GENE THERAPY OF THE SHEEP CORNEA

FOR THE PROLONGATION OF

CORNEAL GRAFT SURVIVAL

Alison Jayne Clarke
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Faculty of Health Sciences
School of Medicine
Flinders University of South Australia
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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≤ 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≤ 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

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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**

≥ greater than or equal to

≤ less than or equal to

°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

CO₂ carbon dioxide

CPPT central polypurine tract

Ct threshold cycle

CTL cytotoxic lymphocyte

CTLA-4 cytotoxic lymphocyte antigen-4

Da Dalton

DEPC diethylyrocarbonate
dH₂O  distilled water
DMEM  Dulbecco's modified essential medium
DMSO  dimethyl sulphoxide
DNA   deoxyribonucleic 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
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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin-10</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton (10³ Da)</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>log</td>
<td>logarithm</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeats</td>
</tr>
<tr>
<td>LV</td>
<td>lentivirus</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>µg</td>
<td>microgram (10⁶)</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLV</td>
<td>Molony murine leukaemia virus</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre (10⁻³)</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar (10⁻⁶)</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>multiplicity of infection</td>
</tr>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
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<tr>
<td>NEB</td>
<td>New England Biolabs</td>
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<tr>
<td>NTC</td>
<td>no template controls</td>
</tr>
<tr>
<td>OCT compound</td>
<td>optimal cutting temperature compound</td>
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<td>Term</td>
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<td>------------</td>
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</tr>
<tr>
<td>oBcl-xL</td>
<td>ovine anti-apoptosis factor of Bcl2 family</td>
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<tr>
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<td>plasmid...ABC</td>
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<tr>
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<td>plaque forming units</td>
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<td>quantitative reverse transcription real time PCR</td>
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<tr>
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<td>rev response element</td>
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<td>reverse transcription</td>
</tr>
<tr>
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<td>sFlt-1</td>
<td>soluble fms-like tyrosine kinase 1 (soluble VEGF receptor 1)</td>
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<tr>
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</tr>
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<tr>
<td>VEGF</td>
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