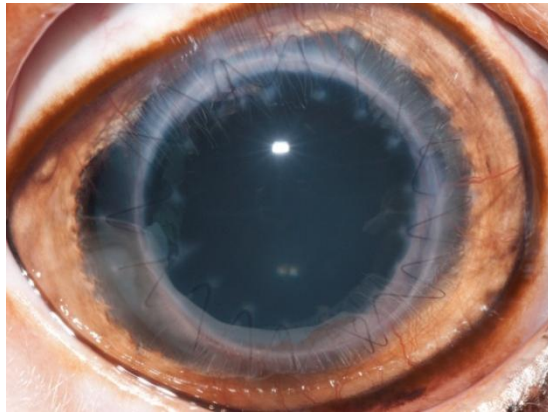


**GENE THERAPY OF THE SHEEP CORNEA
FOR THE PROLONGATION OF
CORNEAL GRAFT SURVIVAL**



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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

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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

\geq	greater than or equal to
\leq	less than or equal to
$^{\circ}\text{C}$	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	diethylpyrocarbonate

dH ₂ O	distilled water
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulphoxide
DNA	dexoyribonucleic acid
dNTP	deoxynucleotide phosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
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
μ g	microgram (10^{-6})
MHC	major histocompatibility complex
MLV	Molony murine leukaemia virus
μ L	microlitre (10^{-3})
μ M	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