Nil	0	0	0
4	NTNC 3	Nil	Nil	0	0	0
5	Ad 5E7/2H/4D 1	Ad-GFP-IL10	40	2	4	0
6	Ad 5E7/2H/4D 2	Ad-GFP-IL10	40	2	4	0
7	Ad 5E7/2H/4D 3	Ad-GFP-IL10	40	2	4	0
8	LV 5E7/24H/4D 1	LV-SV40-IL10	40	24	4	0
9	LV 5E7/24H/4D 2	LV-SV40-IL10	40	24	4	0
10	LV 5E7/24H/4D 3	LV-SV40-IL10	40	24	4	0
11	MOCK 2H/15D	Nil	Nil	2	15	0
12	LV 3.5E7/2H/15D	LV-SV40-IL10	25	2	15	0
13	LV 7E7/2H/15D	LV-SV40-IL10	50	2	15	0
14	MOCK 24H/15D	Nil	Nil	24	15	0
15	LV 3.5E7/24H/15D	LV-SV40-IL10	25	24	15	0
16	LV 7E7/24H/15D	LV-SV40-IL10	50	24	15	0
17	MOCK 3H/15D	Nil	Nil	3	15	0
18	LV 7E7/3H/15D NO PB	LV-SV40-IL10	50	3	15	0
19	LV 7E7/3H/15D +PB 4	LV-SV40-IL10	50	3	15	4
20	LV 7E7/3H/15D +PB 40	LV-SV40-IL10	50	3	15	40

***Footnotes: MOI multiplicity of infection; TP transduction period; OC organ culture; NA not applicable; NTNC nontransduced, noncultured cornea

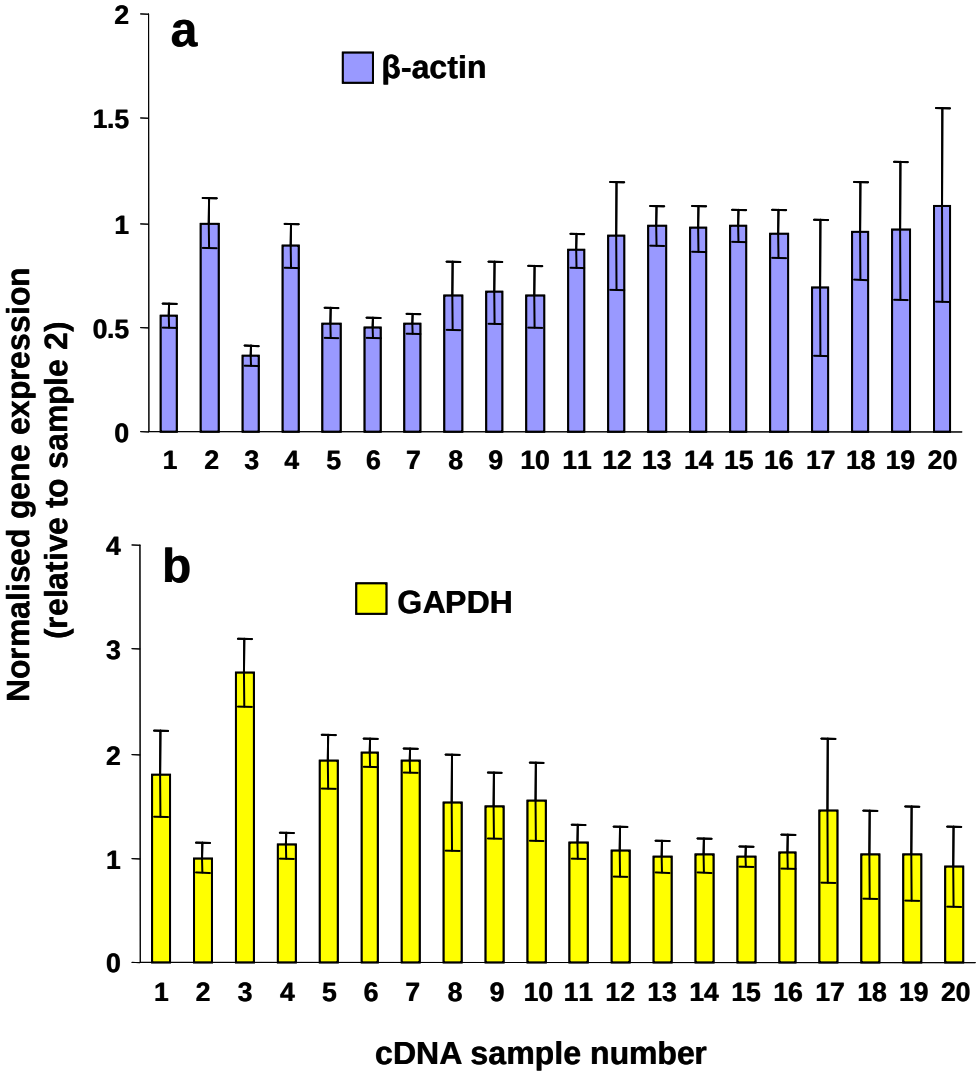


Figure 4.8: Normalised expression of reference genes in ovine corneal cDNA samples

Gene expression in each sample (as listed in Table 4.1) was normalised by dividing the relative expression value—determined by real-time PCR—by the geometric mean of the expression levels of the two reference genes, β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in the same sample.[166] Columns represent the mean±SD of triplicates performed for every sample on each real-time PCR run. The coefficients of variation for (a) β-actin and (b) GAPDH, after normalisation, were 28% and 35% respectively. Expression levels have been scaled with sample 2 (nontransduced, noncultured corneal cDNA) set to 1. SD: standard deviation of the mean.

noncultured control cornea (Figure 4.9). Normalised expression of IL10 mRNA in corneas by transduced with Ad-GFP-IL10 was approximately 10^3-fold higher than in the LV-SV40-IL10-transduced corneas and 10^7-fold higher than in the control. The difference between relative lentiviral and adenoviral expression levels was highly statistically significant (2-tailed, unpaired t-test: $p<0.0001$).

Effect of transduction period and multiplicity of infection on lentivirus-mediated IL10 mRNA expression in ovine corneal endothelium

Next, the influence of transduction period duration and MOI on IL10 mRNA expression was assessed (Figure 4.10). An analysis of RNA extracted from ovine corneas 15 d after transduction showed that a cornea transduced with 3.5×10^7 iu per cornea of LV-SV40-IL10 (MOI 25) for only 2 h, attained an expression level approximately seven-fold lower than that achieved in a cornea transduced for 24 h at the same MOI. The experiment was then repeated using double the dose (MOI 50). In the cornea transduced for 2 h, no increase in expression was observed, compared to the lower dose. However, expression in the cornea transduced for 24 h approximately doubled compared to that measured at the lower dose.

Influence of polybrene on lentivirus-mediated IL10 mRNA expression in ovine corneal endothelium

An experiment was then performed to determine whether the addition of polybrene to the transduction mixture could increase the transduction rate and hence the levels of IL10 expression achieved after a short transduction period. The length of the transduction period was set to 3 h, since this was expected to be the longest period practicable to allow *ex vivo* transduction and transplantation to be carried out on the

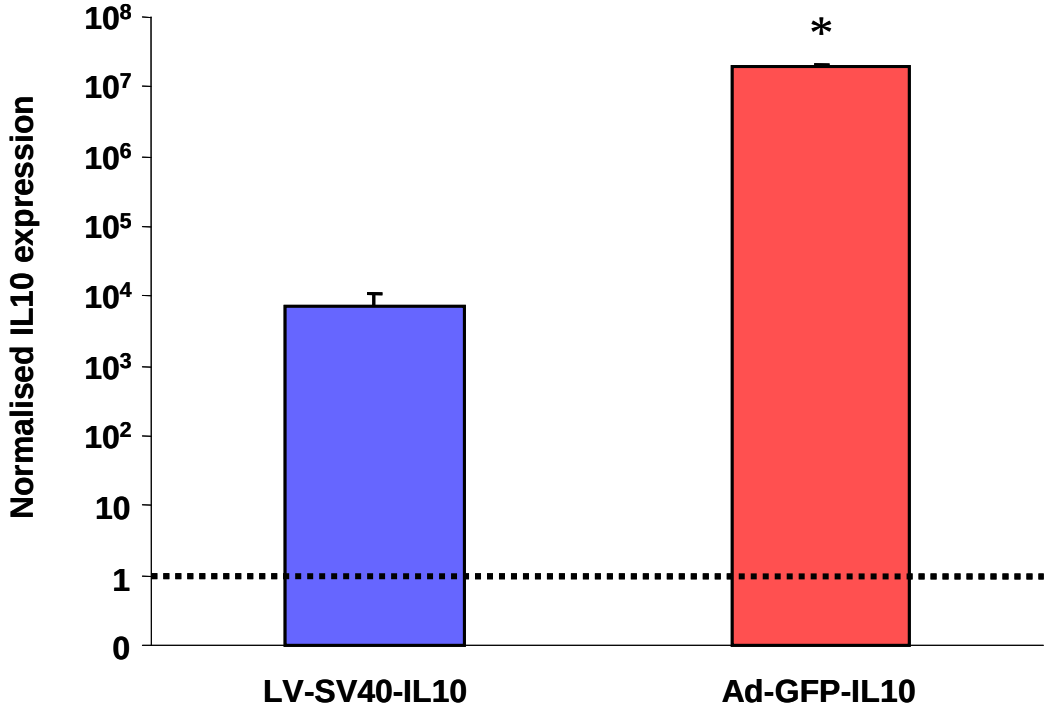


Figure 4.9: Comparison of IL10 mRNA expression in lentivirus - and adenovirus-transduced ovine corneal endothelium, measured by qRT-PCR

Corneas were transduced for 2 h with 5×10^7 pfu per cornea (Ad-GFP-IL10), or for 24 h with 5×10^7 iu per cornea (LV-SV40-IL10), cultured for 4 d, and the endothelia then harvested for qRT-PCR. This equated to a multiplicity of infection of approximately 40. Columns represent the relative expression level of ovine interleukin-10 (IL10) normalised to ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ovine β-actin (mean±SD of three corneas), where expression in nontransduced, noncultured control cornea is 1 (dotted line). Note log scale. * 2-tailed, unpaired t-test : $p<0.0001$. SD: standard deviation of the mean.

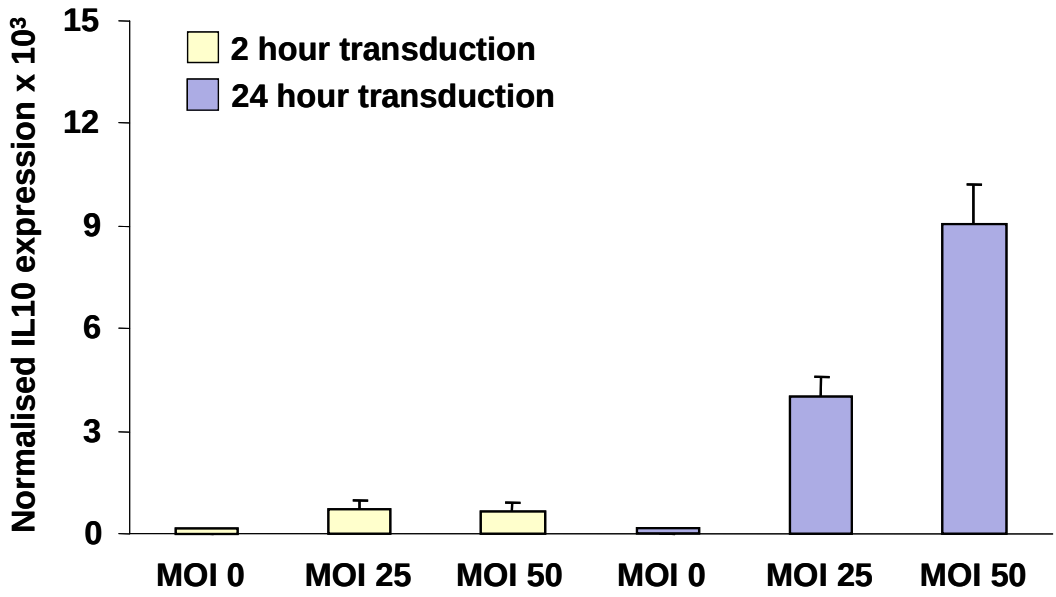


Figure 4.10: Effect of transduction period duration and multiplicity of infection (MOI) on IL10 mRNA expression in lentivirus-transduced ovine corneal endothelium, measured by qRT-PCR

Corneas underwent a 2 h or 24 h transduction period with no virus (MOI 0) or LV-SV40-IL10 (MOI 25 and MOI 50). Each column represents expression levels of ovine interleukin-10 (IL10) normalised to ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ovine β-actin (mean±SD) in the endothelium of a single cornea, 15 d after transduction (where 1 represents the expression level in nontransduced, noncultured cornea). Error bars represent the standard deviation of the mean for three replicates per cornea. Columns representing 'MOI 0' corneas are not to scale, since actual levels were so low as to be invisible in the figure.

same day in later *in vivo* studies. Polybrene was added to the transduction mixture at 4 µg / ml or 40 µg / ml, with 7×10^7 iu per cornea of LV-SV40-IL10 (MOI 50). Corneas were incubated for 3 h followed by a 15 d period of *in vitro* organ culture, whereupon endothelial RNA was extracted and qRT-PCR performed. Corneas transduced for 3 h in the presence of polybrene exhibited higher rates of IL10 expression than those transduced without polybrene and the effect appeared to be dose-dependent (Figure 4.11). However, the differences between groups did not reach statistical significance (2-tailed, unpaired t-test: $p>0.05$).

4.4.4 Quantification of IL10 secretion from transduced ovine corneas by ELISA of organ culture supernatants

To investigate expression of transgenic IL10 at the level of secreted protein, supernatants were collected at three-day intervals from corneas transduced with either LV-SV40-IL10 or Ad-GFP-IL10, following the completion of the transduction period. The relative quantity of ovine IL10 present in corneal culture supernatants was measured by sandwich ELISA. The protocol is described in detail on pages 90–91. Absolute concentrations of ovine IL10 in organ culture supernatants were not determined, because no purified and quantified ovine IL10 was available as a standard. For this reason, all IL10 protein levels are presented as relative optical density values at 450 nm as determined by ELISA. In many of the experiments described below, measurements were made on supernatants collected at 3, 6, 9, 12 and 15 d post-transduction. It should be noted that at each of these time points the supernatant was completely removed from the cornea, and the culture medium then replaced with fresh medium. Therefore, the IL10 concentration at each point represents the accumulation of the protein in the supernatant over the preceding 3 d of organ culture only, not the cumulative concentration since transduction.

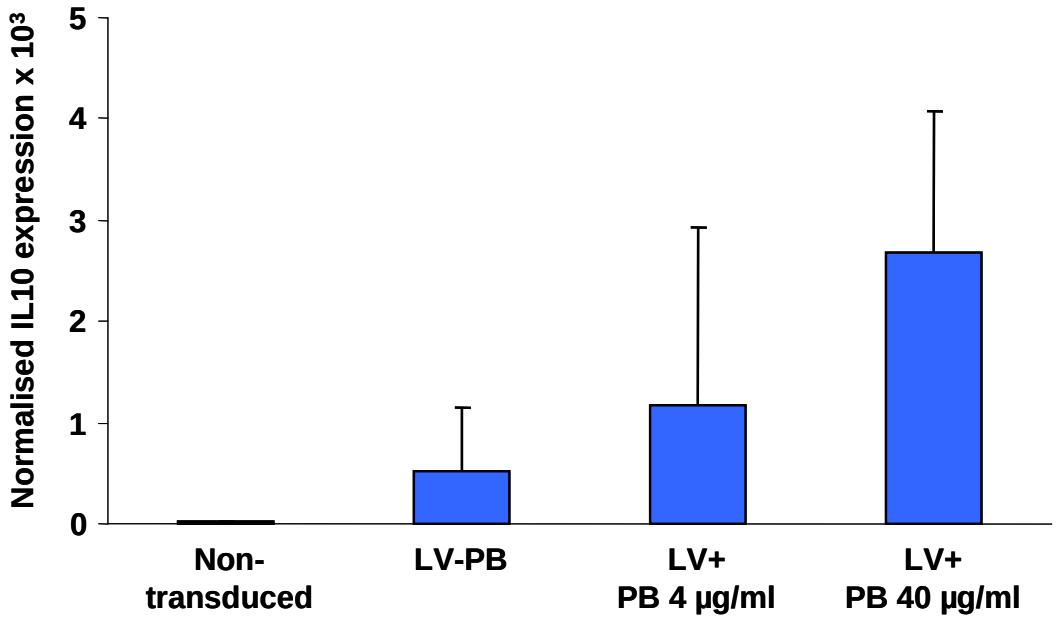


Figure 4.11: Effect of polybrene on IL10 mRNA expression in lentivirus-transduced ovine corneal endothelium, measured by qRT-PCR

Ovine corneas transduced for 3 h with LV-SV40-IL10 at 7×10^7 iu per cornea (MOI 50) in the absence (LV-PB) or presence of polybrene (LV+PB), were subjected to *in vitro* organ culture. At 15 d post-transduction, endothelia were harvested for RNA extraction and qRT-PCR. Columns represent relative expression of ovine interleukin-10 (IL10) normalised to ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ovine β-actin (mean±SD of three corneas), where 1 represents the expression level in nontransduced, noncultured cornea. The column representing the 'Non-transduced' group is not to scale, since actual levels were so low as to be invisible in the figure. SD: standard deviation of the mean.

Kinetics of IL10 protein secretion from lentivirus-transduced ovine corneal endothelium

Initially, organ culture supernatants were collected from a cornea transduced for 24 h with LV-SV40-IL10 at 1.3×10^8 iu per cornea (MOI 100), and from control corneas transduced with medium alone or LV-SV40-eYFP. The high MOI was chosen with the intention of maximising IL10 secretion, and was not expected to cause toxicity, for reasons discussed on pages 151 and 153. The ELISA demonstrated a steady increase in the rate of IL10 secretion over the period of organ culture in the LV-SV40-IL10-transduced sample (Figure 4.12). From 6 d onwards, the level of IL10 in the transduced sample was higher than that in supernatant from nontransduced cornea or in supernatant from the cornea transduced with LV-SV40-eYFP. At the 15 d collection point, the level of secretion had increased approximately 20-fold compared to control corneas, and had not reached a plateau. In the controls, IL10 levels remained low and stable throughout the culture period. Endpoint fluorescence microscopy of the LV-SV40-IL10-transduced cornea demonstrated an intact endothelium, using a Hoechst-33258 nuclear stain (Figure 4.13).

Effect of multiplicity of infection on IL10 secretion by lentivirus-transduced ovine corneal endothelium

Having established the kinetics of lentivirus-mediated IL10 protein secretion at a single dose, the effect of changing the MOI was then investigated. Corneas were transduced for 24 h with LV-SV40-IL10 at 3.5×10^7 or 7×10^7 iu per cornea, equating to MOIs of 25 and 50 respectively. Once again, supernatants were collected at three-day intervals for 15 d. A time-dependent increase in expression rate was reproduced

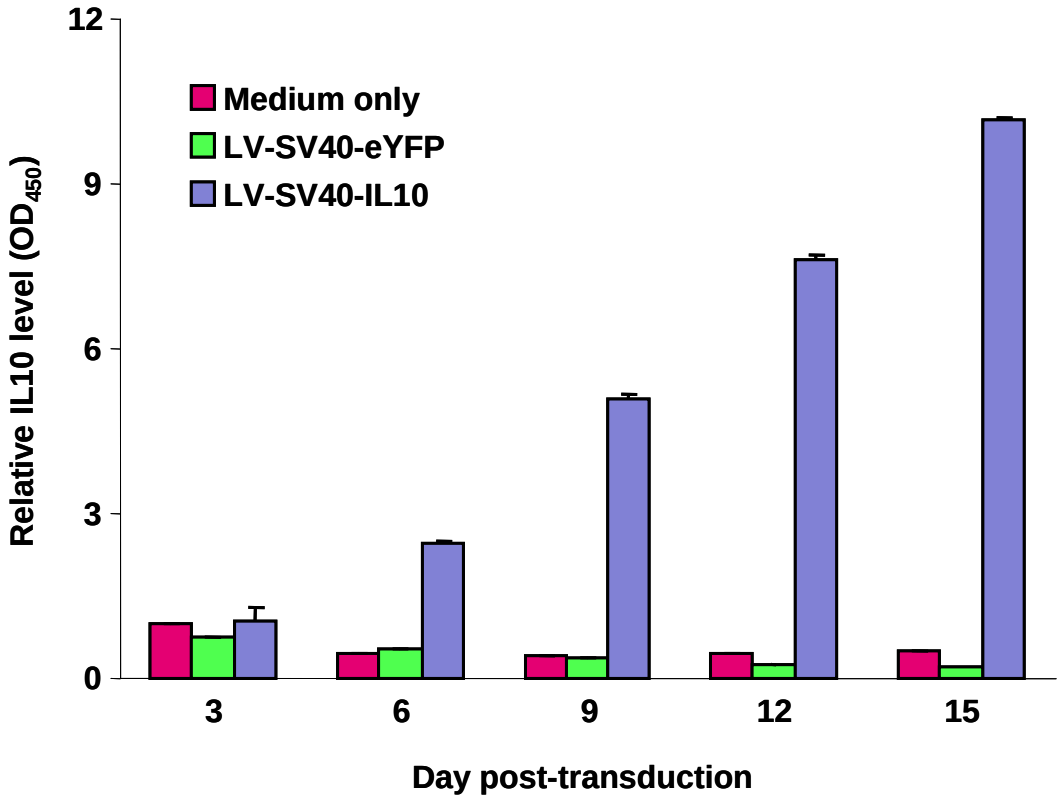


Figure 4.12: Kinetics of IL10 secretion by lentivirus-transduced ovine corneal endothelium

Columns represent relative optical density (OD) levels at 450 nm (mean±range of duplicates) following ELISA on supernatants collected from transduced endothelia at three-day intervals of *in vitro* organ culture. For each treatment, one cornea was transduced for 24 h with: medium only, 2.5×10^{7} TU per cornea LV-SV40-eYFP (MOI 20), or 1.3×10^{8} iu per cornea LV-SV40-IL10 (MOI 100), respectively. The final collection of supernatant was made at 15 d because beyond this point viability of cultured corneas was significantly compromised. Supernatants were tested in an ELISA specific for ovine interleukin-10 (IL10). Relative OD levels have been rescaled with that of a 'Medium only' cornea at 3 d set to 1. MOI: multiplicity of infection.

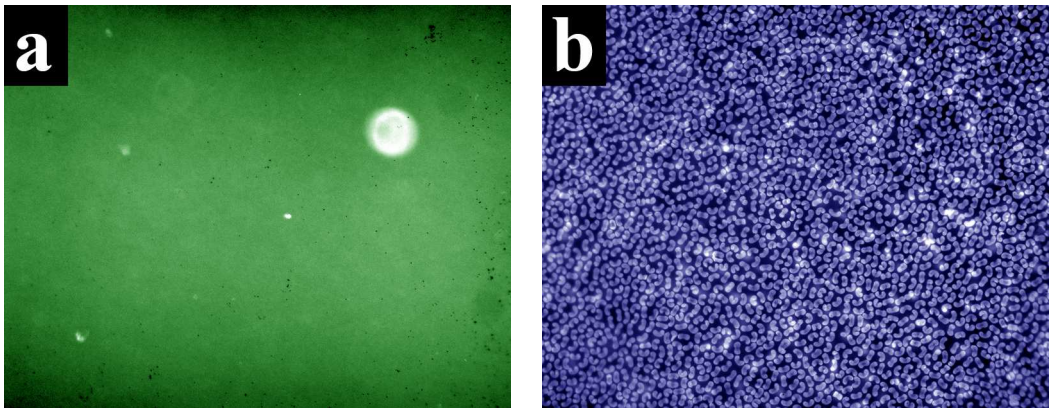


Figure 4.13: Fluorescence microscopy of LV-SV40-IL10-transduced ovine corneal endothelium following 15 d of *in vitro* organ culture

Fluorescence micrographs show a representative central field of the ovine cornea transduced with LV-SV40-IL10 at MOI 100 and subjected to 15 d of *in vitro* organ culture (presented in Figure 4.12). (a) Blue light confirms no enhanced yellow fluorescent protein expression (spots are artefacts). (b) Ultraviolet illumination of Hoechst-33258-stained nuclei shows intact endothelium in the same field. Original magnification 10×.

at both doses tested (Figure 4.14). In addition, the higher MOI resulted in a correspondingly higher rate of expression at each time point. This recapitulated the results of the qRT-PCR experiment showing a relationship between dose and expression (Figure 4.10).

Kinetics of IL10 protein secretion from adenovirus-transduced ovine corneal endothelium

Next, experiments were conducted using the adenoviral vector Ad-GFP-IL10 in ovine corneas. Supernatants were collected from ovine corneas transduced for 2 h with Ad-GFP-IL10 at 5×10^7 pfu per cornea (MOI 40). Supernatants were also collected from control corneas transduced with medium only under the same conditions. The ELISA demonstrated a rapid increase in the rate of protein secretion over the first 6 d of organ culture in Ad-GFP-IL10-transduced samples (Figure 4.15). Protein secretion peaked during the 13–15 d collection period at 10^4-fold higher than control levels. In the control samples, IL10 levels remained low and stable throughout the culture period. In the transduced corneas, endpoint fluorescence microscopy demonstrated widespread eGFP expression, and an intact endothelium, using a Hoechst-33258 nuclear stain (Figure 4.16).

Effect of transduction period duration on IL10 secretion by lentivirus-transduced ovine corneal endothelium

As a follow-up to the real-time qRT-PCR experiment demonstrating the effect of transduction period duration on IL10 mRNA expression, supernatants were collected from ovine corneas transduced for either 2 h or 24 h with LV-SV40-IL10 at 7×10^7 iu per cornea (MOI 50). Supernatant collected from the cornea transduced for 24 h

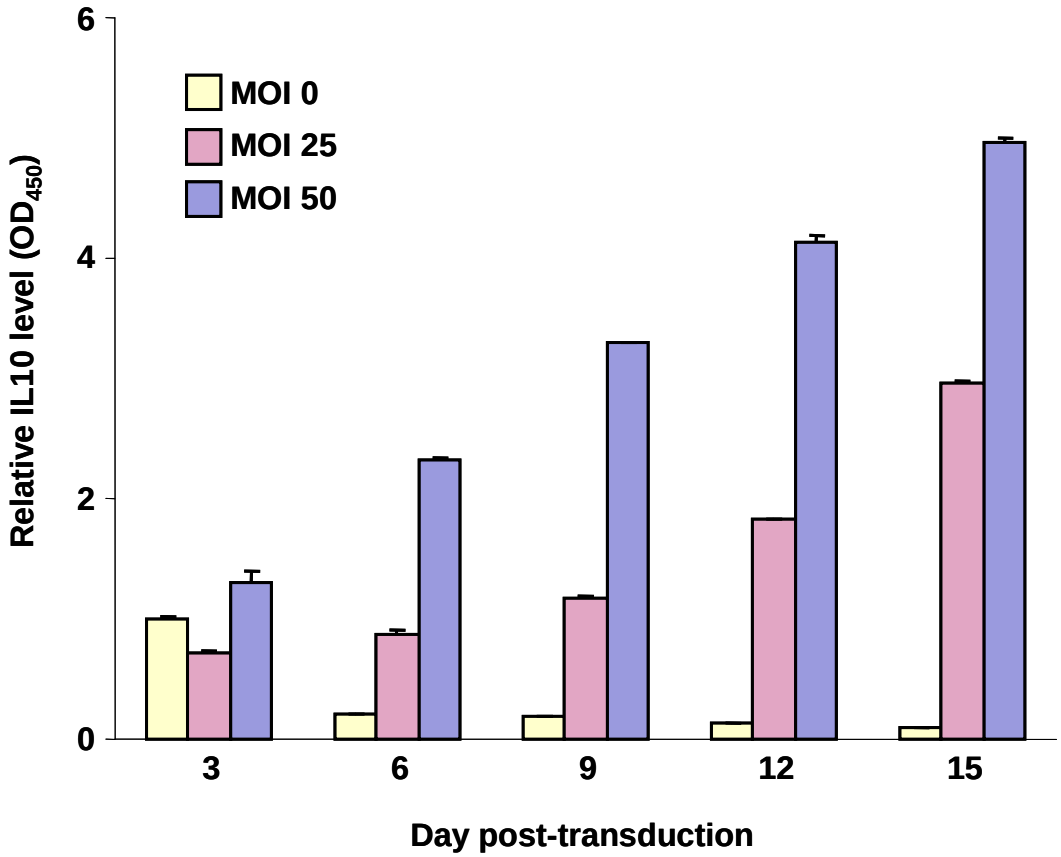


Figure 4.14: Effect of multiplicity of infection (MOI) on levels of secreted IL10 protein in supernatants collected from lentivirus-transduced ovine corneal endothelium

Columns represent relative optical density (OD) levels at 450 nm (mean±range of duplicates) following ELISA on supernatants collected from transduced ovine corneas at three-day intervals of *in vitro* organ culture. Three corneas were transduced for 24 h: one with medium only (MOI 0), one with 3.5×10^7 iu of LV-SV40-IL10 (MOI 25), and one with 7×10^7 iu of LV-SV40-IL10 (MOI 50). The final collection of supernatant was made at 15 d because beyond this point viability of cultured corneas was significantly compromised. Supernatants were tested by an ELISA specific for ovine interleukin-10 (IL10). Relative OD levels have been rescaled with that of an 'MOI 0' cornea at 3 d set to 1.

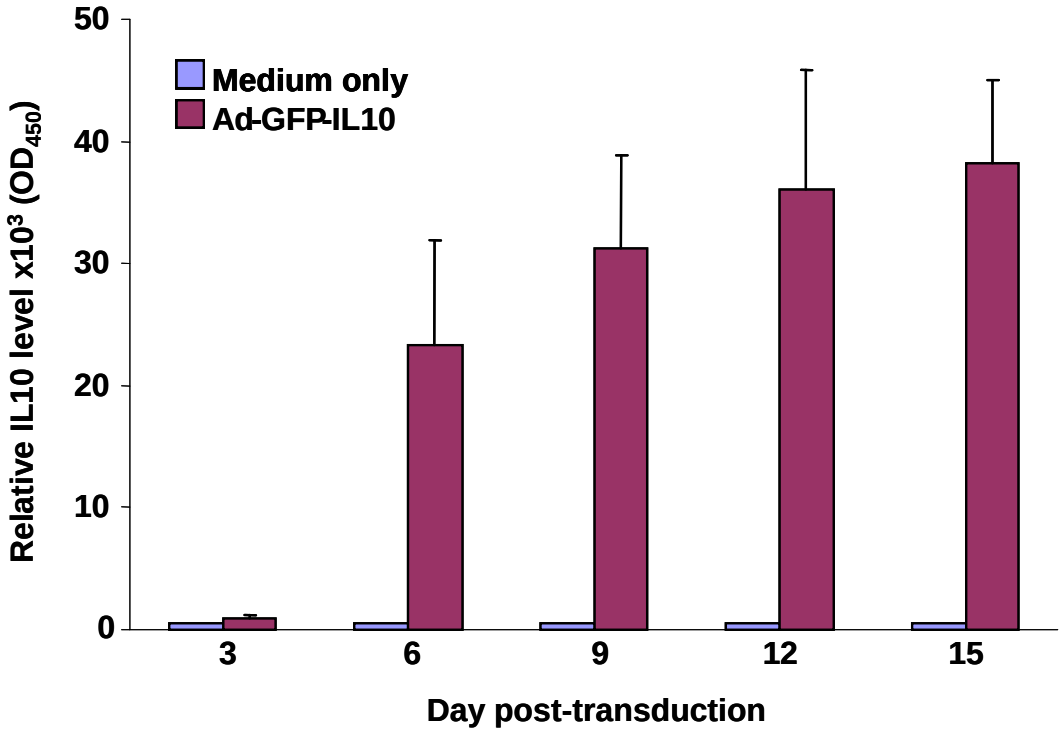


Figure 4.15: Kinetics of IL10 secretion by adenovirus-transduced ovine corneal endothelium

Columns represent relative optical density (OD) at 450 nm (mean±SD of three corneas) following ELISA on supernatants collected from corneas transduced with Ad-GFP-IL10 for 2 h at a dose of 5×10^7 pfu per cornea (MOI 40), after increasing periods of *in vitro* organ culture. Control corneas were transduced for 2 h with medium only. The final collection of supernatant was made at 15 d because beyond this point viability of cultured corneas was significantly compromised. Supernatants were tested in an ELISA specific for ovine interleukin-10 (IL10). Relative OD levels have been rescaled with that of control corneas at 3 d set to 1. Columns representing control corneas are not to scale, since actual levels were so low as to be invisible in the figure. MOI: multiplicity of infection, SD: standard deviation of the mean.

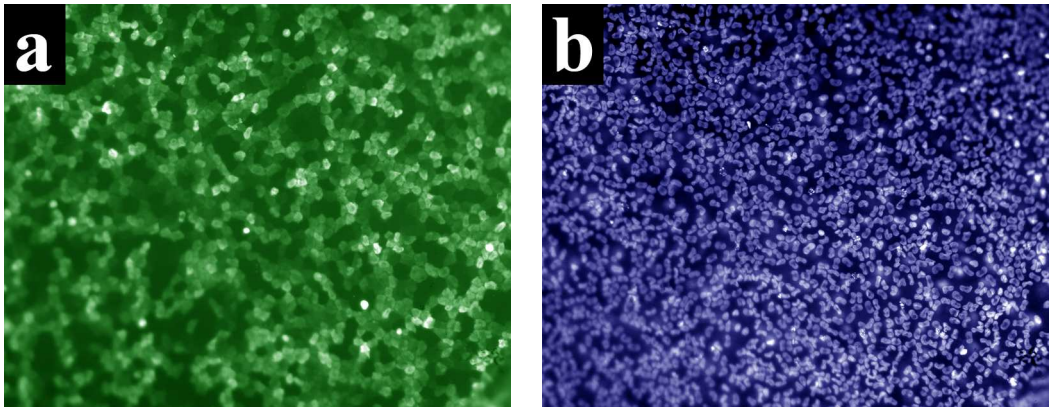


Figure 4.16: Fluorescence microscopy of Ad-GFP-IL10-transduced ovine corneal endothelium following 15 days of *in vitro* organ culture

Fluorescence micrographs of a representative central field show (a) enhanced green fluorescent protein (eGFP) expression, and (b) Hoechst-33258-stained nuclei under ultraviolet illumination, in an ovine cornea transduced with Ad-GFP-IL10 at 5×10^7 pfu (MOI 40) and subjected to 15 d of *in vitro* organ culture. These pictures are of one of the corneas presented in Figure 4.15. Original magnification 10×.

contained approximately five-fold more IL10 protein than the 2 h-transduced sample (Figure 4.17). This result supported the data from the qRT-PCR experiment (Figure 4.10).

Effect of polybrene on IL10 secretion by lentivirus-transduced ovine corneal endothelium

In view of the real-time qRT-PCR data suggesting an increase in IL10 mRNA expression after transduction in the presence of polybrene, supernatants were collected from corneas transduced with LV-SV40-IL10 in the presence of 4 µg / ml and 40 µg / ml polybrene, and subjected to an ELISA for IL10. Consistent with the data from qRT-PCR (Figure 4.11), corneas transduced for 3 h in the presence of polybrene exhibited higher rates of IL10 expression after 15 d, at the level of secreted protein (Figure 4.18). The effect was dose dependent, with the lower dose of polybrene (4 µg / ml) showing only a marginal increase in secretion, but the higher dose of polybrene (40 µg / ml) producing a rate of IL10 secretion approximately nine-fold higher than that observed in corneas transduced with the lentivirus alone. The difference between expression levels achieved with the 4 µg / ml and 40 µg / ml doses of polybrene was statistically significant (2-tailed, unpaired t-test: $p=0.037$).

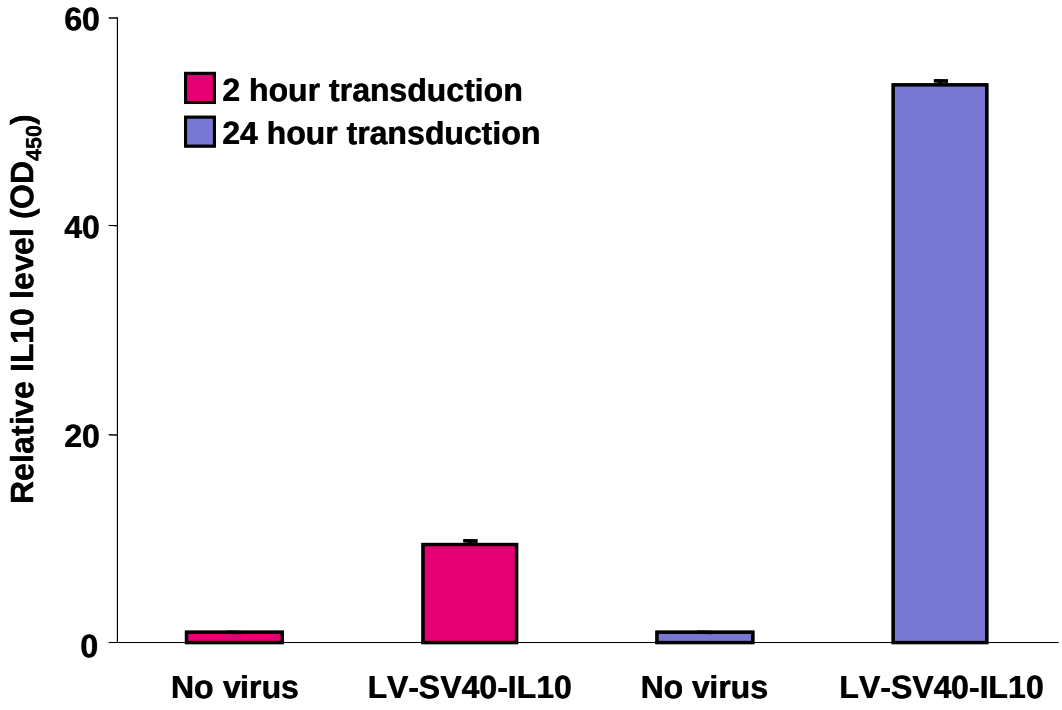


Figure 4.17: Effect of transduction period duration on levels of secreted IL10 in supernatants collected from lentivirus-transduced ovine corneal endothelium

Ovine corneas transduced for 2 h or 24 h with LV-SV40-IL10 at 7×10^7 iu per cornea (MOI 50) were subjected to 15 d of *in vitro* organ culture. Supernatants were collected after 15 d of *in vitro* organ culture, from media replenished at 12 d. An ELISA for ovine interleukin-10 (IL10) was carried out on collected supernatants. Columns represent mean±range of optical density (OD) at 450 nm, in duplicates for a single cornea at each treatment. OD values have been rescaled with the '2 h No virus' sample set to 1.

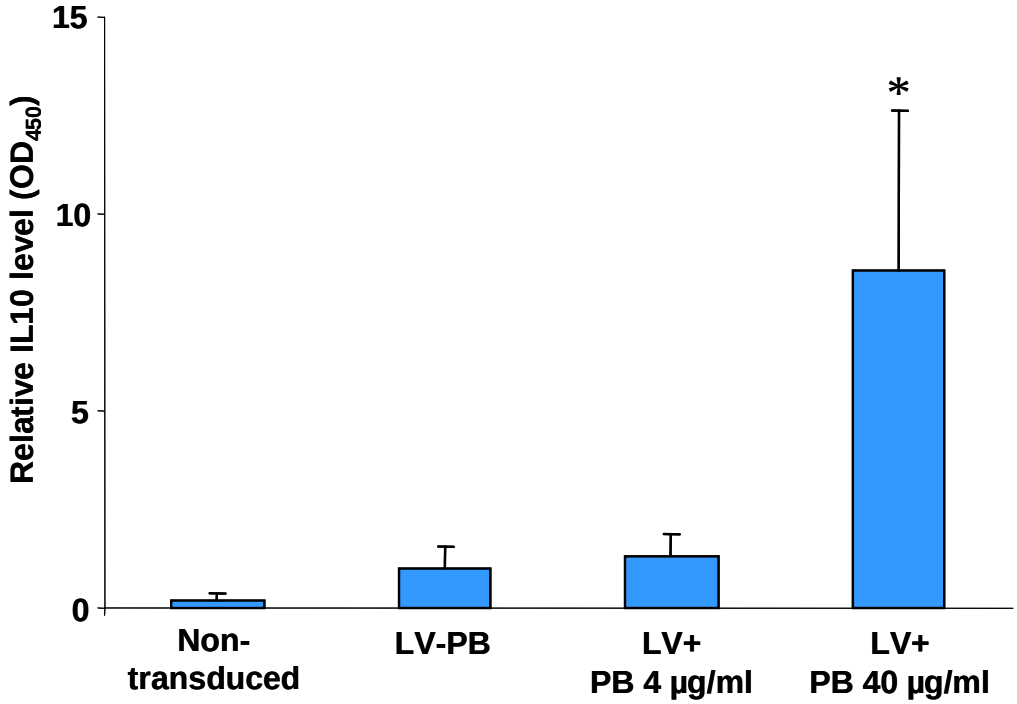


Figure 4.18: Effect of polybrene on levels of secreted IL10 protein in supernatants collected from lentivirus-transduced ovine corneal endothelium

Ovine corneas transduced for 3 h with LV-SV40-IL10 at 7×10^7 iu per cornea (MOI 50) in the absence (LV-PB) or presence of polybrene (LV+PB), were subjected to 15 d of *in vitro* organ culture. Supernatants were collected after 15 d of *in vitro* organ culture, from media replenished at 12 d. An ELISA for ovine interleukin-10 (IL10) was carried out on collected supernatants. Columns represent mean±SD of optical density (OD) at 450 nm (n=3 corneas). Levels have been rescaled with 'LV-PB' samples set to 1. * 2-tailed, unpaired t-test: p=0.037 compared to LV+PB 4 µg/ml, SD: standard deviation of the mean.

4.4.5 Quantification of IL10 secretion from lentivirus-transduced human corneas by ELISA of organ culture supernatants

In the same manner as experiments on ovine corneas, studies were conducted on human corneas to assess the expression of ovine IL10 protein following transduction with LV-SV40-IL10. A total of four corneas were tested (Table 4.2). The doses of 1×10^7 and 2×10^7 iu per cornea corresponded to MOIs of 50 and 100, the former being equivalent to the highest dose tested in experiments on ovine corneas. The same time-points for collection of the supernatants were used (3, 6, 9, 12, and 15 d post-transduction). A control cornea was transduced with medium only.

Table 4.2: Human corneas examined in LV-SV40-IL10 transduction assay

Sample	Treatment	Dose (iu per ml)	MOI	Transduction Period (h)
1	LV-SV40-IL10	1×10^7	50	3
2	LV-SV40-IL10	1×10^7	50	24
3	LV-SV40-IL10	2×10^7	100	24
4	Medium only	Nil	Nil	24

***Footnotes: MOI multiplicity of infection, h hours

Kinetics of IL10 secretion from lentivirus-transduced human corneal endothelium

An ELISA on supernatants from a human cornea transduced with 1×10^7 TU of LV-SV40-IL10 (MOI 50) for 24 h showed that the rate of IL10 secretion increased over the culture period, as seen in the ovine cornea (Figure 4.19). However, in contrast to the ovine cornea, in which the rate of secretion was continuing to rise at 15 d, the secretion rate from the human cornea had reached a plateau at 12 d. The overall level of IL10 detected in the supernatant from the human cornea (MOI 50) was approximately 10-fold higher than that from the ovine cornea (MOI 50) at the 15 d time-point.

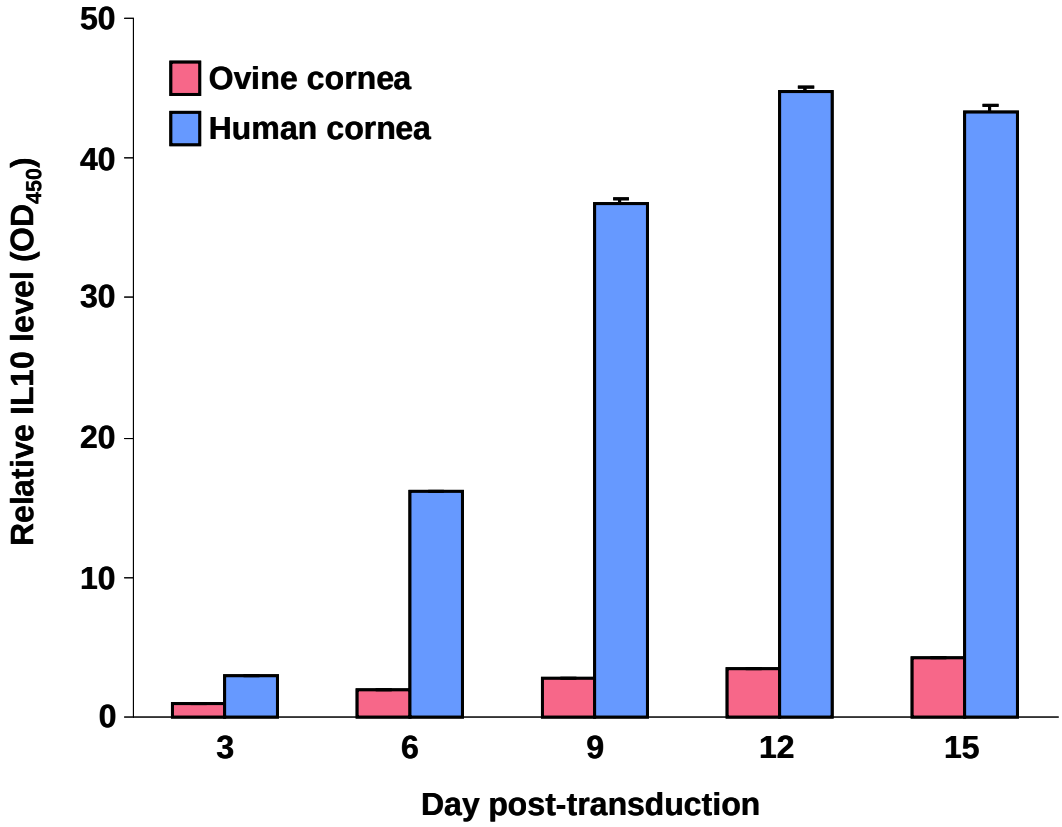


Figure 4.19: Kinetics of IL10 secretion in lentivirus-transduced human corneal endothelium

A human cornea, transduced for 24 h with 1×10^7 iu of LV-SV40-IL10 (MOI 50), was subjected to 15 d of *in vitro* organ culture. Supernatants were collected, and culture medium completely replenished, every 3 d. An ELISA for ovine interleukin10 (IL10) was carried out on collected supernatants. Columns represent mean±range of duplicates for relative optical density (OD) at 450 nm. Relative OD levels for an ovine cornea also transduced for 24 h (MOI 50) are presented for comparison (as in Figure 4.14). OD levels have been rescaled with the 3 d sample from the ovine cornea set to 1. No ovine IL10 was detected at any time point in supernatants collected from a nontransduced human cornea. MOI: multiplicity of infection.

Effect of multiplicity of infection on IL10 secretion by lentivirus-transduced human corneal endothelium

An experiment in which an MOI of 100 (double the previous dose) was tested in another human cornea, showed that the secretion rate was at least 3–4-fold higher than that of the MOI 50 cornea, at all time points (Figure 4.20). Since the dose was higher by a factor of only two, this may reflect that the cornea transduced with the higher dose had a higher endothelial cell density than the other. This was unable to be confirmed. However, the data suggested that the higher dose was well tolerated by human corneal endothelium for at least 15 d. No IL10 protein was detected in supernatants collected from a nontransduced control cornea, demonstrating the specificity of the assay for ovine IL10.

Effect of transduction period duration on IL10 secretion by lentivirus-transduced human corneal endothelium

In view of the reduction in transgene expression observed in the ovine cornea after shortening the transduction period, a human cornea was transduced for 3 h with 1×10^7 iu of LV-SV40-IL10 (MOI 50). Supernatants were collected every 3 d and tested in an ELISA for IL10. As seen in the ovine cornea, the shorter transduction period resulted in a 5–10-fold reduction in the secretion of IL10 after 15 d of *in vitro* organ culture (data not shown).

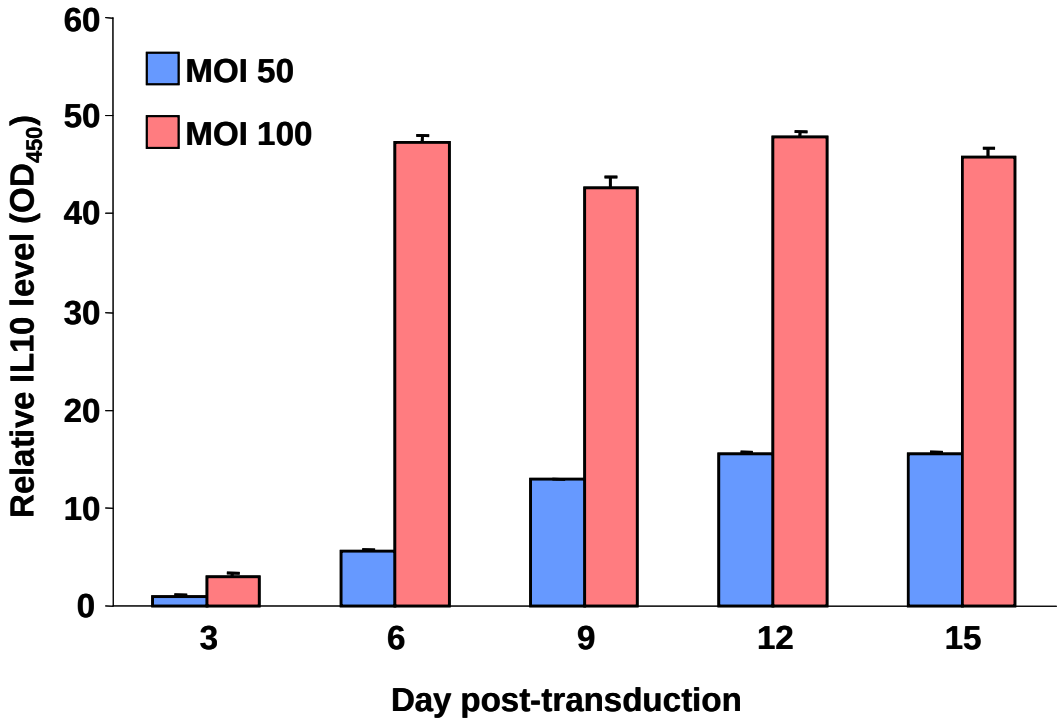


Figure 4.20: Effect of multiplicity of infection (MOI) on IL10 secretion from lentivirus-transduced human corneal endothelium

Individual human corneas transduced for 24 h with LV-SV40-IL10 at 1×10^7 iu per cornea (MOI 50) or 2×10^7 iu per cornea (MOI 100) respectively, were subjected to 15 d of *in vitro* organ culture. Supernatants were collected and culture medium completely replenished, every 3 d. An ELISA for ovine interleukin-10 (IL10) was carried out on collected supernatants. Columns represent mean±range of optical density (OD) at 450 nm. OD levels have been rescaled with the MOI 50 sample at 3 d set to 1.

4.4.6 Assay to confirm the function of transgenic IL10

To confirm the biological activity of the secreted transgenic IL10, an experiment was performed to investigate whether or not it retained its ability to inhibit the synthesis of the proinflammatory cytokine TNF-α. This assay was kindly carried out by Professor Prue Hart and Dr Cecilia Prele at the Telethon Institute for Child Health Research, Subiaco, WA. The method is described in detail in Section 2.3.2 on pages 65–66. Supernatant collected from A549 cells transduced with LV-SV40-IL10 was added to cultures of primary human monocytes. These were stimulated with lipopolysaccharide (500 ng / ml) to induce secretion of TNF-α. A 90% inhibition of TNF-α secretion was found in these samples compared to control monocytes stimulated with LPS alone (Figure 4.21).

4.4.7 Characterisation of a steroid-inducible lentiviral vector

A series of experiments were carried out to investigate the performance and inducibility of the steroid-inducible vector LV-GRE-IL10 in A549 cells, ovine corneas and human corneas. Dexamethasone sodium phosphate was used as the induction agent.

Determination of optimal dose of dexamethasone for induction in A549 cells transduced with LV-GRE-IL10

To confirm expression of IL10 by the LV-GRE-IL10 vector construct an initial experiment was conducted in tissue culture cells. Wells of a 24-well plate (Nunc, Rosklide, Denmark) were seeded with 0.25×10^6 A549 cells and the cells left to adhere for 3 h. The cells then underwent a 24 h transduction period with 7.6×10^4 iu per well of LV-GRE-IL10 in the presence of polybrene (4 µg / ml). At the end of the

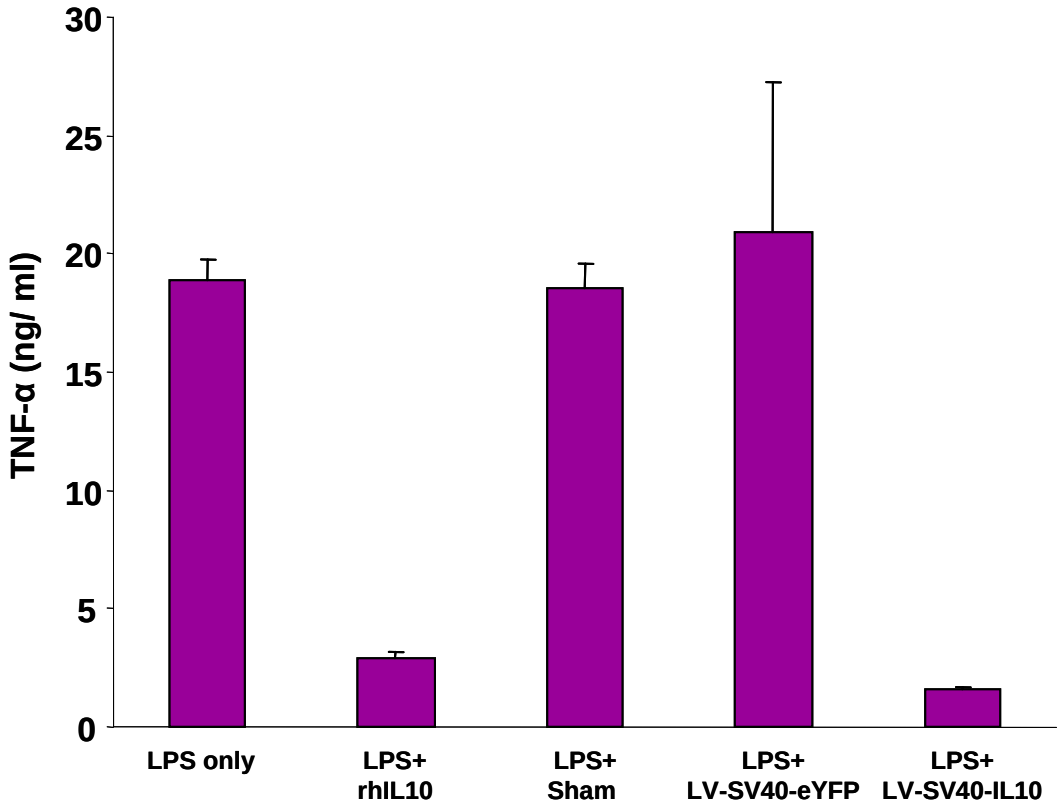


Figure 4.21: TNF-α-inhibition assay to demonstrate function of transgenic IL10

Supernatants were collected from A549 cells transduced with medium only (Sham), LV-SV40-eYFP or LV-SV40-IL10. These were then added to cultures of primary human monocytes, which were stimulated by the addition of lipopolysaccharide (LPS) (500 ng / ml). At 24 h post-stimulation, monocyte culture supernatants were assayed for the concentration of TNF-α by an ELISA. Columns represent the mean±range of duplicates tested. Controls included monocytes stimulated with LPS only (LPS only) and cells co-treated with LPS and recombinant human interleukin-10 (IL10) (10 ng / ml) (LPS+rhIL10). The assay was conducted by Professor Prue Hart and Dr Cecilia Prele at the Telethon Institute for Child Health Research, Subiaco, WA.

transduction period, medium was replaced with culture medium not containing polybrene, but containing varying concentrations of dexamethasone (0, 2.5 nM, 50 nM or 1000 nM). Treatments were performed in triplicate. After 48 h of culture, supernatants were collected and subjected to an ELISA for IL10. Levels of IL10 were approximately 45-fold higher in samples from cells cultured in the presence of 50 nM and 1000 nM dexamethasone compared to samples from transduced cells cultured in the absence of dexamethasone (Figure 4.22). Further experiments were performed using the 50 nM dose.

Effect of adding and withdrawing dexamethasone in A549 cells transduced with LV-GRE-IL10

Next, an experiment was carried out to determine whether withdrawal of dexamethasone from cells transduced with LV-GRE-IL10 would result in a reduction of IL10 expression to basal levels. Initially, A549 cells were transduced with 7.6×10^4 iu per well of LV-GRE-IL10 as done in the previous assay. After the transduction, cells were cultured in the presence of dexamethasone for 2 d, whereupon they were split 1:10 and then cultured for another 4 d, again in the presence of dexamethasone. On day six, cells were again split 1:10 and then cultured for another 4 d but dexamethasone was withdrawn. Cells transduced with LV-GRE-IL10 but not exposed to dexamethasone at any time were included as controls. On days two, six and 10, culture supernatants were collected prior to splitting cells and stored at –80°C. The supernatants were subsequently subjected to an ELISA for IL10. The assay showed approximately 30–35-fold upregulation of IL10 in supernatants collected from cells cultured with dexamethasone and collected on days two and six (Figure 4.23). Samples collected from cells at 10 d, after withdrawal of dexamethasone, showed a return to control levels.

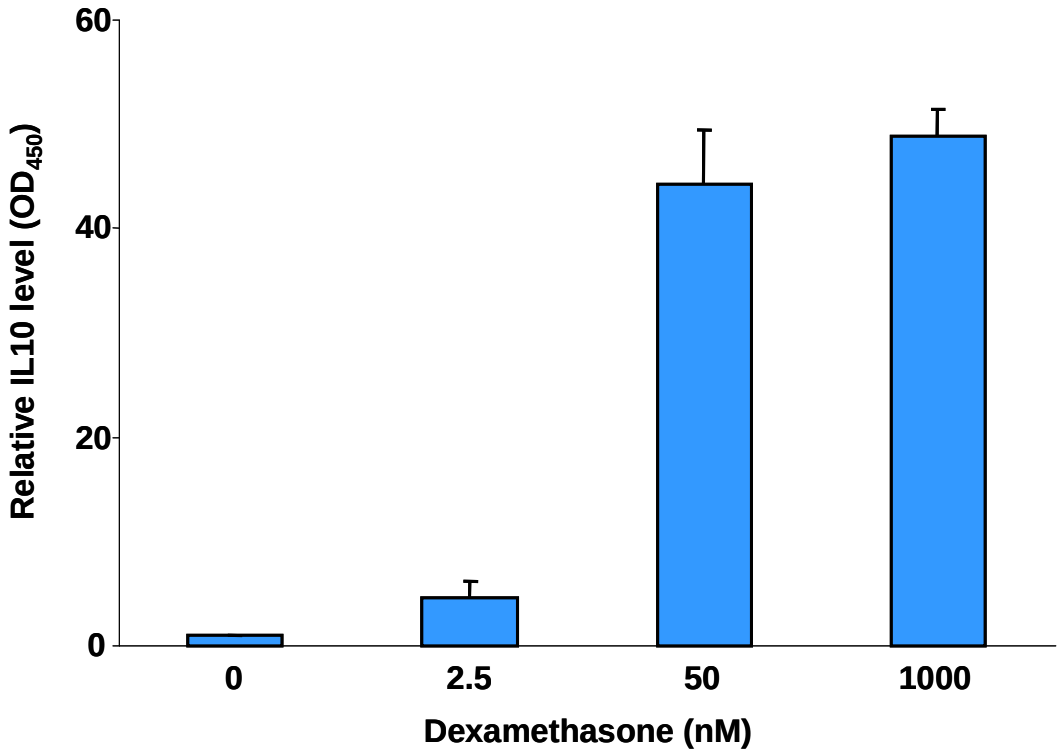


Figure 4.22: Titration of dexamethasone dose for induction of IL10 expression in A549 cells transduced with LV-GRE-IL10

Supernatants were collected from A549 cells transduced with LV-GRE-IL10 and cultured in the presence of increasing doses of dexamethasone. These were then subjected to an ELISA for ovine interleukin-10 (IL10). Columns represent mean±SD of optical density (OD) at 450 nm for three replicates. OD levels have been rescaled with the zero dose samples set to 1. SD: standard deviation of the mean.

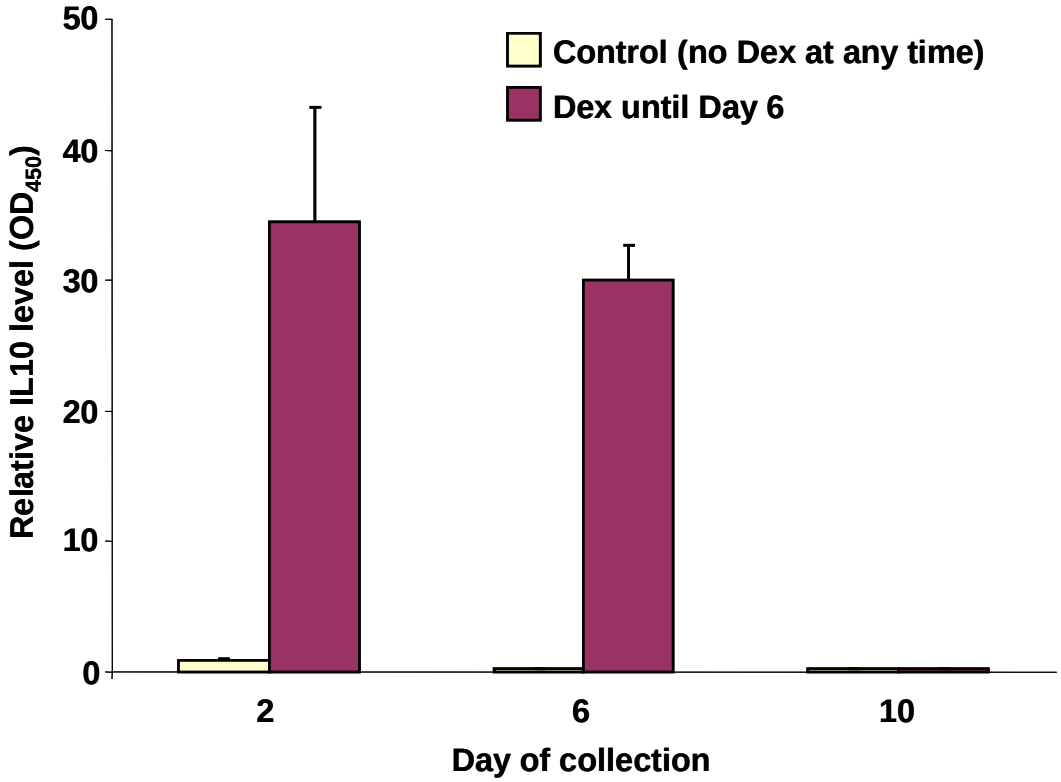


Figure 4.23: Effect of the addition and withdrawal of dexamethasone on IL10 secretion by A549 cells transduced with LV-GRE-IL10

Supernatants were collected from A549 cells transduced with LV-GRE-IL10 and cultured for 6 d in the presence of 50 nM dexamethasone (Dex) and a further 4 d in its absence. Control cells were transduced with LV-GRE-IL10 and cultured for 10 d without any dexamethasone. Samples were collected at 2, 6 and 10 d post-transduction prior to splitting cells 1:10. These were then subjected to an ELISA for ovine interleukin-10 (IL10). Columns represent mean±SD of optical density (OD) at 450 nm for three replicates. OD levels have been rescaled with control samples from day two set to 1. SD: standard deviation of the mean.

Titration of optimal dose of dexamethasone for induction in ovine corneal endothelium transduced with LV-GRE-IL10

The first experiment performed in ovine corneas using the LV-GRE-IL10 vector was a study to determine the dose of dexamethasone required to achieve maximal induction of IL10 expression. Corneas were transduced with LV-GRE-IL10 and subjected to *in vitro* organ culture in the presence of increasing doses of dexamethasone (nil, 2.5 nM, 50 nM, 1000 nM). Supernatants were collected and subjected to an ELISA for IL10. As seen in the experiments on A549 cells, 1000 nM dexamethasone was found to have no advantage over the 50 nM dose (data not shown). Therefore further experiments were conducted using 50 nM dexamethasone.

Secretion of IL10 by ovine corneal endothelium transduced with LV-GRE-IL10

To measure the inducibility of IL10 driven by LV-GRE-IL10 in ovine corneal endothelium, corneas were transduced for 24 h with 1×10^7 iu per cornea of LV-SV40-IL10 or LV-GRE-IL10 (MOI 8), and subjected to *in vitro* organ culture in the presence or absence of 50 nM dexamethasone. After 9 d of organ culture, supernatants from corneas transduced with LV-GRE-IL10 and cultured in the presence of the glucocorticoid were found to contain approximately nine times the concentration of IL10 detected in supernatants from control corneas (Figure 4.24). Those supernatants were also found to have approximately three times the concentration of IL10 detected in supernatants collected from corneas transduced with an equal dose of LV-SV40-IL10. Supernatants from corneas transduced with the same dose of LV-GRE-IL10 but cultured in the absence of dexamethasone did not contain more IL10 than those from control corneas.

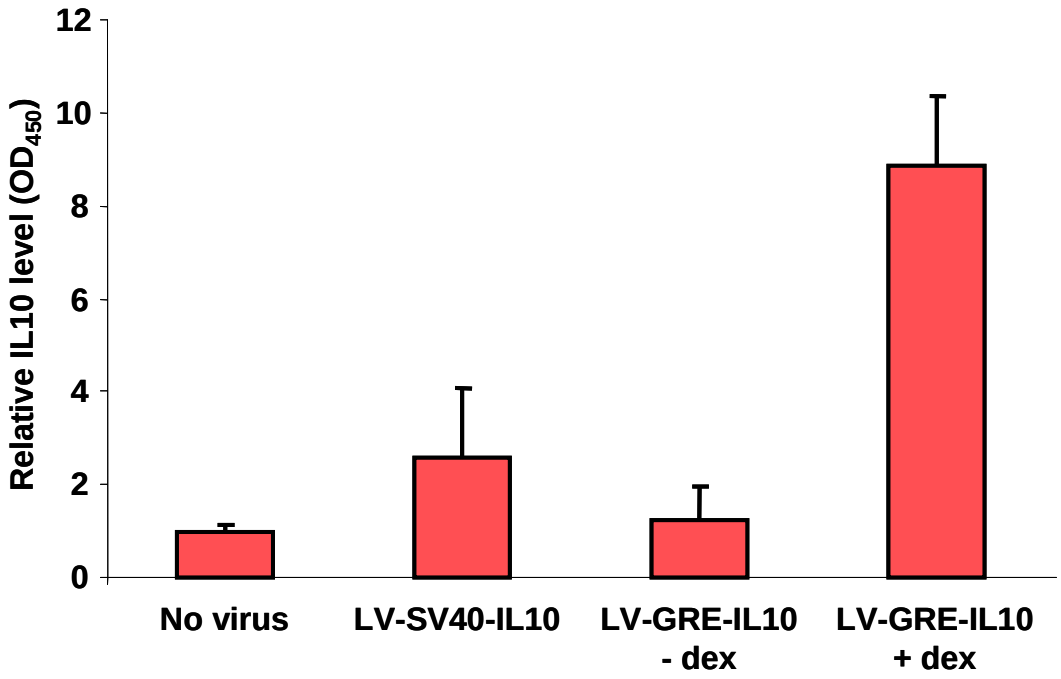


Figure 4.24: Secretion of IL10 by ovine corneal endothelium transduced with LV-GRE-IL10

Ovine corneas were transduced for 24 h in the presence of either: medium alone (no virus), 1×10^7 iu per cornea LV-SV40-IL10 alone (MOI 8), 1×10^7 iu per cornea LV-GRE-IL10 alone (- dex), or 1×10^7 iu per cornea LV-GRE-IL10 with 50 nM dexamethasone (+ dex). Corneas were subjected to *in vitro* organ culture for 9 d. Media were changed at 3 d and 6 d post-transduction. At 9 d the supernatants were collected and subjected to ELISA for ovine interleukin-10 (IL10). Columns represent mean±SD of optical density (OD) at 450 nm for three corneas in each treatment group. OD levels have been rescaled with the 'No virus' samples set to 1. MOI: multiplicity of infection, SD: standard deviation of the mean.

Secretion of IL10 by human corneal endothelium transduced with LV-GRE-IL10

To investigate expression of IL10 from human corneal endothelia transduced with LV-GRE-IL10, six human corneas were procured for a similar experiment to that described above for ovine corneas. Corneas were transduced with 1×10^7 iu per cornea of LV-SV40-IL10 or LV-GRE-IL10 (MOI 50) and subjected to *in vitro* organ culture in the presence or absence of 50 nM dexamethasone. After 9 d of *in vitro* organ culture, supernatants were collected and subjected to an ELISA for ovine IL10. Levels of IL10 in supernatants from corneas transduced with LV-GRE-IL10 and cultured in the presence of dexamethasone were found to be comparable to levels in supernatants from corneas transduced with LV-SV40-IL10. Those levels were approximately 14 times the concentration of IL10 detected in supernatants from corneas transduced with LV-GRE-IL10 and cultured without dexamethasone (Figure 4.25). As seen previously for LV-SV40-IL10 (Figure 4.19), the levels of IL10 produced by LV-GRE-IL10 in the presence of dexamethasone were approximately 12 times higher than in the supernatant from an ovine cornea transduced with LV-SV40-IL10 at the same MOI.

4.5 Discussion

These studies characterised the expression of transgenic IL10 by lentiviral vectors containing the SV40 or GRE5 promoter, in ovine and human corneal endothelium. The findings reflected those of the reporter gene studies described in Chapter 3. Transgene expression was more rapid and higher levels after transduction were attained with the adenoviral vector, compared to the lentiviral vector. Lentivirus-mediated expression was relatively delayed, and of a lower level, in ovine corneal

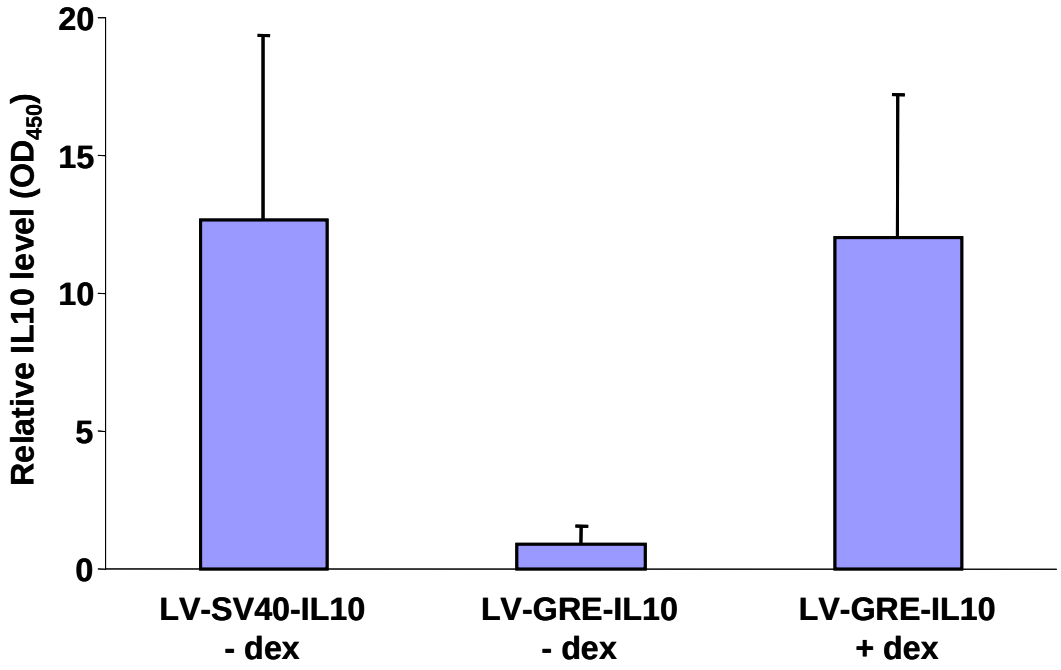


Figure 4.25: Secretion of IL10 by human corneal endothelium transduced with LV-GRE-IL10

Human corneas were transduced for 24 h in the presence of either 1×10^7 iu per cornea LV-SV40-IL10 alone (MOI 50), 1×10^7 iu per cornea LV-GRE-IL10 alone (- dex), or 1×10^7 iu per cornea LV-GRE-IL10 with 50 nM dexamethasone (+ dex). Corneas were subjected to *in vitro* organ culture for 9 d. Media were changed at 3 d and 6 d post-transduction. At 9 d the supernatants were collected and tested in an ELISA for ovine interleukin-10 (IL10). Columns show mean±range of optical density (OD) at 450 nm for two corneas in each treatment group. OD levels have been rescaled with 1 representing the level detected in supernatant from an ovine cornea transduced for 24 h with LV-SV40-IL10 at the same MOI (50) and also harvested after 9 d (as presented in Figure 4.19). MOI: multiplicity of infection.

endothelium compared to human. Multiplicity of infection, the duration of the transduction period, and the use of polybrene (40 µg / ml) during transduction, were found to influence expression in lentivirus-transduced corneal endothelium. Importantly, the transgenic IL10 protein was shown to retain the immunomodulatory function of cellular IL10. Finally, transduction with a lentiviral vector containing the GRE5 steroid-inducible promoter resulted in low basal expression and approximately 10-fold upregulation by dexamethasone, in both ovine and human corneal endothelium.

Adenoviral vectors achieved earlier and higher expression than lentiviral vectors

In ovine corneal endothelium, the adenoviral vector achieved expression levels at least 10^3-fold higher than the lentiviral vector, at both the transcriptional and secreted protein levels. The difference was evident as early as four days post-transduction, and persisted at 14 days. Whilst lentivirus-mediated secretion of IL10 was 10-fold higher in human corneas compared to ovine corneas at the same multiplicity of infection, expression levels were still logarithmically lower than those attained by the adenovirus. Therefore, the current studies demonstrated that lentivirus-mediated IL10 expression in both ovine and human corneal endothelium is much lower than that achieved by adenovirus in the first two weeks following transduction.

There have been numerous reports of adenovirus-mediated expression of secreted proteins by corneal endothelium. Production of the protein has been detected for up to a month *in vitro.*[76,84,97,106,108] Comer and colleagues, investigating the time course of CTLA4-Ig expression by rat corneas, reported maximal production of secreted protein in the first seven days and ongoing production to 27 days post-transduction.[76] Rayner and co-workers detected a transgenic tumour

necrosis factor receptor fusion protein (TNFR) 28 days after adenoviral transduction of rabbit corneal segments.[84] However, *in vivo* adenovirus-mediated transgene expression has been found to be relatively short-lived. After injection of adenovirus expressing β-galactosidase into the anterior chambers of rats, Lai and colleagues reported strong expression at four and seven days post-transduction, a reduction in intensity at seven days, and no detectable expression after 10 days.[103] Larkin et al. transduced rabbit corneas with an adenoviral vector and detected expression for 21 days *in vitro*, but for a maximum of 14 days after transplantation into outbred recipients.[104] A report by Qian and co-workers described adenovirus-mediated expression of a reporter gene in the corneal endothelium of syngeneic grafts in mice for less than two weeks after transduction at 37°C.[99] Interestingly, syngeneic grafts transduced at 4°C manifested expression for up to 12 weeks, the longest period of *in vivo* adenovirus-mediated expression in corneal endothelium that I am aware of.

Conversely, there have been numerous reports of long-term *in vivo* expression in corneal endothelium following lentiviral transduction. For example, Bainbridge and colleagues detected reporter gene expression 12 weeks following anterior chamber injection of mice with an HIV-1-based vector,[141] and more recently the same group reported transgene expression 10 months after delivery of an EIAV vector, also following anterior chamber injection of mice.[139] Thus, despite the lower expression relative to adenovirus, chromosomal integration and the low immunogenicity of lentiviral vectors promise long-term transgene expression. In the case of the Anson lentiviral vector, persistence of reporter gene expression was demonstrated for 21 days (Chapter 3) and transgenic IL10 for 15 days (herein), in ovine corneal endothelium *in vitro*. Due to the limited viability of ovine corneas *in*

vitro, studies of *in vivo* transgene expression were required to demonstrate transgene expression for even longer periods.

Secreted transgenic IL10 was biologically active

The function of the transgenic IL10 was confirmed by a cytokine synthesis inhibition assay on supernatants collected from A549 cells transduced with the lentiviral vector. However, the relative biological activity of supernatants from adenovirus- and lentivirus-transduced corneas was not compared. Whilst production of IL10 was shown to be much higher from adenovirus-transduced ovine corneas compared to lentivirus, the optimal expression level of immunomodulatory cytokines required to achieve prolongation of corneal allograft survival is not known. *Ex vivo* adenovirus-mediated gene transfer of p40-IL12[85] and IL10[75] have been shown to prolong corneal allograft survival in the sheep. However, *ex vivo* adenovirus-mediated gene transfer of the prototypic immunomodulatory cytokine IL4 resulted in eosinophilia, inflammation and rejection.[85] Excessive production of certain immunomodulatory cytokines may be counterproductive. It remained to be seen whether the relatively low, but potentially long-term, expression of IL10 in ovine corneal endothelium—achieved by the Anson lentiviral vector—would result in a prolongation of corneal allograft survival in the outbred sheep.

Variability was observed in ovine reference gene expression

Variability in the expression of ovine GAPDH and β-actin was observed across ovine corneas treated with adenovirus or lentivirus, and in the presence or absence of polybrene. This may be attributable to several factors. In dissecting the endothelium and Descemet's membrane away from the underlying stroma it was difficult to exclude all traces of stroma, and it is likely that variable amounts were carried over

to individual samples. Difficulty extracting large amounts of high quality RNA from highly collagenous tissues like the cornea has been reported previously,[197] and some degree of RNA degradation during the culture and subsequent dissection process is probably unavoidable. In addition to technical factors, the variability observed may accurately reflect substantial inter-animal or inter-treatment variation of reference gene expression in ovine cornea. Both GAPDH and β-actin are virtually ubiquitously expressed at moderately abundant levels, but transcription of both genes has been found to vary with experimental treatments and between individuals.[196] I am unaware of any reports of adenoviral or lentiviral transduction influencing the expression of these two reference genes, and no clear relationship was evident between their expression and experimental conditions in the present studies. Thus, the variation observed may represent inter-animal variation and noise, attributable to technical aspects of the RNA extraction process. The variation would certainly render these genes inappropriate for the accurate quantification of low abundance transcripts, where high sensitivity is vital. However, in the current studies, vector transduction induced a logarithmic increase in IL10 expression and thus the variability was unlikely to have influenced conclusions. For future transduction experiments in ovine cornea, where heightened sensitivity may be required, it will probably be necessary to perform a wider screen to identify more stable reference genes.

Expression from the steroid-inducible vector was regulated by dexamethasone

The ability to regulate transgene expression in a time and dose-dependent manner *in vivo* is a key feature of the ideal vector. Such a system could be well exploited in the cornea, given its accessibility to topical medications. In view of the central role of topical corticosteroids in preventing and treating corneal allograft rejection, I cloned

the GRE5 steroid-inducible promoter into the Anson lentiviral vector and assessed its ability to drive IL10 expression in response to dexamethasone. The vector was found to drive IL10 expression in A549 cells, as well as in ovine and human corneas, in the presence of dexamethasone. Importantly, expression levels supported by the GRE5 promoter during induction with dexamethasone, appeared comparable or superior to levels achieved by the same vector carrying the SV40 early promoter.

I am not aware of any reports to date describing gene delivery to the cornea under the control of a steroid-inducible promoter. However, such vector technology has been described in other systems. The GRE5 promoter was developed by Mader and White, who described a synthetic minimal promoter containing five glucocorticoid response elements (GREs) located upstream of the adenovirus 2 major late promoter TATA box/initiation site.[156] When propagated stably in HeLa cells in an Epstein-Barr virus episomal vector, the GRE5 promoter was more than 50-fold inducible and its activity was strictly dependent on the presence of dexamethasone. The same system has since been used in a number of other studies of gene expression, both *in vitro* and *in vivo*. Halaby and colleagues developed a glucocorticoid-regulated plasmid vector for modulating vascular endothelial growth factor (VEGF) gene expression in a model of rabbit hindlimb ischaemia.[198] They showed not only increased VEGF expression after treatment with dexamethasone, but also collateral vessel development—only in the presence of the steroid. Narumi and co-workers described an adenoviral vector containing the GRE5 promoter, which was used to deliver the chloramphenicol acetyltransferase (CAT) reporter gene to the livers of mice.[158] The vector achieved a 100-fold increase in CAT expression following intraperitoneal dexamethasone. Several other studies also provide support for the use of steroid-inducible promoters in controlling gene expression.[157,199]

In the current studies, the GRE5 promoter resulted in a 30–40-fold upregulation of IL10 expression in A549 cells, but expression levels increased only approximately 10-fold in transduced ovine and human corneas. Whilst this was lower than that reported in some other systems, the expression levels observed were equivalent to (in human), or superior to (in sheep) levels achieved by the vector containing the SV40 promoter. Importantly, the promoter showed low leakiness in the absence of dexamethasone, although ovine IL10 was still detectable in supernatants from transduced human corneas. In these studies, the steroid was added to the culture medium. With a view to clinical use, further studies should be performed *in vivo*, or using an artificial anterior chamber device such as that described by Thiel and colleagues,[200] to determine if topical glucocorticoid formulations can induce adequate transgene expression.

Other inducible promoters available for regulating lentivirus-mediated gene expression in the eye include the Tet-inducible systems.[147] In the original "Tet-Off" system the presence of tetracycline or doxycycline (dox) maintains the system in an "Off" state, whereas in the modified "Tet-On" system transcription is activated in the presence of dox. The incorporation of a "Tet-On" system into an adenoviral vector has recently been described in transducing Muller cells of the rat retina.[201] Various modifications have been introduced into the Tet-inducible systems in an attempt to reduce basal expression and increase maximal transgene expression. Though widely used in laboratory and animal research, concerns remain about their ultimate application in the clinical context. The Tet transactivators are derived from bacterial and viral sources, which are known to elicit humoral and cell-mediated immune responses.[202] Little is known about what immunological responses might be provoked by delivery of lentiviral vectors incorporating Tet-inducible systems *in*

vivo. The immune-privileged eye may be a preferred target for the delivery of such a vector.[203] However, ocular immune privilege in potential recipients of vector-transduced corneal allografts is certain to be compromised, diminishing the clinical potential of the system.

A high dose of polybrene was required to improve expression in lentivirus-transduced ovine corneal endothelium significantly

As found in the reporter gene studies, a shortening of the transduction period from 24 hours to 2 hours resulted in a decrease in transgene expression in lentivirus-transduced ovine corneal endothelium. The addition of polybrene to the transduction mixture resulted in an improvement in expression but this was only significant at the higher dose tested (40 µg / ml). This dose is 10-fold higher than that routinely used to improve retroviral transduction in tissue culture. However, it seems reasonable to speculate that, had the dose been directly toxic to corneal endothelial cells over the 15 days of organ culture, expression might have been reduced accordingly. On this basis, *in vivo* studies described in Chapter 5 utilised the higher dose of polybrene.

Further studies

The studies described in Chapter 3 showed that the Anson lentiviral vector achieved impressive transduction rates in both ovine and human corneal endothelium, and that expression was stable for at least 14 days *in vitro*. Non-quantitative data in those studies suggested that the adenoviral vector achieved higher expression more rapidly than the Anson vector. In this chapter I have presented quantitative data showing that transcription and secretion of transgenic IL10 in corneal endothelium was much higher following adenoviral transduction than it was following lentiviral transduction. In addition, expression in the ovine corneal endothelium was delayed

and lower than that attained in the human. Furthermore, reducing the transduction period from 24 hours to 2 hours, compromised expression levels—although they could be improved by the addition of high-dose polybrene. The implications of these results for *ex vivo* transduction and corneal transplantation in the ovine model are significant. In order to achieve maximal lentivirus-mediated expression it may be necessary to subject ovine donor corneas to long transduction periods and subsequent organ culture, or exposure to high-dose polybrene. The viability of the corneas after such treatment is not known. These issues were addressed by *in vivo* studies described in Chapter 5.

CHAPTER 5

LENTIVIRUS-MEDIATED GENE TRANSFER TO OVINE CORNEAL ENDOTHELIUM *IN VIVO*

5.1 Abstract

Aims

(i) To determine the *in vivo* stability of lentivirus-mediated gene transfer to ovine corneal endothelium, and (ii) to investigate the influence of lentivirus-mediated interleukin-10 (IL10) expression on the survival of donor allografts following orthotopic corneal transplantation in the sheep.

Methods

The stability of enhanced yellow fluorescent protein (eYFP) expression in ovine donor corneas was assessed following *ex vivo* transduction with the lentiviral vector LV-SV40-eYFP, and orthotopic transplantation in outbred sheep. Transduction rates in transplanted donor corneal endothelia and concurrently organ-cultured donor corneal rims were measured by fluorescence microscopy after euthanasia of the animals and harvest of the donor cornea. The viability of organ-cultured ovine donor corneas was examined following orthotopic transplantation in outbred sheep. In studies to assess the influence of lentivirus-mediated expression of ovine IL10 on corneal allograft survival, donor corneas were transduced with a lentiviral vector encoding IL10 under the control of the simian virus type-40 promoter (LV-SV40-IL10), and orthotopic transplantation was performed in outbred sheep. The influence on allograft survival of adding high-dose polybrene (40 μg / ml) to the LV-SV40-IL10 transduction mixture was also investigated. Unmodified donor corneas and corneas transduced with LV-SV40-eYFP constituted controls. Allografts were examined daily using a handheld slit-lamp to identify the day of graft vascularisation and the day of onset of immunological rejection. Endpoint histology was performed on failed allografts.

Results

Following *ex vivo* transduction with LV-SV40-eYFP and orthotopic corneal transplantation, eYFP expression was maintained in ovine corneal endothelial cells for at least 28 d *in vivo*. Corneal rims subjected to *in vitro* organ culture showed lower expression than corresponding transplanted donor buttons at 15 d post-transduction (approximately 10% versus 80% respectively). Nine of twelve unmodified control allografts subjected to a 24 h sham transduction period, followed by time in organ culture, suffered primary graft failure or infection. Unmodified control allografts, subjected to a 2–3 h sham transduction and no organ culture, suffered no primary graft failures, and were rejected at a median of 18 postoperative days (n=11). Allografts transduced for 2–3 h with LV-SV40-eYFP were rejected at a median of 17 d (n=3). Allografts transduced for 2–3 h with LV-SV40-IL10 in the presence of polybrene (40 µg / ml) were rejected at a median of 20 d (n=9), and showed evidence of polybrene-induced toxicity of the epithelium. Allografts transduced for 2–3 h with LV-SV40-IL10 in the absence of polybrene were rejected at a median of 25 d (n=7); the difference between this group and the combined sham transduced and LV-SV40-eYFP groups (n=14) was statistically significant (2-tailed Mann-Whitney U-test, corrected for ties: p=0.026).

Conclusions

Lentivirus-mediated expression in ovine corneal endothelium was stable for 28 d *in vivo*. Ovine donor corneas did not tolerate conventional human corneal organ culture methods. A seven day prolongation of allograft survival was achieved by transduction of donor corneas for 2–3 h with LV-SV40-IL10. Attempts to increase the expression of IL10 by the addition of polybrene (40 µg / ml) to the transduction

mixture resulted in a toxic effect on the cornea which abrogated the beneficial effect of IL10 on graft survival.

5.2 Introduction

Previous studies have shown that the outbred sheep is a high-risk preclinical model of corneal allograft rejection.[70] Ovine corneas are naturally vascularised and undergo immunological rejection at a median of 20 days in the absence of immunosuppression. Adenovirus-mediated gene transfer of several different immunomodulatory cytokines including interleukin-10 (IL10), has been shown to prolong allograft survival in this model (Table 5.1).[75,85] In the IL10 studies, indefinite allograft survival was achieved in two of nine animals treated with Ad-IL10 but tolerance was not achieved in either.[75] Adenoviral transgene expression is known to be limited by immunogenicity and transcriptional silencing.[96] Integrative vectors such as the HIV-1-based Anson lentiviral vector show promise for achieving long-term expression in corneal endothelium.[140]

In Chapter 3, lentiviral vectors expressing a reporter gene were shown to achieve stable expression in transduced ovine corneal endothelium for up to 21 days *in vitro*. However, in Chapter 4 it was shown that adenoviral vectors achieved levels of expression in ovine corneal endothelium 10^3-fold higher than lentiviral vectors, at as early as four days post-transduction. In addition, lentivirus-mediated transgene expression in ovine corneal endothelium was found to be delayed compared to expression in the human tissue.

Table 5.1: Studies of gene-modified corneal allotransplantation in the sheep

Study	Vector	Treatment	n	Day of rejection	Median survival
Klebe et al. (2001)[75]	Adenovirus	Untreated	7	18,19,19,20,20,22,32	20
		Ad-Mock	3	19,21,29	21
		Ad-IL10	9	19,20,30,33,55,66,88,>196,>300	55*
Klebe et al. (2005)[85]	Adenovirus	Untreated	13	18,19,19,19,20,20,21,21,22,22, 23,32,58	21
		Ad-GFP	6	17,18,20,20,21,23	20
		Ad-IL4	6	13,14,18,19,20,22	18.5
		Ad-p40-IL12	9	22,23,32,36,45,58,93,93,121	45**

* p=0.011 compared to untreated group (2-tailed Mann-Whitney U-test, corrected for ties)
** p=0.003 compared to untreated group (2-tailed Mann-Whitney U-test, corrected for ties)

Given the relatively low and delayed expression of IL10 in ovine corneal endothelium transduced with the lentiviral vector compared to an adenoviral vector, strategies were explored to ensure maximal IL10 expression was achieved prior to transplantation. The first approach was to subject donor corneas to a 24 h transduction followed by a period in organ culture. I was not aware of any previous studies describing transplantation of organ-cultured ovine corneas, so the method adopted was based on existing methods used for human corneas. Organ culture is the preferred storage method for human donor corneas in European Eye Banks. Corneas can be stored for up to 30 days at 31–37°C in basic culture solution,[204,205] commonly minimal essential medium (MEM), supplemented with 2% to10% FCS (European Eye Bank Association Directory, 12th Ed., January 2004); and they may be transferred to a deswelling medium containing dextran, for a short period prior to transplantation.[205]

The second strategy undertaken to improve lentiviral expression was to transduce donor corneas for 2–3 hours in the presence of polybrene and to carry out transplantation on the same day.

5.3 Specific aims

- To determine the *in vivo* stability of lentivirus-mediated gene transfer to ovine corneal endothelium following orthotopic allotransplantation;
- To investigate the *in vivo* viability of organ-cultured ovine donor corneas;
- To investigate the survival of ovine corneal allografts following transduction in the presence or absence of polybrene, with a lentiviral vector encoding interleukin-10.

5.4 Results

5.4.1 Expression in ovine corneal allografts following *ex vivo* transduction with LV-SV40-eYFP

Comparison between expression in organ culture and in vivo

To compare lentivirus-mediated reporter gene expression in organ culture and *in vivo*, two ovine corneas were transduced with 2.5×10^7 TU per cornea of LV-SV40-eYFP (MOI 20) and each transplanted to the right eye of outbred sheep. At the time of surgery, the transduced but nontransplanted corneal rims were subjected to concurrent *in vitro* organ culture. The grafts were examined daily using a handheld slit lamp, to detect vascularisation of the donor button and signs of rejection, as described in Section 2.3.6 (pages 92–93). On day 15, before the onset of rejection, the animals were euthanased and both the grafted corneal buttons and organ-cultured rims were harvested into buffered formalin for nuclear staining, micro-dissection and fluorescence microscopy. In both cases, a higher expression rate was evident in the transplanted cornea compared to the respective organ-cultured rim (approximately 80% versus 10% respectively). There was no gross reduction in corneal endothelial

cell density in cultured rims, nor was there evidence of chromatin condensation or nuclear blebbing typical of apoptosis, as judged by inspection of Hoechst-33258-stained nuclei under ultraviolet illumination (Figure 5.1).

Stability of expression in vivo

To investigate the stability of lentiviral expression *in vivo*, three ovine corneas were transduced *ex vivo* for 2–4 h with LV-SV40-eYFP at 2.5–5×10^7 TU per donor cornea (MOI 20–40), and each transplanted to the right eye of outbred sheep. Prior to the onset of rejection, sheep were euthanased, and corneas harvested for fluorescence microscopy. One of the animals, which received no immunosuppression, was killed 20 d postoperatively before immunological rejection supervened. The other two sheep, which each received a single subconjunctival injection of 10 mg methylprednisone acetate immediately after surgery, were killed after 28 postoperative days. In all cases, the donor endothelium appeared intact and expression of eYFP was intense and widespread (Figure 5.2). Examination of the graft-host junction showed that no endothelial cells in the recipient cornea expressed the reporter protein. All three ovine allografts were clear and thin at the time the recipients were euthanased. Dense sheets of fibrous tissue were evident in the anterior chambers of both animals which had received an injection of methylprednisone acetate.

5.4.2 Organ culture of ovine donor allografts

Data from *in vitro* studies indicated that lentivirus-mediated gene expression in ovine corneal endothelium was highest following a 24 h transduction period and increased over time in organ culture. Polybrene (40 µg / ml) was shown to improve the transduction rate achievable during a short transduction period, although it was

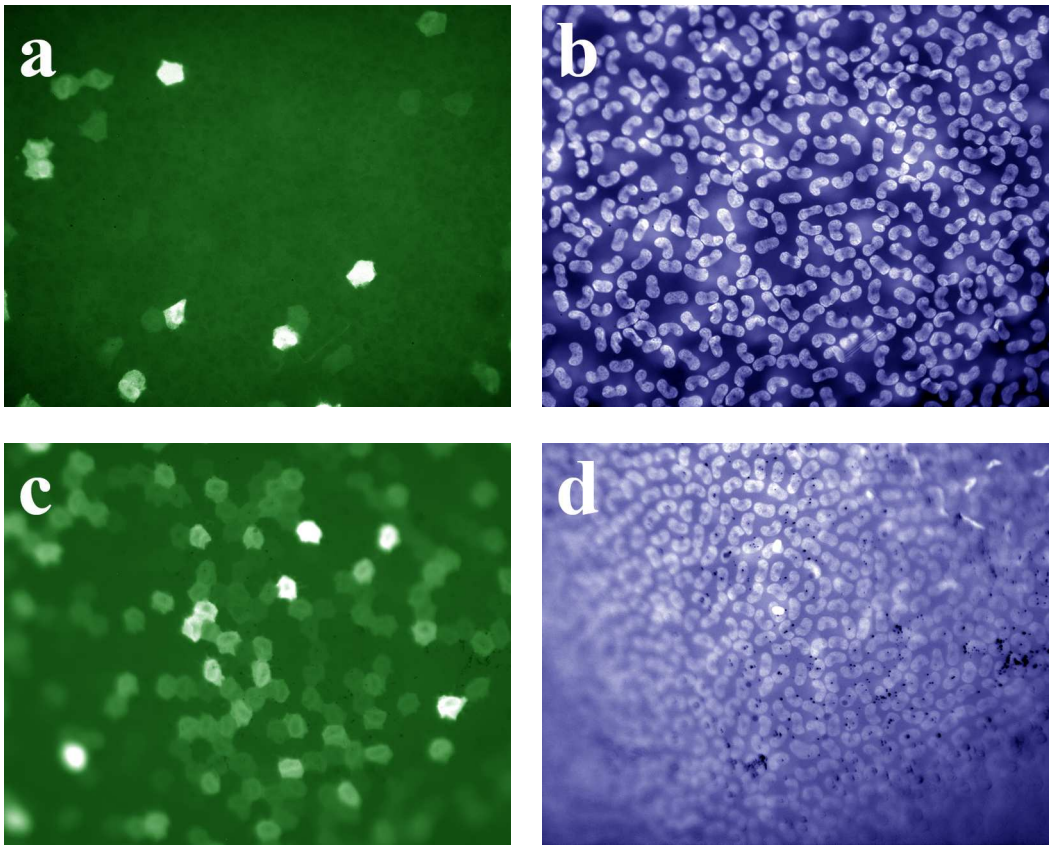
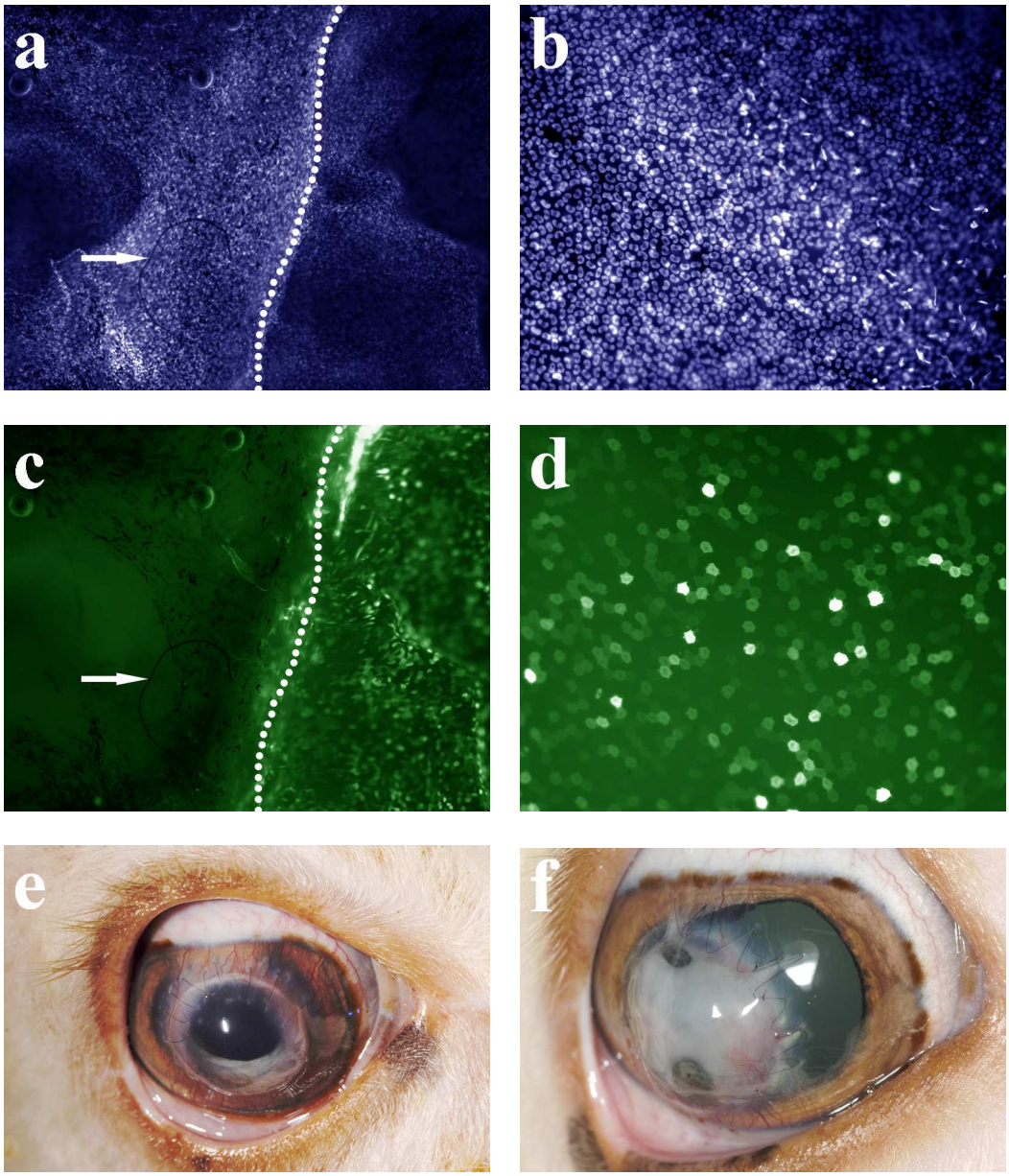


Figure 5.1: Expression of eYFP in organ-cultured ovine corneal rims and corneal allografts after *ex vivo* transduction with LV-SV40-eYFP

Corneal endothelium from (a, b) the rim of the donor cornea following *ex vivo* transduction with 5×10^7 TU of LV-SV40-eYFP (MOI 40) after 15 d of *in vitro* organ culture, and (c, d) from the corresponding transplanted corneal button, after 15 d *in vivo*. Fluorescence micrographs of representative central fields show (a, c) expression of enhanced yellow fluorescent protein (eYFP) under blue light excitation, and (b, d) endothelial cell nuclei stained with Hoechst-33258 under ultraviolet illumination of each respective field. Similar data were generated for a second corneal allograft (not shown). Original magnification 20×.

Figure 5.2: Stability of reporter gene expression after *ex vivo* transduction of ovine corneas with LV-SV40-eYFP and subsequent orthotopic corneal transplantation

Corneal allografts harvested at (a, c, e) 20 days and (b, d, f) 28 days after transplantation. Representative fluorescence micrographs show (a, b) Hoechst-33258-stained endothelial nuclei under ultraviolet illumination and (c, d) expression of enhanced yellow fluorescent protein (eYFP) in identical fields of the same corneas. Note the suture loop (arrow) near the graft-host junction (dotted line) in (a) and (c), and the absence of eYFP expression in host corneal endothelium (left side of field in (c)). The respective lentivirus-transduced ovine corneal allografts *in vivo*, are shown in (e) and (f), shortly before harvesting of tissue. Note dense fibrous tissue in anterior chamber, inferiorly in (e), and forming a sheet in (f); in both cases, corneas were thin and uninflamed. Original magnification (a, c) 4× and (b, d) 10×. [Photographs of allografts taken by J. Johnston]


a
b
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not found to be a prerequisite for expression. Therefore, studies were undertaken to determine the *in vivo* viability of ovine donor allografts following a 24 h transduction period and *in vitro* organ culture. Donor corneas underwent a 24 h sham transduction with 300 µl 2% RPMI at 37°C. Corneas were then immersed in 40 ml of HEPES-buffered RPMI—containing 10% v/v FCS and supplementary L-glutamine (2 mM), penicillin (100 IU / ml), streptomycin (100 µg / ml) and amphotericin B (0.25 µg / ml)—and cultured at 37°C in sealed pots for zero to 14 d. In five cases, corneas were transferred to a deswelling medium containing 5% w/v Dextran T500, for a period of 24 h (two corneas) to 48 h (three corneas) prior to transplantation. No assessment of endothelial cell density or viability was performed before transplantation. The outcomes after orthotopic corneal transplantation are shown in Table 5.2

Table 5.2: Outcomes of organ-cultured ovine donor allografts

No.	Transduction period (h)	Time in organ culture (d)	Time in deswelling medium (h)	Outcome of allograft
1	24	0	0	Primary graft failure
2	24	1	0	Primary graft failure
3	24	1	0	Primary graft failure
4	24	1	0	Immunological rejection (24 d)
5	24	1	0	Immunological rejection (62 d)
6	24	1	0	Infection*
7	24	2	0	Infection**
8	24	8	48	Primary graft failure
9	24	8	48	Immunological rejection (24 d)
10	24	14	24	Primary graft failure
11	24	14	24	Primary graft failure
12	24	14	48	Primary graft failure

Footnotes: h hours, d days, deswelling medium: 5% w/v Dextran T500
* unidentified gram positive bacillus, ** *Moraxella ovis*

In vivo viability of organ-cultured donor allografts

Even short periods (24–48 h) of organ culture resulted in severe swelling of ovine donor corneas, accompanied by the appearance of folds in Descemet's membrane (Figure 5.3). A period in deswelling medium alleviated the swelling only marginally, as judged by gross inspection. Satisfactory apposition of wound margins

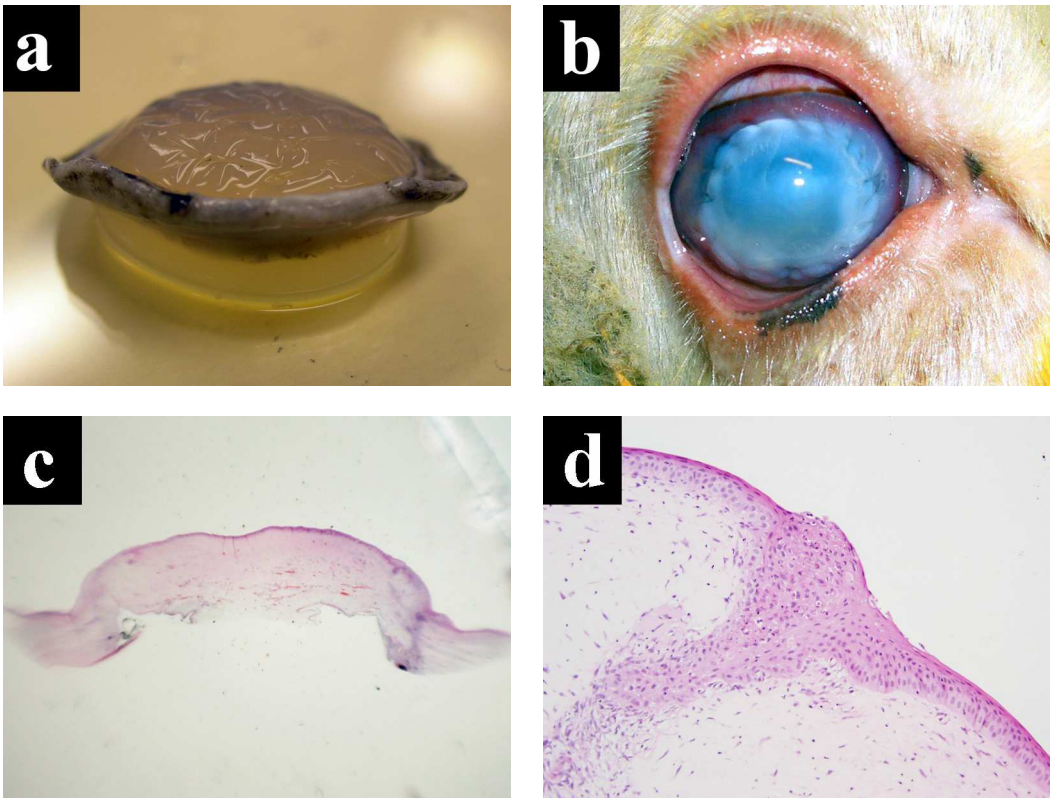


Figure 5.3: Effects of organ culture on ovine donor corneal allografts

(a) A photograph of a cornea (with scleral rim) subjected to *in vitro* organ culture for 22 d, shows severe swelling and folds in Descemet's membrane (endothelium upwards). (b) A photograph taken on postoperative day seven, shows an orthotopic corneal allograft transplanted after a 24 h sham transduction and 14 d of *in vitro* organ culture (including 48 h in deswelling medium). Note the oedematous wound edges in the donor tissue; the donor cornea never recovered normal clarity. (c) A low magnification photomicrograph shows a histological section of an organ-cultured corneal allograft. (d) A photomicrograph shows epithelial downgrowth at the graft-host junction in an organ-cultured allograft. Original magnification 20×.

during surgery was problematical due to the disparity in thickness between host and donor corneas. Following transplantation, grafts were assessed daily using a handheld slit lamp, as described Section 2.3.6 (pages 92–93). The majority of organ-cultured corneas never recovered normal clarity or thickness. Endpoint histology revealed stromal swelling, poor wound edge apposition, epithelial downgrowth at wound margins, and a mixed inflammatory cell infiltrate containing mononuclear cells and neutrophils; endothelial cells were present in all these excised grafts although in most cases their coverage of Descemet's membrane was incomplete. Of 12 donor corneas transplanted, seven were deemed primary graft failures and two developed suture abscesses which were refractory to topical antibiotic (Table 5.2). The remaining three became quite thin and transparent by approximately 14 d after transplantation, but underwent immunological rejection at 24, 24 and 62 postoperative days respectively.

5.4.3 Survival of sham transduced, noncultured donor allografts

Due to the high rate of primary graft failure in donor corneas subjected to a 24 h sham transduction followed by organ culture, the strategy was abandoned and further studies were performed using a transduction period of 2–3 h followed by transplantation on the same day. In the first group, 11 sheep received sham transduced donor allografts, exposed to medium only. Grafts were assessed daily using a handheld slit lamp. There were no infections or primary graft failures in this group. All underwent immunological rejection at a median of 18 postoperative days (range 16–37 d) (Figure 5.4 and Table 5.3). Vascularisation of the donor tissue occurred at a median of 6 d (range 2–12). Endpoint histology showed a predominantly mononuclear cell infiltrate, neovascularisation of the stroma, and destruction of endothelial cells on the donor tissue.

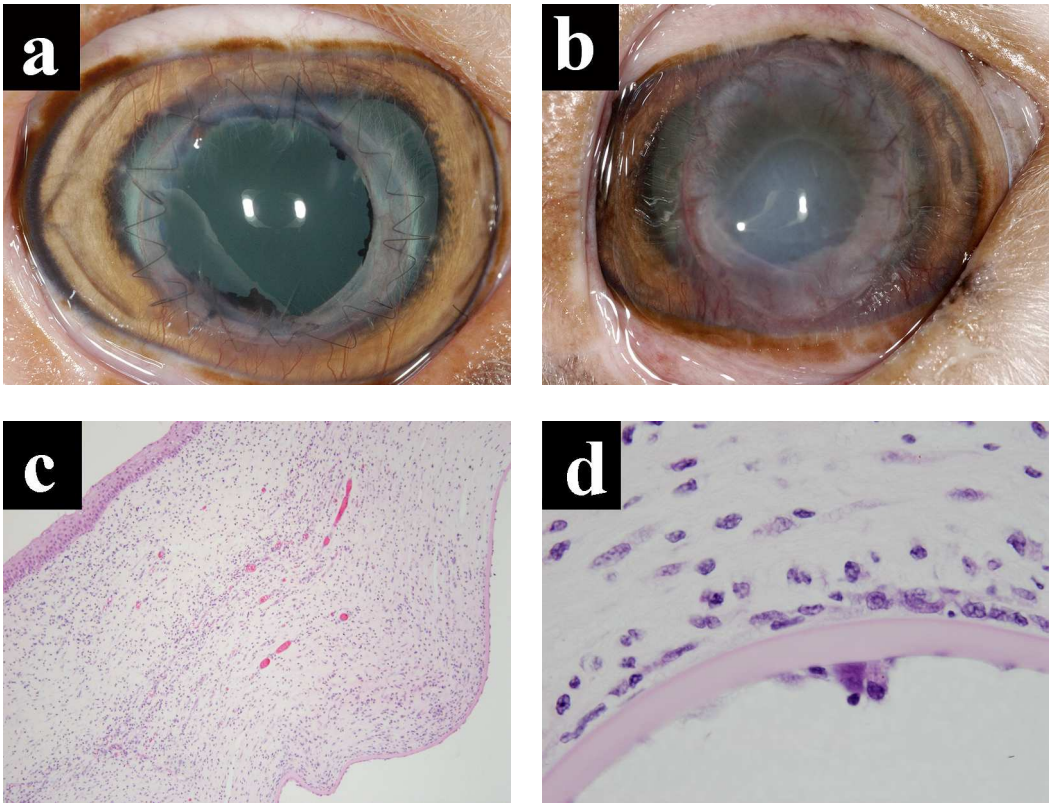


Figure 5.4: Sham transduced ovine corneal allografts

(a) A photograph of an ovine corneal allograft at 20 d after a 2 h sham transduction, and before the onset of immunological rejection; the donor cornea was never subjected to *in vitro* organ culture (the line apparent in the lower left quadrant of the graft is a reflection). (b) A photograph of a sham transduced allograft taken on postoperative day 26, shows evidence of immunological rejection; note stromal oedema, presence of epithelial rejection lines, and dense vascularisation at graft margins. (c) A photomicrograph of a sham transduced allograft undergoing rejection, shows stromal oedema, diffuse inflammatory infiltrate, and neovascularisation. Original magnification 10×. (d) A photomicrograph of a sham transduced allograft shows mononuclear cells on Descemet's membrane. Original magnification 100×.

Table 5.3: Summary of outcomes of gene-modified ovine allografts

Treatment	n	Day of graft vascularisation	Median day of graft vascularisation	Day of rejection	Median survival (days)
Sham transduced (2–3 h)	11	2,2,6,6,6,6,6,6,7,8,12	6	16,17,18,18,18,18,21,21, 22,28,37	18
LV-SV40-eYFP (2–3 h)	3	3,6,6	6	16,17,19	17
Combined controls (Sham transduced and LV-SV40-eYFP)	14	2,2,3,6,6,6,6,6,6,6,6,7,8,12	6	16,16,17,17,18,18,18,18, 19,21,21,22,28,37	18
LV-SV40-IL10 (2–3 h) with polybrene (40 µg / ml)	9	3,5,5,5,5,7,8,9,12	5	12,12,17,18,20,21,23,23, 27	20
LV-SV40-IL10 (2–3 h)	7	6,6,7,9,9,11,13	9[†]	18,19,25,25,29,30,46	25[‡]

***Footnotes: h hours, [†] p=0.046 compared to Sham transduced, [‡] p=0.06 compared to Sham transduced and p=0.026 compared to combined controls (2-tailed Mann-Whitney U-test, corrected for ties)

5.4.4 Survival of LV-SV40-eYFP-transduced donor allografts

Three sheep received allografts subjected to a 2–3 h transduction period with 2.5–5×10^7 TU per cornea of LV-SV40-eYFP (MOI 20–40) and no time in organ culture. All underwent rejection at a median of 17 postoperative days (range 16–19 d), with vascularisation of the graft occurring at a median of 6 d (range 3–6 d) (Table 5.3). Given that the survival of the LV-SV40-eYFP-transduced grafts did not differ from that of the sham transduced grafts, these groups were pooled to form a combined control group for comparison with the LV-SV40-IL10-transduced groups (n=14) (vide infra).

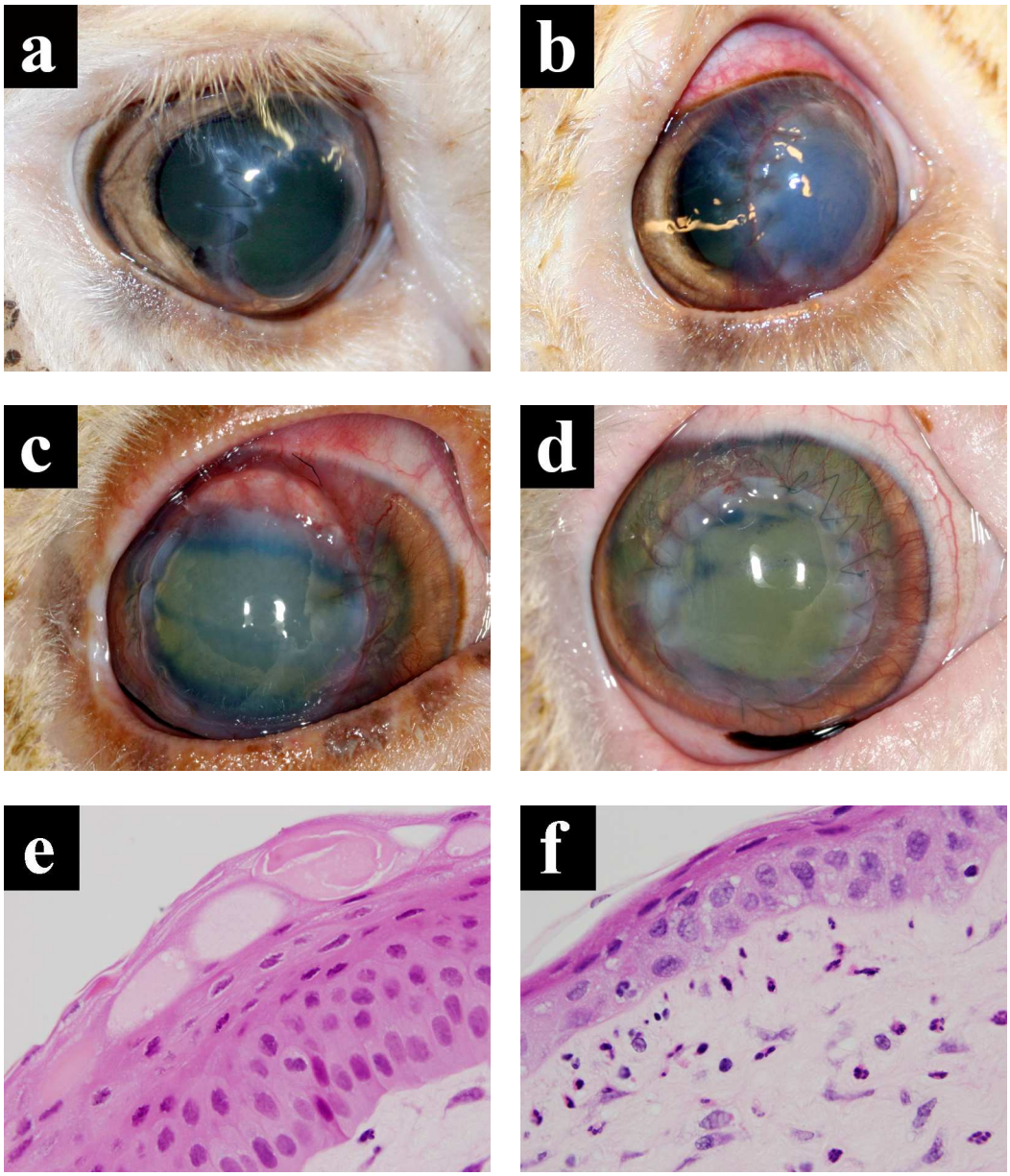
5.4.5 Survival of LV-SV40-IL10-transduced donor allografts

Transduction with polybrene

On the basis of *in vitro* studies demonstrating an increase in the level of IL10 expression achieved with the addition of polybrene (40 µg / ml) to the transduction mixture, nine animals received allografts which had been transduced with 7×10^7 iu per cornea LV-SV40-IL10 (MOI 50) for 2–3 h, in the presence of polybrene (40 µg / ml). The median time until rejection for this group was 20 postoperative days (range 12–27 d). Neither the time to vascularisation nor the survival time of the grafts differed significantly from unmodified controls (Table 5.3). The majority of these allografts endured a fluctuating postoperative course. For the first 10 d, a number of animals exhibited excessive lacrimation, and required topical antibiotic for treatment of suspected infective abscess formation. Host and donor cornea underwent dense vascularisation, and the epithelium appeared roughened (Figure 5.5).

Figure 5.5: LV-SV40-IL10-transduced ovine corneal allografts

(a) A photograph of an allograft transduced with LV-SV40-IL10 in the absence of polybrene, on postoperative day 34. (b) A photograph of the same allograft on postoperative day 49, 3 d after the onset of immunological rejection. (c) A photograph of an allograft treated with LV-SV40-IL10 in the presence of polybrene (40 µg / ml), taken on postoperative day 11. Note rim of residual scleral tissue in superior graft, dense vascularisation of host cornea, and loss of normal corneal clarity. (d) A photograph of another allograft also treated with LV-SV40-IL10 in the presence of polybrene (40 µg / ml), on postoperative day 11. Note dense vascularisation, fibrin in anterior chamber and loss of normal corneal clarity. (e) A photomicrograph of an allograft treated with LV-SV40-IL10 in the presence of polybrene (40 µg / ml), shows cytoplasmic vacuolisation of epithelial cells. Original magnification 100×. (f) A photomicrograph of an allograft treated with LV-SV40-IL10 in the presence of polybrene (40 µg / ml) shows the presence of subepithelial neutrophils and loss of normal epithelial morphology. Original magnification 100×.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Endpoint histology revealed subepithelial aggregations of neutrophils, a stromal mononuclear cell infiltrate, stromal oedema, and marked stromal neovascularisation. Epithelial changes were striking, with prominent cytoplasmic vacuolisation, dyskeratosis, and loss of normal epithelial morphology. Several animals had evidence of concomitant infective abscess formation, localised at the epithelium. Evidence of injury to endothelial cells was not as clear-cut, as specimens were obtained after the onset of immunological rejection. Histopathological sections were examined by a consultant ocular pathologist (Dr Sonja Klebe, Department of Pathology, Flinders Medical Centre, Bedford Park, SA) and were considered to be consistent with acute toxicity of the cornea, predominantly affecting the epithelium.

Transduction without polybrene

In view of the toxic effect of polybrene, a series of allografts were performed in which corneas were transduced with 7×10^7 iu per cornea of LV-SV40-IL10 (MOI 50) for 2–3 h in the absence of polybrene. All grafts eventually underwent rejection, the median being at 25 postoperative days (range 18–46 d) (Figure 5.5), with vascularisation of the graft occurring at a median of 9 d (range 6–13 d). This seven day prolongation of survival did not reach statistical significance when compared with the sham transduced control group alone (2-tailed Mann-Whitney U-test, corrected for ties: Z=-1.878; p=0.06), but was significant in comparison to the combined control group (Z=-2.219; p=0.026) (Table 5.3). Vascularisation of the graft occurred at a median of 3 d later in the LV-SV40-IL10-transduced group compared to the sham transduced group (Z=-1.993; p=0.046). Endpoint histology did not differ from that seen in the case of sham transduced allografts; a predominantly mononuclear cell infiltrate, concentrated at the endothelium and in the stroma at the graft-host junction, was observed in all cases.

5.5 Discussion

Following *ex vivo* transduction with the Anson lentiviral vector and orthotopic corneal transplantation, reporter gene expression was maintained in ovine corneal endothelial cells for at least 28 days *in vivo*. Organ culture of ovine donor corneas resulted in severe swelling and a high rate of primary graft failure after transplantation. *Ex vivo* transduction in the presence of polybrene with the lentivirus encoding IL10, appeared to have a toxic effect on the epithelium of donor corneas, and did not result in a prolongation of allograft survival. Lentivirus-mediated transfer of the gene encoding IL10 to ovine donor allografts in the absence of polybrene, prolonged survival by a median of seven days compared to controls.

Lentivirus-mediated expression was stable in vivo

Several studies have described stable *in vivo* reporter gene expression in the corneal endothelium mediated by lentiviral vectors.[83,139] Balaggan and colleagues injected the anterior chambers of mice with an EIAV vector and detected expression of enhanced green fluorescent protein (eGFP) 10 months later.[139] Barcia and co-workers recently reported expression of eGFP 56 days after transduction of mouse corneas with an HIV-based vector and syngeneic grafting.[83] The detection of widespread reporter gene expression in corneal endothelial cells of two ovine allografts 28 days after transplantation, confirms that the Anson lentiviral vector has the capacity for stable expression *in vivo*. It may be argued that the subconjunctival injection of methylprednisone acetate administered to these allografts postoperatively may have suppressed an immune response against the vector. However, lentiviral vectors have been found to have little or no immunogenicity in the eye,[132,133] and at least one transduced allograft not treated with steroid in the current study, was examined after

20 days *in vivo* and showed widespread reporter gene expression with no preceding clinical inflammation. Importantly, these findings suggested that expression of IL10 in allografts transduced with LV-SV40-IL10, persisted until the onset of immunological rejection.

Lentivirus-mediated expression in vivo was superior to that in organ culture

Studies of *ex vivo*-transduced ovine donor buttons and concurrently organ-cultured rims demonstrated a higher transduction rate in endothelial cells of the transplanted tissue at 15 days post-transduction. This did not appear to be due to a reduction in endothelial cell density in cultured corneas, although formal density counts and vital staining were not performed. However, ovine corneas subjected to *in vitro* organ culture at 37°C did develop severe swelling which was accompanied by corneal folds. In assessing endothelial cell apoptosis in organ-cultured human corneas, Albon and colleagues reported a close correlation between the number and distribution of apoptotic cells, and corneal folding.[206] In the current studies, Hoechst-33258-staining of endothelial cells did not demonstrate nuclear fragmentation, but other apoptosis detection techniques such as the TdT-dUTP terminal nick-end labelling (TUNEL) technique, were not performed. Thus, endothelial cell apoptosis may have been underestimated. Nonetheless, the higher relative expression in the endothelial cells of transplanted corneas may have been due to their exposure to ovine growth factors in the anterior chamber. Lai and colleagues exposed human corneal tissue to rAAV encoding a reporter gene and assessed transduction efficiency and kinetics of expression over 14 days of *in vitro* organ culture.[126] Results were compared in two different culture media. The general condition of endothelial cells was relatively poor in DMEM supplemented with 10% foetal bovine serum (FBS) and 2 mM glutamine, and expression was much lower in that medium than in corneas cultured in an

enriched medium, which contained OPTIMEM-I supplemented with 8% FBS and including α-fibroblast growth factor, nerve growth factor, calcium chloride and chondroitin sulphate. The health of corneal endothelial cells clearly has an impact not only on the maintenance of the endothelial barrier and metabolic pump functions, but also on the efficiency of gene delivery and expression. The finding that lentivirus-mediated eYFP expression was higher *in vivo* than *in vitro* suggested that IL10 expression levels from LV-SV40-IL10 would also be higher *in vivo* than was observed during *in vitro* studies—an important observation given that organ culture proved unsuccessful (vide infra).

Organ-cultured ovine donor corneas were not viable in vivo

The high rate of failure of organ-cultured ovine corneal allografts was primarily due to their severe swelling. This was in turn probably a result of massive imbibition of water, a breakdown in intercellular junctional complexes which comprise the endothelial barrier, and impairment of the endothelial metabolic pump. There was significant difficulty achieving satisfactory wound closure, and normal corneal thickness was never recovered in most cases, indicating irreversible injury to those compensatory mechanisms. In studies described in Chapter 3, endothelial cell density in ovine corneas, as measured by Hoechst-33258-staining apparently remained stable at approximately 3,000 cells per mm^2 for 15 days in organ culture, without evidence of apoptosis. However, those corneas were severely swollen and it is not known what proportion of endothelial cells was viable, since a vital stain was not used. Nor did the assessment by nuclear staining give any indication of the status of junctional complexes.

The specific reasons for the severe swelling observed in ovine corneas compared to what is seen in human corneas are unknown, but structural differences

may play a role. The ovine cornea is much larger and floppier than the human cornea, and different quantities and proportions of proteoglycans may lead to a higher swelling pressure. In addition, the corneal endothelium has specific requirements to maintain normal barrier and pump functions. These may not have been adequately addressed in the culture medium used. Indeed, the ovine corneal endothelium may have particular requirements which are quite distinct from those of the human cornea.

The method adopted for ovine corneal organ culture was based on conventional practices in European Eye Banks, where MEM is commonly supplemented with 2–10% FCS, and corneas are cultured at 31–37°C. An evaluation of the ability of different cell culture solutions to preserve human donor corneas by Moller-Pedersen and colleagues, reported that the use of 8% FCS (in MEM) had a marked protective effect on endothelium—showing the highest RNA synthetic activity and the lowest endothelial cell loss—compared to numerous solutions containing 2% FCS.[207] The medium used in the current studies was HEPES-buffered RPMI containing 10% v/v FCS and supplemented with L-glutamine (2 mM), penicillin (100 IU / ml), streptomycin (100 µg / ml) and amphotericin B (0.25 µg / ml). No osmotic agent was added, except in those cases when Dextran-T500 was added in the final days before transplantation. The hypothermic corneal preservation medium Optisol® GS, which is widely used in Eye Banks in Australia and the United States, contains chondroiton sulphate, Dextran 40, sodium bicarbonate, various amino acids and sodium pyruvate. Glutathione,[208] calcium,[209] and bicarbonate[210] are all known to be critical in maintaining normal function of endothelial cells and their junctional complexes. Future attempts to culture ovine corneas for transplantation will require a trial of different organ culture solutions,

ensuring that these essential components are provided, and perhaps testing a variety of growth factors.

Storage temperature may also have had an effect on corneal swelling. Lowering the storage temperature is classically understood to decrease the endothelial pump function leading to increased corneal thickness.[211] However, a study of the influence of storage temperature on swelling and endothelial morphology in cultured rabbit corneas found a positive correlation between swelling rate and storage temperature.[212] The swelling rate of cultured corneas was significantly higher after culture at 37°C than at 34°C, 31°C or 23°C. The authors considered this attributable to an improved barrier function at lower temperatures. Unlike the 37°C used in the current studies, many European Eye Banks culture corneas at a temperature of 31°C. In future investigations it may be worthwhile attempting culture of ovine corneas at temperatures lower than 37°C.

The failure of ovine corneas to tolerate organ culture, necessitated that the transduction period be reduced and that grafting be carried out on the same day—both factors which had been shown to result in lower transduction rates and levels of lentivirus-mediated transgene expression, during *in vitro* studies (Chapters 3 and 4).

Polybrene was toxic to ovine corneal allografts

An attempt to increase transduction of corneal allografts by the addition of the cationic polymer polybrene to the transduction mixture was unsuccessful. There was clinical and histological evidence of a toxic effect due to polybrene not detected during the *in vitro* studies of endothelial cell density and nuclear morphology described in Chapter 3. The animals exhibited excessive lacrimation and epithelial cells displayed loss of normal morphology, as well as marked dyskeratosis and cytoplasmic vacuolisation. The course of fluctuating graft clarity and oedema

observed was probably the result of breakdown of the epithelial barrier by destruction of epithelial tight junctions, in addition to possible impairment of the endothelial barrier and metabolic pump function. Polybrene is known to have cytotoxic and antiproliferative effects at certain doses in cell culture.[127,184] Importantly, a study of the effects of polycations in perfused rat lungs demonstrated significant extravascular leakage of water and protein in polybrene-treated pulmonary endothelial cells.[213] Electron microscopy of alveolar epithelial cells and pulmonary endothelial cells, after perfusion with the polycation protamine, showed multiple cytoplasmic vacuoles and separation from the basement membrane. These findings were remarkably similar to the changes observed in ovine corneas. In the rat lungs, the mechanism of injury was not clear, but was considered to be related to breakdown of electrochemical gradients—as a result of alteration of the normal negative surface charges of the cells—and subsequent change in the membrane permeability to ions. The dose of polycations tested was 100 µg / ml.

The dose of polybrene used in the current studies was 40 µg / ml, approximately 5–10-fold higher than that routinely used in tissue culture or in a recent report of lentiviral transduction of mouse corneas.[83] Given this dose was unequivocally cytotoxic, lower doses may be better tolerated. However, *in vitro* studies described in Chapter 4, demonstrated no significant increase in transgene expression with 4 µg / ml, and therefore the beneficial effect of lower doses on transduction may need to be carefully titrated against cytotoxicity. An alternative approach might be to trial a different polycation such as protamine sulfate, which is approved for human use by the U.S. Food and Drug Administration, and has been shown to improve retroviral transduction efficiency in human keratocytes.[127] In an interesting development, Landazuri and colleagues have reported that complexation

of retroviruses with a combination of oppositely charged polymers such as polybrene and chondroitin sulphate C (CSC) results in rapid sedimentation onto target cells, and enhances gene transfer.[214,215] This may be because the negatively charged polymer (CSC) protects target cells from what would otherwise be a cytotoxic dose of polybrene. This may constitute another strategy by which to improve transduction of ovine corneal endothelium with the Anson lentiviral vector.

A delay in graft vascularisation was observed in IL10-treated allografts

Vascularisation of the graft bed is a major risk factor for immunological rejection in human corneal allografts.[2] Even in the low risk setting, corneal transplantation is commonly followed by corneal neovascularisation. The limited prolongation of survival observed in allografts transduced with LV-SV40-IL10 and not exposed to polybrene, was accompanied by a modest delay in vascularisation of the graft (a median of three days). There have been a number of reports of inhibition of corneal neovascularisation mediated by strategies targeting the potent angiogenic agent vascular endothelial growth factor (VEGF).[103,216-218] Blockade of VEGF signalling has been shown to inhibit not only corneal haemangiogenesis but also lymphangiogenesis, resulting in a prolongation of allograft survival in a murine model.[219,220] Interestingly, anti-angiogenic properties of IL10 have been observed in models of peripheral vascular disease[221] and tumorigenesis,[222,223] In one case IL10 was found to downregulate VEGF production[221] and in another, to competitively inhibit VEGF.[222] In the current studies no attempt was made to measure VEGF expression. However, the anti-angiogenic effect observed suggests that if the rate of onset and maximal expression levels of IL10 can be improved, the Anson lentiviral vector has the potential to antagonise the potent VEGF-mediated angiogenic component of corneal allograft rejection.

A modest prolongation of survival was achieved in IL10-treated allografts

The survival of unmodified allografts, following same day transplantation, was consistent with the results of previous studies.[70,75,85] However, transduction of donor allografts with the lentiviral vector encoding IL10 in the absence of polybrene, resulted in only a modest prolongation of survival (a median of seven days compared to controls), and several animals did not experience any prolongation whatsoever. This lack of clinical efficacy could not be ascribed to lack of function of the transgenic IL10. Studies described in Chapter 4 demonstrated that transgenic IL10 was secreted from transduced ovine donor corneas *in vitro* and that it retained its immunoinhibitory function (page 180). Nor could it be attributed to loss of expression *in vivo*, since reporter gene expression was detected from the Anson vector 28 days after transduction and allotransplantation (vide supra). The limited therapeutic effect of IL10 observed in the current studies was most likely attributable to the differences in expression levels and kinetics described in Chapter 4. The concentrations of IL10 achieved in the anterior segment were probably insufficient to modulate the alloimmune response.

This is not the first example of the delivery of IL10 failing to prolong corneal allograft survival. A study of subconjunctival injections every second day, following corneal allotransplantation in a MHC-mismatched rat model, failed to delay rejection.[224] In that case, the timing of the dosing, and the route of administration may have played a role. The sole report of a prolongation of corneal allograft survival achieved following *ex vivo* transduction with an IL10-encoding vector came from a study performed in the sheep, where delivery was mediated by adenovirus (Table 5.1).[75] Given the benefit of local IL10 expression is probably attributable to its effects on resident APCs, the collective findings of these studies may indicate that

there is only a short window of opportunity following transplantation during which local expression of IL10 can decisively influence the ultimate outcome of the graft. The relatively low and delayed expression observed in lentivirus-transduced ovine corneas *in vitro* compared to adenovirus-transduced corneas, suggests that secretion probably was not high enough or rapid enough to modulate the alloimmune response significantly—perhaps this was a case of too little, too late.

The modest effect of local immunomodulatory protein production may also be related to the site of expression. Delivery of cytotoxic T-lymphocyte antigen-4 immunoglobulin fusion protein (CTLA4-Ig) is a good example; there have been two reports that systemic administration of adenoviral vectors expressing CTLA4-Ig resulted in significantly greater extensions of survival in rat corneal allografts than *ex vivo* transduction and local expression.[76,79] Further evidence that the site of expression is crucial in modulating the rejection process was reported by Jessup and colleagues, who found that local expression of anti-CD4 single-chain variable-domain antibody fragments—mediated by an adenoviral vector—did not prolong the survival of transduced rat corneal allografts.[49] More recently, Gong and colleagues reported that *ex vivo* adenovirus-mediated transfer of viral IL10 in an MHC-I/II-disparate rat model of corneal allotransplantation did not result in a prolongation of survival; however, systemic delivery did,[86] and was accompanied by modulation of the cytokine mRNA expression profile in draining lymph nodes. Those studies support the hypothesis that a critical component of the alloimmune response—probably T cell activation—occurs in the draining lymph nodes, and that the expression of multiple therapeutic transgenes targeting distinct elements of the graft rejection process at different sites, may be required to achieve success.

CHAPTER 6

FINAL DISCUSSION

6.1 Summary of findings with respect to aims of the thesis

These studies were performed with one overarching aim: to assess the ability of the HIV-1-based Anson lentiviral vector to achieve efficient and stable gene expression in corneal endothelium, thereby evaluating its potential for use in human corneal transplantation. The outbred sheep was chosen as a preclinical model because of the similarities between the corneal endothelium and the corneal allograft rejection process, in sheep and humans.

Lentiviral vectors containing the SV40 or CMV promoters achieved stable transgene expression in the majority of ovine and human corneal endothelial cells, and transduction rates in human corneas were higher than those previously reported for other lentiviral vectors. However, the efficiency of the vectors appeared to be lower than that of adenoviral vectors.

Surprisingly, transgene expression in ovine corneas was delayed compared to expression in human corneas, and lower levels were attained. Another important finding was that ovine corneas were not viable after corneal organ culture; thus, it was not possible to to delay transplantation to allow lentivirus-mediated transgene expression to reach higher levels. These previously unknown characteristics of the ovine model limited the therapeutic effect of lentivirus-mediated IL10 expression in transduced allografts. A delay in vascularisation of the graft and an extension of graft survival were observed, but these effects were modest. Furthermore, an attempt to improve transduction and hence expression—using polybrene—resulted in toxicity for the cornea which nullified the limited therapeutic effect achieved without polybrene.

Despite the limitations identified in the sheep, the stability of expression and higher efficiency observed in human corneas—combined with their capacity for

organ culture—demonstrated the Anson vector's potential for use in human corneal transplantation. However, in order to justify the effort, cost and risk of moving to a clinical trial—and to test the efficacy of potential therapeutic transgenes—it will probably be necessary to perform further preclinical studies. If the ovine model is to be retained, then the mechanisms underlying differences in transgene expression after transduction with lentiviral vectors need to be elucidated, and measures taken to overcome them.

6.2 The Anson vector in the context of clinical gene therapy

Clinical trials involving lentiviral vectors

The early hype surrounding gene therapy has dissipated. Slow progress has curbed expectation, and several significant adverse events have led to tightened regulation of clinical trials. The death of a patient after systemic infusion of an adenoviral vector as a treatment for ornithine transcarbamylase deficiency,[225] the development of T-cell acute lymphoblastic leukaemia-like complications in several patients treated with a gammaretroviral vector for X-linked severe combine immunodeficiency (SCID-X1),[72] and most recently, the death of a patient during a clinical trial involving articular injection of rAAV[226] (now thought to be unrelated to the vector) has kept the spotlight firmly fixed on the potential hazards of gene therapy. Nonetheless, greater scrutiny has led to a more methodical approach, and the field can lay claim to success, the most noteworthy being treatment for SCID. In addition, the treatment of monogenic disorders such as cystic fibrosis and Duchenne muscular dystrophy, as well as complex disorders such as cardiovascular disease and cancer, have now progressed to clinical trials, and early results have been promising.[227]

The ability to transduce nondividing cells, and preclinical studies showing no evidence of insertional oncogenesis, position lentiviral vectors to replace gammaretroviral vectors as the vehicle of choice for gene transfer to haematopoietic stem cells (HSCs). A number of clinical trials using lentiviral vectors have been approved and more are under review. The disorders being targeted include malignant melanoma, leukaemia and lymphoma, β-thalassaemia, and mucopolysaccharidosis (MPS) type VII (www.wiley.co.uk/genetherapy/clinical/ at November 2007). All these trials involve *ex vivo* transduction of autologous HSCs or T lymphocytes.

To date, only one gene therapy clinical trial employing a lentiviral vector has been completed; it evaluated the safety of a conditionally replicating HIV-1-based vector expressing an antisense gene against the HIV envelope (termed VRX496).[228] The trial involved infusion of autologous CD4+ T lymphocytes transduced *ex vivo*, with results showing the treatment was well tolerated. Safety assessments found no evidence for insertional mutagenesis or the development of replication-competent lentiviruses, after 21–36 months of observation. An analysis of integration sites showed integration of the vector was favoured in gene-rich regions, reflecting the pattern seen in the case of wild type HIV. Two follow-on studies are now underway to further evaluate the potential of the vector when given in multiple doses (www.clinicaltrials.gov at November 2007).

Clinical trials of ocular gene therapy

Progress over the last 10 years in experimental models is now beginning to translate into clinical trials of ocular gene therapy, most notably in the areas of angiogenesis and retinal degeneration. A phase I study, in which patients with neovascular age related macular degeneration (AMD) received a single intravitreal injection of an

adenoviral vector encoding pigment epithelium-derived factor, has now been completed. Administration of the E1-, partial E3-, E4-deleted adenovirus was well tolerated, with no serious adverse events identified.[229] The therapeutic efficacy of the vector will be investigated in further trials.

Long-term improvement of visual function has been demonstrated in preclinical models of Leber's congenital amaurosis, using rAAVs encoding the visual cycle protein RPE65.[230-232] Those studies have now culminated in a phase I/II trial of AAV2/2 by subretinal injection for RPE65-dependent severe early-onset retinal dystrophy, which commenced in the United Kingdom this year. Similar trials have now received approval to proceed in the United States.[227]

The eye has been touted as being an ideal target organ for gene therapy for some time.[233,234] Its compartmentalised structure and accessibility allow targeting of the vector to specific ocular tissues, restriction of systemic dissemination, and noninvasive imaging *in vivo*.[234] It is worth noting that whilst intravitreal or subretinal injections of a viral vector may limit its distribution in comparison to systemic administration, and have thus far proved safe, *ex vivo* transduction of a nonproliferative tissue such as the endothelium of a human cornea may constitute the ultimate form of controlled delivery.

To date, no clinical trials of gene therapy have been undertaken in corneal transplantation. This is not due to a lack of promising therapeutic transgenes, since a range of genes with diverse mechanisms have been shown to confer a prolongation of survival in experimental models (Table 1.1, page 22). However, it may in part be related to the need for a vector which has the capacity for efficient transduction and stable transgene expression in human corneal endothelium, but also possesses an acceptable biosafety profile.

Prospects for clinical use of the Anson lentiviral vector

The most significant finding of the current studies was the ability of the HIV-1-based Anson vector to achieve a high transduction rate in human corneal endothelium of Eye Bank corneas which had been in hypothermic storage for up to 25 days. Importantly, expression remained stable for a further 14 days of *in vitro* organ culture, and could be regulated by a steroid-inducible promoter in the presence of dexamethasone. These results clearly demonstrate the potential of the vector to achieve efficient, long-term, controlled expression of therapeutic transgenes in human corneal endothelium. Combined with existing immunosuppression in the form of topical corticosteroids, and by expressing a range of immunomodulatory and cytoprotective factors, the vector may have the capacity to alter the outcome of human corneal transplants. It also bears noting that the stability of expression displayed by the Anson vector shows promise for the long-term genetic modification of endothelial cells in disorders such as congenital hereditary endothelial dystrophy, Fuchs' endothelial dystrophy and posterior polymorphous dystrophy.

In addition to its favourable expression profile, from the all-important standpoint of safety, the Anson vector meets requirements for clinical use. It is a self-inactivating vector, from which all of the HIV-1 accessory genes have been removed; homology between constituent sequences has been minimised; and it has been codon-optimised to achieve high titres without increasing the chance of generating replication-competent lentivirus. The integrated vector sequence does not contain the woodchuck hepatitis virus post-transcriptional regulatory element which improves expression[235] but has been associated with the development of liver tumours in mice,[236,237] and has caused safety concerns.[238] Finally, application of the vector relies on the route of delivery employed by all approved clinical trials involving lentiviral

vectors: *ex vivo* gene transfer to target cells (in this case, the endothelium of human corneas).

Alternatives to the Anson lentiviral vector

Adenoviral vectors, rAAV vectors, and vectors derived from nonprimate lentiviruses such as equine infectious anaemia virus (EIAV) and bovine immunodeficiency virus (BIV), are all potential alternatives to using the Anson vector in a clinical trial of gene therapy to prolong corneal allograft survival. However, each of them has relative limitations. Adenoviral vectors have consistently been shown to be highly efficient in transducing human corneal endothelium, and have demonstrated an ability to prolong allograft survival in several different animal models, including the outbred sheep.[75,85] Their clinical potential is limited by immunogenicity and transient expression. Vectors based on AAV have been shown to express transgenes in human corneal endothelium for at least 14 days[123,126] and promise even longer-term expression, but I am not aware of any reports of an extension of corneal allograft survival following rAAV-driven gene transfer in an experimental model. The possibility of pre-existing neutralising antibodies influencing rAAV-mediated transgene expression is also a consideration. Furthermore, the limited transgene carrying capacity of rAAV detracts from its ability to deliver multiple complementary therapeutic factors, which will be a valuable strategy to improve the success of gene transfer in corneal transplantation. Finally, whilst nonprimate lentiviral vectors have been shown to achieve efficient and stable transgene expression in murine corneal endothelium,[80,100,139,142-144] including prolongation of survival in one case,[80] transduction rates in human corneal endothelium have been less impressive than that achieved by the Anson vector.[144,145] In addition, the clinical safety of nonhuman lentiviruses remains unknown.

6.3 Improving immunomodulatory gene transfer strategies for corneal transplantation

Is there a magic bullet?

Steady improvements in the survival of renal allografts over the last 50 years have not been the result of one therapeutic breakthrough. Rather, the use of living related donor kidneys, the introduction of HLA matching,[239,240] and advancements in immunosuppressive therapies,[241] have each made incremental contributions to improvements in survival. In the case of corneal transplantation, the introduction of corticosteroids approximately 40 years ago doubtless had an impact on success rates, although there are no data available to prove it. An analysis of data within the Australian Corneal Graft Registry shows that no further improvement in survival has been realised over the last 20 years.[2] As we now consider the prospect of gene therapy making an impact on the survival of human corneal allografts, we must not expect to find a magic bullet. Further efforts should be made to achieve stable coexpression of multiple immunomodulatory and cytoprotective agents. The Anson vector would be an ideal candidate for such an approach, in view of its safety profile, high transduction rates in human corneal endothelium, and adequate transgene carrying capacity.

Complex rejection mechanisms demand a combined approach

The CD4+ T cell is well established as the central player in the corneal allograft rejection process, but the precise means by which it acts remain unclear.[32] Efforts to elucidate those mechanisms have served only to demonstrate their complexity and redundancy. With some notable exceptions (vide infra), attempts to prolong corneal allograft survival in experiment models using gene therapy have concentrated on the

overexpression of a single immunomodulatory factor. Such an approach is yet to prove universally successful. A range of transgenes have had varying degrees of success in different models. In contrast, the undisputed success of corticosteroids in preventing and treating corneal allograft rejection is probably due to their broad spectrum of effects.[242] Similarly, the approach required for success of gene therapy in the clinical sphere, may be a combination of immunomodulatory strategies, each targeting different elements of the immune response to the graft.

Expression of multiple therapeutic transgenes

An example of the combined approach to immunomodulation of corneal allografts was provided by Gong and colleagues, who reported that an extension of survival in rat allografts transduced with an adenoviral vector encoding nerve growth factor (NGF)[81] was significantly augmented by concurrent systemic delivery of an adenovirus expressing CTLA4-Ig. This approach was shown to be associated with a reduction in mRNA expression of inflammatory markers in transduced allografts, and the prevention of apoptotic loss of graft endothelial cells. The systemic delivery of an adenoviral vector is unlikely to be acceptable in the clinical scenario, but Gong's study demonstrated that a multifaceted strategy involving gene transfer has potential.

Anti-angiogenic factors which target the neovascular component of allograft rejection, such as sflt-1[103] and E-Kr5[82] show promise, and other potent agents such as pigment epithelium-derived factor are yet to be tested. Studies suggesting that apoptosis may be an important factor in the loss of endothelial cells after corneal transplantation[24,206] make anti-apoptotic agents another strong prospect. Barcia and colleagues recently reported survival in 90% of murine corneal allografts

eight weeks after transduction with an HIV-based vector encoding the anti-apoptotic factor Bcl-xL.[83]

A combined approach to immunomodulation might benefit from the coexpression of multiple therapeutic transgenes, ideally driven at equal levels from a vector containing a single internal promoter. The use of an internal ribosomal entry site may be not be successful, insofar as expression of the transgene encoded by the second cistron can be reduced.[243] An alternative system might utilise the 2A self-processing sequence derived from the foot-and-mouth disease virus, which mediates enzyme-independent cleavage to separate post-translational polypeptides. The system has been employed to demonstrate stoichiometric coexpression of at least four different proteins in mice,[244] and expression of a full-length monoclonal antibody—from a single open reading frame driven by a single promoter—has also been reported, following *in vivo* delivery to mice using rAAV.[245]

Approaches to ovine corneal allograft rejection using the Anson vector

The choice of transgene for prolongation of corneal allograft survival may be dictated by the specific kinetics of expression produced by the vector. Relatively low early expression in ovine allografts by the Anson vector may render it an inappropriate choice for the expression of immunomodulatory cytokines. The previous beneficial effects of IL10 on corneal allograft survival in the sheep were accomplished with an adenoviral vector, which was shown in the current studies to achieve much higher expression levels than the lentiviral vector over the first two weeks following transplantation. If the Anson lentiviral vector encoding IL10 is to prove equally or more successful in the ovine model, further efforts must be made to improve expression levels achieved in the early postoperative period. Measures might include titrating doses of different cationic polymers such as protamine sulfate

to improve transduction rates, or tailoring approaches to improve the viability of ovine corneas during organ culture. Other means of addressing the species-specific difference in expression are discussed below.

Perhaps the stable expression achieved by lentiviral vectors is better suited to other therapeutic transgenes which may not require such high levels of expression so soon after transduction. The success of the HIV-based vector encoding the anti-apoptotic factor Bcl-xL reported by Barcia and colleagues (vide supra), was achieved despite transduction efficiencies of only 15%.[83] A useful strategy may be to perform a cotransduction of ovine donor corneas with an adenovirus expressing IL10 and the lentiviral vector expressing an anti-apoptotic factor such as Bcl-xL. Another means of demonstrating the clinical potential of the Anson vector in the ovine preclinical model may be to combine local therapeutic transgene expression by the vector with short-term corticosteroids. These approaches might provide intense short-term immunosuppression sufficient to modulate the early phase of the rejection response, followed by a longer-term protective effect on endothelial cells. Indeed, this strategy mimics the clinical scenario, in which patients receive topical corticosteroids immediately postoperatively and intermittently throughout the life of the graft.

The analysis of different internal promoters conducted in the current studies demonstrated that the viral SV 40 early promoter and human CMV immediate early promoter resulted in higher expression than the phosphoglycerate kinase and elongation factor-1α promoters; but expression was equally high from the chimeric mammalian/viral GRE5 promoter, in the presence of dexamethasone. An *in vivo* trial of the Anson vector driving transgene expression from this promoter would be instructive. Incorporation of a reporter gene might allow direct visualisation of expression, titration of the necessary dosage of topical corticosteroid required for

induction, and an appraisal of the merits of the steroid-inducible vector approach in the clinical scenario.

6.4 A possible role for retroviral restriction factors

The relative delay and lower expression levels achieved in the ovine corneal endothelium compared to the human, following transduction with the HIV-1-based Anson vector, was an unexpected finding. It probably accounts for the modest therapeutic effect observed in transduced ovine corneal allografts. A satisfactory explanation was not identified during the course of the studies for this thesis, although it was considered that a lack of cofactors such as dNTPs might be involved. However, recent developments in the field of innate antiretroviral immunity may provide clues to the underlying mechanisms, and suggest strategies for improving transgene expression levels in the ovine model.

Innate immunity to retroviruses

A range of cellular factors with the ability to restrict retroviral infection in a species-specific manner, have now been identified. These are referred to as *restriction factors* and include: the Fv1 protein in mice, which controls susceptibility to Friend leukaemia virus disease;[246] the apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) proteins, which inhibit retroviruses in many vertebrates;[247] and most recently, the tripartite motif 5α (TRIM5α) protein, which has emerged as an important restriction factor impacting on retroviral replication—including that of HIV-1—in mammals.[248]

Retroviral restriction by TRIM5α

The TRIM proteins comprise a large family of proteins which are characterised by RING, B-box, and coiled-coil domains.[249] Their functions are poorly understood. Many cytoplasmic TRIM proteins, such as TRIM5α, contain a C-terminal B30.2 (SPRY) domain that is thought to mediate binding to specific ligands. The first TRIM protein shown to have antiviral properties was rhesus macaque TRIM5α, which blocked HIV-1 particles before reverse transcription in the cells of Old World monkeys, but was relatively permissive to simian immunodeficiency virus (SIV).[250] Orthologues of TRIM5α with species-specific antiretroviral activity have since been identified in a range of primates, including humans.[251-253] Sequences in the B30.2 domain from TRIM5α of different primates appear to determine the potency and specificity of the restriction of particular retroviruses. It seems that the sequences encoding this domain have been subject to strong positive selection during primate evolution, possibly as a result of sporadic retroviral epidemics.[254] Differences among TRIM5α orthologues in primate species account for the patterns of restriction observed for particular retroviruses, and studies have shown that the viral capsid protein is the major determinant of susceptibility to these restriction factors.[248] Interestingly, it appears that retroviruses have evolved capsids that are only moderately restricted by the TRIM proteins expressed by the natural host, exemplified by the observation that human TRIM5α at best partially restricts HIV-1 infection.[254]

The mechanisms underlying the retroviral restriction activity of TRIM5α remain unclear. However, TRIM5α is ubiquitinylated and rapidly turned over by the proteasome in a RING domain dependent way.[255] It is thought that restricted virions may be degraded by the proteasome, or that coating of the virus with TRIM5α

trimers may disrupt uncoating of the viral core and subsequent trafficking to the nucleus.[248] Inhibition of the proteasome during restricted infection permits reverse transcription of the virus, but the TRIM5α-virus complex then remains noninfectious.[256]

Importantly, TRIM5 orthologues with antiretroviral activity against several lentiviruses, including HIV-1, have most recently been identified in the cells of both cattle[254,257] and rabbits.[258] In the case of the cow, Madin-Darby bovine kidney cells were found to be efficiently transduced by a BIV-based vector, but not by an HIV-1-based vector. A TRIM5-like protein which was closely related to human and simian TRIM5α sequences was identified, and experiments using siRNAs targeting gene transcripts, demonstrated that at least part of the block to HIV-1 infection in the bovine cells was mediated by the protein.[254]

The likelihood of a TRIM5 orthologue in the sheep

The findings discussed above suggest that TRIM-mediated innate immunity to retroviruses might be widespread in mammals. Indeed, antiviral TRIM proteins may be responsible for the poor infectivity of HIV-1 reported in pig cells.[259] Furthermore, the phylogenetic relationship between cows and sheep raises the possibility that an ovine TRIM5 orthologue with antiretroviral activity against HIV-1, has almost certainly evolved in the sheep. Such a restriction factor may at least be partly involved in the low early expression observed in corneal endothelium transduced with the HIV-1-based Anson lentiviral vector.

It should be noted that in the present studies, transduction of ovine corneal endothelial cells by the HIV-1-based Anson vector was not blocked completely. Expression was very low for the first seven days but appeared widespread within the tissue by 14 days post-transduction; at all times assessed, levels remained lower than

those seen in human tissue. If there were an ovine TRIM5α-like protein targeting incoming capsids for degradation by the proteasome or rendering undegraded virus noninfectious, one might have expected transduction to be prevented completely and no expression seen whatsoever. However, TRIM5α restriction can become saturated at high MOIs, and preinfection with virus-like particles that contain the target for restriction abrogates the restriction, allowing subsequent infection by virus that would otherwise be inhibited.[260] In ovine corneal endothelium, higher expression was achieved earlier using higher MOIs, decreasing the likelihood that the low expression was due purely to an inability of the virus to use host cell cofactors required for its life-cycle. It is possible that the MOIs used in the studies were high enough to saturate a putative restriction factor and achieve some expression in the majority of cells, but that expression was so low that it only reached detectable levels after several days. One must also bear in mind that developments in the field of innate immunity to retroviruses, suggest that TRIM5 is unlikely to be the only factor restricting virus infection—there are probably other as yet unidentified factors at play.

Identification and inhibition of an ovine TRIM5 orthologue

Further studies are required to determine if a TRIM5α orthologue is present in the sheep and whether it is responsible for restriction of Anson vector-mediated expression in ovine corneal endothelial cells. If restriction by a TRIM5-like factor is identified, a strategy could be devised to inhibit its activity, leading to higher levels of HIV-1-mediated expression and facilitating further trials of therapeutic transgenes in the ovine preclinical model. Expression of the gene might be confirmed by RT-PCR on cDNA from ovine corneal endothelium and Anson vector-mediated transgene expression assessed following knockdown using siRNAs. Another

informative experiment would be to transduce bovine corneal endothelium with the Anson vector, and measure the kinetics of expression over 14 days post-transduction. If expression was rapid and high levels were achieved in bovine cornea, it would seem unlikely that an ovine TRIM5 orthologue is responsible for poor expression in the sheep.

6.5 Towards a clinical trial

Alternative animal models

If strategies to improve HIV-1-based lentiviral expression in the sheep are unsuccessful, then preclinical proof-of-principle may need to be tested in a different model of corneal allograft rejection. The choice of large outbred mammal models is limited. Two possibilities are pigs and nonhuman primates. The miniature pig model described by Tavandzi and colleagues is inherently lower-risk than the sheep, and it is not clear whether the endothelium is mitotic.[69] Moreover, the efficiency of HIV-1-vector mediated expression in pig cells has been reported to be very low.[259] The use of nonhuman primates is accompanied by significant ethical issues, as well as high costs of procurement and housing. In addition, Old World monkey cells, such as those of the Rhesus macaque, are resistant to HIV-1,[250] limiting the choice to New World monkeys such as the marmoset. Serious deliberation would be required before embarking on preclinical studies in the monkey.

Has the time come?

Given the need for new therapeutics to improve corneal allograft survival, the success of the Anson vector in transducing human corneas, and the challenges confronting preclinical studies, it is tempting to consider progressing directly to a

clinical trial. After all, a preclinical model is nothing more than an approximation of what *might* happen in people—ultimately the clinical potential of a promising therapeutic can only be truly scrutinised in the context of a clinical trial. The justification for initiating clinical trials is a matter of risk versus benefit. The patients who stand to benefit most from a novel treatment for corneal allograft rejection are those with disabling bilateral disease and multiple previous failed grafts, facing the prospect of systemic immunosuppression. From the point of view of their sight, such patients have little to lose and everything to gain. The issue in justifying a trial of gene therapy in these patients is the same one encountered when considering the use of systemic immunosuppressants—the patient's disease is not life-threatening, and one can not say the treatment is without risk of serious harm (however low that risk may be). The difference lies in the fact that the adverse effects of systemic immunosuppression are well described and treatment can be titrated or stopped, whereas the risks of a gene-modified corneal allograft are less certain, and expression of the therapeutic agent is far less regulated. Factors in favour of the gene therapy approach include restriction of the immunomodulatory agent to the eye, and the ability to examine the gene-modified tissue at the slitlamp, facilitating early detection of a complication; if necessary the graft can be replaced. In addition, the incorporation of an inducible promoter may confer some degree of control over transgene expression levels.

Ex vivo transduction of human corneal endothelium using the Anson lentiviral vector and subsequent transplantation of the gene-modified graft is—in principle—safer than the systemic infusion of lentivirus-transduced autologous lymphocytes, which has already been performed in humans, so far without any significant adverse effects.[228] However, the potential for complications related to

effects of the transgene must not be overlooked. Not only can transgenic proteins provoke an immune response resulting in loss of expression, but sustained immunomodulation by a protein such as IL10 might result in an immune deviant response, manifesting as an increased susceptibility to infection or an auto-immune disorder. Such complications were not observed in current or previous studies of IL10 expression in ovine corneal allografts, but only two of those animals survived for longer than six months.[75]

The studies in this thesis demonstrate the potential of the HIV-1-based Anson lentiviral vector as a means of achieving stable expression of therapeutic transgenes in human corneal transplantation. The steroid-inducible GRE5 promoter shows particular promise for the clinical scenario. However, further work is needed before a clinical trial can be justified. The high-risk ovine model remains a robust representation of the corneal allograft rejection process in humans. To ensure that the Anson vector fulfils its potential in human corneal transplantation a methodical approach is called for. Strategies for improving HIV-1-mediated transgene expression in the ovine model warrant further investigation, including identification and inhibition of putative restriction factors, alternative methods of organ culture, the use of different polycations, the coadministration of corticosteroids, and coexpression of combinations of therapeutic transgenes. The goals should be indefinite survival in the majority of animals, and a follow-up period of one to two years to monitor for adverse effects. Such studies hold the key to undertaking a clinical trial.

6.6 Final remarks

It is now over one hundred years since Eduard Zirm's historic operation ushered in a century of wonderful advances in the field of organ transplantation.[17] The 21st

century promises to be a period of equally spectacular progress in the field of molecular genetics. It would be fitting if corneal transplantation once again took its place in the vanguard of medical innovation by bringing together the adolescent field of gene therapy and the now well-travelled province of transplantation. Successful implementation of gene therapy in corneal transplantation could pave the way for application of the technology in other organs. Thousands of patients worldwide stand to benefit from the success of such work. However, like many worthy scientific ventures, gene transfer to corneal allografts is not without risks, and we must be diligent in ensuring the efficacy and safety of gene transfer strategies before exposing our patients to them. In the words of the Nobel Laureate Marshall W. Nirenburg, 'When man becomes capable of instructing his own cells, he must refrain from doing so until he has sufficient wisdom to use this knowledge for the benefit of mankind.'[261]

APPENDIX 1

LENTIVIRAL VECTOR STOCKS

The following table contains a list of all the lentiviral vector stocks used to transduce corneas in the course of the project.

Virus	Date produced	Promoter	Transgene	Scale of preparation	Titre	Application
LV-SV40-eYFP	30/8/04	SV40	eYFP	Large	1×10^9 A549 TU per ml	*In vitro* experiments (Chapter 3) *In vivo* experiments (Chapter 5)
LV-SV40-eYFP(2)	24/6/05	SV40	eYFP	Large	4×10^9 A549 TU per ml	*In vitro* experiments (Chapter 3) *In vivo* experiments (Chapter 5)
LV-SV40-IL10	22/7/05	SV40	IL10	Large	1.4×10^9 NIH3T3 iu per ml	*In vitro* experiments (Chapter 4) *In vivo* experiments (Chapter 5)
LV-SV40-eYFP(3)	31/8/06	SV40	eYFP	Large	1×10^9 A549 TU per ml	*In vitro* experiments (Chapter 3) *In vivo* experiments (Chapter 5)
LV-SV40-IL10(2)	19/10/06	SV40	IL10	Large	1.4×10^9 NIH3T3 iu per ml	*In vitro* experiments (Chapter 4) *In vivo* experiments (Chapter 5)
LV-PGK-eYFP	2/11/06	PGK	eYFP	Medium	4×10^9 A549 TU per ml	*In vitro* experiments (Chapter 3)
LV-EF-eYFP	17/1/07	EF	eYFP	Medium	4×10^8 A549 TU per ml	*In vitro* experiments (Chapter 3)
LV-CMV-eYFP(2)	15/3/07	CMV	eYFP	Medium	1×10^9 A549 TU per ml	*In vitro* experiments (Chapter 3)
LV-GRE-IL10	30/3/07	GRE5	IL10	Medium	1×10^9 NIH3T3 iu per ml	*In vitro* experiments (Chapter 4)

Footnotes: TU transducing units, iu infectious units, SV40 simian virus type-40, eYFP enhanced yellow fluorescent protein, IL10 interleukin-10, CMV cytomegalovirus, PGK phosphoglycerate kinase, EF elongation factor-1α, GRE5 five glucocorticoid response elements

APPENDIX 2

RECORD SHEETS OF OVINE ALLOGRAFTS

SHEEP CORNEAL GRAFT OPERATION RECORD

Animal ID:

Date of graft:

Experimental group:

Surgeon:

Gender:

Weight:

Eye grafted:

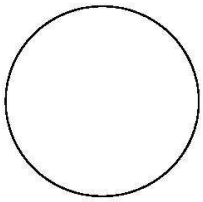
Other sheep grafted:

Size of graft:

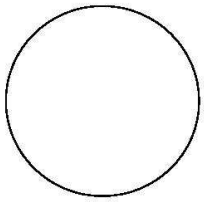
Preoperative treatment:

Prevascularization/remarks at time of grafting:

Recipient:



Donor:



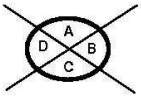
Anaesthesia:

Operation notes:

Post-operative treatment:

SHEEP CORNEAL GRAFT ASSESSMENT SHEET

Animal ID:



Date of graft:

DATE	PO DAY	CLARITY 0-4	OEDEMA 0-4	CELLS 0-4	FIBRIN 0-4	INFLAMM 0-4	VASC A	B	C	D	COMMENTS

APPENDIX 3

PUBLICATION ARISING FROM THIS THESIS

Gene Therapy (2007) 14, 760–767

www.nature.com/gt

ORIGINAL ARTICLE

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

DGA Parker[1,4], C Kaufmann[1,4,5], HM Brereton[1], DS Anson[2,3], L Francis-Staite[1], CF Jessup[1], K Marshall[1], C Tan[2], R Koldej[2,3], DJ Coster[1] and KA Williams[1]

[1]*Department of Ophthalmology, Flinders University of South Australia, Adelaide, Australia;* [2]*Department of Genetic Medicine, Women's and Children's Hospital, Adelaide, Australia and* [3]*Department of Paediatrics, Women's and Children's Hospital, Adelaide, Australia*

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.
Gene Therapy (2007) **14**, 760–767. doi:10.1038/sj.gt.3302921; published online 15 February 2007

Keywords: *corneal transplantation; corneal endothelium; lentiviral vector*

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,

Correspondence: Dr KA Williams, Department of Ophthalmology, Flinders Medical Centre, Bedford Park, SA 5042, Australia.
E-mail: keryn.williams@flinders.edu.au
[4]These authors contributed equally to this work.
[5]Current address: Department of Ophthalmology, University of Zürich, Zürich, Switzerland
Received 1 September 2006; revised 18 December 2006; accepted 18 December 2006; published online 15 February 2007

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).

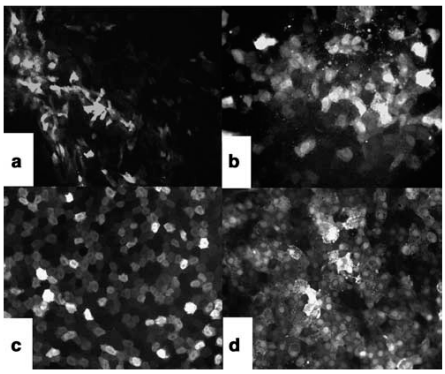


Figure 1 Transduction of corneas with 2.5×10^7 TU LV-SV40-eYFP per cornea. (**a**) Rat corneal epithelium transduced with LV-SV40-eYFP (MOI 400) and examined after 6 days of *in vitro* organ culture. (**b**) Rat corneal endothelium transduced with LV-SV40-eYFP (MOI 400) and examined en-face after 6 days 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 ×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.

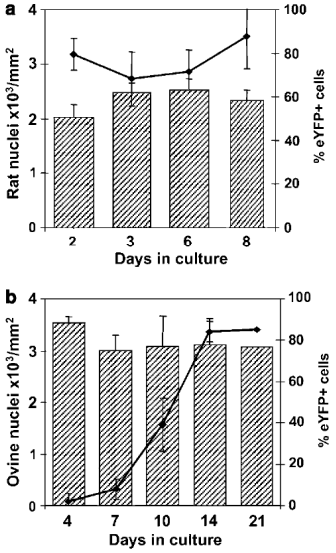


Figure 2 Kinetics of eYFP expression in rat and ovine corneal endothelium following corneal transduction with 2.5×10^7 TU of LV-SV40-eYFP and subsequent *in vitro* organ culture. (**a**) Rat corneal endothelium, MOI 400. (**b**) Ovine corneal endothelium, MOI 20. Hatched columns display the corneal endothelial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

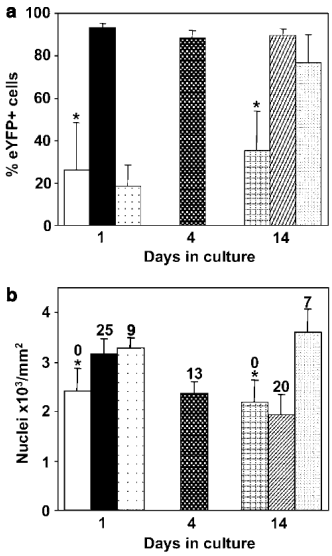


Figure 3 Kinetics of eYFP expression in human corneal endothelium following corneal transduction with two different doses of LV-SV40-eYFP and subsequent *in vitro* organ culture. (**a**) Percentage corneal endothelial cells (mean±s.d.) expressing eYFP. (**b**) Corresponding corneal endothelial nuclei density (mean±s.d.). A total of seven corneas were examined; each column represents the mean±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±4% of rat corneal endothelial cells after 2 days of *in vitro* organ culture in corneas transduced with LV-PGK-eYFP, compared with 13±2% of cells in corneas transduced with LV-SV40-eYFP and 16±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±9% of ovine corneal endothelial cells in corneas transduced with LV-PGK-eYFP, compared with 4±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±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-

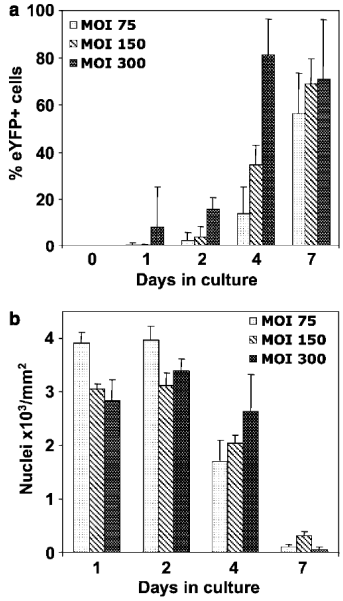


Figure 4 Effect of varying the MOI on (**a**) eYFP expression, and (**b**) corneal endothelial cell density, following 24 h transduction of sheep corneas with LV-SV40-eYFP and *in vitro* organ culture. Each column represents the mean±s.d. of five fields examined for a single cornea.

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

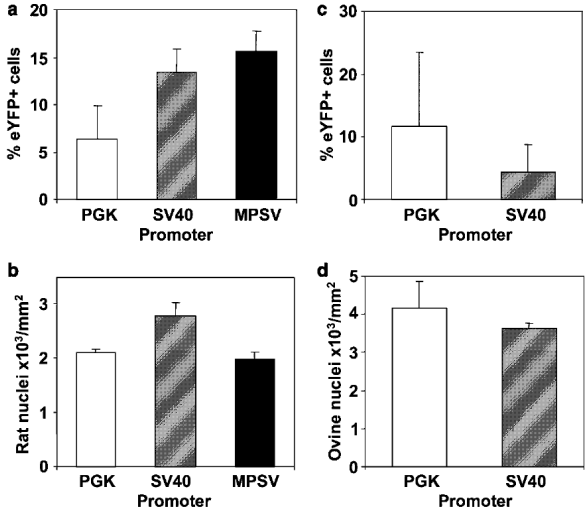


Figure 5 Expression of eYFP driven by different internal promoters after lentiviral transduction of corneas and subsequent *in vitro* organ culture. (**a**, **b**) Rat corneas transduced with 5.5×10^5 TU/cornea (MOI 50) and examined after 2 days of *in vitro* organ culture; (**c**, **d**) sheep corneas 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

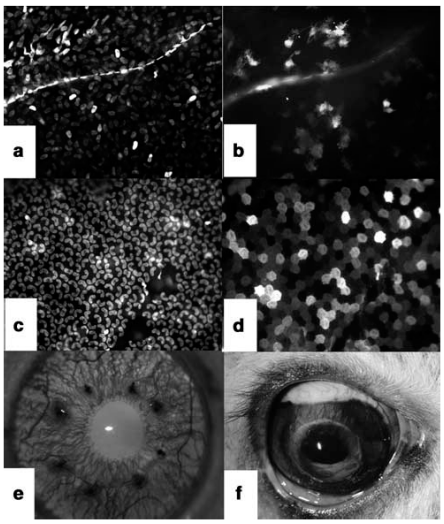


Figure 6 Stability of reporter gene expression after *ex vivo* transduction of the cornea with LV-SV40-eYFP and subsequent orthotopic corneal transplantation. (**a**, **b**) Rat corneal isograft harvested 60 days after transplantation; (**c**, **d**) sheep corneal allograft harvested 20 days after transplantation. (**a**) and (**c**) Show staining with Hoechst 33258 dye; (**b**) and (**d**) show eYFP expression in identical fields of the same corneas. Original magnification ×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 ± s.d. Transduction efficiency was defined as the percentage of corneal endothelial cells expressing eYFP.[48]

Acknowledgements

This work was supported by the National Health & Medical Research Council of Australia, the Ophthalmic Research Institute of Australia and the Flinders Medical Centre Foundation. CK was supported by Nachwuchsfoerderungskredit der Universitaet Zürich, by the EMDO Stiftung Zürich, and by the Schweizerischer Fonds zur Bekaempfung und Verhuetung der Blindheit. The authors thank Mr Ray Yates for assistance with animal husbandry and Mr Joel Johnston for photography.

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