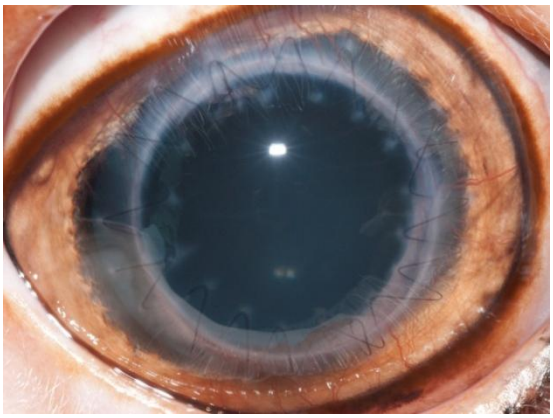
GENE THERAPY OF THE SHEEP CORNEA

FOR THE PROLONGATION OF

CORNEAL GRAFT SURVIVAL



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BBTech (Hons)

Thesis submitted for the degree of

Doctor of Philosophy

April 2013

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Abstract

Although corneal transplants enjoy good short-term survival, their long-term survival is poor. The eye has long been heralded as an immune-privileged site, however this privilege is in a constant state of balance and, if tipped too far by inflammatory forces, corneal transplants will undergo irreversible rejection. This is the major cause of graft failure. Gene therapy has shown potential in experimental transplantation, to reduce the rejection response. Previous studies in our laboratory have shown prolongation of sheep corneal graft survival, one such using an adenoviral vector expressing the interleukin-10 (IL-10) therapeutic transgene under the control of a cytomegalovirus promoter (CMV), and another using the lentiviral vector expressing the same transgene but under the control of the Simian virus type 40 early promoter (SV40).

The aim of this study was to investigate gene therapy with a cocktail of vectors designed to induce long-term transplant survival in a sheep model of corneal transplantation.

In a direct comparison of internal promoters in a lentiviral vector, gene expression induced by a CMV promoter and the SV40 promoter and was measured from transduced sheep corneas *in vitro*. The CMV promoter induced significantly higher transgene expression than the SV40 promoter at both the mRNA and protein level ($p= 0.006$, $p\leq 0.001$, respectively).

Thus the lentivirus vector with the CMV promoter and transgene interleukin-10 was then tested *in vivo* in an outbred sheep model of orthotopic, penetrating corneal transplantation with high risk of rejection. This single gene therapy applied to the

donor cornea significantly prolonged corneal graft survival, with treated grafts surviving a median of 26 days compared with 21 days for the control allografts ($p= 0.043$).

The polycation protamine sulphate was investigated as a possible non-toxic virus transduction enhancer to improve gene expression from the lentiviral vector. It was found to increase transgene expression 14-fold *in vitro* ($p\leq 0.001$), however was deemed not to be successful enough to warrant pursuing *in vivo*.

Therapeutic transgenes IL-10, indoleamine 2,3-dioxygenase, endostatin::kringle5 fusion gene (EK5), soluble fms-like tyrosine kinase 1 (sFlt-1), and Bcl-2 family protein, Bcl-xL, had previously been shown to prolong corneal graft survival in animal models, or to reduce corneal neovascularisation. Individual lentiviral vectors expressing these transgenes, each with CMV promoters, were constructed, viruses prepared and tested *in vitro* for gene expression by qRT-PCR analysis of mRNA and protein expression from sheep corneal endothelial cells. Individual activity assays were performed in cell culture to confirm biologic function of the transgenes. One individual adenoviral vector expressing IL-10 was previously prepared and tested in the laboratory.

A cocktail of lentiviral and adenoviral vectors was investigated, initially *in vitro* to test for vector interference, and finally *in vivo* for prolongation of corneal graft survival. *In vitro* cocktail results of transgene expression showed no vector interference occurred when the viruses were used together in a transduction combination. *In vivo* cocktail therapy with an adenoviral vector expressing IL-10 and two lentiviral vectors expressing EK5 and Bcl-xL did not significantly prolong

corneal graft survival, with cocktail therapy-treated and mock-vector treated allografts both having a median survival of 22 days ($p= 0.68$).

The cocktail approach was chosen to utilise the best features of both viral vectors; the adenovirus to give early and strong gene expression, and the lentivirus to give long-term gene expression. However, with a good pre-clinical model, proven therapeutic genes and a combination of useful vectors, prolongation of graft survival could not be achieved. Gene therapy of the cornea for allograft prolongation has some obstacles to overcome before it can reach its full potential.

Declaration

I certify that this thesis does not incorporate without acknowledgment 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.

Alison Clarke

Date

Acknowledgements

I would like to acknowledge and thank my principle supervisor Keryn Williams for offering me the wonderful opportunity to work in her laboratory and become part of the Flinders Ophthalmology family. Thank you for the guidance you have given me throughout this project, helping me to shape my skills as a scientist and always making me try harder. Thank you for helping me to produce the best thesis I could and especially for your support in the last few months.

To my two supervisors, Helen Brereton and Sonja Klebe, thank you for your support and care. Helen, I am very grateful to you for patiently teaching me a wealth of molecular biology techniques, having an encyclopaedic knowledge on biology related matters, and for painstakingly reviewing my thesis. Sonja, thank you for your constant enthusiasm for the project, for teaching me how to handle the sheep eyes and corneas, for your expertise in reviewing many histology slides for me and for helping me to score the IHC slides. I really appreciate the time you spent with me and all your explanations.

I would like to thank the two ophthalmologists who performed all of the penetrating keratoplasties in the sheep presented in this thesis, Professor Doug Coster and A/Professor Richard Mills. Thank you for making yourselves so readily available for this research amongst all your other commitments and lending your expertise to the project.

Thanks must go to A/Professor Donald Anson and his team in the Department of Genetic Medicine at the Women's and Children's Hospital for teaching us how to

produce lentivirus preparations, providing us with the appropriate packaging cell line, allowing us to use your laboratory to produce lentivirus, and always being there with advice and answers to our questions.

I would like to acknowledge and thank Dr Claudine Bonder and her team at the Centre for Cancer Biology, SA Pathology, for your kind donations of human umbilical vascular endothelial primary cells and advice on their culture conditions.

My thanks go to Sheree Bailey at the Flow Cytometry Facility, Flinders Medical Centre for her assistance and advice in setting up the gating for the Annexin V-FITC flow cytometry assay for the functional activity assessment of the Bcl-xL transgenic protein.

Lauren Mortimer is a fantastic research assistant for Keryn Williams. Lauren set up the lentivirus production procedure at Flinders, helped me to prepare many, many lentivirus preparations, performed the EK5 and sFlt-1 molecular cloning, the sFlt-1 transgenic protein functional activity assay, performed all of the endotoxin LAL tests, conducted several ELISAs for me and helped me with cell cultures. Lauren is an extremely thorough scientist, a comrade and a good friend. It was wonderful sharing this time with you, Lauren, thank you for all your support.

My thanks go to Kirsty Kirk, Gemma Lowe and Madison Helm for their assistance with the sheep handling, surgeries, graft examinations and scoring. Thank you to Gemma for preparing the H&E sections for the IL-10 sheep and to Madi for preparing the H&E and the IHC sections for the cocktail sheep, a huge effort. Thank you to Melinda Tea, who helped with some DNase-treatment of RNA samples, and Yazad Irani, who performed some of the molecular cloning.

I would like to thank the Flinders School of Medicine Animal Facility and staff for the hard work housing the sheep, assisting with surgeries and performing anaesthetics and euthanasia on the animals. I would like to thank Stuart Lisk and Craig Dawe in particular for the expert help and care provided.

I must thank Margaret Philpott and the Eye Bank of SA for her information and support. The generous gift of corneas from the donors and from their families for research was always gratefully and respectfully accepted.

I am very grateful to both Normanville Meatworks and Lobethal Australia. These two abattoirs have kindly donated many sheep eyes to us over the years, and without their ongoing support, this project would have been extremely difficult, if not impossible to achieve.

I would like to acknowledge the support of everyone involved with Flinders Ophthalmology. To Joel Johnston and Angela Chappell the ophthalmic photographers, thank you for donning the wellies and taking many hundreds of photos of my sheep grafts. To the wonderful people in the laboratory: Melinda, Lauren, Sarah, Kirsty, Madi, Dave, Paul, Yazad- thanks for making the lab a great place to come to work every day. Thanks to everyone in the office for the friendly smiles and the people in the eye clinic for kindly helping when I came down for supplies.

To my Mum, Dad and Zoltan, thank you for your constant love and support. I couldn’t have done it without it you all. Love you.

Thank you to my sheep. I’ll miss you all. Except maybe Horace.

Abbreviations

≥	greater than or equal to
≤	less than or equal to
^{o}C	degrees Celsius
A549	human lung adenocarcinoma epithelial cell line
AAV	adeno-associated virus
AC	anterior chamber
AdV	adenovirus
AE	amplification efficiency
APC	antigen presenting cell
ARBP	acidic ribosomal phosphoprotein
Bcl-xL	anti-apoptosis factor of Bcl2 family
bECGF	bovine endothelial cell growth factor
bp	base pairs
BSS	balanced salt solution
CAM	chloramphenicol
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus immediate early promoter
CO_2	carbon dioxide
CPPT	central polypurine tract
Ct	threshold cycle
CTL	cytotoxic lymphocyte
CTLA-4	cytotoxic lymphocyte antigen-4
Da	Dalton
DEPC	diethypyrocarbonate

dH_2O	distilled water
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulphoxide
DNA	dexoyribonucleic acid
dNTP	deoxynucleotide phosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene-diamine-tetraacetic-acid
EK5	human endostatin::kringle5 fusion protein
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
eYFP	enhanced yellow fluorescent protein
FBS	foetal bovine serum
g	gram
g	unit of gravity
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
HEK-293A	human embryonic kidney cell line with E1-region of adenovirus 5
HEK-293T	human embryonic kidney cell line that constitutively expresses the SV40 large T cell antigen
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HRP	horseradish peroxidase
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
IL-10	interleukin-10
kDa	kilo Dalton (10^3 Da)
L	litre
LB	Luria Bertani medium
LC	Langerhans cell
log	logarithm
LTR	long terminal repeats
LV	lentivirus
M	molar
µg	microgram (10^{-6})
MHC	major histocompatibility complex
MLV	Molony murine leukaemia virus
µL	microlitre (10^{-3})
µM	micromolar (10^{-6})
mL	millilitre (10^{-3})
mM	millimolar (10^{-3})
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
n	sample size
ng	nanogram (10^{-9})
NEB	New England Biolabs
NTC	no template controls
OCT compound	optimal cutting temperature compound

oBcl-xL	ovine anti-apoptosis factor of Bcl2 family
oIDO	ovine indoleamine 2,3-dioxygenase
oIL-10	ovine interleukin-10
p…ABC	plasmid…ABC
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pg	picogram (10^{-12})
PLP	paraformaldehyde lysine periodate fixative
qRT-PCR	quantitative reverse transcription real time PCR
RLT	lysis buffer in Qiagen RNeasy mini-kit for RNA extraction
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RREXt	rev response element
RT	reverse transcription
SAP	shrimp alkaline phosphatase
sFlt-1	soluble fms-like tyrosine kinase 1 (soluble VEGF receptor 1)
SIN	self inactivating
SV40	simian virus type 40 early promoter
TU	transducing units
v/v	volume per volume
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor
VSV-G	vesicular stomatitis virus glycoprotein G
w/v	weight per volume

Chapter 1

Introduction

1.1 The Cornea

1.1.1 Anatomy

The cornea is the transparent, refractive tissue covering the anterior chamber of the eye. A diagram of the eye can be seen in Figure 1.1 (A). The front surface of the cornea is convex and slightly elliptical, whereas the posterior surface is concave and circular.[1] The corneal tissue is avascular and comprises several layers: the epithelium; Bowman's layer; the stroma; Descemet's membrane; and the endothelium, from anterior to posterior respectively, as indicated in Figure 1.1 (B).[2] The epithelium comprises five or six layers of nucleated cells, which regenerate by means of stem cells located at the limbus, the border where the cornea meets the sclera.[1, 3] Bowman's layer is an acellular homogeneous membrane underlying the epithelium.[1] The stromal section constitutes the main bulk of the cornea and is composed of collagen fibrils, which are particularly aligned to maintain transparency of the cornea, interspersed with fibroblastic-like keratocytes.[1] Descemet's membrane is synthesised by the cells of the endothelium *in utero*.[4] The corneal endothelium is a monolayer of cells lying on Descemet's membrane, lining the inner surface of the cornea.[5]

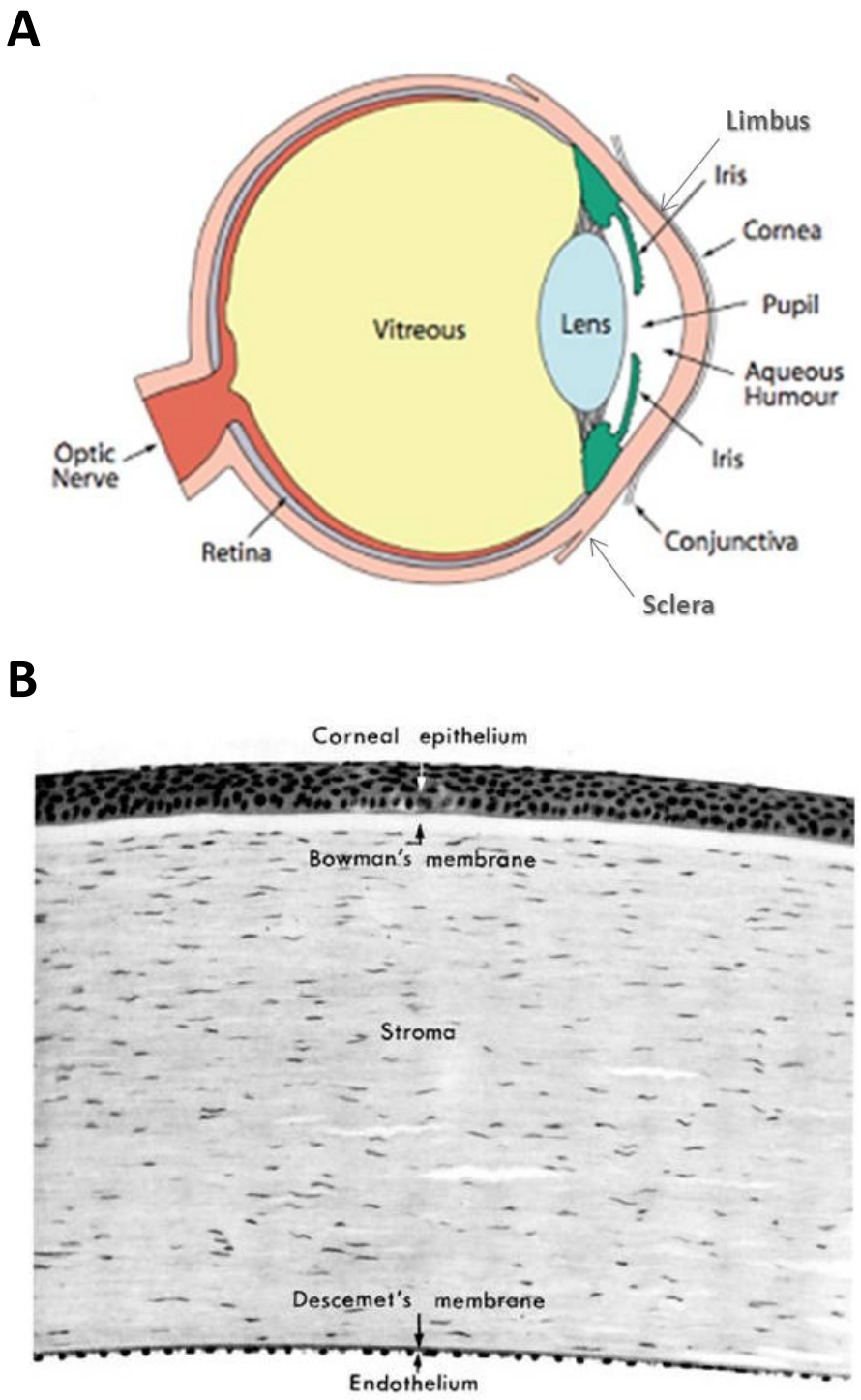


Figure 1.1: (A) Diagram of the side view cross-section of the human eye. Figure adapted from Williams and Coster, 2007.[6] **(B) A histological cross-section of the human cornea showing the cellular layers from anterior to posterior, top to bottom.** Image sourced from The Biology of the Eye, 2005.[7]

1.1.2 Physiology

The cornea has several functions. The curvature of the cornea is responsible for the majority of the refraction of light entering the eye to give visual acuity.[7] The cornea is also a physical barrier, protecting the anterior chamber and preserving the anterior chamber fluid, and has a role in maintaining intraocular pressure.[1]

1.1.3 The Corneal Endothelium

The human corneal endothelium is a monolayer of replication-deficient cells. Joyce *et al.* demonstrated that corneal endothelial cells are arrested in G1 phase of the cell cycle, with the endothelial cell density gradually declining throughout life as the cells die.[2] The endothelium maintains an important metabolic role as an electrolytic barrier, actively pumping water from the corneal stroma into the anterior chamber, preventing the cornea from swelling and becoming opaque. Maintaining the stroma in this dehydrated state is required to preserve the transparency of the cornea required for vision.[2, 4, 8]

1.1.4 Conditions Affecting the Cornea

Corneas may become damaged by several means and, in severe instances of any of these following examples, vision may be improved by having a corneal transplant. Ulcers can be caused by bacterial infection, herpes simplex virus or trauma.[5] There are numerous corneal disorders,[9] usually affecting both eyes symmetrically, such as keratoconus (corneas in young adults become thin and conical in shape) and Fuchs' endothelial dystrophy (corneal oedema and opacity; dystrophic changes in endothelium).[5] Non-ulcerative keratitis (patch of vessels with corneal opacity, sometimes caused by congenital syphilis) can be caused by corneal infection.[5] Bullous keratopathy is a condition involving corneal oedema, usually due to a

damaged endothelium, and includes the appearance of sight-inhibiting and potentially uncomfortable bullae in the epithelium.[10] Keratomalacia is a dryness of the conjunctiva caused by a vitamin A deficiency when young, and which can then lead to perforation of the cornea.[11] Ocular injuries may result from chemicals, flying particles, sharp instruments, blunt injury, injury associated with head trauma and welding burns, to name a few.[5]

1.2 Corneal Transplantation

1.2.1 History

The history of corneal transplantation (keratoplasty) goes back over one hundred years. The first graft recorded was conducted in 1838, when Richard Kissam transplanted a section of pig cornea into a human recipient. This graft remained clear for a fortnight before becoming opaque and was eventually absorbed after one month.[12] The first human corneal graft to a human recipient was conducted successfully on a patient with severely blinding lime burns to both of his eyes, and was performed by Eduard Zirm in 1906.[13] The left allograft remained clear for several months without any immune suppression or topical steroids and allowed the recipient to return to his employment.

Corneal transplantation is currently the most frequently performed transplant, with well over 30,000 grafts performed per annum in the US, at least 16,000 in Europe and approximately 1,250 in Australia.[6, 14, 15] Corneal transplants are performed primarily in order to improve vision, to alleviate pain, for structural repair, and occasionally for aesthetic reasons. Grafting is usually considered as the last option available to the patient with visual impairment secondary to corneal disease, as once a graft fails there are no further treatment options other than to perform another graft.

1.2.2 Corneal Graft Procedures

Donor eyes are collected post mortem by eye banks and may be stored as whole eyes or as excised corneas. The storage time of corneas can range from as little as one hour to twenty eight days prior to transplantation, depending on the storage technique.[16] Protocols vary in different regions as to the use of cold-storage at 4^{o}C or organ culture at 34^{o}C, the latter of which has the advantage of a longer viable storage time.[16, 17] Sera from cadaveric donors are tested for human immunodeficiency virus (HIV) 1 & 2 antibodies, hepatitis B virus surface antigen and hepatitis C virus antibodies, with only corneas from negative screenings released for transplantation.[18] The corneas are examined for transplantation suitability, with the endothelia evaluated to ensure sufficient cell numbers (greater than about 2200 cells/mm^2) are present.[19, 20] In the majority of cases donors and recipients are not matched for blood type or, except in some centres, for human leukocyte antigen (HLA).[6, 17, 21] There are conflicting reports on the benefits of these matches, however there are at least some studies showing minor histocompatibility antigen matching and ABO matching could potentially improve human corneal graft survival.[22-25]

There are several types of corneal transplantation: penetrating, in which a full-thickness of the cornea is excised and replaced; lamellar, in which only a partial thickness of the cornea is transplanted; and limbal, where epithelial stem cells from partial thickness grafts of the limbal region are transplanted.[21, 26, 27] The focus of this thesis will be on full-thickness, penetrating grafts, an example of which may be seen in Figure 1.2.

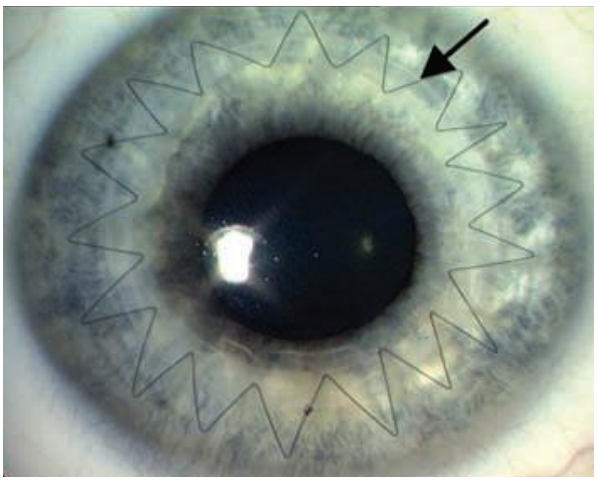


Figure 1.2: Photograph of a functioning penetrating corneal graft. The arrow indicates the graft-host margin. Image sourced from George and Larkin, 2004.[21]

1.2.3 Corneal Graft Management

Despite the lack of tissue typing and blood matching, recipients of corneal allografts are typically given only topical glucocorticosteroids such as prednisolone acetate or dexamethasone, to be administered in varying regimens, up to several times a day, in order to prevent and treat inflammation and graft rejection.[28, 29] In contrast to other organ transplants, systemic immune suppression is only used for grafts with a high risk of rejection and when the co-morbidities of systemic immune suppression are considered worthwhile, as the sight is considered salvageable.[29, 30]

1.2.4 Corneal Graft Survival

According to the 2012 report from the Australian Corneal Graft Registry, corneal grafts have an average survival rate of 87% at one year post graft, however this figure drops to less than 50% at fifteen years, with the mean survival of a corneal graft being only 12.9 years.[16] Graft survival is demonstrated in the Kaplan-Meyer survival plot in Figure 1.3. The long-term survival statistics of corneal transplants are no better than the outcome for other organ transplants such as kidneys and lungs.[31-33]

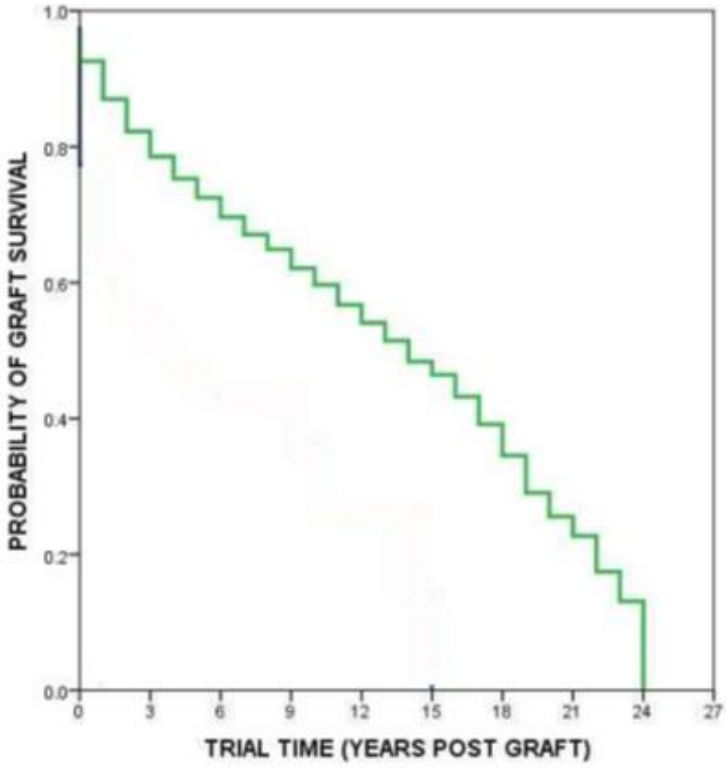


Figure 1.3: Kaplan-Meier plot showing the survival of penetrating corneal grafts. From the Australian Corneal Graft Registry Report 2012.[16]

Corneal grafts are at risk of failure for reasons such as endothelial cell failure, infection and glaucoma, but the most common cause of graft failure is irreversible immunological rejection.[16, 17] The risk factors for graft failure include vascularisation of the recipient cornea, previous inflammation or inflammation at the time of the graft, graft neovascularisation, one or more rejection episodes and previous graft failure.[16, 34] When a graft is considered a failure, it may be removed and a second graft performed, however each subsequent graft has a reduced survival time.[35] An example of a failed corneal graft is shown in Figure 1.4. Corneal grafts are constantly at risk of rejection and may reject at any time, even after years of quiescence.[36]

The number of corneal endothelial cells decreases over a person's lifetime. Transplanted corneas lose endothelial cells steadily, until a critical point where the graft fails due to endothelial failure. The rate at which grafts fail due to slow endothelial failure is dependent on the number of endothelial cells present in the donor tissue at the time of the graft.[20, 37]

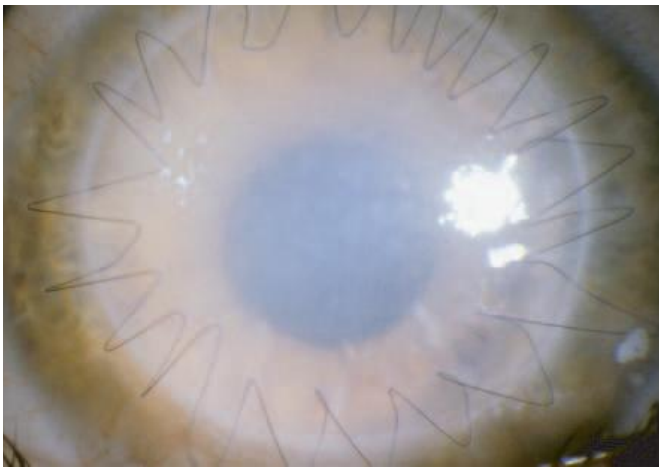


Figure 1.4: Photograph of a failed corneal graft due to immunological rejection. Image sourced from Williams and Coster, 2007.[6]

New therapy options are required to give patients at high risk of corneal graft rejection better treatment options with minimal side effects, which will allow longer graft survival and fewer surgical interventions.

1.3 Immunology of the Cornea

1.3.1 Immune Privilege

Immune privilege is the concept of a grafted foreign tissue or organ exhibiting prolonged survival in certain sites in the host, such as the eye, brain and testes, relative to other transplant sites.[38] The cornea is considered by some to be an immune privileged site due to the success rate of corneal grafts even in the absence of tissue

matching, however the high incidence of immunological rejection of corneal allografts seemingly contradicts this claim.[22]

Tissues and cells need protection from pathogens, however the inflammatory immune response elicited can itself be damaging to the tissues it is striving to protect. Human tissues such as the corneal endothelium and the neural retina require protection from immune responses since the loss of these cells can adversely affect vision, due to their non-replicative nature. Therefore the eye has several means of suppression of immune responses to protect ocular tissues from immune-mediated damage, summarised in Table 1.1. Three main mechanisms contribute to the relative immune privilege enjoyed by corneal allografts: blockade of the afferent immune response; deviation of the allo-immune response; and blockade of the efferent immune response. All three mechanisms need to work collectively in order to sustain immune privilege.[36]

There is a consensus that whilst corneal allografts appear to enjoy immune privilege, this privilege is considered an active process, not merely a constant, passive state.[39]

Table 1.1: Mechanisms of immune privilege in the human eye.

Mechanism	Description	References
Blood-eye barrier	A barrier to restrict free diffusion of solutes and cells into the tissue spaces of the eye.	40, 41
Blockade of the afferent immune response pathway	Lack of blood and lymphatic vessels in the corneal graft bed and relative paucity of MHC class II-positive antigen presenting cells prevents immune response induction.	36, 42
Anterior chamber-associated immune deviation (ACAID)	Down-regulation of systemic delayed-type hypersensitivity. Removal of antigens in the anterior chamber without inflammation by excluding responses by type 1 (Th1) and 2 (Th2) effector $CD4^+$ T cells. This requires the presence of the spleen.	43-46
Blockade of the efferent immune response pathway	Membrane-bound and soluble molecules of the cornea and aqueous humour interfere with the infiltrating, destructive immune cells to induce cell death e.g. Fas ligand expression on ocular cells binds Fas on infiltrating immune cells and causes apoptosis of the immune cells.	39, 47, 48
Immuno-modulatory cytokines and other soluble factors	Present in the normal aqueous humour, for example somatostatin, which induces regulatory T cell activation.	49, 50

1.3.2 Immunology of Graft Rejection

Corneal grafts may fail due to immunological rejection at any stage after surgery, therefore the grafts are at risk indefinitely.[27] Grafts may be categorised as being at normal risk or high risk of graft failure due to immunological rejection. Typically, grafts transplanted into a non-inflamed and avascular host bed have a normal risk of immunological rejection, whereas corneal buttons transplanted into a vascularised recipient bed or a host that is pre-sensitised by previous graft rejection, stand much greater risk of immunological rejection.[51] Immunological rejection may be reversed with treatment, or may be irreversible resulting in the loss of the graft. Each rejection episode results in the loss of endothelial cells and therefore takes the graft further along the path towards eventual graft failure by endothelial cell loss.[20, 27, 37]

There is an enormous amount of information, research and reviews on the subject of corneal graft rejection. The majority of what is known about the immunology of allograft rejection has been obtained from a number of orthotopic penetrating keratoplasty studies in experimental animal models, for example the rabbit, cat and sheep.[52-54] The small rodent species, rat and mouse, in particular, have contributed a wealth of information of the subject over the past fifteen years,[55, 56] and these following sections are drawn from the information elucidated from these experimental studies.

1.3.2.1 A Cell-Mediated Response: Antigen Presentation Cells

There are three conditions necessary for the initiation of an immune response to an allograft: the presence of foreign antigens; antigen presenting cells (APCs); and host immune system cells.[57] The corneal allograft rejection process is primarily a cell-mediated response.[6, 58] The cells include APCs such as macrophages, Langerhans

cells and dendritic cells and, once activated, they present the foreign epitope on their cell surface in the context of major histocompatibility complex (MHC) class II antigens. The APCs stimulate primed host T cells in order to elicit a specific alloimmune response to the donor graft.[51, 59]

Langerhans cells (LC) are dendritic cells with a dominant role in the processing and presentation of MHC class II antigens.[59] A single dendritic cell is capable of stimulating from 100 to 3000 T cells.[60] LCs are present in the limbal region and, upon stimulation, will migrate to the central regions of the cornea and become active.[58] LCs are also present in the central cornea in an immature state, with the ability to capture antigens, however these immature cells are incapable of stimulating T cells.[51]

Macrophages are cells of the innate immune system with phagocytic activity and can function as APCs.[59, 61] These cells are present in the iris, trabecular meshwork, ciliary body and conjunctiva, where they have a major role capturing soluble antigen shed from the donor corneal allograft, and are capable of stimulating T cells.[6, 61] In mice, it has been demonstrated that macrophages have an essential role in the initiation of the corneal allograft rejection response, however not such a critical role in rejection.[62] An experimental study in rats showed that local depletion of macrophages via a sub-conjunctival injection of clodronate liposomes was effective at prolonging graft corneal allograft survival when delivered at an early time point post-transplantation.[63] Macrophages are also found in the corneal allograft cellular infiltrate, indicating a possible effector role for these cells.[21]

According to experimental evidence, Langerhans cells and macrophage populations are required to induce CD4+ T cell-mediated corneal allograft rejection. The absence of one of these populations inhibits the initiation of the alloimmune response and long-term corneal allograft survival will be promoted, reviewed by Niederkorn, 2007.[61]

1.3.2.2 The Direct and Indirect Pathways of Alloresponse Stimulation

There are two pathways by which the alloresponse can be initiated; the direct pathway, whereby APCs from the donor graft tissue display foreign peptide to naïve host T cells, or the indirect pathway, where the host APCs migrate to the donor graft, process the alloantigen and display to naïve host T cells.[51, 58, 59] A study using a mouse model has shown that corneal allografts containing a higher proportion of donor-derived APCs than allografts with fewer donor-derived APCs exhibit a greater risk of immunological rejection.[64] While the direct pathway has been shown to induce a stronger, more efficient immune response to an allograft, the indirect pathway of alloresponse stimulation is considered the most important pathway that leads to corneal allograft rejection.[58] The evidence for this includes the immunological rejection of donor mouse corneal allografts devoid of LCs,[64] and the reduction of corneal allograft rejection in a mouse model when the migration of limbal LCs was inhibited.[65]

Minor histocompatibility antigens are relatively more important in the cornea than in solid vascularised organ transplants.[66] This is possibly due to the significance of the indirect pathway of alloresponse stimulation in corneal transplantation and the relative paucity of mature MHC class II APCs present in the normal central cornea. Experiments in the rat have shown that minor antigen mismatches have more

significance in determining allograft survival than do major histocompatibility antigen mismatches.[67]

1.3.2.3 T Cell Receptor and Co-Stimulation

APCs stimulate naïve T cells by presenting to them foreign peptide, with the aid of the MHC class II antigens, and also with co-stimulatory molecules, which stabilise the immunological synapse with the T cell receptors. The T cell receptor forms a complex with the MHC complex and other molecules that can interact and help to stabilise the bond.[59] The T cell receptor ensures an antigen-specific response.[59] Co-stimulatory molecules include the T cell surface molecule CD28. This molecule interacts with CD80 and CD86 on the surface of activated APCs. This interaction allows the full activation of the T cell.[51, 59, 68]

1.3.2.4 The Cellular Infiltrate of Corneal Graft Rejection

The cellular response to a corneal allograft is predominantly mononuclear, however consists of a heterogeneous inflammatory infiltrate, including CD4+ T lymphocytes, CD8+ T lymphocytes, macrophages, neutrophils and natural killer cells.[6, 58] These cells have been found in the anterior chamber of the eye and adhering to the endothelial surface of the cornea during allograft rejection in animal models.[69] The cells enter from vessels in the peripheral recipient cornea and from vessels in the recipient iris through the anterior chamber.[69] The evidence indicates that the predominant cells controlling the rejection response are the CD4+ T cells. Experimental rat and mouse studies have demonstrated that the depletion of CD4+ T cells has a significant role in preventing allograft rejection in corneal transplantation.[70, 71]

CD4+ T cells induce delayed-type hypersensitivity (DTH) responses to the donor histocompatibility antigens rather than cytotoxic lymphocyte (CTL) responses.[58] DTH responses elicit widespread damage on cells in a non-specific manner as opposed to CTL responses, where only target cells expressing the foreign antigen are specifically killed.[58] CD4+ T cells produce an array of cytokines, chemokines and toxic molecules such as interferon-γ, tumour necrosis factor-α, nitric oxide and super oxide radicals, which have necrotic and apoptotic effects on local cells.[6, 58, 61]

CD8+ T cells, known as cytotoxic lymphocytes, have an unresolved role in corneal allograft rejection. These cells produce CTL responses to foreign antigen, which will readily kill allogeneic cells in cell contact-dependent cytolysis *in vitro*.[72] CD8+ T cells are not considered necessary elements for corneal allograft rejection, as immunological rejection of corneal allografts has been demonstrated to occur in CD8-deficient mice and in mice treated with anti-CD8 antibodies,[73, 74] however CD8+ T cells have been shown to be capable of mediating allograft rejection and inducing graft damage.[6, 61, 72]

Although CD4+ T cells are important mediators of corneal allograft rejection, it is known that CD4+ T cell-independent mechanisms can mediate graft rejection.[61] In a study involving orthotopic corneal allografts in CD4-knockout mice, 45-50% of the allografts underwent immunological rejection.[71, 72] A population of CD4-/CD8+ T lymphocytes were transferred to mice incapable of producing T cells (nude mice) and corneal allografts in these mice were found to undergo swift immunological rejection.[72] Corneal allografts in nude mice also underwent rejection after a transfer of a double negative population of CD4-/CD8- T cells. Interestingly, neither of the two treated mouse groups (both CD4-) showed any CTL responses, yet significant

apoptosis of the donor corneal endothelial cells was observed in both groups. These results indicate that effector cells independent of CD4+ can be generated to mediate corneal allograft rejection.[72]

1.3.2.5 The Location of Antigen Presentation

It is not yet clear where the presentation of corneal-derived alloantigen and sensitisation occurs. Antigen presentation may possibly occur in the cornea itself or in surrounding ocular tissues including the uvea, or in conjunctiva-associated lymphoid tissue, locoregional lymph nodes, draining lymph nodes, more distant secondary lymphoid tissue, or elsewhere.[6, 66] An experimental study using fluorescently labelled antigen injected intracamerally into rat eyes showed the majority of antigen leaves the eye in soluble form, but some are carried by antigen presenting cells.[75] The antigen was extensively distributed in lymph nodes and the spleen.

Local intraocular production of interleukin-10 and CTLA4-Ig by corneal endothelial cells in experimental sheep and rat studies, respectively, elicited increased allograft survival, indicating that some antigen presentation occurs locally.[76, 77] An experimental study using a rat model demonstrated no significant difference in the tempo or nature of the corneal allograft rejection in rats lacking multiple neck and thoracic lymph nodes compared with control rats, indicating that, at least in the rat, antigen presentation does not only occur in the locoregional lymph nodes.[78] This conflicts with evidence from the mouse model of corneal transplantation in which it has been shown that sensitisation occurs in the draining cervical lymph nodes.[79] There is no information as to where sensitisation occurs in the sheep or in humans.

1.3.2.6 Allograft Destruction

Primed T cells undergo clonal expansion into memory and effector T cells. The effector T cells distribute around the body and predominantly target the corneal graft endothelium.[51] Leukocytes are released from the iris and ciliary body into the anterior chamber, and the influx of mononuclear cells results in the local production of some inflammatory cytokines.[6] The target cells expressing the foreign antigen are destroyed by a cellular arsenal: macrophages, some CD8+ cytotoxic lymphocytes, and neutrophils, and by the cytotoxic products they and CD4+ T cells produce.[6, 59] Corneal endothelial cells on the allograft, the survival of which are crucial for the structure and function of that graft, are induced to undergo apoptosis in the early stages of the host immune response.[72, 80] This all results in irreparable endothelial cell damage, which leads to graft oedema and loss of vision.[66]

1.3.2.7 The Role of Alloantibody

The role of allospecific antibody in the response to the corneal allograft is uncertain. It does not appear to be an essential element in the alloresponse, as it is not always produced and some patient studies have shown no link between corneal allograft rejection and the presence of alloantibody.[6, 81, 82] However a large patient study has reported some correlation between alloantibody in sera and corneal allograft rejection in high risk patients,[83] therefore the evidence is mixed. Alloantibody may exacerbate the T cell attack of an allograft and can cause injury to the allograft.[6, 58, 61] An experimental study of penetrating corneal keratoplasty in B-cell deficient mice demonstrated a slight but significant increase in allograft survival, and further, administering antibody to T cell-deficient mice resulted in corneal opacification but no increase in graft rejection.[84] These results indicated that alloantibody plays a minor role in corneal allograft rejection.

1.3.2.8 The Role of Neovascularisation in Graft Rejection

The growth of new vessels into a newly grafted cornea or the presence of pre-existing vessels in the normally avascular human cornea prior to corneal transplantation, are key risk factors for corneal graft rejection. Statistics from the Australian Corneal Graft Registry Report 2012 demonstrate that full-thickness corneal grafts performed in recipients with a high degree of pre-vascularisation have a much shorter average survival time than grafts performed in recipients with no pre-vascularisation.[16] This is shown in Figure 1.5, a Kaplan-Meier survival plot of corneal graft survival over time, where recipients without any degree of pre-graft corneal vascularisation (blue line) show much better probability of graft survival over time than any of the pre-vascularised recipients (green, orange, purple and red lines). The higher the degree of pre-vascularisation, in these cases the more quadrants of the eye experiencing pre-vascularisation, the shorter the probability of graft survival.

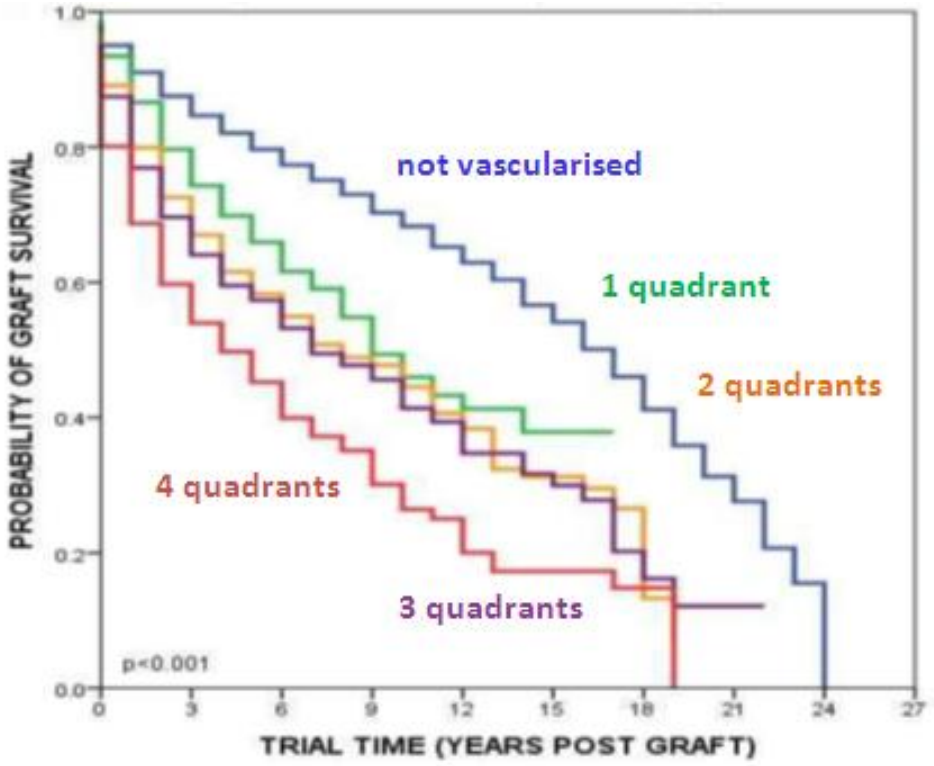


Figure 1.5: Kaplan-Meier survival plot over time for penetrating corneal grafts with varying degrees of pre-vascularisation in the recipient eye prior to transplantation. Pre-vascularisation range: not vascularised, one quadrant (least) through to all four quadrants of the eye vascularised (most). Figure from the Australian Corneal Graft Registry Report 2012.[16]

Several studies have shown a correlation between inhibition of neovascularisation and prolongation of corneal graft survival in animal models.[85-87] Successful inhibition of new vessel infiltration into corneal grafts is a much needed therapy to prolong the survival of grafts at high-risk of rejection. Currently, treatments for corneal neovascularisation are still in experimental stages and there is an urgent need to develop therapies for better outcomes for patients at high-risk of graft rejection.

1.4 Animal Models of Corneal Transplantation

Various animals have been proven useful for experiments involving corneal transplantation of the cornea, including the rat, mouse, rabbit, cat, sheep and the miniature pig.[52-56, 88-90] Mice and rats are valuable experimental animals as they are easy to handle, genetically inbred strains are readily available and a corneal allograft performed in these animals will undergo a similar process of immunological rejection to a human corneal allograft.[91] However rodent species have some disadvantages that impair their use as pre-clinical models, including an eye size and anatomy that differs from the human eye, having comparatively a much larger lens relative to the other ocular components of the anterior segment than the human eye.[91] The other major disadvantage offered by rodent species as a model for corneal transplantation is a replicative corneal endothelial cell layer, as opposed to the human scenario, where the corneal endothelial cells are amitotic.[2, 91] Further, the small rodents rarely show the epithelial and endothelial lines of rejection (aggregates of small inflammatory cells, which arrange in a linear manner with either damaged epithelial or endothelial corneal cells), that can be seen during human corneal graft rejection.[91, 92]

Rabbit and cat models are also useful outbred models, with the cat having a relatively amitotic corneal endothelium,[93] however they have disadvantages experimentally as the reagents needed to dissect the immune response in these animals are limited. Further, the fact that these animals are domestic pets can make euthanasia distressing for the animal handlers.

Whilst no non-human animal is a perfect substitute for clinical research, I used an animal model that can give useful results as they pertain to humans. The sheep is an

outbred animal readily available in Australia and of economic importance. The size, anatomy and physiology of the sheep eye are similar to the human eye, with a non-replicative corneal endothelial layer.[76] Sheep corneal grafts undergo immunological rejection in a manner that is histologically similar to human rejection and the grafts are reliably rejected after approximately three weeks.[53] Corneal grafting in sheep is a similar process to the human clinical procedure, with the assessment of the grafts an easy task as no anaesthetic is required and sheep are mostly docile animals.[91]

For the work presented in this thesis, the well-established sheep model of corneal transplantation was chosen as the pre-clinical animal model in which to test prolongation of corneal allograft survival by gene therapy. A good pre-clinical model is necessary in order to develop appropriate hypotheses from the results which can eventually lead to translation into clinical therapies.

1.5 Current Interventions and Research

The current method of preventing corneal allograft rejection at the clinical level is by applying topical glucocorticosteroids to the eye. In some cases, systemic immune suppression is used, however the limited benefit potentially given by this treatment, comes at the cost of morbidities to the patient.[94, 95] Some of the current experimental research directions and clinical trials designed to prevent corneal allograft rejection are outlined in the following sections.

1.5.1 Modulation of Host T Cell Responses

Therapeutic modulation of host T cell responses has been addressed in several studies of corneal transplantation. Qian *et al.* showed that the topical administration of a soluble tumour necrosis factor receptor type 1 to mouse corneal allografts

resulted in the prolongation of graft survival compared to controls.[96] The depletion of host T cells using anti-CD4 antibodies has been shown to prolong allograft survival in a rat model of corneal transplantation.[70] The modification of the inflammatory response by altering cytokine production has been addressed in experimental studies, with the aim of reducing the local activity of pro-inflammatory molecules such as tumour necrosis factor α, IL-1, and -12 or increasing the concentration of local immuno-modulatory cytokines such as interleukin-10 (IL-10) and IL-4.[76, 97-99]

The following sections focus on three molecules, interleukin-10, indoleamine 2,3-dioxygenase, and cytotoxic lymphocyte antigen-4. These molecules were selected for discussion as the over-expression of all three individually has been demonstrated to increase corneal allograft survival in experimental models, as described below.

1.5.1.1 Interleukin-10

The biology and function of the cytokine interleukin-10 has been comprehensively reviewed by Moore *et al.*, 2001.[100] IL-10 is an immune-modulatory cytokine which has anti-inflammatory activities and, under some circumstances, down-regulates cell mediated immune responses. The expression of cytokines and cell surface molecules by cells of myeloid origin may be modulated by IL-10, and this affects the ability of these cells to participate in inflammatory responses.[100] The presence of immuno-modulatory cytokines such as IL-10 influences the immune system towards an immune-modulatory response to corneal allografts.[66]

IL-10 is naturally produced by most leukocytes, with monocytes and macrophages being the main synthesizers of the cytokine, however T cells, B cells, dendritic cells, natural killer cells, mast cells, neutrophils and eosinophils also synthesise IL-10 in

some tissues at various times.[101] Following T cell receptor triggering, activated T cells will secrete IL-10. A subset of CD4+ T cells known as type 1 regulatory T cells (Tr1) develop from naïve T cells and almost exclusively synthesise IL-10, suppressing the function of antigen presenting cells.[101]

Interleukin-10 prevents the synthesis of many pro- and anti-inflammatory cytokines and inflammatory chemokines by antigen presenting cells, and induces the up-regulation of anti-inflammatory molecules by these cells.[100] In a study by Fiorentino *et al.*, the authors demonstrated that murine IL-10 acts on antigen presenting cells and impairs their function in stimulating Th1 T cell clones to produce inflammatory cytokines such as interferon-γ, however IL-10 did not impair APC stimulation of Th2 T cell clones.[102]

IL-10 affects the function of T cells both directly, by inhibiting IL-2, IL-4 and IL-5 and interferon-γ production from T cells, and indirectly, by its down-regulatory effects on antigen presenting cell functions.[100, 101] Interestingly, IL-10 has been reported to have a stimulatory effect on CD8+ T cells, inducing their cytotoxicity and proliferation.[103]

There has been mixed success therapeutically when using the over-expression of IL-10 to prevent an immune response to allografts in animal models of transplantation. Torres *et al.* demonstrated that over-expression of mouse IL-10, delivered by either sub-conjunctival or intra-peritoneal injection in a rat model of corneal transplantation, did not prolong allograft survival and may have even accelerated the rejection process.[104] All animals received IL-10 doses on the day of surgery, some the day before surgery, and all on staggered days post-operatively. Similarly, Brandt

et al. found rat renal and cardiac allografts treated with viral IL-10-expressing T lymphocytes did not show prolonged survival, even with sub-optimal doses of the immunosuppressive agent cyclosporine.[105] In contrast to these studies, Li *et al.* showed a significant prolongation of mouse cardiac allografts by pre-treatment of the recipient mouse with IL-10, and also significant prolongation with IL-10 pre-treatment of the donor mouse, albeit not to the same extent as with recipient pre-treatment.[106] The authors demonstrated no prolongation of allograft survival with post-transplantation treatment with IL-10; in fact this treatment appeared to accelerate the rejection process. Of particular relevance here, Klebe *et al.* found significant prolongation of sheep corneal allografts when donor corneas were treated *ex vivo* with an adenoviral vector expressing ovine IL-10 prior to transplantation.[76] The authors reported a median allograft rejection day of 55 for the animals with allografts expressing IL-10 post-surgery, with two individual sheep supporting allografts with survival times greater than 196 and 300 days, respectively, compared with a median rejection day of 20 for the combined control groups (unmodified allografts and mock-vector treated controls). This last study demonstrates the ability of IL-10 to locally suppress the immune response to the sheep corneal allografts, however it was not effective across all of the treated animals, with almost half of the treated animals rejecting their allografts in a similar time frame to the control animals.[76]

It appears the timing of IL-10 expression during the immune response is a critical factor in determining the success and use of this cytokine as a therapeutic agent in transplantation. Given the significant prolongation of corneal allografts induced by IL-10 in the pre-clinical sheep model shown by Klebe *et al.*, it was considered

worthwhile to investigate this cytokine further in the context of corneal transplantation.

1.5.1.2 Indoleamine 2,3-dioxygenase

Indoleamine 2,3-dioxygenase (IDO) is an inducible intracellular enzyme that catalyses the initial stages of tryptophan degradation. Tryptophan is an essential amino acid required by T cells for proliferation, therefore a reduction in tryptophan levels leads to a suppression of T cell responses.[107, 108] It has been suggested that IDO plays a role in maintaining the immune privilege of the eye, since it is induced by interferon-γ.[109] Several animal studies have shown that an over-expression of IDO can prolong allograft survival in many transplantation models (for review see Quan *et al.* [108]). In a study by Beutelspacher *et al.*, a lentiviral vector was used to over-express the IDO transgene in mouse corneal allografts.[107] The authors demonstrated a significant prolongation of graft survival by the IDO-treated grafts compared with control allografts. IDO may not have therapeutic potential for all organ transplants, however, as Laurence and colleagues demonstrated that the over-expression of IDO using an adeno-associated viral vector in rat liver allografts did not prolong the survival of the grafts when compared to reporter gene-transduced control allografts.[110]

The pathway of tryptophan degradation catalysed by IDO produces downstream metabolites called kynurenines.[111] Kynurenines have also been shown to have an inhibitory effect on T cell responses.[112-114] Zaher *et al.* administered four kynurenines topically and systemically to mice with corneal allografts and demonstrated a significant prolongation of allografts treated with two of the kynurenines compared with control allografts.[115] Further to this work, Zaher *et al.* have recently found a

novel molecule, which may be of benefit to future corneal allografts.[116] N-(3,4-dimethoxycinnamonyl) anthranilic acid (DAA) is an anti-allergy drug which is an analogue to tryptophan metabolites, sharing the same anthranilic core structure. The authors found that systemic administration of DAA to mice with corneal and skin allografts had significantly delayed onset of rejection. Further, it was demonstrated that the mode of action of DAA was immune suppression of the allogeneic responses by cell-cycle arrest rather than by T cell death, as is the case with kynurenine-mediated inhibition of immune responses.[115]

1.5.1.3 Cytotoxic Lymphocyte Antigen-4 (CTLA-4)

Antigen presenting cells interact with T cells via T cell receptors and major histocompatibility complexes (MHC), however secondary signalling or co-stimulation also occurs, which stimulates the activation of naïve T cells.[117] This co-stimulation of T cells by CD28-CD80-CD86 interactions with APCs has been a target for immunomodulation.[118-120] Cytotoxic lymphocyte antigen-4 (CTLA-4) competitively binds CD80 and CD86 receptors on APCs thus blocking their interaction with CD28 on the surface of T cells, preventing the stimulation and clonal expansion of the T cells.[68] Mice corneal allografts have shown prolonged survival upon ballistic gene therapy treatment with DNA encoding the extracellular fragment of CTLA-4, and rat allografts treated either with the protein CTLA4-Ig or with an adenoviral vector encoding CTLA4-Ig also showed prolonged survival compared with controls.[77, 121] CTLA-4 has been proven effective at graft prolongation in animal organ transplant studies, for review see Larsen *et al.*, 2006.[117]

Fusion proteins, abatacept and belatacept, which consist of the extracellular domain of CTLA-4 and an IgG1 fragment, are newly commercially available as biologics for

blocking T cell activation.[122, 123] Abatacept is mainly used for patients with rheumatoid arthritis and juvenile idiopathic arthritis (JIA), however this agent has also been demonstrated to be effective in several animal studies of ocular inflammation including uveitis and uveitis associated with JIA, reviewed by Dastiridou *et al.*, 2012.[124] The findings from a clinical trial of abatacept reported in 2010 by Zubian *et al.* showed that of the 7 patients with JIA with severe bilateral chronic anterior uveitis that was refractory to both classic immunosuppressive and anti-TNFα treatment, all responded to abatacept treatment and uveitis improved.[125] Belatacept, which differs from abatacept by only two amino acid residues, has a higher binding affinity for CD80 and CD86 receptors than abatacept and has approval for clinical use in renal transplantation.[122] A three year follow-up study has shown the efficacy of belatacept at providing sustained renal function in the recipients of kidney transplants,[126] and a study conducted by Larsen *et al.* demonstrated that belatacept out-performed abatacept in terms of enhanced immunosuppressive activity and increased allograft survival of kidney transplants in a non-human primate model.[127] Further pre-clinical investigations are required in order to characterise this biologic, before its use is approved for immune suppression in other solid organ transplants.

The host T cell response is the main instigator of the host immunological response to the foreign allograft. T cell modulation has the potential for prolonging corneal allograft survival indefinitely. This could lead to localised therapeutic treatments for corneal transplant patients.

1.5.2 Anti-neovascularisation of the Cornea

Current treatments in the clinic for corneal neovascularisation usually involve the use of anti-inflammatory agents such as steroids or cyclosporine A in topical doses or sub-conjunctival injections.[128, 129] Anti-inflammatory treatment has been shown to be effective at inhibiting the proliferation and migration of vascular endothelial cells, however the efficacy is limited when neovascularisation is present without inflammation.[129] Steroidal anti-inflammatory treatments have significant side effects such as the potential for cataract and glaucoma development, and the increased risk of infection.[128] Depending on the growth stage of the vessels, in some instances of established vessels fine needle cauterisation can be implemented to help reduce vessels and inflammation.[130]

Vascular endothelial growth factor-A (VEGF-A) is a potent angiogenesis stimulator, and the first therapy against this factor was the cancer therapy drug bevacizumab, a humanised anti-VEGF neutralising antibody (Figure 1.6).[131] Since VEGF is an important mediator of vessel growth in the body, blocking VEGF in a localised manner to treat unwanted neovascularisation is essential to reduce side-effects. Antibodies against VEGF therefore have potential for localised use in the eye, due to the blood-eye barrier, which restricts the free diffusion of molecules through the ocular tissues to the blood stream.[41] Delivery of anti-VEGF antibodies to the eye is problematic, however, because of their large size. For example, bevacizumab is 148 kDa. Whole antibodies do not penetrate the corneal epithelium when administered topically, and sub-conjunctival injection, whilst effective, comes with potential side effects following prolonged administration in this manner.[128] A recent clinical trial reported by Petsoglou *et al*. demonstrated that monthly sub-conjunctival injections of bevacizumab for three months caused a significant reduction in newly formed

corneal neovascularisation in 15 patients compared with an increase in neovascularisation in the saline-treated control patients.[132] Another intervention is the delivery of anti-sense nucleotides to the cornea to target the protein insulin receptor substrate-1 (IRS-1), to target actively growing new vessels.[129] A clinical trial has shown topical eye drops containing an oligonucleotide to inhibit IRS-1 stabilised vessel growth when given at a low dosage, and at a medium dosage caused significant inhibition and regression of corneal neovascularisation in treated patients.[133] Clinical trials are continuing for these therapeutics.

1.5.2.1 Soluble Fms-like Tyrosine Kinase 1 (sFlt-1)

The cornea is purposely avascular in order to preserve sight, and this function is maintained by the expression of soluble fms-like tyrosine kinase (sFlt-1), otherwise known as soluble vascular endothelial growth factor receptor 1.[134] This is a soluble receptor specific for vascular endothelial growth factor-A (VEGF-A), an important regulator of angiogenesis in the body.[135, 136] sFlt-1 acts like a trap to bind VEGF in the normal eye and prevent its interaction and signalling through membrane-bound VEGF receptors (Figure 1.6). sFlt-1 may also form heterodimers with VEGF receptors 1 and 2 to prevent VEGF signalling and in this way inhibit the angiogenic activity of VEGF.[134, 137] Lai *et al.* demonstrated that in a rat model of induced vascularisation of the cornea, over-expression of sFlt-1 using an adenoviral vector injected into the anterior chamber induced decreased levels of VEGF in rat corneas, and an inhibition of new vessel formation compared with controls.[138] Iriyama *et al.* used an injection of a polyplex micelle to deliver DNA encoding the sFlt-1 gene to the sub-conjunctival space of mice with induced corneal neovascularisation.[139] The authors found significantly less neovascularisation in the sFlt-1 treated mouse corneas than control treated corneas.

Recently, Cho *et al.* demonstrated that by decreasing levels of membrane-bound Flt protein and increasing levels of soluble Flt protein using synthetic morpholino molecules (similar to DNA oligonucleotides) in the corneas of mice with suture-induced vascularisation, the area and volume of corneal neovascularisation was significantly decreased compared with controls, where neovascularisation was seen to increase.[140] There is a good deal of interest in sFlt and its potential as an anti-angiogenic factor for the treatment of retinal disorders involving unwanted vascularisation in the posterior segment of the eye.[141-143]

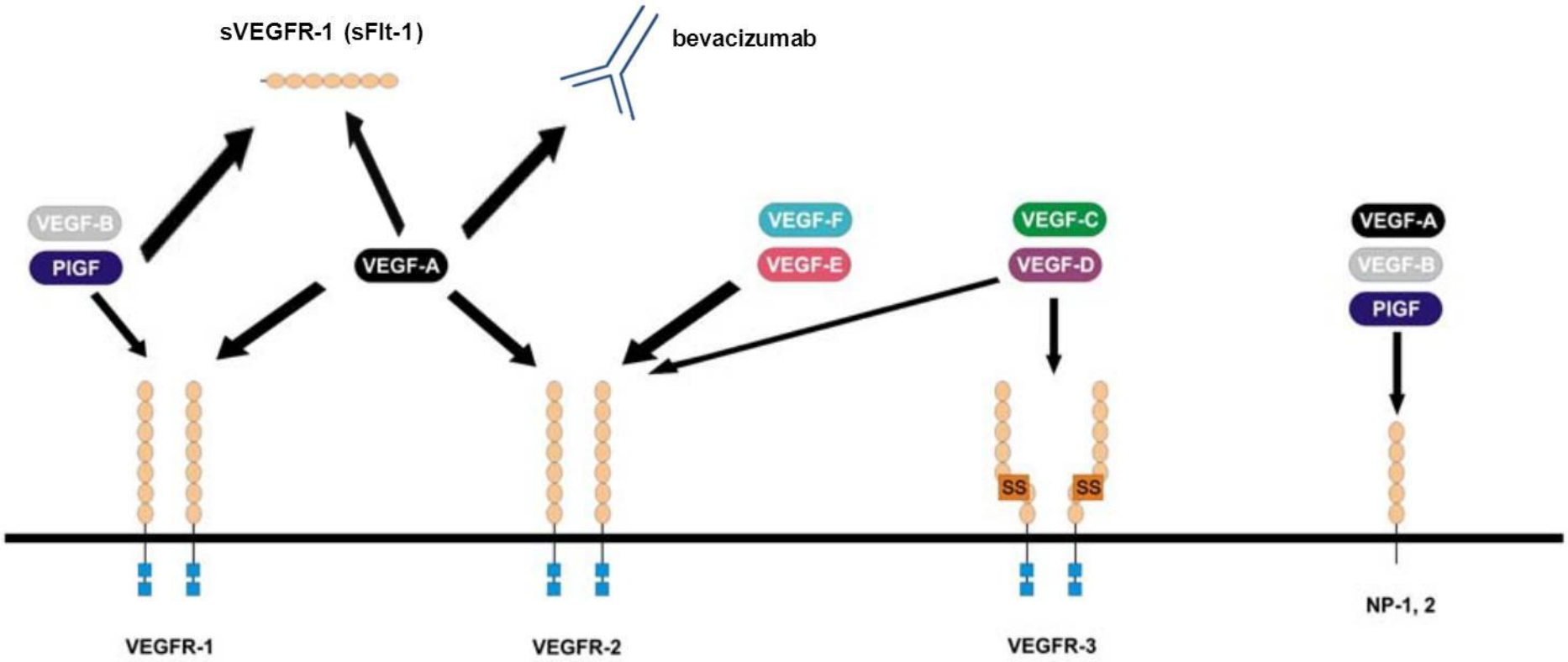


Figure 1.6: Vascular endothelial growth factor (VEGF) isoforms and interactions with membrane-bound receptors, soluble receptors, and anti-VEGF-A neutralising antibody bevacizumab. NP-1,2 – neuropilins 1 & 2, sVEGFR-1 (sFlt-1) – soluble VEGF receptor 1 (soluble Fms-like tyrosine kinase 1), PlGF – Placental growth factor. Figure modified from Otrock *et al*., 2007 [136].

1.5.2.2 Endostatin::Kringle5

A different mechanism of preventing angiogenesis can be to inhibit the action of vascular endothelial cells forming tubular vessel structures. Kringle-5 is a domain of the human plasminogen gene, which is a potent angiogenesis inhibitor. This domain specifically prevents the cellular proliferation and migration of vascular endothelial cells, to inhibit the formation of new vasculature.[87, 144, 145] Endostatin is a fragment of collagen XVIII and is also a strong inhibitor of angiogenesis. It specifically down-regulates genes controlling functions such as anti-apoptosis and cell growth in endothelial cells.[87, 146, 147] A soluble fusion protein consisting of kringle-5 and endostatin was created by Murthy *et al.* and used to treat rabbit corneas *ex vivo* prior to corneal transplantation.[87] All of the treated allografts showed a significant decrease in corneal neovascularisation compared with controls, and prolonged survival with no graft failure, in contrast to 100% rejection of reporter gene-treated control allografts.[87]

As neovascularisation is a key risk factor for graft rejection, developing preventative therapies to counter new vessel growth in the cornea after corneal grafting is important. The use of pre-clinical animal models to trial potential anti-neovascularisation therapies can give useful insights as to their potential efficacy in clinical trials.

1.5.3 Improving the Survival of the Corneal Endothelium

The efferent arm, or effector stage, of the immune response is responsible for the destruction of the foreign cells, and is largely directed towards the non-replicative corneal endothelium in allograft rejection.[148] Graft endothelial cells undergo programmed cell death (apoptosis) in response to the cytotoxic cells produced by the

host immune system,[72, 80] and the death of these cells ultimately means the endothelium loses its functional barrier properties, resulting in the loss of corneal clarity.[2]

One mechanism by which apoptosis may be initiated is by the release of cytochrome-c from the mitochondrial membrane into the cytosol.[149] Once in the cytosol, a caspase cascade is triggered leading to cell death. Bcl-2 is an anti-apoptosis factor found in the outer membrane of mitochondria. It has a possible role in preventing apoptosis by inhibiting the release of cytochrome-c from the mitochondrial membrane.[150] The anti-apoptosis factor of the bcl-2 family, Bcl-xL, has been found to prevent the loss of corneal endothelial cells and prolong the survival of corneal allografts, when over-expressed in a mouse model of corneal transplantation.[80]

Given that the human corneal endothelium is a non-replicative monolayer of cells,[2] prolonging the survival of these cells is important to the health and survival of corneal grafts.

1.6 Gene Therapy

Gene therapy describes the process of introducing functional copies of genes into a system, in order to restore normal gene expression and function.[151, 152] Gene therapy may also be used to induce atypical gene expression to serve a specific purpose, for example increasing the expression of immunomodulatory cytokines to aid the control of an inflammatory response.[76] There are many approaches to gene delivery, including virus-mediated gene delivery and non-viral methods such as naked DNA transfer, oligonucleotides, cationic lipids, polycationic carriers and nanoparticles.[153-155] One of the limitations of this technology currently is developing an efficient and non-toxic gene delivery system.[156, 157]

1.6.1 Viral Vectors

The ideal gene therapy delivery system will transduce target cells with high specificity, high efficiency, and induce high transgene expression for the duration required for a therapeutic effect, without having a toxic effect on the target cells.[151, 158] Viral vectors are considered the most efficient gene delivery system. Viruses have naturally evolved to deliver genes to target cells and, at the start of this project in 2008, had been utilised in 70% of gene therapy clinical trials.[152, 157] Viral vectors used for gene therapy are modified to remove non-essential viral genes, increase their specificity for target cells, and to increase their safety.[152, 153] There are issues with using viral vectors as delivery systems in humans because of their potential adverse effects, such as immunogenicity, risk of homologous recombination, insertional mutagenesis and, in some cases, the pathogenic nature of the viruses.[151] Table 1.2 summarises the main viral vectors used for gene therapy.

Table 1.2: Summary of selected viral vectors used in gene therapy clinical trials.

Vectors	Transgene Capacity	Host Genome Integration Status	Peak Expression Time	Inflammatory Potential	Refs
Herpes simplex virus	30-50 kb	Episomal	Transient	high	151, 152, 158
Adenovirus	7 - 8 kb	Episomal	Transient	high	151, 152, 158-160
Adeno-associated virus	4.9 kb	Episomal, some integration	Medium to long term	low	152, 153, 157, 160, 161
Retrovirus	<8 kb	Integrated	Long term	low	151, 158, 162
Lentivirus (complex retrovirus)	~8 kb	Integrated	Long term	low	151, 153, 158, 160, 163

Adeno-associated virus vectors (AAV) are derived from human parvoviruses and have no known disease association, meaning they are non-pathogenic.[157, 158] AAV vectors have a broad target cell tropism, will transduce non-dividing cells, and have the potential for long-term transgene expression with some ability for random integration into the host genome.[151, 158, 161] AAV vectors do have the disadvantages of requiring helper viruses during AAV vector preparation, and also that there is evidence of a pre-existing immunity to AAVs in human populations.[157, 164]

Herpes simplex virus (HSV) vectors have a notably large transgene capacity, with over 30 kbp available in the genome for transgene expression.[151, 158] HSV vectors have the ability to transduce dividing and non-dividing cells and have a broad cell tropism, however they do not integrate into the host genome and therefore transgene expression is only transient.[151] Hudde *et al.* trialled an AAV vector and an HSV vector for the *ex vivo* transduction of rabbit and human corneas.[165] The authors found long-term transgene expression induced by the AAV vector, however much poorer transgene expression and evidence of cytotoxicity was shown from the HSV vector. Klebe *et al.* tested HSV-1 for delivery of transgene to the ovine cornea and found inefficient gene transfer, with less than 0.01% of the endothelial cells expressing the reporter gene.[155]

Retroviral vectors are widely used in gene therapy clinical trials as they have a high transduction efficiency, will infect a broad range of cell types and can mediate high transgene expression.[151] The disadvantages of some retroviral vectors include the ability to only transduce dividing cells and the potential for insertional mutagenesis.[151]

The adenoviral vector and the lentiviral vector, which were selected for use in the work described in this thesis, are described in more detail below.

1.6.1.1 The Adenoviral Vector

Adenoviral vectors are double-stranded DNA gene therapy vectors. The vector has been modified over generations and the vectors used in the majority of more recent gene therapy studies are second generation or later, replication deficient, and with significant genome deletions for increased safety.[157]

The adenoviral vector is a non-integrative gene therapy vector, which means that after infection and nuclear import into the host cell, the viral genome remains episomal, and expression of the viral DNA is only transient.[151, 158] Another potential drawback of the adenoviral vector when considering its use for *in vivo* gene therapy, is the strong innate inflammatory response elicited by the body towards the vector.[159, 160, 166] The adenoviral vector activates inflammatory cytokines, complement signalling, natural killer and endothelial cells, and induces up-regulation of type 1 interferons, as responses directed against the capsid proteins, the double-stranded DNA genome, the viral proteins or the transgenes themselves.[160] However, it has been shown to be a very successful gene therapy vector, with the ability to transduce a wide variety of cell types, dividing and non-dividing, and the ability to

induce high levels of transgene expression both *in vitro* and *in vivo*.[151, 158] Bainbridge *et al*. have demonstrated the use of an adenoviral vector to successfully transduce corneal endothelial cells, iris, ciliary body, and the trabecular meshwork after intra-vitreal injection in a mouse model.[167] *Ex vivo* transduction of human corneal endothelial cells using adenoviral vectors was also achieved by Oral *et al*.[168]

An adenoviral vector was selected for work described in this thesis, as this vector transduces non-dividing cells such as the corneal endothelium, and delivers strong initial transgene expression.

1.6.1.2 The Lentiviral Vector

The lentivirus is a single-stranded RNA virus of the retroviridae genus.[151, 169] Retroviridae viral vectors include the gamma-retroviral vectors, which have been used in several clinical instances of gene therapy of haematopoietic stem cells.[170-172] The retroviridae are integrative viruses, meaning that once infection, reverse transcription and nuclear import have occurred, the viral genome will integrate into the host genome for long-term expression of the viral DNA.[151]

The focus of this thesis was on the primate lentivirus, human immunodeficiency virus type 1 (HIV-1). This virus naturally infects T cells due to the specificity of the outer envelope protein of the virus.[173] Engineered vectors may be modified for improved safety characteristics and to enhance features for better performance as gene therapy vectors. One way in which this has been achieved with the lentiviral vector is by modifying the envelope protein to a vesicular stomatitis virus glycoprotein G (VSV-G), which alters the tropism of the vector from CD4 T-lymphocytes, macrophages, dendritic cells and microglia, to a much broader range of

cell types, allows target cell entry via endocytosis and also adds stability during the vector manufacturing process.[173-175] Another modification of the lentiviral gene therapy vector has been the deletion of enhancer sequences in the 3' U3 region. In the wild-type virus, these enhancer elements aid in the generation of replication competent viral particles, therefore deletions of these elements create self-inactivating (SIN) vectors.[153, 176]

Lentiviral vectors can transduce dividing and non-dividing cells, making them versatile vectors for gene therapy.[151, 158] It has also been demonstrated that lentiviral vectors have a low inflammatory potential in the eye in comparison with adenoviral vectors.[166] The ability of lentiviral vectors to transduce the various ocular cell layers of the eye in several animal models has been reviewed by Balaggan and Ali, 2011.[177] Parker *et al.* demonstrated the ability of an HIV-1 lentiviral vector to transduce rat, sheep and human corneas *ex vivo* and successfully express transgenic protein.[178] Whilst the adenoviral vector gives strong early transgene expression, such expression is transient. The lentiviral vector, with its integrative mechanisms, can give long-term, stable transgene expression, for a lasting therapeutic effect, while exerting less inflammation in the eye.

Previous gene therapy studies in our laboratory have utilised both adenoviral and lentiviral vectors as gene delivery systems to the ovine cornea.[91, 98, 178] In order to investigate the potential for the stable long-term expression of therapeutic transgenes, an integrative lentiviral vector was chosen as the primary delivery system for the work described in this thesis. The vector uses an adapted HIV-1 lentiviral transfer plasmid from the laboratory of Associate Professor Donald Anson,[179] which enables

the creation of a non-replicative, self-inactivating lentiviral vector that will infect non-dividing cells.

1.6.2 Current Status of Gene Therapy

Gene therapy is a promising technology that has been proven in principle; however there are limitations that currently prevent the achievement of long term genetic reconstitution. For example, gene delivery to tissues that are difficult to access, therapy with multiple genes, and sustained gene correction are issues that need to be overcome. Studies into *ex vivo* delivery, the preparation of safe viral vectors, integrating multiple therapeutic approaches and harnessing the immune system are leading to promising results.[157] There have, however, been two deaths and significant morbidities during human gene therapy clinical trials, summarised in Table 1.3. As the technology of vectors progresses, for example by lowering inflammatory potential and the creation of self-inactivating viral vectors, the prospects for safer gene therapy trials improve.[180] Information provided by The Journal of Gene Medicine, updated in January 2013, shows that the majority of the vectors used in human gene therapy trials are adenoviral vectors, closely followed by retroviral vectors (www.wiley.co.uk/genmed/clinical).[181]

Table 1.3: Summary of significant incidents that have occurred during human gene therapy clinical trials.

Date	Patient	Disease/ Illness	Vector	Administration	Outcome	Comment	Ref
1999	Jesse Gelsinger	18 year old male with an inherited ornithine-transcarbamylase deficiency	AdV	Direct infusion of liver through hepatic artery	Patient died of multi-organ failure caused by a massive inflammatory immune response against the large dose of virus (38 trillion viral particles).	First patient to die from the gene therapy itself	**162, 182**
2000, 2003	Clinical trials with a total of 20 children treated	Children with X-linked severe combined immunodeficiency (SCID-X1)	γ-retroviral	Re-infusion of *ex vivo* transduced haematopoietic CD34 stem cells from bone marrow	The majority of the children were successfully treated, but five developed lympho-proliferative disorders (leukaemia-like rapid expansion of T cell clones) after treatment. No deaths attributed to the gene therapy; one death occurred after allogeneic stem cell transplantation, and a second death in a separate trial due to pulmonary failure caused by SCID-X1.	Gene therapy has been shown to be a highly effective treatment for this disease, however this vector appeared to have non-random insertion near the proto-oncogene LMO2 promoter	183-186
2007	Jolee Mohr	36 year old female with rheumatoid arthritis, taking immunosuppression	AAV	Intraarticular injection into patient's right knee	Patient died from a fungal infection, but an immune response resulting from the vector was not ruled out as a contributing factor.	Immune suppressed patient lived in a region where fungus was endemic	**187, 188**

AdV – adenovirus vector; AAV – adeno-associated virus vector

Understanding the molecular basis of vector interactions with the human body is of great importance as it leads to improvements in vector safety, efficiency and specificity. Viral vectors have some concerning limitations, as may be seen in Table 1.3, such as systemic inflammatory potential and risk of insertional mutagenesis, which must to be addressed before their escalated use in clinical trials. Already a shift can be seen in pre-clinical gene therapy investigations from the more inflammatory adenoviral vectors to adeno-associated viral and lentiviral vectors, with their lower inflammatory potential.[189, 190] Further, the use of oncoviral retroviral vectors such as the mouse leukaemia virus (MLV) vectors, which were commonly used to transduce haematopoietic stem cells and used in the gene therapy trial to treat the SCID-X1 children, is being phased out in favour of lentiviral retroviral vectors, as it has been demonstrated that the former have a much higher preference for transcription start sites than the latter, sites where the strong MLV LTR enhancer elements are able to activate nearby genes.[174, 191-193]

1.6.3 Gene Therapy and the Eye

The eye is a good candidate organ for the potential use of gene therapy, as the blood-eye barrier prevents most systemic migration of any administered vector.[41] There are many ocular disorders and malignancies that could potentially benefit from gene therapy, for example retinoblastoma, corneal neovascularisation, age-related macular degeneration, and retinal and choroid neovascularisation.[194] Several cell layers and structures in the eye have been identified as potential gene therapy targets and successfully transduced with viral vectors in animal studies, such as the retinal pigment epithelium, iris pigment epithelium, trabecular meshwork, and the corneal endothelium.[177, 195-197]

1.6.4 Gene Therapy for Long-term Corneal Graft Survival

Gene therapy is a potentially useful system to induce long-term human corneal allograft prolongation, since, unlike other solid organ transplants, the donor tissue can be viably maintained for up to 14 days or several weeks in cold storage medium or organ culture, respectively, prior to transplantation, enabling *ex vivo* manipulation of the tissue.[21, 198] The target cells selected for gene therapy of the cornea are the corneal endothelial cells, an easily accessible monolayer on the donor cornea. Due to the fact that the target cells are a monolayer, the gene therapy vectors have access to the majority of the target cells, which increases the efficacy of the transduction and the chances of therapy success.[199] The corneal endothelial cells are also one of the major targets of the host immune response, as mentioned previously, therefore by transducing the corneal endothelium, the therapeutic transgenes are being delivered directly to the site of the immune response. The fact that the viral transduction may be conducted *ex vivo*, with no application, systemic or otherwise, of virus to the patient, localises the treatment and increases the safety of the procedure by reducing the overall dosage of virus required. The transparency of the cornea allows for visual observation and monitoring of graft health *in vivo* post-transplantation.[91, 154, 198, 200]

Therapeutic transduction of the cornea prior to penetrating keratoplasty has previously proven effective for significantly improving allograft prolongation using lentiviral and adenoviral vectors, and mouse, rat, rabbit and sheep animal models.[76, 87, 107, 201, 202]

1.6.5 Enhancing the Success of Gene Therapy

As mentioned previously, human corneas may be stored for varying periods of time before transplantation, ranging from one hour to up to 28 days, depending on the style of storage employed. Sheep corneas on the other hand, do not survive well if

incubated in culture for 24 hours prior to transplantation, with these grafts often resulting in technical failures (results not published).[203] Further, the transduction of human corneas with lentivirus has been shown to lead to rapid and high levels of transgene expression, whereas lentiviral transduction of ovine corneas has shown a slower response of transgene expression.[178]

In order to improve gene therapy of sheep corneal transplants, a shorter transduction time of the corneas *ex vivo* is necessary to ensure successful grafts, and earlier transgene expression needs to be induced. This may be achieved by improving the viral transduction efficiency. There are various methods of achieving an increase in transduction efficiency such as increasing the viral dosage, using centrifugal forces, coating polymers and lipofective and charge neutralising agents to achieve enhanced contact between the viral particles and the target cell surfaces.[204-208]

1.6.5.1 Polycations for Improved Transduction Efficiency of the Lentivirus

Many studies have shown that helper agents such as cell-permeable peptides and polycations can be successfully used to improve the success rate of viral infection.[205, 206, 209] Polycations aid the internalisation process of the particles by reducing the electrostatic repulsion of the negatively charged viral particles from the negatively charged cell membrane.[210, 211] Polybrene (hexadimethrine bromide) is a cationic polymer that has been shown to increase viral transduction efficiencies.[212-214] In his PhD thesis, during studies investigating the use of the polycation polybrene to increase the transduction efficiency of viral particles into ovine corneal endothelial cells, Dr Douglas Parker found that polybrene had a toxic effect on ovine endothelial cells at a dose of 40 μg/mL and led to the technical failure of sheep corneal allografts

(results not published).[203] Due to the apparent toxicity of polybrene on the ovine corneal transplantation model, an alternative polycation was sought.

Protamine sulphate is a polycation that has been approved by the United States Food and Drug Administration for human use. Cornetta *et al.*, 1989, suggested this molecule as an alternative to polybrene for the purpose of increasing the efficiency of viral transduction.[215] Protamine sulphate has been shown to be effective in this capacity *in vitro* and *in vivo*, however the results have been mixed.[212, 216] Thus, for example, Haas *et al.* found the addition of 4μg/mL protamine sulphate to a lentiviral transduction of CD34+ cells *in vitro* did not make any difference to the percentage transgene expression by the cells.[217] Lanuti *et al.* demonstrated that, whilst protamine sulphate successfully improved reporter gene transfer to intraperitoneal mouse tumours when complexed with an adenoviral vector, this did not correspond to an enhanced animal survival when using therapeutic transgenes.[216]

I argued that the polycation protamine sulphate might enhance the efficacy of lentiviral transduction in the sheep cornea and thus promote earlier and stronger transgene expression by the host cells. This could enhance the success of the gene therapy whilst reducing the viral load on the target cells.

1.6.5.2 Increasing the Multiplicity of Infection

The term ‘multiplicity of infection’ (MOI) refers to the number of viral particles used to transduce the target cells, a prospective, calculated number, rather than a retrospective number of particles determined to have successfully transduced the target cells. The MOI essentially describes the viral dose delivered to the target cells. An increase in the MOI should increase the number of viral particles transducing the

cells, which should theoretically increase transgene expression. This has been demonstrated by Wang *et al.* in primary human keratocytes using a lentiviral vector expressing a reporter gene.[218] Increasing the viral vector dosage for an increase in transgene expression cannot continue *ad infinitum*; at some point the number of viral particles will become overwhelming for the target cells and will lead to abnormal cellular growth, morphology or even cell death.[219] Finding the optimal vector MOI for the target cells in order to achieve a high level of transgene expression, while maintaining target cell health, is an important consideration for effective gene therapy.

1.6.5.3 Characterising Internal Transgene Promoters

Promoters are regions of DNA upstream of transgenes, which regulate the transcription of the transgenes. The therapeutic transgenes encoded in lentiviral and adenoviral vectors are expressed under the control of internal promoter sequences. The lentivirus transfer plasmid used in this thesis utilises the Simian virus type 40 (SV40) early promoter to induce transgene expression and the adenoviral vector utilises the cytomegalovirus (CMV) promoter to induce transgene expression. The SV40 and CMV promoters are both constitutive promoters, meaning they induce continuous transcription of their respective transgenes. SV40 and CMV promoters have each been used with success to initiate transgene expression in the sheep cornea.[76, 178] CMV promoters have been shown to be strong inducers of initial transgene expression, however can be prone to silencing due to methylation.[220]

Characterising a constitutive transgene promoter to induce early, strong and consistently high amounts of therapeutic transgenic protein from a gene therapy vector is desirable, in order to maximise possible therapeutic effects.

1.6.6 Multigenic Therapeutic Approach

Several studies of corneal allograft survival in animal models have shown significant prolongation of transplants with various single transgene transductions,[70, 87, 107] however in experiments using animals with an amitotic corneal endothelium, none of these studies showed consistent and indefinite survival of corneal grafts across all treated animals.[76, 202] As discussed earlier, the host immune response to a corneal allograft has several stages and involves multiple cellular processes, neovascularisation and corneal endothelial cell apoptosis. Because targeted therapy of any of these responses individually has been shown to prolong allograft survival,[70, 80, 87, 107] we argued that combating multiple pathways of the immune response simultaneously with several therapeutic transgenes might induce prolongation of corneal allograft survival in a greater proportion of the treated animals, and might increase the overall success of the therapy.

When transducing donor corneas *ex vivo*, there are several options for delivery, in order to express several transgenes simultaneously. Multigenic vectors present a desirable option, as several transgenes may be expressed with only one vector application to the target cells, keeping the multiplicity of infection and chances of toxicity low. Decisions must be made about how to express multiple transgenes in one cassette, with options including internal ribosomal entry sites, protein cleavage sequences, or individual internal promoters for each transgene. All these methods have limitations, such as unequal transgene expression depending on the coding order of the transgenes and the target expression locations for each of the transgenes.[221-223] Alternatively, cocktails of vectors may be used, each expressing individual transgenes. For this method, care must be taken not to over-burden the target cells with a toxic viral load.

Adenoviral and adeno-associated viral vectors have been successfully used in combination cocktail transductions of *in vivo* mouse tumour models for enhanced therapeutic effect.[189, 224] Nie *et al.* successfully induced a significantly higher therapeutic effect when using a combination of an adenoviral and an adeno-associated viral vector cocktail than with single vectors alone in a colon cancer mouse model.[224] Gao *et al.* also found a combination of an adenoviral vector and an adeno-associated viral vector significantly increased the inhibition of glioblastoma growth in a mouse model, compared with the adeno-associated vector alone.[189] Moon *et al.* demonstrated that three therapeutic transgenes could be used to transduce human intervertebral disc cells *in vitro* in a triple cocktail combination using individual adenoviral vectors, minimising the individual viral doses, and gave the greatest increase in proteoglycan synthesis compared with single or double transductions.[225]

I chose to use an approach involving several monogenic viral vectors in a cocktail transduction, to best utilise the features of the individual vectors.

1.7 Project Aims

The overarching aim of this project was to use lentiviral-mediated gene therapy to deliver therapeutic transgenes to donor corneas in a preclinical outbred sheep model of orthotopic corneal transplantation, to achieve significantly prolonged corneal allograft survival. This was developed into the individual aims, listed below.

1. To characterise two internal transgene promoters in lentiviral vectors for efficacy of therapeutic transgene expression, and test the optimal vector in a sheep model of orthotopic corneal transplantation for prolongation of allograft survival.

2. To further develop the pre-clinical sheep model of corneal transplantation for gene therapy experimentation, by investigating the enhancement of lentivirus transduction of the sheep corneal endothelium.

3. To investigate the efficacy of multigenic therapy in the targeting of multiple arms of the host immune response simultaneously, in a sheep model of orthotopic corneal transplantation.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were of analytical reagent grade unless otherwise specified. A list of general chemicals and solutions can be found in Appendix A1.1. Water was double glass distilled (dH_2O), except where otherwise stated.

2.1.1 Tissue Culture Reagents

Tissue culture flasks and disposable pipettes were sourced from Greiner Bio-One GmbH (Frickenhausen, Germany). Tissue culture well-plates were sourced from Falcon® (Franklin Lakes, NJ USA) and Corning Inc (Corning, NY USA). Disposable graduated measuring pots and tubes were sourced from Techno Plas (SA), Lab Serv Inc (Paramount, CA USA) and Sarstedt Australia Pty Ltd (SA). Powdered media used to prepare liquid media for tissue culture were obtained from Gibco (Invitrogen Corporation, NJ USA). High-glucose Dulbecco's Modified Essential Medium, Hepes-buffered RPMI 1640 and Medium 199 were prepared according to the recipes in Appendix A2.1.17, A2.1.15 and A2.1.22 and 0.22 µm filter sterilised before use. Miscellaneous tissue culture reagents are listed in Table 2.1.

Table 2.1: Tissue culture reagents

Reagent	Description	Company
Amphotericin B	Antifungal agent 250 µg/mL	Sigma, St Louis, MO USA
Bovine endothelial cell growth factor (bECGF)	Endothelial cell growth supplement	Roche Diagnostics Australia, Castle Hill, NSW
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt	Sigma, St Louis, MO USA
Foetal bovine serum (FBS)	Australian characterised Hyclone-Serum	Thermo Scientific, Scoresby, VIC
Gentamicin solution	Antibiotic used in tissue cultures containing virus	Sigma, St Louis, MO USA
Heparin Sodium	DBL® 1000 IU/mL	Hospira Australia, Mulgrave, VIC
HEPES	Buffer solution	Invitrogen, CA USA
L-Glutamine	ReagentPlus™ ≥99%	Sigma, St Louis, MO USA
Lipofectamine™ 2000 Reagent	Transfection mediator	Invitrogen, CA USA
Non-essential Amino Acids	10 mM solution	Invitrogen, CA USA
Penicillin	Penicillin G (benzylpenicillin) potassium salt	Sigma, St Louis, MO USA
Polybrene	Hexadimethrine bromide ≥ 94%	Sigma, St Louis, MO USA
Sodium Pyruvate	100 mM solution	Invitrogen, CA USA
Streptomycin	Streptomycin sulphate 765 IU/mg	Sigma-Aldrich, St Louis, MO USA
Trypan Blue	Used at 1% w/v in dH_2O	Sigma, St Louis, MO USA
Trypsin	from porcine pancreas 1000-2000 BAEE units/mg	Sigma, St Louis, MO USA
VEGF	Recombinant human	ProSpec-Tany, Rehovot, Israel

2.1.2 Media

All media used in molecular cloning processes and tissue culture are listed in Table 2.2.

Table 2.2: Media for molecular cloning and tissue culture

Reagent	Description	Company
Tissue Culture Media		
Dulbecco's Modified Eagle Medium	High glucose, see Appendix A2.1.17 for recipe	Gibco, Invitrogen Corporation, NJ USA
RPMI Medium 1640	Hepes-buffered, see Appendix A2.1.15 for recipe	Gibco, Invitrogen Corporation, NJ USA
Medium 199	See Appendix A2.1.22 for recipe	Gibco, Invitrogen Corporation, NJ USA
Opti-Mem®	Reduced serum medium	Gibco, Invitrogen Corporation, NJ USA
OptiPRO™ SFM	Serum free medium	Gibco, Invitrogen Corporation, NJ USA
Bacterial Media		
Luria Bertani medium	Microbial broth medium	See recipe, Appendix A2.1.19
SOC	Super Optimal broth with Catabolite repression	See recipe, Appendix A2.1.28

2.1.3 Molecular Reagents

All molecular reagents used in molecular cloning processes are listed in Table 2.3.

Table 2.3: Molecular cloning reagents

Reagent	Description	Company
2-log DNA ladder	Molecular marker used in gel electrophoresis (0.1 – 10 kb)	New England Biolabs, MA USA
Agarose	Used for agarose gel electrophoresis	Promega, WI USA
Bovine serum albumin	Used in restriction endonuclease digests	New England Biolabs, MA USA
6x Gel Loading Buffer	Used in gel electrophoresis	See recipe in Appendix A2.1.12
GelRed™	Used to visualise DNA in gel electrophoresis, 10,000x	Biotium Inc, CA USA
Glycogen	For molecular biology 20 mg/mL	Roche Diagnostics Australia, Castle Hill, NSW
10x Ligase buffer	Reaction buffer used with T4 DNA ligase	New England Biolabs, MA USA
Magnesium chloride	50 mM used in endpoint PCR reactions	Invitrogen, CA USA
10x NEBuffers	Restriction endonuclease reaction buffers	New England Biolabs, MA USA
One-Phor-All buffer	Universal restriction endonuclease reaction buffer	USB Corporation, Ohio USA
10x PCR Reaction buffer	Endpoint PCR buffer	Invitrogen, CA USA
PCR Nucleotide Mix	10 mM dNTPS used in endpoint PCR reactions	Roche Diagnostics Australia, Castle Hill, NSW
Platinum® *Taq* DNA Polymerase	Used in endpoint PCR reactions	Invitrogen, CA USA
***Power* SYBR® Green**	PCR master mix used in real time PCR reactions	Applied Biosystems, Warrington, UK

2.1.4 Enzymes

Restriction endonucleases and other enzymes used in molecular cloning processes are listed in Table 2.4.

Table 2.4: Enzymes

Restriction Enzyme	Sequence Specificity	Company
Ase1	AT/TAAT TAAT/TA	New England Biolabs, MA USA
BamH1	G/GATCC CCTAG/G	New England Biolabs, MA USA
Cla1	AT/CGAT TAGC/TA	New England Biolabs, MA USA
EcoR1	G/AATTC CTTAA/G	New England Biolabs, MA USA
HindIII	A/AGCTT TTGCA/A	New England Biolabs, MA USA
Kpn1	GGTAC/C C/CATGG	New England Biolabs, MA USA
Nde1	CA/TATG GTAT/AC	New England Biolabs, MA USA
Sal1	G/TCGAC CAGCT/G	New England Biolabs, MA USA
Spe1	A/CTAGT TGATC/A	New England Biolabs, MA USA
Enzyme	**Function**	**Company**
Shrimp alkaline phosphatase	Removes 5' phosphates from DNA fragment ends to prevent re-ligation	USB Corporation, Ohio USA
T4 DNA Ligase	Ligation of DNA fragments	New England Biolabs, MA USA

2.1.5 End-point PCR Primers for Cloning and Sequencing

All end-point PCR primers used for amplification of transgenes for cloning and sequencing were constructed by GeneWorks, SA. These are listed in Table 2.5.

Table 2.5: End-point PCR primers

Primer	Sequence 5' – 3'	Use
CPPTfor1	GTAGACATAATAGCAACAGAC	Upstream of the promoter in the lentivirus shuttle vector. Forward sequencing of insert
pHIV1SVrev2	GAGGGTTAATTGCGCGCTTG	Downstream of the transgene in the lentivirus shuttle vector. Reverse sequencing of insert
Internal hu sFlt-1 forward	ACG CCC AGT CAA ATT ACT TA	Forward sequencing of central section of human sFlt-1 gene
Internal hu sFlt-1 reverse	ATC TGG GGT TTC ACA TTG ACA	Reverse sequencing of central section of human sFlt-1 gene
Internal oBcl-xL forward	GAT GAG TTT GAA CTG AGG TAC CGA CG	Forward sequencing of central section of oBcl-xL gene
Internal oBcl-xL reverse	CGC ACA GTG CCC CAC CGA AG	Reverse sequencing of central section of oBcl-xL gene
pHIV1-CMV-oIDO Cloning		
oIDO forward with Spe1	GGA CTA GTA TGG CAG CTG ACA AGT CAT CT	Amplifying oIDO adding Spe1 site
oIDO reverse with EcoR1	CGG AAT TCA ATT TAC TTT CTT GGA TTA CA	Amplifying oIDO adding EcoR1 site
oIDO forward with Kpn1	GAG GGT ACC GCA AGA ATG GCA GCT GAC AAG TCA	Amplifying oIDO adding Kpn1 site, sequencing
oIDO reverse with Sal1	CGC CTC GTC GAC TCA ATT ACT TTC TTG GAT	Amplifying oIDO adding Sal1 site
pHIV1-CMV-oIL-10 Cloning		
CMV forward with BamH1	CGC GGA TCC AAT AGT AAT CAA TTA CGG	Amplifying CMV adding BamH1 site, sequencing
CMV reverse with Cla1	CCA TCG ATG GAT CTG ACG GTT CAC TA	Amplifying CMV adding Cla1 site, sequencing
oIL-10 forward	CGC AGG TAC CTC CGC CAT GCC CAG CAG CTC AGC CGT	Amplifying oIL-10 adding Kpn1 site
oIL-10 reverse	ATC GCA AGC TTA GAT TAG TGG TGG TGG TGG TGG TGC ATC TTC GTT GTC ATG TAG G	Amplifying oIL-10 adding HindIII site
pHIV1-CMV-BclxL Cloning		
oBcl-xL forward with Spe1	GGA CTA GTA TGT CTC AGA GCA ACC GGG AG	Amplifying oBcl-xL adding Spe1 site
oBcl-xL reverse with EcoR1	CGG AAT TCT CAT TTC CGA CTG AAG AGC GA	Amplifying oBcl-xL adding EcoR1 site
oBcl-xL forward with Kozak, Sal1	GCG TCA GTC GAC GAC ACT ATG TCT CAG AGC AAC CGG GAG CTG	Adding a Kozak sequence and Sal1 site
oBcl-xL reverse with Stop, Sal1	CTG CAG GTC GAC TCA TTT CCG ACT GAA GAG CGA GCC	Adding a stop codon and Sal1 site
pHIV1-CMV-hu sFlt1 Cloning		
hu sFlt-1 forward with Cla1	TTA TCG ATA TGG TCA GCT ACT GGG ACA C	Amplifying human sFlt-1 adding Cla1 site
hu sFlt-1 reverse Ase1	TTA TTA ATT TAA TGT TTT ACA TTA CTT T	Amplifying human sFlt-1 adding Ase1 site
pHIV1-CMV-EK5 Cloning		
EK5 forward with Cla1	CTG AGT TAA TTA AGG CGA AGG AG	Amplifying EK5 adding Cla1 site
EK5 reverse with Nde1	CAA TGT ATC CAT ATG TGT CGA GCT AGC	Amplifying EK5 adding Nde1 site

2.1.6 Real-time PCR Primers

All real-time primers used for reference gene and transgene amplification and quantification were constructed by GeneWorks, SA. These are listed in Table 2.6. Primers were designed to overlap exon and intron boundaries to ensure amplification of cDNA only and produce amplification products of approximately 100 base pairs. The optimal G/C content of the primers was 40-60% and they were optimally 20 base pairs in length.

Table 2.6: Real-time PCR primers

Primer	Sense/ Antisense	Sequence 5' – 3'
Ovine IL-10 forward	Sense	CAA GCC TTG TCG GAA ATG AT
Ovine IL-10 reverse	Antisense	TTC ACG TGC TCC TTG ATG TC
Human β-actin forward	Sense	TCC CTG GAG AAG AGC TAC GA
Human β-actin reverse	Antisense	AGC ACT GTG TTG GCG TAC AG
Human 18S forward	Sense	TAG AGG GAC AAG TGG CGT TC
Human 18S reverse	Antisense	TTC CTC GTT CAT GGG GAA TA
Human ARBP forward	Sense	TCA TCA ACG GGT ACA AAC GA
Human ARBP reverse	Antisense	GCA GAT GGA TCA GCC AAG A
Ovine β-actin forward	Sense	CAC CCA GCA CGA TGA AGA T
Ovine β-actin reverse	Antisense	ACA TCT GCT GGA AGG TGG AC
Ovine GAPDH forward	Sense	ATC AAT GGA AAG GCC ATC AC
Ovine GAPDH reverse	Antisense	CAC GTA CTC AGC ACC AGC AT
Ovine IDO forward	Sense	CAA TGA GTG CGT GGA AGC TA
Ovine IDO reverse	Antisense	TTC TTT CCA CCT TGG ATT GC
Human sFlt-1 forward	Sense	AGT TTA AAA GGC ACC CAG CA
Human sFlt-1 reverse	Antisense	CCA TTT CAG GCA AAG ACC AT
Ovine Bcl-xL forward	Sense	GGA ACT CTA CGG GAA CAA CG
Ovine Bcl-xL reverse	Antisense	AGA ACC ACA CCA GCC ACA GT
Human EK5 forward	Sense	CCA TCG TCA ACC TCA AGG AC
Human EK5 reverse	Antisense	CAC AGT AGC TCT CGG TCA GC
Gag forward	Sense	AGC TAG AAC GAT TCG CAG TTG AT
Gag reverse	Antisense	CCA GTA TTT GTC TAC AGC CTT CTG A
Human transferrin forward	Sense	GCC CTG CCT GCC TAC A
Human transferrin reverse	Antisense	CAG GTT GTG CTT CTG ACT CAC T

2.1.7 Ophthalmic Materials, Reagents and Eye Drops

Table 2.7 lists the ophthalmic reagents used for orthotopic penetrating keratoplasty in the sheep.

Table 2.7: Ophthalmic reagents

Reagent	Description	Company
Atropt eye drops	Atropine sulphate 1% (w/v)	Sigma Pharmaceuticals, Rowville, VIC
Balanced Salt Solution (BSS)	Sterile irrigating solution	Alcon Laboratories, Inc, TX USA
Chlorsig™ eye drops	Chloramphenicol 5 mg/mL	Sigma Pharmaceuticals, Rowville, VIC
Chlorsig™ eye ointment	Chloramphenicol 10 mg/g	Sigma Pharmaceuticals, Rowville, VIC
Corneal trephines	11 mm, 12 mm diameter	Tecfen Corporation, CA USA
Cyclogyl™ eye drops	Cyclopentolate 1% (w/v)	Alcon Laboratories (Australia), Frenchs Forest, NSW
Dexamethasone	Dexamethasone sodium phosphate injection USP 4 mg/mL	Mayne Pharma, Mulgrave VIC
Eye Spears	bvi Weck-Cel®	Beaver Visitec International, Inc, Waltham, MA USA
Minims® Phenylephrine hydrochloride eye drops	Phenylephrine hydrochloride 2.5% (w/v)	Chauvin Pharmaceuticals, UK
Mydriacyl® eye drops	Tropicamide 1% (w/v)	Alcon Laboratories (Australia), Frenchs Forest, NSW
Ophthalmic knife	Mani® Straight 15° Recta	Mani, Inc, Tochigi, Japan
Surgical sutures, 9.0	Monofilament nylon	Alcon Laboratories, Inc, TX USA

2.1.8 Commercial Kits

The commercial kits used for methods including RNA extraction, DNA purification, cDNA synthesis, cloning methods, p24 detection and transgene detection are listed in Table 2.8.

Table 2.8: Commercial Kits

Kit	Use	Company
TACS® Annexin V-FITC kit	Apoptosis detection by flow cytometry	R&D Systems, Minneapolis, MN USA
DuoSet IC Human/ Mouse Total Bcl-xL	Bcl-xL detection ELISA kit	R&D Systems, Minneapolis, MN USA
QIAGEN mini, midi and mega, QIAfilter mega, QIAprep Miniprep and EndoFree Plasmid Purification Kits	Plasmid purification	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	DNA gel extraction	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	PCR product clean up	Qiagen, Hilden, Germany
QIAshredder homogenizers	Homogenisation of cell and tissue lysates	Qiagen, Hilden, Germany
Quantikine® Endostatin Immunoassay	Endostatin detection ELISA kit	R&D Systems, Minneapolis, MN USA
Quantikine® Immunoassay VEGF R1	hu sFlt-1 detection ELISA kit	R&D Systems, Minneapolis, MN USA
RNeasy mini-kit	RNA extraction	Qiagen, Hilden, Germany
SuperScript® III First-Strand Synthesis System for RT-PCR	cDNA synthesis	Invitrogen, CA USA
Turbo DNA-*free*™	DNase removal	Ambion, TX USA
Wizard® SV Genomic DNA Purification System	Genomic DNA extraction	Promega, WI USA
XpressBio P24 ELISA Assay	HIV-1 p24 ELISA	Express Biotech International, MD USA

2.1.9 Instruments and Equipment

A list of instruments and equipment used can be seen in Table 2.9.

Table 2.9: Instruments and Equipment

Instrument	Description	Company
Allegra™ X-12R Centrifuge	Refrigerated centrifuge	Beckman Coulter, Inc, Indianapolis, IN USA
Barron Artificial Anterior Chamber	Artificial anterior chamber	Barron Precision Instruments, Grand Blanc, MI USA
BD Accuri® C6 Flow Cytometer	Flow cytometer	Accuri Cytometers, Ann Arbor, MI USA
Bio-Line Platform Rocker	Platform Rocker 4100	Edwards Instrument Company, Narellan, NSW
Bio-Rad Micropulser	Electroporation apparatus	Bio-Rad Laboratories, Hercules, CA USA
Bio-Rad Power Pac 200	Power pack for eletrophoresis	Bio-Rad Laboratories, Hercules, CA USA
BX50 microscope	Upright fluorescence microscope	Olympus America, PA USA
Cryocut 1800	Cryostat microtome	Reichert-Jung Leica, Berlin, Germany
Electrophoresis tanks		Plaztek Scientific, Berwick, VIC
Heraeus® Pico™ Microcentrifuge	Bench-top centrifuge	Kendro Laboratory Products, Langenselbold, Germany
IKA® Vibrax® VXR	Plate shaker	Janke & Kunkel GmBH &Co., Staufen, Germany
Leica Wild M690 Operating microscope	Surgical ophthalmic microscope	Wild Leitz Ltd, Switzerland
Microtome	RM2135	Leica Biosystems, North Ryde, NSW
NanoDrop 8000	Spectrophotometer	Thermo Scientific, Scoresby, Vic
Palm Cycler (CG1-96)	Endpoint PCR amplification	Corbett Research, Sydney, NSW
Peristaltic pump	Series 323	Watson-Marlow, Cornwall, England
Pressure display unit with pressure transducers	Pressure monitor for 750kDa hollow-fibre membrane	GE Healthcare, Westborough, MA USA
Rotor Gene 6000 PCR machine	Real-time rotary analyser	Corbett Research, Sydney, NSW
Slit lamp	Hand-held slit lamp SO-801	Scan Optics, SA
Sorvall® RC-6™ Centrifuge	Refrigerated centrifuge	Kendro, Asheville, NC USA
StepOnePlus™ real-time PCR system	Real-time plate and tube analyser	Applied Biosystems, Warrington, UK
Ultracentrifuge	L8-70M, Class H	Beckman Coulter, Inc, Indianapolis, IN USA
VERSAmax™ tunable microplate reader	Absorbance microplate reader	Molecular Devices, Sunnyvale, CA USA

2.2 Methods

2.2.1 Molecular Cloning Methods

2.2.1.1 Preparation of Electrocompetent Cells

Electrocompetent cells were required for transformation of plasmid vectors into bacterial stocks, for growth and storage. Ten millilitres of low salt LB medium (Appendix A2.1.21) was inoculated with a single *E. coli* DH5α (Table 2.10) colony and incubated at 37^{o}C with shaking overnight. This overnight starter culture was used to inoculate 500 mL of low salt LB medium and this was incubated at 37^{o}C with shaking until the optical density, measured at 600 nm, was greater than 0.6. The suspension was chilled on ice for fifteen minutes in pre-chilled centrifuge bottles and then centrifuged at 6000 g at 2^{o}C for twenty minutes, with six minutes deceleration. The supernatant was carefully poured off and cells resuspended in 10 mL of ice cold, distilled and autoclaved water. The cells were washed with 500 mL of ice cold dH_2O and then centrifuged as before. The supernatant was carefully decanted until the pellet was seen to shift, then the cells were centrifuged again for ten minutes to tighten the cell pellet, before the removal of the remaining supernatant with a Pasteur pipette and suction. This washing step was repeated one more time. After removal of supernatant, the cell pellet was resuspended in 10 mL of chilled 10% (v/v) glycerol, transferred to a 50 mL tube, and a further 40 mL of 10% (v/v) glycerol was added. Cells were centrifuge at 2500 g at 2^{o}C for ten minutes. The supernatant was removed and cell pellet resuspended in 1 mL of 10% (v/v) glycerol. The electrocompetent cells were placed in 60 μL aliquots into pre-chilled, labelled tubes, snap frozen in liquid nitrogen and stored at -80^{o}C.

Table 2.10: *Escherichia coli* strain

E. coli Strain	Genotype	Description
DH5α	SupE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	A recombination-deficient strain used for growth and amplification of recombinant plasmids

2.2.1.2 Lentiviral Transfer Plasmid

The lentiviral transfer plasmid used was derived from the human immunodeficiency virus type 1 (HIV-1), with several modifications to change the tropism and create a self-inactivating, replication-deficient vector. This plasmid is denoted pAF2m2Δ and was obtained from our collaborator Associate Professor Donald Anson at the Department of Genetic Medicine, Women's and Children's Hospital, Adelaide SA.[179] The primary reporter vector contains a 5' long terminal repeat (LTR) and contiguous sequences up until a Gag initiation codon, a polypurine tract, a 3' LTR with U3 deletions, and a reporter gene, enhanced yellow fluorescence protein (eYFP), under the control of the Simian virus type 40 early promoter (SV40), see Figure 2.1.[179]

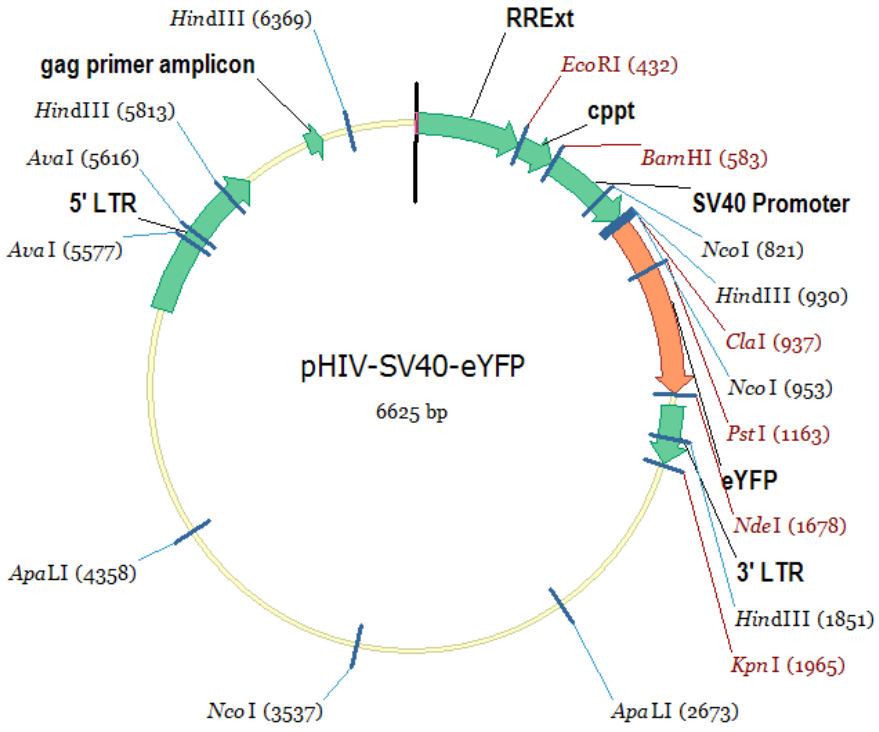


Figure 2.1: Map of the lentivirus transfer plasmid.

In order to produce viral particles, a multi-plasmid system was used, with the transfer plasmid containing the transgene of interest, and four helper plasmids to provide necessary viral proteins in trans; pcDNA3Tat101ml (encoding HIV-1 Tat), pHCMVRevmlwhvpre (encoding HIV-1 Rev), pHCMVgagpolmllstwhv (encoding HIV-1 GagPol) and pHCMV-G (encoding vesicular stomatitis virus glycoprotein G), see Table 2.12 in Section 2.2.4.1.[226, 227]

2.2.1.3 Plasmid Purification

Plasmid DNA was purified from DH5α *E.coli* cells using commercially available plasmid purification kits (Mini-, Midi- kits) according to the manufacturer's instructions. Endotoxin-low preparations were required for virus production and for these, the Qiagen EndoFree Plasmid Purification Mega kit was used. Plasmid DNA was quantified using spectrophotometry at 260 nm on the NanoDrop 8000.

2.2.1.4 Insert Amplification

Inserts, either promoter or transgene sequences, were prepared for ligation into plasmids by polymerase chain reaction (PCR) amplification from purified plasmids, with the PCR addition of vector-specific restriction endonuclease sites at both ends of the insert sequence. The PCR products were separated by agarose gel electrophoresis (Section 2.2.1.6) and bands of correct size were excised. The commercially available QIAquick Gel Extraction kit from Qiagen was used to purify the DNA from the excised band.

2.2.1.5 Restriction Enzyme Digests

DNA constructs were digested with restriction endonucleases from New England Biolabs (NEB, MA USA) (Table 2.4). These enzymes were used in a range of molecular cloning techniques including the creation of DNA cohesive ends for ligation reactions, and the cutting of plasmids to confirm vector identities. Restriction enzymes were stored in 50% (v/v) glycerol and in the digestion reactions, care was taken to ensure less than 5% (v/v) glycerol was present. Appropriate reaction buffers were obtained from NEB and USB Corporation (Ohio, USA), and bovine serum albumin (BSA) from NEB (Table 2.3). Typically, between 0.6 and 5 µg of DNA was added to the restriction digests, and the enzymes were used either individually for single digests or in pairs for double digests at 10 or 20 units per microgram of DNA.

Depending on individual enzyme requirements, reactions were performed at 37^{o}C for 1-2 hours before placing on ice. A sample from the reactions was visualised by gel electrophoresis (Section 2.2.1.6) to confirm successful digestion of DNA.

2.2.1.6 Agarose Gel Electrophoresis

1% (w/v) agarose in 0.5x Tris-borate gels were used to confirm the size of DNA fragments, PCR products, restriction digests, ligations and plasmids. GelRed™ was used at a 1:10,000 dilution to visualise DNA. Gels were run at 100 volts for 60 minutes in tanks 21 cm in length. 6x Gel-loading buffer (Appendix A2.1.12) was added to each DNA sample in a 1:6 dilution for visibility during gel loading and to determine the progress of the electrophoresis.

2.2.1.7 Dephosphorylation of Linearised Plasmids

Shrimp Alkaline Phosphatase (SAP) enzyme removes the phosphate groups from the 5' ends of DNA and RNA fragments. This prevents re-ligation of a linearised plasmid without an insert. SAP has activity in restriction enzyme reaction buffers, and was used on linearised plasmid DNA at one unit of SAP per pmol of DNA ends, immediately after restriction digest. 5 µg of DNA was used in cloning restriction digests of 6 kb plasmids, which gave 2.5 pmol of DNA ends. This required the addition of 2.5 U of SAP and the samples were incubated at 37^{o}C for thirty to sixty minutes. The reactions were terminated by heat inactivation at 65^{o}C for fifteen minutes.

2.2.1.8 DNA Ligation

The DNA of the genes of interest was ligated individually into the linearised HIV-1 lentiviral transfer plasmid. After dephosphorylation, the digested plasmid was purified using a PCR purification kit and the gene to be inserted was purified from an agarose gel using a gel extraction kit. The ligation reactions were carried out with the plasmid and insert at a molar ratio of 1:1 and also at 1:3 (plasmid: insert). The following reactions and control reactions were set up:

Single cut plasmid without ligase	(control for uncut DNA)
Single cut plasmid with ligase	(control for ligase efficacy)
Double cut plasmid without ligase	(control for uncut DNA)
Double cut plasmid with ligase	(control for SAP efficacy)
Double cut plasmid with ligase and insert (1:1)	(target colonies)
Double cut plasmid with ligase and insert (1:3)	(target colonies)

Reactions consisted of 10x ligase buffer, 400ng of digested plasmid, DNA insert, in appropriate quantity for the molar ratios, and water. 1 µL of 2 x 10^{6} U/mL T4 DNA ligase was added last for a total reaction volume of 40 µL. Reactions were mixed and spun briefly to collect the contents and incubated at 4^{o}C overnight. 5 µL of each

ligation reaction was subjected to 1% (w/v) agarose gel electrophoresis for an indication of ligation success.

Ligated DNA was purified by ethanol precipitation. Reactions were first diluted to 200 µL with water for injection and 20 µg of glycogen was added as a DNA carrier. 3 M NaOAc pH 5.2 (Appendix A2.1.30) was added at one-tenth of the total reaction volume (20 µL). 500 µL of ice-cold 100% (v/v) ethanol was added and samples were incubated at -20^{o}C for thirty minutes. The samples were centrifuged at 12,000 g for fifteen minutes at 4^{o}C and the supernatants carefully removed. The DNA pellets were washed twice with 70% (v/v) ethanol using the same centrifugation conditions as previously. The DNA was air dried and resuspended in 10 µL of water for injection. The purified DNA was checked again on a 1% (w/v) gel for recovery and the appearance of correct ligation before electroporation into electrocompetent bacterial cells.

2.2.1.9 Electroporation of Plasmid DNA into Electrocompetent Cells

In order to utilise bacteria for clonal expansion of the DNA constructs, electroporation was used to facilitate the uptake of DNA by the bacteria. 5 pg – 500 ng (1 µL) of plasmid DNA was applied to 60 µL of electrocompetent cells (*E. coli* DH5α) on ice for 10 minutes with intermittent flick mixing. 55 µL of this cell-plasmid mix was placed in a chilled 0.1 cm electroporation cuvette (BioRad, CA USA) and the cuvette was placed in the sample chamber of a micropulser (BioRad, CA USA). A pulse was administered at 1.8kV, 200Ω and the time constant of the pulse was recorded in milliseconds. Within one minute of the pulse, 1 mL of warm SOC medium (Appendix A2.1.28) was added to the cuvette and the whole suspension was removed to a clean tube and incubated at 37^{o}C for one hour. The

suspension was centrifuged for one minute at 3000 g and 800 µL of supernatant removed. Cells were resuspended in the remaining liquid and spread onto a pre-warmed LB plate with the appropriate selective antibiotic in the medium for the lentiviral plasmid vectors (34 µg/mL chloramphenicol, Appendix A2.1.6). The plate was incubated at 37^{o}C overnight. Colonies were tested to ensure presence of the correct insert by PCR or restriction digest analysis.

2.2.1.10 Analysis of Potential Colonies by Endpoint PCR

Potential colonies containing constructed lentiviral plasmid DNA were tested for the presence of the correct plasmid by performing endpoint PCR on the DNA. Colonies were plucked from selective medium agar plates and dipped into 10 µL 10 mM Tris-chloride pH 8.5 (Appendix A2.1.35), or alternatively grown overnight in 3 mL LB broth cultures and 10 µL of the culture added to 10 µL Tris-chloride. These colony suspensions were then heated at 95^{o}C for ten minutes to release the plasmid DNA before being chilled on ice. A PCR master mix was prepared for final reactions of 25 µL:

	Stock conc.	**Final conc.**	**Vol (µL)**
10x Buffer	10x	1x	2.5
dNTPs	10 mM	100 µM	0.25
$MgCl_2$	50 mM	1.5 mM	0.75
Forward primer	20 µM	0.4 µM	0.5
Reverse primer	20 µM	0.4 µM	0.5
Platinum taq-polymerase	5 U/µL	0.5 U	0.1
Milli-Q H_20			18.4
		total	23 µL

2 µL of the colony suspension was added as the template DNA to 23 µL of the PCR master mix. Two reactions were set up containing Milli-Q water instead of DNA template (negative controls) and two positive control reactions were set up using known plasmid DNA containing the correct insert. PCR reactions were performed on a Palm Cycler (CG1-96, Corbett Research, Sydney, NSW) using the following

cycling conditions: 94^{o}C for five minutes, thirty five cycles of 94^{o}C for thirty seconds, 59^{o}C for thirty seconds and 72^{o}C for one minute, followed by 72^{o}C for five minutes. Primers used in end-point PCR reactions are listed in Table 2.5. The annealing temperature sometimes varied from 59^{o}C depending on the melting temperatures of the primers being used. The resulting PCR products were run on a 1% (w/v) agarose gel to visualise amplified bands, giving an indication of the colonies likely to contain the plasmid of interest.

2.2.1.11 DNA Sequencing

All DNA constructs were sequenced using primers to create multiple overlapping amplicons. The sequencing reactions were performed on a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA USA), purified using CleanSeq Magnetic Beads and a SPRI plate according to the manufacturer's protocol (Agencourt, Beckman Coulter Inc, Indianapolis, IN USA), and the extension products were then resolved using the 3130XL Genetic Analyser (Applied Biosystems, Warrington, UK). All sequencing reactions were performed by SouthPath and Flinders Sequencing Facility in the Flinders Medical Centre, Bedford Park SA.

2.2.2 Tissue Culture Methods

2.2.2.1 Propagation of Cells

The cell lines used can be seen in Table 2.11. A549, HEK-293T and HEK-293A cells were cultured in Dulbecco's modified essential medium (DMEM, Appendix A2.1.17) with 10% (v/v) foetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine. CHO-K1 cells were cultured in Hepes-buffered RPMI 1640 medium with the same additives as just described. Cells were maintained in 75 cm^2 tissue culture flasks. When confluent, the adherent cells were passaged by washing with 4 – 5 mL of 1x phosphate buffered saline (PBS, Appendix A2.1.25) and detached from the plastic flask surface using 2 mL of 0.05% (w/v) trypsin-EDTA (Appendix A2.1.36) for 5 minutes at 37^oC. 10 mL of fresh medium was added to the detached cells to terminate the trypsin activity and a single cell suspension was created. 1.2, 1.5 or 2 mL of the suspension was taken into a fresh flask to create a 1:10, 1:6 or 1:8 split, with fresh medium to a total volume of 15 mL. The cells were incubated at 37^oC with 5% CO_2 in air.

Human umbilical vascular endothelial cells (HUVECs) were primary cells used up to a maximum passage of four. The cells were cultured in Medium 199 (see Appendix A2.1.22 for recipe) with 20% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 100 µM non-essential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine. Heparin sodium salt (1000 IU/mL) and bovine endothelial cell growth factor (10 mg/mL) were added separately to culture medium immediately prior to use, at 2.1 µL per mL of medium and 1.5 µL per mL of medium, respectively. The cells were grown in bovine gelatin-coated 75 cm^2 or 25 cm^2 tissue culture flasks or 96-well plates to aid cell attachment and growth, see Appendix A2.1.2 for bovine gelatin recipe. HUVECs were passaged by washing with 1x PBS and detached from

plastic surfaces using 0.05% (w/v) trypsin-EDTA (Appendix A2.1.36) for 5 minutes at 37^{o}C. Medium was added to terminate the trypsin reaction and the cells were centrifuged at 233 g for five minutes and resuspended in fresh medium.

Table 2.11: Cell lines

Cell Line	Description	Use	Source
A549	Adenocarcinomic human alveolar basal epithelial cell line	Transfection and transduction testing, viral titring	American Type Culture Collection (ATCC), Manassas, VA USA, CCL 185
CHO-K1	Chinese hamster ovary cell line	Transfection and transduction testing	ATCC, CCL-61
HEK-293A	Human embryonic kidney cells with E1- region of adenovirus 5	Transfection and transduction testing	Qbiogene Inc, Carlsbad, CA USA
HEK-293T	Human embryonic kidney cell line constitutively expressing the SV40 large T cell antigen	Transduction testing	ATCC, CRL 11269
HEK-293T-DA	Human embryonic kidney cell line constitutively expressing the SV40 large T cell antigen	Virus production, transduction testing	Obtained from Assoc. Prof. Donald Anson, Dept. Genetic Medicine, Women's and Children's Hospital, Adelaide, SA
HUVEC	Human umbilical vascular endothelial primary cells	Gene activity testing; proliferation assays	Kind donation from Dr Claudine Bonder, Vascular Biology & Cell Trafficking Laboratory, Human Immunology, Centre for Cancer Biology, Adelaide, SA

2.2.2.2 Transfection of Cells with Plasmid DNA

All viral plasmid constructs were tested for correct transgene expression prior to virus production. Adherent human embryonic kidney 293A cell line cells (HEK-293A, Qbiogene Inc, Carlsbad, CA USA), were seeded into wells of a 6-well plate at 0.5 x 10^{6} cells/ mL. When the cells were approximately 80% confluent, they were washed with 1x sterile phosphate-buffered saline (PBS) and 960 µL of Opti-MEM® medium (Gibco, Invitrogen Corporation, NJ USA) was applied to the cells before

further incubation at 37°C for less than ten minutes. Plasmid DNA, 1.6 μg, was added to 200 μL of pre-warmed Opti-MEM medium and 8 μL of 1 mg/mL Lipofectamine® 2000 (Invitrogen, CA USA) was added to the Opti-MEM/ DNA mix. This was mixed by inversion and incubated at room temperature for twenty minutes. The Opti-MEM/ DNA/ Lipofectamine mix was added to the cells and incubated for six hours or overnight. The cell culture supernatant was then removed and replaced with 3 mL high glucose DMEM (Appendix A2.1.17) with 5% (v/v) FBS, penicillin, streptomycin, and incubated at 37°C with 5% CO_2 for five days. The supernatant was harvested on the fifth day after transfection, filter sterilised (0.22 μm filter) and stored at 4°C or -20°C long term. Supernatant samples were tested for the presence of transgenes at the protein level, Sections 2.2.13.10 - 14.

Transient Transfection to Produce Conditioned Medium Containing EK5 Protein

In order to obtain conditioned medium containing transgenic endostatin::kringle5 protein for use in functional activity assays, a transient transfection of HEK-293T cells was performed using the plasmid expressing the EK5 transgene.

HEK-293T cells were seeded in two tissue culture round plates (diameter 8.5cm) at 5 x 10^5 cells/ mL in culture medium as described in Section 2.2.2.1. The medium was removed from the plates and serum-free Opti-MEM® medium (Gibco, Invitrogen Corporation, NJ USA) was placed on the cells for 10 minutes at 37°C. A DNA mix was created with 1.5 mL of Opti-MEM and 15 μL (24μg) of the endotoxin-low preparation of the lentivirus shuttle plasmid containing the EK5 transgene under the control of a CMV promoter (pHIV1-CMV-EK5). A second mix was created with 1.5 mL of Opti-MEM and 60 μL of Lipofectamine™ 2000 Reagent (Invitrogen, CA USA). Both mixes were incubated for 5 minutes at room temperature before being

added together, mixed by inversion and incubated for a further 20 minutes at room temperature. 3 mL of this DNA/ Lipofectamine mix was added to one tissue culture plate containing the 293T cells and 3 mL of Opti-MEM was added to the other plate of 293T cells as a transfection control. Both plates were swirled gently and incubated at 37^{o}C overnight. The medium was removed from both plates and 14 mL of high glucose, endotoxin-low DMEM containing 10% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine was replaced on both plates. The cells were incubated at 37^{o}C for a further five days, after which the supernatants were collected and sterile filtered.

2.2.2.3 Cell Proliferation MTS Assay

The medium of HUVECs plated in 96-well plates was replenished and 20 µL of Cell Titer 96$^{®}$AQ$_{ueous}$ One Solution Reagent (Promega, Madison, WI USA) was added to each well. The plate was kept in the dark at 37^{o}C for 4 hours before the addition of 25 µL of 10% (w/v) sodium dodecyl sulphate (SDS) in dH_2O to terminate the reaction. The plate was gently mixed and the absorption from each well was assayed in a microplate reader at the 490 nm wavelength. The absorbance at this wavelength is directly proportional to the number of living cells in the culture.

2.2.3 Adenovirus Preparation

The adenoviral vector used to deliver transgenes ovine interleukin-10 (oIL-10) and Green Fluorescent Protein (GFP) to corneal cells was a second generation, E1-, E3-deleted, replication defective serotype 5 adenovirus. This vector was previously produced and purified in our laboratory by Dr Douglas Parker (Department of Ophthalmology, Flinders University, Bedford Park, SA) using the AdEasy system and AdTrack-CMV backbone plasmid.[228] The vector contained a cytomegalovirus

(CMV) constitutive viral promoter upstream of the ovine inteleukin-10 (oIL-10) transgene. The virus was purified from lysates of HEK-293A cells over caesium chloride gradients and titred to determine plaque-forming units per millilitre using the tissue culture infectious dose method ($TCID_{50}$) and fluorescence microscopy.[76, 202, 229, 230]

2.2.3.1 Titration of the Adenovirus Preparation

In order to determine the number of plaque forming units per millilitre in the adenovirus preparation, the 50% tissue culture infectious dose ($TCID_{50}$) method was used. The adenovirus construct has a green fluorescent protein (GFP) reporter gene upon which the titration process was centred (AdV-CMV-IL10-GFP). HEK-293A cells were cultured in DMEM with 2% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine. Cells were seeded at 1 x 10^5 cells/ mL into 96-well plates. An aliquot of the adenovirus was serially diluted with culture medium to gain dilutions from 10^{-2} through to 10^{-14}. The virus dilutions were applied to the cells with an n-value of 10 wells per dilution. The cells were incubated for ten days at 37^{o}C with 5% CO_2. Wells were scored on the tenth day for GFP expression; a patch of fluorescing cells was a positive score and no visible fluorescence was a negative score. The number of positive wells for a dilution was divided by the total number of wells for that dilution. The sum of all the dilution fractions was totalled and added to 1. This gave the 'S' value. The equation to estimate the viral titre was based on Poisson distribution:

$$\textbf{Titre} = 10^{S+0.8}\ \textbf{pfu/mL}$$

2.2.4 Lentivirus Production

2.2.4.1 Medium-Scale Lentivirus Production

For the production of approximately 400 µL of concentrated lentivirus stock, medium-scale preparations were performed. All solutions and glassware used were endotoxin-low. Packaging cells, human embryonic kidney 293T (HEK-293T-DA), were cultured in high glucose endotoxin-low DMEM containing 10% (v/v) FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin (Table 2.1). Cells were seeded into seven or eight 500 cm^2 square tissue culture plates (Corning, NY, USA) at 0.374 x 10^6 cells per millilitre, with 105 mL per plate, and incubated at 37^oC and 5% CO_2.

The confluent 293T cells were transfected 24 hours after seeding with four helper plasmids separately containing individual genes encoding the virion components tat, rev, gag-pol, and the coat protein VSV-G (Section 2.2.1.2), along with the transfer plasmid containing the therapeutic or reporter transgene of interest. The transfection was carried out using calcium chloride precipitation. The plasmid DNAs were combined according to the amounts stated in Table 2.12, with the transfer plasmid added last. This was made up to a final concentration of 250 mM $CaCl_2$ in a 50 mL Falcon tube with the addition of 2.5M $CaCl_2$ and water for injection. For one plate, 3.2 mL of this DNA mix was added over a 5 second period, to an equal volume of 2x Hepes-buffered saline, with constant vortexing of the saline. The mixture was vortexed for a further 25 seconds before resting for 90 seconds. The mix was then poured carefully down the side of one culture plate containing the packaging cells. The plate was rested for twenty to thirty seconds before swirling gently to distribute. This was repeated for all plates in the preparation. The plates were incubated at 37^oC with 5% CO_2 for eight hours. The transfection was terminated with the complete removal of the medium from the plates and careful replacement with 150 mL per

plate of serum-free medium, OptiPro™ SFM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine. The plates were incubated at 37°C with 5% CO_2 for forty hours.

Table 2.12: Transfer and helper plasmid DNA amounts required, per culture plate, for transfection of 293T packaging cells plated in 500 cm^2 tissue culture plates with 3.94 x 10^7 cells. Information courtesy of Associate Professor Donald Anson, Department of Genetic Medicine, Women's and Children's Hospital, SA.

Plasmid	Description	DNA Required Per Culture Plate (µg)
pHIV-CMV-gene of interest	Transfer plasmid	158
pcDNA3Tat101ml	Helper plasmid with *tat* gene	3.16
pHCMVRevmlwhvpre	Helper plasmid with *rev* gene	3.16
pHCMVgagpolmllstwhv	Helper plasmid with *gag-pol* gene	15.8
pHCMV-G	Helper plasmid encoding VSV-G glycoprotein	7.9

The virus-containing supernatants from the tissue culture plates were pooled together and weighed. 2.5% (w/v) BSA in PBS was added to make a final concentration of 0.1% (w/v) BSA and the supernatant was re-weighed and a small sample taken. The viral supernatant was filtered using an in-line 0.45 µm Polydisc filter (Whatman, GE Healthcare, Westborough, MA USA) into a sterile endotoxin-low collection bottle (Figure 2.2). The container which held the supernatant was rinsed with 50 mL 0.1% (w/v) BSA in PBS, which was also filtered into the holding bottle. A small sample was taken after this filtration.

Figure 2.2: The 0.45 µm filtration process of the viral supernatant using a peristaltic pump. Image courtesy of Associate Professor Donald Anson, Department of Genetic Medicine, Women's and Children's Hospital, SA.

The virus supernatant was then concentrated using a 750 kDa hollow fibre cartridge membrane (GE Healthcare, Westborough, MA USA) (Figure 2.3), keeping the membrane gauge pressure between 1.0 – 1.2 bar and the waste flow rate at approximately 8 – 10 mL per minute. When approximately 200 mL of supernatant remained in the reservoir, 500 mL of 0.1% (w/v) BSA in PBS was added in a diafiltration process. The supernatant was concentrated to a volume of approximately 55 mL.

The concentrate was then filtered through a series of Minisart filters (Sartorius Stedim Biotech, Goettingen, Germany): 5 µm, 1.2 µm and 0.8 µm. This concentrated virus was then carefully placed into two Quick-Seal Polyallomer Konical™ centrifuge tubes (Beckman Instruments Inc, CA USA) and ultra-centrifuged at 20,000 g for ninety minutes at 4^{o}C. The supernatant was removed and the pellets gently resuspended in a total volume of approximately 400 µL of ophthalmic balanced salt solution (BSS). This final concentrated virus was filtered through a

final 0.45 µm Millex®-HV low binding Durapore filter (Millipore, Cork, Ireland) and the concentrated virus stored in aliquots at -80^{o}C.

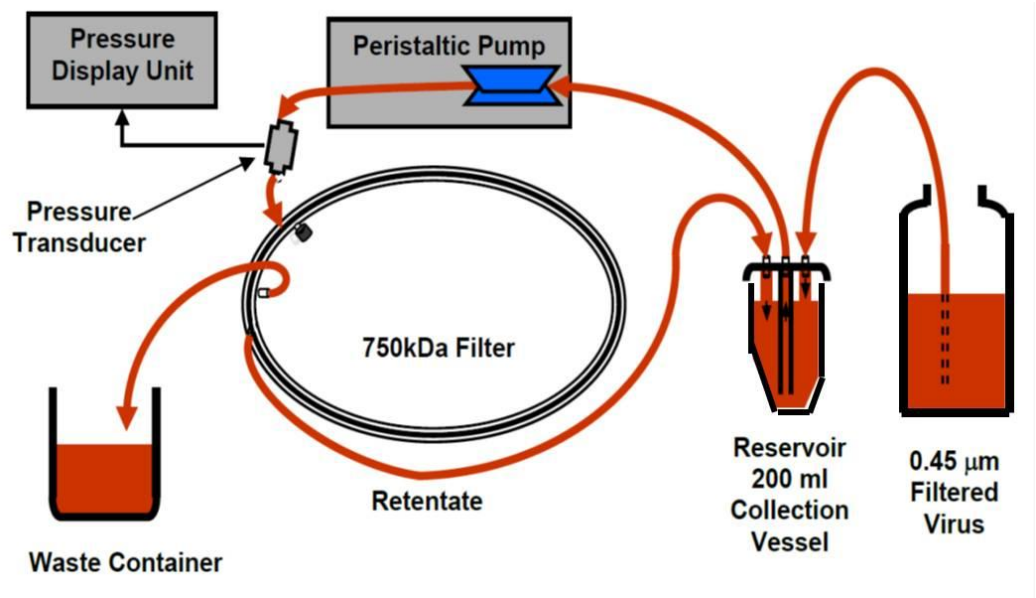


Figure 2.3: Diagram of the 750 kDa diafiltration and concentration process set up during lentivirus production.

2.2.4.2 Titration of Lentivirus Preparations

In order to determine the number of infective lentiviral particles per millilitre or transducing units per millilitre in a lentivirus preparation, a titration of transduced cells was performed and assayed by either real time PCR or flow cytometry.

Adenocarcinomic human alveolar basal epithelial cells (A549 cells) were seeded into a 24-well plate at 0.25x10^{6} cells per well in 0.5 mL 10% (v/v) FBS high glucose DMEM with penicillin and streptomycin at 100 IU/mL and 100 µg/mL respectively. The cells were incubated at 37^{o}C for approximately three hours to allow adherence to the well surface. The medium was then changed to fresh DMEM as just described, but also containing 4 µg/mL polybrene and 50 µg/mL gentamicin (Table 2.1). Concentrated lentivirus was diluted in transduction medium to 1:100 and 1 µL, 0.5 µL and 0.1 µL amounts of this dilution were each added to cells in triplicate wells,

with untransduced cells used as controls. The cells were transduced for 24 hours before a medium change to 10% (v/v) FBS in high glucose DMEM with penicillin, streptomycin and gentamicin. The cells were cultured for four weeks, with passaging every two to three days.

Titre Cell Collection

After the four week culture, cells were washed once with 1x PBS and trypsinised to resuspend. The cells were then placed in 1x PBS with 1% (v/v) FBS and centrifuged at 1500 g for five minutes. The supernatant was removed and the cells washed in 1x PBS and centrifuged again with the same conditions. The supernatant was removed and the cell pellets were stored at -80^{o}C.

Genomic DNA purification

Genomic DNA (gDNA) was purified from the frozen titre cell pellets using the Wizard® SV Genomic DNA Purification System. The manufacturer's instructions for gDNA extraction from tissue culture cells using a microcentrifuge were followed. Briefly, Wizard® SV Lysis Buffer was added to the thawed cell pellets and these mixed lysates were transferred to spin columns and centrifuged at 13,000 g for three minutes. The columns underwent four washes with Wizard® SV Wash Solution before drying and the gDNA was eluted with warm nuclease-free water. The gDNA was stored overnight at -20^{o}C.

Titration of Lentiviral Preparations by Quantifying Proviral Integration using Real-Time PCR

Real time PCR was conducted on the gDNA purified from the titre cells, to determine the viral titre of lentiviral preparations in the absence of a reporter gene. The beginning of the 'gag' sequence, which encodes the HIV Gag-polyprotein, was amplified. Primers were designed to amplify the gag sequence and the human

transferrin sequence, a reference gene with one copy per haploid genome, therefore two copies per cell. *Power* SYBR® Green (Table 2.3) was used to create master mixes. A sample of gDNA purified from A549 cells containing one copy number or one viral genome per cell was used as a standard sample in each real time run. Template-free controls containing UltraPure water instead of DNA template were included in each run as a contamination control.

20 µL reaction mixtures were created with 2 µL each of 5mM forward and reverse primers (Table 2.6), 10 µL of *Power* SYBR® Green, 1 µL of UltraPure water and 5 µL of gDNA sample, standard sample or UltraPure water, for the template-free controls. The real time PCR assays were carried out on a real-time rotary analyser, Rotor Gene 6000 (Corbett Research, Sydney, NSW) or on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK), using the provided software.

The titres of concentrated lentivirus preparations were determined using the $2^{-\Delta\Delta Ct}$ quantification method to quantify the amount of viral vector genomic DNA.[231] The threshold cycle (Ct) for each genomic DNA sample amplified for the Gag sequence was taken when in the linear, exponential phase of the amplification curve in triplicate. The Ct values for the reference gene, human transferrin, were subtracted from the Gag Ct values to obtain a change in Ct value (ΔCt). The change in Ct value for the standard sample was then subtracted from the ΔCt values of the samples to obtain a ΔΔCt value for each sample. To obtain a copy number for the gag sequence, the equation was used:

$$\boldsymbol{No.\,of\ viral\ genomes\ per\ cell} = \frac{1}{2^{\Delta\Delta Ct}}$$

The actual titre of the concentrated virus was determined by multiplying the number of cells seeded in the well by the copy number and dividing by the volume of virus used to transduce the cells. Thus, a final titre in transducing units per millilitre was obtained.

$$Viral\ titre = \frac{no.of\ cells\ seeded * copy\ number}{viral\ volume}$$

Titration of Lentiviral Preparations by Flow Cytometry

Cells transduced with lentiviral constructs containing a fluorescence reporter gene, such as enhanced Yellow Fluorescent Protein (eYFP), can be used to give a quantification of the viral titre, without the need for viral titring by real-time PCR.

A549 cells were seeded into 24-well plates at 0.25 x 10^6 cells per well in 0.5 mL of DMEM with 10% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine and left to adhere for at least three hours. The medium was then changed to the DMEM with 10% (v/v) FBS mentioned above, also containing 4 µg/mL polybrene and 50 µg/mL gentamicin. Concentrated lentivirus containing the eYFP transgene was added to the wells in doses of 0.1 µL, 0.5 µL and 1 µL in triplicate, with non-transduced cell controls. The transduction was ended with a medium change twenty four hours later, to the above medium without polybrene.

The cells were passaged the following day and harvested forty eight hours later, by trypsinisation. Cells were placed in 1% FBS in PBS (v/v) and centrifuged for five minutes at 520 g and resuspended in FACSfix, a fixative solution used with flow cytometry, see Appendix A2.1.11 for recipe. Untransduced control cells were also analysed to provide a background control. The BD Accuri® C6 Flow Cytometer (Accuri Cytometers, Ann Arbor, MI USA) and accompanying software were used to

determine the percentage of eYFP positive cells in the lentivirus-transduced cell suspension samples, corrected against the average percentage of positives in the untransduced control cells. The titre of the lentivirus preparation was determined by the formula:

$$\frac{\text{[no. of seeded cells x ((\% gated eYFP cells – \% gated control cells)/100)x1000]}}{\text{virus added to the cells (µL)}}$$

This gives the number of transducing units per millilitre. Table 2.13 shows the titres and endotoxin levels of the lentivirus and adenovirus preparations described in this thesis. See Section 2.2.4.3 for the endotoxin assay methods.

Table 2.13: Titres of lentivirus and adenovirus preparations used for *in vitro* and *in vivo* experiments, and the level or range of endotoxin contamination detected in the preparation using the LAL test, if tested.

Preparation	Transgene	Titre (TU/mL)	Titre Method	Endotoxin Level (EU/mL)
LV-SV40-eYFP (1)	enhanced yellow fluorescent protein	4×10^9	Flow cytometry and real time PCR	240 - 360
LV-SV40-eYFP (2)	enhanced yellow fluorescent protein	2.4×10^9	Flow cytometry and real time PCR	> 720
LV-SV40-eYFP (3)	enhanced yellow fluorescent protein	3.2×10^9	Flow cytometry and real time PCR	600 - 720
LV-CMV-oIL10	ovine interleukin-10	6.17×10^9	Real time PCR	420 - 480
LV-CMV-oIDO (1)	ovine indoleamine 2,3 dioxygenase	1.9×10^9	Real time PCR	not tested
LV-CMV-oIDO (2)	ovine indoleamine 2,3 dioxygenase	1.1×10^9	Real time PCR	not tested
LV-CMV-BclxL	ovine Bcl-xL	4×10^9	Real time PCR	120 - 180
LV-CMV-EK5	human endostatin::kringle5	2×10^{10}	Real time PCR	240 - 300
LV-CMV-sFlt1	human soluble fms-like tyrosine kinase 1	2.3×10^9	Real time PCR	not tested
AdV-CMV-oIL10	ovine interleukin-10; green fluorescent protein	7.9×10^9 pfu/mL	$TCID_{50}$	1.8 - 3
AdV-CMV-GFP	green fluorescent protein	2×10^{10} pfu/mL	$TCID_{50}$	6 - 30

Number in brackets refers to batch number when multiple preparations were created

2.2.4.3 Endotoxin Assay

All gene therapy virus batches were prepared using endotoxin-low techniques and reagents to reduce contamination from endotoxin. Gram-negative bacterial endotoxin contamination of viral preparations was determined using the Limulus Amebocyte Lysate (LAL) test. All reagents for the test were sourced from Associates of Cape Cod, Inc (East Falmouth, MA USA), and the test carried out according to the manufacturer's instructions. Briefly, the LAL test is based on the ability of endotoxin to activate a pro-enzyme in the LAL, which hydrolyses bonds within coagulin, resulting in self-association of the coagulin and the formation of a clot.[232]

The labelled sensitivity of the test of 0.06 endotoxin units (EU) per mL was confirmed with a LAL test on a series of standard endotoxin concentrations that bracket the labelled sensitivity: lyophilised endotoxin standard was reconstituted with LAL reagent water, according to the specific certificate of analysis for the combined endotoxin standard and LAL reagent lot numbers, and dilutions were created in depyrogenated borosilicate dilution tubes (0.12, 0.06, 0.03 and 0.016 EU/mL). LAL reagent water was used as a negative control.

The virus preparations used in *in vivo* applications were diluted to varying degrees using LAL reagent water, in depyrogenated borosilicate dilution tubes, in order to characterise the amount of endotoxin present in each preparation. Two positive controls were used: the endotoxin standard at 0.12 EU/mL; and the lowest dilution of the virus being tested spiked with endotoxin standard up to a final concentration of 0.12 EU/mL, to rule out a false negative result. LAL reagent water was used as a negative control.

Equi-volumes (100 µL each) of reconstituted Pyrotell® (LAL reagent) and the diluted virus samples were thoroughly mixed by vortexing in depyrogenated soda-lime reaction tubes and incubated at 37°C for 60 minutes. The tubes were then smoothly inverted by hand in one motion to test for the formation of a gel clot (a positive result) or collapse of the gel upon inversion (a negative result). The tests were performed in duplicate. The concentration of endotoxin in each viral preparation was calculated by multiplying the LAL sensitivity (0.06 EU/mL) by the lowest dilution of the viral preparation with a negative result. Table 2.13 shows the range of endotoxin contamination levels detected in tested virus preparations.

2.2.4.4 p24 Assay for the Detection of Replication-Competent Lentiviral Particles

The presence of the viral capsid protein, p24, was used to determine the replication competency of the prepared lentivirus stocks. Reduced expression of p24 in lentivirus-transduced cultured cells over time indicated viruses which were non-replicative and thus safe to use.

HEK-293T cells were seeded into 12-well plates at 5 x 10^5 cells/ mL in DMEM supplemented with 10% (v/v) FBS. After four hours, the medium was replaced with DMEM containing 10% (v/v) FBS with 4 µg/mL polybrene and 50 µg/mL gentamicin. The final concentrated virus was added to the wells in triplicate, 1 µL per well, leaving three wells as untransduced controls. The cells were incubated at 37°C and 5% CO_2 overnight. The transduction duration was 24 hours before the medium was replenished without polybrene. The cells were cultured for a total of 12 or 18 days, with supernatant samples collected on days 6, 12 and 18. The cells were passaged every three days.

An HIV-1 p24 ELISA assay (XpressBio, Express Biotech International, MD USA) was used as per the manufacturer's instructions to detect the p24 protein in the cell culture supernatant samples. A viral batch was considered non-replicative and therefore safe to use when the p24 levels detected in the culture supernatant by the ELISA were seen to significantly decrease over the duration of the 18 day culture.

2.2.5 Calculation of the Multiplicity of Infection

The multiplicity of infection (MOI) refers to the number of infective viral particles present during a transduction relative to the number of target cells exposed. This is a simplified way of describing the viral dose on cells in culture, or on ovine or human corneal endothelial cells. The ovine cornea was estimated to have an average of 1.4 x 10^6 endothelial cells. This number was determined by multiplying the surface area of the corneal endothelium by the endothelial cell density. The surface area of the endothelium side of the sheep cornea was approximated from calculations of the surface area of an ellipsoidal dome. Ovine endothelial cell density was estimated by specular microscopy to be 4,000 cells per mm^2.[155] The human cornea was estimated to have an average of 2 x 10^5 endothelial cells. The surface area of the human corneal endothelium was calculated based on the surface area of a spheroidal dome and the cell density was assumed to be 2,500 cells per mm^2.[19]

2.2.6 Transduction of A549 Cells with Lentivirus and Adenovirus

A549 cells were seeded into 24-well plates and allowed to adhere, as per Section 2.2.4.2. The medium was freshened to high glucose DMEM with 10% (v/v) FBS, penicillin and streptomycin at 100 IU/mL and 100 µg/mL respectively, 4 µg/mL polybrene and 50 µg/mL gentamicin. Concentrated lentivirus or adenovirus was added to the wells at an MOI of approximately 1.6. After twenty four hours the

transduction was ended with a change to fresh medium, without polybrene, but still containing gentamicin. The cells were passaged every two to three days, with culture supernatant collected at strategic time points for protein assays and the cells collected at the conclusion of the culture for mRNA assessment, see Section 2.2.4.2 for cell collection method.

2.2.7 In Vitro Culture of Human Corneas

Human corneas found to be unsuitable for clinical transplantation and approved for research use, were generously donated by the Eye Bank of South Australia. This was done with the permission from the next-of-kin and with approval from the Southern Adelaide Clinical Human Research Ethics Committee. The corneas had been stored at 4^{o}C for at least two weeks in Optisol GS anti-swelling medium with gentamicin sulphate (100 µg/mL) and streptomycin sulphate (200 µg/mL) (Bausch and Lomb, Rochester, NY, USA). After transduction with viral constructs, the corneas were cultured at 37^{o}C in RPMI 1640 medium containing 10% (v/v) FBS, 100IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 2.5 µg/mL of the anti-fungal agent amphotericin B, with the medium completely replenished every two to three days.

2.2.8 In Vitro Culture of Sheep Corneas

Whole sheep eyes were donated by local abattoirs and were collected within hours post mortem. The eyes were immersed in Betadine® Antiseptic Solution (Appendix A1.1) for three to five minutes to disinfect. The eyes were then transferred to sterile sodium chloride 0.9% (w/v) for irrigation (Appendix A1.1) for one minute before a final wash in fresh sterile sodium chloride for irrigation for at least one minute. Surgical instruments were sterilised in autoclave for fifteen minutes at 120^{o}C with

100-120 kPa pressure prior to use. Corneas were excised from the globes in an aseptic manner, leaving a scleral ring of approximately 5 mm surrounding the cornea. Corneas were cultured in pre-warmed RPMI 1640 medium with 10% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 2.5 µg/mL of the anti-fungal agent amphotericin B, and incubated at 37^{o}C. Medium was completely refreshed every two days.

2.2.9 Transduction of Human and Sheep Corneas with Lentivirus and Adenovirus for Organ Culture

Human and sheep corneas were placed endothelium side up in sealed containers, on top of sterilised concave supportive eye caps, Figure 2.4. Concentrated lentivirus or adenovirus was diluted with RPMI 1640 medium containing 2% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine. Sheep corneas were transduced with a total volume of 330 µL and human corneas with a total volume of 200 µL, with the lentivirus or adenovirus at MOIs ranging from 20 through to 100 particles per cell. The corneas were incubated at 37^{o}C for three hours before the removal of the eye caps and the addition of 10 mL of RPMI 1640 medium with 10% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine and 2.5 µg/mL amphotericin B. The corneas were cultured at 37^{o}C for several days, with fresh medium every two to three days. Culture supernatant was collected for protein assays and the corneal endothelia were collected at the conclusion of the culture for flat-mounting or for mRNA analysis.

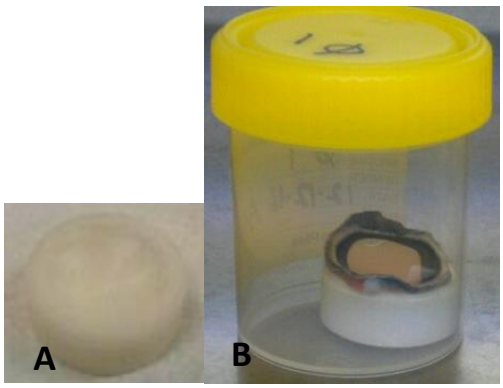


Figure 2.4: (A) Example image of an eye cap used to support excised corneas. (B) Image of a sheep cornea, endothelium upwards, on a supportive eye cap in a sealed storage pot.

2.2.10 In Vivo Sheep Orthotopic Penetrating Keratoplasty

2.2.10.1 Sheep

The animals used for *in vivo* studies were adult cross-breed Merino ewes and wethers from various farm sources in South Australia and Victoria. The animals were housed in paddocks in the grounds of Flinders University and allowed unlimited grass, lucerne hay and water. In the animal facility within the hospital, the animals were housed in crates and pens, with a minimum of two animals kept together at all times. The housing room contained a large mirror to increase the apparent number of animals. The sheep were given chaff, lucerne hay and water and their health and wellbeing were monitored daily. Sheep were acclimatised to physical handling and the indoor conditions for one week prior to surgery. The right eye only of each sheep was operated on to ensure the animals had constant clear vision. All experiments conducted on the animals were approved by the Animal Welfare Committee of Flinders University South Australia.

2.2.10.2 Orthotopic Penetrating Keratoplasty with Corneas Treated with Viral Vectors

The procedure of orthotopic penetrating keratoplasty in the sheep is well established in our laboratory.[53] Recipient sheep were fasted overnight prior to penetrating keratoplasty. In order to induce pupillary dilation, ophthalmic dilating eye drops were administered to a single eye only in the manner of: 1% (w/v) atropine sulphate twenty hours before surgery and hourly from four hours prior to surgery. One percent tropicamide, 1% (w/v) cyclopentolate and 2.5% (w/v) phenylephrine hydrochloride were administered hourly from two hours prior to surgery. See Table 2.7 for sources of ophthalmic eye drops.

Donor corneas were obtained from sheep eyes collected from local abattoirs within hours post mortem, disinfected, and the corneas excised as described in Section 2.2.8. The donors corneas were placed endothelium-up on autoclaved eye caps for support, and transduced for three hours at 37^{o}C with lentivirus or adenovirus in RPMI medium 1640 (Appendix A2.1.15), containing 2% (v/v) FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin. The corneas were thoroughly rinsed three times in serum-free RPMI medium 1640.

Sheep were anaesthetised by intravenous injection of sodium thiopentone 25 mg/kg into the external jugular vein and anaesthesia was maintained with intubation and 3% isofluorane in 4L oxygen. This was performed by a trained animal technician and pre-surgery blood samples were taken. Penetrating keratoplasty was performed by an ophthalmic surgeon. A 12 mm diameter button was cut from the donor cornea using a surgical corneal trephine (Table 2.7) and a single punching action against a Teflon block. Alternatively, the cornea was mounted on a Barron Artificial Anterior

Chamber (Table 2.9) and the trephine and ophthalmic curved scissors were used to remove the donor button. An 11 mm trephine was used to score the central cornea of the recipient sheep. The recipient button was removed with the aid of a diamond knife and curved corneal scissors. The donor button was secured in place on the recipient sheep cornea using 9.0 monofilament nylon sutures (Table 2.7), with four interrupted sutures and a continuous stitch. The eye was treated with a topical chloramphenicol ointment (Table 2.7) and the sheep was allowed to recover from the anaesthetic. Once standing, the animal was allowed food and water under supervision. See Appendix A3.1 for graft operation record sheet.

2.2.10.3 Post-operative Treatment and Monitoring of Animals

The welfare of the animal and of the corneal graft was monitored on a daily basis, with topical Chlorsig™ (chloramphenicol) antibiotic eye drops (Table 2.7) given for three days post-operatively and upon the onset of any adverse events that may have indicated infection.[53]

The grafts were scored daily for clarity, inflammation, swelling, neovascularisation, fibrin presence and inflammatory cell presence, see Appendix A3.2 for graft scoresheet. Grafts were considered technical failures if the central cornea did not become thin and clear by ten days post-operatively. Immunological graft rejection was determined as a loss of corneal clarity (a score of two or more on a scale from zero to four), or the appearance of a corneal epithelial or endothelial rejection line. After the onset of graft rejection, post-operative blood samples were taken and the sheep were euthanised with an overdose of sodium pentobarbitone (170 mg/kg) injected intravenously. Both eyes were enucleated for histology and anterior chamber fluid was harvested.

2.2.10.4 Culture of Corneal Rims for Transgene Expression

To gain an indication of transduction success and transgene expression from the donor cornea buttons used for penetrating keratoplasty in the sheep, the corneal rims remaining once the donor buttons were removed were collected for *in vitro* culture. The rims were cultured in sterile Hepes-buffered RPMI 1640 medium with 10% (v/v) FBS, penicillin and streptomycin at 100 IU/mL and 100 µg/mL respectively, 2 mM glutamine, 2.5 µg/mL of the anti-fungal agent amphotericin B at 37^{o}C for five or six days post surgery. The culture supernatant was collected at day 2 and fresh medium replaced on the rims. At day 5 or 6, the supernatant was collected and the rims discarded. The collected supernatant was assayed for the expression of the transgenes used to transduce the donor corneas (Sections 2.2.13.10 - 14).

2.2.11 Histology

2.2.11.1 Dehydration and Paraffin Embedding of Corneal and Iris Tissue

Corneal and iris samples from grafted and non-grafted sheep eyes were fixed in 10% (v/v) buffered formalin (Medvet Science Pty Ltd, SA) for at least 24 hours prior to dehydration. The tissues were cut down to 5 mm wide strips and any residual vitreous humour was removed from iris samples. The samples were then situated in individual plastic meshed cassettes and immersed with the following dehydration stages: one hour in 70% (v/v) ethanol, one hour in 80% (v/v) ethanol, one hour in 90% (v/v) ethanol, followed by three 30 minute immersions in 100% (v/v) ethanol, before submersion in chloroform (Ajax Finechem, NSW) over night. The cassettes were then placed in a glass dish containing hot molten paraffin wax (Paraplast® Tissue Embedding Medium, Leica Biosystems, St Louis, Netherlands) and incubated in a wax oven at approximately 60^{o}C for 45 minutes. The wax was then changed for fresh molten wax and the dish of samples was incubated in a vacuum (-25kpa) for 45

minutes. The wax was changed again and placed back in the vacuum for a further 45 minutes. The samples were then removed from their cassettes and placed in molten wax-filled metal moulds, oriented on edge with the graft edge down, or the internal iris edge down. The labelled parts of the plastic cassettes were then placed on top of the mould and further molten wax applied to adhere the cassettes. The moulds were then placed on a cold block to set the wax. After approximately 20 minutes when the wax blocks were set, they were removed from the metal moulds and stored at room temperature for sectioning.

2.2.11.2 Sectioning Tissue Samples

Sections from paraffin-embedded corneal graft specimens were cut in 5 µm thick sections on a microtome. These were floated on to microscope slides and dried at 37^{o}C ready for staining.

2.2.11.3 Haematoxylin and Eosin Staining

Cornea tissue sections from paraffin blocks were stained with a standard haematoxylin and eosin staining protocol. The sections were dewaxed in fresh xylene (Merck, VIC) with two immersions each for four minutes. Two washes in absolute alcohol (Merck, VIC) for two minutes each were conducted, before two further minutes in 90% (v/v) alcohol and two minutes in 70% (v/v) alcohol. The sections were then rinsed in deionised water. Haematoxylin (Appendix A2.1.13) staining followed for 10 minutes and then slides were washed under a running tap for one minute. The sections were then differentiated with one quick dip in acid alcohol (Appendix A2.1.1) and immediately washed under a running tap for one minute. Sections were then immersed in lithium carbonate (Merck, Darmstadt, Germany) for two minutes and rinsed with deionised water. Staining with eosin (Appendix

A2.1.10) then followed for two minutes, before washing under a running tap for one minute. A ten second immersion in absolute alcohol was repeated for a total of three times, then the sections were placed in fresh xylene twice for two minutes. The sections were then cover-slipped using DePex mounting medium (BDH Laboratory Supplies, Poole England) and viewed the following day.

2.2.12 Immunohistochemistry

2.2.12.1 Paraformaldehyde-Lysine-Periodate Fixing of Corneal Graft Tissue

Corneal samples from grafted and non-grafted sheep eyes were cut down to 5mm wide strips were fixed in paraformaldehyde-lysine-periodate solution (Appendix A2.1.24) for 2 hours at 4^{o}C. The samples were removed to 7% (w/v) sucrose solution for 18 hours at 4^{o}C and 4 hours in 15% (w/v) sucrose solution at 4^{o}C (Appendices A2.1.31, A2.1.32). The corneal blocks were then mounted graft-edge down in plastic cassettes in optimal cutting temperature (O.C.T.) embedding medium (Tissue-Tek®, Sakura Finetechnical, Tokyo, Japan) and snap frozen, and stored at -80^{o}C prior to sectioning.

2.2.12.2 Sectioning Frozen Tissue Samples

8µm sections were cut from the frozen O.C.T. corneal blocks on a cryostat, placed on chrome alum subbed microscope slides (Appendix A2.1.5) and stored for a maximum of five days at -20^{o}C wrapped tightly in aluminium foil.

2.2.12.3 Immunoperoxidase Labelling of Corneal Sections

Corneal frozen sections were thawed for 30 minutes at 31^{o}C and sections ringed with a diamond pencil. In a humidified chamber the sections were flooded with 10% (v/v) normal swine serum for 10 minutes before this was tipped off and the primary

monoclonal antibodies were applied to the sections in duplicate, see Table 2.14 for the antibody details. Sections were incubated at room temperature overnight in the humidified chamber.

Table 2.14: Antibodies used for immunoperoxidase labelling of sheep corneal graft frozen sections. (Ig, Immunoglobulin)

Designation	Specificity	Antibody	Format	Source
X63	Unknown (negative control)	Monoclonal mouse IgG_1	Hybridoma supernatant	H. Zola, Women's and Children's Health Research Institute, North Adelaide, SA
Anti-CD4	Sheep CD4	Monoclonal mouse IgG_1	Purified	AbD Serotec, Oxford, UK
Anti-CD8	Sheep CD8	Monoclonal mouse IgG_{2a}	Hybridoma supernatant	University of Melbourne, School of Veterinary Science, VIC
Anti-CD45	Sheep CD45	Monoclonal mouse IgG_1	Purified	AbD Serotec, Oxford, UK
Anti-MHC class II	Sheep MHC class II	Monoclonal mouse IgG_1	Purified	AbD Serotec, Oxford, UK
Biotinylated goat anti-mouse	Mouse Ig	Polyclonal Goat Ig	Purified, solid phase absorbed with human Ig and cow serum proteins	DakoCytomation, Glostrup, Denmark

Sections were washed individually with 2-3 pipettefuls of PBS-gelatin (Appendix A2.1.26) then completely immersed twice for 5 minutes in PBS-gelatin with agitation to wash. The slides were carefully dried with tissues around the sections before the secondary antibody, biotinylated goat anti-mouse Ig, was applied to sections in a 1/500 dilution made up in sheep diluent (Appendix A2.1.27). Sections were incubated for 30 minutes in the humidified chamber. Sections were washed twice and dried as before by immersion in PBS-gelatin. Streptavidin/HRP was diluted 1/1000 in PBS and applied to sections for 30 minutes at room temperature in the humidified chamber. Sections were washed twice and dried as before by immersion in PBS-gelatin. A 1x working solution of DAB (3,3 diaminobenzidine)

was prepared by adding 4 mL Tris-azide (Appendix A2.1.33), 40 µL 1M imidazole (Appendix A2.1.18) and 10 µL 30% (w/v) hydrogen peroxide (Chem Supply, Gillman, SA) to 400 µL 10x DAB stock (Appendix A2.1.7). Sections were covered with 1x DAB for at least 5 minutes then washed thoroughly in running tap water for at least 5 minutes. Sections were counterstained for 3 seconds in haematoxylin and washed in running tap water, then dehydrated through a series of ethanol washes (70%, 100%, 100%, 100%, all v/v) for at least 2 minutes in each wash. Sections were cleared in xylene for 5 minutes and cover-slipped using DePex mounting medium (BDH Laboratory Supplies, Poole England).

2.2.13 Transgene Detection

2.2.13.1 Staining of Endothelial Cell Nuclei with Hoechst 33258

After organ culture, sheep corneal endothelia were examined for endothelial cell appearance and density. Corneas were fixed for 10 minutes in 10% (v/v) buffered formalin (Medvet Science Pty Ltd, SA). They were then rinsed in balanced salt solution (BSS) and 300 µL of 10 µg/mL Hoechst 33258 stain (Appendix A1.1) was applied to the corneas, endothelium-up on supportive eye caps. The corneas were protected from light and incubated with this nuclei stain for 30 minutes. BSS was used to rinse the corneas and, under a microscope, the endothelial cells were then removed in intact layers and flat-mounted using Bartels Buffered Glycerol Mounting Medium (Trinity Biotech plc, Wiclow, Ireland) on chrome alum subbed microscope slides, endothelium upwards. Cover slips were sealed over the flat mounts whilst still protecting from the light. The flat mounts were viewed on an upright fluorescence microscope (BX50, Olympus America, PA USA) using an Olympus U-MNUA UV filter (excitation between 360 – 370 nm, emission 420 – 460 nm) to visualise the stained endothelial nuclei. Five representative images were taken from each flat

mount using a digital camera (CoolSNAP high resolution cooled CCD, 1.0X tube) and image analysis software (AnalySIS® FIVE, Software Imaging System; Olympus Soft Imaging Solutions GmbH, Münster, Germany), each with a field size of 0.15 mm^2. The stained cell nuclei were counted to obtain an indication of endothelial cell density.

2.2.13.2 Reporter Gene Detection in Stained Corneal Endothelial Cells

In order to detect and quantify reporter gene eYFP in transduced corneal cells, the corneas were fixed, stained with Hoechst 33258 and flat-mounted onto slides as described previously. Expression of eYFP was visualised on the fluorescence microscope BX50 using a blue light filter (Chroma 31001) (excitation 465 – 495 nm, emission 515 – 555 nm). The same representative fields were taken while using this filter as were taken with the UV filter (Olympus U-MNUA). The cell nuclei and eYFP expression from these fields were counted and the average percentage fluorescence determined.

2.2.13.3 RNA Extraction from Transduced Corneal Endothelia

RNA was extracted from corneal endothelial cells, which had been harvested from organ culture or from rejected ovine penetrating keratoplasty graft tissue. All surfaces and instruments were sprayed with RNaseZap, soaked for several minutes and rinsed with DEPC-treated water for irrigation. Cultured corneas were removed from the medium and rinsed with chilled DEPC-treated saline. Endothelial layers were removed from the corneas and each placed in a 1.8 mL nuclease-free cryotube (Nunc, Roskilde Denmark) containing 600 µL of lysis buffer (RLT, RNeasy Mini kit) with 1% (v/v) 2-mercaptoethanol. The tubes were vortexed for two minutes to

dissociate endothelial cells from Descemet's membrane, then snap frozen in liquid nitrogen prior to storage at -80^{o}C.

RNA was extracted from the endothelial cells using QIAshredder columns and RNeasy Mini kit using the manufacturer's instructions. Briefly, the thawed lysate was pipetted into QIAshredder spin columns without any bulk tissue transfer and centrifuged in a bench-top microcentrifuge for two minutes at 13,000 g. One volume of 70% (v/v) ethanol was mixed with the homogenised lysates and the samples transferred to RNeasy Mini kit spin columns. The purification procedure was carried out as per the manufacturer's instructions and purified RNA was eluted from the columns with RNase-free water. The RNA was quantified by spectrophotometric analysis on the NanoDrop 8000.

2.2.13.4 RNA Extraction from Cell Culture

RNA was extracted from stored cell pellets prepared from cultured cells (refer to Section 2.2.4.2 for cell pelleting procedure). Cell pellets were thawed and disrupted with the addition of lysis buffer (Buffer RLT) containing 2-mercaptoethanol. This lysate was pipette into QIA-Shredder spin columns for homogenisation. One volume of 70% (v/v) ethanol was mixed with the homogenised lysates and the samples transferred to RNeasy Mini kit spin columns. The purification procedure was carried out as per the manufacturer's instructions and purified RNA was eluted from the columns with RNase-free water.

2.2.13.5 DNase Treatment of Extracted RNA

RNA extracted from cultured cells or ovine endothelial cells was treated to remove DNA contamination from the samples using a DNase Treatment Kit (TURBO DNA-

free™, Ambion, TX USA) as per the manufacturer's instructions. RNA was quantified by spectrophotometric analysis on the NanoDrop 8000 pre- and post-DNase treatment. Rigorous DNase treatments were performed to remove DNA from samples containing more than 200 ng/μL of nucleic acid. To each sample 2 μL of 2 U/μL TURBO DNase and 0.1x volume of 10x TURBO DNase Buffer was added and incubated at 37^{o}C for 20 - 30 minutes. After the incubation, 0.2x volume of DNase Inactivation Reagent was added to each sample and incubated at room temperature for two minutes with intermittent mixing. The samples were then centrifuged at 10,000 g for 90 seconds and the top aqueous layer of treated RNA was taken into fresh tubes for cDNA synthesis.

2.2.13.6 Complementary DNA Synthesis

RNA samples were reverse transcribed to create complementary DNA (cDNA) using Superscript III First Strand Synthesis System for RT-PCR (Invitrogen, CA USA), as per the manufacturer's instructions. Briefly, either 1 μg or 0.5 μg of RNA, depending of total RNA quantity, in a maximum volume of 8 μL was added to a reaction mix consisting of 1 μL of dNTPs and 1 μL of oligo dT, made up to a total volume of 10 μL with water. The reaction mixture was placed at 65^{o}C for five minutes and then placed on ice for at least one minute. A synthesis mix consisting of 2 μL 10x RT Buffer, 4 μL 25 nM $MgCl_2$, 2 μL 0.1 M DTT, 1 μL RNaseOUT and 1 μL of Superscript III reverse transcriptase was prepared and 10 μL added to each reaction. In order to create 'no reverse transcription' controls, the reverse transcription enzyme was replaced with water in the reactions. The tubes were incubated at 50^{o}C for 50 minutes and then 85^{o}C for five minutes. One microlitre of RNase H was added to each reaction and incubated for a further 20 minutes at 37^{o}C. The tubes were then cooled on ice and stored at -20^{o}C.

2.2.13.7 Real-Time PCR for Transgene Quantification

Real-time PCR reactions were performed to detect the expression of transgenes at the message level from cell culture cells and ovine corneal endothelial cells, which had been transduced with viral vectors. A Rotor Gene 6000 (Corbett Research, Sydney, NSW) or a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK) with accompanying softwares were used for the PCR amplification and analysis.

Real-time primers were designed to be specific for the transgenes of interest, have a G-C base content of between 40-60%, a melting temperature of approximately 56^{o}C, and to create approximately 100 base pair products. A list of the genes and their corresponding primers can be seen in Table 2.6.

A PCR master mix was created so that one PCR reaction contained 2 µL of each forward and reverse primer at 5 µM, and 10 µL of *Power* SYBR® Green (Applied Biosystems, Warrington, UK). To this 14 µL, 6 µL of the cDNA sample, standard sample (Section 2.2.13.8) or UltraPure water, for template-free controls, was added. The cDNA was diluted 1:20 prior to use in the PCR reactions. The cycling conditions were as follows: 95^{o}C for 10 minutes, 40 cycles of 95^{o}C for 10 seconds, 52^{o}C for 15 seconds and 72^{o}C for 20 seconds, followed by a hold at 72^{o}C for one minute. Dissociation curves were created in a slow melt from 60^{o}C through to 99^{o}C, with five seconds hold at each 0.5^{o}C increment.

2.2.13.8 Real-Time PCR Standards and Determination of Primer Pair Amplification Efficiency

A standard sample was created using 5 µL of cDNA from all representative samples, treatment and controls. A second standard sample was created from 5 µL of each of the 'no reverse transcription' control cDNA samples. The standard samples were 3-fold serially diluted seven times and these dilutions were used in real-time PCR reactions in triplicate with primers specific for the two reference genes, ovine β-actin and ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For human cell lines, reference genes ARBP (acidic ribosomal phosphoprotein) and β-actin were used. The mean threshold cycle values for the serially diluted cDNA were plotted against the $\log_e$ of the relative dilution of the cDNA to create a trend line of linear regression. The slope of this regression line was used to determine the primer efficiency by using the calculation:

$$\textbf{Primer efficiency} = [(10^{-(1/\text{slope})}) - 1] \times 100 \quad \text{[ref 233]}$$

An efficiency value as close as possible to one hundred was desirable. These standard curves were prepared in duplicate and the average primer efficiency calculated.

2.2.13.9 Gene Expression Analysis

Real-time PCR gene expression was analysed using the $2^{-\Delta\Delta Ct}$ relative quantification method from Livak and Schmittgen as described in Section 2.2.4.2.[231] To normalise the data by incorporating the primer amplification efficiencies and allow multiple reference genes, modifications of this method were made based on the quantification algorithm described in the freely available geNorm software.[234] The standard sample

used in the primer efficiency standard curve reactions was included in triplicate for each primer set in the PCR reactions on sample cDNA.

The following equations were used for quantification and normalisation:

$$\mathbf{Q = (AE+1)^{(Cts-CtA)}}$$

$$\mathbf{X_A = Q/NF}$$

Q is the relative quantity of each gene

AE is the amplification efficiency

Cts is the geometric mean threshold cycle of the standard sample replicates

CtA is the threshold cycle of a sample of interest

X_A is the normalised relative quantity of each gene

NF is the normalisation factor (the geometric mean of the relative quantities (Q) of the reference genes

2.2.13.10 Ovine Interleukin-10 Detection by Sandwich ELISA

An oIL-10 standard sample was prepared by pooling cell culture supernatants from HEK-293A cells transfected with plasmids expressing the oIL-10 transgene. This standard sample was assayed for IL-10 concentration using a Human IL-10 Time Resolved Fluorescence Assay. This was kindly conducted by Melinda Judge under the supervision of Professor Prue Hart at the Telethon Institute for Child Health Research, Perth, WA. This standard sample was used in all oIL-10 ELISAs conducted.

Ovine interleukin-10 was detected at the protein level in tissue culture supernatant and sheep anterior chamber fluid using bovine IL-10 antibodies in a sandwich ELISA. A 96-well plate was coated with 100 µL of the capture antibody, mouse anti-bovine IL-10 monoclonal antibody (AbD Serotec, UK) diluted 1:167 with 0.1 M

sodium carbonate. The coated plate was stored at 4^{o}C overnight. Wells were then washed three times with ELISA wash buffer (0.05% (v/v) Tween-20 in PBS). 200 µL of blocking solution, consisting of 10% (v/v) heat inactivated FBS in PBS, was added to each well and the plate incubated for one hour at room temperature. The plate was washed with the wash buffer three times before 100 µL of supernatant samples (pre-diluted appropriately with 10% (v/v) FBS in PBS) were applied to the wells, with 10% (v/v) FBS in PBS used for negative controls. To generate a standard curve, serial dilutions of the standard sample were prepared using 10% (v/v) FBS in PBS. Eight dilution points were used, from 1:400 to 1:256,000. 100 µL of each of the serial dilutions were added to triplicate wells. The plate was incubated for two hours at room temperature. The plate was then washed five times and 100 µL of biotinylated mouse anti-bovine IL-10 monoclonal detection antibody (AbD Serotec, UK), diluted 1 in 500 with 10% (v/v) FBS in PBS, was applied to wells. The plate was incubated for one hour at room temperature. The plate was washed five times and 100 µL of streptavidin-HRP (DakoCytomation, Glostrup, Denmark), diluted 1 in 1000 with 10% (v/v) FBS in PBS, was applied to wells and the plate was incubated at room temperature for one hour. The plate was washed five times and 100 µL of TMB Substrates A and B in an equal ratio were added to all wells. The plate was then incubated in the dark for 30 minutes. Fifty microlitres of 1 M H_2SO_4 was added to all wells to terminate the reaction and the plate was read at 450 nm on a microplate reader. A standard curve was plotted of absorbance versus standard sample concentration and the equation of a linear regression line used to determine oIL-10 concentration in the unknown samples.

2.2.13.11 Indoleamine 2,3-dioxygenase Activity Assay

A colourimetric assay to detect L-kynurenine, a by-product of indoleamine 2,3-dioxygenase-induced (IDO) degradation of tryptophan, was used to measure IDO activity. This method was adapted from Nisapakultorn *et al.* 2009 and from a protocol from PhD candidate Nesrine Kamal Bassal under the supervision of Dr Maurizio Costabile, School of Pharmacy and Medical Sciences, University of South Australia.[235]

To generate a standard curve, dilutions were prepared from a 10mM stock solution of L-kynurenine (0, 6.25, 12.5, 25, 50, 100 μM) using Milli-Q water or tissue culture medium. Tissue culture supernatant samples were centrifuged at 16,000 g for five minutes to settle cell debris. 40 μL of 30% (w/v) trichloroacetic acid was added to 100 μL of standards and supernatant samples, mixed and centrifuged at 16,000 g for five minutes at room temperature. 100 μL of supernatant from each standard and sample was placed in a 96-well plate. 100 μL of 6% (w/v) Ehrlich's reagent (p-dimethylamino benzaldehyde) dissolved in 100% (v/v) glacial acetic acid was added to each well. The plate was incubated in the dark for no more than 10 minutes, mixed gently and read at 492 nm. A standard curve of absorbance versus L-kynurenine concentration was plotted and used to determine L-kynurenine concentration in the unknown samples.

2.2.13.12 Bcl-xL Detection by Sandwich ELISA

Bcl-xL protein expression from concentrated tissue and organ culture medium was assayed using DuoSet IC Human/ Mouse Total Bcl-xL (R&D Systems, Minneapolis, MN USA) in a sandwich ELISA, using the manufacturer's instructions. A Nunc 96-well plate was coated with the capture antibody at 8μg/mL and incubated at room

temperature overnight. The plate was washed three times with wash buffer (PBS and 0.05% (v/v) Tween-20) and blocked with a 1% (w/v) bovine serum albumin solution made up with PBS and 0.05% (w/v) sodium azide for 1 to 2 hours. The plate was washed again three times and 100 µL of samples and standards were applied to the wells. The reconstituted standard provided (160 ng/mL) was serially diluted using 1 mM EDTA in PBS solution with 0.5% (v/v) Triton X-100, and 2-fold dilutions from 20,000 pg/mL through to 312.5 pg/mL were used to form a standard curve. The plate was incubated at room temperature for two hours before washing three times. The detection antibody was reconstituted using 10 mg/mL BSA in PBS and applied to all wells at a concentration of 2 µg/mL, then the plate was incubated for a further two hours at room temperature. The plate was washed three times and the provided streptavidin-HRP was applied to all wells in a 1:200 dilution, using the 1mM EDTA diluent. The plate was incubated for 20 minutes at room temperature and protected from the light. After three washes, equal amounts of TMB colour reagents A and B were applied to all wells and the plate again incubated in the dark for 20 minutes. 1 M H_2SO_4 was added to all wells to terminate the reaction and the plate was analysed on a plate reader, subtracting the absorbance readings at 540 nm from 450 nm to obtain an absorbance reading corrected for optical imperfections in the plate. A standard curve was plotted of absorbance versus standard sample concentration and the equation of a linear regression line used to determine Bcl-xL concentration in the unknown samples.

2.2.13.13 Human Endostatin Detection by Sandwich ELISA

Neat and concentrated tissue and organ culture medium was assayed for the presence of the human endostatin protein using a sandwich ELISA Quantikine® kit: Human Endostatin Immunoassay (R&D Systems, Minneapolis, MN USA), using the

manufacturer's instructions. This ELISA was used for the detection of the endostatin part of a fusion protein consisting of human endostatin and the kringle-5 domain of the human plasminogen gene.[87] The protocol was as follows: the provided assay diluent was added to wells before the addition of 50 µL of samples and standards. The standard provided was reconstituted with 1 mL dH_2O to 100 ng/mL and a dilution series from 10 ng/mL to 0.31 ng/mL was created. The plate was incubated for two hours at room temperature, with shaking at approximately 500 rpm. The wells were then washed four times using the provided Wash Buffer Concentrate, diluted 1:25 with dH_2O. 200 µL of the Endostatin Conjugate provided was added to each well and the plate incubated for two hours at room temperature, with shaking at approximately 500 rpm. The wells were washed four times and 200 µL of the provided Substrate Solution (a mix of colour reagents A and B in equal amounts) was added to each well, and the plate incubated for thirty minutes at room temperature, protected from light. 50 µL of the provided Stop Solution (2 N H_2SO_4) was added to each well to terminate the reaction, and the plate gently tapped to mix. The plate was analysed on a plate reader, subtracting the absorbance readings at 570 nm from 450 nm to obtain an absorbance reading corrected for optical imperfections in the plate. A standard curve was plotted of absorbance versus standard sample concentration and the equation of a linear regression line used to determine human endostatin concentration in the unknown samples.

2.2.13.14 Human Soluble Fms-like Tyrosine Kinase 1 (hu sFlt-1) Detection by Sandwich ELISA

Human sFlt-1 protein expression from concentrated tissue and organ culture medium was assayed using a sandwich ELISA Quantikine® kit: Human soluble VEGF-R1/Flt-1 Immunoassay (R&D Systems, Minneapolis, MN USA), using the

manufacturer's instructions. The provided assay diluent was added to wells before the addition of 100 µL of samples and standards. The standard provided was reconstituted with dH_2O to 20,000 pg/mL and a dilution series from 2000 pg/mL to 31.25 pg/mL was created. The plate was incubated for two hours at room temperature, with shaking at approximately 500 rpm. The wells were then washed four times using the provided Wash Buffer Concentrate, diluted 1:25 with dH_2O. 200 µL of VEGF-R1 conjugate was added to each well and the plate incubated for two hours at room temperature, with shaking at approximately 500 rpm. The wells were washed four times and 200 µL of a mix of colour reagents A and B in equal amounts was added to each well, and the plate incubated for thirty minutes protected from light. 50 µL of the stop solution (2 N H_2SO_4) was added to each well to terminate the reaction, and the plate gently tapped to mix. The plate was analysed on a plate reader, subtracting the absorbance readings at 570 nm from 450 nm to obtain an absorbance reading corrected for optical imperfections in the plate. A standard curve was plotted of absorbance versus standard sample concentration and the equation of a linear regression line used to determine hu sFlt-1 concentration in the unknown samples.

2.2.13.15 Concentration of Samples for Detection

Cell culture and organ culture supernatants from cells and corneas transduced with viral constructs containing secreted genes, occasionally required concentrating in order to detect the protein. This was done by pooling the collected supernatants for a given treatment group to obtain a large starting volume and concentrating by centrifugal filtration, using Amicon® Ultra-15 Centrifugal Filter Devices according to the manufacturer's instructions (Millipore Ireland, Cork, Ireland). The protein sizes for all the transgenes were between twenty kilodaltons (kDa) and 70 kDa,

therefore filters with a 10,000 Da molecular weight cut off were used to ensure the proteins were preserved. The initial supernatant was weighed before being subjected to several centrifuge spins at 3000 g for approximately fifteen minutes. A final weighing of concentrated samples gave the number of times concentrated, usually somewhere between 13 - 30-fold. Both treated and mock-treated samples were concentrated to a similar extent. This number was factored in when performing assays with the concentrated samples.

2.2.14 Annexin V-FITC Apoptosis Assay by Flow Cytometry

The TACS® Annexin V-FITC kit (Table 2.8) was used to determine the biological functional activity of the transgenic ovine Bcl-xL protein. HEK-293T cells were seeded into a 24-well plate at 5 x 10^5 cells/ mL in culture medium as described in Section 2.2.2.1. The cells were transduced with LV-CMV-oBclxL at an MOI of 2, for 24 hours in a transducing medium containing 50 µg/mL gentamicin and 4 µg/mL polybrene. Apoptosis of the cultured adherent HEK-293T cells was induced at day 15 post-transduction with the addition of the cytotoxic agent staurosporine (Sigma, St Louis, MO USA) at a final concentration of 1 µM for 6 hours. The cell supernatant was collected into 1 mL 1% (v/v) FBS in PBS and the adherent cells were washed with 1x PBS, gently trypsinised and also collected. The cells were centrifuged at 300g for 5 minutes at room temperature. One millilitre of ice cold 1x PBS was used to wash the cells and they were centrifuged as before. Care was taken to remove all PBS from the cells by blotting on clean paper towel.

The TACS® Annexin V-FITC kit (Table 2.8) was used to label the apoptotic cells, which were then quantified by flow cytometry. This kit enables fluorescent labelling of cells undergoing early apoptosis, as defined by their cell surface chemistry. Using

the manufacturer's instructions, a 100 µL mix of annexin V-FITC and propidium iodide (PI) was prepared to label each cell sample:

10 µL 10x binding buffer

10 µL propidium iodide

1 µL annexin V conjugate

79 µL dH_2O

The cells were resuspended in the labelling mix and kept in the dark for fifteen minutes. 100 µL of ice cold 1x binding buffer was added to each sample and the cells were assessed by flow cytometry on the BD Accuri® C6 Flow Cytometer.

Two photomultiplier tube channels were calibrated to avoid spectral overlap, with cells in binding buffer alone (a control for auto-fluorescence), cells labelled with PI, and cells labelled with annexin, to define the boundaries for each population. The cell population of interest was gated by Ms Sheree Bailey (Flow Cytometry Facility, Flinders Medical Centre), a flow cytometrist experienced with the Annexin V-FITC assay, to exclude cellular debris. Within this population, a dot plot of the labelled cells was prepared for each sample assayed with Annexin labelling detection represented on the x-axis and PI labelling on the y-axis. Only cell populations labelled annexin positive and PI negative were considered to be in early apoptosis, Figure 2.5.

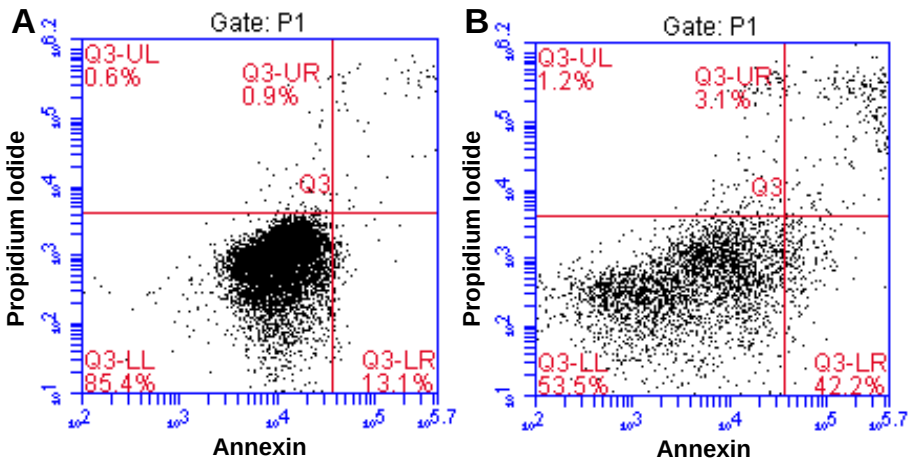


Figure 2.5: Examples of apoptosis dot plots of cells labelled with Annexin V-FITC and propidium iodide. Lower left quadrant shows normal viable cells, lower right quadrant shows early apoptotic cells, and upper right quadrant shows late apoptotic or necrotic cells. **(A)** Untreated cells. **(B)** Cells treated with 1 µM staurosporine, an apoptosis-inducing agent, for 6 hrs prior to fluorescent labelling.

2.2.15 Statistical Analysis

Statistical analysis was performed using PASW Statistics software version 18.

Differences in gene expression at the protein level in cell culture and cornea culture promoter comparison experiments and vector cocktail interference tests were determined by one-way ANOVA comparison between groups with unequal variance assumed. If homogeneity of variance was assumed (above significant level) then Tukey's post-hoc test was used or if homogeneity of variance was not assumed, i.e. below 0.05 (significant) then Dunnett's T3 post-hoc test was used. The statistical significance level was set at a p-value of ≤ 0.05. Differences in gene expression at the mRNA level in cell culture promoter comparison experiments were determined by independent samples t-Test for each time point of the culture. The Mann-Whitney

U test was used to determine statistical differences in gene expression at the mRNA level in cornea culture promoter comparison experiments.

One-way between-groups ANOVA analysis was used to analyse variance of eYFP expression between protamine sulphate treated lentivirus transductions in ovine corneas. This was testing for enhancement of transduction. Post-hoc comparisons made using the Dunnett T3 test. The statistical significance level was set at a p-value of ≤ 0.05.

The Kruskal-Wallis Test was used for one-way between-groups analysis of variance of kynurenine expression by lentivirus-treated cells in the assay for biological activity of the IDO gene. The statistical significance level was set at a p-value of ≤ 0.017 after Bonferroni correction for multiple comparisons.

One-way between-groups ANOVA analysis was used to analyse variance of EK5-treated and mock-treated HUVE cell proliferation in the test for biological activity of the EK5 gene, with post-hoc comparisons made using the Tukey HSD test. This same statistical method and post-hoc test was used to analyse variance between the survival of Bcl-xL-treated and untreated HEK-293T cells in the test for biological activity of the Bcl-xL gene, with the file first split by apoptosis inducement. In both cases the statistical significance level was set at a p-value of ≤ 0.05.

In the test for biological activity of the sFlt-1 gene, a reduction in human umbilical vein endothelial cell proliferation mediated by the sFlt-1 protein was determined statistically using one-way between-groups ANOVA analysis, with post-hoc

comparisons made using the Tukey HSD test. The statistical significance level was set at a p-value of ≤ 0.05.

Long-term ovine corneal graft survival was determined statistically using the Mann-Whitney U non-parametric test for independent samples, corrected for ties. The statistical significance level was set at a p-value of ≤ 0.05. Kaplan-Meier survival plots were analysed for Log-Rank significance using the Peto statistic, with a significance level set at ≤ 0.05.

Chapter 3

Construction, Characterisation and Therapeutic Testing of a Lentiviral Vector

3.1 Abstract

3.1.1 Aims

The aims of the work described in this chapter were to create and characterise a lentiviral vector containing the therapeutic transgene interleukin-10 under the control of a constitutive cytomegalovirus (CMV) internal promoter, to investigate methods to increase transgene expression from ovine corneal endothelial cells using the lentiviral vector, and to use the lentiviral vector therapeutically in a sheep model of orthotopic penetrating keratoplasty.

3.1.2 Methods

Using molecular cloning techniques, a lentiviral construct was created containing the ovine IL-10 transgene under the control of a CMV internal promoter. The construct was tested *in vitro* for transgene expression (oIL-10) at both the protein and message level in a comparison with a lentiviral construct with the Simian virus type 40 (SV40) early promoter inducing IL-10 expression. The constructs were used to transduce two human cell lines (A549, HEK-293A), and ovine corneas in organ culture. Transgene expression was measured in the culture supernatant using a sandwich ELISA and in the mRNA of corneal endothelial cells by qRT-PCR.

To investigate transduction enhancement, the polycation protamine sulphate was used as a charge neutraliser to minimise electrostatic repulsion between lentiviral particles and the target cell membranes, to allow better viral contact and transduction of the target ovine corneal endothelial cells. Reporter gene expression, enhanced yellow fluorescent protein (eYFP) in the lentiviral vector, was used to determine transduction efficiency by fluorescence microscopy. The multiplicity of infection (MOI) was increased to determine whether a significant increase in transgene expression could be achieved, and whether the corneal endothelial cells would

tolerate a high viral load. Detection of the lentiviral transgene interleukin-10 (IL-10) in the culture supernatant of corneas was used to determine expression efficacy at the varying MOIs, and counts of the endothelial cell nuclei were used to determine microscopically any toxic effects from the viral loads. The lentiviral vector containing a CMV promoter and the IL-10 transgene was used to transduce ovine corneas *ex vivo*, prior to orthotopic corneal transplantation in a sheep model.

3.1.3 Results

The lentiviral construct containing the IL-10 transgene under the control of a CMV promoter was successfully created. This construct was found to induce higher transgene expression levels than the SV40-containing vector at both the protein and message level. Protamine sulphate gave a modest but significant increase in early transgene expression (14-fold) at the highest dosage tested, 100 μg/mL, compared with lentivirus treatment alone. Increasing the MOI from 40 to 100 viral particles per target cell gave a modest increase in transgene expression at the protein level (1.2- to 2.3-fold), but was not significant across all time points of the culture. The increased viral load did not have a toxic effect on the endothelial cell nuclei. Lentiviral over-expression of ovine IL-10 in donor sheep corneas significantly prolonged allograft survival, however individual instances of allograft rejection at a similar time to control allografts were still seen.

3.1.4 Conclusions

Protamine sulphate did not increase early transgene expression enough to warrant further investigation. While transducing corneas with lentivirus at an MOI of 100 did not adversely affect the number of endothelial cells, an MOI of 40 still achieved useful transgene expression. The lentiviral vector induced a modest increase in median allograft survival day with the IL-10 therapeutic transgene. Based on

previous adenoviral vector studies, I speculated that earlier and higher transgene expression is required to increase the therapeutic potential of IL-10 in the lentiviral vector.

3.2 Introduction

3.2.1 Corneal graft rejection

The eye enjoys a degree of immune privilege, with many mechanisms functioning to create an environment that minimises inflammatory potential.[43] Corneal allografts have an excellent *initial* survival rate, especially when considering there is generally no HLA matching or systemic immune suppression.[21] However the long-term survival of corneal allografts is *not* excellent when compared to other organ transplants, with Australian corneal allografts in humans having an average survival of 12.9 years.[16] The major cause of allograft failure is irreversible immunological rejection. The immune privileged state of the eye therefore is a finely balanced dynamic situation. Recipients with pre-operation factors such as a poor visual acuity, prevascularised or inflamed host cornea, or corneal trauma have a high risk of immunological rejection.[16] Immune modulation is required for long-term survival of corneal allografts at high risk of immunological rejection. However the current regimens of topical and systemic immunosuppressive agents for prophylaxis and treatment of allograft rejection will not always prevent graft loss.[6]

3.2.2 Gene Transfer to the Eye

For over ten years therapeutic genes have been successfully transferred to ocular tissues under experimental conditions, using viral and non-viral methods.[236] The compartmentalised anatomy of the eye enables localised delivery of gene therapy and lower dose requirements compared with systemic treatment.[194] Viral vectors such as

adenovirus, adeno-associated virus and lentivirus have successfully transduced ocular tissues such as the corneal stroma, corneal endothelium, trabecular meshwork and the retinal pigment epithelium.[166, 177, 194, 195, 197, 201, 236, 237] Non-viral gene transfer techniques such as ballistic transfer, use of nanoparticles, electroporation and lipofection have also been demonstrated to induce gene expression in targeted ocular tissues, with little or no immune response, however achieving only low levels of transient gene expression.[199, 238] Preliminary results from clinical trials involving viral vector gene transfer to the human eye are beginning to emerge, mainly for, but not limited to, therapies targeting abnormal neovascularisation in retinal eye disorders, in tumours such as retinoblastoma, and in age-related macular degeneration.[177, 194, 239]

3.2.3 The Lentiviral Vector

Lentiviral vectors are widely used in gene therapy applications and have been successfully used in animal models of penetrating keratoplasty to deliver therapeutic genes to the cornea.[87, 201] The lentivirus used in this study for gene therapy was constructed from a transfer vector kindly donated by Associate Professor Donald Anson at the Women's and Children's Hospital, Adelaide Australia.[179] This self-inactivating integrative vector has been used in previous gene therapy experiments in our laboratory.[178] This chapter describes the molecular cloning techniques that were employed to remove the existing internal promoter and replace it with a CMV promoter to induce constitutive transgene expression.

3.2.4 The Role of Interleukin-10 in Graft Acceptance

The primary transgenic protein examined in the experiments described here was interleukin-10 (IL-10). IL-10 is an immune-modulatory cytokine affecting the function of many immune system cells, having an inhibitory effect on antigen presentation by monocytes and macrophages. It also inhibits CD4+ T cell proliferation, cells which have a controlling role in corneal allograft rejection.[6, 101] IL-10 has been used in several instances of experimental allograft survival trials.[76, 240, 241] Previously in our department, it has been shown that IL-10 can have an immuno-modulatory effect and prolong corneal graft survival in a sheep model of corneal transplantation.[76, 203] This graft prolongation was demonstrated using an adenovirus gene therapy vector with a CMV internal promoter and, to a lesser extent, using a lentiviral vector with the SV40 promoter.

3.2.5 Polycations as Transduction Enhancers

Incubating viral particles with positively charged molecules called polycations has been shown to increase viral transduction efficiency.[206] This is believed to be due to the enhanced contact between the viral particles and target cells, as the polycation reduces the electrostatic repulsion between these two negatively charged surfaces.[242] Polybrene is a well-known polycation that has been shown to increase lentiviral transduction.[211, 214] Parker *et al.* found a significant increase in polybrene-mediated lentiviral transgene expression in the ovine cornea *in vitro*, however discovered the agent was toxic when used on ovine corneas for penetrating keratoplasty *in vivo* (results not published).[203] Another study found polybrene to have toxic effects on primary human keratocytes.[212] Protamine sulphate is a polycation which has U.S. Food and Drug Administration approval and thus may be more clinically useful than polybrene as a transduction enhancer.[210] Several studies have shown protamine

sulphate to successfully enhance transduction efficiency *in vitro*.[212, 215] In this chapter, I investigate protamine sulphate as an alternative to polybrene as a lentiviral transduction enhancer in ovine corneal gene therapy.

3.2.6 The Effect of Viral Multiplicity of Infection on Transgene Expression

The multiplicity of infection (MOI) is the number of viral particles present per target cell. Increasing the MOI increases the chances of a higher proportion of target cells being successfully transduced with a viral vector. Wang *et al.* used a lentiviral vector at increasing MOIs to steadily increase transgene expression in primary keratocytes.[218] Too high a viral load on target cells can lead to cellular morbidities such as abnormal growth, morphology and even death.[219] Increasing the viral dose without inducing morbidities in the target cells could be an effective way to improve the success of the lentivirus as a gene therapy vector in ovine corneas. In this chapter, the effect of increasing the lentivirus MOI on transgene expression and target cell density in ovine corneal endothelial cells was investigated.

3.2.7 Sheep model

The sheep is a useful pre-clinical model for penetrating keratoplasty. The physiology of the sheep eye is similar to the human eye, most notably having a non-replicative corneal endothelial layer, and sheep corneal grafts undergo corneal graft rejection in a manner that is similar histologically to the human experience.[53, 76] The LV-CMV-IL10 construct was tested in the sheep model of corneal transplantation in order to determine whether the internal transgene promoter plays a significant role in prolonging the survival of the corneal graft by enhancing the success of the gene therapy.

3.3 Specific Aims

1) To create a construct containing the interleukin-10 transgene under the control of the cytomegalovirus constitutive promoter, in the HIV-1 lentiviral vector.
2) To compare the *in vitro* expression of the interleukin-10 transgene in the lentiviral gene therapy vector when under the control of two constitutively expressed viral promoters.
3) To investigate the efficacy of the polycation protamine sulphate to enhance lentivirus transduction of the sheep cornea.
4) To investigate the effect of increasing the lentivirus multiplicity of infection on transgene expression and endothelial cell density in the sheep cornea.
5) To transduce donor sheep corneas with the lentiviral vector containing the interleukin-10 transgene with the appropriate promoter, perform orthotopic penetrating keratoplasty on recipient sheep using these transduced corneas, and monitor for prolongation of graft survival compared with mock-transduced control grafts.

3.4 Results

3.4.1 Construction of Lentiviral Shuttle Plasmid Containing Ovine Interleukin-10

A lentiviral shuttle plasmid containing the transgene interleukin-10 under the control of a cytomegalovirus (CMV) constitutive viral promoter was prepared from an existing lentiviral plasmid in the laboratory, containing oIL-10 with the SV40 promoter (pHIV-SV40-oIL10).[202] Figure 3.1 shows a map of the constructed pHIV-CMV-oIL10 plasmid. The SV40 promoter was swapped for the CMV promoter, which was obtained by restriction digest of another existing lentivirus plasmid in the laboratory containing eYFP with the CMV promoter (pHIV-CMV-eYFP). Both plasmids were digested with restriction endonucleases BamH1 and Cla1 to remove the promoter regions, as described in Section 2.2.1.5 (Figure 3.2). The digestion reaction of plasmid containing oIL-10 was treated with shrimp alkaline phosphatase (SAP) to remove the 5' phosphate groups and prevent self re-ligation (Section 2.2.1.7). The vector and CMV insert were then purified using a PCR purification kit and a gel purification kit, respectively (Figure 3.3).

The CMV insert was then ligated into the oIL-10-containing plasmid. Potential clones were isolated after electroporation of the construct into DH5α *E. coli* electrocompetent cells and culturing on selective media (Section 2.2.1.9). Endpoint PCR was performed on the clones with primers designed to amplify the CMV promoter region in order to screen for colonies containing the pHIV-CMV-oIL10 construct. An agarose gel of the PCR products is shown in Figure 3.4. A positive clone was identified by showing a DNA band at approximately 580 base pairs. Of the twenty colonies screened, nineteen contained the CMV DNA of the correct size and one showed a negative result. Four of the positive clones were selected for sub-

culture and were confirmed as housing the correct construct by restriction digest, with a BamH1 and Cla1 double digest and an EcoR1 and Kpn1 double digest, giving DNA bands at 580 bp and 730 bp, respectively (Figure 3.5). Restriction site positions used to verify clone identities can be seen in the vector map in Figure 3.1.

Clones 2 and 3 were randomly selected for sequencing and clone 2 was selected as the ideal candidate after alignment against existing sequencing of lentiviral constructs containing the CMV promoter and the ovine IL-10 gene showed identical sequence (Figure 3.4).

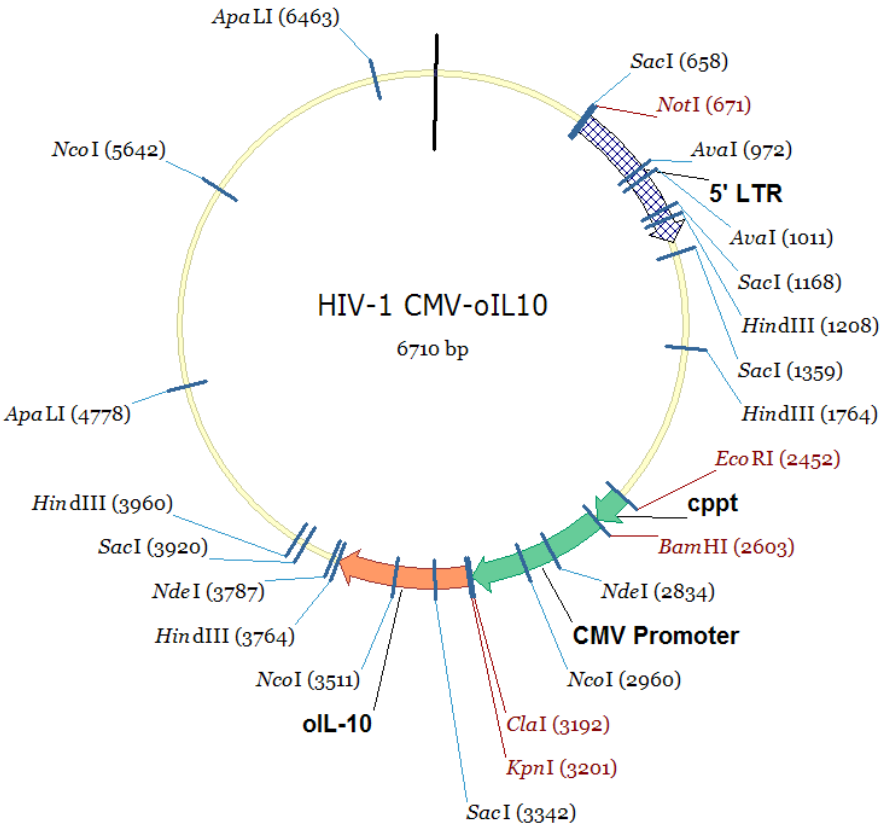


Figure 3.1: Vector map of proposed new lentivirus shuttle plasmid containing the ovine IL-10 transgene with the internal CMV promoter.

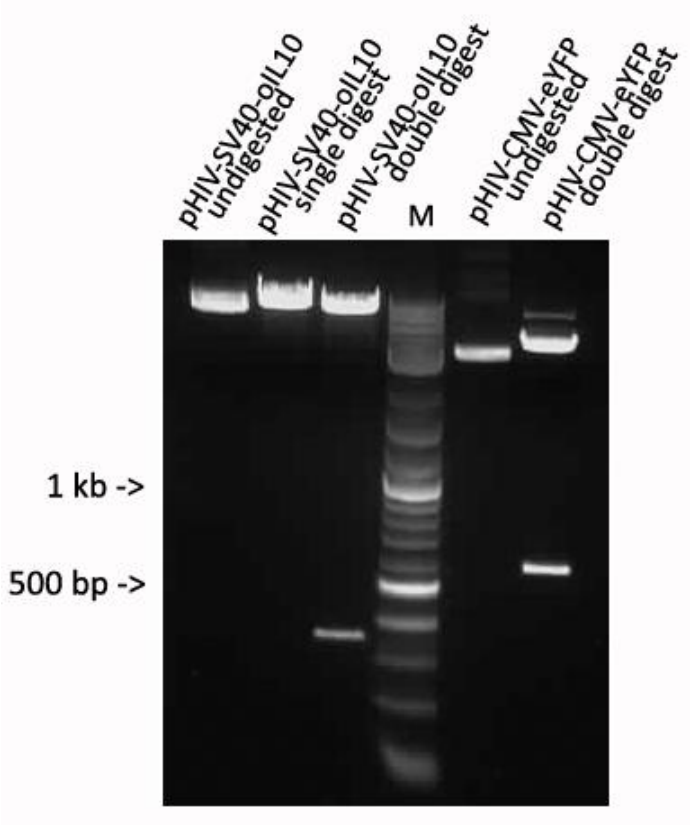


Figure 3.2: 1% (w/v) agarose gel of the restriction digest reactions of pHIV-SV40-oIL10 and pHIV-CMV-eYFP with restriction endonucleases BamH1 and Cla1 to remove promoter regions. Double digests of plasmids show the excised ~580 bp CMV promoter region and the ~359 bp excised SV40 promoter region. Lane denoted 'M' indicates the 2-log DNA ladder.

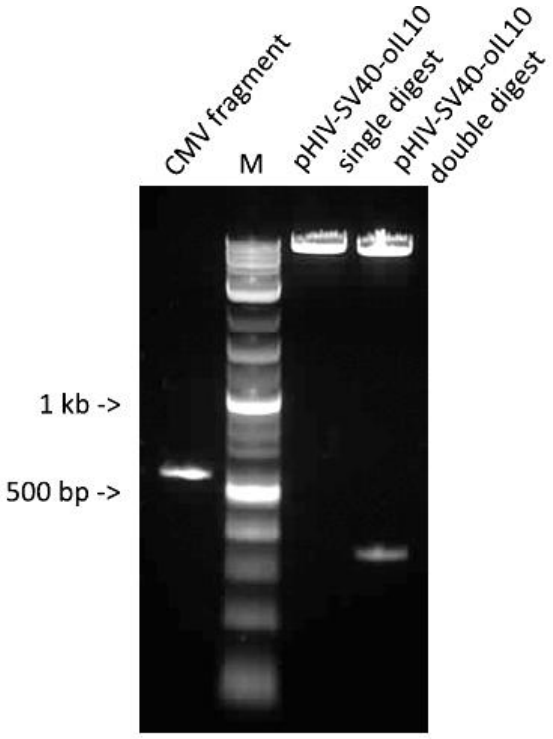


Figure 3.3: 1% (w/v) agarose gel of the purified plasmid and CMV promoter fragments. The purified CMV fragment can be seen at ~580 bp and the purified double digest of the plasmid pHIV-SV40-oIL10 shows the excised SV40 promoter region at ~359 bp. Lane denoted 'M' indicates the 2-log DNA ladder.

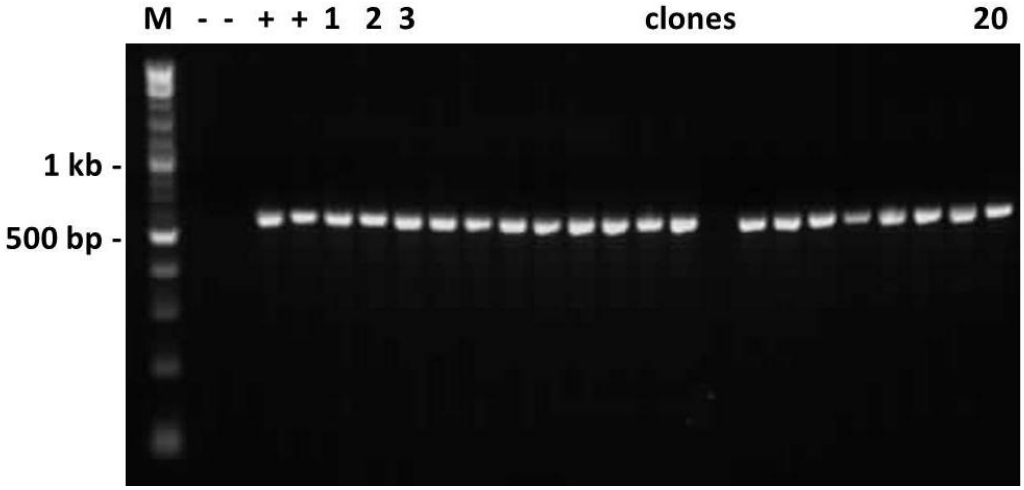


Figure 3.4: 1% (w/v) agarose gel of PCR reaction products, screened for amplification of the CMV promoter region in clones potentially containing the pHIV-CMV-oIL10 plasmid. A positive product was expected at ~580 bp. Lanes 2 and 3 show no-template control reactions (NTC); Lanes 4 and 5 show positive control plasmid template reactions; Lanes 6 through 25 show amplification reactions from clones 1 through to 20. Clones 2 and 3 correspond to the clones selected for sequencing. Nineteen out of the 20 clones tested were positive for the CMV promoter region. Lane denoted 'M' indicates the 2-log DNA ladder.

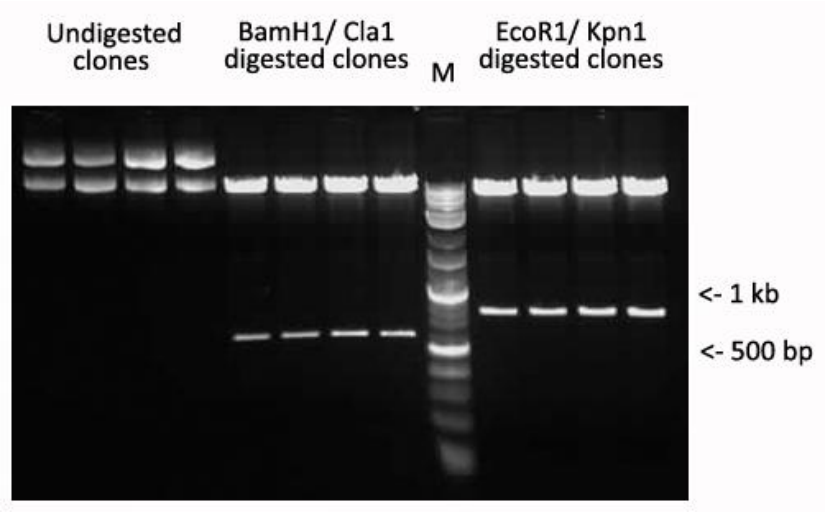


Figure 3.5: 1% (w/v) agarose gel of the restriction digest products of the potential pHIV-CMV-oIL10 mini plasmid preparations. Restriction digestion reactions of mini plasmid preparations of clones 2, 3, 4 and 5 from Figure 3.4 are shown in this figure. All four clones showed the correct sized fragment bands in both of the double digests; 580 bp for the BamH1 /Cla1 digest and 730 bp for the EcoR1 /Kpn1 digest. Lane denoted 'M' indicates the 2-log DNA ladder.

3.4.2 Test of Transgene Expression by Transient Transfection

HEK-293A cells were transiently transfected with the new plasmid, pHIV-CMV-oIL10 as described in Section 2.2.2.2, to investigate whether the correct gene product would be produced at the protein level. A reporter gene plasmid, pHIV-SV40-eYFP, and the pHIV-SV40-oIL10 plasmid were used as transfection controls, along with untransfected cells.

At three days post transfection, the reporter gene was visualised by fluorescence microscopy to be present in approximately 95% of the cells transfected. A sandwich ELISA was performed on the culture supernatant samples at day 5 post transfection, to assay for the presence of IL-10. This was performed as described in Section 2.2.13.10. Ovine interleukin-10 was found to be present in the supernatant of both of the IL-10 constructs (Figure 3.6).

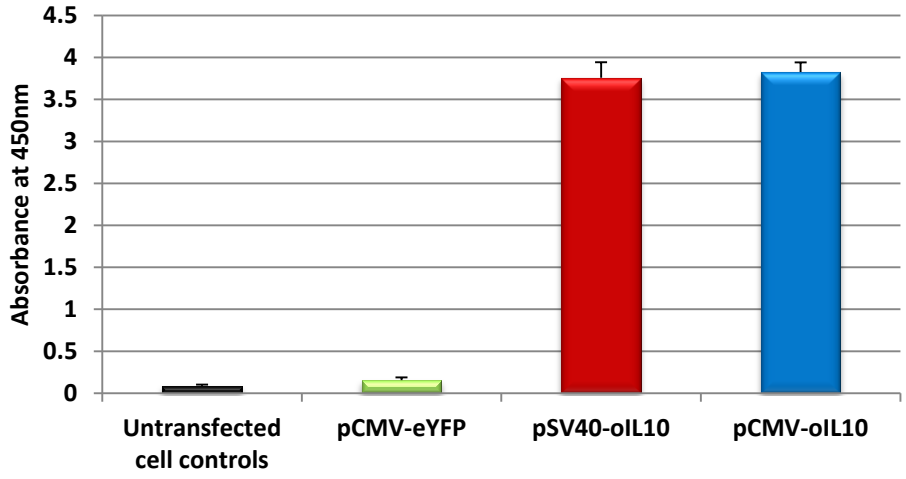


Figure 3.6: Bars show mean ovine interleukin-10 detected at the protein level by sandwich ELISA from HEK-293A transfection culture supernatants five days post transfection with 1.6 µg of plasmid DNA (biological replicates n= 1). Supernatant samples were tested at a 1:50 dilution. Error bars are standard deviation of the technical replicates, n= 3. The absorbance sensitivity of the microplate reader range reached a maximum at approximately 4 on the scale, therefore these results are a qualitative indication that the new construct, pHIV-CMV-oIL10, expressed IL-10.

3.4.3 In vitro Testing of Transgene Expression from Two Lentivirus Preparations using Mammalian Cell Culture

Lentivirus preparations were made using the lentiviral shuttle plasmids, pHIV1-CMV-oIL10 and pHIV1-SV40-oIL10, as described in Section 2.2.4.

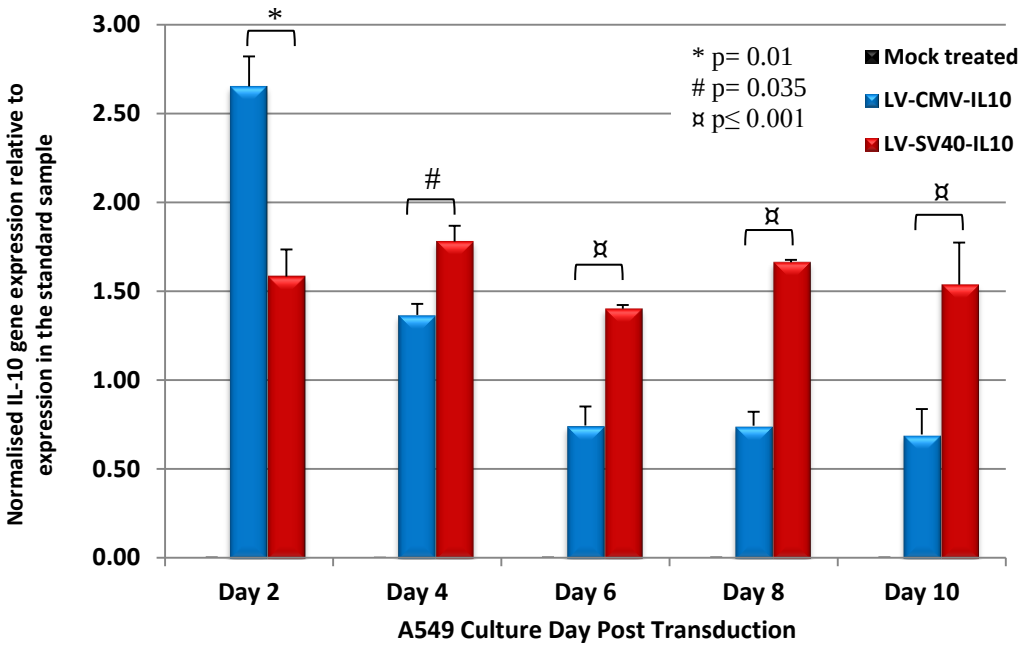
Two adherent human cell lines were used to examine interleukin-10 transgene expression from the two lentivirus preparations. In one preparation, transgene expression was controlled by a constitutive viral promoter CMV (LV-CMV-oIL10), and in the other transgene expression was controlled by the constitutive viral promoter SV40 (LV-SV40-oIL10). A549 and HEK-293A cells were transduced in triplicate with the two lentivirus preparations at an MOI of 1.6 (4 $x10^5$ TU/well) for 24 hours, the cells cultured for 10 days and passaged every two days (Section 2.2.2.1). The cell culture supernatants were assayed for IL-10 protein every two days (Section 2.2.13.10) and mRNA was extracted from the cells ten days post-transduction, and triplicates pooled for real-time quantification of ovine IL-10 expression in the cDNA, using reference genes human acidic ribosomal phosphoprotein (ARBP) and human β-actin (Sections 2.2.13.4 – 2.2.13.9).

In the A549 cell line, the CMV promoter induced high levels of IL-10 mRNA initially, significantly higher than the SV40 promoter at day 2 ($p= 0.01$, independent-samples t-test), however the CMV-induced IL-10 mRNA levels then dropped to a stable but significantly lower level than SV40-induced expression at all subsequent time points (day 4 $p= 0.035$, days 6, 8, 10 $p\leq 0.001$) (Figure 3.7(A)). In contrast, the HEK-293A cells showed excellent expression of IL-10 induced by the CMV promoter, giving significantly higher expression than SV40 across all time points of the culture (days 2, 10 $p\leq 0.001$, days 4, 6 $p= 0.001$, day 8 $p= 0.002$) (Figure 3.7(B)).

The mRNA expression of IL-10 by these two cell lines was reflected at the protein level. It was found that the SV40 promoter controlled stable transgene expression for the culture duration in both the A549 and HEK-293A cells, producing similar quantities of IL-10 protein in both cell lines (Figure 3.8). Strong IL-10 expression was seen to have been induced by the CMV promoter in the A549 cells at the two early time points (day 2 and day 4), however this expression was not maintained for the duration of the culture, with SV40-induced expression being significantly higher than CMV-induced expression at days 8 and 10 ($p= 0.045$, $p= 0.026$) (Figure 3.8(A)). This decline in transgene expression in the LV-CMV-oIL10 treated cells over time was not seen in the HEK-293A culture, where CMV-induced IL-10 expression was significantly higher than the expression driven by the SV40 promoter across all five culture time points (p-values = 0.003, 0.004, 0.006, 0.008, 0.006, days 2 through to 10, respectively) (Figure 3.8(B)). The IL-10 protein detected in the HEK-293A cell culture supernatants was notably logs higher than in the A549 culture supernatants.

Figure 3.7: Bars represent normalised ovine IL-10 expression at the mRNA level by (A) A549 cells and (B) HEK-293A cells 10 days after transduction with either a lentivirus containing the oIL-10 transgene under the control of a CMV promoter or under the control of the SV40 promoter (log scale). Transgene quantification by relative quantitative RT-PCR. No bars are visible for the mock-treated A549 cells as ovine IL-10 was undetected in the cDNA. Ovine IL-10 was not detected in either human cell line, bars are only visible for mock-treated HEK-293A cells in (B) due to the log scale. Biological replicates n= 6 (A549 cells) n= 3 (HEK-293A cells). Error bars represent the standard deviation in the PCR technical replicates, n= 3. P-values (A) *p= 0.01 (day 2), #p= 0.035 (day 4), ¤p≤ 0.001 (days 6, 8, 10); (B) *p≤ 0.001 (days 2, 10), #p= 0.001 (days 4, 6), ¤p= 0.002 (day 8).

A


3.00
2.50
2.00
1.50
1.00
0.50
0.00
Normalised IL-10 gene expression relative to expression in the standard sample
* p= 0.01
# p= 0.035
¤ p≤ 0.001
Mock treated
LV-CMV-IL10
LV-SV40-IL10
*
#
¤
¤
¤
Day 2
Day 4
Day 6
Day 8
Day 10
A549 Culture Day Post Transduction

B

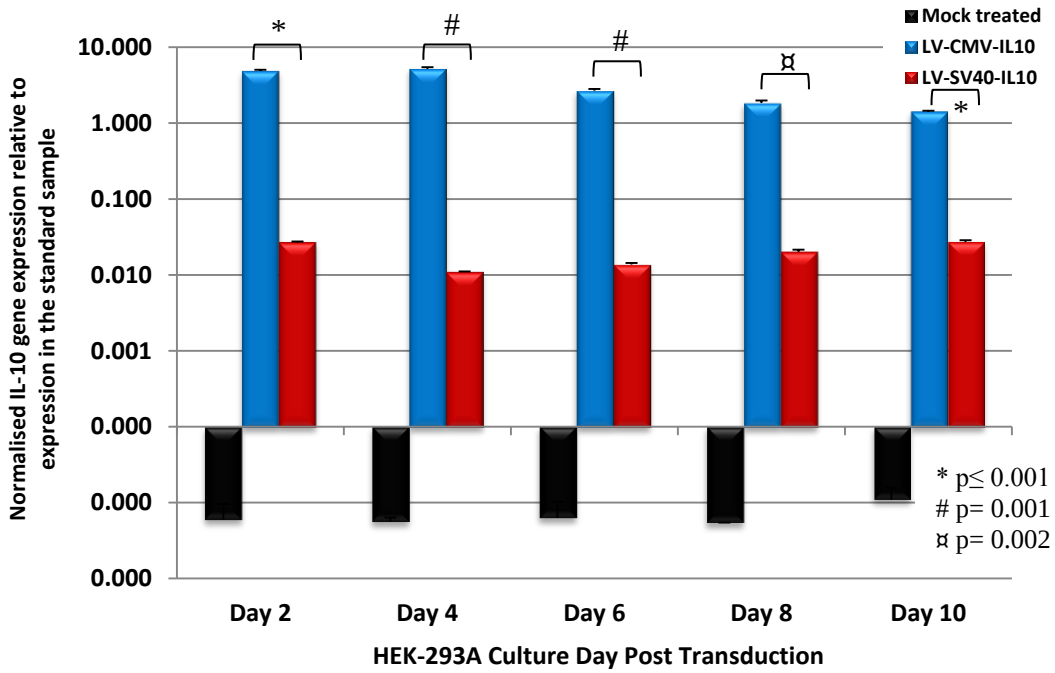
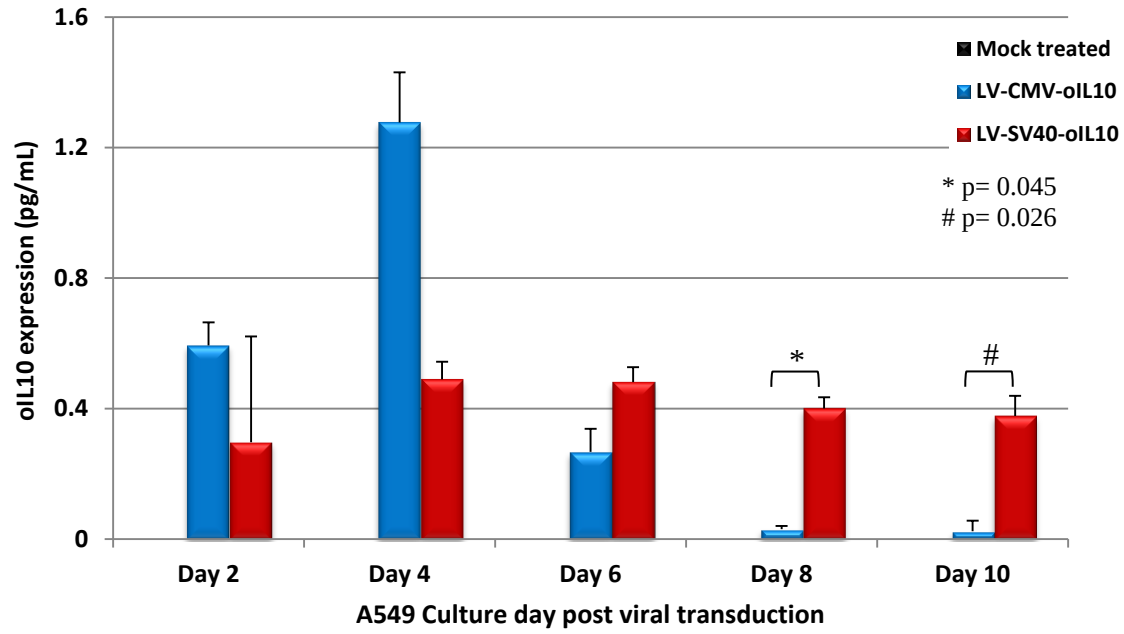
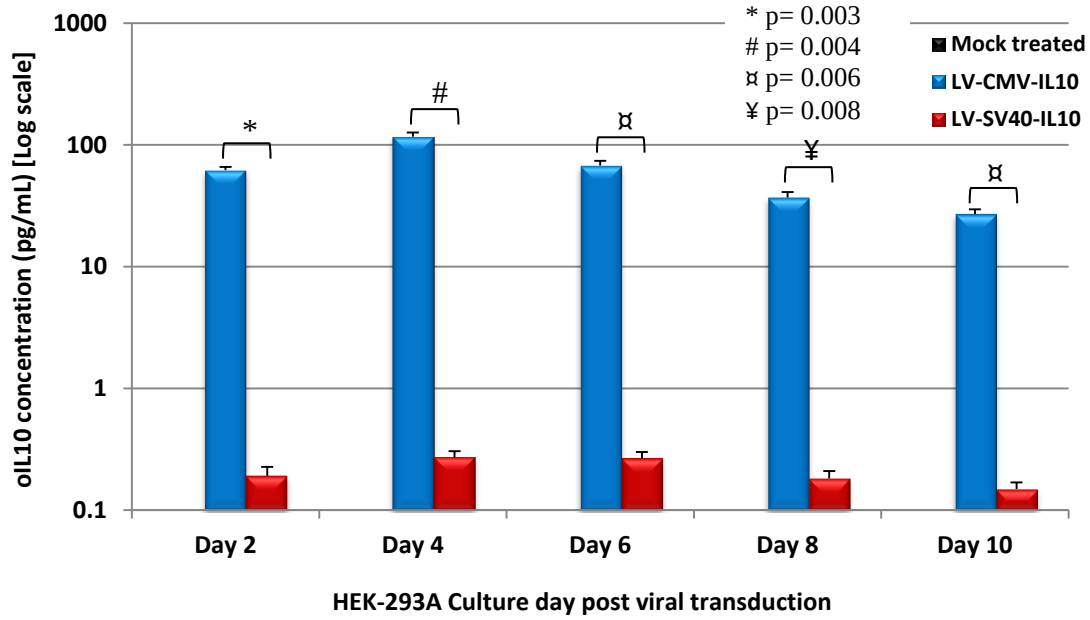

10.000
1.000
0.100
0.010
0.001
0.000
0.000
0.000
Normalised IL-10 gene expression relative to expression in the standard sample
Mock treated
LV-CMV-IL10
LV-SV40-IL10
*
#
#
¤
*
* p≤ 0.001
# p= 0.001
¤ p= 0.002
Day 2
Day 4
Day 6
Day 8
Day 10
HEK-293A Culture Day Post Transduction

Figure 3.8: Bars show mean ovine interleukin-10 protein expression (pg/mL), measured by sandwich ELISA, in the supernatant of (A) A549 cells and (B) HEK-293A cells transduced with LV-CMV-oIL10 or LV-SV40-oIL10 over a ten day culture period. Mock-treated control cells were not transduced with lentivirus. These samples were tested for oIL-10, however the bars do not appear on the graphs as the human cell lines did not express ovine IL-10. Error bars show the standard deviations of the biological replicates, n = 3. This experiment was conducted twice using the A549 cell line and a similar trend in oIL-10 expression was seen across the culture time points in both replications. P-values (A) *p= 0.045 (day 8), #p= 0.026 (day 10); (B) *p= 0.003 (day 2), #p= 0.004 (day 4), ¤p= 0.006 (days 6, 10), ¥p= 0.008 (day 8).

A


1.6
1.2
0.8
0.4
0
oIL10 expression (pg/mL)
Mock treated
LV-CMV-oIL10
LV-SV40-oIL10
* p= 0.045
# p= 0.026
*
#
Day 2
Day 4
Day 6
Day 8
Day 10
A549 Culture day post viral transduction

B


1000
100
10
1
0.1
oIL10 concentration (pg/mL) [Log scale]
* p= 0.003
# p= 0.004
¤ p= 0.006
¥ p= 0.008
Mock treated
LV-CMV-IL10
LV-SV40-IL10
*
#
¤
¥
¤
Day 2
Day 4
Day 6
Day 8
Day 10
HEK-293A Culture day post viral transduction

3.4.4 In vitro Testing of Transgene Expression from Two Lentivirus Preparations using Ovine Corneas

Excised corneas from donated sheep eyes were prepared as described in Sections 2.2.8 - 9. The corneas were transduced with the lentivirus containing the gene IL-10 under the control of a CMV promoter (LV-CMV-oIL10), n= 5, or the lentivirus containing IL-10 under the control of the SV40 promoter (LV-SV40-oIL10), n= 6, at an MOI of 20, 2.8 x 10^7 transducing units/ cornea. Control corneas were mock-transduced with Hepes-buffered RPMI 1640 medium with 2% (v/v) FBS, PS and glutamine, n= 5. The transduction duration was 3 hours at 37^{o}C. The corneas were then cultured in 10 mL of Hepes-buffered RPMI 1640 medium with 10% (v/v) FBS, PS, glutamine and amphotericin B for 10 days, with the medium completely replenished every two days.

At day 10 the corneal endothelial layer was dissected off each cornea. Quantitative real-time PCR was performed on the cDNA prepared from the extracted RNA from the endothelial cells, for the quantification of transgene expression, using reference genes ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ovine β-actin, as described in Sections 2.2.13.3, 2.2.13.5 to 2.2.13.9. Expression of oIL-10 in LV-CMV-oIL10-treated corneal endothelial cells from combined corneas (n= 5) was 4.4-fold higher than expression in the standard sample. This was significantly higher expression than the 0.12-fold increase in oIL-10 expression seen with the LV-SV40-oIL10-treated corneal endothelial cells from combined corneas (n= 6), (p= 0.006, Mann-Whitney U Test), see Figure 3.9. Ovine IL-10 mRNA expression levels were seen to vary considerably between individual corneas, particularly with the CMV promoter.

Supernatant samples were collected every two days and assayed for IL-10 expression by sandwich ELISA. The lentivirus containing the CMV promoter was found to induce significantly higher transgene expression than SV40-induced expression in ovine corneas at every time point of the culture ($p < 0.001$ at each time point, one-way between-groups ANOVA, with Dunnett T3 post-hoc analysis) (Figure 3.10).

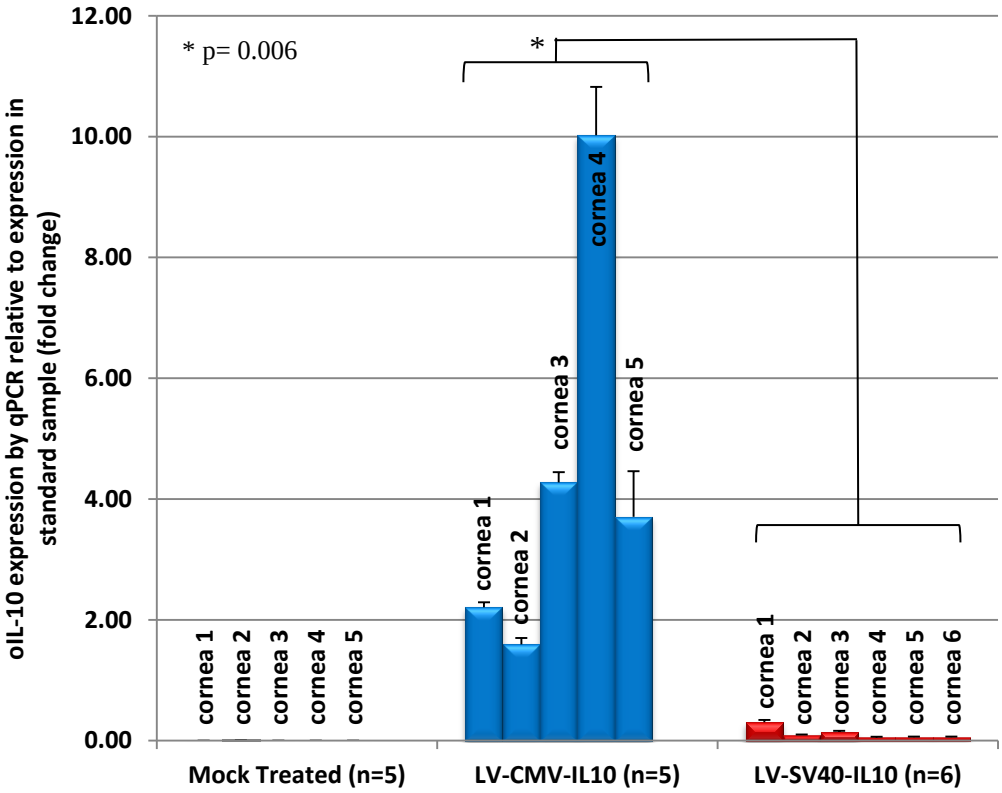


Figure 3.9: Bars show ovine interleukin-10 mRNA expression in ovine corneal endothelial cells from individual cultured corneas transduced with LV-CMV-oIL10 or LV-SV40-oIL10 harvested at day 10 post transduction. Expression was measured at the message level by quantitative real-time PCR, and fold-change normalised relative to the expression in the standard sample. LV-CMV-oIL10-treated corneas showed significantly higher transgene expression than expression driven by the SV40 promoter ($p = 0.006$). Mock-treated control corneas were sham-transduced with medium alone. Error bars show the standard deviations of the technical qPCR replicates, n= min 3, max 6.

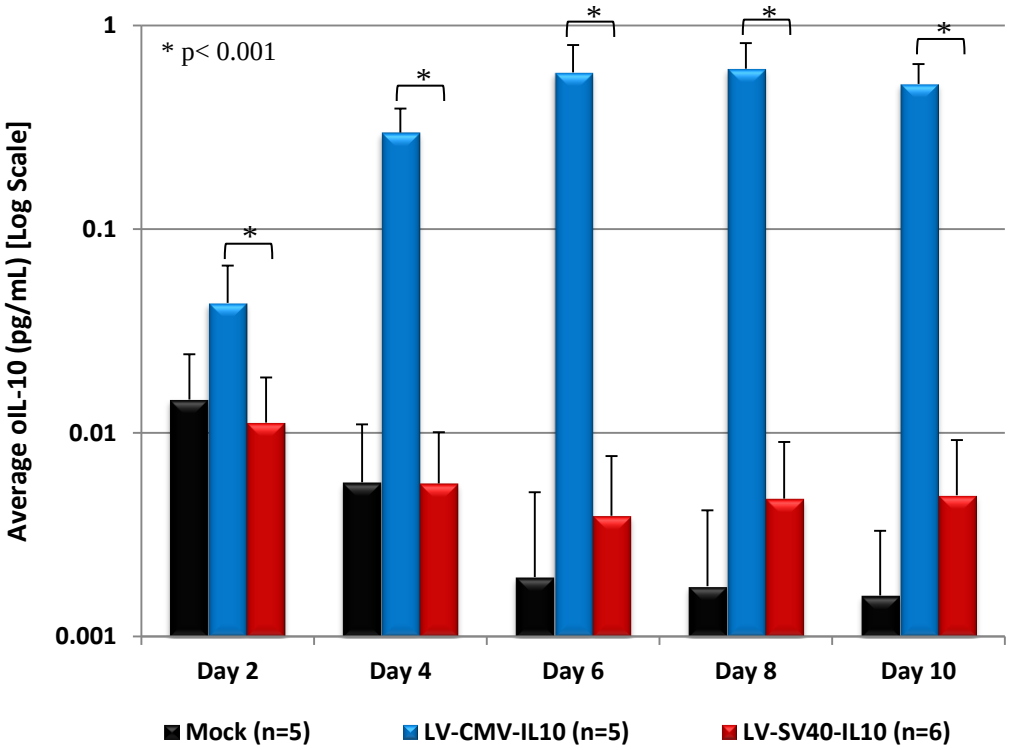


Figure 3.10: Bars show mean ovine interleukin-10 protein expression (pg/mL), measured by sandwich ELISA, in the supernatant of cultured ovine corneas transduced with LV-CMV-oIL10 or LV-SV40-oIL10 over a ten day culture period. LV-CMV-oIL10-treated corneas showed logs higher transgene expression than endogenous levels and expression driven by the SV40 promoter. Mock-treated control corneas were sham-transduced with medium alone. Error bars show the standard deviations of the biological replicates, n= 5 (Mock-treated, LV-CMV-oIL10) or 6 (LV-SV40-oIL10). *p< 0.001.

3.4.5 Protamine Sulphate to Enhance Lentivirus Transduction in the Ovine Cornea

Corneal transduction medium was prepared as described in Section 2.2.9. The lentivirus containing the reporter gene enhanced yellow fluorescent protein (eYFP) under the control of the SV40 promoter (LV-eYFP), was used at an MOI of 20. Protamine sulphate was added to the transduction medium at a final concentration of 0, 8, 40 or 100 μg/mL. The transduction medium containing LV-eYFP and 100 μg/mL protamine sulphate was pre-incubated at 37^{o}C for 15 minutes prior to application onto corneas.

Excised corneas from abattoir sheep eyes were prepared as described in Sections 2.2.8 - 9. The corneas were transduced with the transduction media for three hours at 37^{o}C. The corneas were then cultured and were harvested at either day 5 or day 10. Each time point and treatment included three corneas (n= 3), with nine control corneas transduced without protamine sulphate (n= 9). Corneas were fixed with buffered formalin for 10 minutes prior to staining with Hoechst 33258 for 30 minutes. The endothelium was then dissected from each of the corneas and flat-mounted on a microscope slide for eYFP expression analysis using a fluorescence microscope, as described in Section 2.2.13.1 - 2. Five representative field images were taken from each endothelial flat-mount. The Hoechst-stained endothelial nuclei and the eYFP expressing cells were counted for each field to gain an average number of cells successfully transduced. Figure 3.11 shows representative images of Hoechst-stained ovine cornea endothelial cell nuclei and the corresponding eYFP expression from that same image field.

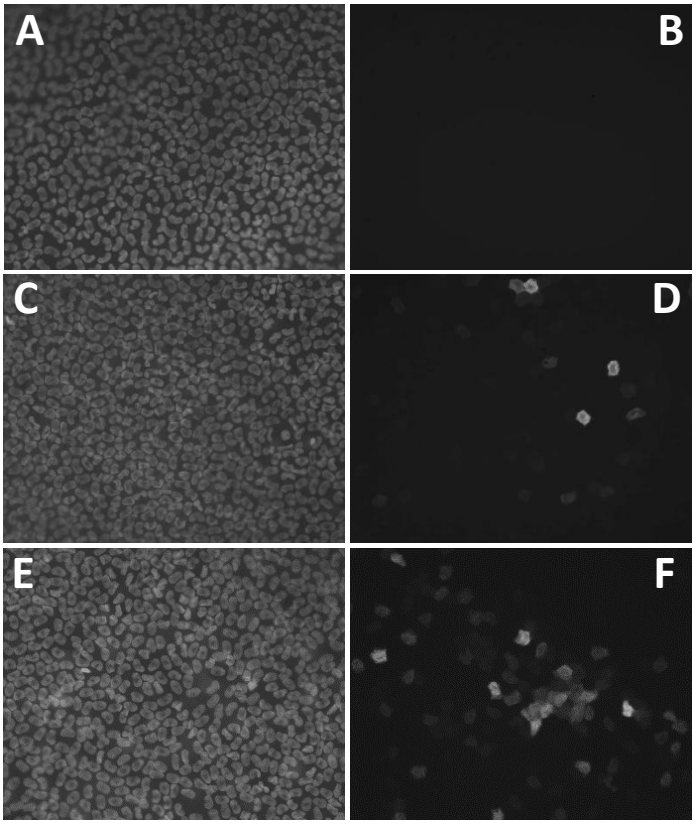


Figure 3.11: Representative microscope field images of Hoechst 33258-stained endothelial cell nuclei from ovine corneas (A, C, E) and the corresponding eYFP expression from the same field (B, D, F). A and B untransduced, C and D transduced with LV-eYFP, E and F transduced with LV-eYFP and protamine sulphate (100 μg/mL) in transduction medium. All images 200x original magnification.

The percentage of corneal endothelial cells expressing eYFP with increasing concentrations of protamine sulphate is shown in Table 3.1, at an early transduction time point (day 5) and at a late transduction time point (day 10), A and B, respectively.

At the early time point, five days post-transduction, the treated endothelial cells showed significantly enhanced eYFP expression at the protamine sulphate concentration of 100 μg/mL, with 7% of endothelial cells fluorescing compared with virus alone at 0.5% ($p < 0.001$, one-way between-groups ANOVA, Dunnett T3 post-hoc analysis). At the later time point, ten days post transduction, two of the protamine sulphate treatments showed significantly higher percentages of successfully transduced endothelial cells with 9% and 10% of cells fluorescing at concentrations 40 and 100 μg/mL, respectively, compared with the virus alone treatment, which achieved 2% of endothelial cells expressing eYFP ($p = 0.01, 0.001$, respectively).

Table 3.1: Mean cell counts with standard deviations of ovine corneal endothelial cells transduced with LV-eYFP and protamine sulphate in the transduction media at concentrations of 0, 8, 40 and 100 µg/mL. Cell count numbers represent average counts of endothelial cell nuclei from five image fields per cornea.

	Day 5								Day 10							
	Protamine sulphate concentration								Protamine sulphate concentration							
	0 µg/mL		8 µg/mL		40 µg/mL		100 µg/mL		0 µg/mL		8 µg/mL		40 µg/mL		100 µg/mL	
Corneas (n)	9		3		3		3		9		3		3		3	
Mean endothelial cells	523	±73.8	484.5	±26.6	507.6	±37.2	624.5	±75.8	543.9	±65	497.8	±23.1	524.2	±51.3	580.6	±51.8
Mean eYFP cells	2.6	±3.8	0.33	±1	11.2	±14.4	42.7	±27.2	16	±11.3	28.1	±20	45.9	±29.9	58.7	±34.2
% Transduction	0.5%		0.001%		2%		7%*		2.3%		5.6%		9%#		10%¤	

*p< 0.001, #p= 0.01, ¤p= 0.001

3.4.6 Increasing Multiplicity of Infection of the Lentivirus in the Ovine Cornea

Excised corneas from donated sheep eyes were prepared as described in Section 2.2.8. The corneas were transduced with the lentivirus containing the transgene ovine interleukin-10 (oIL-10) under the control of a CMV promoter (LV-oIL10), in a transduction medium prepared as described in Section 2.2.9. The transduction media contained the lentivirus at multiplicity of infections of 40 and 100 (5.6×10^7 and 1.4×10^8 transducing units/ cornea, respectively). The corneas were transduced for three hours at 37^{o}C and cultured for ten days in 10 mL of Hepes-buffered RPMI 1640 medium with 10% (v/v) FBS, PS, glutamine and amphotericin B. IL-10 expression was measured every two days at the protein level by sandwich ELISA. At day 10, corneas were fixed with buffered formalin for 10 minutes prior to staining with Hoechst 33258 for 30 minutes. The endothelium was then dissected from each of the corneas and flat-mounted on a microscope slide for visualisation with a fluorescence microscope. Five representative field images were taken from each endothelial flat-mount, see Figure 3.12 for an example field image. The Hoechst-stained endothelial nuclei in each field were counted to obtain an average number of cells per field.

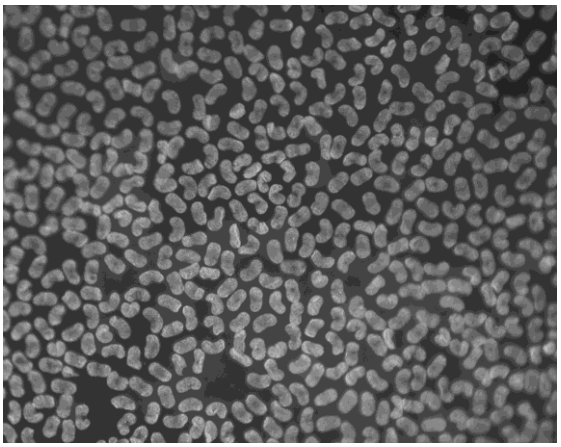


Figure 3.12: Example field image of Hoechst 33258-stained untransduced ovine cornea endothelial cell nuclei (200x original magnification).

Figure 3.13 shows the IL-10 detected in the culture supernatant of ovine corneas transduced with the lentivirus containing the oIL-10 transgene at varying MOIs. The viral dose of MOI 100 resulted in a 1.2- to 2.3-fold increase in protein expression across the culture time points compared with the dose of MOI 40. No significant difference was found between expression levels with an MOI of 40 or with an MOI of 100 at time points day 2 and day 8 (p= 0.17, p= 0.28, respectively, one-way ANOVA between-groups analysis, Dunnett T3 and Tukey HSD post-hoc analysis), however a significant difference in IL-10 protein expression was found between the two treatments at days 4, 6 and 10 (p= 0.001, p= 0.01 and p= 0.002, respectively).

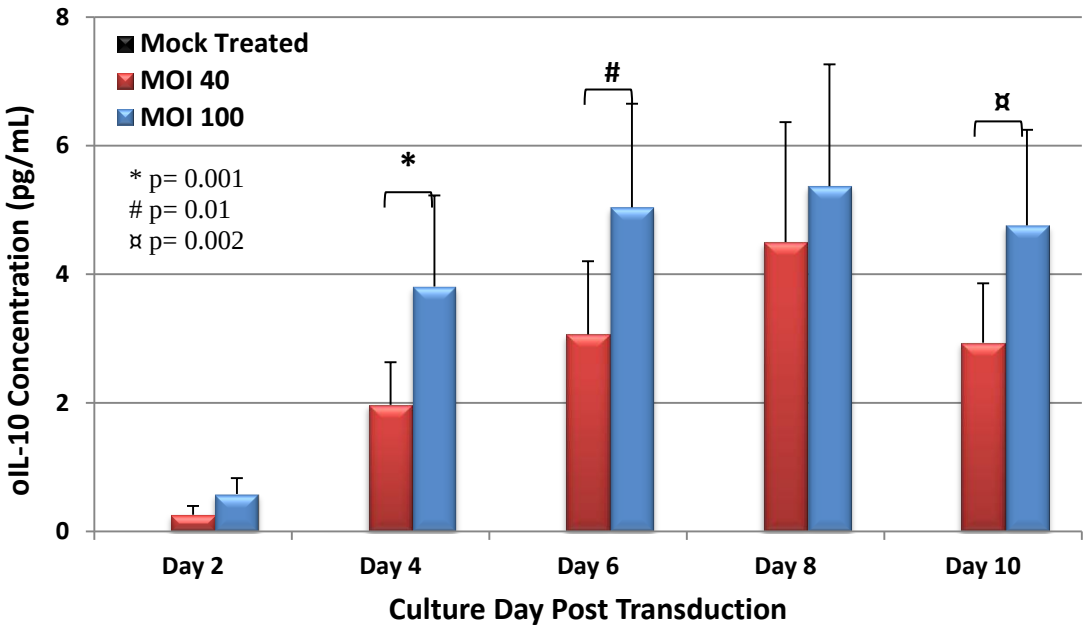


Figure 3.13: Bars represent mean ovine IL-10 protein detected in the culture supernatant of ovine corneas transduced with LV-CMV-oIL10, measured by sandwich ELISA. Mock treated corneas were found to have very low levels of IL-10, which are not visible in this figure. The experiment comprised three biological corneal replicates for each treatment (n= 3). This experiment was repeated with the same number of biological replicates, and a similar trend in oIL-10 protein expression was observed. Error bars show the mean ± standard deviation of the biological replicates. * p= 0.001 (day 4), # p= 0.01 (day 6), ¤ p= 0.002 (day 10).

Table 3.2 shows the endothelial cell counts from mock transduced ovine corneas and ovine corneas treated with the lentivirus containing the IL-10 transgene at an MOI of 40 or 100 transducing units per cell. There was no significant difference between the mock transduced and the virus-treated cell nuclei counts (p= 0.49, one-way between-groups ANOVA), and an increase in viral dosage did not adversely affect the average cell nuclei numbers, showing that an MOI of 100 with this virus did not have a microscopically visible toxic effect on the endothelial cells.

Table 3.2: Average number of corneal endothelial cells per image field in mock transduced or lentivirus-treated ovine corneas at two different MOIs.

Cornea Treatment	Biological n-value	Total Fields	Endothelial Cell Count (field average)	Std Dev (between fields)
Mock transduced	6	30	**457**	84
LV-IL10 MOI 40	7	35	**479**	44
LV-IL10 MOI 100	7	35	**495**	34

p= 0.49 (one-way between-groups ANOVA)

3.4.7 Therapeutic Testing of the Lentivirus in vivo

Based on *in vitro* testing, the lentivirus containing the CMV promoter controlling transgene expression was selected for subsequent therapeutic testing, at an MOI of 40 viral particles per target cell. The ovine interleukin-10 transgene had been previously tested for biological activity.[202] The lentivirus preparation was tested for level of endotoxin contamination using the LAL test described in Section 2.2.4.3 and determined to contain between 420 – 480 endotoxin units per millilitre. This test was performed by Ms Lauren Mortimer (Dept. Ophthalmology, Flinders University SA). The total amount of endotoxin units in the lentiviral treatment medium was between 3.8 and 4.3 units per cornea.

Donor sheep corneas were prepared as described in Sections 2.2.8 - 9. Corneas were transduced for 3 hours with LV-CMV-oIL10 at 5.6 x 10^7 TU/cornea (MOI 40) in Hepes-buffered RPMI medium 1640 containing 2% (v/v) FBS and PS. Control corneas were mock-transduced with medium alone. Penetrating keratoplasties were performed on the sheep by an ophthalmologist, as described in Section 2.2.10. The lentiviral treatment group and the mock-transduced control group each consisted of ten animals. The remaining donor corneal rims were cultured in RPMI 1640 with 10% (v/v) FBS, PS, FZ for five days, with supernatant samples taken on days 2 and 5 to assay for transgene expression at the protein level (Sections 2.2.10.4 and 2.2.13.10).

Grafts were assessed daily with a hand-held slit lamp for inflammation, neovascularisation, clarity, oedema, presence of inflammatory cells and fibrin formation. Score sheets are shown in Appendix A3.2. The onset of immunological rejection was judged to be upon the presence of a rejection line or a corneal clarity score of 2, on a scale of 0 - 4. Grafts were considered technical failures if they did not become thin and clear by 10 days post-operatively and the survival days of these sheep grafts were not considered in the results. Two experimental animals were considered technical failures out of a total of 26 animals for this reason. Representative images of corneal grafts in the sheep can be seen in Figure 3.14.

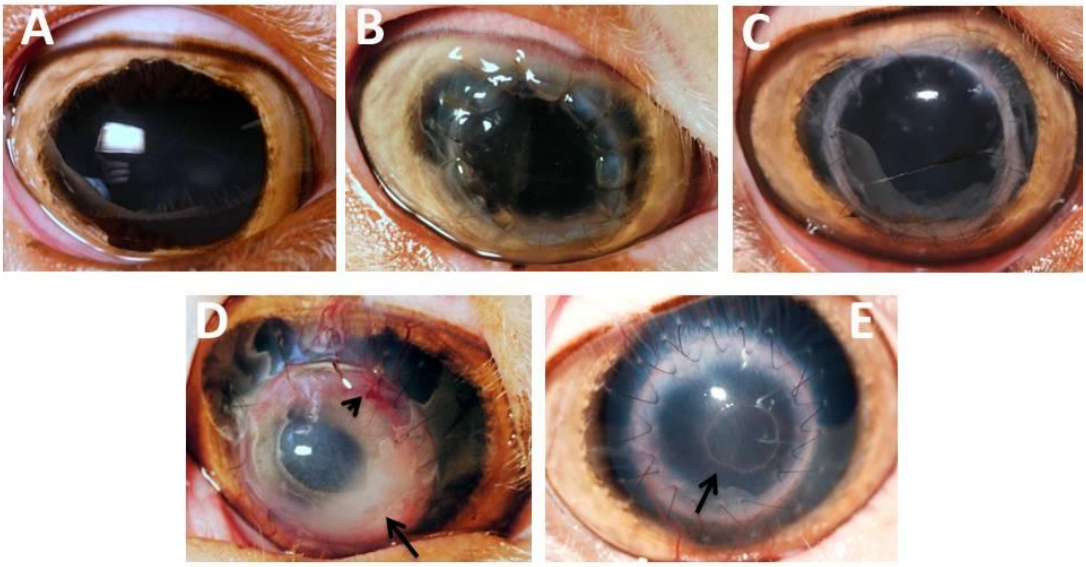


Figure 3.14: Example images of sheep eyes at varying stages of penetrating keratoplasty. A) unmodified sheep eye **B)** graft at 4 days post-operative (p.o.) **C)** clear healed graft at 21 days p.o. **D)** rejecting graft at 43 days p.o., note swelling of the graft, loss in clarity (arrow) and infiltrating vessels (arrow head) **E)** rejecting graft at 68 days p.o., note appearance of circular rejection line (arrow). Photographs courtesy of Ms Angela Chappell and Mr Joel Johnston, Ophthalmic Imaging, Department of Ophthalmology, Flinders Eye Centre.

Histological examination of the graft tissue post-mortem was conducted by an ophthalmic pathologist, A/Professor Sonja Klebe, on haematoxylin and eosin-stained specimens. Any grafts showing a neutrophilic cellular infiltrate that was not consistent with immunological rejection, instead indicating a possible infection, were not considered in the results. Two animals out of a total of 26 were removed from results due to histological examination, in one case due to an inverted graft button and the other having graft tissue with an aberrant, crumpled appearance. Figure 3.15 shows example images of haematoxylin and eosin-stained sheep cornea sections.

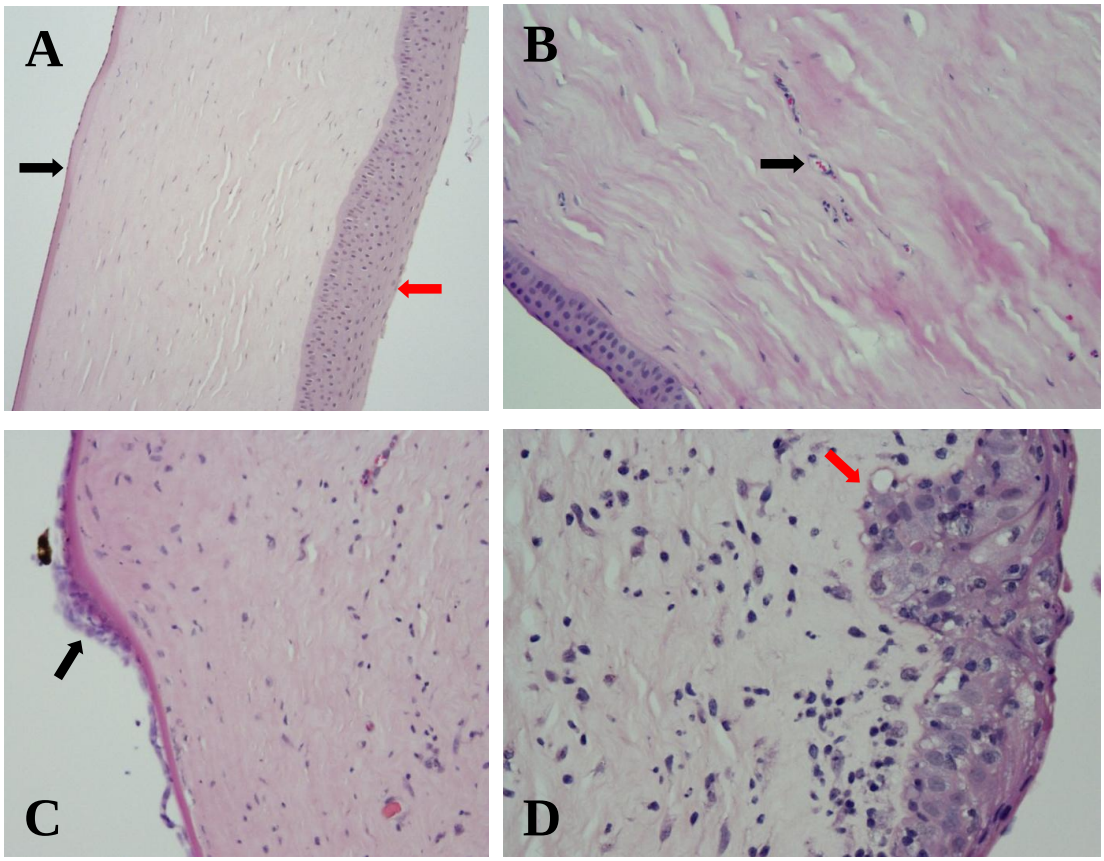


Figure 3.15: Haematoxylin and eosin stained sheep cornea sections (5µm). (A) Normal sheep cornea, 100x original magnification. Black arrow indicates the corneal endothelium on Descemet's membrane, red arrow indicates the corneal epithelium. **(B)** Vascularised sheep cornea, 200x original magnification. Black arrow shows vessel growth in the corneal stroma. **(C)** Endothelial rejection of sheep corneal graft, 200x original magnification. Black arrow indicates the leucocytes attacking the endothelial cells. **(D)** Epithelial rejection of sheep corneal graft, 400x original magnification. Stromal infiltrate is also visible. Red arrow indicates cellular infiltrate into the epithelium.

The culture supernatants of the rims from donor corneas used in sheep corneal graft surgery were assayed for the expression of ovine IL-10. This was done to investigate whether transgene expression should be expected from the graft tissue. Table 3.3 shows the levels of oIL-10 detected by sandwich ELISA at days two and five post-operatively. At day 2, an average 4-fold increase in oIL-10 was observed in corneas transduced with LV-CMV-oIL10 relative to the mock-transduced corneas, and at day 5 this rose to an average of approximately 300-fold higher expression. This demonstrated the successful secretion of transgenic IL-10 by the transduced corneal endothelial cells in the donor rims.

After the onset of immune rejection and euthanasia of the animals, anterior chamber (AC) fluid was collected from both the treated eye and the untreated eye and assayed for the presence of IL-10 by sandwich ELISA. Table 3.3 shows the levels of IL-10 detected in the anterior chamber fluid for each animal. These results show an average 8-fold increase in IL-10 expression in LV-treated grafts compared to the mock-treated grafts.

Table 3.3 indicates that six out of the nine LV-treated sheep grafts that were tested showed increased oIL-10 expression in the anterior chamber fluid of the treated eye, and all ten of the cultured rims showed over expression of oIL-10 compared with the mock-transduced rims. IL-10 protein levels detected in the anterior chamber fluid of the treated sheep grafts were quite low, possibly reflecting aqueous humour turnover. Earlier anterior chamber sampling might have indicated higher levels, but this would have been conducted with the risk of inflaming the graft area and inducing rejection onset. There was, however, no association between the graft survival time and oIL-10 expression levels seen. One of the treated sheep was not tested for the presence of IL-10 expression in the anterior chamber fluid of the treated and untreated eyes as this animal did not undergo rejection of the corneal allograft and was not euthanised before the conclusion of the project.

Table 3.3: Ovine IL-10 protein detected in the anterior chamber fluid of lentivirus-treated and untreated sheep eyes and in the donor rim culture supernatants, measured by sandwich ELISA. AC – anterior chamber; n/t – not tested, as sheep has not yet rejected allograft

Recipient Sheep			Donor Eye					
I.D.	Treatment	Sex	IL-10 detected in AC fluid (pg/mL)		IL-10 detected in Rim culture (pg/mL)			
			Treated eye	Untreated eye	Day 2	Day 5	Rejection day	AC fluid collection day
Sheep 1	LV-CMV-IL10	F	0.002	0	0.047	1.074	19	25
Sheep 2	LV-CMV-IL10	F	0.0003	0	0.052	0.969	21	30
Sheep 3	LV-CMV-IL10	F	0	0	0.102	2.080	23	30
Sheep 4	LV-CMV-IL10	F	0.002	0	0.011	0.669	24	31
Sheep 5	LV-CMV-IL10	F	0.019	0	0.145	2.273	24	30
Sheep 6	LV-CMV-IL10	M	0	0	0.011	0.244	28	29
Sheep 7	LV-CMV-IL10	F	0	0	0.042	0.708	39	50
Sheep 8	LV-CMV-IL10	F	0.001	0	0.035	0.549	43	44
Sheep 9	LV-CMV-IL10	F	0.0002	0	0.016	0.435	52	65
Sheep 10	LV-CMV-IL10	M	n/t	n/t	0.016	0.030	>151	not taken
Sheep 11	Mock-transduced	F	0	0	0.011	0.003	16	23
Sheep 12	Mock-transduced	F	0	0	0.005	0.000	18	22
Sheep 13	Mock-transduced	M	0.002	0	0.018	0.002	19	23
Sheep 14	Mock-transduced	M	0.001	0	0.012	0.006	19	20
Sheep 15	Mock-transduced	F	0	0	0.012	0.005	20	23
Sheep 16	Mock-transduced	F	0	0	0.018	0.003	22	28
Sheep 17	Mock-transduced	M	0	0	0.014	0.007	24	25
Sheep 18	Mock-transduced	F	0	0	0.013	0.002	27	29
Sheep 19	Mock-transduced	F	0	0	0.005	0.000	27	30
Sheep 20	Mock-transduced	F	0	0	0.003	0.002	28	31

Table 3.4 shows the graft survival of the individual sheep in days post-surgery; the median survival days and the median day of vessel migration into the graft tissue. The results showed a significant prolongation of graft survival in the therapeutic lentivirus-treated animals ($p= 0.043$, Mann-Whitney U test). The survival of the corneal allografts by treatment is presented in a Kaplan-Meier survival plot in Figure 3.16. The Log-Rank (Mantel-Cox) significance of this survival plot had a value of $p= 0.025$, which showed there was a significant difference of survival distributions between the lentivirus IL-10 treated sheep corneal allografts and the untransduced allografts. The values of p from the Mann-Whitney U test and the Log-Rank significance scores differ due to the differing data analysis methods, but in both cases, the data returned a significance level less than 0.05.

Treatment with IL-10 did not affect the day of vessel cross-over into the graft. Six individual sheep corneal allografts were removed from results out of a total of 26 animals, four for reasons described above, and two further cases were considered technical failures due to the snapping of the continuous suture.

Table 3.4: Graft survival of penetrating keratoplasties performed in sheep with donor corneas transduced with the lentiviral vector expressing ovine IL-10 induced by the CMV promoter. Median day of rejection and median day of vessels crossing the graft-host junction are shown for each treatment group.

Treatment Group	Day Vascularisation Crossed into Graft	Median Day of Vascularisation	Graft Survival Days	Median Day of Rejection
Mock-Transduced (n=10)	7,7,7,8,11,11,13,13,14,14	11	16,18,19,19,20,22,24,27,27,28	**21**
LV-CMV-oIL10 (n=10)	7,7,9,10,11,11,13,15,18,20	11	19,21,23,24,24,28,39,43,52,>151	**26***

* p= 0.043 compared to mock-transduced (Mann-Whitney U test)

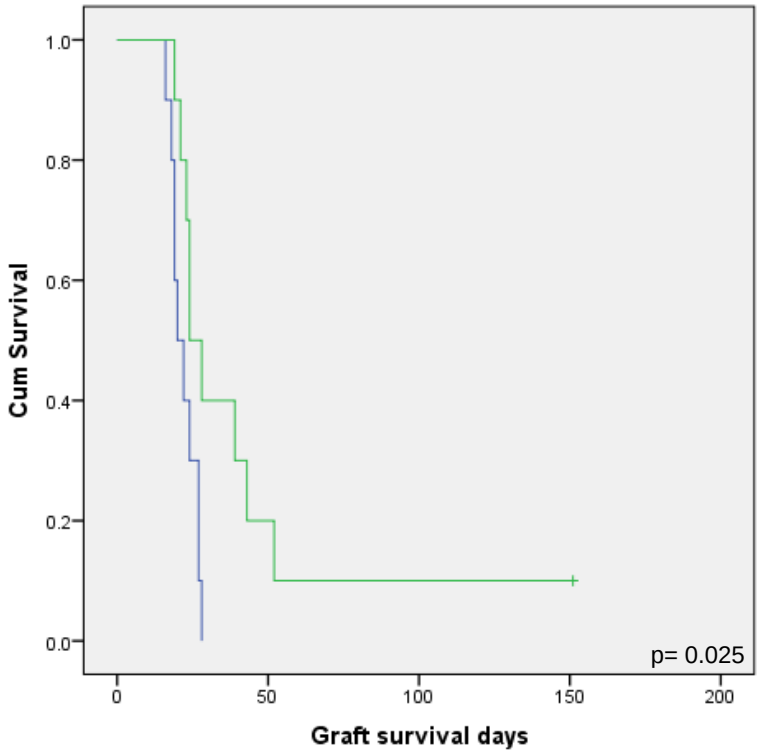


Figure 3.16: Kaplan-Meier survival plot showing the cumulative graft survival in days of the LV-IL10 treated sheep corneal grafts (green line) and the mock-transduced sheep corneal grafts (blue line). Log-rank (Mantel-Cox) statistic p= 0.025.

3.5 Summary and Discussion

3.5.1 Summary of Findings

The experiments presented in this chapter describe the outcomes of the therapeutic testing of a lentiviral gene therapy vector containing the transgene interleukin-10. The lentiviral vector was made, tested and trialled in a sheep model of penetrating keratoplasty. *In vitro* testing of the vector revealed that transgene expression induced by a CMV promoter was significantly higher than expression induced by the SV40 promoter in transduced 293A cells and ovine corneas. This was shown at both the mRNA and the protein level. The enhancement of lentiviral transduction by pre-

incubation with the polycation protamine sulphate gave a significant but modest increase in early (day 5) transgene expression at the highest dosage tested, 100 μg/mL. By day 10, the two higher dosages of protamine sulphate achieved modest but significantly higher transgene expression than lentivirus treatment alone. Increasing the multiplicity of infection from 40 to 100 viral particles per cell resulted in a modest increase in transgene expression, and no loss in ovine cornea endothelial cell density. When tested for therapeutic effect *in vivo*, although not effective at prolonging corneal allograft survival in all treated animals, the lentiviral vector containing IL-10 with a CMV promoter induced an overall significant prolongation of corneal graft survival in the sheep.

3.5.2 Discussion

3.5.2.1 Internal Transgene Promoter Comparison

Although touted as a strong promoter and commonly used for gene expression in gene therapy applications, the CMV promoter gave mixed transgene expression results. *In vitro* transgene expression in both human cell lines tested showed, at both the mRNA and protein levels, excellent initial expression driven by CMV, peaking at day 2 or 4, before a continued decline in expression. Other studies have noted this same decrease in transgene expression under the control of a CMV promoter.[243, 244] Zhou *et al.* reported a decrease in transgene IL-10 expression in plasmid electroporation of mice corneas, after an initial peak at day 1 when using a CMV promoter.[245] The possible causes for the decline in transgene expression *in vivo* could be due to intracellular degradation, lack of nuclear localisation signals, transcriptional silencing, an immune response to the transgene, or promoter inactivation. In the study by Zhou *et al.*, the authors ruled out the possibility that an immune response to the gene product was reducing expression, as re-dosing of the

therapy increased the transgene expression levels to slightly higher than original levels.[245] Needless to say this is also unlikely to be the case in our *in vitro* cultures. A lack of nuclear localisation signal is not likely to be the cause of reduced transgene expression in our scenario as the lentiviral vector has a nuclear localisation signal in the matrix protein encoded by the gag gene.[246, 247]

The expression drop is also unlikely to be a cellular response to the vector or transgene, since I observed that transgene expression induced by the SV40 promoter was reasonably stable for the duration of all *in vitro* cultures, although a slight decrease in expression was seen at the protein level.

CMV promoters can be subject to methylation and transcriptional silencing *in vivo* and *in vitro*.[220] Mehta *et al.* report that the site of integration is a critical factor in determining the degree of promoter inactivation. In our work, the CMV promoter may be experiencing some degree of epigenetic regulation in the form of promoter silencing. Schmitt *et al.* demonstrated differing expression levels induced by the CMV promoter in a variety of different tissues.[248] The markedly differing transgene expression levels induced by the CMV promoter in the two human cell lines I tested could be due to the cell lines having different organ origins, or perhaps the viral integration occurred in sites of varying transcriptional activity and consequently differing degrees of promoter silencing occurred.

An altogether different expression profile was observed, however, when ovine corneas were transduced, with CMV-induced transgene expression increasing over the duration of the culture, peaking at day 8. The major difference between cultured cell lines and organ cultured corneas is the replicative capacity of the target cells.

Unlike the human cell lines, ovine corneal endothelial cells are amitotic.[76] Iyer *et al.* have shown that transcription can occur from pre-integrated viral DNA in HIV-1 lentivirus-treated cells.[249] The authors mention that the high level of non-integrative transcription is not stand-alone but dependent on the integration of the provirus. In other words, integration needs to occur for the pre-integration transcription to occur at significant levels. Non-integrative expression from the lentivirus could explain why I observed excellent transgene expression from the non-replicative corneal endothelium. It is feasible that the drop in transgene expression in cell culture is due to the non-integrated virus subsequently being diluted out during cell divisions and passaging. This phenomenon was observed by Haas *et al.* in 293 cells transduced with a non-integrative lentiviral vector expressing GFP under the control of a CMV promoter.[217] However, although I saw a slight decrease in expression at the protein level with the SV40 promoter, the relatively stable expression of IL-10 by SV40 at the message level does not support this hypothesis. I suggest it is the nature of CMV promoters in cell culture to give an initial high expression peak before decreasing to a stable level. This has been seen in at least one other cell culture study, and two *in vivo* studies.[244, 245, 250] The cell culture experiments outlined in this chapter were taken to only ten days. Perhaps transgene expression would have stabilised with longer culture duration, however the health and integrity of the ovine corneal endothelial cells reduces with long culture periods.

The expression of IL-10 induced by the CMV promoter in the HEK-293 cells was higher than in the A549 cells at the protein level. This effect may have been be due to the use of HEK-293 cells, widely known for being highly transfectable; they accept and rapidly express plasmid DNA. Turner *et al.* showed that HEK-293 cells transduced with an adenoviral vector gave significantly higher viral expression than

similarly transduced A549 cells.[251] The significant difference between the cell lines, noted by the authors, is that the 293 cells are an E1-complementing cell line, supporting the replication of the E1-deleted adenoviral vector, and the A549 cells are non-complementing. This specific property however, should not have a significant impact on the uptake of the lentiviral vector. The lentiviral coat protein, vesicular stomatitis virus glycoprotein G (VSV-G), may have a higher abundance of receptor regions in the 293 cell plasma membrane than in the A549 cell, although this is speculative and difficult to investigate, since the VSV-G receptor has yet to be clearly defined.[252]

In contrast to the results with the CMV promoter, the transgene expression levels induced by the SV40 promoter at the protein level remained at similar levels in both cell lines. This may indicate a promoter-specific effect. The A549 carcinoma cell line may induce stronger promoter methylation events on the CMV promoter than the human embryonic kidney cell line.

It was noteworthy that a degree of variability was observed in transgene expression at the mRNA level in individually transduced ovine corneas (Figure 3.9). This was particularly evident with the transgene expression induced by the CMV promoter. These varying expressions from otherwise identically treated corneas indicates uncontrolled transduction efficiency, which may have led to varied *in vivo* treatment results.

Despite possible methylation events and varying transduction efficiencies, the CMV promoter was chosen for transgene expression in the subsequent *in vivo* work. This was due to the high expression of IL-10 induced by the CMV promoter in HEK-

293A cells and in ovine corneas *in vitro,* which were logs higher when compared with the expression induced by the SV40 promoter.

3.5.2.2 Enhancing Transduction Efficiency

The transduction of human corneas with lentiviral vectors *in vitro* resulted in strong early transgene expression.[178, 202] This was encouraging, considering the eventual point of this lentiviral gene therapy was for clinical treatment. There are several intervening stages, however, before the realisation of treatments in a clinical setting. The creation of a robust pre-clinical animal model is important when aiming to advance a therapy from the laboratory to the clinic. The sheep model of penetrating keratoplasty is a good clinical model in many respects, as discussed in chapter 1, however a lag in early transgene expression when sheep corneas are transduced with lentiviral vectors impedes the success of gene therapy of the ovine donor cornea.

The polycation investigated in this chapter, protamine sulphate, was shown to be weakly effective at enhancing the success of transgene expression. A study by Seitz *et al.* showed both protamine sulphate and polybrene to have similar transduction enhancement efficiencies to that shown by protamine sulphate in this chapter, in human keratocytes transduced with a retroviral vector.[212] However a 7% increase in the number of transduced target cells at ten days post treatment, although a statistically significant increase, was not a potentially clinically relevant increase in expression. A transduction enhancer needs to have much more impressive biological effectiveness, achieving levels of transgene expression comparable to those induced by adenoviral transduction to be worthy of continuing investigation *in vivo*. Previous *in vitro* findings from Parker (unpublished) with the polycation polybrene have shown significantly increased transgene expression, nine times higher at the protein

level in polybrene treated ovine corneas than in the virus alone.[203] Unfortunately polybrene has the toxic *in vivo* side effects previously mentioned (Section 3.2.5).

Conti *et al.* showed that mode of action of protamine sulphate as a transduction enhancer was cell line dependent: increasing vesicular stomatitis virus uptake in a human cell line, but decreasing viral uptake in a chicken fibroblast cell line.[253] In a study to enhance adenovirus transduction, Lanuti *et al.* found that the addition of protamine sulphate significantly increased gene expression in *in vitro* and *in vivo* experiments, however the results varied depending on the cell line examined.[216] The authors found the extent of the enhancement by protamine sulphate was inversely proportional to the amount of coxsackievirus and adenovirus receptor expression in the cell line. These studies indicate that although electrostatic interactions are important in viral particle and cell contact and uptake, the cell line and composition of the cell membrane receptors influences the efficacy of transduction enhancing agents.

It is clear there are other mechanisms and specific structural features involved with viral attachment to target cells. Overcoming electrostatic interactions between viral particles and cells with current polycation adjuvants is not a sufficient strategy to use to improve transduction enhancement in the sheep cornea.

3.5.2.3 Increasing the Multiplicity of Infection

Increasing the dose of viral treatments in gene therapy is a balance between achieving good transduction and over-burdening cells with the viral load. A larger viral dose has the potential to increase gene expression, but this relationship is not indefinitely linear. Gene expression will reach a plateau and begin to decrease when

the target cells are overcome by the toxic effects of too many viral particles. It is desirable to use the lowest MOI possible, to reduce the viral load on the target cells, but a high enough dosage to induce significant transgene expression. This will reduce the required volume of viral preparations and thus production costs, no insignificant consideration in lentivirus production.

In the results presented in this chapter, whilst increasing the MOI did make a modest increase in transgene expression, it did not increase early transgene expression, which, for interleukin-10, is possibly the critical time point necessary for therapy success.

When using an integrating vector such as the lentivirus, as the number of transducing units per target cell increases, the risk of multiple copies of the lentiviral genome per cell also increases and with that, the risk of insertional mutagenesis from random retroviral integration.[254] This may or may not have phenotypic consequences on the cells. In 2003 Hacein-Bay *et al.* reported that in a gene therapy trial for children with severe combined immunodeficiency (SCID), two of the ten children exhibited aberrant gene transcription and expression at an unrelated locus, despite the gene therapy being effective.[170] This trial involved the *ex vivo* transfer of the SCID-X1 gene to autologous bone marrow-derived CD34+ cells using a gamma retroviral vector, and the patients received injections of these modified cells. After 30 and 34 months, respectively, two children exhibited uncontrolled proliferation of mature T cells with leukaemia-like characteristics. The authors investigated the integration sites of the vector and detected multiple integration sites (≥ 50) in peripheral T cells before the onset of proliferation, however at the onset of the leukaemia-like proliferation, a single insertion site was predominant. The retroviral vector was

mapped to a region close to the promoter of the oncogene LMO2 locus in one patient and upstream of the first LMO2 exon in the second patient. This study induced significant concern about the safety of gene therapy, since prior to this no gene therapy clinical trials had observed cases of insertional mutagenesis.[170] It appeared that the risks of gene therapy with gamma retroviral vectors needed to be re-evaluated.

Retroviruses can be classified into simple or complex viruses.[174] The MLV vector used in the SCID-X1 trial is an example of a simple gamma retrovirus, containing genes encoding only structural proteins *gag*, *pol* and *env*, as opposed to the HIV vector, a complex lentivirus, which also encodes genes responsible for non-structural proteins such as *tat* and *rev*.[174, 255, 256] It is known that the different retroviridae families have varying preferences for integration sites in the host cell genome, with the simple gamma retroviral vector favouring sites around transcriptional start sites and CpG islands, whereas the complex lentiviral vector (HIV) typically integrates into sites of high transcriptional activity.[257, 258, 192] These non-random integration site preferences have been found to be due to the long terminal repeats (LTR) U3 region enhancer and the different integrase genes expressed by the different members of the retroviridae families.[192, 256, 259] These observations have led to a trend in gene therapy moving away from the simple gamma retroviral vectors to the increasing use of complex lentiviral vectors, the safety characteristics of which are considered improved in terms of minimising the risk of insertional gene activation. These studies are helping to make better gene therapy vectors and lower the risks of insertional mutagenesis in gene therapy trials.

A further modification of the lentiviral vector is the removal of enhancer elements to create a self-inactivating vector (SIN). SIN vectors are modified in the 3' LTR region of the retroviral genome where promoter and enhancer elements normally present have been deleted.[176] The deletion of these elements reduces the propensity of SIN vectors to induce cellular transformation, reducing insertional mutagenesis.[260]

In more recent times, an integrative, replication-defective HIV-1 lentiviral vector was used in a gene therapy clinical trial for X-linked adrenoleukodystrophy (ALD), a severe brain demyelinating disease of the central nervous system in boys.[261] This disease is caused by a deficiency in the ALD protein, encoded by the *ABCD1* gene. Autologous CD34+ haematopoietic stem cells from two patients were transduced with the lentiviral vector expressing the wild type *ABCD1* gene and re-infused back into the patients after the patients received a full myeloablation treatment. At 14 to 16 months after re-infusion, the progressive cellular demyelination had come to a halt in both patients, and remained stable for 36 months post treatment.[262] In the wake of the SCID-X1 gene therapy trial with several of the patients developing leukaemia-like characteristics due, in part, to insertional mutagenesis, Cartier *et al.* performed extensive screening on the transduced haematopoietic cells to map lentiviral insertion sites and determine the occurrence of any vector-induced dominance of individual clones.[261] The authors found insertions sites typical of the HIV-1 lentivirus; distributed mainly in gene coding regions, and that the clonal distribution varied, with no emergence of dominance among active haematopoietic clones or reappearance of a frequent clone with an increasing count in the two patients. Biffi *et al.* have shown in their own work and in meta-analysis of gene therapy studies including the ALD clinical trial, that there is evidence for lentiviral

integrations at common insertion sites in transduced haematopoietic stem cells to be biased towards specific genomic regions, rather than having an oncogenic bias.[263] Almost a decade since the SCID-X1 trial, gene therapy trials are continuing to return positive results to patients with no other treatment options, and the benefit to be gained by pursuing gene therapy seems too great to be dismissed.[185, 264-267]

It is noteworthy that this discussion of insertional mutagenesis in trials and studies involved the transduction of primary haematopoietic stem cells, aimed at creating clonal populations of transgenic cells, in principle for the re-infusion into gene therapy patients. This methodology is not used in the work described in this thesis, where the gene therapy target cells (corneal endothelial cells) were not progenitor or stem cells but rather somatic cells, and, given their amitotic and end-stage differentiated state, unlikely to become highly proliferative.[76]

Reducing the viral copy number per cell in my experiments is perhaps a consideration for keeping the viral dose as low as possible, whilst still aiming to achieve significant gene expression for a therapeutic effect from the transgene.

3.5.2.4 Corneal Graft Survival Prolongation Using IL-10

For the *in vivo* experiments, the CMV promoter was chosen to induce transgene expression, as, of the two promoters examined *in vitro*, it produced higher amounts of transgenic protein from transduced ovine corneas. Protamine sulphate was not used as an adjuvant to enhance the transduction of the lentivirus *in vivo*, as the *in vitro* results did not show a noteworthy boost to transduction expression. A multiplicity of infection of 40 particles per target cell was chosen as the *in vivo* lentivirus transduction dosage, as this was well within the bounds of the doses testing

negative for toxic effects on the target cells. This MOI was high enough to produce similar levels of transgenic protein to an MOI of 100 (Figure 3.13), and was an efficient amount to use in terms of laboratory supplies.

Considering previous work conducted in our laboratory by Parker *et al.* [203], in which a modest prolongation in sheep corneal graft survival was observed following treating of donor corneas with an IL-10-expressing lentivirus with the SV40 promoter, it was reasonable to assume that the new lentivirus construct, LV-CMV-oIL10, which showed superior expression potential *in vitro*, might lead to a modest prolongation of corneal graft survival, if not better.

Penetrating keratoplasty in the sheep with donor corneas transduced with the lentivirus expressing IL-10 *did* significantly prolong the survival of the grafts compared with the untransduced control corneas. However, there were instances of treated allografts rejecting at the same time as the control allografts. It would have been ideal to have a vector control as well as the untransduced control, to investigate the potential for vector-induced immunogenicity causing earlier onset of graft rejection. It is possible that variable lentiviral transduction efficiency between individually treated corneas was a contributing factor to the varying graft survival outcomes.

The *in vitro* work suggested that the CMV promoter would induce stronger transgenic IL-10 protein expression than the SV40 promoter, as discussed previously, with higher expression induced by the lentivirus containing the CMV promoter than the SV40 promoter in both a human cell line and in sheep corneal endothelial cells. As mentioned earlier, in previous work, a lentivirus containing the

IL-10 transgene and the SV40 promoter was used to transduce sheep corneas prior to penetrating keratoplasty.[202] A modest but significant prolongation of graft survival resulted, with the median day of rejection increasing from 18 (combined controls) to 25 (treated allografts). Given these results, a larger increase in the median day of rejection of the treated corneal allografts was expected with a CMV promoter in this study, however this did not occur. These results suggest the amount of transgenic IL-10 protein produced was not the critical factor involved in prolongation of graft survival, however factors such as low viral vector transduction efficiency or vector-induced immunogenicity may have been responsible for the lack of significant improvement in graft survival prolongation.

Previous work by Klebe *et al.* [76] has shown that significant prolongation of corneal graft survival in the sheep can be induced by over expression of IL-10 in the donor cornea using an adenoviral vector and a CMV promoter. The authors demonstrated a median day of corneal allograft rejection of 55 in the treated sheep compared to 20 in the control animals. These data together suggest that the promoter is not the critical element involved in inducing a therapeutic effect in this system.

Table 3.5 shows the combined graft survival data in days post-surgery of the individual sheep treated with LV-SV40-oIL10 (Parker *et al.*) and the results of corneal graft surgery in sheep with LV-CMV-oIL10 treatment (this study). These combined results show a modest but significant prolongation of graft survival in the therapeutic lentivirus-treated animals ($p = 0.003$, Mann-Whitney-U test, corrected for ties).

Table 3.5: Median survival data of sheep corneal grafts treated with lentivirus expressing the ovine IL-10 transgene irrespective of the constitutive promoter inducing expression. Graft survival days shown in red are the results from the mock- and LV-CMV-IL10-treated sheep from the study described in this chapter. Graft survival days shown in black are the results from Parker *et al*.[202]

Treatment Group	Survival Days	Median Day of Rejection
Mock-Transduced (n=21)	16,16,17,18,18,18,18,18,19,19,20,21, 21,22,22,24,27,27,28,28,37	**20**
LV-IL10 (n=17)	18,19,19,21,23,24,24,25,25,28,29,30, 39,43,46,52,>151	**25***

* p= 0.003 compared to mock-transduced (Mann-Whitney U test, corrected for ties)

These results show that the promoter swap in the lentiviral vector made no difference to the prolongation of corneal allograft survival in the sheep, and since the study by Klebe *et al.*, mentioned previously, with the adenoviral vector expressing ovine IL-10 gave a much longer prolongation of allograft survival than either of these two studies with lentiviral vectors. These results suggest that the viral vector is the critical element that is determining the prolongation of graft survival. Previous work investigating the kinetics of transgene expression indicated that transgene expression occurs at a much earlier time point when using the adenoviral vector compared with the lentiviral vector.[202] The transgene mRNA expression was also logs higher than the expression induced with lentivirus-mediated transduction. In a separate study by Fleury *et al.*, a comparison of adenoviral and lentiviral transduction of rat myocytes showed earlier and higher reporter gene expression in the adenovirus-treated cells.[250] The adenovirus vector therefore has some advantage over the lentivirus vector at inducing early therapeutic gene expression, perhaps due to its different mechanism of infectivity.

The type 5 adenovirus enters target cells via receptor-mediated endocytosis. The virions dock at the plasma membrane via the coxsackievirus and adenovirus receptor and integrin co-receptors, accumulate in clathrin-coated pits, and move into endocytic vesicles.[268-270] The endosomes disassemble in the cytosol due to acidic conditions and integrin interactions and the contents are directed to the nuclear pore complex.[270] The viral DNA is released from the disassembling virus particles, enters the nucleus via the nuclear pore and expression of the viral genes occurs.[271] Adenovirus infection is a rapid process, with Trotman *et al.* demonstrating *in vitro* results of adenoviral transduction of A549 cells taking approximately 15 minutes from outer cell contact to the release of the viral particles within the transduced cells.[272]

The lentivirus, pseudotyped with a VSV-G coat, exhibits a broad tropism of target cells as the cell surface receptor for VSV-G is a widely distributed lipid component of the plasma membrane.[273, 274] The VSV-G coated lentivirus virions enter the target cells by clathrin-mediated endocytosis.[256, 275-277] The viral complex is released from the endosome when low pH conditions are encountered by fusion of the viral envelope with the endosome membrane. The capsid and capsid proteins are discarded during uncoating in the cytoplasm and reverse transcription of the viral RNA occurs during nuclear translocation.[256, 275, 278] The newly formed double-stranded viral DNA in a large nucleoprotein complex is termed the pre-integration complex (PIC). This complex allows entry into the nucleus via the nuclear pore, rather than having to wait for mitosis and the breakdown of the nuclear envelope, and hence the lentivirus can infect non-dividing cells.[256] Nuclear import occurs and the provirus is integrated into the host chromatin. Johannsdottir *et al.* found that internalisation of vesicular stomatitis virus with the VSV-G coat in HeLa cells was a

process that took approximately five minutes, and acid-induced fusion of the membranes following internalisation was also rapid, taking only eight to ten minutes.[277] The authors found similar infection kinetics when using a different mammalian cell line.

It is not clear from the current literature exactly why the difference between adenoviral and lentiviral vectors occurs, as the internalisation and nuclear importation processes are reported to be quite rapid in both cases.[272, 277] I speculate that the cause of the expression delay with the lentivirus is likely to occur after nuclear import, and may be due to the integration mechanisms, epigenetic regulation, or cellular defences against retroviral infection.

Considering that this gene therapy technology is directed towards clinical practice in the future, it is useful to examine the potential of the lentiviral vector in human tissue. Parker *et al.* showed that human corneas transduced with a lentiviral vector had ten-fold higher transgene expression than the similarly transduced ovine corneas, when detected at an early time point.[202] This further suggests that the initial expression lag observed in ovine corneas with the lentiviral vector may be species-specific.

3.5.2.5 IL-10 Transgenic Protein

There was no association observed between the IL-10 levels detected in the anterior chamber fluid and corneal graft survival. This may have been due to the fact that the animals were not euthanised at the same time point after the onset of rejection, as indicated in Table 3.3. Given the rapid turnover of anterior chamber fluid and the degradation of the corneal endothelium including the loss and death of endothelial

cells during the rejection process, the levels of IL-10 could fluctuate greatly from animal to animal.[279] However, the detection of IL-10 at increased levels in the anterior chamber fluid in eight out of ten treated sheep confirms that the gene was successfully over-expressed by the corneal endothelial cells. The question remains as to how much therapeutic protein is necessary to induce a modulation of the immune response and at what point in time this protein is required in order to be effective at prolonging graft survival.

It is instructive to consider the mechanism of action of the cytokine interleukin-10, to understand why early expression of this protein might make the difference between graft survival and rejection. Interleukin-10 is an anti-inflammatory cytokine. It has a role in preventing immune-mediated tissue damage by limiting strong immune responses and promoting tolerance, by regulating many different cells of the immune system. The roles of this cytokine have been comprehensively reviewed by Moore *et al.* 2001 and Sabat *et al.* 2010.[100, 101] IL-10 has the capacity to inhibit the activation of antigen presenting cells, which in turn reduces the ability of Th1 cells (helper T cells) to produce inflammatory cytokines such as interferon-γ.[102] Groux *et al.* showed that the presence of IL-10 induces populations of CD4+ T cells which have a low proliferative capacity and have a modulatory effect on the immune response. These cells are designated Tr1 cells (T regulatory subset 1).[280] IL-10 can also affect immature or semi-mature antigen presenting cells, which are found in the normal central cornea.[281, 282] These cells then have a reduced ability to stimulate CD4+ T cells, the crucial mediators of the immune response and corneal allograft rejection.[61, 72, 283] Hattori *et al.* showed that administering IL-10-induced tolerogenic dendritic cells significantly prolonged corneal allograft survival in a mouse model.[284] In addition to the immature and semi-mature antigen-presenting cells in the cornea,

mature dendritic cells found in immune privileged sites in the body have been observed to bias naïve T cells into becoming Tr1 cells, which will themselves produce IL-10.[280, 285, 286]

Given these modes of action of IL-10, it seems that the over-expression of this cytokine would be necessary at an early time point in order to successfully inhibit the initial stimulation and clonal expansion of the CD4+ T cells, and thus prevent sensitisation. Several studies support this theory, with mouse heart allografts, rat liver allografts and human bone marrow transplants all showing increased survival with *pre-treatment* of the host with systemic IL-10, reviewed by Moore *et al.* 2001.[100] In a study by Li *et al.*, mouse cardiac allografts exhibited enhanced survival with IL-10 pre-treatment of the recipient.[106] Interestingly, the authors also found that post-graft treatment of the recipient with IL-10 actually increased the speed of allograft rejection. As discussed in Chapter 1, Section 1.5.1.1, a study by Torres *et al.* showed prolongation of rat corneal allograft survival was not enhanced by regional or systemic doses of recombinant IL-10 to the recipient rat.[104] However, in a recent study by Takiishi *et al.*, it was found that expression of IL-10 in a *donor* mouse skin graft as opposed to the *host* mouse was the more critical indicator for graft acceptance.[241] These findings suggest that pre-treatment of the donor tissue is perhaps a better regimen than local or systemic treatment of the recipient animal. The authors also mention that early production of IL-10 is necessary for graft survival in the isografts they investigated.

These findings lead to the conclusion that early immune suppression before the stimulation of the T cells is beneficial for allograft survival.

One advantage of the human cornea is the ease of access to the tissue for *in vitro* modification prior to transplant surgery. This means the therapy may be applied directly to the donor cornea and need not be delivered systemically to the recipient. Further, as mentioned previously, the cornea is avascular, is a site of immune privilege and has a relative paucity of antigen presenting cells (APCs), therefore treatment with IL-10 directly post graft surgery may be early enough to prevent the activation of APCs in this region without the necessity for pre-treatment. For these reasons, and also with the evidence of Klebe *et al.* 2001, who showed corneal graft prolongation with the use of IL-10 expressed post-surgery in a sheep model where there was even some pre-vascularisation of the cornea, there is reasonable evidence that post-surgical expression IL-10 should not increase the speed of allograft rejection in corneal transplantation.

The sheep model that I am using, like any model, has limitations. Unlike the human cornea, the donor sheep cornea cannot be successfully transplanted after organ culture. This means that pre-treatment of the donor cornea is limited to hours rather than days. Pre-treatment of the recipient animal would be achievable with systemic doses of the gene therapy, but this is an undesirable option as large vector dosages are required for systemic administration, and systemic treatment is much less safe than *ex vivo* treatment.

3.5.2.6 The Lentiviral Vector

The integrative lentivirus has a distinct advantage over the adenoviral vector in allowing long-term gene expression.[218, 287] This long-term gene expression is highly desirable in our model of corneal transplantation given that, currently, the clinical procedure of penetrating keratoplasty has an excellent survival prognosis in the early

stages but the long-term survival outcomes of the grafts are poor.[16, 288] Long-term expression in the sheep cornea can be induced by the adenovirus vector,[76] however this long-term expression was not seen across all of the treated animals, therefore clinically this might create the need for further treatments. It is also known that the adenovirus vector can itself cause a greater immune response than the lentivirus both systemically and at the site of transduction, which is highly undesirable in a model of the regulation of allograft rejection.[99, 166, 289]

The lentiviral vector has already been used in several examples of gene therapy of the cornea and other ocular tissues. In a study by Auricchio *et al.*, stable, long-term reporter gene expression was detected in the retinae of mice treated with a subretinal injection of a lentiviral vector.[290] Beutelspacher *et al.* demonstrated that an HIV lentiviral vector with VSV-G pseudotyping was able to stably transduce both rabbit and human cornea tissue e*x vivo*.[291] Significantly, Murthy *et al.*, reported successful transduction of rabbit corneas with a lentiviral vector containing the anti-neovascularisation fusion gene, endostatin::Kringle5, which resulted in an inhibition of both neovascularisation and graft failure after penetrating keratoplasty in the treated animals.[87] Recently Nosov *et al.* showed that *ex vivo* modification of rat corneas to overexpress programmed death-ligand 1 mediated by a lentiviral vector resulted in a prolongation of allograft survival compared to controls in a model of penetrating keratoplasty.[201] The use of lentiviral vectors for gene therapy of *human* corneas shows promise with both Parker *et al.* and Wang *et al.,* along with Beutelspacher *et al.* mentioned above, demonstrating the successful transduction of human corneal tissue with lentiviral vectors.[178, 218]

3.5.3 Conclusions

It seems a balanced decision must be made between the adenoviral and the lentiviral vectors in this sheep model of corneal transplantation, based on the desired outcome of transduction, be it early, strong gene expression or prolonged expression of the gene. It is clear that the dose, timing, kinetics and location of the IL-10 therapy are all crucial to the success of the cytokine as a means to prolong graft survival. Combined data from therapeutic lentiviral treatments of penetrating keratoplasty in the sheep show success in prolonging graft survival, however it seems that earlier expression of the therapeutic transgene is needed more rapidly post surgery, since greater therapeutic success has been shown with the adenoviral vector, which gives earlier and stronger transgene expression. Addressing this issue is the key to highly significant prolongation of corneal transplantation in this sheep model using lentivirus-mediated transduction with therapeutic genes, for their long-term, integrated expression.

Given the results presented in this chapter, the decision was made to discontinue efforts towards increasing the effectiveness of the lentiviral vector as a single unit and instead approach the gene therapy with both the adenoviral vector and the lentiviral vector in a cocktail combination with multiple therapeutic transgenes.

Chapter 4

Multigenic, Vector Cocktail Therapy of the Sheep Cornea

4.1 Abstract

4.1.1 Aims

To construct several lentiviral vectors, each expressing a therapeutic transgene to target either corneal neovascularisation (sFlt-1, EK5) or a particular aspect of the immune response against corneal allotransplantation (Bcl-xL, IDO), to characterise these constructs *in vitro*, and utilise viral preparations of these constructs along with an adenoviral vector expressing IL-10 in a cocktail treatment of ovine orthotopic keratoplasties.

4.1.2 Methods

Four lentiviral vectors were constructed by molecular cloning and tested *in vitro* in sheep cornea endothelial cells for transgene expression at both the messenger RNA and protein level. The biological functions of the four transgenes were determined *in vitro* using an apoptosis assay to test for increased cell survival with Bcl-xL expression, vascular endothelial cell proliferation assays to test for inhibition by sFlt-1 and EK5 proteins, and testing for the presence of a down-stream metabolite of tryptophan degradation from the reaction catalysed by the transgenic enzyme IDO. To investigate whether vector interference occurred during transduction with a cocktail of different viral vectors, the sheep cornea was simultaneously transduced *in vitro* with both a lentivirus and an adenovirus, each encoding a different transgene, EK5 and IL-10, respectively. Expression of the individual transgenes was assessed at the mRNA level by real-time qPCR and at the protein level by sandwich ELISAs. Orthotopic penetrating keratoplasties were performed on sheep using donor sheep corneas transduced with a cocktail therapy containing two lentiviral preparations and one adenovirus, each expressing individual transgenes, EK5, Bcl-xL and IL-10, respectively. Sheep grafts were monitored daily and the day of graft rejection in the

treated allografts was compared with that of mock-vector treated control allografts. The median day of neovascularisation crossing the graft-host margin into the graft was used to determine if significant inhibition of neovascularisation was achieved in the treated allografts compared with the mock-vector control allografts.

4.1.3 Results

All four transgenes were determined to have functional biological activity *in vitro*. All four of the lentivirus preparations successfully transduced ovine cornea endothelial cells and showed over-expression of the transgenes at the mRNA level at day 10 of organ culture. Two of the four lentivirus preparations, LV-BclxL and LV-EK5, demonstrated strong transgenic protein expression from transduced ovine corneas across all culture time points. *In vitro* cocktail transduction of ovine corneas with a lentivirus and an adenovirus indicated no evidence of vector interference when transgene expression was investigated, at either the mRNA or protein levels. A cocktail treatment of donor ovine corneas with AdV-IL-10, LV-EK5 and LV-BclxL did not prolong corneal allograft survival in the sheep, nor did it have an effect on the median day of neovascularisation crossing the graft-host junction of the corneal allografts.

4.1.4 Conclusions

It is likely that increased inflammation, mis-targeted expression of the transgenic proteins *in vivo* or contradictory therapeutic actions were responsible for the lack of prolongation of allograft survival in vector cocktail-treated corneas.

4.2 Introduction

4.2.1 Multigenic and Vector Cocktail Treatment of Corneal Allografts

Given the moderate success of achieving prolongation of corneal allograft survival using the lentiviral vector with a single therapeutic transgene, IL-10, as described in chapter 3, the work described in this chapter investigated the use of multigenic therapy targeting multiple arms of the host immune response. The lentivirus has significant benefits over the adenovirus as a gene therapy vector, such as inducing a low inflammatory response from the host and integration into the host genome for long-term transgene expression.[160] However, the adenoviral IL-10 vector has achieved much longer prolongation of corneal allograft survival in the sheep.[76, 202] I argued that using a combination of vectors to express therapeutic transgenes exploits the benefits of the individual vectors whilst minimising their limitations: utilising one adenoviral vector minimises the inflammatory response, but will induce early, strong transgene expression, and utilising four lentiviral vectors takes advantage of the low inflammatory response of this vector and the long-term integrated transgene expression.

Viral vector cocktails have been used in many other contexts for enhanced therapeutic effects, as discussed in Chapter 1, Section 1.6.6.[189, 224, 225] The aim of this chapter was to investigate the use of a cocktail of adenoviral and lentiviral vectors, each expressing a different therapeutic transgene, to prolong corneal allograft survival.

4.2.2 Inhibition of T cell Responses to Improve Corneal Allograft Survival

Important mediators of the host immune response to corneal allografts are CD4+ T cells.[59, 61, 71, 148] These cells are stimulated by antigen presenting cells and undergo

proliferation and clonal expansion into memory and effector T cells.[51, 66] T cells distribute around the host via the lymphatic system and the T effector cells have a major role in destroying the foreign graft cells.[51] Inhibition of host T cell responses has induced significant prolongation of corneal allograft survival in animal models.[70, 73, 292]

Interleukin-10 (IL-10) is an anti-inflammatory cytokine, known to prolong corneal allograft survival in a sheep model of corneal transplantation.[76] As previously discussed in Section 3.5.2.5, IL-10 tends to tolerise immature or semi-mature antigen presenting cells, which lends the cells a reduced ability to stimulate CD4+ T cells.[281, 283] Results from previous studies [76] and those described in chapter 3 of this thesis indicate that early expression of the IL-10 transgenic protein is necessary for therapeutic efficacy in prolongation of sheep corneal allografts. In consequence, an adenoviral vector with the ability to induce early and higher transgene expression than the lentiviral vector [202, 250] was chosen as the optimal expression vector for the therapeutic protein ovine IL-10 as part of a vector cocktail.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that catalyses the degradation of the amino acid tryptophan.[107] Tryptophan is an essential amino acid necessary for T cell proliferation and clonal expansion. When tryptophan levels are depleted, T cells are arrested in G1 phase and induced into apoptosis.[111, 112, 293] A study by Beutelspacher *et al.*, has demonstrated that the over-expression of IDO can significantly prolong the survival of mouse corneal allografts compared with control allografts.[107] As discussed in Chapter 1, Section 1.5.1.2, there is evidence to show that the down-stream metabolites of tryptophan degradation, kynurenines, themselves also have a modulatory effect on T cell responses.[112-114]

The over-expression of ovine IL-10 and ovine IDO in sheep corneal endothelial cells using an adenoviral and a lentiviral vector, respectively, is investigated in this chapter, with the aim of minimising the host T cell responses to sheep corneal allografts *in vivo*.

4.2.3 Corneal Endothelial Cell Death During Graft Rejection

The donor cornea endothelial cell layer is one of the major target sites of the immune response during corneal graft rejection.[148] When corneal endothelial cells come under immune attack, an apoptotic response is initiated.[80, 294] The loss of corneal endothelial cells significantly affects the integrity of the cornea, as the cells are amitotic, and the metabolic function of the endothelium is compromised.[2, 27] This results in the loss of corneal clarity and leads to graft failure.[2]

Bcl-xL is an anti-apoptosis factor of the Bcl2 family of pro- and anti-survival factors.[295] Bcl-xL resides in the mitochondrial membrane and inhibits cytochrome-c release from the mitochondrion.[149, 150] Cytochrome-c release induces a caspase cascade leading to apoptosis of the cell.[149, 296] Barcia *et al.* used a lentiviral vector to over-express the Bcl-xL transgene in a mouse model of corneal transplantation and demonstrated significantly improved survival of the treated allografts compared with the control allografts.[80] An *in vitro* study by Fuchsluger *et al.* involving the storage of human corneas demonstrated a significant decrease in corneal endothelial cell loss by corneas treated with a lentiviral vector expressing the Bcl-xL transgene compared with untreated corneas.[297]

This chapter describes the use of a lentiviral vector to over-express Bcl-xL in donor sheep cornea endothelial cells to prolong cell survival after penetrating keratoplasty in the sheep.

4.2.4 Neovascularisation a Risk Factor for Corneal Graft Rejection

The normal human cornea is avascular.[128, 134, 298] New vessel growth into the cornea is a key risk factor for corneal graft rejection.[85] The 2012 Australian Corneal Graft Registry Report showed the median survival of a corneal graft transplanted into a recipient with extensive pre-vascularisation (all four corneal quadrants) was 4 years, as compared with the median survival of a graft transplanted into a recipient without any pre-vascularisation, which was significantly longer at 17 years.[16] In human and animal studies, both blood vessels and lymphatic vessels have been identified to infiltrate the avascular corneal graft tissue after transplantation, and exhibit a strong correlation with graft rejection.[85, 86]

Endostatin::kringle5 (EK5) is a synthetic fusion gene comprising human endostatin, a protein component from the C-terminus of collagen XVIII, and the kringle5 domain of human plasminogen gene.[87] Endostatin and kringle5 are potent anti-neovascularisation factors, preventing vessel growth by specifically inhibiting vascular endothelial cell proliferation and migration.[147] Murthy *et al.* demonstrated a significant decrease in the corneal neovascularisation of rabbit corneal transplants transduced with a lentiviral vector expressing EK5, compared with control allografts.[87] The EK5 treated animals also showed significantly prolonged corneal allograft survival compared with mock-vector treated control allografts.

Soluble fms-like tyrosine kinase (sFlt-1), also known as soluble VEGF-R1, is the soluble receptor for vascular endothelial growth factor (VEGF). Increased expression levels of VEGF in the human eye have been linked to increased levels of vascularisation and inflammation of corneas.[299] As discussed in Chapter 1, Section 1.5.2.1, the action of sFlt-1 in binding VEGF inhibits angiogenesis, and the over-

expression of sFlt-1 has been demonstrated to inhibit corneal neovascularisation in several animal studies.[134, 137-139]

This chapter investigates the lentivirus-mediated over-expression of the two transgenes, human EK5 and human sFlt-1, in sheep corneal endothelial cells, and the ability of the EK5 transgenic protein to reduce corneal neovascularisation in sheep corneal allografts.

4.3 Specific Aims

1) To construct four lentivirus vectors containing transgenes encoding ovine indoleamine 2,3-dioxygenase, human endostatin::kringle5 fusion protein, human soluble vascular endothelial growth factor receptor 1 (sFlt-1) and ovine Bcl-xL.
2) To test the virus preparations for specific biological function, and for transgene expression in the ovine cornea at the mRNA and protein level.
3) To determine if any vector-related transgene expression interference occurs in ovine corneas transduced *in vitro* with a combination of a therapeutic adenovirus and a lentivirus.
4) To perform ovine orthotopic keratoplasty with donor corneas treated *ex vivo* with a cocktail of the lentiviral preparations and one adenovirus and investigate the effect on corneal graft survival compared to mock-vector-transduced control grafts.

4.4 Specific Methods and Results

4.4.1 Construction of Lentivirus Containing Indoleamine 2,3-dioxygenase

In previous work conducted by Dr Helen Brereton (Department of Ophthalmology, Flinders University), the ovine indoleamine 2,3-dioxygenase (oIDO) gene was cloned into the plasmid pBluescriptII SK+ (Thermo Scientific, Vic). The gene was PCR amplified from sheep lymph node cDNA using primers based on *Ovis aries* Expressed Sequence Tags (GenBank Accession numbers EE792499 and FE023194). The complete coding sequence for the ovine IDO gene has the GenBank Accession number FJ380928.

I transferred the oIDO gene into a lentivirus shuttle plasmid containing an inducible promoter and a multiple cloning site. This plasmid was kindly provided by Mr Yazad Irani (Department of Ophthalmology, Flinders University). The promoter was then swapped for a CMV promoter by restriction digest of this oIDO-containing plasmid and the previously prepared plasmid pHIV-1-CMV-oIL10 (Section 3.4.1), with endonucleases BamH1 and Kpn1 to remove the promoter regions. The oIDO plasmid was SAP-treated and purified using a PCR purification kit. The CMV promoter fragment was gel purified and ligated into the oIDO plasmid, and this construct was electroporated into *E.coli* DH5α electrocompetent cells. The construct was sequenced. Figure 4.1 shows a vector map of this shuttle plasmid (pHIV1-CMV-oIDO). This plasmid was used by me to produce lentivirus preparations, LV-CMV-oIDO, as described in Section 2.2.4. The titres of these lentivirus preparations are given in Table 2.13, Section 2.2.4.2.

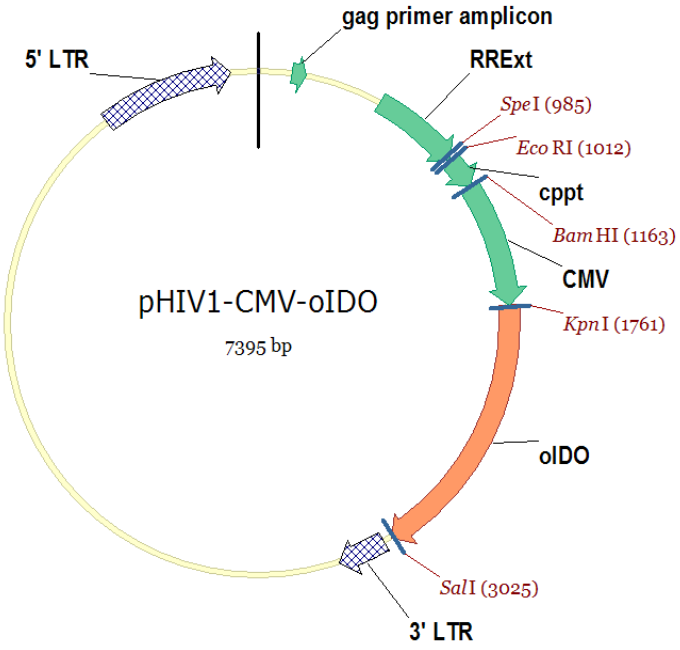


Figure 4.1: Vector map of lentivirus shuttle plasmid containing the ovine indoleamine 2,3-dioxygenase gene (oIDO) with a CMV promoter. LTR – long terminal repeats, RREXt – Rev response element in the env coding region, cppt – central polypurine tract.

4.4.1.1 Assay to Test for the Biological Activity of Transgenic Ovine IDO Protein

HEK-293T cells were seeded into 6-well plates at 5 x 10^5 cells/ mL (1.25 x 10^6 cells per well) in culture medium as described in Section 2.2.2.1. A transduction medium was prepared containing polybrene and gentamicin, and the cells were transduced with LV-CMV-oIDO or LV-SV40-eYFP at an MOI of 2 (2.5 x 10^6 transducing units) for 24 hrs. Half of the cells received supplemental L-tryptophan to a total medium concentration of 200 µM. Supplemental tryptophan was added to ensure the IDO enzyme was not limited for lack of substrate material. The cells were passaged the following day and culture supernatant was collected on this day (day 2) and at day 5.

The supernatant was assayed for the presence of the metabolite L-kynurenine, a stable down-stream product of tryptophan degradation catalysed by IDO. Over-expression of kynurenine in the culture supernatant of LV-CMV-oIDO treated cells was indicative of the activity of the transgenic IDO protein. A colourimetric assay was used to detect kynurenine levels as described in Chapter 2, Section 2.2.13.11.

Figure 4.2 shows the kynurenine detected in the culture supernatants of LV-oIDO transduced cells. At day 2 no difference was observed in the kynurenine levels of the untransduced, the LV-eYFP transduced and the LV-oIDO transduced cells. At day 5 however, a Kruskal-Wallis test revealed a significant difference in kynurenine levels between the treatment groups, both with and without the increased tryptophan supplement ($p< 0.003$ in both cases), even after the significance threshold was set at 0.017 following Bonferroni correction.

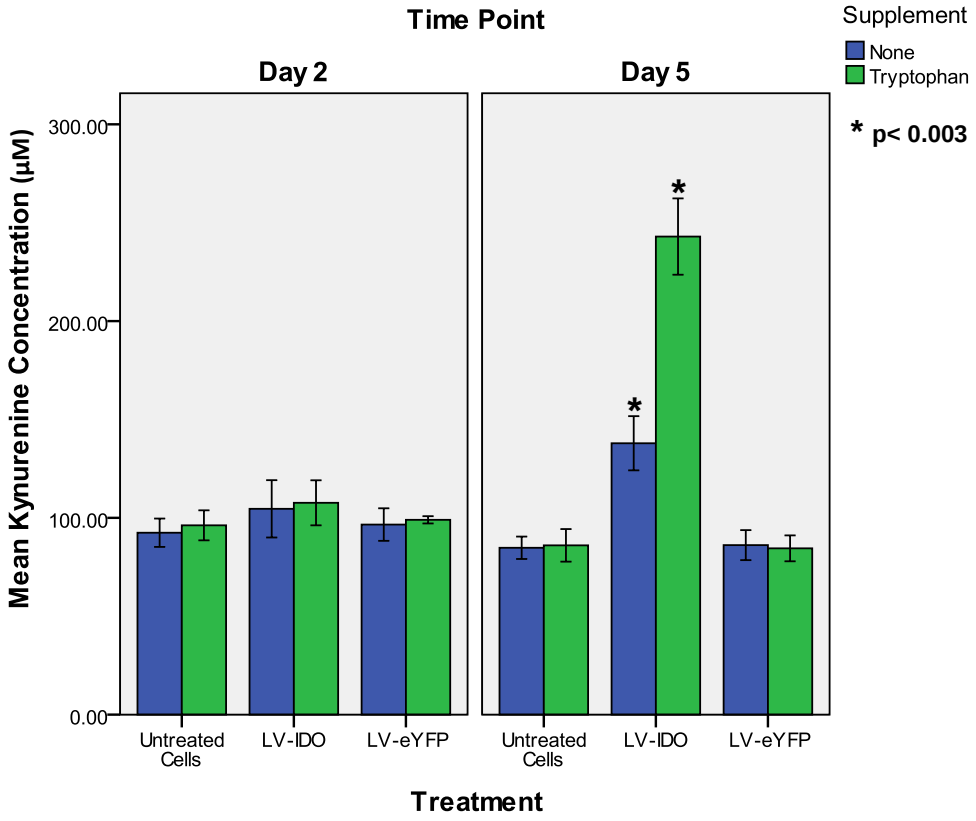


Figure 4.2: Average kynurenine (µM) detected in the culture supernatant of 293T cells transduced with LV-CMV-oIDO. Tryptophan was an added supplement to some treatments at a final concentration of 200 µM. Detection of the kynurenine metabolite was significantly higher in the supernatant of LV-IDO treated cells. The culture experiment was conducted twice, with three pooled biological replicates (cell culture wells) on each occasion for each treatment. Error bars represent the mean ± the standard deviation of technical replicates (assay wells, n= 6). * p< 0.003 (Kruskal-Wallis, with Bonferroni correction for significance set at p≤ 0.017).

4.4.2 Construction of Lentivirus Containing Ovine Bcl-xL

The ovine Bcl-xL gene was PCR amplified from sheep lymph node cDNA using primers based on *Ovis aries* Bcl-xL complete coding sequence (GenBank Accession number AF164517), with the addition of a Kozak consensus sequence at the 5' end. The Bcl-xL gene was transferred to a lentiviral shuttle plasmid containing a CMV promoter and sequenced to confirm identity and correct insertion. This work was conducted previously by Dr Helen Brereton, Mr Yazad Irani and Ms Lauren Mortimer (Department of Ophthalmology, Flinders University).

Figure 4.3 shows a vector map of this construct (pHIV1-CMV-oBclxL). This plasmid was used by me to produce a lentivirus preparation, LV-CMV-oBclxL, as described in Section 2.2.4. The titre of this lentivirus preparation is given in Table 2.13, Section 2.2.4.2.

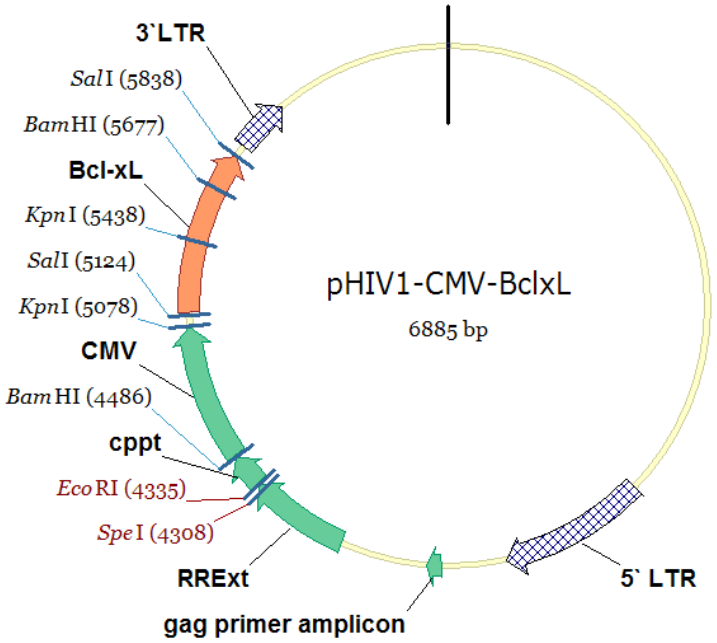


Figure 4.3: Vector map of lentivirus shuttle plasmid containing the ovine Bcl-xL gene with a CMV promoter. LTR – long terminal repeats, RREXt – Rev response element in the env coding region, cppt – central polypurine tract.

4.4.2.1 Assay to Test for the Biological Activity of Transgenic Ovine Bcl-xL Protein

HEK-293T cells were cultured as described in Section 2.2.2.1, and transduced with LV-CMV-oBclxL at an MOI of 2 for 24 hours as described in Section 2.2.14. The cells were passaged every two to three days, with the supernatant collected on days 8, 10 and 13, pooled and concentrated (7.2- and 8.9-fold for Bcl-xL transduced cells and untransduced cells, respectively) in order to test for oBcl-xL expression at the protein level.

Quantification of oBcl-xL Protein

A sandwich ELISA was performed to detect for oBcl-xL in the culture supernatant of the transduced HEK-293T cells used in this apoptosis assay, as described in Section 2.2.13.12. The supernatant from the Bcl-xL transduced cells contained 945-fold more Bcl-xL protein than the untransduced cell supernatant, Figure 4.4.

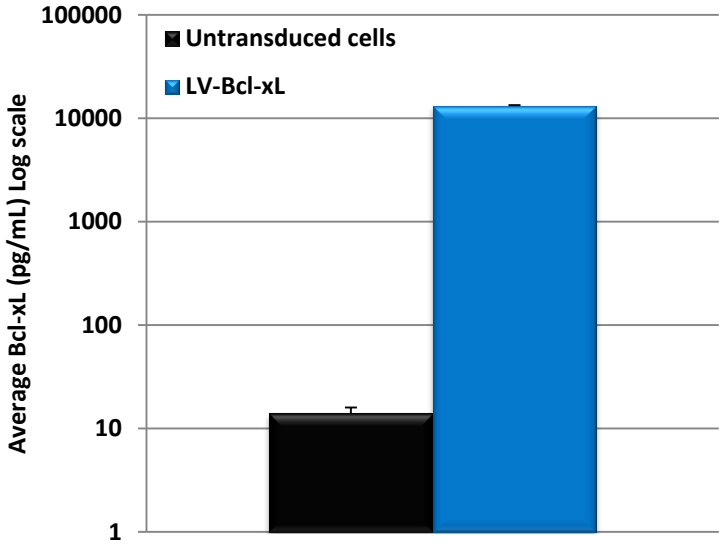


Figure 4.4: Bcl-xL detected in the pooled, concentrated supernatant of HEK-293T cells by sandwich ELISA. Cells were untransduced, or transduced with the lentivirus containing the oBcl-xL transgene, and were used in the apoptosis activity assay. Biological replicates (culture wells) n= 6. Error bars represent the error range in the technical replicates (n= 2).

Verification of Transgenic oBcl-xL Protein Biological Activity

At 15 days post-transduction, apoptosis was induced with the addition of the cytotoxic agent staurosporine (Sigma, St Louis, MO USA) at a final concentration of 1 µM for 6 hours, and the number of cells in early apoptosis was determined by flow cytometry using the Annexin V-FITC labelling system as described in Section 2.2.14.

Figure 4.5 shows the average number of viable, early apoptotic and late apoptotic/ necrotic cells from the cells treated with the lentivirus containing the oBcl-xL gene compared with untreated cells and cells treated with a non-therapeutic (control) lentivirus (LV-eYFP). The cells transduced with LV-oBclxL had significantly fewer cells in early apoptosis than untransduced cells (p= 0.001, one-way between-groups

ANOVA), likewise significantly fewer than the LV-eYFP transduced cells (p= 0.000), after treatment for six hours with apoptosis inducer 1 μM staurosporine. It is suggested that the low proportion of viable LV-eYFP transduced cells compared with untransduced and LV-BclxL transduced cells might have been due to the fluorescent eYFP protein being quite large and possibly toxic to the cells as it is produced.

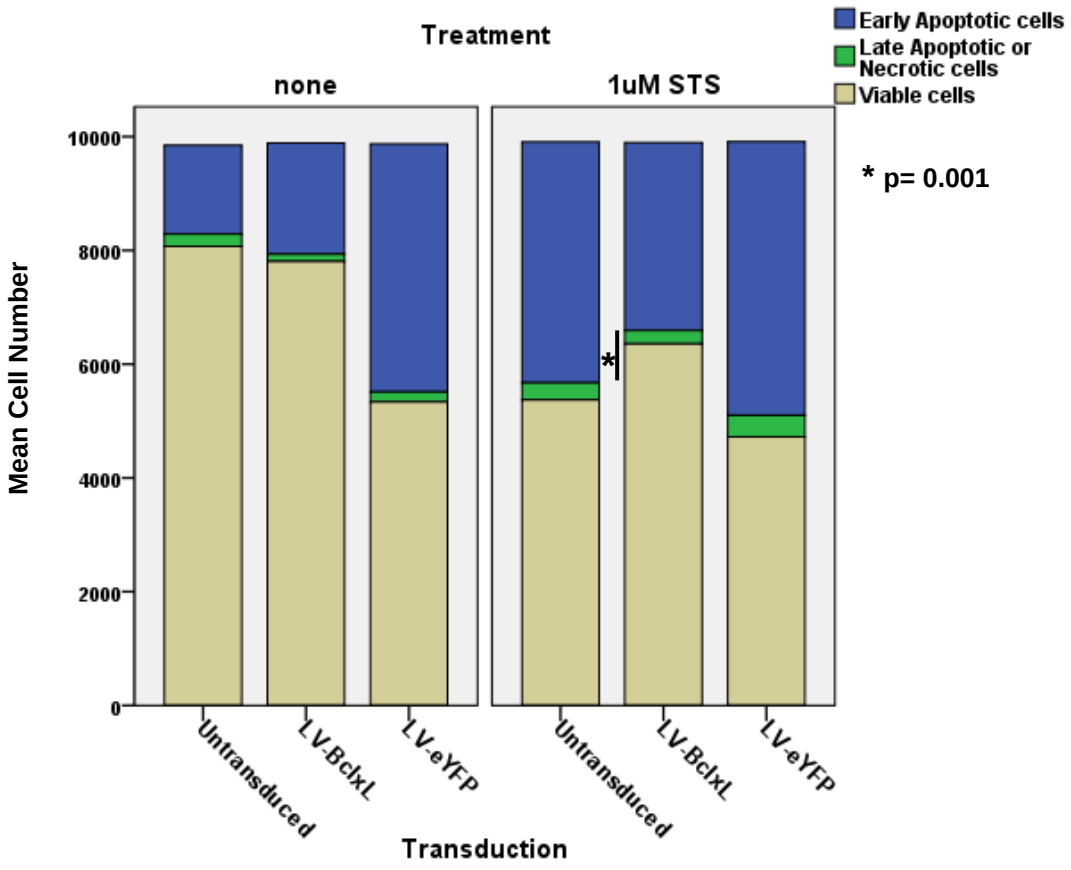


Figure 4.5: Average number of viable, early apoptotic and late/ necrotic cell populations in HEK-293T cells that were untransduced, transduced with lentivirus expressing transgene Bcl-xL, or transduced with lentivirus expressing reporter gene eYFP, and underwent apoptotic inducement (1 μM STS) for six hours, n= 3. Cells were labelled with Annexin V-FITC and propidium iodide; only cells which were Annexin positive and PI negative were considered early apoptotic. * p= 0.001 (early apoptotic cells; one-way between-groups ANOVA).

4.4.3 Construction of Lentivirus Containing Human Endostatin::Kringle5 Fusion Protein

In order to prepare a lentiviral shuttle plasmid containing the transgene encoding the endostatin::kringle5 (EK5) fusion protein, the open reading frame of the EK5 sequence was obtained from the pBlast41-hEndoKringle5 expression plasmid (Integrated Sciences, Chatswood NSW). The EK5 coding region was transferred to a lentiviral shuttle plasmid and the existing SV40 promoter was replaced with a CMV promoter using restriction endonucleases BamH1 and Cla1, as described in Section 2.2.1.5. This work was performed by Ms Lauren Mortimer (Department of Ophthalmology, Flinders University).

Figure 4.6 shows a vector map of this construct (pHIV1-CMV-EK5). This plasmid was used by me to produce a lentivirus preparation, LV-CMV-EK5, as described in Section 2.2.4. The titre of this lentivirus preparation is given in Table 2.13, Section 2.2.4.2.

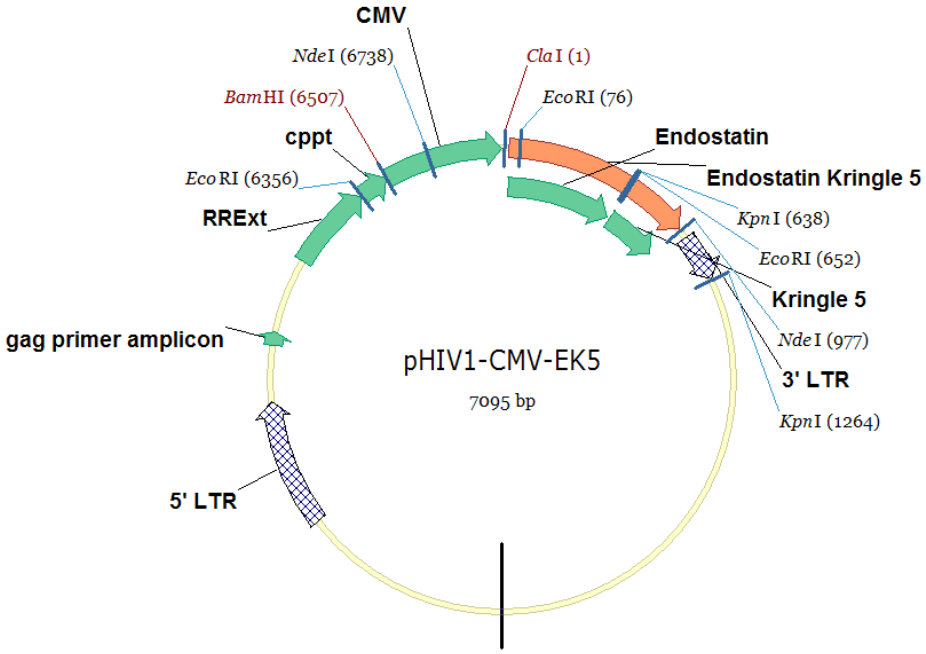


Figure 4.6: Vector map of lentivirus shuttle plasmid containing the fusion gene endostatin::kringle5 (EK5) with a CMV promoter. LTR – long terminal repeats, RREXt – Rev response element in the env coding region, cppt – central polypurine tract.

4.4.3.1 Assay to Test for the Biological Activity of Transgenic EK5 Protein

A cell-based assay was used to determine the biological efficacy of the endostatin::kringle5 fusion protein at preventing endothelial cell proliferation.

Transient Transfection of Mammalian Cells to Obtain Conditioned Medium Containing the EK5 Protein

A conditioned medium sample containing transgenic endostatin::kringle5 protein and a transfection control sample (created using medium only, no plasmid) were obtained by transient transfection of HEK-293T cells, as described in Section 2.2.2.2 'Transient Transfection to Produce Conditioned Medium Containing EK5 Protein'.

Quantification of the EK5 Conditioned Medium Samples

The conditioned media samples were tested for endostatin expression by sandwich ELISA as described in Section 2.2.13.13. The control transfection supernatant and the EK5 transfection supernatant were determined to contain approximately 6 ng/mL and 20 μg/mL endostatin protein, respectively, ELISA technical replicates n= 2.

HUVECs Proliferation Assay

Primary human umbilical vein endothelial cells (HUVECs) were kindly donated by Dr Claudine Bonder and her staff at the Centre for Cancer Biology, SA Pathology, Adelaide SA. The HUVECs were cultured to promote proliferation as described in Section 2.2.2.1. At an early passage (P2 - 4), the cells were seeded in the same culture medium, but without bECGF (bovine endothelial cell growth factor), into gelatin-coated 96-well plates at 2 x 10^3 cells per well. See Appendix A2.1.2 for bovine gelatin recipe. The cells were incubated for 24 hours at 37^{o}C in 5% CO_2 in air.

Medium was replenished on the cells and the cells treated with either the conditioned medium containing EK5 at a final endostatin concentration of 10 μg/mL, or the same volume of the transfection control medium. The cells were incubated for 48 hours at 37^{o}C and 5% CO_2. Cell proliferation MTS assays were performed at 24 hours post cell seeding and at 48 hours post sample application as described in Section 2.2.2.3.

Figure 4.7 shows the cell proliferation of untreated HUVECs, HUVECs treated with EK5 transfection supernatant with 10 μg/mL endostatin, or HUVECs treated with the control transfection supernatant. The EK5 treated cells showed a significant decrease in proliferation when compared with the control treated cells (p= 0.004, one-way

ANOVA). The increase in proliferation shown in the transfection control and the EK5 conditioned medium sample compared to the HUVECs alone at 48 hours post-sample addition is due to the extra serum in these samples.

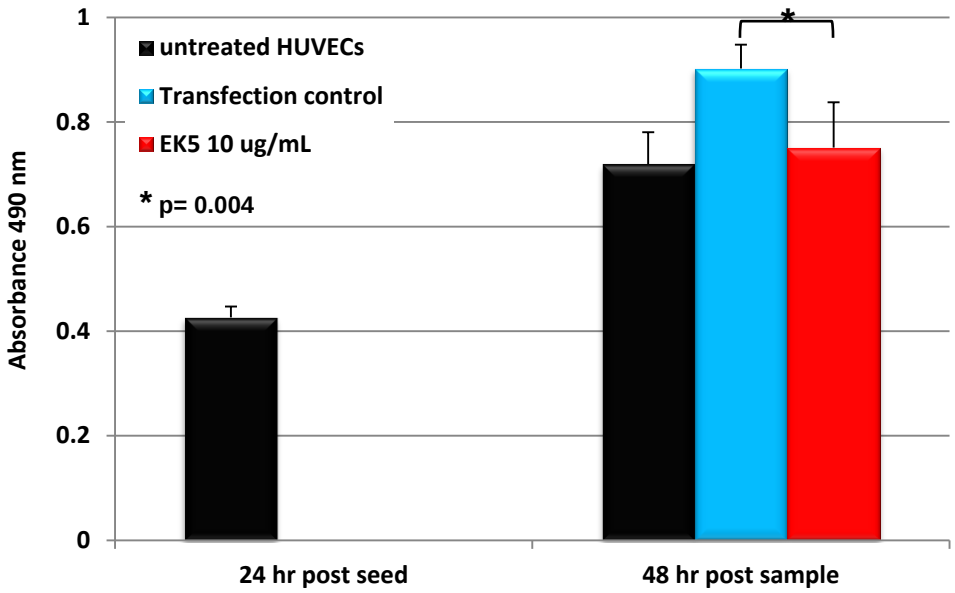


Figure 4.7: MTS proliferation assay on HUVECs treated for 48 hours with EK5-containing supernatant from cell culture transfection. EK5 conditioned medium contained 10 μg/mL endostatin. Both treatments had a biological sample size of n= 6. * p= 0.004 (one-way ANOVA)

4.4.4 Construction of Lentivirus Containing Human Soluble Fms-like Tyrosine Kinase 1

The human soluble Fms-like tyrosine kinase 1 (sFlt-1) gene, also known as soluble vascular endothelial growth factor receptor 1 (sVEGF-R1) was previously transferred from plasmid pCDNA-sFlt (a kind gift from Winthrop Professor Elizabeth Rakoczy, Centre for Ophthalmology and Visual Science, University of Western Australia, Nedlands WA), to a lentiviral shuttle plasmid containing the SV40 promoter, which was then swapped for a CMV promoter as described (Sections 2.2.1.4 - 11), using the BamH1 and Cla1 restriction sites. The construct was sequenced to confirm identity and correct insertion. This work was conducted by Ms Lauren Mortimer (Department of Ophthalmology, Flinders University). A vector map of this plasmid (pHIV1-CMV-sFlt1) is shown in Figure 4.8. This plasmid was used by me to produce a lentivirus preparation, LV-CMV-sFlt1, as described in Section 2.2.4. The titre of this lentivirus preparation is given in Table 2.13, Section 2.2.4.2.

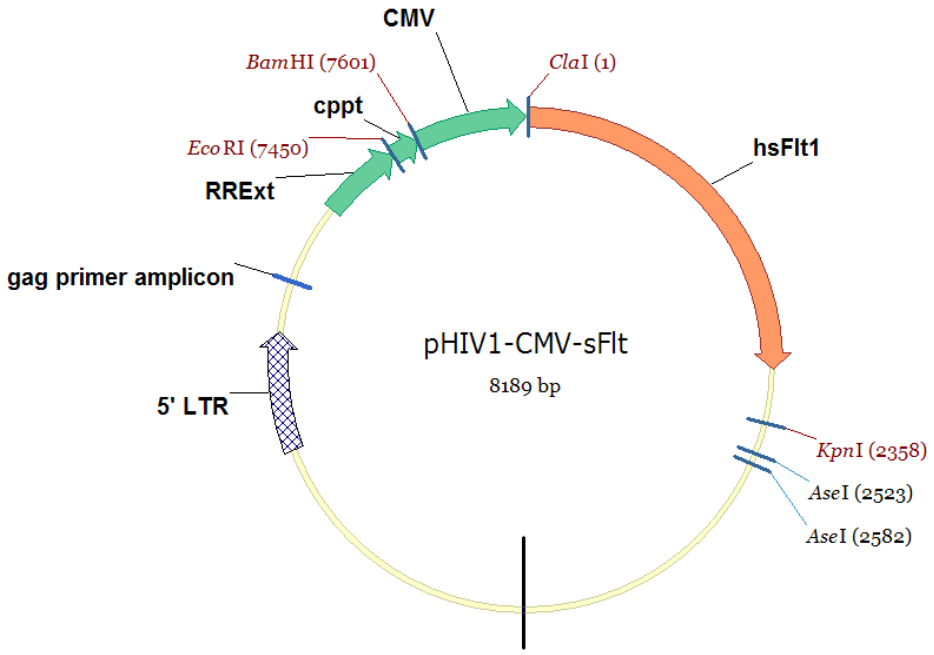


Figure 4.8: Vector map of lentiviral shuttle plasmid containing the human soluble vascular endothelial growth factor receptor 1 (sFlt-1) gene with a CMV promoter. LTR – long terminal repeats, RRExt – Rev response element in the env coding region, cppt – central polypurine tract.

4.4.4.1 Assay to Test for the Biological Activity of Transgenic Human sFlt-1 Protein

A cell-based assay was used to determine the biological efficacy of the human soluble Flt-1 protein at preventing endothelial cell proliferation. This work was conducted by Ms Lauren Mortimer (Department of Ophthalmology, Flinders University).

Transduction of Mammalian Cells to Obtain Conditioned Medium Containing the human sFlt-1 Protein

A conditioned medium sample containing human sFlt-1 protein and a transduction control were obtained from HEK-293T cells transduced LV-sFlt1 at an MOI of 20 or medium, respectively. The conditioned media samples were concentrated approximately 12-fold as described in Section 2.2.13.15, and the LV-sFlt1-transduced medium was determined to contain 129 ng/mL sFlt-1 by sandwich ELISA as described in Section 2.2.13.14.

HUVECs Proliferation Assay

The HUVECs proliferation assay was performed in a manner similar to that described for EK5 (Section 4.4.3.1) except the HUVECs were initially seeded at 1 x 10^3 cells per well in a starvation medium: deficient of heparin, bECGF and 10% (v/v) FBS. Proliferation was stimulated after 24 hours with the addition of recombinant human vascular endothelial growth factor (rhVEGF) at 5 ng per well.

50 μL of either the sterile filtered conditioned medium containing the sFlt-1 protein or the same volume of a similarly-concentrated transduction control conditioned medium was added to the HUVECs, making a total volume of 150 μL per well. On days 4 and 6 post seeding, the starvation medium with VEGF and the conditioned medium were replenished. The relative proliferation of the HUVECs was measured by MTS assays performed at 24 hours and 196 hours post cell seeding as described in Section 2.2.2.3.

Figure 4.9 shows some representative images of HUVECs. Figure 4.10 shows the cell proliferation of untreated HUVECs in starvation medium, HUVECs in starvation

medium with VEGF additive, HUVECs treated with VEGF and conditioned medium containing approximately 43 ng/mL sFlt-1, or HUVECs treated with VEGF and the transduction control conditioned medium. All cells showed an increase in proliferation from the 24 hour time point. The sFlt-1 treated cells showed a significant decrease in proliferation when compared with the transduction control treated cells and compared to the VEGF treated cells at 196 hours ($p \leq 0.0001$ in both cases, one-way between-groups ANOVA). The increase in proliferation shown in the transduction control compared to the cells treated with VEGF alone is due to the extra serum contained in the transduction sample.

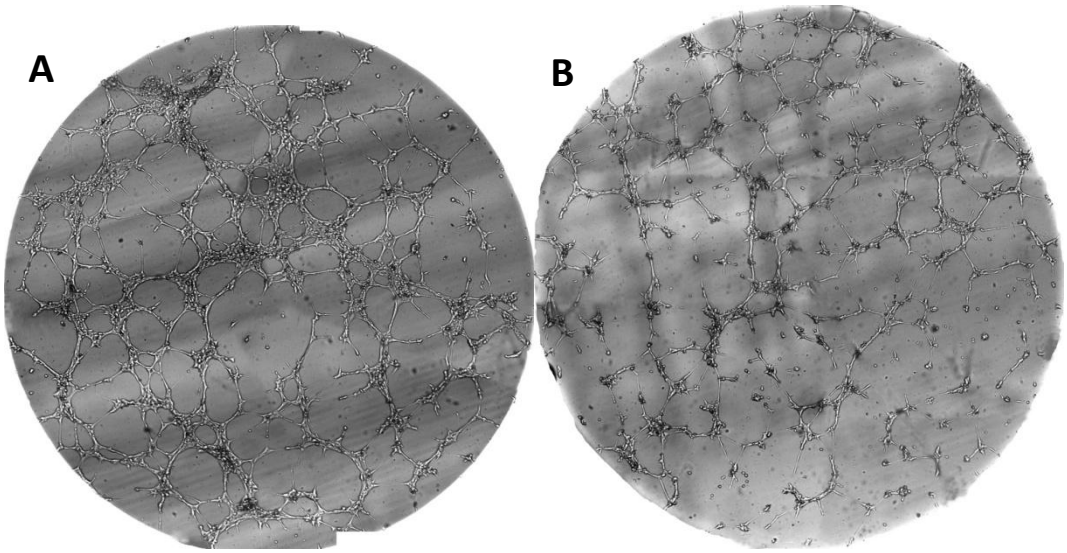


Figure 4.9: *In vitro* images of tube-forming primary human umbilical vein endothelial cells (HUVECs). Spindle-shaped endothelial cells can be seen forming branches and loops for the beginnings of tube-like structures in culture. **(A)** HUVECs growing in proliferation medium **(B)** HUVECs growing in proliferation medium treated with a conditioned medium containing sFlt-1. Inhibition of tube formation is apparent, with fewer branches and loops compared with image (A). These images were kindly provided by Ms Lauren Mortimer.

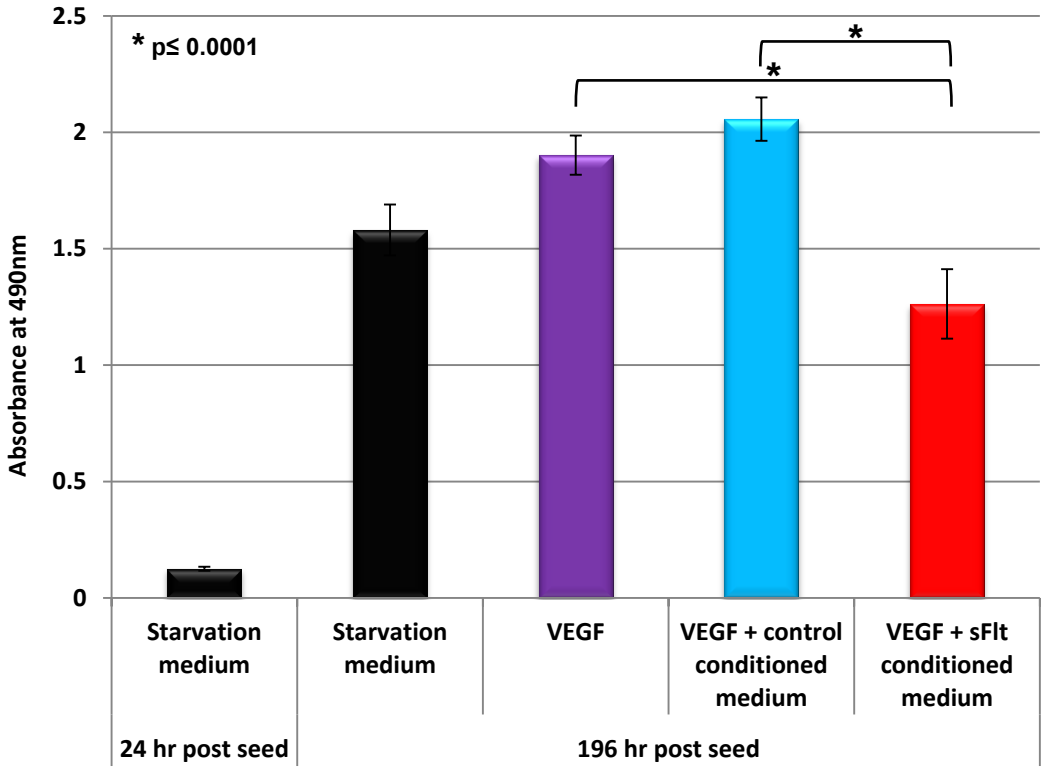


Figure 4.10: MTS proliferation assay on HUVECs treated for 7 days with sFlt-1-containing supernatant from cell culture transduction. sFlt-1 conditioned medium contained approximately 43 ng/mL sFlt-1. All treatments had a biological sample size of n= 6 except sFlt-1 and control conditioned media samples, which had biological sample sizes of n= 3. * p≤ 0.0001 (one-way between-groups ANOVA). This figure was kindly provided by Ms Lauren Mortimer.

4.4.5 Testing of Transgene Expression in the Sheep Cornea

Ovine corneas were transduced with the four lentivirus constructs, individually, at 37^{o}C for 3 hours at an MOI of 20 transducing units per cell, and were cultured *in vitro* for 10 days. Supernatant samples were taken every two days and the corneal endothelial cells were harvested at day 10 for gene expression testing at the protein and mRNA levels, respectively.

4.4.5.1 Transgene mRNA Determinations

Quantitative real-time PCR was performed on the cDNA prepared from the RNA extracted from the corneal endothelial cells. 0.5 μg of RNA was used in the cDNA synthesis reactions, however when this amount could not be achieved due to low RNA yield, the maximum amount of RNA possible was used. A standard sample was created as described in Section 2.2.13.8, with cDNA from all representative samples. Primer set efficiencies were calculated as described in Section 2.2.13.8, and these efficiencies were factored into the gene expression analysis (Section 2.2.13.9).

Figure 4.11 shows the qPCR results of individual transgene expression at the mRNA level, from mock transduced sheep corneas and sheep corneas transduced with the lentiviral vectors encoding transgenes human sFlt-1, ovine IDO, ovine Bcl-xL or human EK5, all with a CMV promoter inducing expression, relative to expression in the standard sample. Over-expression of the transgenes was demonstrated at the mRNA level for all four lentiviral vectors compared with the mock transduced controls. The bars in the figure represent individual corneas, with three to six corneas treated per group. The varying expression levels occurring between identically treated corneas indicated a quite variable transduction efficiency of individual corneas by each lentiviral vector.

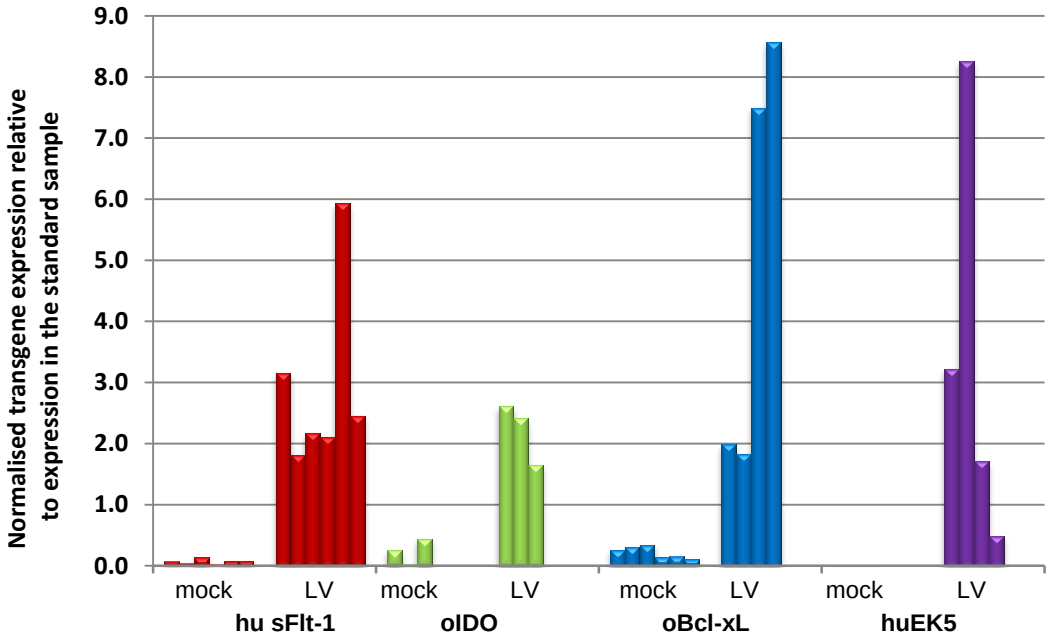


Figure 4.11: mRNA expression of transgenes by endothelial cells from individual cultured ovine corneas ten days post-transduction, as measured by quantitative real-time PCR. Bars represent transgene expression relative to expression in the standard sample for each treated cornea. Three to six individual corneas were transduced with each lentiviral vector and the resulting cDNA amplified for transgene expression (LV-sFlt1 n= 6, LV-IDO n= 3, LV-BclxL n= 4, and LV-EK5 n= 4). cDNA from mock treated corneas was amplified for human sFlt-1, ovine IDO, ovine Bcl-xL and human EK5 expression (n= 6, 3, 6 and 3, respectively).

4.4.5.2 Transgene Protein Determinations

The culture supernatant was assayed at five time points across the ten day cultures, for the presence of the proteins human endostatin, human sFlt-1, ovine Bcl-xL and the metabolite L-kynurenine, a stable down-stream product of tryptophan degradation catalysed by IDO. Sandwich ELISAs were used to detect the three

proteins and a colourimetric assay was used to detect kynurenine levels, all as described in Sections 2.2.13.10 - 14. Supernatant samples from LV-sFlt1, LV-BclxL and LV-IDO treated corneas were concentrated as described in Section 2.2.13.15 prior to use in detection assays.

Human sFlt-1

The lentiviral vector containing the human sFlt-1 transgene was used to transduce sheep corneas and the culture supernatants from six mock-treated and six LV-sFlt1-treated corneas were pooled and concentrated approximately 20-fold and assayed by sandwich ELISA for human sFlt-1 protein expression. Figure 4.12 shows the expression of sFlt-1 protein by transduced corneas compared with control mock transduced corneas, which do not express human sFlt-1. sFlt-1 was detected in the supernatant across all time points of the culture in the treated corneas, however peak expression occurred at day 2 before a 13-fold decrease to day 4 and a continuous decline across the remaining culture time points. This experiment was conducted twice and this figure shows the results of the combined experiments. The total number of corneas for each treatment was six.

The lentiviral vector encoding the sFlt-1 transgene was able to transduce ovine cornea endothelial cells, however the decline in transgenic sFlt-1 protein over the culture period suggested transgene expression might be transient *in vivo*.

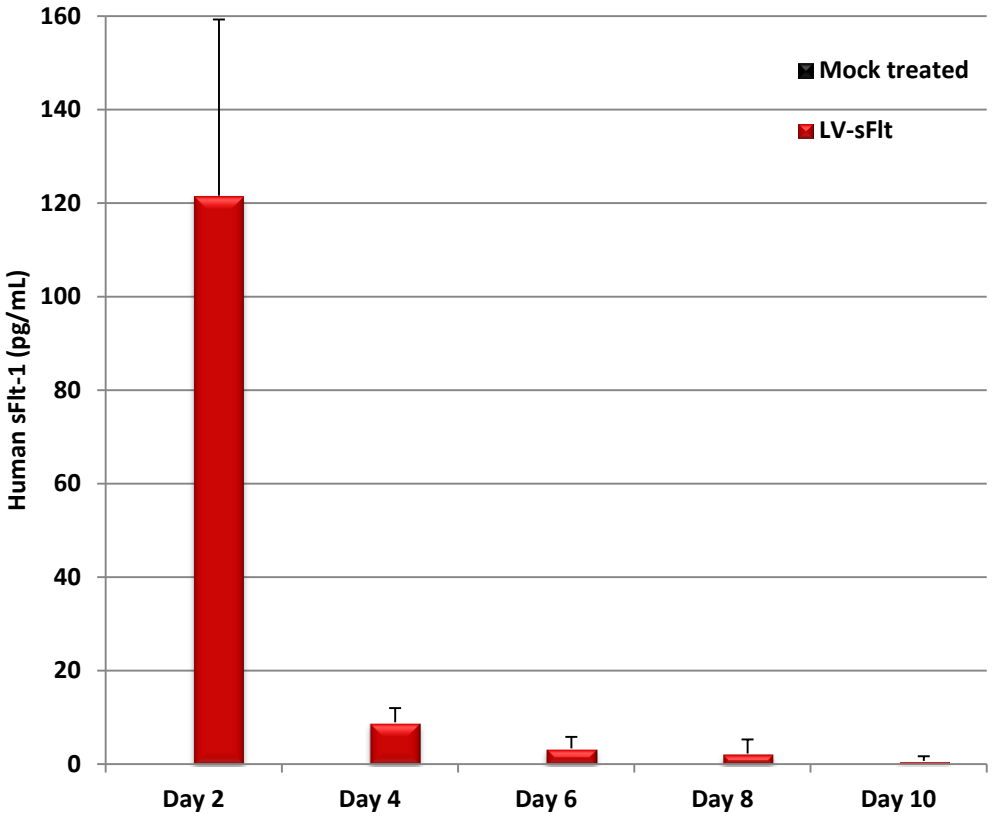


Figure 4.12: Mean sFlt-1 protein detected in the concentrated culture supernatant of sheep corneas transduced with the lentiviral vector LV-CMV-sFlt1, measured by sandwich ELISA. Pooled biological replicates (corneas) n= 6. Error bars represent the standard deviation in the technical replicates (minimum n= 5). Human sFlt-1 was undetectable in all mock transduced corneas assayed.

Ovine IDO

The metabolite kynurenine was *not* detected at higher levels in the culture supernatant of the corneas treated with the lentiviral vector expressing IDO compared with the supernatant of the mock treated control corneas (Figure 4.13 (A)). When L-tryptophan was added to the culture media as a substrate for the IDO enzyme, the levels of kynurenine were still not detected at higher levels in the culture supernatant of the corneas treated with the lentiviral vector expressing IDO compared with the supernatant of the mock treated control corneas (Figure 4.13 (B)). The lentiviral vector encoding the IDO transgene was shown by mRNA expression to transduce ovine cornea endothelial cells (Figure 4.11), however the activity of the transgenic IDO protein in lentivirus-treated ovine corneas was not shown to be higher than in the mock-treated corneas.

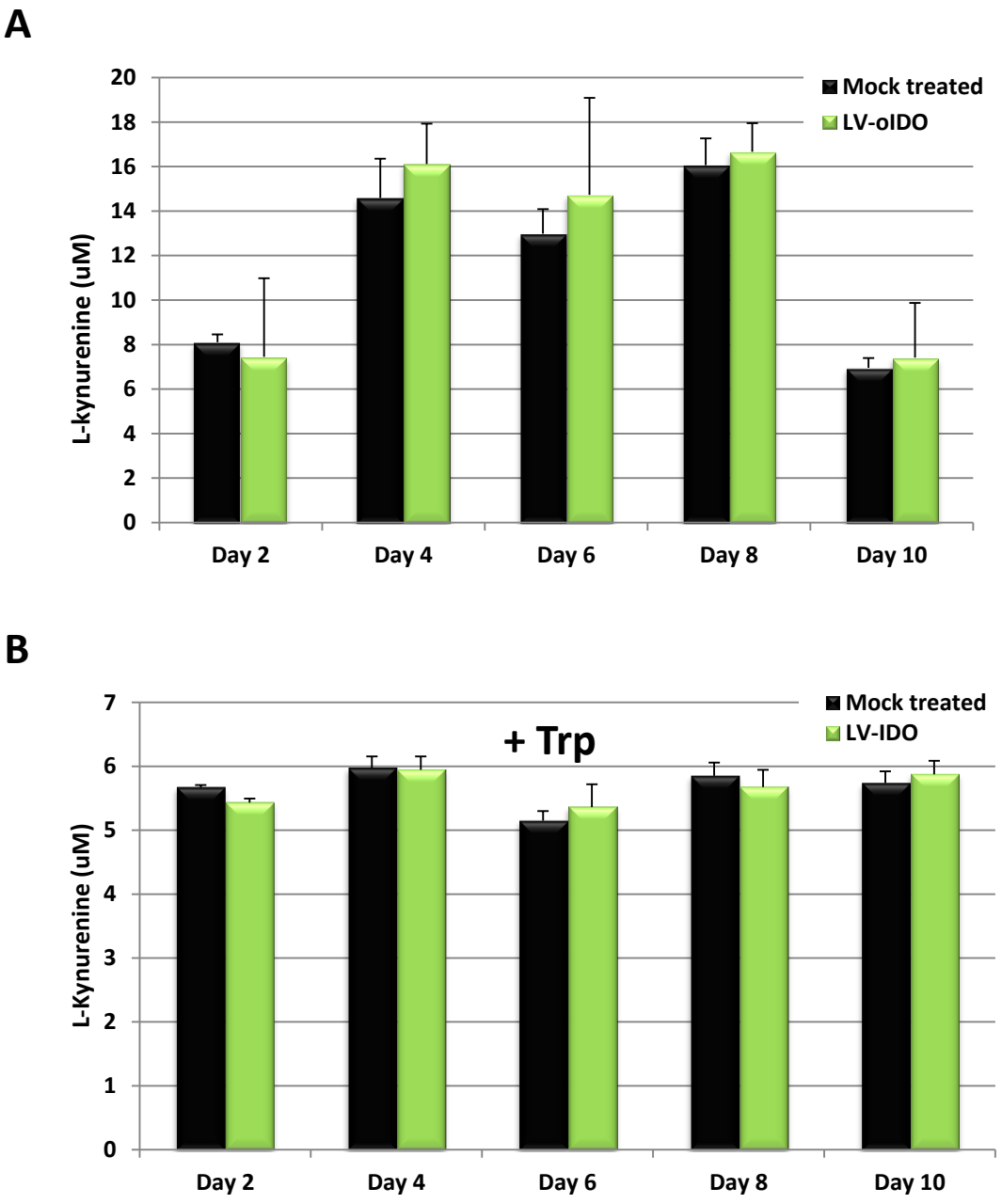


Figure 4.13: L-kynurenine detected in the concentrated culture supernatant of sheep corneas transduced with the lentiviral vector LV-CMV-oIDO. (A) Pooled biological replicates (corneas) n= 6. Error bars represent the standard deviation in the assay technical replicates n= 6. **(B) Culture medium was supplemented with L-tryptophan to a final concentration of 200 µM.** Pooled biological replicates (corneas) n= 4. Error bars represent the standard deviation in the assay technical replicates n= 3.

Ovine Bcl-xL

The lentivirus containing the ovine Bcl-xL transgene was used to transduce sheep corneas and both the mock-treated and the LV-BclxL-treated cornea culture supernatants were concentrated approximately 20-fold and assayed in a sandwich ELISA for ovine Bcl-xL protein expression. Figure 4.14 shows over-expression of Bcl-xL by the transduced corneas compared with control mock-treated corneas, which show the endogenous levels of Bcl-xL secreted by the corneal cells. A high level of Bcl-xL protein was detected in the supernatant across all time points of the culture, greatly increased from the endogenous expression levels shown. This culture experiment was conducted twice and the results are the combination of two experiments. The total number of corneas included for each treatment was six. The lentiviral vector encoding the ovine Bcl-xL transgene was able to transduce ovine cornea endothelial cells and induce stable transgene expression.

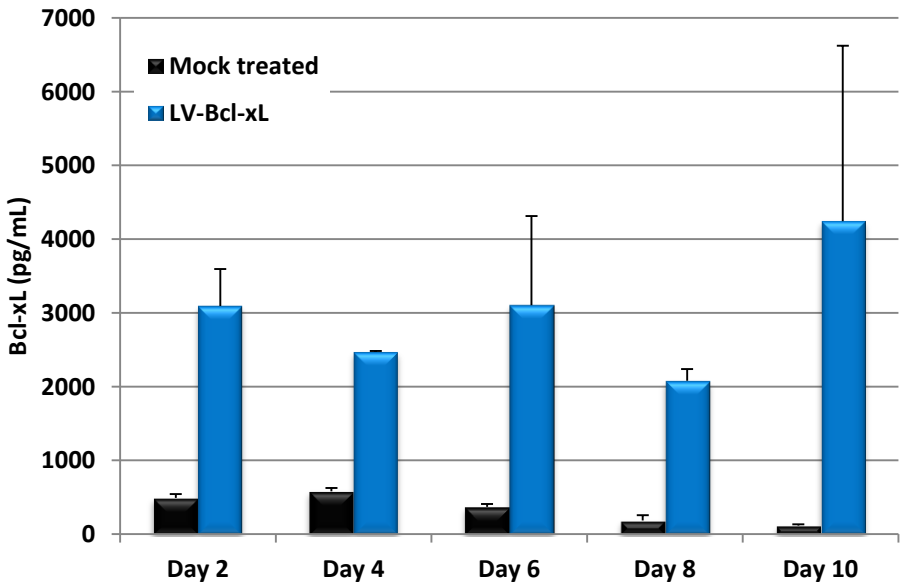


Figure 4.14: Average ovine Bcl-xL detected in the concentrated culture supernatant of sheep corneas transduced with the lentivirus containing the construct LV-CMV-oBclxL, by sandwich ELISA. Biological replicates (pooled corneas) n= 6, error bars represent the standard deviation in the technical replicates n= 4.

Human EK5

The lentivirus containing the human EK5 fusion gene was used to transduce sheep corneas and the culture supernatant was assayed in a sandwich ELISA for human endostatin expression. Figure 4.15 shows over-expression of human endostatin protein by the lentivirus transduced corneas compared with control mock-transduced corneas, which do not express human endostatin. Endostatin protein was detected in the supernatant of the lentiviral vector-treated corneas across all time points of the culture, increasing to day 10. The total number of corneas included for each treatment was three. The lentiviral vector encoding the EK5 transgene was able to transduce ovine cornea endothelial cells and induce stable transgene expression.

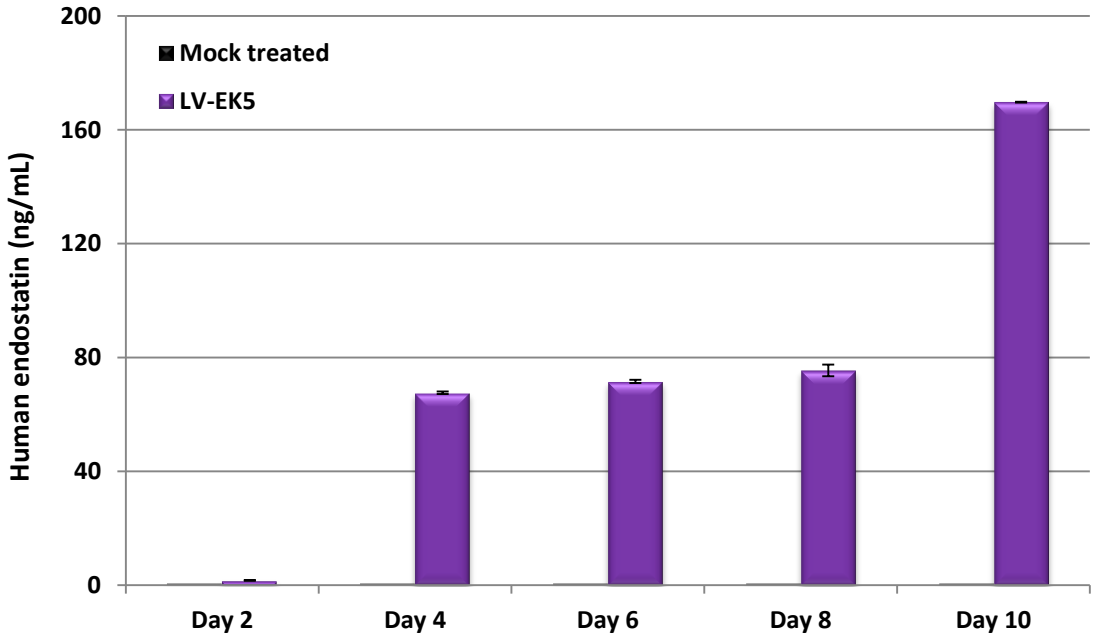


Figure 4.15: Mean human endostatin protein detected in the culture supernatant of sheep corneas transduced with the lentiviral vector LV-CMV-EK5, measured by sandwich ELISA. Pooled biological replicates (n= 3). Error bars represent the error range in the technical replicates (n= 2). Mock treated bars are not visible in figure as no human endostatin protein was detected in culture supernatant.

4.4.5.3 Summary of Vector Characterisation

The four lentiviral vectors each successfully transduced ovine corneas *in vitro* and over-expression of all four transgenes was detected at the mRNA level in ovine corneal endothelial cells. The lentiviral vectors encoding Bcl-xL and EK5 produced detectable transgenic protein in the ovine cornea culture supernatant across all culture time points, therefore these two vectors were selected for *in vivo* use in a cocktail therapy.

4.4.6 Adenoviral and Lentiviral Vector Cocktail Interference Test in Sheep Corneas

To determine whether the transduction of corneal cells using viruses with different transduction mechanisms would cause any vector interference, *in vitro* cocktail testing was conducted. For this experiment and further experiments, the adenoviral vector previously constructed by Dr Douglas Parker (Department of Ophthalmology, Flinders University) was used, see Section 2.2.3 and vector map Figure 4.16. Ovine corneas were transduced with the adenoviral vector containing the oIL-10 transgene at an MOI of 35 plaque forming units (pfu) per cell, simultaneously with the lentiviral vector containing the human EK5 transgene at an MOI of 40 transducing units (TU) per cell. The corneas were transduced at 37^{o}C for 3 hours, before culturing for six days. On days three and six, supernatant samples were taken for protein expression analysis and on day six the corneal endothelial cells were harvested for gene expression analysis at the mRNA level. All EK5-treated cornea supernatant samples were pooled and some of these were concentrated between 25- and 38-fold prior to ELISA assays. Due to loss of corneas owing to culture fungal infections and low RNA recovery, extra corneas were transduced and cultured for

either protein or mRNA analysis, therefore the n-values between Figures 4.17 and 4.18 may not directly correspond.

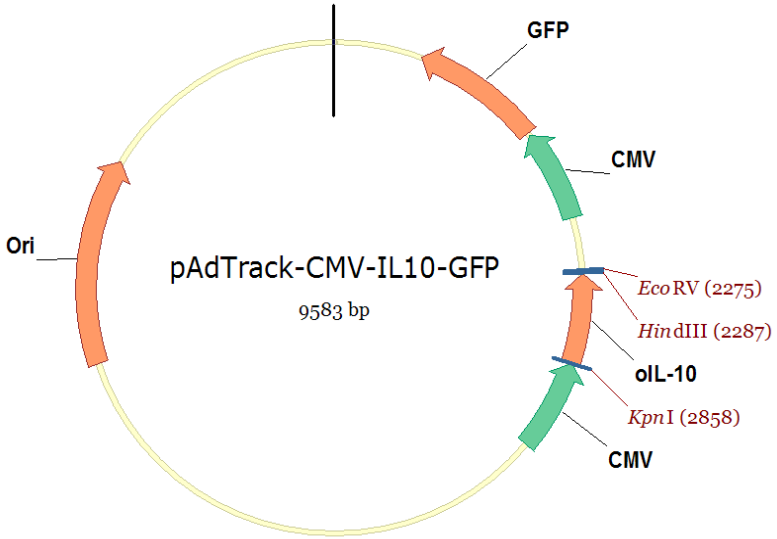


Figure 4.16: Vector map of the adenoviral vector expressing transgenes ovine interleukin-10 and green fluorescent protein (GFP), each with an internal CMV promoter.

4.4.6.1 Transgene mRNA Determinations

Figure 4.17 shows the qRT-PCR results of human EK5 and ovine IL-10 mRNA expression by corneal endothelial cells of corneas treated with the individual lentiviral and adenoviral vectors and with the vector cocktail treatment. Over-expression of EK5 was observed for both the lentiviral vector alone-treated corneas and the corneas treated with the cocktail of the lentiviral vector and the adenoviral vector at day 6, compared with the mock treated corneas, where transgene expression was undetected. The same result was seen for the adenoviral vector alone-treated corneas and the corneas treated with the cocktail containing both the adenoviral vector and the lentiviral vector: ovine IL-10 mRNA over-expression was evident for

both of these treatment groups. No expression of oIL-10 was detected in the mock treated corneas. It is noteworthy that the variability in transgene expression was again evident for individual corneas, indicating ovine cornea individual transduction efficiencies were quite variable.

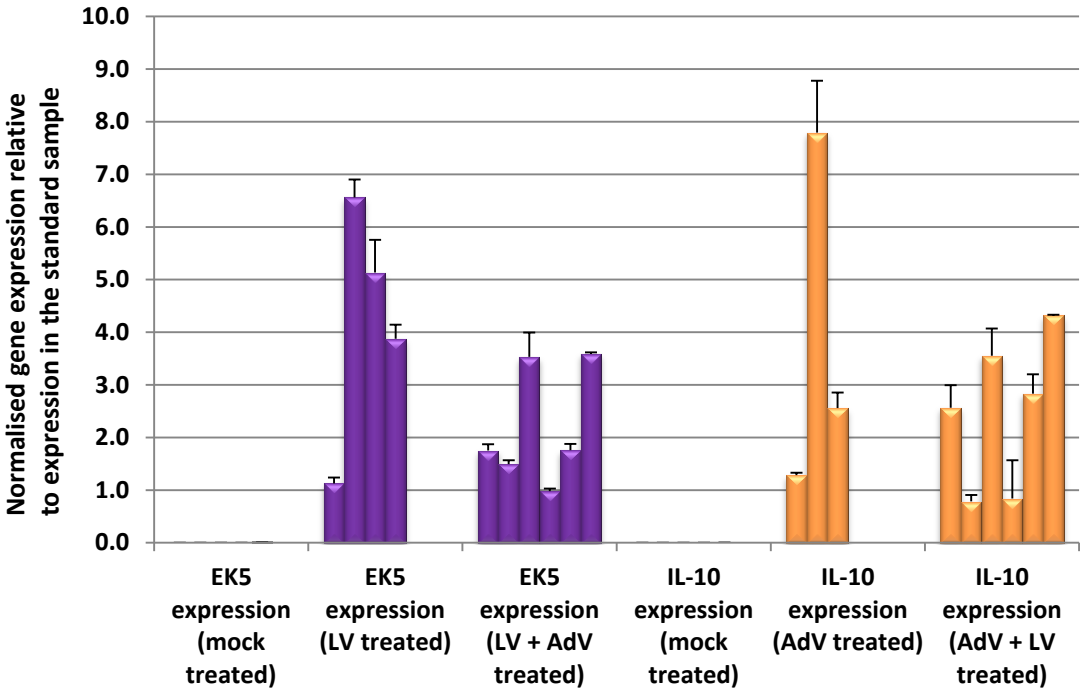


Figure 4.17: Ovine IL-10 and human EK5 mRNA expression by ovine corneal endothelial cells from individually transduced corneas at day 6 post transduction. Corneas were treated with LV-EK5 (n= 4) or AdV-IL10 (n= 3) alone, or with a cocktail of the two virus preparations (n= 6). Gene expression was measured by qRT-PCR and expression was relative to the expression in the standard sample. No expression of huEK5 or oIL-10 was detected in the mock treated corneas (n= 5). Error bars represent the standard deviation from the qRT-PCR technical replicates (n= 3).

4.4.6.2 Transgene Protein Determinations

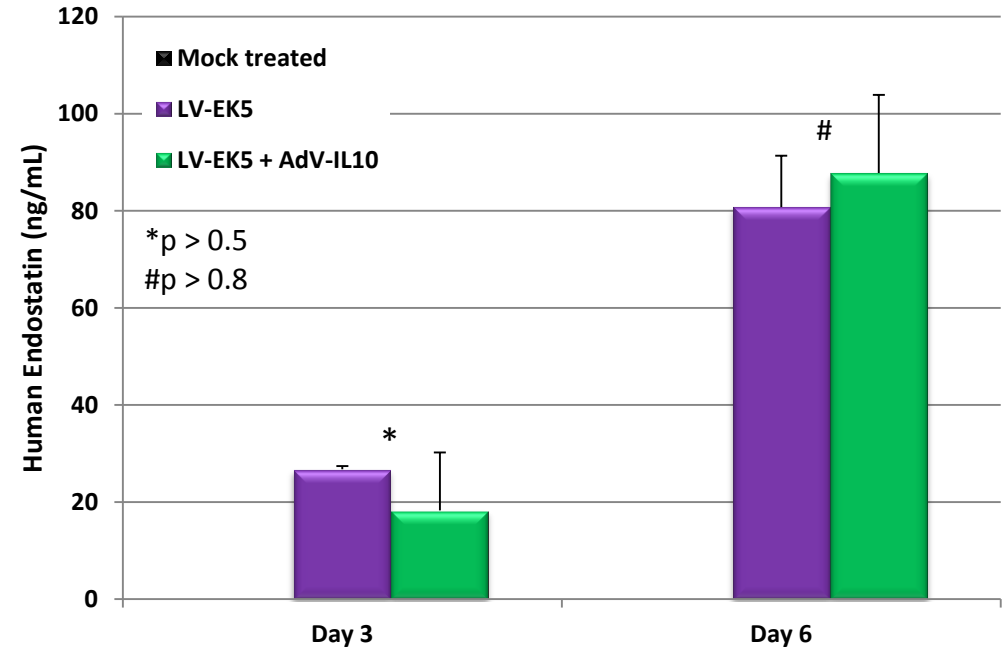
Figures 4.18 (A) and (B) show the human endostatin and ovine IL-10 expression, respectively, detected at the protein level from the culture supernatant of the cocktail-treated ovine corneas. Similar levels of human endostatin protein were detected from corneas treated with the lentiviral vector alone and corneas treated with the cocktail containing the lentiviral vector and the adenoviral vector at both day 3 and day 6 (Figure 4.18 (A)). Similarly, comparable levels of ovine IL-10 protein were detected from corneas treated with the adenoviral vector alone and corneas treated with the cocktail containing the adenoviral vector and the lentiviral vector at both culture time points (Figure 4.18 (B)).

The mRNA and protein results from the vector cocktail experiments indicated that the lentiviral vector and the adenoviral vector did not exhibit interference, and that transgene expression was not affected in simultaneous transduction of sheep corneas with a vector cocktail.

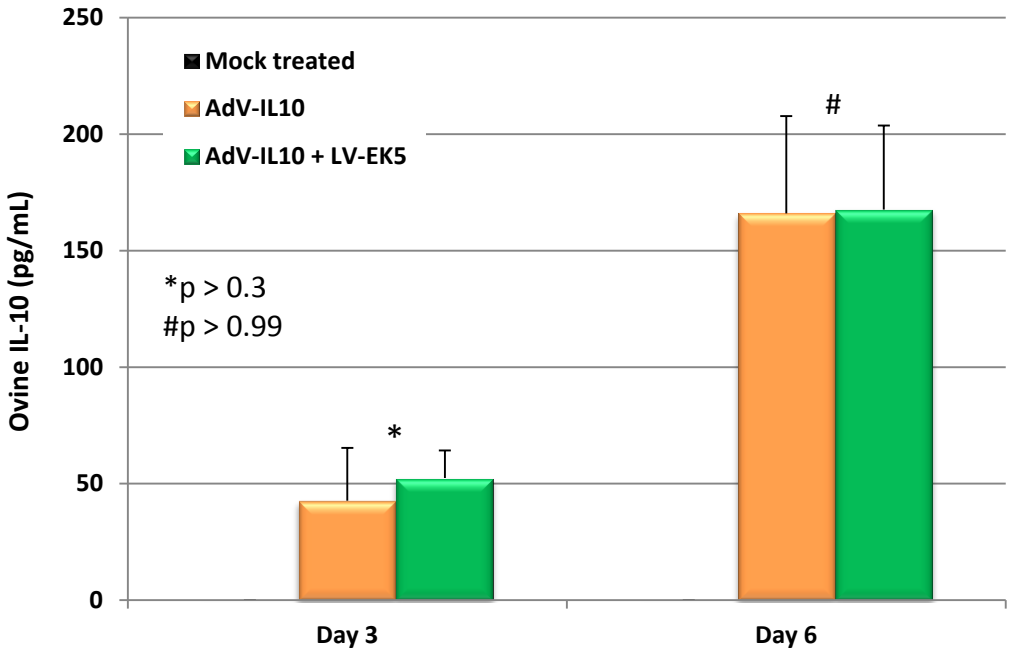
Figure 4.18: Mean protein detected in culture supernatant of sheep corneas transduced with the either (A) the lentiviral vector encoding the human EK5 transgene alone, (B) the adenoviral vector encoding the ovine IL-10 transgene alone, or (A) & (B) a cocktail containing both the lentiviral and adenoviral vectors. (A) Human endostatin detected in culture supernatant, minimum biological replicates (corneas) n= 5. (*p > 0.5, #p > 0.8 one-way ANOVA, Dunnett T3 Post Hoc test). **(B) Ovine IL-10 detected in culture supernatant**, minimum biological replicates (corneas) n= 5. (*p > 0.3, #p > 0.99 one-way ANOVA, Dunnett T3 Post Hoc test).

Bars for mock-transduced corneas are not visible in the figures as both the human endostatin and oIL-10 detected in mock-transduced samples was either very low or undetected. Protein levels were measured by sandwich ELISAs. Error bars represent the standard deviation of the means, from the technical replicates (endostatin ELISA min n= 4; IL-10 ELISA min n= 15, max n= 18).

A


120
100
80
60
40
20
0
Human Endostatin (ng/mL)
Mock treated
LV-EK5
LV-EK5 + AdV-IL10
*p > 0.5
#p > 0.8
*
#
Day 3
Day 6

B


250
200
150
100
50
0
Ovine IL-10 (pg/mL)
Mock treated
AdV-IL10
AdV-IL10 + LV-EK5
*p > 0.3
#p > 0.99
*
#
Day 3
Day 6

4.4.7 Adenoviral and Lentiviral Vector Cocktail Interference Test in Human Corneas

To determine whether the transduction of corneal cells using viruses with different transduction mechanisms would cause any vector interference in *human* corneas, *in vitro* cocktail testing was conducted. Human corneas were a generous gift from the Eye Bank of South Australia following consent from donor families, and were prepared as described in Sections 2.2.7 and 2.2.9. The corneas were transduced with the adenoviral vector containing the oIL-10 transgene at an MOI of 35 pfu per cell simultaneously with the lentiviral vector containing the EK5 transgene at an MOI of 40 TU per cell. As with the ovine corneas, the human corneas were transduced at 37^{o}C for 3 hours, before culturing for six days. On days three and six, supernatant samples were taken for protein expression analysis. Due to the limited availability of human corneas for research, only the viral cocktail was tested on human corneas and no individual vector transductions were performed.

Figures 4.19 (A) and (B) show the average transgenic protein detected in the culture supernatant of human corneas transduced simultaneously with AdV-IL10 and LV-EK5. The human endostatin protein was detected at a 4-fold higher level in the supernatant of the cocktail treated human corneas compared with endogenous level in the mock treated control cornea at day 6, demonstrating successful transduction of the human corneas with the LV-EK5 vector (Figure 4.19 (A)). Figure 4.19 (B) shows a marked increase in IL-10 expression from day 3 to day 6 during the culture, demonstrating a successful transduction of all three human corneas with the AdV-IL10 vector. The IL-10 protein expression was lower than from ovine cornea culture (Figure 4.18 (B)), however the human corneas had been stored for a long period of time (at least 14 days in cold storage) prior to transduction and the endothelial cell density was likely to have been poor in comparison.

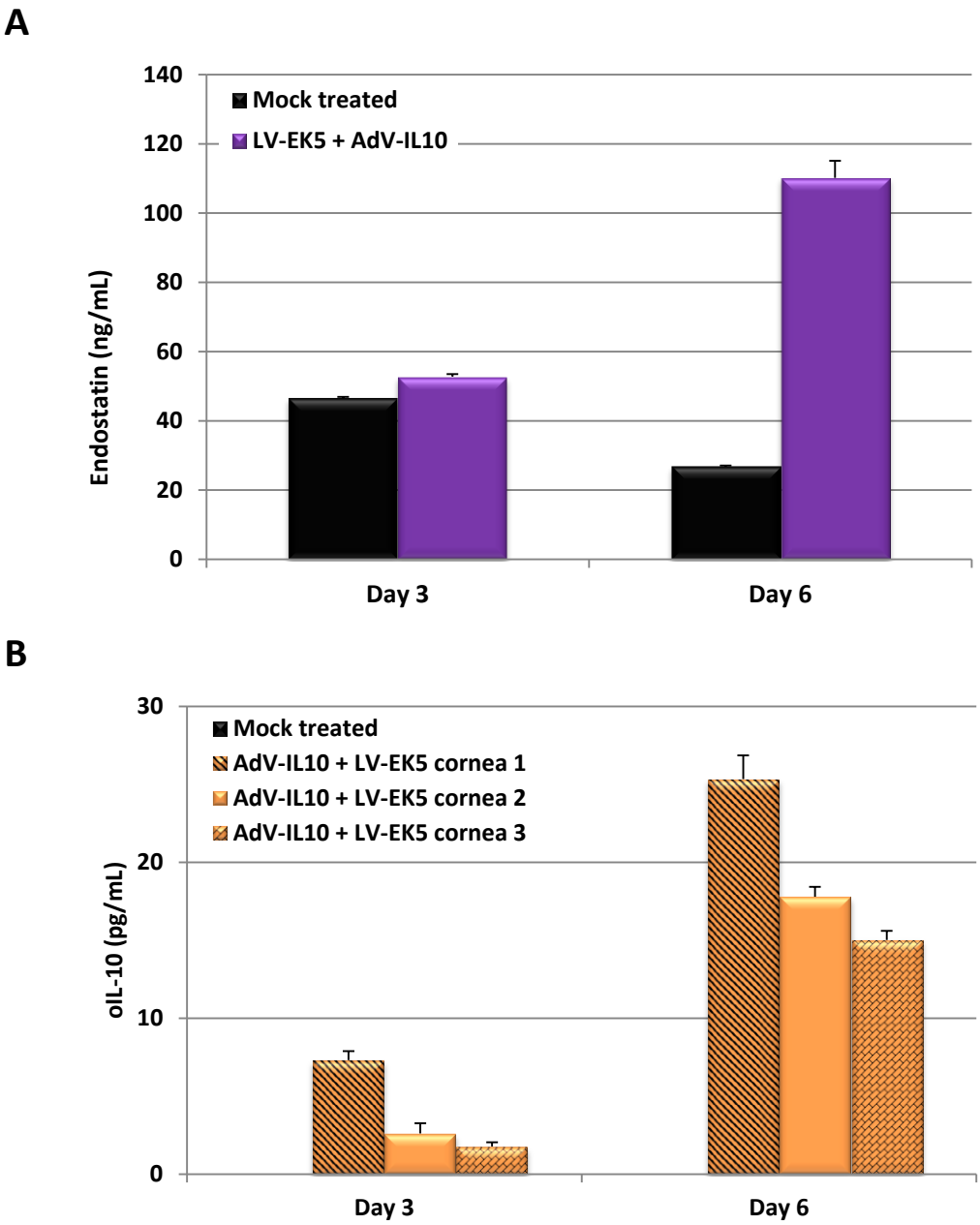


Figure 4.19: Mean protein detected in the culture supernatant of human corneas transduced with vector cocktail LV-EK5 and AdV-oIL10. (A) Human endostatin protein. (B) Ovine IL-10 protein. Measured by sandwich ELISA. Biological replicates (corneas) mock-treated n= 1, cocktail-treated n= 3. Error bars represent the standard deviation of the technical replicates (n= 3). Individual diluted supernatant samples used in IL-10 ELISA. Samples pooled and concentrated prior to endostatin ELISA assay.

4.4.8 In Vivo Therapeutic Testing of Viral Cocktail to Prolong the Survival of Sheep Corneal Allografts

Based on *in vitro* testing, the lentiviral vectors expressing Bcl-xL and EK5 were selected for subsequent therapeutic testing, as both of these lentiviral vectors induced detectable expression of the transgenes at both the mRNA and protein levels in ovine corneas. An MOI of 40 viral particles per target cell was chosen for each lentiviral vector to ensure the total lentivirus MOI in the cocktail therapy would not exceed 100 particles per target cell, the highest amount tested *in vitro* without adverse effects on endothelial cell health (Section 3.4.6). The ovine interleukin-10 transgene had been previously tested for biological activity.[202] All of the lentivirus and adenovirus preparations used *in vivo* were tested for level of endotoxin contamination using the LAL test described in Section 2.2.4.3. This test was performed by Ms Lauren Mortimer (Dept. Ophthalmology, Flinders University). The endotoxin contamination levels for the virus preparations are listed in Table 2.13, Section 2.2.4.2. The total amount of endotoxin units in the cocktail therapy (LV-BclxL, LV-EK5, and AdV-IL10) was between 2.4 and 3.4 units per cornea. Due to the high MOI (80) required for the LV-eYFP in the mock-vector control treatment, three preparations of this lentivirus were required and the level of endotoxin contamination varied between preparations. Of the 12 mock-vector-treated sheep (AdV-GFP, LV-eYFP), 5 animals had between 6.7 and 10.2 endotoxin units (EU) per cornea, 5 sheep had between 21 and 25.3 EU per cornea, and 2 sheep had a minimum of 33.6 EU per cornea.

Donor sheep corneas were prepared as described in Sections 2.2.8 - 9. Cocktail therapy corneas were transduced for 3 hours at 37^{o}C with AdV-CMV-oIL10-CMV-GFP at 5 x 10^{7} pfu/ cornea (MOI 35), LV-CMV-oBclxL and LV-CMV-EK5 each at

5.6 x 10^7 TU/cornea (MOI 40) in transducing medium as described in Section 2.2.10.2. The control corneas were transduced with AdV-GFP at 5 x 10^7 pfu/ cornea (MOI 35) and LV-eYFP at 1.12 x 10^8 TU/cornea (MOI 80). Penetrating keratoplasties were performed on the sheep by an ophthalmologist, as described in Section 2.2.10. The cocktail therapy group and the mock vector control group each consisted of twelve animals. The remaining donor corneal rims were cultured for six days, with supernatant samples taken on days 2 and 6 to assay for transgene expression at the protein level (Sections 2.2.10.4, 2.2.13.10 and 2.2.13.13). On day 6 the donor corneal rims were fixed, the endothelial cells stained with Hoechst-33258 dye and this monolayer flat-mounted to visualise transduction success via eYFP/ GFP expression by endothelial cells, using fluorescence microscopy (Sections 2.2.13.1 and 2).

Grafts were assessed daily with a hand-held slit lamp for inflammation, neovascularisation, clarity, oedema, presence of inflammatory cells and fibrin formation. Score sheets are shown in Appendix A3.2. The onset of immunological rejection was judged to be upon the presence of a rejection line or a corneal clarity score of 2, on a scale of 0 - 4. Grafts were considered technical failures if they did not become thin and clear by 10 days post-operatively and the survival days of these sheep grafts were not considered in the results. One experimental animal was considered a technical failure out of a total of 25 animals for this reason.

4.4.8.1 Histology

Endpoint haematoxylin and eosin staining was performed on buffered formalin-fixed, paraffin wax-embedded corneal graft sections post-mortem as described in Section 2.2.11, and indirect immunohistochemical (IHC) labelling of the corneal graft tissue post-mortem was conducted using monoclonal antibodies to sheep CD4, CD8, CD45 and MHC class II expressing cells as described in Section 2.2.12. Figure 4.20 shows representative images of labelled sheep cornea sections. The endpoint histology results demonstrated localised cellular infiltrates consisting of predominantly mononuclear cells in all rejected grafts. Stronger inflammation, in terms of the number of inflammatory cells present, was observed in the majority of cocktail-treated rejected grafts than in the mock-vector treated rejected grafts. Of particular note, was an increased frequency in the formation of retrocorneal membranes behind Descemet's membrane in the cocktail-treated sheep allografts, occurring in six out of the twelve animals, compared with one occurrence in the mock-vector treated group. The formation of the retrocorneal membranes may have been the result of inflammation. Interestingly, the two mock-vector-treated sheep with the highest levels of endotoxin contamination in the transduction media (Table 4.1, sheep 15 and sheep 18) exhibited comparatively low inflammation in terms of the number of inflammatory cells present, suggesting that endotoxin contamination was not the cause of excessive inflammation in the cocktail-treated sheep corneas.

Immunohistochemical labelling of paraformaldehyde-lysine-periodate (PLP) fixed frozen corneal sections from rejected allografts revealed a population of CD45-positively labelled cells in the corneal stroma, at the graft margin and suture sites. The inflammatory infiltrate consisted of a higher proportion of CD4-labelled cells than CD8-labelled cells in both the cocktail and the mock-vector treatment groups.

There were slightly higher numbers of CD4-positive labelled infiltrating cells in the cocktail-treated allografts compared with the mock-vector treated allografts. The cocktail-treatment group also showed higher numbers of CD8-labelled cells in the allograft sections than the mock-vector treated allografts. Many CD45-positive cells also labelled positively with the MHC class II monoclonal antibody. A higher proportion of MHC class II-positive cells was observed in the cocktail-treated allograft sections than in the mock-vector treated allograft sections. No labelling was observed in the negative control antibody sections. These results are shown in Table 4.1.

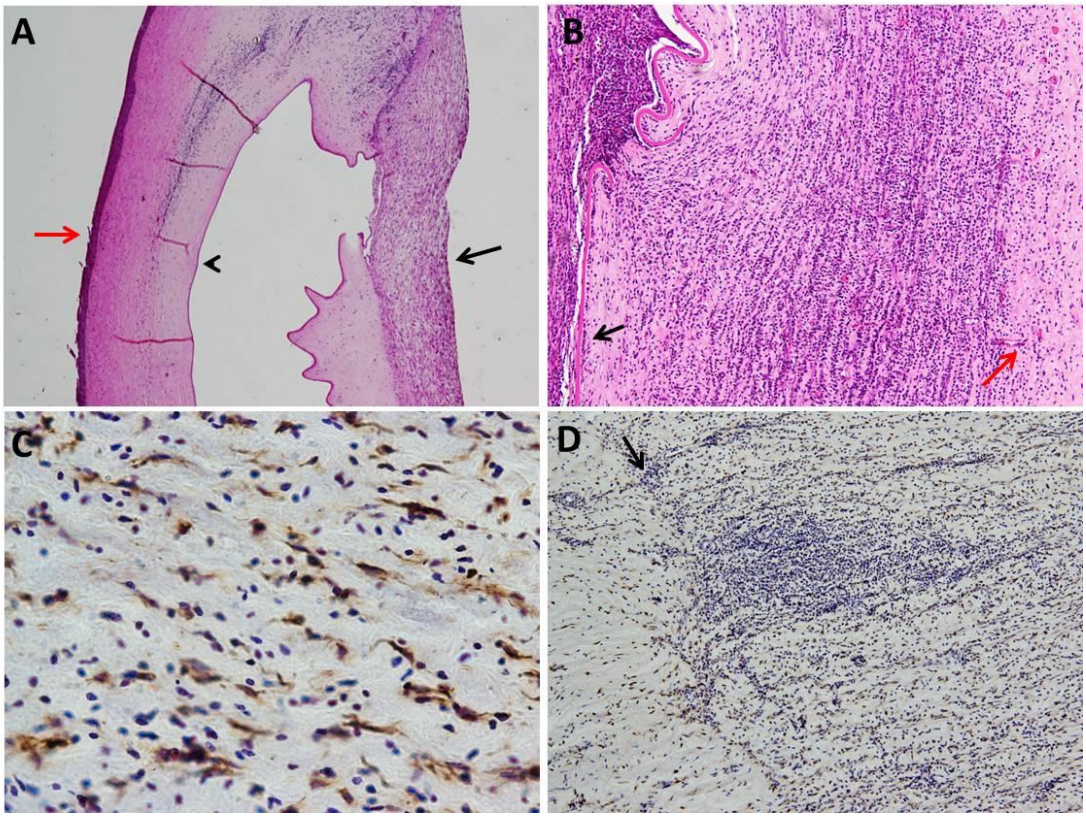


Figure 4.20: Representative images of immunohistochemical cell labelling in sheep corneal allograft sections (8µm). A) haematoxylin and eosin stained section from a sheep allograft showing retrocorneal membrane formation, red arrow indicates corneal epithelium, arrow-head indicates Descemet's membrane, black arrow indicates the retrocorneal membrane. 40x original magnification **B)** haematoxylin and eosin stained section from a sheep allograft showing heterogeneous inflammatory cell infiltrate, black arrow indicates Descemet's membrane, red arrow indicates example of blood vessel. 100x original magnification **C)** MHC class II (brown-labelled) cells in a sheep allograft, 400x original magnification **D)** CD4-labelled section from a sheep allograft showing the labelled cellular infiltrate at the graft margin, indicated with the arrow. 100x original magnification.

Table 4.1: Immunohistochemical labelling of rejected sheep corneal allograft sections. Percentage of cells relates to the number of positively labelled cells for each monoclonal antibody, from the total cell population which were also CD45 positive.

Recipient Sheep	Treatment	Cells labelled with anti-CD4 (%)	Cells labelled with anti-CD8 (%)	Cells labelled with anti-MHC class II
Sheep 1	Cocktail therapy	50	30	++
Sheep 2	Cocktail therapy	50	30	++
Sheep 3	Cocktail therapy	50	25	++
Sheep 4	Cocktail therapy	30	10	++
Sheep 5	Cocktail therapy	50	20	++
Sheep 6	Cocktail therapy	50	50	++
Sheep 7	Cocktail therapy	40	40	++
Sheep 8	Cocktail therapy	30	20	++
Sheep 9	Cocktail therapy	40	10	++
Sheep 10	Cocktail therapy	50	30	++
Sheep 11	Cocktail therapy	20	30	+
Sheep 12	Cocktail therapy	20	10	+
Sheep 13	Mock-vector	50	20	+
Sheep 14	Mock-vector	5	20	+
Sheep 15	Mock-vector	10	<10	+
Sheep 16	Mock-vector	<10	<10	-/+
Sheep 17	Mock-vector	30	20	+
Sheep 18	Mock-vector	20	20	++
Sheep 19	Mock-vector	40	<10	+
Sheep 20	Mock-vector	50	20	++
Sheep 21	Mock-vector	50	10	+
Sheep 22	Mock-vector	20	<10	+
Sheep 23	Mock-vector	30	<10	+
Sheep 24	Mock-vector	<10	<10	-/+

-/+ very rare positive labelling; + few positive cells; ++ moderate positive labelled cells

4.4.8.2 Transgenic Protein Expression in Donor Rims

The culture supernatants of the rims from donor corneas used in sheep corneal graft surgery were assayed for the expression of ovine IL-10 and human endostatin protein. This was done to investigate whether transgene expression should be expected from the graft tissue, i.e. whether transduction had been successful. Tables

4.2 and 4.3 show the levels of ovine IL-10 and human endostatin, respectively, detected by sandwich ELISAs at days two and six post-operatively from the cultured donor corneal rims. At day 2, an average of an almost 800-fold increase in oIL-10 was observed in corneas transduced with the cocktail therapy containing the AdV-CMV-oIL10 vector, relative to the mock-vector control transduced corneas, and at day 6 this rose to an average of over 3000-fold higher expression relative to control corneas. Endostatin was also detected in the culture supernatant of all donor corneal rims transduced with cocktail therapy containing the LV-CMV-EK5 vector by day 6 compared with the mock-vector control corneal rims, which did not express the transgenic protein.

4.4.8.3 Transgenic Protein Expression in Recipient Anterior Chamber Fluid

After the onset of immune rejection and euthanasia of the animals, anterior chamber (AC) fluid was collected from both the treated eye and the untreated eye and assayed for the presence of ovine IL-10 and human endostatin proteins by sandwich ELISAs. Table 4.2 shows the level of IL-10 detected in the AC fluid. These results show no detection of ovine IL-10 expression in the AC fluid of any of grafted eyes of the mock-vector control sheep, however all twelve of the sheep eyes that received a graft treated with the cocktail therapy showed a detectable level of IL-10 in the AC fluid, with the mean being 0.25 pg/mL. There was, however, no association between the graft survival time and oIL-10 expression levels seen.

Table 4.3 shows the levels of human endostatin protein detected in the AC fluid of sheep after the onset of rejection. Endostatin was detected in the AC fluid of eight out of the twelve sheep eyes receiving cocktail therapy transduced corneal grafts, with the mean being 0.82 ng/mL, compared with no detection in any of the AC fluid

samples from sheep with mock-vector control treated corneal grafts. There was no association between the graft survival time and endostatin expression levels seen, nor was there any association seen between endostatin levels and the day of vessels crossing the graft-host junction.

The Bcl-xL ELISA was not sensitive enough to detect transgenic Bcl-xL in the AC fluid from sheep, therefore no Bcl-xL expression data could be obtained.

Table 4.2: Ovine IL-10 protein detected in the anterior chamber fluid of cocktail therapy treated and mock-vector treated sheep eyes and in the donor rim culture supernatants, measured by sandwich ELISA. AC – anterior chamber

Recipient Sheep					Donor Eye			
I.D.	Treatment	Sex	IL-10 detected in AC fluid (pg/mL)		IL-10 detected in Rim culture (pg/mL)			
			Treated eye	Untreated eye	Day 2	Day 6	Rejection day	AC fluid collection day
Sheep 1	Cocktail therapy	M	0.002	0	0.07	1.06	14	15
Sheep 2	Cocktail therapy	M	0.001	0	2.80	6.29	18	20
Sheep 3	Cocktail therapy	M	0.002	0	1.11	6.83	19	20
Sheep 4	Cocktail therapy	M	0.04	0	4.41	19.04	19	20
Sheep 5	Cocktail therapy	M	0.06	0	3.51	15.28	19	20
Sheep 6	Cocktail therapy	M	0.001	0	1.04	4.47	21	25
Sheep 7	Cocktail therapy	M	2.47	0	0.90	6.45	23	25
Sheep 8	Cocktail therapy	M	0.15	0	0.57	4.83	24	27
Sheep 9	Cocktail therapy	M	0.001	0	2.17	9.04	25	26
Sheep 10	Cocktail therapy	M	0.27	0	2.80	11.19	32	34
Sheep 11	Cocktail therapy	M	0.001	0	0.77	7.46	40	41
Sheep 12	Cocktail therapy	M	0.03	0	1.20	6.88	122	123
Sheep 13	Mock-vector	F	0	0	0	0	14	18
Sheep 14	Mock-vector	M	0	0	0	0	19	21
Sheep 15	Mock-vector	M	0	0	0	0	19	21

Table 4.2: continued

Recipient Sheep					Donor Eye			
I.D.	**Treatment**	**Sex**	**IL-10 detected in AC fluid (pg/mL)**		**IL-10 detected in Rim culture (pg/mL)**			
			Treated eye	**Untreated eye**	**Day 2**	**Day 6**	**Rejection day**	**AC fluid collection day**
Sheep 16	Mock-vector	M	0	0	0	0	21	27
Sheep 17	Mock-vector	M	0	0	0	0	21	25
Sheep 18	Mock-vector	M	0	0	0.01	0.01	22	23
Sheep 19	Mock-vector	M	0	0	0.004	0.004	22	25
Sheep 20	Mock-vector	M	0	0	0	0.01	24	25
Sheep 21	Mock-vector	M	0	0	0.01	0.01	28	32
Sheep 22	Mock-vector	M	0	0	0	0	38	39
Sheep 23	Mock-vector	M	0	0	0.01	0.004	44	48
Sheep 24	Mock-vector	F	0	0	0	0	60	69

Table 4.3: Human endostatin protein detected in the anterior chamber fluid of cocktail therapy treated and mock-vector treated sheep eyes and in the donor rim culture supernatants, measured by sandwich ELISA. AC – anterior chamber, GHJ – graft-host junction

Recipient Sheep		Donor Eye						
I.D.	Treatment	Endostatin in AC fluid (ng/mL)		Endostatin in Rim culture (ng/mL)				
		Treated eye	Untreated eye	Day 2	Day 6	Vessel cross GHJ day	Rejection day	AC fluid collection day
Sheep 1	Cocktail therapy	0.45	0	0	6.69	6	14	15
Sheep 2	Cocktail therapy	0	0	1.22	14.43	11	18	20
Sheep 3	Cocktail therapy	0.001	0	0.53	24.1	11	19	20
Sheep 4	Cocktail therapy	0	0	1.02	11.18	11	19	20
Sheep 5	Cocktail therapy	1.72	0	0.79	14.97	11	19	20
Sheep 6	Cocktail therapy	0	0	0	8.42	7	21	25
Sheep 7	Cocktail therapy	4.43	0	0	6.97	8	23	25
Sheep 8	Cocktail therapy	1.17	0	0.4	3.25	9	24	27
Sheep 9	Cocktail therapy	0	0	0.3	8.32	7	25	26
Sheep 10	Cocktail therapy	1.34	0	0.75	13.12	10	32	34
Sheep 11	Cocktail therapy	0.69	0	0	8.06	7	40	41
Sheep 12	Cocktail therapy	0.03	0	0	16.21	43	122	123
Sheep 13	Mock-vector	0	0	0	0	3	14	18
Sheep 14	Mock-vector	0	0	0	0	7	19	21
Sheep 15	Mock-vector	0	0	0	0	9	19	21

Table 4.3: continued

Recipient Sheep				Donor Eye				
I.D.	Treatment	Endostatin in AC fluid (ng/mL)		Endostatin in Rim culture (ng/mL)				
		Treated eye	Untreated eye	Day 2	Day 6	Vessel cross GHJ day	Rejection day	AC fluid collection day
Sheep 16	Mock-vector	0	0	0	0	8	21	27
Sheep 17	Mock-vector	0	0	0	0	13	21	25
Sheep 18	Mock-vector	0	0	0	0	8	22	23
Sheep 19	Mock-vector	0	0	0	0	7	22	25
Sheep 20	Mock-vector	0	0	0	0	10	24	25
Sheep 21	Mock-vector	0	0	0	0	6	28	32
Sheep 22	Mock-vector	0	0	0	0	4	38	39
Sheep 23	Mock-vector	0	0	0	0	8	44	48
Sheep 24	Mock-vector	0	0	0	0	11	60	69

Table 4.4 shows the evidence for successful, albeit variable, transduction of the donor corneas by the expression of the GFP and eYFP reporter genes in the cocktail transduced (AdV-IL10-GFP) and mock-vector (AdV-GFP and LV-eYFP) transduced corneal rims, cultured for six days post-operatively, and flat-mounted for eYFP and GFP visualisation.

Table 4.4: GFP and eYFP reporter genes expression from transduced donor corneal rims cultured and flat-mounted six days post-operatively for an indication of transgene expression.

I.D.	Treatment	Approximate GFP/ eYFP Fluorescence (%)	Transduction Comment	Allograft Survival Days
Sheep 1	Cocktail therapy	<5%	very poor	14
Sheep 2	Cocktail therapy	>80%	good/excellent	18
Sheep 3	Cocktail therapy	>50%	good	19
Sheep 4	Cocktail therapy	>90%	excellent	19
Sheep 5	Cocktail therapy	>50%	good	19
Sheep 6	Cocktail therapy	>50%	good	21
Sheep 7	Cocktail therapy	>30%	acceptable	23
Sheep 8	Cocktail therapy	<10%	poor	24
Sheep 9	Cocktail therapy	>80%	good/excellent	25
Sheep 10	Cocktail therapy	>90%	excellent	32
Sheep 11	Cocktail therapy	>30%	acceptable	40
Sheep 12	Cocktail therapy	>30%	acceptable	122
Sheep 13	Mock-vector	(not tested)	(not tested)	14
Sheep 14	Mock-vector	>30%	acceptable	19
Sheep 15	Mock-vector	>70%	very good	19
Sheep 16	Mock-vector	>30%	acceptable	21
Sheep 17	Mock-vector	>40%	acceptable/good	21
Sheep 18	Mock-vector	>80%	good/excellent	22
Sheep 19	Mock-vector	>80%	good/excellent	22
Sheep 20	Mock-vector	>40%	acceptable/good	24
Sheep 21	Mock-vector	<10%	poor	28
Sheep 22	Mock-vector	>30%	acceptable	38
Sheep 23	Mock-vector	>30%	acceptable	44
Sheep 24	Mock-vector	(not tested)	(not tested)	60

4.4.8.4 *In Vivo* Allograft Survival with Vector Cocktail Therapy

Table 4.5 shows the graft survival of the individual sheep in days post-surgery; the median survival days and the median day of vessel migration into the graft tissue. The results showed no significant prolongation of graft survival in the cocktail therapy-treated animals (p= 0.68, Mann-Whitney U test). The survival of the corneal grafts by treatment is presented in a Kaplan-Meier survival plot in Figure 4.21. The Log-Rank (Mantel-Cox) significance was 0.94, which confirmed there was no significant difference of survival distributions between the cocktail therapy treated sheep corneal allografts and the mock-vector treated allografts.

Treatment with the cocktail therapy including the lentiviral vector expressing the endostatin::kringle5 transgene did not affect the day of vessel cross-over into the graft, with the median day of vessels crossing the graft-host junction not significantly different to the median day of cross-over in the mock vector control treatment group (p= 0.4, Mann-Whitney U test).

In the five days leading up to the day of graft rejection, the visible appearance of ocular inflammation in the grafted sheep eyes was very low, with a score of 1 given as the highest inflammation score (on a scale of 0 – 4, with 4 being the most inflamed). This score was achieved by only two sheep out of the 24; one mock-vector treated allograft and one cocktail-treated allograft. The visual inflammation scores were very similar between both treatment groups.

Table 4.5: Corneal graft survival of penetrating keratoplasties performed in sheep with donor corneas transduced with vector cocktail therapy expressing therapeutic transgenes ovine IL-10, ovine Bcl-xL and human EK5. Median day of rejection and median day of vessels crossing the graft-host junction are shown for each treatment group.

Treatment Group		Day Vascularisation Crossed into Graft	Median Day of Vascularisation	Graft Survival Days	Median Day of Rejection
Mock-Vector (n=12)	AdV-GFP LV-eYFP	3,4,6,7,7,8,8,8,9,10,11,13	8	14,19,19,21,21,22,22,24,28,38,44,60	22
Cocktail Therapy (n=12)	AdV-IL10 LV-BclxL LV-EK5	6,7,7,7,8,9,10,11,11,11,11,43	9.5#	14,18,19,19,19,21,23,24,25,32,40,122	22*

* p= 0.68, # p= 0.4 compared to mock-vector controls (Mann-Whitney U test)

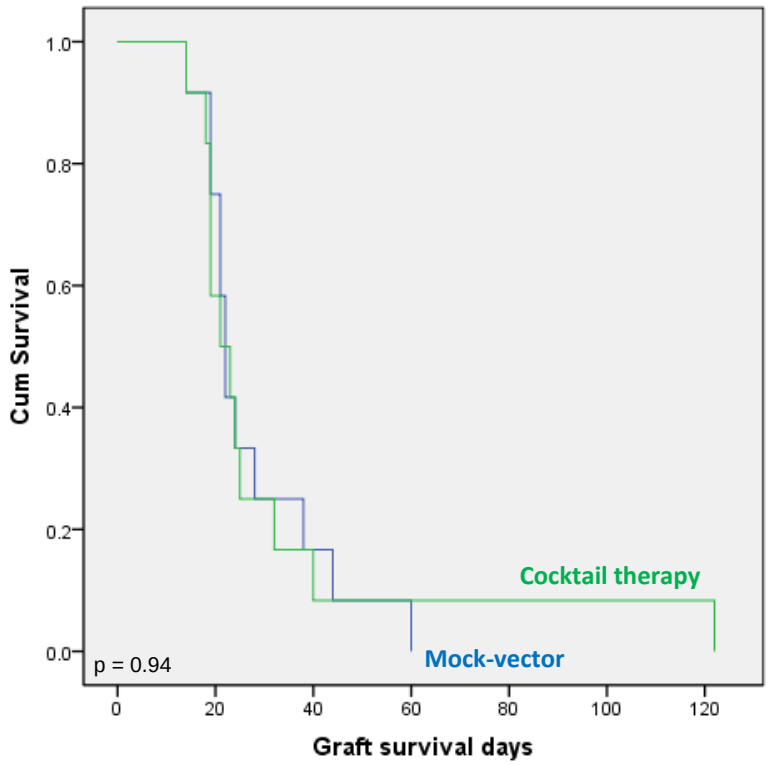


Figure 4.21: Kaplan-Meier survival plot showing the cumulative graft survival in days of the cocktail-therapy treated sheep corneal grafts (green line) and the mock-vector control sheep corneal grafts (blue line). Log-rank (Mantel-Cox) significance p= 0.94.

4.5 Summary and Discussion

4.5.1 Summary of Findings

Four lentiviral vectors were constructed encoding transgenes ovine IDO, ovine Bcl-xL, human EK5 and human sFlt-1, each with a CMV constitutive promoter inducing transgene expression. The biological activity of each of the transgenic proteins was confirmed by *in vitro* testing. Each of the lentiviral vectors successfully transduced ovine corneas *in vitro* and over-expression of all four transgenes was detected at the mRNA level in ovine corneal endothelial cells. Bcl-xL and EK5 were successfully detected at the protein level in the ovine cornea culture supernatant across all culture time points.

Transduction of individual ovine corneas with a cocktail containing a lentiviral vector and an adenoviral vector showed no evidence of vector interference when transgene expression was investigated, at either the mRNA or protein levels. Human corneas were also successfully transduced with both the adenoviral vector and the lentiviral vector in a cocktail transduction and transgene expression was detected from both constructs at the protein level.

A vector cocktail treatment of donor ovine corneas with three therapeutic transgenes, ovine IL-10, human EK5 and ovine Bcl-xL, did not prolong corneal allograft survival in the sheep nor did it have an effect on the median day of vessels crossing the graft-host junction of the corneal grafts.

4.5.2 Discussion

4.5.2.1 Vector Cocktail Approach to Transplant Therapy

The multigenic cocktail approach was taken in this chapter as the literature and the experimental evidence suggested that targeting a single pathway of the rejection process was not sufficient to induce long-term allograft survival.[300] Some effort was put into the construction of multigenic vectors, however these did not come to fruition and single vector constructs were used to express the individual transgenes. The lentiviral vector was the main therapeutic vector of choice due to its integrative capacity and the induction of a low inflammatory response.[166, 218, 289] Considering the *in vivo* results presented in chapter 3 and the previous corneal allograft prolongation success by Klebe *et al.* [76], the decision was made to use the adenoviral vector to express the IL-10 transgene, in order to induce early, high expression of this protein. The four other transgenes; two targeting neovascularisation, one targeting T cell responses and one targeting pro-survival of graft corneal endothelial cells, were expressed in lentiviral vectors in order to maintain their long-term expression. It was hypothesised that the pathways mediated by these transgenes might not require transgene expression immediately post-surgery in order to modulate the immune response, therefore the lentiviral vector, with its slower kinetics of expression in ovine corneas compared with the adenoviral vector,[202] would be an appropriate vector for these transgenes.

4.5.2.2 In vitro Transgene Expression Variability at mRNA Level

A great deal of variability was seen in transgene expression by qRT-PCR at the mRNA level from individual corneas transduced with lentiviral vectors, an event also noted in chapter 3. This variability indicates the transduction efficiency cannot be controlled. The implication is that some of the treated corneal allografts may have

rejected due to very low transgene expression, resulting from poor transduction. The transduced donor corneal rims were examined post-surgery for GFP expression (Table 4.4). As expected, the transduction success was seen by this measure to be variable; with some rims showing excellent (> 90%) endothelial cell transduction at day 6 post-surgery, and others poor (< 10%) transduction. It is noteworthy that these corneas were prepared for surgery on a Barron Artificial Anterior Chamber (Table 2.9), which involves the compression of a tight plastic screw-thread against the corneal endothelial cells of the rim, and the health of the endothelial cells may have been compromised during this process. Therefore the resulting flat mounts at day 6 may not in all instances be an accurate representation of transduction.

White *et al.* demonstrated an unexpected and striking degree in transduction variability whilst using both HIV-1 and EIAV lentiviral vectors in a controlled study investigating the transduction of the striata of rat and pig brains, a variability not seen in previous studies utilising variously serotyped AAV vectors.[301]

Similarly, Jessup *et al.,* when using an adenoviral vector to transduce human corneas, found a degree of transduction variation that did not correlate with the age of the corneal donor, endothelial cell density nor to the length of corneal storage time prior to transduction.[302] All of the ovine eyes used for *in vitro* and *in vivo* experiments in this thesis were obtained from local abattoirs, and corneas were transduced within hours post-mortem, therefore the transgene expression variability was not due to long-term storage reducing the health of corneal endothelial cells. It appears that corneal endothelial cells in conjunction with lentiviral vectors in particular exhibit quite variable transduction efficiencies and this should be taken into account when interpreting results of interventions *in vivo*.

4.5.2.3 Expression Loss of Transgenic sFlt-1 Protein *In Vitro*

The protein levels of sFlt-1 detected in the ovine cornea culture supernatant dropped from day 2 to day 4, and were at almost undetectable levels by day 10 of the culture (Figure 4.12). This protein expression profile was different to all other transgenes in the lentiviral vector tested in ovine cornea culture, which suggests that the cause of the down-regulation was unlikely to have been due to the vector or promoter silencing, but was likely to be a transgene specific issue. mRNA expression of the sFlt-1 transgene was detected in the ovine cornea endothelial cells at day 10, indicating that transcription was occurring throughout the culture, but very little protein was produced after day 2. In a mouse study of tumour angiogenesis, Sivanandam *et al.* assayed the serum levels of mice injected with an adenoviral vector expressing sFlt-1 under the control of an inducible promoter for the expression of the transgenic sFlt-1 protein.[303] Similar to the results presented in this chapter, the authors detected the earliest sFlt-1 protein by ELISA at 4 hours post injection, found peak expression at 10 hours, a decline to low protein levels by 24 hours, and by 48 hours the sFlt-1 protein was undetectable in the mice sera.[303] As this study utilised inducible transgene expression, it demonstrated the short half-life of the sFlt-1 protein. The detection of transgenic sFlt-1 mRNA in the corneal endothelial cells at day 10 of the culture and the continued decrease in sFlt-1 protein expression over the culture time points observed in the results presented in this chapter, suggests there was a problem with transgenic protein production, rather than a natural degradation of the protein.

No specific testing was conducted to determine the cause of the sFlt-1 protein expression drop in ovine cornea culture; however it is speculated that sFlt-1 protein was initially functionally produced for a very short period of time, before a

translational or post-translational interruption, perhaps micro-RNA regulation, occurred to halt protein production.

4.5.2.4 Kynurenine Undetected in IDO-Transduced Ovine Corneal Supernatant *In Vitro*

L-kynurenine, the down-stream metabolite of tryptophan degradation mediated by IDO, was detected to confirm the presence of the IDO protein in cell and organ cultures *in vitro*. The IDO protein was not identified specifically itself as an antibody to ovine IDO could not be commercially sourced, high performance liquid chromatography was considered to be outside of my time and expense restraints, and I had set up the kynurenine assay, which was simple, cost-effective and a functional indicator of the biological activity of the IDO protein.

L-kynurenine was successfully detected in the supernatant of LV-IDO transduced mammalian cells at significantly higher levels than untransduced cells, with and without the addition of tryptophan substrate (Figure 4.2). However, L-kynurenine was not detected in the culture supernatant of LV-IDO transduced ovine corneas at levels different to untransduced corneas, even upon addition of supplemental tryptophan substrate (Figure 4.13 (A) and (B)). This was unexpected, as over-expression of the IDO transgene was demonstrated at the mRNA level in the LV-IDO transduced ovine cornea endothelial cells at day 10 post-transduction (Figure 4.11).

Several studies have shown very low IDO detection levels in the culture of human corneal primary cells, cell lines and in dendritic cells at the protein level, without stimulation by the cytokine interferon-gamma (IFN-γ).[109, 114, 304] Serbecic *et al.* found

during the culture of human corneal endothelial cells that expression of IDO could be detected at the mRNA level, however no kynurenine was detected at the protein level without IFN-γ stimulation.[114]

IDO is an intracellular enzyme[107] and requires access to the tryptophan in the culture supernatant to catalyse the degradation reaction. For the detection of kynurenine, this metabolite must be transported out of the cells into the culture supernatant. Serbecic *et al.* also reported the presence of a large amino acid transporter 1 (LAT-1) transporter protein, which enables the exchange of tryptophan to the internal compartment and kynurenine to the outside compartment.[114] The authors demonstrated a four-fold increase in gene expression of LAT-1 upon stimulation with IFN-γ by microarray analysis, and confirmed this up-regulation at the mRNA and protein levels. As mentioned in the introduction, tryptophan is an essential amino acid for T cell proliferation and kynurenine has been shown to have an inhibitory effect on T cell proliferation. The authors proposed the LAT-1 transporter may act as part of a negative feedback loop to reduce local immune responses by promoting a two-armed T cell inactivation process.[114]

It is possible that the reason for the low detection of kynurenine in the culture supernatant of the transduced ovine corneas reported in this chapter was due to the lack of stimulation of the LAT-1 transporter, disabling the exchange of tryptophan and kynurenine across the plasma membrane of the corneal endothelial cells.

There is evidence that expression of LAT-1 may be linked with cell proliferation. Ichinoe *et al.* demonstrated a correlation of LAT-1 expression intensity with increasing proliferative capacities of human gastric adenomas and carcinomas.[169]

Higher expression of LAT-1 in more proliferative cells could explain why kynurenine was detected in the supernatant of the highly proliferative immortalised cell line, HEK-293T, transduced with LV-IDO (Figure 4.2) and not in the supernatant of ovine corneas, with amitotic corneal endothelial cells transduced with LV-IDO (Figure 4.13). The observed delay from day 2 to day 5 in kynurenine detection in the supernatant of the transduced 293T cells (Figure 4.2) could have been due to the time taken to transport the kynurenine from the intracellular space to the culture supernatant. For future *in vivo* application, it would need to be considered that the therapeutic effects of this molecule could be delayed unless LAT-1 stimulation is also induced.

4.5.2.5 Therapeutic Cocktail to Prolong Allograft Survival *In Vivo*

Inflammation

The histology results demonstrating the high incidence of retrocorneal membrane formation and slightly higher ratio of infiltrating CD4+ and CD8+ T cells suggests a higher degree of inflammation that may have occurred in the cocktail-treated sheep allografts compared with the mock-vector control allografts, although this was not observed visually at the slit lamp.[305] Since both treatments involved the same overall dosage of lentivirus and adenovirus, it may be reasoned that a reaction to the vectors was not the cause of the increased degree of inflammation. However, various transgenes and even different viral preparations may induce different host responses.

It is possible that the cocktail-treated allografts had sufficient endotoxin contamination in the viral preparations to induce an inflammatory response, which could potentially have caused the formation of the retrocorneal membranes. Bacterial endotoxin is a lipopolysaccharide found in the cell wall of gram-negative bacteria

and is usually released from bacteria upon lysis.[306] Endotoxin can induce severe ocular inflammation.[306] The total amount of endotoxin units in the cocktail therapy was between 2 and 3 units per cornea, however was as high as 34 units for two of the mock-vector-treated control sheep allografts. The limit of bacterial endotoxin contamination for ophthalmic irrigating solutions is 0.5 endotoxin units per mL, as specified in the International Organisation for Standardization 16671 (Ophthalmic Implants – Irrigation Solutions for Ophthalmic Surgery)[306] and for medical devices such as intraocular lenses the limit is lower, at 0.2 EU or less.[307] In 2005 a significant outbreak of toxic anterior segment syndrome occurred, involving 112 cataract surgery patients in the US.[308] The cause of the inflammation was considered due to endotoxin contamination in a brand of ophthalmic balanced salt solution at levels exceeding the 0.5 EU/mL limit. Sakimoto *et al.* studied the endotoxin levels required to induce inflammation in the anterior segment of rabbit eyes after intracameral injection.[309] The authors demonstrated the need for 0.23 – 0.6 EU to induce inflammation. The level of endotoxin contamination present in the cocktail therapy in this chapter was much higher than these standard and experimental limits.

Interestingly, the sheep allografts treated with LV-CMV-IL10, performed in Chapter 3, had higher endotoxin exposure than the cocktail-treated allografts in the results presented in this chapter, with the endotoxin units ranging between 3.8 and 4.3 units per cornea (Section 3.4.7), however no retrocorneal membranes were observed in any of these sheep, treated or mock-transduced. Only one retrocorneal membrane formation was observed from the mock-vector-treated allografts group, and these treated grafts were exposed to much higher endotoxin contamination levels than the cocktail therapy allografts.

Viral vectors were placed on the corneas *ex vivo*, washed three times from the corneas with endotoxin-low medium prior to surgery and were not delivered directly to the intraocular compartment, which may or may not have reduced the contamination potential.

There is evidence to show that endotoxin can increase the speed of rejection of corneal allografts. Pillai and colleagues demonstrated that mouse corneal allografts treated *ex vivo* with lipopolysaccharide underwent faster rejection than untreated allografts.[310] However, whilst it is always desirable to keep endotoxin contamination below recommended levels especially when working towards a clinical therapy, in this case I do not think endotoxin was responsible for the lack of treatment success. It is possible that the transgene combination caused an increased inflammatory response in the cocktail-treated sheep, which may have reduced efficacy of the treatment and increased the speed of the allograft rejection. The sheep system may have elicited an immune response against the human EK5 protein, although Murthy *et al*. did not report a strong inflammatory response to this human transgenic protein in the rabbit cornea [87] and, as is discussed in Section 4.5.2.6, the sheep shares more identity with the human plasminogen protein than does the rabbit, suggesting the sheep is likely to tolerate this human protein.

Why Did the Vector Cocktail Not Prolong Graft Survival?

My results showing no significant prolongation of corneal allograft survival were unexpected, considering the significant prolongation of graft survival achieved with IL-10 in the results presented in chapter 3, and also the highly successful graft survival shown by Klebe *et al.*, using an adenoviral vector and the IL-10 transgene in the same animal model.[76] The authors used an E1, E3 deleted, second generation,

serotype 5 adenoviral vector, as in this chapter, expressing the ovine IL-10 transgene under the control of a CMV promoter, at the same viral dose, and achieved a median allograft survival day of 55 compared with 20 days for the untreated allografts.[76]

The one difference between the two vectors used in the two studies, is the presence of the green fluorescent protein (GFP) reporter gene along with its own constitutive CMV promoter in the AdV-oIL10 vector used in this chapter, whereas this extra transgene and promoter were absent from the adenoviral vector used in the work by Klebe *et al*. It has been reported that when expressing multiple transgenes in a single vector, a lack of efficiency in transgene expression can occur.[311, 312] The adenoviral vector used in this chapter, however, was constructed from a commercial plasmid and each transgene had its own internal promoter, which makes poor transgene expression unlikely.[223] Furthermore, both transgenic proteins, GFP and IL-10, were successfully detected in the corneal rim cells and culture supernatant, respectively, and IL-10 was detected in the AC fluid of sheep eyes with treated corneal allografts. Klebe *et al.* did not test for IL-10 in the AC fluid of their treated sheep, so a direct comparison of their vector and the adenoviral vector used in this chapter, in terms of transgenic protein production, cannot be made.

ELISA results on anterior chamber fluid samples from the vector cocktail treated sheep eyes determined over-expression of the IL-10 protein compared with control eyes, indicating successful viral transduction and transgene expression. However, the average IL-10 protein amount detected in the AC fluid of the AdV-IL10 cocktail treated sheep was ten times the average amount detected in the fluid of the LV-IL10 treated sheep in chapter 3, yet a greater increase in median survival days of the treated corneal allografts was seen with the LV-IL10 treated sheep. From these data,

I suggest that the amount of IL-10 protein being produced may not be the critical factor for therapeutic benefit, instead there are potentially some other factors at play such as the timing of expression, the location of the expression and the interaction of the transgenic proteins in the vector cocktail.

4.5.2.6 Therapeutic Cocktail of Transgenic Proteins *In Vivo*

The properties of each of the three transgenic proteins expressed in the cocktail therapy of the sheep corneal allografts in this chapter are shown in Table 4.6. Ovine specific transgenes were used for IL-10 and Bcl-xL. A human-specific EK5 transgene was used as this was a previously prepared synthetic fusion gene. According to direct protein alignments using a basic local alignment search tool (BLASTp; National Center for Biotechnology Information), the human and sheep plasminogen proteins (containing kringle5) share a maximum 83% identity, and the human and sheep collagen XVIII proteins (containing endostatin) share a maximum 80% identity. As previously described, Murthy *et al.* demonstrated the use of the human EK5 transgene in a rabbit model of corneal transplantation to successfully inhibit neovascularisation and prolong allograft survival.[87] Given that the human and rabbit plasminogen proteins share a maximum 78% identity, I argued that the human EK5 transgene should be biologically functional in the sheep, but this has yet to be formally tested.

Table 4.6: Information on the three transgenic proteins expressed *in vivo* in the cocktail therapy of penetrating sheep corneal allografts.

Transgene	Protein size	Half-life	Targeted for expression	Ref
oIL-10	18.4 - 20.2 kDa	~2 hr*#	Secreted; aqueous humour	313-315
oBcl-xL	29 kDa	>4 hr*	Corneal endothelial cells; intracellular compartment	316, 317
huEK5	34.1 kDa#	~30 hr#	Secreted; aqueous humour	318 #

* human protein half-life

information from ProtParam,[318] a web-based tool to predict protein size and half-life based on amino acid sequence.

4.5.2.7 Expression Locations of Transgenic Proteins *In Vivo*

Immunohistochemistry labelling of corneal sections of rejected allografts revealed no evidence of reduced numbers of CD4+ T cells in therapeutically treated allografts, in fact the allografts treated with IL-10 had slightly more CD4-labelling than the mock-vector control allografts. There was no clinical evidence of reduced vessel ingrowth, or statistical increase of the median day blood vessels crossed the graft-host margin into the donor allograft in the cocktail therapy-treated animals. Table 4.6 describes the target location of each of the three transgenic proteins used in the cocktail. The transgenic proteins endostatin::kringle5 and interleukin-10 were targeted for secreted expression to the aqueous humour. Given the size of the molecules, 34.1 kDa and ~20 kDa, respectively, it is possible that these proteins were able to pass through Descemet's membrane: the bovine membrane has been shown to allow passage of a molecule approximately 48 kDa in size *in vitro*,[319] and be active in a different compartment, such as the corneal stroma. Data from ELISAs conducted on the anterior chamber fluid detected the presence of both the endostatin and IL-10 transgenic proteins in the aqueous humour of treated sheep eyes, demonstrating the proteins were successfully secreted to this compartment at least. The longevity of

protein expression, with detection after the onset of allograft rejection, and reasonably long protein half-lives (predicted, in the case of EK5), indicate that it is unlikely that a lack of therapeutic effect was due to loss of transgene expression.

It is likely that the site where the EK5 protein needs to be expressed for therapeutic effect is not the aqueous humour, but rather the region where the new vessels are forming, specifically the limbal region.[139] This may involve transducing the limbal stem cells. Chen *et al.* proved in principle that this could be done with adenoviral vectors and a population of limbal cells grown out in culture,[320] and Igarashi *et al.* determined that a lentiviral vector could be used to induce long-term reporter gene expression *in vivo* in rat corneal epithelial progenitor cells.[321] The reasons for targeting the corneal endothelial cell layer in this project were because the cells are highly accessible. Because they are a monolayer, there was a good chance of transducing the majority of the cells.[199] These cells can secrete transgenic proteins directly into the aqueous humour where some of the host immune response gains access to the area.[154, 322, 323] Further, endothelial cells are active metabolically but slow to divide, so are able to support long-term gene expression,[2] and the endothelium is a major target of the host immune response, so that treating these cells directly to help prevent an immune response was desirable.[27, 324]

As Table 4.6 shows, the Bcl-xL transgenic protein, unlike the other two transgenic proteins, was not targeted for secretion to the aqueous humour. However this protein was detected by ELISA in the concentrated culture supernatant of ovine corneas transduced with LV-BclxL (Figure 4.14). This suggests that not all of the Bcl-xL protein may have remained as an intracellular protein *in vivo* and functioned to protect the corneal endothelial cells as expected, some may have been secreted to the

AC fluid and bathed the endothelial cells to some extent. Given the half-life of the human Bcl-xL protein is estimated to be longer than four hours, and the human and ovine Bcl-xL proteins have 98% identity (National Center for Biotechnology Information online Basic Local Alignment Search Tool), with the turn-over of the aqueous humour having a rate of 3 μL/min in humans and expecting a similar rate in sheep, continued expression of the protein would be expected.[325] There is no evidence to suggest, however, that the Bcl-xL protein would function to reduce apoptosis of the corneal endothelial cells as a secreted protein in the anterior chamber fluid. Figure 4.4 shows the detection of Bcl-xL protein in the culture supernatant of HEK-293T cells transduced with LV-BclxL. These transduced 293T cells demonstrated a protection from staurosporine-induced apoptosis (Figure 4.5) despite the secretion of the Bcl-xL protein, suggesting some retention of functional intracellular Bcl-xL. This suggests that even if some transgenic Bcl-xL protein was inadvertently secreted to the anterior chamber in the *in vivo* cocktail-transduced sheep allografts, some transgenic Bcl-xL may have remained in the intracellular compartment and been functional. No *in vivo* expression data was obtained for transgenic Bcl-xL at either the mRNA or protein level.

4.5.2.8 Therapeutic Expression of Transgenic Proteins *In Vivo*

There remains the question of 'how much is enough?' when it comes to the amount of transgenic protein required for a therapeutic effect. As the results described, with the adenoviral vector expressing IL-10, an average of ten times the amount of transgenic protein was detected in the anterior chamber fluid of the treated sheep eyes compared with the lentiviral vector-treated sheep in chapter 3. Despite the large increase in protein production, no increase in allograft prolongation was observed; in fact the median survival time was reduced. This was probably due to other factors as

there were other vectors and transgenes involved in the adenovirus-treated animals, however, these results suggest the current levels of transgene expression are probably therapeutic and the reason for the treatment failure is unlikely to be due to the need for higher transgene expression. When considering the two lentiviral vectors and their respective transgenes, EK5 and Bcl-xL, however, there is no comparative measure to use to determine a therapeutic expression level yet. As mentioned, there was no evidence of reduced vessel ingrowth or delay in median day of vessels crossing the graft-host margin, and only one treated animal out of the twelve treated showed any notable delay in this cross-over day. A possible explanation for these results could be that the expression of the EK5 transgene was not sufficient for a therapeutic effect. Transgenic endostatin protein was detected at an average level of 0.82 ng/mL in the anterior chamber fluid of cocktail therapy treated sheep eyes after the onset of allograft rejection. Ergun *et al.* demonstrated the anti-angiogenic properties of human endostatin *in vitro* and *in vivo* in a SCID mouse tumour model, using the recombinant protein at levels of 150 ng/mL and higher to achieve clear reductions in endothelial tube numbers and inhibition of cell migration, which was logs higher than levels detected in the results presented here.[326]

4.5.2.9 Timing of Transgenic Protein Expression *In Vivo*

The results indicate that transgenic protein was produced for at least two out of the three transgenes in the vector cocktail therapy, albeit possibly not at therapeutic locations or levels. An alternative reason for the lack of allograft prolongation could be in the timing of the protein expression.

IL-10

From *in vitro* adenoviral vector results, excellent GFP reporter gene expression was observed in several rim culture flat mounts at day 6 post-transduction (Table 4.4), and over-expression of IL-10 transgenic protein was detected in the rim culture supernatant at day 2 post-transduction (Table 4.2). Figure 4.18 (B) also shows IL-10 protein detection in the culture supernatant of AdV-IL-10 transduced ovine corneas at day 3 and a marked increase by day 6. These *in vitro* results suggest that strong transgenic expression of IL-10 *in vivo* should have occurred by at least day 6 post-operatively. Wang *et al.* demonstrated the first appearance of CD4+ T lymphocytes occurred after three days post-operatively in mouse corneal allografts by immunohistochemistry.[323] Banerjee *et al.* found corneal allografts in rats could induce leukocyte aggregates as early as three days post-operatively.[327] Using the same sheep model of penetrating keratoplasty, the same oIL-10 transgene, and a similar adenoviral vector and dosage, Klebe *et al.* demonstrated a significant prolongation of allograft survival compared with mock-vector-treated allografts.[76] The *in vitro* data and these studies suggest that the timing of the expression of transgenic IL-10 in the sheep corneal allografts in my work was likely to have occurred at the right time to help prevent T cell stimulation.

EK5

In vitro results with the lentiviral vector expressing the EK5 transgene show very little transgenic endostatin protein detected in the culture supernatant of transduced ovine corneas at day 2, a slight increase to stable levels by day 4 and a significant increase at day 10 to levels over 160 ng/mL (Figure 4.15). Figure 4.18 (A) shows the levels of transgenic endostatin protein detected in the culture supernatant of transduced ovine corneas increasing from day 3 to day 6. These results indicate that

expression of the EK5 transgenic protein *in vivo* would be expected at strong levels after at least 6 to 10 days post-operatively. Cursiefen *et al.*, in a mouse model of corneal transplantation, showed evidence of new infiltrating vessels at day 3 post-allograft surgery.[85] This mouse study is not directly comparable to the sheep system, however it does give an indication that new vessel formation and infiltration occurs reasonably rapidly within the first several days post-allograft surgery.

Given the *in vitro* expression kinetics of transgenic endostatin protein by transduced ovine corneas, it is possible that significant neovascularisation had already occurred *in vivo* before strong levels of the EK5 transgenic protein were produced, and therefore the timing of this transgene expression may not have been optimal to prevent neovascularisation.

Bcl-xL

Studies in mice have shown that corneal endothelial cells undergo apoptosis when under immune attack by the host during graft rejection, and this apoptotic response is initiated between weeks one and two post-operatively and prior to the onset of clinical rejection.[80] The data presented here show secreted Bcl-xL transgenic protein detected in the culture supernatant of transduced ovine corneas at reasonably consistent levels from day 2 of culture through to a slight increase at day 10 (Figure 4.14). This data suggests the expression of transgenic Bcl-xL protein *in vivo* was probably present at the right time to allay the apoptotic response of the corneal endothelial cells.

4.5.2.10 Transgenic Protein Interactions *In Vivo*

All three transgenes used in the therapeutic vector cocktail *in vivo* work were proven to prolong corneal allografts in previous animal studies.[76, 80, 87] If time had permitted, these transgenes should have been individually tested for their effect on sheep corneal allograft survival during this project. Given the lack of allograft prolongation in the results presented in this chapter, the question arises as to whether these three treatments can work in combination.

For our purposes, the actions of the Bcl-xL transgene compared with the EK5 and IL-10 transgenes could seem contradictory, with the former a pro-survival factor, and the latter two, in broad terms, anti-survival factors. IL-10 causes anergy of T cells and induces them on the path to apoptosis, and kringle5 inhibits neovascularisation by inducing apoptotic death in endothelial cells.[100, 144] However it is an important distinction that the EK5 transgenic protein is active specifically on vascular endothelial cells and that corneal endothelial cells are distinct from vascular endothelial cells; having different embryonic origins.[144, 146, 328] The transgenic proteins were targeted for expression to different compartments (Table 4.6), therefore interactions between the Bcl-xL and the other two proteins should have been limited, however as mentioned earlier, secreted Bcl-xL protein was detected by ELISA in the concentrated culture supernatant of transduced ovine corneas *in vitro* (Figure 4.14), suggesting that at least some of the transgenic Bcl-xL could have been secreted to the aqueous humour *in vivo*. Since transgenic Bcl-xL protein levels could not be measured in the anterior chamber fluid, it cannot be ruled out that Bcl-xL was expressed in this compartment. However, the Bcl-xL protein is required intracellularly and there is no evidence to suggest that external bathing of T cells or vascular endothelial cells with Bcl-xL would have a pro-survival effect.

With hindsight, and if time had permitted, the levels and location of transgenic Bcl-xL protein should have been measured intracellularly, and also the reason for the secretion of the transgenic Bcl-xL protein in to the culture medium should have been investigated.

Summary

The therapy-treated allografts had significant histological indications of inflammation compared with the mock-vector control allografts. This may have contributed to faster allograft rejection times and reduced the efficacy of the treatment.

The endostatin::kringle5 transgenic protein was successfully detected in the anterior chamber fluid of the treated animals, however the lack of therapeutic effect of this transgene suggests that the target site may not have been optimal for the secretion of protein where it was functionally relevant, that protein expression possibly occurred too late for therapeutic good, or that the amount of transgenic protein produced was not sufficient for a therapeutic effect.

The interleukin-10 transgenic protein was detected at high levels in what was considered the appropriate target site for expression in the therapy treated sheep allografts.

The Bcl-xL transgene *in vitro* data suggested the timing of expression was likely to be suitable for a protective response. The transgene was considered targeted appropriately, to the corneal endothelial cells, however the *in vitro* data suggests some of this protein may have been secreted inadvertently to the anterior chamber fluid.

4.5.3 Conclusions

It is possible that a strong inflammatory response to the transgenes caused the early rejection of sheep corneal allografts. Mis-targeted expression of the transgenic proteins *in vivo* could have led to contradictory therapeutic actions, which could have been responsible for the lack of prolongation of sheep corneal allograft survival.

The combination of transgenes and their target expression locations is an important consideration for future applications of this therapy, as remains the therapeutic amount of transgenic protein produced when using the lentiviral vector, and the timing of the transgenic protein expression.

Chapter 5

Final Discussion

5.1 Summary of Major Thesis Findings

The overarching aim of my project was to use lentiviral-mediated gene therapy to deliver therapeutic transgenes to donor corneas in a preclinical outbred sheep model of orthotopic corneal transplantation, to achieve significantly prolonged corneal allograft survival. Within this broad aim, I endeavoured to characterise two internal transgene promoters in lentiviral vectors for efficacy of therapeutic transgene expression, and to investigate the efficacy of multigenic therapy in the targeting of multiple arms of the host immune response simultaneously.

The constitutive cytomegalovirus promoter was demonstrated to be the stronger internal promoter of transgene expression in lentiviral vector-mediated transduction of corneal endothelial cells *in vitro*, when compared with the simian virus type 40 early promoter. The cytomegalovirus promoter was used as the internal transgene promoter for all subsequent lentiviral vectors created for the work in this thesis.

I found that lentiviral gene therapy using a single therapeutic transgene, interleukin-10, induced statistically significant prolongation of corneal allograft survival in an outbred pre-clinical sheep model. This prolongation was modest and not seen across all experimental animals.

Multigenic therapeutic gene therapy using a combination of viral vectors did not prolong corneal allograft survival in a pre-clinical sheep model of corneal transplantation. The potential of multigenic therapy to prevent the host immune responses to the corneal allograft warrants further investigation, but requires careful consideration of candidate therapeutic transgene selection, in order to reduce the potential for conflict during functional expression.

5.2 Gene Therapy of the Eye

According to information provided by the Journal of Gene Medicine (www.wiley.co.uk/genmed/clinical)[181], as of January 2013 there have been 1902 human gene therapy clinical trials worldwide, with 28 (1.5%) classified as pertaining to 'ocular diseases'. Four viral vectors were approved for use in these trials: adeno-associated viral vectors made up the majority, with 13 trials utilising this vector, the latest gaining approval in December of 2011. Three trials were approved using lentiviral vectors (complex retroviral vectors), the latest in 2011; two trials using adenoviral vectors were approved, the latest in 2003; and one trial using a retroviral vector was approved in 2002. No poxvirus or herpes simplex virus human clinical trials have been approved for ocular interventions. These statistics are indicative of the movement in viral vector-mediated gene therapy from the adenoviral to the adeno-associated viral vector as a key vector in ocular gene therapy. The remaining 9 clinical trials of the 28 classified for ocular diseases involved the use of non-viral vectors: 5 were trials of small interfering RNA molecules and 4 were trials of naked DNA transfer, all of which were approved by 2006 in the latest instance.

5.2.1 Adeno-Associated Virus (AAV) Vectors

As can be seen from the clinical trial statistics above, adeno-associated viral vectors (AAV) are becoming the vectors of choice for many gene therapy studies. Lacking pathogenicity, having a low inflammatory potential, and inducing sustained gene expression, AAV vectors are a versatile alternative to adenoviral vectors.[161, 329, 330] AAV vectors have been used in several successful phase I and II clinical trials, transducing many different cell types including lung, muscle, central nervous system, eye and liver, reviewed by Xiao *et al*.[330]

Seven gene therapy clinical trials have been approved for Leber's congenital amaurosis since 2004 (www.wiley.co.uk/genmed/clinical), all of them utilising AAV vectors.

Leber's congenital amaurosis is an autosomal recessive blinding disease of the retina.[331] At least three independent clinical trials have demonstrated the efficacy of sub-retinal injections of AAV vectors to transduce retinal cells with the RPE65 gene, although an adverse event occurred in an initial trial and a transient rise in neutralising anti-AAV capsid human antibodies was detected. The RPE65 gene is delivered to replace the mutated disease-causing gene in one form of this blinding disease (LCA2). Results of a clinical trial reported by Maguire and colleagues, in which an AAV2 vector encoding the RPE65 gene was delivered to three patients by sub-retinal injection, demonstrated improvement in retinal function and gains in visual acuity for all patients, in one case for up to six months of follow up.[332] Using the same vector, transgene and delivery method, Simonelli *et al.* reported increased visual acuity and pupillary light reflexes in a clinical trial involving three LCA2 patients.[333] This trial investigated the safety as well as the efficacy of the gene therapy and the authors found persistence of functional amelioration of dystrophy (1.5 years), with no serious adverse events and minimal immune responses to the vector. Ashtari *et al.* showed that a sub-retinal injection of an AAV2 vector encoding the RPE65 transgene in three LCA2 patients induced the visual cortex to become responsive to visual stimuli after years of visual deprivation, and stable retinotopic improvement was demonstrated for at least two years.[334] Overall, results from these trials suggested that at low-vector doses, improvements of visual function after gene therapy were significant.[332-334]

Recently, Cideciyen and colleagues reported some important caveats into the use of gene therapy to treat Leber's congenital amaurosis.[335] The RPE65 gene-mutated form of the dystrophy involves the dysfunction and degeneration of the retinal photoreceptor cells, both characteristics researchers originally thought would be improved by gene therapy using wild type cDNA.[336] As the clinical trial results showed, the dysfunction of the cells was indeed improved by the gene therapy, with long-term improved patient vision reported, however it has recently been discovered that the degeneration of the photoreceptor cells showed continual progression.[335] It appears that once the degeneration process has begun, gene therapy is unable to halt or reverse this progress. This has implications for the timing of gene administration for this dystrophy, and for the gene therapy of other retinal degenerative diseases. It follows from this, that early detection of disease is essential in order to deliver earlier gene therapy intervention before cellular degeneration occurs.

AAV vectors have also been trialled successfully for safety in several pre-clinical models for the treatment of the retinal neovascularisation that occurs with conditions such as the wet form of age-related macular degeneration. MacLachlan *et al*. used a non-human primate pre-clinical model to test the safety of an AAV2 vector encoding the sFlt-1 transgene.[142] The authors delivered the vector to one eye of each animal via intravitreal injection at one of two doses: 2.4×10^9 vector genomes (vg) or 2.4×10^{10} vg. Variable transgene expression was observed within each dosing group, likely due to the method of delivery necessitating slow progress of the vector towards the target cells through the viscous vitreous. A mild to moderate inflammatory response was induced towards the AAV2 vector capsid in 14 out of the 18 animals treated in the higher vector dosage group. No T cell response was directed towards the sFlt-1 protein at any time in any treatment group. More recently Lai and

colleagues used an AAV2 vector encoding sFlt-1 in a non-human primate safety trial of the vector.[337] The animals underwent subretinal injections of the AAV vector (8 x 10^{11} particles). The results demonstrated that no visual impairment occurred and that retinal morphology and function were not affected. Higher sFlt-1 levels were detected in the vitreous of the AAV2-sFlt1-treated eyes than the control eyes, but the vector was not detected in non-ocular tissues. No cellular immune response was elicited against the vector, therefore the authors concluded that AAV2-sFlt1 was a well-tolerated gene therapy vector.

Five gene therapy clinical trials have been approved for age-related macular degeneration since 2001, three of them utilising AAV vectors.

AAV vectors are rapidly having a significant impact on the treatment design in clinical studies of ocular disorders such as retinal neovascularisation. Of the total 13 approved clinical trials using AAV vectors for ocular disorders, 10 were approved in the last five years alone, during the course of my PhD candidature. An AAV vector was not selected as a gene therapy vector for the work presented in this thesis as the smaller transgene capacity limits the usefulness of this vector for the delivery of large therapeutic genes or a multi-genic cassette, which was a consideration at the start of the project.[153, 157]

5.2.2 Gene Therapy Clinical Trials for the Anterior Segment

As discussed in chapter one, the eye is a promising candidate organ for gene therapy, with the blood-ocular barrier preventing much systemic migration of vector and several cell layers, such as the retinal pigment epithelium, iris pigment epithelium, trabecular meshwork and the corneal endothelium, being accessible for

transduction.[41, 177, 195-197] Most ocular gene therapy studies are in pre-clinical stages, with only three vectors in phase I/II clinical trials.[338] Of these trials, the majority are focussed on the posterior segment of the eye, and diseases such as Leber's congenital amaurosis, age-related macular degeneration and retinal neovascularisation, as mentioned earlier. Up until January 2013, there had been only 2 of 28 human gene therapy clinical trials for the anterior segment of the eye, which involved treatment for corneal scarring and glaucoma. Results from these trials are yet to be published. There are currently no human clinical trial data published for corneal dystrophies.

Pre-clinical gene therapy studies in the anterior segment using AAV vectors have given mixed results. In 2010, Buie *et al.* used an unmodified AAV vector and a self-complementary AAV vector, to deliver GFP reporter genes intracamerally to the eyes of rats (both vectors) and cynomolgus monkeys (self-complementary AAV vector only).[339] The self-complementary AAV vector encodes both the sense and the complementary cDNA of the transgene, thereby single strands of the vector genome can pair with each other upon entry into the host cell and bypass the need for second strand DNA synthesis.[340] Second strand DNA synthesis is a function that does not occur in all cell types, so this modified vector offers great versatility.[341] The unmodified AAV vector failed to transduce the anterior segment tissues of the rat eye, however the modified self-complementary AAV vector induced transgene expression detectable in the trabecular meshwork for more than 3.5 months and over 2 years in the rat and monkey eyes, respectively.

AAV5 serotyped vectors were found to effectively transduce primary fibroblasts when topically applied to cells cultured from equine corneas,[342] and primary canine corneal fibroblast *in vitro*.[343] An AAV5 serotyped vector was also shown to

transduce keratocytes in the stroma of rabbit corneas *in vivo*, giving prolonged and high levels of transgene expression.[344] In the study of rabbit corneas and during the successful transduction of mouse corneas with AAV vectors in a study by Sharma *et al*., the corneas underwent epithelial removal prior to transduction in order to localise the gene therapy to the cells of the stroma.[344, 345] Epithelial debridement is not an ideal technique to use *in vivo* as it as can lead to morbidity, such as an increased risk of corneal infection.[236, 346]

A study by Hippert *et al*. investigated AAV vector serotypes 1, 2, 5 and 8.[237] The authors demonstrated that AAV serotype 8 was the most efficient of those tested at transducing both mouse and human keratocytes. Prolonged reporter transgene expression in the mouse cornea was shown up to 17 months after *in vivo* intrastromal injection of the AAV serotype 8 vector.

Sharma, Buss, Mohan and colleagues have recently published the results of several studies investigating various AAV vector serotypes and their ability to transduce corneal cells safely and effectively.[342, 344, 345] The authors demonstrated that the AAV vectors with serotype 6, 8 or 9 will all safely and effectively transduce both the mouse and human cornea, *in vivo* and *ex vivo*, respectively, with serotype 9 vectors achieving the greatest transduction efficiencies and serotype 6 vectors achieving the lowest transduction efficiencies.[345]

These studies indicate that AAV vectors have the potential to successfully transduce the cells of the cornea, giving safe and prolonged transgene expression. AAV vectors could be tested for efficacy of sheep cornea endothelial cell transduction in future

experiments, for the delivery of smaller therapeutic transgenes during penetrating keratoplasty in the sheep model, as an integrative alternative to lentiviral vectors.

5.3 Gene Therapy of the Cornea

5.3.1 Enhancers of Lentivirus-mediated Gene Therapy

A lentiviral vector, with its large transgene capacity, was selected as a gene therapy vector for the work described in this thesis.[151, 158] During transduction of the sheep cornea, a significant lag in transgene expression is observed that is not observed during similar transduction of the human cornea.[178, 202] It can take up to 14 days before peak transgene expression is detected from lentivirus-transduced ovine corneas compared with 1 – 6 days for lentivirus-transduced human corneas.[178, 202] Since the experimental animal model of penetrating keratoplasty in the sheep reflected the high-risk human situation very well, and the lentiviral vector provided many benefits over other vectors, the aim was to improve the transduction efficiency of the lentiviral vector in the sheep cornea, rather than change the vector or animal model.

Protamine sulphate is a small, positively charged polypeptide used successfully to increase the transduction efficiency of gene therapy viral vectors by neutralising the electrostatic repulsion between the viral particles and the target cell membranes.[212, 215, 242] The *in vitro* results described in this thesis showed protamine sulphate did significantly enhance the lentiviral transduction of sheep corneal endothelial cells at the maximum dose tested, with 7% transduction of target cells compared with 0.5% transduction in controls, however this enhancement was not sufficient to warrant further *in vivo* investigation.

Achieving high transgene expression in order to enhance the success of gene therapy has been, and still is, a major focus of many studies. Many different methods have been employed to this end, focusing on enhancing the cellular uptake of the viral particles or the DNA packages. Recombinant liposome complexes with DNA as delivery vehicles,[347] cationised gelatin complexed with enveloped viruses for an improved complex,[208] small cell-permeable polybasic peptides to increase viral entry,[205] and synthetic peptides forming complexes with DNA to enable better transfection of cells,[348] have all been used to increase gene expression *in vitro*.

More recently, two interesting substances have come to light as potential gene transduction enhancers. The first substance came to the fore after it was reported that particular peptides in semen formed amyloid fibrils, which captured HIV particles and aided their attachment to cells.[349] The fibrils are known as SEVI or semen-derived enhancer of virus infection. The transduction-enhancing property has been found to be due to the strong cationic properties of SEVI.[350] Wurm *et al.* found that in direct comparison with polycations polybrene and protamine sulphate, SEVI gave a consistent and higher increase in gene transfer efficiency using retroviral vectors, with no major toxic effects detected.[351]

The second group of substances of note are poloxamers. Poloxamers are non-ionic polymers, easily customisable, and have surfactant properties.[352] It is not known exactly by what mechanism poloxamers enhance viral transduction and improve transgene expression. They have been used in cell culture to prevent cell shearing and are found in many cosmetics and pharmaceuticals.[353, 354] A cosmetic ingredient review Expert Panel described 34 poloxamers as safe cosmetics ingredients within their stated parameters.[354] Hofig *et al.* showed that the poloxamer synperonic F108

was able to induce enhanced levels of lentivirus-mediated transgene expression.[355] The authors demonstrated that synperonic F108 increased the transduction rate in HEK-293T cells from 32% to 61%, which was a much larger increase than induced by the agent polybrene, which increased the transduction rate from 32% to 48% in a direct comparison. The potential of poloxamers is of particular interest, as two separate studies have shown improved survival of corneal endothelial cells in human and bovine corneas cultured *ex vivo* with poloxamers in the storage media.[356, 357] It would be useful to investigate the potential of novel agents such as poloxamers and SEVI to achieve enhanced viral transduction of the ovine cornea in the future.

5.3.2 Therapeutic Transgenes

The five therapeutic transgenes used in this thesis were each selected for their potential immuno-modulatory ability or effect on neovascularisation, when expressed by graft corneal endothelial cells after penetrating keratoplasty in the sheep. Each of the transgenes: IL-10, EK5, sFlt-1, IDO and Bcl-xL, had previously demonstrated efficacy for their proposed role for this project during *in vivo* animal studies.[76, 80, 87, 107, 138] Two transgenes were selected which are involved in suppressing T cell responses, and two transgenes were selected which are involved in the inhibition of neovascularisation. In both cases, the mechanisms by which these transgenic proteins act are quite distinct. The two transgenes ultimately suppress the same endpoint response, however they do so by separate pathways and therefore it was considered useful to include multiple transgenes to combat an individual, complex host system response. Three of the five transgenes were tested *in vivo* for prolongation of sheep corneal allografts and two of these, IL-10 and EK5, are discussed in this section, in light of what the *in vivo* results revealed and recent findings in the literature.

5.3.2.1 Interleukin-10 (IL-10)

The IL-10 protein was used to supress T cell immune responses. Over-expression of IL-10 using lentivirus vector-mediated transduction of donor sheep corneal endothelial cells *in vivo* resulted in statistically significant prolongation of corneal allograft survival, however only a modest overall increase from day 21 to day 26 for the median day of allograft rejection was achieved with the treatment. Adenovirus vector-mediated over-expression of IL-10 in the same sheep model of penetrating keratoplasty, in a cocktail of two other therapeutic genes, did not result in the prolongation of sheep corneal allograft survival. The adenoviral vector is noted for its ability to induce early and high transgene expression.[151] This vector encoding IL-10 was used in the *in vivo* therapeutic cocktail in order to induce earlier expression of this protein for an earlier intervention in the immune response to the allograft. As the results demonstrated, this approach was not effective, even though a previous study with the adenoviral vector encoding IL-10 in the sheep model of corneal transplantation found longer allograft prolongation than with the lentiviral vector, albeit without the additional cocktail of vectors involved.[76, 202]

As discussed in chapter 3, the timing of IL-10 administration for the prolongation of allograft survival is complex. Li *et al.* treated recipient mice receiving cardiac allografts with intra-peritoneal injections of recombinant IL-10.[106] The IL-10 was given either prior to transplantation or post-transplantation. The authors found a significant prolongation of allograft survival with *pre-treatment* of the recipient animals with IL-10, and either no effect on survival or an acceleration rate of rejection when the recipients were treated post-transplantation with IL-10. The authors speculated that the late administration of IL-10 after antigen presenting cells have matured and are already stimulated may enhance humoral and cytotoxic

immune responses. Torres *et al.* treated recipient rats receiving corneal allografts with either sub-conjunctival or intra-peritoneal injections of recombinant IL-10 or both.[104] The IL-10 was given either on the day before surgery or on the day of grafting, and every second day post-operatively until day 6 or 10. This treatment did not result in any prolongation of allograft survival and may have contributed to the acceleration of graft rejection. The results of these two studies suggest pre-treatment of the recipient animals with IL-10 is perhaps the most effective timing regimen for the protein expression in terms of preventing the immune response to the allografts.

In a recent study by Takiishi *et al*., the role of IL-10 in skin graft acceptance was investigated.[241] The authors discovered that syngeneic skin grafts from IL-10 knockout mice transplanted into wild-type mice were not well accepted, however grafts from wild-type donor mice transplanted into IL-10 knockout mice *were* accepted, suggesting that early, localised IL-10 expression was required for graft acceptance. In the work presented in this thesis, by using viral-mediated transgene expression, in effect, only localised post-graft treatment of the recipient cornea with IL-10 was conducted, with mixed results achieved.

Overall, these results suggest the best method to use in order to achieve optimal use of IL-10 for prolongation of corneal allograft survival may be to pre-treat the recipient animals. This does, however, abrogate the very advantage that eye banking of human corneas allows, which is *ex vivo* manipulation of corneas. In the sheep model, this would entail localised delivery to the eye, as systemic delivery of a therapeutic would entail an extremely high vector dose. Injection of a viral vector encoding IL-10 intracamerally for transduction of corneal endothelial cells and IL-10 expression prior to penetrating keratoplasty could be an option for pre-treatment of

recipient sheep. However this approach may lead to inflammation at the site of the injection or as a response to the vector, undesirable as inflammation is a risk factor for corneal graft rejection.[16, 34, 177] The subsequent penetrating keratoplasty would entail the loss of a good deal of the transduced cells with the removal of the recipient button. For this reason and the increased risk of inflammation, I would not propose to test this idea. It would be of value to gain further information about the anatomic site of sensitisation in the sheep, as an animal model closer in size and anatomy to the human than the mouse or rat, and determine the optimal location to direct the IL-10 therapy. Removing the spleen and performing corneal allografts to measure graft survival duration could be a method to investigate sensitisation location in the sheep, however this would be complex, high risk surgery, inappropriate for translation to humans.

5.3.2.2 Endostatin::Kringle5

In the *in vitro* results described in this thesis, the potent anti-angiogenesis, fusion transgene endostatin::kringle5 was shown to successfully inhibit the proliferation of tube-forming vascular endothelial cells. When tested *in vivo* in the lentiviral expression vector (along with a cocktail of other therapeutic transgenes) for the ability to inhibit corneal neovascularisation after orthotopic penetrating keratoplasty in the sheep, no inhibition of allograft vascularisation was observed and no prolongation of corneal allograft survival was achieved compared with mock-vector treated control allografts. However I suggest that further investigation of this gene is warranted, considering the potentially confounding effects of the cocktail treatments and the ocular inflammation. This could be achieved by individual testing of this transgene in a lentiviral vector using the sheep model of corneal transplantation,

increasing the MOI from the dosage described in this thesis, and delivering the vector by sub-conjunctival injection in order to transduce cells of the limbal region.

Results are continuing to be published showing the efficacy of endostatin in modulating angiogenic activity in the cornea, with Ge *et al.* recently demonstrating significant inhibition of neovascularisation in rabbit corneas after sub-conjunctival injection of both native and modified endostatin genes.[358] In another recent study by Tan *et al.*, the role of endostatin was investigated in a mouse model of corneal allograft transplantation.[359] The authors discovered that the levels of endogenous endostatin present in mouse cornea allografts significantly declined ten days post-operatively, with a noteworthy increase in endogenous VEGF levels at the same time point. Allografts exhibited increased graft rejection, as compared with syngeneic grafts, which experienced no decline in endostatin levels at day 10 and had 100% survival. Immunohistochemistry on the corneal allografts demonstrated that infiltrating CD3+ T cells clustered around the endostatin-producing cells and were present in inversely-proportional numbers to the endostatin-producing cells. Further investigation using a mouse model deficient in mature T cells, revealed the infiltrating T cells were specific in targeting the endostatin-producing cells for destruction, leading to neovascularisation and the rejection of the allografts. This information, which suggests the need for immunosuppressive therapies in conjunction with anti-neovascularisation therapies for more effective corneal allograft survival, lends further support for the hypothesis of a multi-armed therapeutic strategy.

A recent study by Li *et al.* has shown that the anti-neovascularisation activity of kringle 5 can be improved with a small modification to the amino acid composition

at the N-terminal domain of the protein.[360] The authors demonstrated a significant decrease in inflammation and area of vascularisation in corneas of rats with induced corneal vascularisation when treated topically with the mutant kringle 5 compared with the normal kringle 5.

5.3.2.3 Summary

In the context of future approaches with the transgenes discussed, multigenic therapy seems likely to be a useful technology, considering the recent results presented for the endostatin gene by Tan *et al*.[359] There has been some recent research investigating the modification of the amino acid composition of genes leading to enhanced gene activity, mentioned here with both endostatin and kringle 5, Ge *et al.* and Li *et al.*, respectively.[358, 360] This is a development that could increase the chances of success of gene therapy, if other issues can be addressed, such as reduction of endotoxin contamination, the potential for contradictory transgene interference in a multigenic cocktail therapy, and transgene expression timing for optimal biological function when required during the immune response. Contradictory transgene interference would require *in vitro* testing to determine the cellular locations of the transgenic proteins and their abilities to functionally interact. Timing of transgene expression, especially in the case of IL-10 appears to be a significant factor for determining the outcome of graft survival.

5.3.3 Gene Therapy of the Cornea in Pre-Clinical Animal Models

Gene therapy to prevent corneal allograft rejection may not be progressing to human clinical trials because the treatments used in pre-clinical animal models are not sufficiently successful. Appleby and colleagues demonstrated a statistically significant prolongation of rat corneal allograft survival with lentiviral treatment to over-express anti-CD4 single-chain antibody fragment, in order to inhibit host T cell responses.[292] This result suggests that the prolonged expression of the single-chain anti-body fragment induced by the integrated lentiviral therapy may have delayed the kinetics of the T effector cell response. Although significant, the prolongation was only modest, from a median of 17 days (control allografts) to 22 days (treated allografts). Jessup *et al.* transduced rat corneal allografts with an adenoviral vector encoding anti-CD4 single-chain antibody fragment, however found no increase in corneal allograft survival was induced by this therapy.[361] This result suggests that the transient transgene expression induced by the adenoviral vector was not sustained sufficiently for a therapeutic effect. The inflammatory potential of the adenoviral vector may also have reduced the therapeutic immunosuppression of the anti-CD4 single-chain antibody fragment. The results from these two studies together suggest that in the rat model of corneal transplantation, the major site of sensitisation is not likely to be the cornea itself, instead at distant locations such as the lymph nodes or spleen. As discussed in Section 1.3.2.5, it is not yet clear where antigen presentation and sensitisation occurs in humans or in sheep.

There have been some gene therapy studies in the mouse [80, 107] and also in the larger sheep model,[76, 202] in which the eye anatomy and physiology is more similar to humans, where allograft prolongation mediated by gene therapy has been found to be statistically significant, including in some of the work described in this thesis.

However, in the case of the sheep studies, the therapies were not consistent enough across all of the treated animals to warrant human clinical trials.

5.3.4 Gene Therapy of the Cornea Versus the Current Regimen

Topical steroids are given to all corneal graft patients at varying regimens. Steroidal anti-inflammatories are successful at reducing the frequency of graft rejection episodes.[362, 363] Penetrating keratoplasty performed in a patient with a history of ocular inflammation, or inflammation at the time of surgery, has a 50% chance of graft survival at 5 years compared with 91% for a patient with no ocular inflammation at the time of surgery or history of inflammation.[16] It is these patients at high-risk of graft rejection who require an alternative therapy to topical steroids. The administration of systemic steroids alongside topical steroids provided no additional benefit to patients in terms of preventing graft rejection episodes,[364] and from a 2004 survey of The Cornea Society members, it was reported that only 16% of respondents would prescribe oral systemic immune suppression for routine management of high-risk corneal grafts.[30]

The pre-clinical sheep model of corneal transplantation is a high-risk model. Without any form of immune suppression, corneal allografts in the sheep will undergo immunological rejection at approximately 3 weeks post-graft. Topical glucocorticosteroids are the current standard therapeutic used to treat inflammation and episodes of graft rejection in human corneal transplants.[28, 29] In a separate experiment not reported on in this thesis, six outbred sheep were given daily topical glucocorticoid steroid eye drops after penetrating keratoplasty in one eye. These sheep had a median day of graft rejection of 64.5. Even though this was a very small experimental group, these observations suggest that the current treatment used in the

clinic for corneal graft rejection episodes, topical steroidal anti-inflammatories, are more effective than any experimental gene therapy we have investigated in our laboratory, although results from Klebe *et al*. using the adenoviral vector encoding IL-10 were close (median allograft survival day of 55).[76]

Despite the degree of immune privilege enjoyed by the eye, the immune response mounted by the host against a corneal allograft is strong and multi-faceted. Gene therapy for monogenic disorders, designed to correct a single, mutated disease-causing gene, is quite a different scenario from gene therapy to prevent an overwhelming, immune response to an allograft. I argued that the optimal way to prevent such a multi-faceted response might be by using a multi-faceted therapy, however, as I have demonstrated in this thesis, determination of the correct combination and delivery of a multi-armed therapy requires careful consideration, and may still be insufficient to prevent rejection.

5.4 Multi-vector Cocktail Therapies

Although multiple gene therapy vectors in combination did not prolong corneal graft survival in the sheep model, my *in vitro* results demonstrated that cocktail therapy with the lentiviral and adenoviral vector resulted in good transduction, and transgene expression from both vectors. As introduced in chapters 1 and 4, combinations of viral vectors have been used simultaneously in previous studies to good effect,[189, 224, 225] usually with a combination of an adenoviral and an adeno-associated viral vector. These results are continuing to appear, with a recent study by Liu *et al.* utilising an adenoviral and an adeno-associated viral vector to express the same transgene: an inhibitor of the growth and tumourigenicity of human glioblastoma and melanoma cells.[365] The authors found the combination treatment of the two vectors was more

effective than treatment with either vector alone at reducing tumour growth in a mouse model, utilising the strong initial transgene expression from the adenoviral vector and the long-term expression from the adeno-associated viral vector.

There have been three multi-vector clinical trials approved using a combination of an adenoviral vector and a retroviral vector (www.wiley.co.uk/genmed/clinical). Two of these trials are for phase I lymphoma from the same group of investigators in the United States, with the latest approval given in 2005. There are no published results from any of these trials although it appears the investigators in the lymphoma trials may be utilising the adenoviral vector in a single vector approach.[366, 367]

Taking a slightly different method to multigenic therapy, Verrier *et al.* recently used a bi-cistronic lentiviral vector, which utilised a viral 2A-like cleavage peptide between a therapeutic transgene and a transgene encoding a fluorescent protein. This cleavage peptide allows co-translational ribosomal 'skipping' and equal expression of both transgenes.[368] This vector was successfully delivered to the retinas of an avian model of Leber's congenital amaurosis, and transduction of the cells resulted in the mutated gene correction and a subsequent improvement in vision in the birds.[369] This cleavage peptide could be used to enable multigenic transduction of the sheep cornea and reduce the viral load and the potential for endotoxin contamination that come with the use of individual viral vectors.

5.5 Current Status of Gene Therapy

5.5.1 Gene Therapy Clinical Trials

Ex vivo retroviral vector-mediated gene therapy of haematopoietic stem cells and reinfusion back into patients has been used to successfully treat severe life-threatening diseases such as X-linked severe combined immunodeficiency (SCID-X1), adenine deaminase deficiency, chronic granulomatous disease, X-linked adrenoleukodystrophy and Wiskott-Aldrich syndrome.[171, 172, 261, 370, 371] There remain issues with the safety of this technology, with many recent studies showing these integrative viral vectors have distinct preferences for proviral genomic insertion sites, Table 5.1, which can impact the likelihood of causing adverse genotoxic events such as insertional mutagenesis, oncogenesis and clonal dominance.[372]

Table 5.1: Summary of proviral insertion site preferences for selected integrative simple and complex retroviral vectors.

Retroviral Vector	Insertion Site Preference	References
MLV	Transcription start sites; CpG Islands	191, 192
HIV	Transcription units; active genes	257, 258

MLV – murine leukaemia virus; HIV – human immunodeficiency virus

Lentiviral vectors, which are complex retroviral vectors, have an improved safety profile in comparison with simple gamma retroviral vectors. In a comparative study by Montini *et al.*, the authors demonstrated the importance of transcriptionally active long terminal repeat sequences (LTRs) in driving genotoxicity, as well as insertion site selection of the viral vectors for substantially modulating genotoxicity.[373] The study showed the relative oncogenic risk associated with an integration of a self-inactivating lentiviral vector is much less than with a gamma retroviral vector. The investigators of the SCID-X1 trial in France are also investigating self-inactivating human immunodeficiency virus (HIV) lentiviral vectors as potential replacements to

the gamma retroviral vector previously used, and investigating the potential of these vectors to treat SCID-X1 disease in a safe manner.[374]

While the self-inactivating lentiviral vector may be an improvement on the gamma retroviral vector, there are still integration issues to investigate. Heckl *et al.* found acute leukaemia was induced in one mouse out of seven after transplantation with bone marrow cells transduced with a self-inactivating lentiviral vector.[375] The investigators discovered proviral integration had occurred at an intron in the *Ebf1* tumour suppressor gene, causing transcriptional down-regulation of this gene. The human gene therapy clinical trial treating X-linked adrenoleukodystrophy using the lentiviral vector to transduce *ex vivo* haematopoietic stem cells has, however, reported no signs of genotoxicity.[261]

Greater understanding of the vector integration preferences is leading to safer gene therapy vectors, however the ultimate viral transfer vector is still on the horizon. At this point in time, utilising a self-inactivating HIV lentiviral vector with internal promoters for *ex vivo* transduction of the cornea is one of the safest and most efficient methods for integrated transgene expression.

5.6 Thoughts for the Future

In order to continue the investigations of this project, in immediate experiments, it would be ideal to test the individual transgenes EK5 and Bcl-xL in lentiviral vectors for prolongation of sheep corneal allograft survival. Delivery of EK5 by sub-conjunctival injection to the limbal region would be of interest to investigate whether an alternate delivery location can enhance the efficacy of the treatment, with limbal stem cells as the target for transduction. The location of the transgenic protein

expression and any functional interference between proteins when used in a cocktail could be determined by *in vitro* immunohistochemical investigations and functional activity assays. In the longer term, providing more information about the location of antigen presentation in the sheep would be of use as it pertains to the human scenario.

5.7 Concluding Remarks

Viral vectors are becoming safer, more specific, efficient, and better characterised for transduction of ocular cells. Our understanding of the host immune responses to corneal allografts is improving and selection of possible therapeutic transgenes is broadening. Ultimately, however, the immune response to corneal allografts may overwhelm any therapeutic intervention mediated by gene therapy.

For gene therapy to become the champion of corneal graft prolongation, consistent transduction of corneas is necessary for more predictable survival results across animal groups. Long-term transgene expression at therapeutic levels is required at specifically targeted locations, with transgenic protein expression timed for optimal therapeutic relevance. A multi-faceted treatment with a combination of therapeutic transgenic proteins must be considered.

Gene therapy has great potential for several ocular disorders and diseases, but these obstacles must be overcome before it may reach its full potential for the prolongation of penetrating corneal transplants.

Appendix 1

General Chemicals and Solutions

A1.1 General Chemicals and Solutions

Table A1.1: General chemicals and laboratory reagents.

Reagent	Description	Company
Absolute alcohol	Ethanol absolute	Merck, Kilsyth, VIC
Acetic acid	Glacial acetic acid	Ajax Finechem, Taren Point, NSW
Albumin, from bovine serum	≥98% purity, catalogue #A7906	Sigma-Aldrich, St Louis, MO USA
Bacto™ Agar	Solidifying agent	Becton, Dickson & Co., NJ, USA
Bacto™ Tryptone	Peptone for use in culture media	Becton, Dickson & Co., NJ, USA
Bacto™ Yeast Extract	Extract of autolysed yeast cells	Becton, Dickson & Co., NJ, USA
Betadine® Antiseptic Solution	10% (w/v) Povidone-Iodine	Betadine, Virginia, QLD
Boric Acid	catalogue #101	Ajax Finechem, Taren Point, NSW
Calcium chloride dihydrate	Transfection reagent, DNA precipitation	VWR International, Leuven, Belgium
CellTiter 96® AQ_{ueous} One Solution Reagent	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium	Promega, Madison, WI USA
Chloramphenicol	Antibiotic for microbial selection	Sigma, St Louis, MO USA
D-Glucose (anhydrous)	dextrose anhydrous, catalogue #GA018	Ajax Finechem, Taren Point, NSW
DEPC	Diethyl pyrocarbonate	Sigma, St Louis, MO USA
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt hydrate	Sigma, St Louis, MO USA
Ehrlich's Reagent	4-(dimethyamino)-benzaldehyde	Sigma, St Louis, MO USA
Ethanol	Ethanol absolute ACS	Merck, Kilsyth, VIC
E-Toxa-Clean®	Cleaning glassware for endotoxin-low preparations	Sigma, St Louis, MO USA
Formalin	Formaldehyde aqueous solution (10% v/v)	Medvet Science Pty Ltd, SA
Glycerol	analytical reagent, catalogue #A242	Ajax Finechem, Taren Point, NSW
Hepes	≥ 99.5% purity	Sigma, St Louis, MO USA
Hoechst 33258	BisBenzimide, nuclear stain, blue dye	Sigma, St Louis, MO USA
Hydrochloric acid	36% (v/v), analytical reagent	Ajax Chemicals, Auburn, NSW
Hydrogen peroxide (30% w/v)	H_2O_2	Chem-Supply, Gillman, SA
L-Kynurenine	catalogue #K8625	Sigma, St Louis, MO USA
Lysine hydrochloride	L-lysine mono-hydrochloride	BDH Chemicals, Poole, England
2-Mercaptoethanol	catalogue #M3148	Sigma, St Louis, MO USA

Table A1.1 continued

Reagent	Description	Company
OCT compound	(Optimal Cutting Temperature) Embedding medium for frozen tissue specimens	Tissue-Tek, Tokyo, JPN
Paraffin	Paraplast tissue embedding medium	McCormick Scientific, St Louis, MO USA
Paraformaldehyde	catalogue #P6148	Sigma, St Louis, MO USA
Potassium chloride	KCl, catalogue #10198	BDH Chemicals Australia, Kilsyth, Vic
Potassium dihydrogen orthophosphate anhydrous	KH_2PO_4, catalogue #PA009	Chem-Supply, Gillman, SA
2-Propanol	For molecular biology ≥ 99% purity	Sigma, St Louis, MO USA
Protamine sulphate	Protamine sulphate salt from salmon, Grade X	Sigma, St Louis, MO USA
Sodium azide	extra pure	Merck, Kilsyth, VIC
Sodium carbonate	Na_2CO_3	Ajax Chemicals, Auburn, NSW
Sodium chloride	NaCl	Chem-Supply, Gillman, SA
Sodium chloride 0.9% for irrigation	0.9% (w/v) isotonic, nonpyrogenic, sterile	Baxter, Old Toongabbie, NSW
Sodium dodecyl sulphate	SDS, for molecular biology. Approx. 99% purity	Sigma, St Louis, MO USA
Sodium hydrogen carbonate (sodium bicarbonate)	$NaHCO_3$	Ajax Finechem, Taren Point, NSW
Sodium hydroxide	NaOH	VWR International, Leuven, Belgium
Di-sodium hydrogen orthophosphate	Na_2HPO_4 (anhydrous)	Chem-Supply, Gillman, SA
Sodium di-hydrogen orthophosphate dihydrate	$NaH_2PO_4.2H_2O$	Chem-Supply, Gillman, SA
Sodium m-periodate	$NaIO_4$	Sigma, St Louis, MO USA
Streptavidin/HRP	Streptavidin conjugated to horseradish peroxidise. Binds to biotin or biotinylated antibodies	DakoCytomation, Glostrup, Denmark
Sucrose	catalogue #10274.4B	Merck, Kilsyth, VIC
Trichloroacetic Acid	catalogue #T6399	Sigma-Aldrich, St Louis, MO USA
Tris base	Trizma® Base, minimum 99.9% purity (titration)	Sigma-Aldrich, St Louis, MO USA
Tween-20	Polyoxyethylenesorbitan monolaurate	Sigma, St Louis, MO USA
UltraPure water	Used in quantitative real time PCR	Fisher Biotec Australia, Wembley, WA
Water for injections BP	Sterile, used in endotoxin-low methods	Phebra, Lane Cove, NSW
Water for irrigation	Sterile, nonpyrogenic, used in endotoxin-low methods	Baxter, Old Toongabbie, NSW
Xylene	catalogue #1023	BDH Chemicals Australia, Kilsyth, Vic

Appendix 2

Formulations of Reagents, Buffers, Media and Solutions

A2.1 Formulation of Reagents, Buffers, Media and Solutions

A2.1.1 Acid Alcohol

Used in haematoxylin and eosin histological staining. 1 mL of concentrated hydrochloric acid was added to 100 mL of 70% (v/v) ethanol.

A2.1.2 Bovine Gelatin

Water for irrigation (sterile)	750 mL
Bovine gelatin (Sigma, St Louis, MO USA) granular	15.39 g
Foetal bovine serum	32.6 mL
7.5% (w/v) sodium bicarbonate (sterile)	19.6 mL
10x Hepes buffered HBSS with glucose (sterile) (see A2.1.14 for 10x Hepes-buffered HBSS recipe)	65.2 mL

Water was poured into a sterile endotoxin-low tissue culture bottle and warmed on a hot plate until just warm (not hot). Gelatin was added and the bottled swirled to mix. With occasional swirling, the mixture was gently heated until gelatin dissolved. The solution was then autoclaved and placed at 4^{o}C to ensure the gelatin sets. The gelatin was thawed in a 37^{o}C water bath and the remaining reagents were added. The solution was aliquotted into 10 mL tubes and stored at 4^{o}C.

A2.1.3 2.5% (w/v) Bovine Serum Albumin (endotoxin-low)

Albumin, bovine serum	5 g
PBS (endotoxin-low)	up to 200 mL

Albumin dissolved and the solution was 0.22 µm sterile filtered.

A2.1.4 2.5M Calcium Chloride (endotoxin-low)

36.76 g calcium chloride was made up to 100 mL using water for irrigation and 0.22 µm sterile filtered.

A2.1.5 Chrome Alum Subbing Solution for Slides

Chrome alum	0.5 g	$CrK(SO_4)_2.12H_2O$ (Ajax Chemicals, Sydney, NSW)
Gelatin	5 g	(BDH Laboratory Supplies, Poole, England)

Gelatin was dissolved in 500 mL of distilled water, warmed to no more than 60^{o}C. The chrome alum was added and the solution made up to 1 L with distilled water. Filtered through Whatman number 1 filter paper (Whatman, Maidstone, England).
Glass microscope slides were washed overnight in Extran® soap solution (Merck, Darmstadt, Germany) then rinsed for 30 minutes in running deionised water. Slides were dried before being dipped for 2 – 4 minutes into warmed subbing solution in racks, drained on paper towel then dried overnight with loose covers to prevent dust adhering to surfaces.

A2.1.6 Chloramphenicol for Antibiotic Selection

0.34 g chloramphenicol was made up to 10 mL with 100% (v/v) Ethanol (AR grade) and filter sterilised for 34 mg/mL aliquots. Used at 34 µg/mL in selective media.

A2.1.7 10x DAB stock

1 g of 3,3-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO USA) was made up to 6 mg/mL with 160 mL of 0.1M Tris-azide pH7.6 (Appendix A2.1.33), rested for 30 minutes and filtered through Whatman number 1 filter paper (Whatman, Maidstone, England).

A2.1.8 Diethylpyrocarbonate (DEPC) Treatment of Solutions

Solutions to be made RNase-free were treated with diethylpyrocarbonate (DEPC). DEPC was added to solutions at 0.1% (v/v) volume, shaken thoroughly and left to stand overnight. The DEPC was then inactivated by autoclaving.

A2.1.9 0.5M EDTA

113 g of EDTA was made up to 500 mL with dH_2O. The pH was adjusted to 8 using 1 M hydrochloric acid and the solution autoclaved.

A2.1.10 Eosin

Eosin stock was made by dissolving 1 g of eosin Y (Chroma-Gesellschaft Schmid GmbH & Co, Köngen/N, Germany) in 20 mL of dH_2O and adding 80 mL 95% (v/v) ethanol. 25 mL of this stock was taken and added to 75 mL of 80% (v/v) ethanol. Immediately before use 0.5 mL glacial acetic acid was added.

A2.1.11 FACS Fixative

D-Glucose	10 g
Formaldehyde	13 mL
4 M Sodium azide	0.625 mL

The solution was made up to 500 mL with 1x PBS and the pH adjusted to 7.3. Solution stored at 4^oC and protected from the light.

2.1.12 6x Gel Electrophoresis Loading Buffer

Bromophenol blue	0.25% (w/v) (Sigma, St Louis, MO USA)
Xylene cyanol	0.25% (w/v) (Sigma, St Louis, MO USA)
Ficoll-Paque™ PLUS	15% (v/v) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

Dissolved on low heat for 1-2 hrs until solution turned green. Method from Sambrook *et al.* 1989.[376]

A2.1.13 Haematoxylin

Haematoxylin powder	1.25 g
(Aldrich, Milwalkee, WIS USA)	
Absolute ethanol	2 mL
Glycerol	75 mL
Sodium iodate	0.25 g
(Koch-Light Laboratories, Colnbrook, Buckinghamshire England)	
Aluminium potassium sulphate	12.5 g
(Ajax Finechem, Taren Point, NSW)	
Glacial acetic acid	0.5 mL
dH_2O	175 mL

The haematoxylin powder was dissolved in absolute ethanol. Aluminium potassium sulphate was added to 60 mL of distilled water, heated to dissolve and cooled. The remaining water was added to the aluminium potassium sulphate with the dissolved haematoxylin, sodium iodate, acetic acid and glycerol. The resulting solution was filtered and stored in the dark.

A2.1.14 10x Hepes-buffered Hank's Balanced Salt Solution (HBSS) with glucose

KCl	4 g
KH_2PO_4	0.6 g
NaCl	80 g
$NaHCO_3$	3.5 g
Na_2HPO_4	0.465 g
D-glucose	10 g

Made up to 1 L using water for irrigation and pH adjusted to 7.3. Sterile filtered through a 0.22 μm filter. Endotoxin-low glassware was used.

A2.1.15 Hepes-buffered RPMI 1640 (endotoxin-low)

RPMI medium 1640	1 sachet
(with L-glutamine)	
(with 25mM HEPES buffer)	
(without $NaHCO_3$)	
$NaHCO_3$	2 g

Made up to 1 L using water for irrigation and pH adjusted to 7.1-7.2 with 1 M NaOH. Sterile filtered with a 0.22 μm filter. Additives included foetal bovine serum (FBS) at 2-10% (v/v), penicillin and streptomycin at 100 IU/mL and 100 μg/mL concentrations, respectively, glutamine at 2 mM concentration and amphotericin B, used at 2.5 μg/mL. Endotoxin-low glassware was used.

A2.1.16 2x Hepes-buffered Saline (endotoxin-low)

Na_2HPO_4 (1.5mM)	0.11 g
Hepes (50mM)	5.96 g
NaCl (0.28mM)	8.2 g

Made up to 500 mL using water for irrigation and pH adjusted to 7.05. 0.22 µm sterile filtered.

A2.1.17 High Glucose DMEM (endotoxin-low)

Dulbecco's Modified Essential Medium (with L-glutamine, sodium pyruvate, phenol red) (without HEPES buffer, without $NaHCO_3$)	1 sachet
$NaHCO_3$	3.7 g
D-glucose (anhydrous)	3 g

Made up to 1 L with water for irrigation and pH adjusted to 7.2 with 1 M NaOH. Sterile filtered with a 0.22 µm filter. Additives include FBS at 2-10% (v/v), penicillin and streptomycin at 100 IU/mL and 100 µg/mL concentrations, respectively, and glutamine at 2 mM concentration. Endotoxin-low glassware was used.

A2.1.18 1M Imidazole

6.81 g of imidazole (Sigma, St Louis, MO USA) was added to distilled water and made up to 100 mL.

A2.1.19 Luria Bertani (LB) Broth Medium

Bacto™ Tryptone	10 g
Bacto™ Yeast Extract	5 g
NaCl	10 g

Made up to 1 L using dH_2O and pH adjusted to 7 with 10 M NaOH.

A2.1.20 LB Agar

Bacto™ Agar was added to LB broth medium at 15 g/L prior to autoclaving.

A2.1.21 LB for Preparation of Electrocompetent cells (low salt)

As for normal LB broth medium, containing 5 g sodium chloride.

A2.1.22 Medium 199

M199 powder	1 sachet
(with L-glutamine, phenol red; without HEPES, sodium bicarbonate)	
Sodium bicarbonate powder	2.25 g

Made up to 1 L with water for irrigation and pH adjusted to 7.2. Sterile filtered with a 0.22 µm filter. Additives included 20% (v/v) FBS, 20 mM HEPES, penicillin and streptomycin at 100 IU/mL and 100 µg/mL concentrations, respectively, 2 mM glutamine, 100 µM non-essential amino acids, 1 mM sodium pyruvate, heparin sodium salt and bovine endothelial cell growth factor. Endotoxin-low glassware was used.

A2.1.23 Normal Swine Serum

Normal swine serum (Trace Biosciences, Castle Hill, NSW), heat inactivated at 56^{o}C for 30 minutes prior to use. Used at 10% (v/v), made up in PBS (Appendix A2.1.25).

A2.1.24 Paraformaldehyde-Lysine-Periodate Fixative

0.1 M Lysine hydrochloride in 0.05M phosphate buffer

Solution A 0.2 M Lysine

Lysine hydrochloride	18.27 g

Made up to 500 mL with dH_2O.

Solution B 0.1 M Phosphate

0.2 M $NaH_2PO_4.2H_2O$	47.5 mL
0.2 M Na_2HPO_4	202.5 mL

Made up to 500 mL with dH_2O.

Solution A (500 mL) was added to Solution B (500 mL) and 0.22 µm sterile filtered.

8% (w/v) Paraformaldehyde

8 g paraformaldehyde was mixed with approximately 60 mL dH_2O and heated gently on a stirrer in the fume hood to 60^{o}C. 10 M NaOH was added drop-wise until the solution cleared and the solution was allowed to cool. Made up to 100 mL using dH_2O and carefully 0.22 µm sterile filtered.

0.1 M Lysine hydrochloride (0.075 M)	15 mL
8% (w/v) Paraformaldehyde (2% final concentration)	5 mL
Sodium periodate (0.01 M)	0.043 g

The pH was adjusted to 7.2 – 7.4 with 10 M NaOH. Solution made up fresh from stocks for use within twelve hours.

A2.1.25 Phosphate Buffered Saline

Na_2HPO_4 (anhydrous)	2.29 g
$NaH_2PO_4.2H_2O$	0.63 g
NaCl	7 g

The solution was made up to 1 L with dH_2O and the pH adjusted to 7.3-7.4 with 1 M NaOH and the solution autoclaved prior to use. Endotoxin-low preparations use E-Toxa-Clean® glassware and sterile water for irrigation.

A2.1.26 Phosphate Buffered Saline – Gelatin (1 L)

Gelatin	2 g	(BDH Laboratory Supplies, Poole, England)
Na_2HPO_4	0.85 g	
KH_2PO_4	0.54 g	(BDH Laboratory Supplies, Poole, England)
NaCl	8.7 g	

Gelatin was dissolved in approximately 500 mL of distilled water with gently heat. Salts were dissolved in remaining water to make up to 1 L. The gelatin solution was added to the salt solution.

A2.1.27 Sheep diluent

Sheep diluent was prepared containing 1% (v/v) foetal bovine serum (Thermo Scientific, Scoresby, VIC), 1% (v/v) normal swine serum (Trace Biosciences, Castle Hill, NSW) and 1% (v/v) normal sheep serum (separated from whole sheep blood sample by centrifugation) (all heat inactivated at 56^oC for 30 minutes) in PBS. This was filtered through a 0.22 μm membrane and stored at 4^oC.

A2.1.28 SOC Medium

Base medium

Bacto-tryptone	6 g	(2% w/v)	
Yeast extract	1.5 g	(0.5% w/v)	
NaCl	0.18 g	(10 mM)	
KCl	0.24 g	(2.5 mM)	
$MgSO_4.7H_2O$	0.75 g	(10 mM)	(Merck, Kilsyth, Vic)

Made up to 300 mL with dH_2O and pH 7 before autoclaving.

1M $MgCl_2.6H_2O$

$MgCl_2.6H_2O$	20.33 g	(Chem-Supply, Gillman, SA)

Made up to 100 mL with dH_2O and 0.22 μm sterile filtered.

1M Glucose

Glucose	18.02 g

Made up to 100 mL with dH_2O and 0.22 μm sterile filtered.

1 mL of 1 M $MgCl_2.6H_2O$ and 2 mL 1 M glucose added to 100 mL SOC medium prior to use.

A2.1.29 0.1 M Sodium Carbonate

2.12 g sodium carbonate was made up to 200 mL with dH_2O and pH adjusted to 9.5 with 1 M acetic acid. Used with monoclonal capture antibody as a plate coating buffer for the IL-10 ELISA.

A2.1.30 3 M Sodium acetate pH 5.2

$NaOAc.3H_2O$	408.1 g (Ajax Chemicals, Sydney, NSW)

Dissolved in water and made up to 1 L. pH adjusted to 5.2 with acetic acid, solution autoclaved. Method from Sambrook *et al*. 1989.[376]

A2.1.31 7% (w/v) Sucrose in PBS

7 g sucrose was dissolved in 100 mL PBS. 500 µl of 4 M sodium azide was added and the solution stored at 4°C.

A2.1.32 15% (w/v) Sucrose in PBS

15 g sucrose was dissolved in 100 mL PBS. 500 µl of 4 M sodium azide was added and the solution stored at 4°C.

A2.1.33 Tris-azide (0.1 M pH 7.6)

6.055 g of Tris base was dissolved in distilled water and made up to 500 mL. pH adjusted to 7.6 with 11.5 N HCl. 1.5 g sodium azide added and stored at 4^oC.

A2.1.34 5x Tris-Borate EDTA

Tris Base	54 g
Boric Acid	27.5 g
0.5M EDTA pH 8	20 mL

Made up to 1 L with dH_2O and autoclaved. Used at a working concentration of 0.5x in agarose gels and in the gel electrophoresis tanks.

A2.1.35 0.5 M Tris-Chloride pH 8.5

0.2 M Tris solution was prepared by dissolving 24.23 g of Tris base in 1 L dH_2O. 50 mL of this solution was mixed with 16.5 mL of 0.1 M HCl and made up to 200 mL with dH_2O. This final solution was diluted to 10 mM before use.

A2.1.36 Trypsin-EDTA (endotoxin-low)

Trypsin powder 1:250	0.5 g
EDTA.4Na (0.05% w/v)	0.2 g
10x PBS (endotoxin-low)	100 mL

Made up to 1 L with water for irrigation and sterile filtered with a 0.22 µm filter. Endotoxin-low glassware was used.

Appendix 3

Sheep Orthotopic Keratoplasty Surgical Record and Graft Scoring Sheets

A3.1 Sheep Corneal Graft Operation Record

Animal ID: **Date of Graft:**

Experimental group: **Surgeon:**

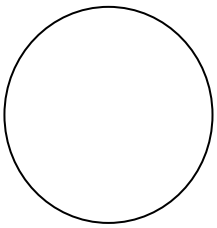
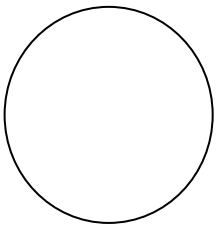
Sex: **Weight:**

Eye grafted:

Size of graft:

Pre-operative treatment:

Prevascularisation/ remarks at time of grafting:

Recipient:  **Donor:** 

Anaesthesia:

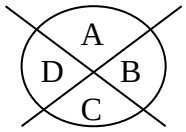
Operation notes:

Post-operative treatment:

A3.2 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	Vascularisatn				Comments
							A	B	C	D	

References

1. Bron, A.J., R.C. Tripathi, B.J. Tripathi, and E. Wolff, Wolff's anatomy of the eye and orbit. 8th ed. 1997, London: Chapman & Hall Medical.
2. Joyce, N.C., Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res*, 2003. **22**(3): p. 359-89.
3. Van Buskirk, E.M., The anatomy of the limbus. *Eye*, 1989. **3 (Pt 2)**: p. 101-8.
4. Tuft, S.J. and D.J. Coster, The corneal endothelium. *Eye*, 1990. **4 (Pt 3)**: p. 389-424.
5. Lim, A.S.M. and I.J. Constable, Colour atlas of ophthalmology. 7th ed. 1979, London: Kimpton.
6. Williams, K.A. and D.J. Coster, The immunobiology of corneal transplantation. *Transplantation*, 2007. **84**(7): p. 806-13.
7. Fischbarg, J., The Biology of the eye, in Advances in organ biology ; v. 10. 2005, Elsevier: Amsterdam.
8. Mergler, S. and U. Pleyer, The human corneal endothelium: new insights into electrophysiology and ion channels. *Prog Retin Eye Res*, 2007. **26**(4): p. 359-78.
9. Klintworth, G.K., The molecular genetics of the corneal dystrophies--current status. *Front Biosci*, 2003. **8**: p. d687-713.
10. Donaldson, D.D., Atlas of external diseases of the eye / vol. 3. Cornea and sclera. 2nd ed. 1980, St. Louis: Mosby.
11. Sen, K., Keratomalacia: causes, diagnosis, treatment and prevention. *J Indian Med Assoc*, 1954. **24**(1): p. 17-20.
12. Kissam, R.S., Ceratoplastice in Man. *N Y J Med*, 1844. **2**: p. 281-282.
13. Zirm, E.K., Eine erfolgreiche totale Keratoplastik (A successful total keratoplasty). 1906. *Refract Corneal Surg*, 1989. **5**(4): p. 258-61.
14. Transplant Activity in the UK, U.T. Statistics and Audit Directorate, Editor. 2008, NHS Blood and Transplant: Bristol. p. 90.
15. Aiken-O'Neill, P. and M.J. Mannis, Summary of comeal transplant activity Eye Bank Association of America. *Cornea*, 2002. **21**(1): p. 1-3.
16. The Australian Corneal Graft Registry 2012 Report, K.A. Williams, M.T. Lowe, M.C. Keane, V.J. Jones, R.S. Loh, and D.J. Coster, Editors. 2012, The Australian Corneal Graft Registry: Adelaide.
17. Vail, A., S.M. Gore, B.A. Bradley, D.L. Easty, C.A. Rogers, and W.J. Armitage, Influence of donor and histocompatibility factors on corneal graft outcome. *Transplantation*, 1994. **58**(11): p. 1210-6.
18. Glasser, D.B., Serologic testing of cornea donors. *Cornea*, 1998. **17**(2): p. 123-8.
19. Wiffen, S.J., L.R. Nelson, A.F. Ali, and W.M. Bourne, Morphologic assessment of corneal endothelium by specular microscopy in evaluation of donor corneas for transplantation. *Cornea*, 1995. **14**(6): p. 554-61.
20. Armitage, W.J. and D.L. Easty, Factors influencing the suitability of organ-cultured corneas for transplantation. *Invest Ophthalmol Vis Sci*, 1997. **38**(1): p. 16-24.
21. George, A.J. and D.F. Larkin, Corneal transplantation: the forgotten graft. *Am J Transplant*, 2004. **4**(5): p. 678-85.
22. The collaborative corneal transplantation studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group. *Arch Ophthalmol*, 1992. **110**(10): p. 1392-403.

23. Katami, M., P.W. Madden, D.J. White, P.G. Watson, and N. Kamada, The extent of immunological privilege of orthotopic corneal grafts in the inbred rat. *Transplantation*, 1989. **48**(3): p. 371-6.
24. Sano, Y., B.R. Ksander, and J.W. Streilein, Murine orthotopic corneal transplantation in high-risk eyes. Rejection is dictated primarily by weak rather than strong alloantigens. *Invest Ophthalmol Vis Sci*, 1997. **38**(6): p. 1130-8.
25. Bohringer, D., E. Spierings, J. Enczmann, S. Bohringer, R. Sundmacher, E. Goulmy, and T. Reinhard, Matching of the minor histocompatibility antigen HLA-A1/H-Y may improve prognosis in corneal transplantation. *Transplantation*, 2006. **82**(8): p. 1037-41.
26. Kenyon, K.R. and S.C. Tseng, Limbal autograft transplantation for ocular surface disorders. *Ophthalmology*, 1989. **96**(5): p. 709-22; discussion 722-3.
27. Claerhout, I., H. Beele, and P. Kestelyn, Graft failure: I. Endothelial cell loss. *Int Ophthalmol*, 2008. **28**(3): p. 165-73.
28. Rinne, J.R. and R.D. Stulting, Current practices in the prevention and treatment of corneal graft rejection. *Cornea*, 1992. **11**(4): p. 326-8.
29. Barker, N.H., T.R. Henderson, C.A. Ross, D.J. Coster, and K.A. Williams, Current Australian practice in the prevention and management of corneal allograft rejection. *Clin Experiment Ophthalmol*, 2000. **28**(5): p. 357-60.
30. Randleman, J.B. and R.D. Stulting, Prevention and treatment of corneal graft rejection: current practice patterns (2004). *Cornea*, 2006. **25**(3): p. 286-90.
31. Hassanzadeh, J., A.A. Hashiani, A. Rajaeefard, H. Salahi, E. Khedmati, F. Kakaei, S. Nikeghbalian, and A. Malek-Hossein, Long-term survival of living donor renal transplants: A single center study. *Indian J Nephrol*, 2010. **20**(4): p. 179-84.
32. Williams, K.A., S.M. Muehlberg, R.F. Lewis, and D.J. Coster, How successful is corneal transplantation? A report from the Australian Corneal Graft Register. *Eye*, 1995. **9 (Pt 2)**: p. 219-27.
33. Lodhi, S.A. and H.U. Meier-Kriesche, Kidney allograft survival: the long and short of it. *Nephrol Dial Transplant*, 2011. **26**(1): p. 15-7.
34. Armitage, W.J., HLA matching and corneal transplantation. *Eye*, 2004. **18**(3): p. 231-2.
35. Williams, K.A., A.J. Esterman, C. Bartlett, H. Holland, N.B. Hornsby, and D.J. Coster, How effective is penetrating corneal transplantation? Factors influencing long-term outcome in multivariate analysis. *Transplantation*, 2006. **81**(6): p. 896-901.
36. Niederkorn, J.Y., The immune privilege of corneal grafts. *J Leukoc Biol*, 2003. **74**(2): p. 167-71.
37. Bourne, W.M., Cellular changes in transplanted human corneas. *Cornea*, 2001. **20**(6): p. 560-9.
38. Barker, C.F. and R.E. Billingham, Immunologically privileged sites. *Adv Immunol*, 1977. **25**: p. 1-54.
39. Griffith, T.S., T. Brunner, S.M. Fletcher, D.R. Green, and T.A. Ferguson, Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science*, 1995. **270**(5239): p. 1189-92.
40. Williams, K.A. and D.J. Coster, Rethinking immunological privilege: implications for corneal and limbal stem cell transplantation. *Mol Med Today*, 1997. **3**(11): p. 495-501.
41. Cumha-Vaz, J.G., The blood-ocular barriers. *Invest Ophthalmol Vis Sci*, 1978. **17**(11): p. 1037-9.

42. Cursiefen, C., Immune privilege and angiogenic privilege of the cornea. *Chem Immunol Allergy*, 2007. **92**: p. 50-7.
43. Niederkorn, J.Y., Immune privilege and immune regulation in the eye. *Adv Immunol*, 1990. **48**: p. 191-226.
44. Sonoda, Y. and J.W. Streilein, Impaired cell-mediated immunity in mice bearing healthy orthotopic corneal allografts. *J Immunol*, 1993. **150**(5): p. 1727-34.
45. Niederkorn, J.Y., Anterior chamber-associated immune deviation. *Chem Immunol*, 1999. **73**: p. 59-71.
46. Streilein, J.W., Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation. *J Leukoc Biol*, 2003. **74**(2): p. 179-85.
47. Yamagami, S., H. Kawashima, T. Tsuru, H. Yamagami, N. Kayagaki, H. Yagita, K. Okumura, and D.S. Gregerson, Role of Fas-Fas ligand interactions in the immunorejection of allogeneic mouse corneal transplants. *Transplantation*, 1997. **64**(8): p. 1107-11.
48. Williams, K.A., S.D. Standfield, J.R. Smith, and D.J. Coster, Corneal graft rejection occurs despite Fas ligand expression and apoptosis of infiltrating cells. *Br J Ophthalmol*, 2005. **89**(5): p. 632-8.
49. Niederkorn, J.Y., Regulatory T cells and the eye. *Chem Immunol Allergy*, 2007. **92**: p. 131-9.
50. Taylor, A.W. and D.G. Yee, Somatostatin is an immunosuppressive factor in aqueous humor. *Invest Ophthalmol Vis Sci*, 2003. **44**(6): p. 2644-9.
51. Chong, E.M. and M.R. Dana, Graft failure IV. Immunologic mechanisms of corneal transplant rejection. *Int Ophthalmol*, 2008. **28**(3): p. 209-22.
52. Bahn, C.F., R.F. Meyer, D.K. MacCallum, J.H. Lillie, E.J. Lovett, A. Sugar, and C.L. Martonyi, Penetrating keratoplasty in the cat. A clinically applicable model. *Ophthalmology*, 1982. **89**(6): p. 687-99.
53. Williams, K.A., S.D. Standfield, R.A. Mills, T. Takano, D.F. Larkin, R. Krishnan, G.R. Russ, and D.J. Coster, A new model of orthotopic penetrating corneal transplantation in the sheep: graft survival, phenotypes of graft-infiltrating cells and local cytokine production. *Aust N Z J Ophthalmol*, 1999. **27**(2): p. 127-35.
54. Khodadoust, A.A., Penetrating keratoplasty in the rabbit. *Am J Ophthalmol*, 1968. **66**(5): p. 899-905.
55. Williams, K.A. and D.J. Coster, Penetrating corneal transplantation in the inbred rat: a new model. *Invest Ophthalmol Vis Sci*, 1985. **26**(1): p. 23-30.
56. She, S.C., L.P. Steahly, and E.J. Moticka, A method for performing full-thickness, orthotopic, penetrating keratoplasty in the mouse. *Ophthalmic Surg*, 1990. **21**(11): p. 781-5.
57. Coster, D.J., C.F. Jessup, and K.A. Williams, Mechanisms of corneal allograft rejection and regional immunosuppression. *Eye (Lond)*, 2009. **23**(10): p. 1894-7.
58. Niederkorn, J.Y., Immunology and immunomodulation of corneal transplantation. *Int Rev Immunol*, 2002. **21**(2-3): p. 173-96.
59. Pleyer, U., H. Dannowski, H.D. Volk, and T. Ritter, Corneal allograft rejection: current understanding. I. Immunobiology and basic mechanisms. *Ophthalmologica*, 2001. **215**(4): p. 254-62.
60. Banchereau, J. and R.M. Steinman, Dendritic cells and the control of immunity. *Nature*, 1998. **392**(6673): p. 245-52.
61. Niederkorn, J.Y., Immune mechanisms of corneal allograft rejection. *Curr Eye Res*, 2007. **32**(12): p. 1005-16.

62. Hegde, S., C. Beauregard, E. Mayhew, and J.Y. Niederkorn, CD4(+) T-cell-mediated mechanisms of corneal allograft rejection: role of Fas-induced apoptosis. *Transplantation*, 2005. **79**(1): p. 23-31.
63. Slegers, T.P., P.F. Torres, L. Broersma, N. van Rooijen, G. van Rij, and R. van der Gaag, Effect of macrophage depletion on immune effector mechanisms during corneal allograft rejection in rats. *Invest Ophthalmol Vis Sci*, 2000. **41**(8): p. 2239-47.
64. He, Y.G. and J.Y. Niederkorn, Depletion of donor-derived Langerhans cells promotes corneal allograft survival. *Cornea*, 1996. **15**(1): p. 82-9.
65. Dana, M.R., J. Yamada, and J.W. Streilein, Topical interleukin 1 receptor antagonist promotes corneal transplant survival. *Transplantation*, 1997. **63**(10): p. 1501-7.
66. Coster, D.J., C.F. Jessup, B. Biotech, and K.A. Williams, Mechanisms of corneal allograft rejection. *Ocul Surf*, 2005. **3**(4 Suppl): p. S165-8.
67. Nicholls, S.M., B.B. Bradley, and D.L. Easty, Effect of mismatches for major histocompatibility complex and minor antigens on corneal graft rejection. *Invest Ophthalmol Vis Sci*, 1991. **32**(10): p. 2729-34.
68. Vincenti, F., Costimulation blockade in autoimmunity and transplantation. *J Allergy Clin Immunol*, 2008. **121**(2): p. 299-306; quiz 307-8.
69. Larkin, D.F., V.L. Calder, and S.L. Lightman, Identification and characterization of cells infiltrating the graft and aqueous humour in rat corneal allograft rejection. *Clin Exp Immunol*, 1997. **107**(2): p. 381-91.
70. Ayliffe, W., Y. Alam, E.B. Bell, D. McLeod, and I.V. Hutchinson, Prolongation of rat corneal graft survival by treatment with anti-CD4 monoclonal antibody. *Br J Ophthalmol*, 1992. **76**(10): p. 602-6.
71. Yamada, J., I. Kurimoto, and J.W. Streilein, Role of CD4+ T cells in immunobiology of orthotopic corneal transplants in mice. *Invest Ophthalmol Vis Sci*, 1999. **40**(11): p. 2614-21.
72. Niederkorn, J.Y., C. Stevens, J. Mellon, and E. Mayhew, CD4+ T-cell-independent rejection of corneal allografts. *Transplantation*, 2006. **81**(8): p. 1171-8.
73. He, Y.G., J. Ross, and J.Y. Niederkorn, Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. *Invest Ophthalmol Vis Sci*, 1991. **32**(10): p. 2723-8.
74. Hegde, S. and J.Y. Niederkorn, The role of cytotoxic T lymphocytes in corneal allograft rejection. *Invest Ophthalmol Vis Sci*, 2000. **41**(11): p. 3341-7.
75. Camelo, S., A. Shanley, A.S. Voon, and P.G. McMenamin, The distribution of antigen in lymphoid tissues following its injection into the anterior chamber of the rat eye. *J Immunol*, 2004. **172**(9): p. 5388-95.
76. Klebe, S., P. Sykes, D. Coster, Krishnan, and K. Williams, Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. Transplantation 2001; 71: 1214. *Transplantation*, 2001. **71**(9): p. 1207-1209.
77. Comer, R.M., W.J. King, N. Ardjomand, S. Theoharis, A.J. George, and D.F. Larkin, Effect of administration of CTLA4-Ig as protein or cDNA on corneal allograft survival. *Invest Ophthalmol Vis Sci*, 2002. **43**(4): p. 1095-103.
78. Brice, S.L., K. Kirk, H.M. Brereton, D.J. Coster, and K.A. Williams, The influence of cervical and thoracic lymphadenectomy on corneal allograft rejection in inbred rats. *Br J Ophthalmol*, 2012. **96**(3): p. 448-50.

79. Yamagami, S. and M.R. Dana, The critical role of lymph nodes in corneal alloimmunization and graft rejection. *Invest Ophthalmol Vis Sci*, 2001. **42**(6): p. 1293-8.
80. Barcia, R.N., M.R. Dana, and A. Kazlauskas, Corneal graft rejection is accompanied by apoptosis of the endothelium and is prevented by gene therapy with bcl-xL. *Am J Transplant*, 2007. **7**(9): p. 2082-9.
81. Jager, M.J., A. Vos, S. Pasmans, R. Hoekzema, L. Broersma, and R. van der Gaag, Circulating cornea-specific antibodies in corneal disease and cornea transplantation. *Graefes Arch Clin Exp Ophthalmol*, 1994. **232**(2): p. 82-6.
82. Grunnet, N., T. Kristensen, F. Kissmeyer-Nielsen, and N. Ehlers, Occurrence of lymphocytotoxic lymphocytes and antibodies after corneal transplantation. *Acta Ophthalmol (Copenh)*, 1976. **54**(2 p): p. 167-73.
83. Hahn, A.B., G.N. Foulks, C. Enger, N. Fink, W.J. Stark, K.A. Hopkins, and F. Sanfilippo, The association of lymphocytotoxic antibodies with corneal allograft rejection in high risk patients. The Collaborative Corneal Transplantation Studies Research Group. *Transplantation*, 1995. **59**(1): p. 21-7.
84. Hegde, S., J.K. Mellon, S.L. Hargrave, and J.Y. Niederkorn, Effect of alloantibodies on corneal allograft survival. *Invest Ophthalmol Vis Sci*, 2002. **43**(4): p. 1012-8.
85. Cursiefen, C., J. Cao, L. Chen, Y. Liu, K. Maruyama, D. Jackson, F.E. Kruse, S.J. Wiegand, M.R. Dana, and J.W. Streilein, Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. *Invest Ophthalmol Vis Sci*, 2004. **45**(8): p. 2666-73.
86. Bachmann, B.O., F. Bock, S.J. Wiegand, K. Maruyama, M.R. Dana, F.E. Kruse, E. Luetjen-Drecoll, and C. Cursiefen, Promotion of graft survival by vascular endothelial growth factor a neutralization after high-risk corneal transplantation. *Arch Ophthalmol*, 2008. **126**(1): p. 71-7.
87. Murthy, R.C., T.J. McFarland, J. Yoken, S. Chen, C. Barone, D. Burke, Y. Zhang, B. Appukuttan, and J.T. Stout, Corneal transduction to inhibit angiogenesis and graft failure. *Invest Ophthalmol Vis Sci*, 2003. **44**(5): p. 1837-42.
88. Tavandzi, U., R. Prochazka, D. Usvald, J. Hlucilova, M. Vitaskova, J. Motlik, A. Vitova, M. Filipec, J.V. Forrester, and V. Holan, A new model of corneal transplantation in the miniature pig: efficacy of immunosuppressive treatment. *Transplantation*, 2007. **83**(10): p. 1401-3.
89. Funaki, T., N. Ebihara, A. Murakami, and A. Nakao, Ex vivo transfer of Smad7 decreases damage to the corneal endothelium after penetrating keratoplasty. *Jpn J Ophthalmol*, 2008. **52**(3): p. 204-10.
90. Cohen, K.L., N.K. Tripoli, G. Cervantes, and D. Smith, Cat endothelial morphology after corneal transplant. *Curr Eye Res*, 1990. **9**(5): p. 445-50.
91. Williams, K.A., H.M. Brereton, and D.J. Coster, Prospects for genetic modulation of corneal graft survival. *Eye*, 2008. **23**(10): p. 1904-9.
92. Cho, B.J., S.J. Gross, D.R. Pfister, and E.J. Holland, In vivo confocal microscopic analysis of corneal allograft rejection in rabbits. *Cornea*, 1998. **17**(4): p. 417-22.
93. Van Horn, D.L., D.D. Sendele, S. Seideman, and P.J. Buco, Regenerative capacity of the corneal endothelium in rabbit and cat. *Invest Ophthalmol Vis Sci*, 1977. **16**(7): p. 597-613.
94. Coster, D.J. and K.A. Williams, Management of high-risk corneal grafts. *Eye*, 2003. **17**(8): p. 996-1002.

95. Fu, H., D.F. Larkin, and A.J. George, Immune modulation in corneal transplantation. *Transplant Rev (Orlando)*, 2008. **22**(2): p. 105-15.
96. Qian, Y., I. Dekaris, S. Yamagami, and M.R. Dana, Topical soluble tumor necrosis factor receptor type I suppresses ocular chemokine gene expression and rejection of allogeneic corneal transplants. *Arch Ophthalmol*, 2000. **118**(12): p. 1666-71.
97. Yamada, J., M.R. Dana, S.N. Zhu, P. Alard, and J.W. Streilein, Interleukin 1 receptor antagonist suppresses allosensitization in corneal transplantation. *Arch Ophthalmol*, 1998. **116**(10): p. 1351-7.
98. Klebe, S., D.J. Coster, P.J. Sykes, S. Swinburne, P. Hallsworth, J.P. Scheerlinck, R. Krishnan, and K.A. Williams, Prolongation of sheep corneal allograft survival by transfer of the gene encoding ovine IL-12-p40 but not IL-4 to donor corneal endothelium. *J Immunol*, 2005. **175**(4): p. 2219-26.
99. Rayner, S.A., D.F. Larkin, and A.J. George, TNF receptor secretion after ex vivo adenoviral gene transfer to cornea and effect on in vivo graft survival. *Invest Ophthalmol Vis Sci*, 2001. **42**(7): p. 1568-73.
100. Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra, Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 2001. **19**: p. 683-765.
101. Sabat, R., G. Grutz, K. Warszawska, S. Kirsch, E. Witte, K. Wolk, and J. Geginat, Biology of interleukin-10. *Cytokine Growth Factor Rev*, 2010. **21**(5): p. 331-44.
102. Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore, and A. O'Garra, IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol*, 1991. **146**(10): p. 3444-51.
103. Groux, H., M. Bigler, J.E. de Vries, and M.G. Roncarolo, Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J Immunol*, 1998. **160**(7): p. 3188-93.
104. Torres, P.F., A.F. de Vos, B. Martins, and A. Kijlstra, Interleukin 10 treatment does not prolong experimental corneal allograft survival. *Ophthalmic Res*, 1999. **31**(4): p. 297-303.
105. Brandt, C., J. Yang, I. Schmitt-Knosalla, A. Siepert, M.H. Hammer, K. Vogt, B. Sawitzki, M. Lehmann, H.D. Volk, and T. Ritter, Allo-specific T-cells encoding for viral IL-10 exert strong immunomodulatory effects in vitro but fail to prevent graft rejection. *Am J Transplant*, 2005. **5**(2): p. 268-81.
106. Li, W., F. Fu, L. Lu, S.K. Narula, J.J. Fung, A.W. Thomson, and S. Qian, Recipient pretreatment with mammalian IL-10 prolongs mouse cardiac allograft survival by inhibition of anti-donor T cell responses. *Transplant Proc*, 1999. **31**(1-2): p. 115.
107. Beutelspacher, S.C., R. Pillai, M.P. Watson, P.H. Tan, J. Tsang, M.O. McClure, A.J. George, and D.F. Larkin, Function of indoleamine 2,3-dioxygenase in corneal allograft rejection and prolongation of allograft survival by over-expression. *Eur J Immunol*, 2006. **36**(3): p. 690-700.
108. Quan, J., P.H. Tan, A. MacDonald, and P.J. Friend, Manipulation of indoleamine 2,3-dioxygenase (IDO) for clinical transplantation: promises and challenges. *Expert Opin Biol Ther*, 2008. **8**(11): p. 1705-19.
109. Ryu, Y.H. and J.C. Kim, Expression of indoleamine 2,3-dioxygenase in human corneal cells as a local immunosuppressive factor. *Invest Ophthalmol Vis Sci*, 2007. **48**(9): p. 4148-52.
110. Laurence, J.M., C. Wang, M. Zheng, S. Cunningham, J. Earl, S.S. Tay, R.D. Allen, G.W. McCaughan, I.E. Alexander, G.A. Bishop, and A.F. Sharland, Overexpression of indoleamine dioxygenase in rat liver allografts using a

high-efficiency adeno-associated virus vector does not prevent acute rejection. *Liver Transpl*, 2009. **15**(2): p. 233-41.
111. Grohmann, U., F. Fallarino, and P. Puccetti, Tolerance, DCs and tryptophan: much ado about IDO. *Trends Immunol*, 2003. **24**(5): p. 242-8.
112. Frumento, G., R. Rotondo, M. Tonetti, G. Damonte, U. Benatti, and G.B. Ferrara, Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J Exp Med*, 2002. **196**(4): p. 459-68.
113. Terness, P., T.M. Bauer, L. Rose, C. Dufter, A. Watzlik, H. Simon, and G. Opelz, Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med*, 2002. **196**(4): p. 447-57.
114. Serbecic, N., I. Lahdou, A. Scheuerle, R. Hoftberger, and F. Aboul-Enein, Function of the tryptophan metabolite, L-kynurenine, in human corneal endothelial cells. *Mol Vis*, 2009. **15**: p. 1312-24.
115. Zaher, S.S., C. Germain, H. Fu, D.F. Larkin, and A.J. George, 3-hydroxykynurenine suppresses CD4+ T-cell proliferation, induces T-regulatory-cell development, and prolongs corneal allograft survival. *Invest Ophthalmol Vis Sci*, 2011. **52**(5): p. 2640-8.
116. Zaher, S.S., D. Coe, J.G. Chai, D.F. Larkin, and A.J. George, Suppression of the allogeneic response by the anti-allergy drug N-(3,4-dimethoxycinnamonyl) anthranilic acid results from T-cell cycle arrest. *Immunology*, 2013. **138**(2): p. 157-64.
117. Larsen, C.P., S.J. Knechtle, A. Adams, T. Pearson, and A.D. Kirk, A new look at blockade of T-cell costimulation: a therapeutic strategy for long-term maintenance immunosuppression. *Am J Transplant*, 2006. **6**(5 Pt 1): p. 876-83.
118. Pearson, T.C., D.Z. Alexander, K.J. Winn, P.S. Linsley, R.P. Lowry, and C.P. Larsen, Transplantation tolerance induced by CTLA4-Ig. *Transplantation*, 1994. **57**(12): p. 1701-6.
119. Onodera, K., A. Chandraker, M. Schaub, T.H. Stadlbauer, S. Korom, R. Peach, P.S. Linsley, M.H. Sayegh, and J.W. Kupiec-Weglinski, CD28-B7 T cell costimulatory blockade by CTLA4Ig in sensitized rat recipients: induction of transplantation tolerance in association with depressed cell-mediated and humoral immune responses. *J Immunol*, 1997. **159**(4): p. 1711-7.
120. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S. Linsley, and J.A. Bluestone, Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science*, 1992. **257**(5071): p. 789-92.
121. Konig Merediz, S.A., E.P. Zhang, B. Wittig, and F. Hoffmann, Ballistic transfer of minimalistic immunologically defined expression constructs for IL4 and CTLA4 into the corneal epithelium in mice after orthotopic corneal allograft transplantation. *Graefes Arch Clin Exp Ophthalmol*, 2000. **238**(8): p. 701-7.
122. El-Charabaty, E., A.S. Geara, C. Ting, S. El-Sayegh, and J. Azzi, Belatacept: a new era of immunosuppression? *Expert Rev Clin Immunol*, 2012. **8**(6): p. 527-36.
123. Charpentier, B., Belatacept: a novel immunosuppressive agent for kidney transplant recipients. *Expert Rev Clin Immunol*, 2012. **8**(8): p. 719-28.
124. Dastiridou, A., C. Kalogeropoulos, P. Brazitikos, C. Symeonidis, and S. Androudi, New biologic-response modifiers in ocular inflammatory disease:

beyond anti-TNF treatment. *Expert Rev Clin Pharmacol*, 2012. **5**(5): p. 543-55.

125. Zulian, F., M. Balzarin, F. Falcini, G. Martini, M. Alessio, R. Cimaz, L. Cimino, and M.E. Zannin, Abatacept for severe anti-tumor necrosis factor alpha refractory juvenile idiopathic arthritis-related uveitis. *Arthritis Care Res (Hoboken)*, 2010. **62**(6): p. 821-5.
126. Schnitzler, M.A., K.L. Lentine, D. Axelrod, A. Gheorghian, M. You, A. Kalsekar, and G. L'Italien, Use of 12-month renal function and baseline clinical factors to predict long-term graft survival: application to BENEFIT and BENEFIT-EXT trials. *Transplantation*, 2012. **93**(2): p. 172-81.
127. Larsen, C.P., T.C. Pearson, A.B. Adams, P. Tso, N. Shirasugi, E. Strobert, D. Anderson, S. Cowan, K. Price, J. Naemura, J. Emswiler, J. Greene, L.A. Turk, J. Bajorath, R. Townsend, D. Hagerty, P.S. Linsley, and R.J. Peach, Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant*, 2005. **5**(3): p. 443-53.
128. Maddula, S., D.K. Davis, M.K. Burrow, and B.K. Ambati, Horizons in therapy for corneal angiogenesis. *Ophthalmology*, 2011. **118**(3): p. 591-9.
129. Menzel-Severing, J., Emerging techniques to treat corneal neovascularisation. *Eye (Lond)*, 2012. **26**(1): p. 2-12.
130. Pillai, C.T., H.S. Dua, and P. Hossain, Fine needle diathermy occlusion of corneal vessels. *Invest Ophthalmol Vis Sci*, 2000. **41**(8): p. 2148-53.
131. Cao, Y., Molecular mechanisms and therapeutic development of angiogenesis inhibitors. *Adv Cancer Res*, 2008. **100**: p. 113-31.
132. Petsoglou, C., K.S. Balaggan, J.K. Dart, C. Bunce, W. Xing, R.R. Ali, and S.J. Tuft, Subconjunctival bevacizumab induces regression of corneal neovascularisation: a pilot randomised placebo-controlled double-masked trial. *Br J Ophthalmol*, 2013. **97**(1): p. 28-32.
133. Cursiefen, C., F. Bock, F.K. Horn, F.E. Kruse, B. Seitz, V. Borderie, B. Fruh, M.A. Thiel, F. Wilhelm, B. Geudelin, I. Descohand, K.P. Steuhl, A. Hahn, and D. Meller, GS-101 antisense oligonucleotide eye drops inhibit corneal neovascularization: interim results of a randomized phase II trial. *Ophthalmology*, 2009. **116**(9): p. 1630-7.
134. Ambati, B.K., M. Nozaki, N. Singh, A. Takeda, P.D. Jani, T. Suthar, R.J. Albuquerque, E. Richter, E. Sakurai, M.T. Newcomb, M.E. Kleinman, R.B. Caldwell, Q. Lin, Y. Ogura, A. Orecchia, D.A. Samuelson, D.W. Agnew, J. St Leger, W.R. Green, P.J. Mahasreshti, D.T. Curiel, D. Kwan, H. Marsh, S. Ikeda, L.J. Leiper, J.M. Collinson, S. Bogdanovich, T.S. Khurana, M. Shibuya, M.E. Baldwin, N. Ferrara, H.P. Gerber, S. De Falco, J. Witta, J.Z. Baffi, B.J. Raisler, and J. Ambati, Corneal avascularity is due to soluble VEGF receptor-1. *Nature*, 2006. **443**(7114): p. 993-7.
135. Olsson, A.K., A. Dimberg, J. Kreuger, and L. Claesson-Welsh, VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*, 2006. **7**(5): p. 359-71.
136. Otrock, Z.K., J.A. Makarem, and A.I. Shamseddine, Vascular endothelial growth factor family of ligands and receptors: review. *Blood Cells Mol Dis*, 2007. **38**(3): p. 258-68.
137. Kendall, R.L. and K.A. Thomas, Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A*, 1993. **90**(22): p. 10705-9.
138. Lai, C.M., M. Brankov, T. Zaknich, Y.K. Lai, W.Y. Shen, I.J. Constable, I. Kovesdi, and P.E. Rakoczy, Inhibition of angiogenesis by adenovirus-

mediated sFlt-1 expression in a rat model of corneal neovascularization. *Hum Gene Ther*, 2001. **12**(10): p. 1299-310.
139. Iriyama, A., T. Usui, Y. Yanagi, S. Amano, M. Oba, K. Miyata, N. Nishiyama, and K. Kataoka, Gene Transfer Using Micellar Nanovectors Inhibits Corneal Neovascularization In Vivo. *Cornea*, 2012. **30**(12): p. 1423-7.
140. Cho, Y.K., X. Zhang, H. Uehara, J.R. Young, B. Archer, and B. Ambati, Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) Morpholino Increases Graft Survival in a Murine Penetrating Keratoplasty Model. *Invest Ophthalmol Vis Sci*, 2012. **53**(13): p. 8458-71.
141. Touchard, E., M. Berdugo, P. Bigey, M. El Sanharawi, M. Savoldelli, M.C. Naud, J.C. Jeanny, and F. Behar-Cohen, Suprachoroidal electrotransfer: a nonviral gene delivery method to transfect the choroid and the retina without detaching the retina. *Mol Ther*, 2012. **20**(8): p. 1559-70.
142. Maclachlan, T.K., M. Lukason, M. Collins, R. Munger, E. Isenberger, C. Rogers, S. Malatos, E. Dufresne, J. Morris, R. Calcedo, G. Veres, A. Scaria, L. Andrews, and S. Wadsworth, Preclinical safety evaluation of AAV2-sFLT01- a gene therapy for age-related macular degeneration. *Mol Ther*, 2011. **19**(2): p. 326-34.
143. Igarashi, T., K. Miyake, I. Masuda, H. Takahashi, and T. Shimada, Adeno-associated vector (type 8)-mediated expression of soluble Flt-1 efficiently inhibits neovascularization in a murine choroidal neovascularization model. *Hum Gene Ther*, 2010. **21**(5): p. 631-7.
144. Nguyen, T.M., I.V. Subramanian, A. Kelekar, and S. Ramakrishnan, Kringle 5 of human plasminogen, an angiogenesis inhibitor, induces both autophagy and apoptotic death in endothelial cells. *Blood*, 2007. **109**(11): p. 4793-802.
145. Veitonmaki, N., R. Cao, L.H. Wu, T.L. Moser, B. Li, S.V. Pizzo, B. Zhivotovsky, and Y. Cao, Endothelial cell surface ATP synthase-triggered caspase-apoptotic pathway is essential for k1-5-induced antiangiogenesis. *Cancer Res*, 2004. **64**(10): p. 3679-86.
146. O'Reilly, M.S., T. Boehm, Y. Shing, N. Fukai, G. Vasios, W.S. Lane, E. Flynn, J.R. Birkhead, B.R. Olsen, and J. Folkman, Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, 1997. **88**(2): p. 277-85.
147. Ramakrishnan, S., T.M. Nguyen, I.V. Subramanian, and A. Kelekar, Autophagy and angiogenesis inhibition. *Autophagy*, 2007. **3**(5): p. 512-5.
148. Panda, A., M. Vanathi, A. Kumar, Y. Dash, and S. Priya, Corneal graft rejection. *Surv Ophthalmol*, 2007. **52**(4): p. 375-96.
149. Hengartner, M.O., The biochemistry of apoptosis. *Nature*, 2000. **407**(6805): p. 770-6.
150. Yang, J., X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.I. Peng, D.P. Jones, and X. Wang, Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, 1997. **275**(5303): p. 1129-32.
151. Walther, W. and U. Stein, Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs*, 2000. **60**(2): p. 249-71.
152. Bouard, D., N. Alazard-Dany, and F.L. Cosset, Viral vectors: from virology to transgene expression. *Br J Pharmacol*, 2009. **157**(2): p. 153-65.
153. Raty, J.K., H.P. Lesch, T. Wirth, and S. Yla-Herttuala, Improving safety of gene therapy. *Curr Drug Saf*, 2008. **3**(1): p. 46-53.
154. George, A.J., C.V. Arancibia-Carcamo, H.M. Awad, R.M. Comer, Z. Fehevari, W.J. King, M. Kadifachi, T. Hudde, C. Kerouedan-Lebosse, F. Mirza, H. Barbaros Oral, S.A. Rayner, P.H. Tan, E. Tay, and D.F. Larkin,

Gene delivery to the corneal endothelium. *Am J Respir Crit Care Med*, 2000. **162**(4 Pt 2): p. S194-200.
155. Klebe, S., P.J. Sykes, D.J. Coster, D.C. Bloom, and K.A. Williams, Gene transfer to ovine corneal endothelium. *Clin Experiment Ophthalmol*, 2001. **29**(5): p. 316-22.
156. Laurence, J.M., R.D. Allen, G.W. McCaughan, G.J. Logan, I.E. Alexander, G.A. Bishop, and A.F. Sharland, Gene therapy in transplantation. *Transplant Rev (Orlando)*, 2009. **23**(3): p. 159-70.
157. Young, L.S., P.F. Searle, D. Onion, and V. Mautner, Viral gene therapy strategies: from basic science to clinical application. *J Pathol*, 2006. **208**(2): p. 299-318.
158. Kay, M.A., J.C. Glorioso, and L. Naldini, Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med*, 2001. **7**(1): p. 33-40.
159. Muruve, D.A., The innate immune response to adenovirus vectors. *Hum Gene Ther*, 2004. **15**(12): p. 1157-66.
160. Nayak, S. and R.W. Herzog, Progress and prospects: immune responses to viral vectors. *Gene Ther*, 2010. **17**(3): p. 295-304.
161. Zentilin, L. and M. Giacca, Adeno-associated virus vectors: versatile tools for in vivo gene transfer. *Contrib Nephrol*, 2008. **159**: p. 63-77.
162. Thomas, C.E., A. Ehrhardt, and M.A. Kay, Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet*, 2003. **4**(5): p. 346-58.
163. Parker, D.G., H.M. Brereton, D.J. Coster, and K.A. Williams, The potential of viral vector-mediated gene transfer to prolong corneal allograft survival. *Curr Gene Ther*, 2009. **9**(1): p. 33-44.
164. Chirmule, N., K. Propert, S. Magosin, Y. Qian, R. Qian, and J. Wilson, Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther*, 1999. **6**(9): p. 1574-83.
165. Hudde, T., S.A. Rayner, M. De Alwis, A.J. Thrasher, J. Smith, R.S. Coffin, A.J. George, and D.F. Larkin, Adeno-associated and herpes simplex viruses as vectors for gene transfer to the corneal endothelium. *Cornea*, 2000. **19**(3): p. 369-73.
166. Bennett, J., Immune response following intraocular delivery of recombinant viral vectors. *Gene Ther*, 2003. **10**(11): p. 977-82.
167. Bainbridge, J.W., A. Mistry, M. De Alwis, E. Paleolog, A. Baker, A.J. Thrasher, and R.R. Ali, Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. *Gene Ther*, 2002. **9**(5): p. 320-6.
168. Oral, H.B., D.F. Larkin, Z. Fehervari, A.P. Byrnes, A.M. Rankin, D.O. Haskard, M.J. Wood, M.J. Dallman, and A.J. George, Ex vivo adenovirus-mediated gene transfer and immunomodulatory protein production in human cornea. *Gene Ther*, 1997. **4**(7): p. 639-47.
169. Ichinoe, M., T. Mikami, T. Yoshida, I. Igawa, T. Tsuruta, N. Nakada, N. Anzai, Y. Suzuki, H. Endou, and I. Okayasu, High expression of L-type amino-acid transporter 1 (LAT1) in gastric carcinomas: comparison with non-cancerous lesions. *Pathol Int*, 2011. **61**(5): p. 281-9.
170. Hacein-Bey-Abina, S., C. Von Kalle, M. Schmidt, M.P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C.S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J.I. Cohen, G. de Saint Basile, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Sigaux, J. Soulier, L.E. Leiva, M. Wissler, C. Prinz, T.H. Rabbitts, F. Le Deist, A. Fischer, and M. Cavazzana-Calvo, LMO2-associated clonal T cell

proliferation in two patients after gene therapy for SCID-X1. *Science*, 2003. **302**(5644): p. 415-9.

171. Boztug, K., M. Schmidt, A. Schwarzer, P.P. Banerjee, I.A. Diez, R.A. Dewey, M. Bohm, A. Nowrouzi, C.R. Ball, H. Glimm, S. Naundorf, K. Kuhlcke, R. Blasczyk, I. Kondratenko, L. Marodi, J.S. Orange, C. von Kalle, and C. Klein, Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med*, 2010. **363**(20): p. 1918-27.
172. Ott, M.G., M. Schmidt, K. Schwarzwaelder, S. Stein, U. Siler, U. Koehl, H. Glimm, K. Kuhlcke, A. Schilz, H. Kunkel, S. Naundorf, A. Brinkmann, A. Deichmann, M. Fischer, C. Ball, I. Pilz, C. Dunbar, Y. Du, N.A. Jenkins, N.G. Copeland, U. Luthi, M. Hassan, A.J. Thrasher, D. Hoelzer, C. von Kalle, R. Seger, and M. Grez, Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med*, 2006. **12**(4): p. 401-9.
173. Cronin, J., X.Y. Zhang, and J. Reiser, Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther*, 2005. **5**(4): p. 387-98.
174. Weiss, R.A., Retrovirus classification and cell interactions. *J Antimicrob Chemother*, 1996. **37 Suppl B**: p. 1-11.
175. Kafri, T., Gene delivery by lentivirus vectors an overview. *Methods Mol Biol*, 2004. **246**: p. 367-90.
176. Yu, S.F., T. von Ruden, P.W. Kantoff, C. Garber, M. Seiberg, U. Ruther, W.F. Anderson, E.F. Wagner, and E. Gilboa, Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc Natl Acad Sci U S A*, 1986. **83**(10): p. 3194-8.
177. Balaggan, K.S. and R.R. Ali, Ocular gene delivery using lentiviral vectors. *Gene Ther*, 2012. **19**(2): p. 145-53.
178. Parker, D.G., C. Kaufmann, H.M. Brereton, D.S. Anson, L. Francis-Staite, C.F. Jessup, K. Marshall, C. Tan, R. Koldej, D.J. Coster, and K.A. Williams, Lentivirus-mediated gene transfer to the rat, ovine and human cornea. *Gene Ther*, 2007. **14**(9): p. 760-7.
179. Anson, D.S. and M. Fuller, Rational development of a HIV-1 gene therapy vector. *J Gene Med*, 2003. **5**(10): p. 829-38.
180. Case of leukaemia associated with X-linked severe combined immunodeficiency gene therapy trial in London. *Hum Gene Ther*, 2008. **19**(1): p. 3-4.
181. Ginn, S.L., I.E. Alexander, M.L. Edelstein, M.R. Abedi, and J. Wixon, Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med*, 2013. **15**(2): p. 65-77.
182. Marshall, E., Gene therapy death prompts review of adenovirus vector. *Science*, 1999. **286**(5448): p. 2244-5.
183. Thrasher, A.J., S. Hacein-Bey-Abina, H.B. Gaspar, S. Blanche, E.G. Davies, K. Parsley, K. Gilmour, D. King, S. Howe, J. Sinclair, C. Hue, F. Carlier, C. von Kalle, G. de Saint Basile, F. le Deist, A. Fischer, and M. Cavazzana-Calvo, Failure of SCID-X1 gene therapy in older patients. *Blood*, 2005. **105**(11): p. 4255-7.
184. Williams, D.A. and C. Baum, Medicine. Gene therapy--new challenges ahead. *Science*, 2003. **302**(5644): p. 400-1.
185. Hacein-Bey-Abina, S., A. Garrigue, G.P. Wang, J. Soulier, A. Lim, E. Morillon, E. Clappier, L. Caccavelli, E. Delabesse, K. Beldjord, V. Asnafi, E. MacIntyre, L. Dal Cortivo, I. Radford, N. Brousse, F. Sigaux, D. Moshous, J. Hauer, A. Borkhardt, B.H. Belohradsky, U. Wintergerst, M.C. Velez, L. Leiva, R. Sorensen, N. Wulffraat, S. Blanche, F.D. Bushman, A. Fischer, and

M. Cavazzana-Calvo, Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*, 2008. **118**(9): p. 3132-42.
186. Qasim, W., H.B. Gaspar, and A.J. Thrasher, Update on clinical gene therapy in childhood. *Arch Dis Child*, 2007. **92**(11): p. 1028-31.
187. Frank, K.M., D.K. Hogarth, J.L. Miller, S. Mandal, P.J. Mease, R.J. Samulski, G.A. Weisgerber, and J. Hart, Investigation of the cause of death in a gene-therapy trial. *N Engl J Med*, 2009. **361**(2): p. 161-9.
188. Caplan, A.L., If it's broken, shouldn't it be fixed? Informed consent and initial clinical trials of gene therapy. *Hum Gene Ther*, 2008. **19**(1): p. 5-6.
189. Gao, Y., S.S. Ng, D.H. Chau, H. Yao, C. Yang, K. Man, P.T. Huang, C. Huang, J.J. Huang, H.F. Kung, and M.C. Lin, Development of recombinant adeno-associated virus and adenovirus cocktail system for efficient hTERTC27 polypeptide-mediated cancer gene therapy. *Cancer Gene Ther*, 2008. **15**(11): p. 723-32.
190. Limberis, M.P., Phoenix rising: gene therapy makes a comeback. *Acta Biochim Biophys Sin (Shanghai)*, 2012. **44**(8): p. 632-40.
191. Wu, X., Y. Li, B. Crise, and S.M. Burgess, Transcription start regions in the human genome are favored targets for MLV integration. *Science*, 2003. **300**(5626): p. 1749-51.
192. Felice, B., C. Cattoglio, D. Cittaro, A. Testa, A. Miccio, G. Ferrari, L. Luzi, A. Recchia, and F. Mavilio, Transcription factor binding sites are genetic determinants of retroviral integration in the human genome. *PLoS One*, 2009. **4**(2): p. e4571.
193. Hu, J., G. Renaud, T.J. Gomes, A. Ferris, P.C. Hendrie, R.E. Donahue, S.H. Hughes, T.G. Wolfsberg, D.W. Russell, and C.E. Dunbar, Reduced genotoxicity of avian sarcoma leukosis virus vectors in rhesus long-term repopulating cells compared to standard murine retrovirus vectors. *Mol Ther*, 2008. **16**(9): p. 1617-23.
194. Liu, M.M., J. Tuo, and C.C. Chan, Republished review: Gene therapy for ocular diseases. *Postgrad Med J*, 2011. **87**(1029): p. 487-95.
195. Lai, C.M., T. Robertson, J. Papadimitriou, W.Y. Shen, N. Daw, I.J. Constable, and P.E. Rakoczy, Controlled production of active cathepsin D in retinal pigment epithelial cells following adenovirus-mediated gene delivery. *Mol Ther*, 2000. **2**(5): p. 476-84.
196. Lai, Y.K., W.Y. Shen, M. Brankov, C.M. Lai, I.J. Constable, and P.E. Rakoczy, Potential long-term inhibition of ocular neovascularisation by recombinant adeno-associated virus-mediated secretion gene therapy. *Gene Ther*, 2002. **9**(12): p. 804-13.
197. Balaggan, K.S., K. Binley, M. Esapa, S. Iqball, Z. Askham, O. Kan, M. Tschernutter, J.W. Bainbridge, S. Naylor, and R.R. Ali, Stable and efficient intraocular gene transfer using pseudotyped EIAV lentiviral vectors. *J Gene Med*, 2006. **8**(3): p. 275-85.
198. Klausner, E.A., D. Peer, R.L. Chapman, R.F. Multack, and S.V. Andurkar, Corneal gene therapy. *J Control Release*, 2007. **124**(3): p. 107-33.
199. Kampik, D., R.R. Ali, and D.F. Larkin, Experimental gene transfer to the corneal endothelium. *Exp Eye Res*, 2012. **95**(1): p. 54-9.
200. Pleyer, U. and T. Ritter, Gene therapy in immune-mediated diseases of the eye. *Prog Retin Eye Res*, 2003. **22**(3): p. 277-93.
201. Nosov, M., M. Wilk, M. Morcos, M. Cregg, L. O'Flynn, O. Treacy, and T. Ritter, Role of Lentivirus-Mediated Overexpression of Programmed Death-Ligand 1 on Corneal Allograft Survival. *Am J Transplant*, 2012. **12**(5): p. 1313-22.

202. Parker, D.G., D.J. Coster, H.M. Brereton, P.H. Hart, R. Koldej, D.S. Anson, and K.A. Williams, Lentivirus-mediated gene transfer of interleukin 10 to the ovine and human cornea. *Clin Experiment Ophthalmol*, 2010. **38**(4): p. 405-13.
203. Parker, D.G., Lentivirus-mediated gene expression in corneal endothelium, in Department of Ophthalmology. 2008, Flinders University: Adelaide. p. 281.
204. Swaney, W.P., F.L. Sorgi, A.B. Bahnson, and J.A. Barranger, The effect of cationic liposome pretreatment and centrifugation on retrovirus-mediated gene transfer. *Gene Ther*, 1997. **4**(12): p. 1379-86.
205. Gratton, J.P., J. Yu, J.W. Griffith, R.W. Babbitt, R.S. Scotland, R. Hickey, F.J. Giordano, and W.C. Sessa, Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and in vivo. *Nat Med*, 2003. **9**(3): p. 357-62.
206. Toyoshima, K. and P.K. Vogt, Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. *Virology*, 1969. **38**(3): p. 414-26.
207. Wasungu, L. and D. Hoekstra, Cationic lipids, lipoplexes and intracellular delivery of genes. *J Control Release*, 2006. **116**(2): p. 255-64.
208. Mima, H., R. Tomoshige, T. Kanamori, Y. Tabata, S. Yamamoto, S. Ito, K. Tamai, and Y. Kaneda, Biocompatible polymer enhances the in vitro and in vivo transfection efficiency of HVJ envelope vector. *J Gene Med*, 2005. **7**(7): p. 888-97.
209. Davis, H.E., M. Rosinski, J.R. Morgan, and M.L. Yarmush, Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. *Biophys J*, 2004. **86**(2): p. 1234-42.
210. Lehmusvaara, S., O. Rautsi, T. Hakkarainen, and J. Wahifors, Utility of cell-permeable peptides for enhancement of virus-mediated gene transfer to human tumor cells. *Biotechniques*, 2006. **40**(5): p. 573-4, 576.
211. Landazuri, N. and J.M. Le Doux, Complexation of retroviruses with charged polymers enhances gene transfer by increasing the rate that viruses are delivered to cells. *J Gene Med*, 2004. **6**(12): p. 1304-19.
212. Seitz, B., E. Baktanian, E.M. Gordon, W.F. Anderson, L. LaBree, and P.J. McDonnell, Retroviral vector-mediated gene transfer into keratocytes: in vitro effects of polybrene and protamine sulfate. *Graefes Arch Clin Exp Ophthalmol*, 1998. **236**(8): p. 602-12.
213. Le Doux, J.M., N. Landazuri, M.L. Yarmush, and J.R. Morgan, Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer. *Hum Gene Ther*, 2001. **12**(13): p. 1611-21.
214. Davis, H.E., J.R. Morgan, and M.L. Yarmush, Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. *Biophys Chem*, 2002. **97**(2-3): p. 159-72.
215. Cornetta, K. and W.F. Anderson, Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene-transfer: implications for human gene therapy. *J Virol Methods*, 1989. **23**(2): p. 187-94.
216. Lanuti, M., C.E. Kouri, S. Force, M. Chang, K. Amin, K. Xu, I. Blair, L. Kaiser, and S. Albelda, Use of protamine to augment adenovirus-mediated cancer gene therapy. *Gene Ther*, 1999. **6**(9): p. 1600-10.
217. Haas, D.L., S.S. Case, G.M. Crooks, and D.B. Kohn, Critical factors influencing stable transduction of human CD34(+) cells with HIV-1-derived lentiviral vectors. *Mol Ther*, 2000. **2**(1): p. 71-80.
218. Wang, X., B. Appukuttan, S. Ott, R. Patel, J. Irvine, J. Song, J.H. Park, R. Smith, and J.T. Stout, Efficient and sustained transgene expression in human

corneal cells mediated by a lentiviral vector. *Gene Ther*, 2000. **7**(3): p. 196-200.
219. Ehlert, E.M., R. Eggers, S.P. Niclou, and J. Verhaagen, Cellular toxicity following application of adeno-associated viral vector-mediated RNA interference in the nervous system. *BMC Neurosci*, 2010. **11**: p. 20.
220. Mehta, A.K., S.S. Majumdar, P. Alam, N. Gulati, and V. Brahmachari, Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. *Gene*, 2009. **428**(1-2): p. 20-4.
221. Mizuguchi, H., Z. Xu, A. Ishii-Watabe, E. Uchida, and T. Hayakawa, IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol Ther*, 2000. **1**(4): p. 376-82.
222. Chinnasamy, D., M.D. Milsom, J. Shaffer, J. Neuenfeldt, A.F. Shaaban, G.P. Margison, L.J. Fairbairn, and N. Chinnasamy, Multicistronic lentiviral vectors containing the FMDV 2A cleavage factor demonstrate robust expression of encoded genes at limiting MOI. *Virol J*, 2006. **3**: p. 14.
223. Yu, X., X. Zhan, J. D'Costa, V.M. Tanavde, Z. Ye, T. Peng, M.T. Malehorn, X. Yang, C.I. Civin, and L. Cheng, Lentiviral vectors with two independent internal promoters transfer high-level expression of multiple transgenes to human hematopoietic stem-progenitor cells. *Mol Ther*, 2003. **7**(6): p. 827-38.
224. Nie, B., Z. Shen, J.B. Wen, O.G. Wong, W.D. Hsueh, L.F. Huo, H.F. Kung, B. Jiang, and M.C. Lin, AAV-HGFK1 and Ad-p53 cocktail therapy prolongs survival of mice with colon cancer. *Mol Cancer Ther*, 2008. **7**(9): p. 2855-65.
225. Moon, S.H., K. Nishida, L.G. Gilbertson, H.M. Lee, H. Kim, R.A. Hall, P.D. Robbins, and J.D. Kang, Biologic response of human intervertebral disc cells to gene therapy cocktail. *Spine (Phila Pa 1976)*, 2008. **33**(17): p. 1850-5.
226. Fuller, M. and D.S. Anson, Helper plasmids for production of HIV-1-derived vectors. *Hum Gene Ther*, 2001. **12**(17): p. 2081-93.
227. Koldej, R., P. Cmielewski, A. Stocker, D.W. Parsons, and D.S. Anson, Optimisation of a multipartite human immunodeficiency virus based vector system; control of virus infectivity and large-scale production. *J Gene Med*, 2005. **7**(11): p. 1390-9.
228. He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein, A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A*, 1998. **95**(5): p. 2509-14.
229. Kanegae, Y., M. Makimura, and I. Saito, A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol*, 1994. **47**(3): p. 157-66.
230. Nyberg-Hoffman, C., P. Shabram, W. Li, D. Giroux, and E. Aguilar-Cordova, Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat Med*, 1997. **3**(7): p. 808-11.
231. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 2001. **25**(4): p. 402-8.
232. Young, N.S., J. Levin, and R.A. Prendergast, An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. *J Clin Invest*, 1972. **51**(7): p. 1790-7.
233. Meijerink, J., C. Mandigers, L. van de Locht, E. Tonnissen, F. Goodsaid, and J. Raemaekers, A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. *J Mol Diagn*, 2001. **3**(2): p. 55-61.
234. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman, Accurate normalization of real-time quantitative

RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, 2002. **3**(7): p. RESEARCH0034.

235. Nisapakultorn, K., J. Makrudthong, N. Sa-Ard-Iam, P. Rerkyen, R. Mahanonda, and O. Takikawa, Indoleamine 2,3-dioxygenase expression and regulation in chronic periodontitis. *J Periodontol*, 2009. **80**(1): p. 114-21.
236. Williams, K.A. and S. Klebe, Gene therapy for corneal dystrophies and disease, where are we? *Curr Opin Ophthalmol*, 2012. **23**(4): p. 276-9.
237. Hippert, C., S. Ibanes, N. Serratrice, F. Court, F. Malecaze, E.J. Kremer, and V. Kalatzis, Corneal Transduction by Intra-Stromal Injection of AAV Vectors In Vivo in the Mouse and Ex Vivo in Human Explants. *PLoS One*, 2012. **7**(4): p. e35318.
238. Marano, R.J. and P.E. Rakoczy, Controlling vascular endothelial growth factor: therapies for ocular diseases associated with neovascularization. *Adv Exp Med Biol*, 2006. **572**: p. 303-7.
239. Jacobson, S.G., A.V. Cideciyan, R. Ratnakaram, E. Heon, S.B. Schwartz, A.J. Roman, M.C. Peden, T.S. Aleman, S.L. Boye, A. Sumaroka, T.J. Conlon, R. Calcedo, J.J. Pang, K.E. Erger, M.B. Olivares, C.L. Mullins, M. Swider, S. Kaushal, W.J. Feuer, A. Iannaccone, G.A. Fishman, E.M. Stone, B.J. Byrne, and W.W. Hauswirth, Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch Ophthalmol*, 2012. **130**(1): p. 9-24.
240. Brikci-Nigassa, L., M. Matsuyama, T. Hase, A. Eljaafari, J. Chargui, K. Sanhadji, F. Inori, T. Nakatani, R. Yoshimura, and J.L. Touraine, Prope tolerance to heart allografts in mice associated with persistence of donor interleukin-10-transduced stem cells. *Transplantation*, 2012. **93**(8): p. 761-8.
241. Takiishi, T., C.E. Tadokoro, L.V. Rizzo, and L.V. de Moraes, Early IL-10 production is essential for syngeneic graft acceptance. *J Leukoc Biol*, 2012. **92**(2): p. 259-64.
242. McTaggart, S. and M. Al-Rubeai, Retroviral vectors for human gene delivery. *Biotechnol Adv*, 2002. **20**(1): p. 1-31.
243. Young, J.L., J.N. Benoit, and D.A. Dean, Effect of a DNA nuclear targeting sequence on gene transfer and expression of plasmids in the intact vasculature. *Gene Ther*, 2003. **10**(17): p. 1465-70.
244. Kachi, S., N. Esumi, D.J. Zack, and P.A. Campochiaro, Sustained expression after nonviral ocular gene transfer using mammalian promoters. *Gene Ther*, 2006. **13**(9): p. 798-804.
245. Zhou, R. and D.A. Dean, Gene transfer of interleukin 10 to the murine cornea using electroporation. *Exp Biol Med (Maywood)*, 2007. **232**(3): p. 362-9.
246. Gallay, P., S. Swingler, J. Song, F. Bushman, and D. Trono, HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell*, 1995. **83**(4): p. 569-76.
247. von Schwedler, U., R.S. Kornbluth, and D. Trono, The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc Natl Acad Sci U S A*, 1994. **91**(15): p. 6992-6.
248. Schmidt, E.V., G. Christoph, R. Zeller, and P. Leder, The cytomegalovirus enhancer: a pan-active control element in transgenic mice. *Mol Cell Biol*, 1990. **10**(8): p. 4406-11.
249. Iyer, S.R., D. Yu, A. Biancotto, L.B. Margolis, and Y. Wu, Measurement of human immunodeficiency virus type 1 preintegration transcription by using Rev-dependent Rev-CEM cells reveals a sizable transcribing DNA

population comparable to that from proviral templates. *J Virol*, 2009. **83**(17): p. 8662-73.
250. Fleury, S., E. Simeoni, C. Zuppinger, N. Deglon, L.K. von Segesser, L. Kappenberger, and G. Vassalli, Multiply attenuated, self-inactivating lentiviral vectors efficiently deliver and express genes for extended periods of time in adult rat cardiomyocytes in vivo. *Circulation*, 2003. **107**(18): p. 2375-82.
251. Turner, P., A. Petch, and M. Al-Rubeai, Encapsulation of viral vectors for gene therapy applications. *Biotechnol Prog*, 2007. **23**(2): p. 423-9.
252. Bloor, S., J. Maelfait, R. Krumbach, R. Beyaert, and F. Randow, Endoplasmic reticulum chaperone gp96 is essential for infection with vesicular stomatitis virus. *Proc Natl Acad Sci U S A*, 2010. **107**(15): p. 6970-5.
253. Conti, C., P. Mastromarino, A. Riccioli, and N. Orsi, Electrostatic interactions in the early events of VSV infection. *Res Virol*, 1991. **142**(1): p. 17-24.
254. Kustikova, O.S., A. Wahlers, K. Kuhlcke, B. Stahle, A.R. Zander, C. Baum, and B. Fehse, Dose finding with retroviral vectors: correlation of retroviral vector copy numbers in single cells with gene transfer efficiency in a cell population. *Blood*, 2003. **102**(12): p. 3934-7.
255. Anson, D.S., The use of retroviral vectors for gene therapy-what are the risks? A review of retroviral pathogenesis and its relevance to retroviral vector-mediated gene delivery. *Genet Vaccines Ther*, 2004. **2**(1): p. 9.
256. Lech, P. and N.V. Somia, Retrovirus vectors. *Contrib Nephrol*, 2008. **159**: p. 30-46.
257. Schroder, A.R., P. Shinn, H. Chen, C. Berry, J.R. Ecker, and F. Bushman, HIV-1 integration in the human genome favors active genes and local hotspots. *Cell*, 2002. **110**(4): p. 521-9.
258. Mitchell, R.S., B.F. Beitzel, A.R. Schroder, P. Shinn, H. Chen, C.C. Berry, J.R. Ecker, and F.D. Bushman, Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol*, 2004. **2**(8): p. E234.
259. Lewinski, M.K., M. Yamashita, M. Emerman, A. Ciuffi, H. Marshall, G. Crawford, F. Collins, P. Shinn, J. Leipzig, S. Hannenhalli, C.C. Berry, J.R. Ecker, and F.D. Bushman, Retroviral DNA integration: viral and cellular determinants of target-site selection. *PLoS Pathog*, 2006. **2**(6): p. e60.
260. Modlich, U., J. Bohne, M. Schmidt, C. von Kalle, S. Knoss, A. Schambach, and C. Baum, Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity. *Blood*, 2006. **108**(8): p. 2545-53.
261. Cartier, N., S. Hacein-Bey-Abina, C.C. Bartholomae, G. Veres, M. Schmidt, I. Kutschera, M. Vidaud, U. Abel, L. Dal-Cortivo, L. Caccavelli, N. Mahlaoui, V. Kiermer, D. Mittelstaedt, C. Bellesme, N. Lahlou, F. Lefrere, S. Blanche, M. Audit, E. Payen, P. Leboulch, B. l'Homme, P. Bougneres, C. Von Kalle, A. Fischer, M. Cavazzana-Calvo, and P. Aubourg, Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*, 2009. **326**(5954): p. 818-23.
262. Cartier, N. and P. Aubourg, Hematopoietic stem cell transplantation and hematopoietic stem cell gene therapy in X-linked adrenoleukodystrophy. *Brain Pathol*, 2010. **20**(4): p. 857-62.
263. Biffi, A., C.C. Bartolomae, D. Cesana, N. Cartier, P. Aubourg, M. Ranzani, M. Cesani, F. Benedicenti, T. Plati, E. Rubagotti, S. Merella, A. Capotondo, J. Sgualdino, G. Zanetti, C. von Kalle, M. Schmidt, L. Naldini, and E. Montini, Lentiviral vector common integration sites in preclinical models and

a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood*, 2011. **117**(20): p. 5332-9.

264. Baum, C., C. von Kalle, F.J. Staal, Z. Li, B. Fehse, M. Schmidt, F. Weerkamp, S. Karlsson, G. Wagemaker, and D.A. Williams, Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. *Mol Ther*, 2004. **9**(1): p. 5-13.
265. von Kalle, C., B. Fehse, G. Layh-Schmitt, M. Schmidt, P. Kelly, and C. Baum, Stem cell clonality and genotoxicity in hematopoietic cells: gene activation side effects should be avoidable. *Semin Hematol*, 2004. **41**(4): p. 303-18.
266. Nowrouzi, A., H. Glimm, C. von Kalle, and M. Schmidt, Retroviral vectors: post entry events and genomic alterations. *Viruses*, 2011. **3**(5): p. 429-55.
267. Baum, C., O. Kustikova, U. Modlich, Z. Li, and B. Fehse, Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum Gene Ther*, 2006. **17**(3): p. 253-63.
268. Philipson, L., K. Lonberg-Holm, and U. Pettersson, Virus-receptor interaction in an adenovirus system. *J Virol*, 1968. **2**(10): p. 1064-75.
269. Seth, P., D. Fitzgerald, H. Ginsberg, M. Willingham, and I. Pastan, Evidence that the penton base of adenovirus is involved in potentiation of toxicity of Pseudomonas exotoxin conjugated to epidermal growth factor. *Mol Cell Biol*, 1984. **4**(8): p. 1528-33.
270. Meier, O. and U.F. Greber, Adenovirus endocytosis. *J Gene Med*, 2004. **6 Suppl 1**: p. S152-63.
271. Fields' Virology, D.M. Knipe, Howley, P.M., Editor. 2006, Philadelphia : Lippincott Williams & Wilkins.
272. Trotman, L.C., N. Mosberger, M. Fornerod, R.P. Stidwill, and U.F. Greber, Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat Cell Biol*, 2001. **3**(12): p. 1092-100.
273. Frecha, C., J. Szecsi, F.L. Cosset, and E. Verhoeyen, Strategies for targeting lentiviral vectors. *Curr Gene Ther*, 2008. **8**(6): p. 449-60.
274. Seganti, L., F. Superti, C. Girmenia, L. Melucci, and N. Orsi, Study of receptors for vesicular stomatitis virus in vertebrate and invertebrate cells. *Microbiologica*, 1986. **9**(3): p. 259-67.
275. Luo, T., J.L. Douglas, R.L. Livingston, and J.V. Garcia, Infectivity enhancement by HIV-1 Nef is dependent on the pathway of virus entry: implications for HIV-based gene transfer systems. *Virology*, 1998. **241**(2): p. 224-33.
276. Miyauchi, K., Y. Kim, O. Latinovic, V. Morozov, and G.B. Melikyan, HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell*, 2009. **137**(3): p. 433-44.
277. Johannsdottir, H.K., R. Mancini, J. Kartenbeck, L. Amato, and A. Helenius, Host cell factors and functions involved in vesicular stomatitis virus entry. *J Virol*, 2009. **83**(1): p. 440-53.
278. Tang, H., K.L. Kuhen, and F. Wong-Staal, Lentivirus replication and regulation. *Annu Rev Genet*, 1999. **33**: p. 133-70.
279. Goel, M., R.G. Picciani, R.K. Lee, and S.K. Bhattacharya, Aqueous humor dynamics: a review. *Open Ophthalmol J*, 2010. **4**: p. 52-9.
280. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo, A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, 1997. **389**(6652): p. 737-42.
281. Rutella, S., S. Danese, and G. Leone, Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood*, 2006. **108**(5): p. 1435-40.

282. Hamrah, P. and M.R. Dana, Corneal antigen-presenting cells. *Chem Immunol Allergy*, 2007. **92**: p. 58-70.
283. Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and A.H. Enk, Induction of tolerance by IL-10-treated dendritic cells. *J Immunol*, 1997. **159**(10): p. 4772-80.
284. Hattori, T., D.R. Saban, P. Emami-Naeini, S.K. Chauhan, T. Funaki, H. Ueno, and R. Dana, Donor-derived, tolerogenic dendritic cells suppress immune rejection in the indirect allosensitization-dominant setting of corneal transplantation. *J Leukoc Biol*, 2012. **91**(4): p. 621-7.
285. Akbari, O., R.H. DeKruyff, and D.T. Umetsu, Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol*, 2001. **2**(8): p. 725-31.
286. Lutz, M.B. and G. Schuler, Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol*, 2002. **23**(9): p. 445-9.
287. Naldini, L., U. Blomer, F.H. Gage, D. Trono, and I.M. Verma, Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*, 1996. **93**(21): p. 11382-8.
288. Williams, K.A., M. Lowe, C. Bartlett, T.L. Kelly, and D.J. Coster, Risk factors for human corneal graft failure within the Australian corneal graft registry. *Transplantation*, 2008. **86**(12): p. 1720-4.
289. Felizardo, T.C., J.C. Wang, R.A. McGray, C. Evelegh, D.E. Spaner, D.H. Fowler, J.L. Bramson, and J.A. Medin, Differential immune responses mediated by adenovirus- and lentivirus-transduced DCs in a HER-2/neu overexpressing tumor model. *Gene Ther*, 2011. **18**(10): p. 986-95.
290. Auricchio, A., G. Kobinger, V. Anand, M. Hildinger, E. O'Connor, A.M. Maguire, J.M. Wilson, and J. Bennett, Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model. *Hum Mol Genet*, 2001. **10**(26): p. 3075-81.
291. Beutelspacher, S.C., N. Ardjomand, P.H. Tan, G.S. Patton, D.F. Larkin, A.J. George, and M.O. McClure, Comparison of HIV-1 and EIAV-based lentiviral vectors in corneal transduction. *Exp Eye Res*, 2005. **80**(6): p. 787-94.
292. Appleby, S.L., C.F. Jessup, L.A. Mortimer, K. Kirk, H.M. Brereton, D.J. Coster, C.K. Tan, and K.A. Williams, Expression of an anti-CD4 single-chain antibody fragment from the donor cornea can prolong corneal allograft survival in inbred rats. *Br J Ophthalmol*, 2013. **97**(1): p. 101-5.
293. Munn, D.H., E. Shafizadeh, J.T. Attwood, I. Bondarev, A. Pashine, and A.L. Mellor, Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med*, 1999. **189**(9): p. 1363-72.
294. Sagoo, P., G. Chan, D.F. Larkin, and A.J. George, Inflammatory cytokines induce apoptosis of corneal endothelium through nitric oxide. *Invest Ophthalmol Vis Sci*, 2004. **45**(11): p. 3964-73.
295. Cory, S. and J.M. Adams, The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*, 2002. **2**(9): p. 647-56.
296. Kim, R., Unknotting the roles of Bcl-2 and Bcl-xL in cell death. *Biochem Biophys Res Commun*, 2005. **333**(2): p. 336-43.
297. Fuchsluger, T.A., U. Jurkunas, A. Kazlauskas, and R. Dana, Anti-apoptotic gene therapy prolongs survival of corneal endothelial cells during storage. *Gene Ther*, 2011. **18**(8): p. 778-87.

298. Iwamoto, T. and G.K. Smelser, Electron microscope studies on the mast cells and blood and lymphatic capillaries of the human corneal limbus. *Invest Ophthalmol*, 1965. **4**(5): p. 815-34.
299. Philipp, W., L. Speicher, and C. Humpel, Expression of vascular endothelial growth factor and its receptors in inflamed and vascularized human corneas. *Invest Ophthalmol Vis Sci*, 2000. **41**(9): p. 2514-22.
300. Klebe, S., D.J. Coster, and K.A. Williams, Rejection and acceptance of corneal allografts. *Curr Opin Organ Transplant*, 2009. **14**(1): p. 4-9.
301. White, E., A. Bienemann, L. Megraw, C. Bunnun, M. Wyatt, H. Taylor, and S. Gill, Distribution properties of lentiviral vectors administered into the striatum by convection-enhanced delivery. *Hum Gene Ther*, 2012. **23**(1): p. 115-27.
302. Jessup, C.F., H.M. Brereton, D.J. Coster, and K.A. Williams, In vitro adenovirus mediated gene transfer to the human cornea. *Br J Ophthalmol*, 2005. **89**(6): p. 658-61.
303. Sivanandam, V.G., S.L. Stephen, R. Hernandez-Alcoceba, P. Alzuguren, M. Zabala, N. van Rooijen, C. Qian, I. Berger, M.L. Gross, J. Prieto, and S. Kochanek, Lethality in an anti-angiogenic tumor gene therapy model upon constitutive but not inducible expression of the soluble vascular endothelial growth factor receptor 1. *J Gene Med*, 2008. **10**(10): p. 1083-91.
304. Yang, J.W., D.S. Ham, H.W. Kim, S.G. Lee, S.K. Park, and S.K. Seo, Expression of Stat3 and indoleamine 2, 3-dioxygenase in cornea keratocytes as factor of ocular immune privilege. *Graefes Arch Clin Exp Ophthalmol*, 2012. **250**(1): p. 25-31.
305. Jakobiec, F.A. and P. Bhat, Retrocorneal membranes: a comparative immunohistochemical analysis of keratocytic, endothelial, and epithelial origins. *Am J Ophthalmol*, 2010. **150**(2): p. 230-242 e2.
306. Buchen, S.Y., D. Calogero, G. Hilmantel, and M.B. Eydelman, Rabbit ocular reactivity to bacterial endotoxin contained in aqueous solution and ophthalmic viscosurgical devices. *Ophthalmology*, 2012. **119**(7): p. e4-e10.
307. US Food and Drug Administration. *Recognized Consensus Standards*. Publication date 2011, site last updated 20/08/2012, accessed 18/03/2013. Available http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfstandards/detail.cfm?standard__identification_no=29285.
308. Kutty, P.K., T.S. Forster, C. Wood-Koob, N. Thayer, R.B. Nelson, S.J. Berke, L. Pontacolone, T.L. Beardsley, H.F. Edelhauser, M.J. Arduino, N. Mamalis, and A. Srinivasan, Multistate outbreak of toxic anterior segment syndrome, 2005. *J Cataract Refract Surg*, 2008. **34**(4): p. 585-90.
309. Sakimoto, A., M. Sawa, T. Oshida, S. Sugaya, T. Hirono, and A. Ishimori, Minimum endotoxin concentration causing inflammation in the anterior segment of rabbit eyes. *Jpn J Ophthalmol*, 2009. **53**(4): p. 425-32.
310. Pillai, R.G., S.C. Beutelspacher, D.F. Larkin, and A.J. George, Upregulation of chemokine expression in murine cornea due to mechanical trauma or endotoxin. *Br J Ophthalmol*, 2008. **92**(2): p. 259-64.
311. Curtin, J.A., A.P. Dane, A. Swanson, I.E. Alexander, and S.L. Ginn, Bidirectional promoter interference between two widely used internal heterologous promoters in a late-generation lentiviral construct. *Gene Ther*, 2008. **15**(5): p. 384-90.
312. Sadaie, M.R., M. Zamani, S. Whang, N. Sistron, and S.K. Arya, Towards developing HIV-2 lentivirus-based retroviral vectors for gene therapy: dual

gene expression in the context of HIV-2 LTR and Tat. *J Med Virol*, 1998. **54**(2): p. 118-28.
313. Dutia, B.M., P. Hunt, D.R. Sargan, R.G. Dalziel, and J. Hopkins, Sequence of the sheep interleukin-10-encoding cDNA. *Gene*, 1994. **149**(2): p. 393-4.
314. Li, L., J.F. Elliott, and T.R. Mosmann, IL-10 inhibits cytokine production, vascular leakage, and swelling during T helper 1 cell-induced delayed-type hypersensitivity. *J Immunol*, 1994. **153**(9): p. 3967-78.
315. Martin, H.M., A.D. Nash, and A.E. Andrews, Cloning and characterisation of an ovine interleukin-10-encoding cDNA. *Gene*, 1995. **159**(2): p. 187-91.
316. Jang, J.H. and Y.J. Surh, Possible role of NF-kappaB in Bcl-X(L) protection against hydrogen peroxide-induced PC12 cell death. *Redox Rep*, 2004. **9**(6): p. 343-8.
317. Li, B., Q. Hu, R. Xu, H. Ren, E. Fei, D. Chen, and G. Wang, Hax-1 is rapidly degraded by the proteasome dependent on its PEST sequence. *BMC Cell Biol*, 2012. **13**: p. 20.
318. Gasteiger E., H.C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A., Protein Identification and Analysis Tools on the ExPASy Server. The Proteomics Protocols Handbook, ed. J.M. Walker. 2005: Humana Press.
319. Fannon, M., K. Forsten-Williams, B. Zhao, E. Bach, P.P. Parekh, C.L. Chu, A.L. Goerges-Wildt, J.A. Buczek-Thomas, and M.A. Nugent, Facilitated diffusion of VEGF165 through descemet's membrane with sucrose octasulfate. *J Cell Physiol*, 2012. **227**(11): p. 3693-700.
320. Chen, Z., H. Mok, S.C. Pflugfelder, D.Q. Li, and M.A. Barry, Improved transduction of human corneal epithelial progenitor cells with cell-targeting adenoviral vectors. *Exp Eye Res*, 2006. **83**(4): p. 798-806.
321. Igarashi, T., K. Miyake, N. Suzuki, K. Kato, H. Takahashi, K. Ohara, and T. Shimada, New strategy for in vivo transgene expression in corneal epithelial progenitor cells. *Curr Eye Res*, 2002. **24**(1): p. 46-50.
322. Flynn, T.H., N.A. Mitchison, S.J. Ono, and D.F. Larkin, Aqueous humor alloreactive cell phenotypes, cytokines and chemokines in corneal allograft rejection. *Am J Transplant*, 2008. **8**(7): p. 1537-43.
323. Wang, F.H., M. Chen, T. Liu, X.J. Zang, H.Q. Gong, and W.Y. Shi, Lymphocyte infiltration and activation in iris-ciliary body and anterior chamber of mice in corneal allograft rejection. *Int J Ophthalmol*, 2012. **5**(6): p. 681-6.
324. Faraj, L.A., K. Hashmani, T. Khatib, M. Al-Aqaba, and H.S. Dua, The changing face of corneal graft rejection. *Br J Ophthalmol*, 2012. **96**(8): p. 1049-50.
325. Urtti, A., Challenges and obstacles of ocular pharmacokinetics and drug delivery. *Adv Drug Deliv Rev*, 2006. **58**(11): p. 1131-5.
326. Ergun, S., N. Kilic, J.H. Wurmbach, A. Ebrahimnejad, M. Fernando, S. Sevinc, E. Kilic, F. Chalajour, W. Fiedler, H. Lauke, K. Lamszus, P. Hammerer, J. Weil, H. Herbst, and J. Folkman, Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes. *Angiogenesis*, 2001. **4**(3): p. 193-206.
327. Banerjee, S., F.C. Figueiredo, D.L. Easty, A.D. Dick, and S.M. Nicholls, Development of organised conjunctival leucocyte aggregates after corneal transplantation in rats. *Br J Ophthalmol*, 2003. **87**(12): p. 1515-22.
328. Yu, W.Y., C. Sheridan, I. Grierson, S. Mason, V. Kearns, A.C. Lo, and D. Wong, Progenitors for the corneal endothelium and trabecular meshwork: a potential source for personalized stem cell therapy in corneal endothelial diseases and glaucoma. *J Biomed Biotechnol*, 2011. **2011**: p. 412743.

329. Mueller, C. and T.R. Flotte, Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther*, 2008. **15**(11): p. 858-63.
330. Xiao, P.J., T.B. Lentz, and R.J. Samulski, Recombinant adeno-associated virus: clinical application and development as a gene-therapy vector. *Ther Deliv*, 2012. **3**(7): p. 835-56.
331. Stone, E.M., Leber congenital amaurosis - a model for efficient genetic testing of heterogeneous disorders: LXIV Edward Jackson Memorial Lecture. *Am J Ophthalmol*, 2007. **144**(6): p. 791-811.
332. Maguire, A.M., F. Simonelli, E.A. Pierce, E.N. Pugh, Jr., F. Mingozzi, J. Bennicelli, S. Banfi, K.A. Marshall, F. Testa, E.M. Surace, S. Rossi, A. Lyubarsky, V.R. Arruda, B. Konkle, E. Stone, J. Sun, J. Jacobs, L. Dell'Osso, R. Hertle, J.X. Ma, T.M. Redmond, X. Zhu, B. Hauck, O. Zelenaia, K.S. Shindler, M.G. Maguire, J.F. Wright, N.J. Volpe, J.W. McDonnell, A. Auricchio, K.A. High, and J. Bennett, Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med*, 2008. **358**(21): p. 2240-8.
333. Simonelli, F., A.M. Maguire, F. Testa, E.A. Pierce, F. Mingozzi, J.L. Bennicelli, S. Rossi, K. Marshall, S. Banfi, E.M. Surace, J. Sun, T.M. Redmond, X. Zhu, K.S. Shindler, G.S. Ying, C. Ziviello, C. Acerra, J.F. Wright, J.W. McDonnell, K.A. High, J. Bennett, and A. Auricchio, Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. *Mol Ther*, 2010. **18**(3): p. 643-50.
334. Ashtari, M., L.L. Cyckowski, J.F. Monroe, K.A. Marshall, D.C. Chung, A. Auricchio, F. Simonelli, B.P. Leroy, A.M. Maguire, K.S. Shindler, and J. Bennett, The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest*, 2011. **121**(6): p. 2160-8.
335. Cideciyan, A.V., S.G. Jacobson, W.A. Beltran, A. Sumaroka, M. Swider, S. Iwabe, A.J. Roman, M.B. Olivares, S.B. Schwartz, A.M. Komaromy, W.W. Hauswirth, and G.D. Aguirre, Human retinal gene therapy for Leber congenital amaurosis shows advancing retinal degeneration despite enduring visual improvement. *Proc Natl Acad Sci U S A*, 2013. **110**(6): p. E517-25.
336. Cepko, C. and L.H. Vandenberghe, Retinal Gene Therapy Coming of Age. *Hum Gene Ther*, 2013. **24**(3): p. 242-4.
337. Lai, C.M., M.J. Estcourt, R.P. Himbeck, S.Y. Lee, I. Yew-San Yeo, C. Luu, B.K. Loh, M.W. Lee, A. Barathi, J. Villano, C.L. Ang, R.G. van der Most, I.J. Constable, D. Dismuke, R.J. Samulski, M.A. Degli-Esposti, and E.P. Rakoczy, Preclinical safety evaluation of subretinal AAV2.sFlt-1 in non-human primates. *Gene Ther*, 2012. **19**(10): p. 999-1009.
338. Kinnunen, K. and S. Yla-Herttuala, Gene therapy in age related macular degeneration and hereditary macular disorders. *Front Biosci (Elite Ed)*, 2012. **4**: p. 2546-57.
339. Buie, L.K., C.A. Rasmussen, E.C. Porterfield, V.S. Ramgolam, V.W. Choi, S. Markovic-Plese, R.J. Samulski, P.L. Kaufman, and T. Borras, Self-complementary AAV virus (scAAV) safe and long-term gene transfer in the trabecular meshwork of living rats and monkeys. *Invest Ophthalmol Vis Sci*, 2010. **51**(1): p. 236-48.
340. McCarty, D.M., H. Fu, P.E. Monahan, C.E. Toulson, P. Naik, and R.J. Samulski, Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. *Gene Ther*, 2003. **10**(26): p. 2112-8.
341. Borras, T., W. Xue, V.W. Choi, J.S. Bartlett, G. Li, R.J. Samulski, and S.S. Chisolm, Mechanisms of AAV transduction in glaucoma-associated human trabecular meshwork cells. *J Gene Med*, 2006. **8**(5): p. 589-602.

342. Buss, D.G., E. Giuliano, A. Sharma, and R.R. Mohan, Gene delivery in the equine cornea: a novel therapeutic strategy. *Vet Ophthalmol*, 2010. **13**(5): p. 301-6.
343. Bosiack, A.P., E.A. Giuliano, R. Gupta, and R.R. Mohan, Canine corneal fibroblast and myofibroblast transduction with AAV5. *Vet Ophthalmol*, 2012. **15**(5): p. 291-8.
344. Mohan, R.R., S. Sinha, A. Tandon, R. Gupta, J.C. Tovey, and A. Sharma, Efficacious and safe tissue-selective controlled gene therapy approaches for the cornea. *PLoS One*, 2011. **6**(4): p. e18771.
345. Sharma, A., J.C. Tovey, A. Ghosh, and R.R. Mohan, AAV serotype influences gene transfer in corneal stroma in vivo. *Exp Eye Res*, 2010. **91**(3): p. 440-8.
346. Dohlman, C.H., The function of the corneal epithelium in health and disease. The Jonas S. Friedenwald Memorial Lecture. *Invest Ophthalmol*, 1971. **10**(6): p. 383-407.
347. Whitemore, M., S. Li, and L. Huang, Liposome vectors for in vivo gene delivery. *Curr Protoc Hum Genet*, 2001. **Chapter 12**: p. Unit 12 8.
348. Collins, L. and J.W. Fabre, A synthetic peptide vector system for optimal gene delivery to corneal endothelium. *J Gene Med*, 2004. **6**(2): p. 185-94.
349. Roan, N.R., J. Munch, N. Arhel, W. Mothes, J. Neidleman, A. Kobayashi, K. Smith-McCune, F. Kirchhoff, and W.C. Greene, The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. *J Virol*, 2009. **83**(1): p. 73-80.
350. Nyberg, P., L. Xie, and R. Kalluri, Endogenous inhibitors of angiogenesis. *Cancer Res*, 2005. **65**(10): p. 3967-79.
351. Wurm, M., A. Schambach, D. Lindemann, H. Hanenberg, L. Standker, W.G. Forssmann, R. Blasczyk, and P.A. Horn, The influence of semen-derived enhancer of virus infection on the efficiency of retroviral gene transfer. *J Gene Med*, 2010. **12**(2): p. 137-46.
352. Karmarkar, A.B. (2008) Poloxamers and their applications. *Pharmacy Student Articles* **http://www.pharmainfo.net, accessed 25th November 2012.**
353. Karleta, V., I. Andrlik, S. Braunmuller, T. Franke, M. Wirth, and F. Gabor, Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles. *ALTEX*, 2010. **27**(3): p. 191-7.
354. Singh-Joy, S.D. and V.C. McLain, Safety assessment of poloxamers 101, 105, 108, 122, 123, 124, 181, 182, 183, 184, 185, 188, 212, 215, 217, 231, 234, 235, 237, 238, 282, 284, 288, 331, 333, 334, 335, 338, 401, 402, 403, and 407, poloxamer 105 benzoate, and poloxamer 182 dibenzoate as used in cosmetics. *Int J Toxicol*, 2008. **27 Suppl 2**: p. 93-128.
355. Hofig, I., M.J. Atkinson, S. Mall, A.M. Krackhardt, C. Thirion, and N. Anastasov, Poloxamer synperonic F108 improves cellular transduction with lentiviral vectors. *J Gene Med*, 2012. **14**(8): p. 549-60.
356. Steinhardt, R.A. and J.M. Alderton, Poloxamer 188 enhances endothelial cell survival in bovine corneas in cold storage. *Cornea*, 2006. **25**(7): p. 839-44.
357. Zhao, M., G. Thuret, S. Piselli, A. Pipparelli, S. Acquart, M. Peoc'h, J.M. Dumollard, and P. Gain, Use of poloxamers for deswelling of organ-cultured corneas. *Invest Ophthalmol Vis Sci*, 2008. **49**(2): p. 550-9.
358. Ge, H.Y., N. Xiao, X.L. Yin, S.B. Fu, J.Y. Ge, Y. Shi, and P. Liu, Comparison of the antiangiogenic activity of modified RGDRGD-endostatin

to endostatin delivered by gene transfer in vivo rabbit neovascularization model. *Mol Vis*, 2011. **17**: p. 1918-28.

359. Tan, Y., F. Cruz-Guilloty, C.A. Medina-Mendez, N.J. Cutrufello, R.E. Martinez, M. Urbieta, D. Wilson, Y. Li, and V.L. Perez, Immunological disruption of antiangiogenic signals by recruited allospecific T cells leads to corneal allograft rejection. *J Immunol*, 2012. **188**(12): p. 5962-9.
360. Li, C., L. Li, R. Cheng, Z. Dai, Y. Yao, T. Zhou, Z. Yang, G. Gao, and X. Yang, Acidic/Neutral Amino Acid Residues Substitution in NH2 Terminal of Plasminogen Kringle 5 Exerts Enhanced Effects on Corneal Neovascularization. *Cornea*, 2013. **32**(5): p. 680-8.
361. Jessup, C.F., H.M. Brereton, P.J. Sykes, M.A. Thiel, D.J. Coster, and K.A. Williams, Local gene transfer to modulate rat corneal allograft rejection. *Invest Ophthalmol Vis Sci*, 2005. **46**(5): p. 1675-81.
362. Nguyen, N.X., B. Seitz, P. Martus, A. Langenbucher, and C. Cursiefen, Long-term topical steroid treatment improves graft survival following normal-risk penetrating keratoplasty. *Am J Ophthalmol*, 2007. **144**(2): p. 318-9.
363. Shimazaki, J., A. Iseda, Y. Satake, and S. Shimazaki-Den, Efficacy and safety of long-term corticosteroid eye drops after penetrating keratoplasty: a prospective, randomized, clinical trial. *Ophthalmology*, 2012. **119**(4): p. 668-73.
364. Hudde, T., D.C. Minassian, and D.F. Larkin, Randomised controlled trial of corticosteroid regimens in endothelial corneal allograft rejection. *Br J Ophthalmol*, 1999. **83**(12): p. 1348-52.
365. Liu, X., X.P. Li, Y. Peng, S.S. Ng, H. Yao, Z.F. Wang, X.M. Wang, H.F. Kung, and M.C. Lin, Suppressing tumor growth of nasopharyngeal carcinoma by hTERTC27 polypeptide delivered through adeno-associated virus plus adenovirus vector cocktail. *Chin J Cancer*, 2012. **31**(12): p. 588-97.
366. Bollard, C.M., K.C. Straathof, M.H. Huls, A. Leen, K. Lacuesta, A. Davis, S. Gottschalk, M.K. Brenner, H.E. Heslop, and C.M. Rooney, The generation and characterization of LMP2-specific CTLs for use as adoptive transfer from patients with relapsed EBV-positive Hodgkin disease. *J Immunother*, 2004. **27**(4): p. 317-27.
367. Bollard, C.M., S. Gottschalk, A.M. Leen, H. Weiss, K.C. Straathof, G. Carrum, M. Khalil, M.F. Wu, M.H. Huls, C.C. Chang, M.V. Gresik, A.P. Gee, M.K. Brenner, C.M. Rooney, and H.E. Heslop, Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood*, 2007. **110**(8): p. 2838-45.
368. de Felipe, P., L.E. Hughes, M.D. Ryan, and J.D. Brown, Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. *J Biol Chem*, 2003. **278**(13): p. 11441-8.
369. Verrier, J.D., I. Madorsky, W.E. Coggin, M. Geesey, M. Hochman, E. Walling, D. Daroszewski, K.S. Eccles, R. Ludlow, and S.L. Semple-Rowland, Bicistronic lentiviruses containing a viral 2A cleavage sequence reliably co-express two proteins and restore vision to an animal model of LCA1. *PLoS One*, 2011. **6**(5): p. e20553.
370. Hacein-Bey-Abina, S., J. Hauer, A. Lim, C. Picard, G.P. Wang, C.C. Berry, C. Martinache, F. Rieux-Laucat, S. Latour, B.H. Belohradsky, L. Leiva, R. Sorensen, M. Debre, J.L. Casanova, S. Blanche, A. Durandy, F.D. Bushman, A. Fischer, and M. Cavazzana-Calvo, Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*, 2010. **363**(4): p. 355-64.

371. Aiuti, A., F. Cattaneo, S. Galimberti, U. Benninghoff, B. Cassani, L. Callegaro, S. Scaramuzza, G. Andolfi, M. Mirolo, I. Brigida, A. Tabucchi, F. Carlucci, M. Eibl, M. Aker, S. Slavin, H. Al-Mousa, A. Al Ghonaium, A. Ferster, A. Duppenthaler, L. Notarangelo, U. Wintergerst, R.H. Buckley, M. Bregni, S. Marktel, M.G. Valsecchi, P. Rossi, F. Ciceri, R. Miniero, C. Bordignon, and M.G. Roncarolo, Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*, 2009. **360**(5): p. 447-58.
372. Trobridge, G.D., Genotoxicity of retroviral hematopoietic stem cell gene therapy. *Expert Opin Biol Ther*, 2011. **11**(5): p. 581-93.
373. Montini, E., D. Cesana, M. Schmidt, F. Sanvito, C.C. Bartholomae, M. Ranzani, F. Benedicenti, L.S. Sergi, A. Ambrosi, M. Ponzoni, C. Doglioni, C. Di Serio, C. von Kalle, and L. Naldini, The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J Clin Invest*, 2009. **119**(4): p. 964-75.
374. Zhou, S., D. Mody, S.S. DeRavin, J. Hauer, T. Lu, Z. Ma, S. Hacein-Bey Abina, J.T. Gray, M.R. Greene, M. Cavazzana-Calvo, H.L. Malech, and B.P. Sorrentino, A self-inactivating lentiviral vector for SCID-X1 gene therapy that does not activate LMO2 expression in human T cells. *Blood*, 2010. **116**(6): p. 900-8.
375. Heckl, D., A. Schwarzer, R. Haemmerle, D. Steinemann, C. Rudolph, B. Skawran, S. Knoess, J. Krause, Z. Li, B. Schlegelberger, C. Baum, and U. Modlich, Lentiviral vector induced insertional haploinsufficiency of Ebf1 causes murine leukemia. *Mol Ther*, 2012. **20**(6): p. 1187-95.
376. Sambrook, J.F., Fritsch, E.F., Maniatis, T., Molecular cloning: a laboratory manual/. J Sambrook, E.F. Fritsch, T. Maniatis. 2nd ed. 1989, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.