Gene and Stem Cell Therapy for Type 1 Diabetes Mellitus

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Certificate of Authorship and Originality

I certify that this thesis has not been previously submitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This research was supported by an Australian Government Research Training Program Scholarship.

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Abstract

Type 1 diabetes (T1D) results from the autoimmune destruction of the insulin-producing pancreatic β-cells. As a result, people with T1D suffer from high blood glucose which requires exogenous insulin therapy to maintain within the normal physiological range. However, exogenous insulin therapy does not mimic the tightly regulated function of the pancreas, and as a result does not prevent the development of diabetic complications. Currently, the only cure is either pancreas or islet transplantation; however these treatments are hampered by a shortage of donors. As a result, the generation of an alternative cell replacement therapy would overcome the aforementioned limitations with current treatments. Gene therapy as a means of generating "artificial" insulin-producing cells (IPCs) is being considered as a potential cure for T1D. Previous research has shown that the viral-mediated transfer of the pancreatic transcription factor *NeuroD1* and human furin-cleavable insulin (*INS-FUR*) genes to hepatocytes resulted in their transdifferentiation into pancreatic-like cells capable of synthesising, storing and secreting insulin in response to fluctuating glucose concentrations. Due to their ease of isolation, ease of genetic modification and immune-modulatory properties, the aim of this study was to evaluate the utility of *ex vivo* expanded murine bone marrow-derived mesenchymal stem cells (BMSCs) as gene therapy targets for the development of a T1D cell replacement therapy following the lentiviral over-expression of murine *NeuroD1* and *Pdx1*, and *INS-FUR*.

Non-invasive bioluminescence imaging (BLI) technology is an established and sensitive tool for accessing cell replacement therapy efficacy and treatment outcome in living preclinical small animal models. Furthermore, preclinical BLI results often serve as the decision point of

a cell replacement therapy's suitability (efficacy and safety) for clinical trial testing in humans. This study utilised the *Firefly luciferase* reporter protein *Luc2*, a *Luc2* specific light producing substrate D-luciferin and an IVIS Lumina II imaging system. First, a unique sub-population of BMSCs were isolated from the bone marrow of non-obese diabetic (NOD) mice. These BMSCs displayed potent clonogenicity and tri-lineage differentiation potential, hallmark characteristics of mesenchymal stem cells, when compared to the plastic adherent bone marrow stromal cell starting population. Second, BMSCs were nucleofected to express the yeast fusion cytosine deaminase uracil phosphoribosyltransferase (*CDUPRT*) and/or *Luc2* genes (BMSC-*Luc2/CDUPRT*; BMSC-*Luc2*). *Luc2* was utilised as a reporter protein for evaluating the *in vitro* and *in vivo* persistence of transgene expression in BMSCs and the *in vivo* persistence of gene-modified BMSCs in immune intact and immune-compromised mice. *CDUPRT* is a pro-drug converting enzyme, otherwise known as a suicide gene that was utilised as a cell therapy experimental 'off' switch. *CDUPRT* converts the non-toxic pro-drug 5-fluorocytosine (5-FC) into the toxic metabolite 5-fluorouracil (5-FU) that causes BMSC death. *In vitro* functional characterisation of *CDUPRT* using a *Luc2* based cytotoxicity assay showed that following exposure to 5-FC, BMSC-*Luc2/CDUPRT* demonstrated increased cell death when compared to BMSC-*Luc2* and parental BMSC controls. A subcutaneous transplant of *Luc2/CDUPRT*-expressing BMSCs in immune-intact (NOD; n=4) and immune-deficient (NOD/*Scid*; n=4) mice persisted for 2 weeks and 12 weeks respectively. These results show a BMSC transplant survival providing an experimental window of 12 weeks in NOD/*Scid* mice and the rapid immune-mediated destruction of BMSC carrying non-mammalian genes in NOD mice.

Ex vivo culture-expanded BMSCs were subsequently transduced with the HMD lentiviral vector (MOI=10) to express *INS-FUR*, and murine *NeuroD1* and *Pdx1* as mediators of β-cell differentiation. Pancreatic transdifferentiation was characterised via reverse transcriptase

polymerase chain reaction (RT-PCR), followed by the assessment of insulin storage and secretion. *INS-FUR*-expressing BMSCs lacked glucose-responsiveness and secreted large amounts of human insulin chronically, whereas *NeuroD1* and *Pdx1*-expressing BMSCs lacked glucose-responsiveness and insulin secretion capabilities. Furthermore, RT-PCR analysis showed that BMSC did not undergo pancreatic transdifferentiation when transduced with pancreatic transcription factors, and did not store insulin within secretory granules as determined by acid/ethanol extraction. A subcutaneous transplant of $1x10^7$ and $5x10^7$ *INS-FUR*-expressing BMSCs were assessed for their ability to reverse diabetes in STZ-NOD/*Scid* mice (n=5), which failed to do so upon transplantation.

This study showed *ex vivo* expanded BMSC multipotential differentiation into fat and bone diminishes with increasing passage, and therefore BMSC may be more useful as gene therapy targets prior to expansion. This correlates with other studies where *ex vivo* expansion of MSCs is associated with a loss of MSC characteristics (phenotype, proliferative capacity, self-renewal, immunomodulation) and negative T1D clinical outcomes.

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2. **Gerace D**, Martiniello-Wilks R, Habib R, Simpson AM. *In vitro* luciferase-based validation of mesenchymal stem cell suicide gene therapy. 2017; *In preparation*.

3. **Gerace D**, Martiniello-Wilks R, Nassif NT, Lal S, Steptoe R, Simpson AM. CRISPR-targeted genome editing of mesenchymal stem cell-derived therapies for type 1 diabetes: a path to clinical success? *Stem Cell Research & Therapy* 2017; **8**(1)**:** 62.

4. **Gerace D**, Martiniello-Wilks R, Simpson AM. Viral-mediated gene therapy for the generation of artificial insulin-producing cells as a therapeutic treatment for type 1 diabetes mellitus. In: A. Hardikar A (ed) *Pancreatic Islet Biology*. Springer International Publishing: Cham, 2016, pp 241-255.

5. **Gerace D**, Martiniello-Wilks R, Simpson AM. Diabetes reversal via gene transfer: building on successes in animal models. *Research and Reports in Endocrine Disorders* 2015; 5: 15-29.

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7. **Gerace D**, Ren B, Hawthorne WJ, Byrne MR, Phillips PM, O'Brien BA *et al.* Pancreatic transdifferentiation in porcine liver following lentiviral delivery of human furin–cleavable insulin. *Transplantation Proceedings* 2013; 45(5): 1869-1874.

List of Presentations

1. **Gerace D**, Martiniello-Wilks R, Nassif NT, Ren B, Simpson AM. *Ex vivo* expanded murine mesenchymal stem cells as targets for the generation of a cell replacement therapy for type 1 diabetes. In: 77th American Diabetes Association (ADA) Scientific Sessions. San Diego, USA, 2017.

2. **Gerace D**, Martiniello-Wilks R, Simpson AM. Persistence of Luciferase Expressing Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs) in Non-Obese Diabetic (NOD) and NOD/*Scid* Mice. In: *8th Islet Society – Australian Islet Study Group Meeting*. Sydney, Australia, 2015

3. **Gerace D**, Martiniello-Wilks R, Simpson AM. Bioluminescent Imaging of Mesenchymal Stem Cell Engraftment in Immune Competent and Immune Deficient Animal Models of Type 1 Diabetes. In: *9th Australasian Gene and Cell Therapy Society (AGCTS) Conference*. Melbourne, Australia, 2015

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5. **Gerace D**, Ren B, Hawthorne WJ, Byrne MR, Phillips P, O'Brien BA *et al.* Reversal of Diabetes in a Porcine Model Following Liver-Directed Gene Therapy. In: *8th AGTS Meeting*. Sydney, Australia, 2013.

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7. **Gerace D**, Ren B, Hawthorne WJ, Byrne MR, Phillips P, O'Brien BA *et al.* Pancreatic Transdifferentiation in Porcine Liver Following Lentiviral Delivery of Human Furin-Cleavable Insulin. In: *The 24th International Congress of the Transplantation Society*. Berlin, Germany, 2012.

8. **Gerace D**, Ren B, Byrne MR, O'Brien BA, Swan A, Simpson AM. Pancreatic Transdifferentiation in the Livers of Non-Obese Diabetic (NOD) Mice Following Lentiviral Delivery of Furin-Cleavable Insulin. In: *RNSH/UTS/USYD/KIMR Annual Scientific Meeting*. Sydney, Australia, 2011.

Abbreviations

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
AAV	Adeno-associated virus
AD-MSCs	Adipose-derived mesenchymal stem cells
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BB	Biobreeding
bFGF	Basic fibroblast growth factor
BLI	Bioluminescence imaging
BMSCs	Bone marrow-derived mesenchymal stem cells
CD	Cluster of differentiation
CD	Cytosine deaminase
CDUPRT	Yeast cytosine deaminase uracil phosphoribosyltransferase fusion pro-drug converting enzyme
CLEC16A	C-type lectin domain containing 16A
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeat
CTLA-4	Cytotoxic lymphocyte antigen 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DPP-4	Dipeptidyl peptidase-4
EDTA	Ethylenediaminetetracetic acid

eGFP	Enhanced green fluorescent protein
EGR1	Early growth response-1
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
ESRF	End-stage renal failure
FACS	Fluorescence-assisted cytometric sorting
FCS	Foetal calf serum
Fcy::Fur	Yeast gene that encodes CDUPRT fusion protein
FoxA1/FoxA2	Forkhead box factors
G1RE	Glucose-responsive element
GD	Gestational diabetes
GFP	Green fluorescent protein
GLUT2	Glucose transporter 2
GSIS	Glucose-stimulated insulin secretion
GWAS	Genome-wide association study
HbA1C	Glycated haemoglobin
HBSS	Hanks buffered salt solution
HDAD	Helper-dependent adenovirus
Hes-1	Hairy and enhancer of split 1
hIPCs	Human islet-derived progenitor cells
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
hTERT	Human telomerase reverse transcriptase
ICA	Islet cell-like aggregates
IDO	Indoleamime 2,3-dioxygenase
IDDM1	Insulin-dependent diabetes mellitus 1
IFN-γ	Interferon gamma

IL	Interleukin
IL2RA	Interleukin 2 receptor subunit alpha
INS-FUR	Furin-cleavable human insulin
IPCs	Insulin-producing cells
IPGTT	Intraperitoneal glucose tolerance test
iPSCs	Induced pluripotent stem cells
IRES	Internal ribosome entry site
ISG	Insulin secretory granules
Isl-1	LIM Homeobox 1
IVC	Individually ventilated cage
KCNJ11	ATP-sensitive potassium channel
LADA	Latent-onset autoimmune diabetes in adults
LB	Luria broth
LPK	L-type pyruvate kinase
Luc2	Firefly luciferase
mAb	Monoclonal antibody
MafA	v-maf musculoaponeurotic fibrosarcoma A
MAP	*Mycobacterium avium* paratuberculosis
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MODY	Mature-onset diabetes of the young
MSCs	Mesenchymal stem cells
NCBI	National Centre for Biotechnology Information
NeuroD1	Neuronal differentiation 1
Neurog3	Neurogenin 3
NK	Natural killer
Nkx2.2	NK2 homeobox 2

Nkx6.1	*NK6 homeobox 1*
P/S/G	Penicillin-Streptomycin-Glutamine
PANDER	Pancreatic derived factor
Pax4	Paired homeobox 4
Pax6	Paired homeobox 6
PBS	Phosphate buffered saline
PD-1	Programmed death 1
Pdx1	Pancreatic and duodenal homeobox 1
PGE2	Prostaglandin E2
PP	Pancreatic polypeptide
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PTPN2	Protein tyrosine phosphatase, non-receptor type 2
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
QTL	Quantitative trait loci
RLU	Relative light units
RO	Reverse osmosis
rSAP	Shrimp alkaline phosphatase
RT-PCR	Reverse transcription polymerase chain reaction
SCA-1	Stem cell antigen 1
Scid	Severe combined immunodeficiency
SSEA-1	Stage-specific embryonic antigen 1
STRO-1	Stromal cell antigen 1
STZ	Streptozotocin
SV40T	Simian virus 40 antigen
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBE	Tris/Borate/EDTA

TCF7L2	Transcription factor 7-like 2
TFF	Tangential flow filtration
TGF-β	Transforming growth factor beta
UC-MSCs	Umbilical cord mesenchymal stem cells
UPRT	Yeast uracil phosphoribosyltransferase pro-drug converting enzyme
UTR	Untranslated region
VNTR	Variable number tandem repeat
VSMCs	Vascular smooth muscle cells