

Examination of Pancreatic Transdifferentiation in the Livers of Animals

by Que Tran La

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degree of

Doctor of Philosophy

under the supervision of:
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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Que Tran La declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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List of Publications

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List of Presentations

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Format of the Thesis

This is a thesis by compilation. The first chapter is the introduction to the research study and the review of the literature. Chapter 2 describes the experiments, general materials and methods, followed by chapter 3, 4 and 5 showing detailed methods, results and the discussion of each subset of experiments. Chapter 4 is a published peer reviewed journal article. The last chapter of this thesis is the general discussion and the recommendation for future studies.

Publication Included in the Thesis

Title: Use of a Hybrid Adeno-Associated Viral Vector Transposon System to Deliver the Insulin Gene to Diabetic NOD Mice

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Ann M. Simpson¹ and Que T. La^{1,2} wrote the draft article and all authors contributed to review of the final article and editing.

Binhai Ren^{1,2} produced the lentiviral vector and with Que T. La^{1,2}, contributed to experimental design, performed the animal experiments, immunohistochemistry, RT-PCR analysis and data analysis.

Grant Logan³ designed the AAV vectors, contributed to experimental design and performed data analysis.

Sharon C. Cunningham³ designed the AAV/*piggyBac* vectors and contributed to experimental design and data analysis.

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Najah T. Nassif^{1,2} contributed to the experimental design and performed primer design and data analysis.

Bronwyn A. O'Brien^{1,2} contributed to experimental design and data analysis.

All authors contributed to the interpretation of the data.

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List of Abbreviations

9330175E14Rik	RIKEN cDNA 9330175E14 gene
AAV	Recombinant adeno-associated virus
Ad	Adenovirus
Adar	adenosine deaminase, RNA-specific
Adar1	Double-stranded RNA-specific adenosine deaminase 1
AIHW	Australian Institute of Health and Welfare
APC	Antigen presenting cells
Apol9a	apolipoprotein L 9a
Apol9b	apolipoprotein L 9b
Arx	Aristaless related homeobox
ATP	Adenosine triphosphate
BGL	Blood glucose levels
bHLH	basic helix-loop-helix
B-H-p-value	Benjamini-Hochberg-P-value
Ca ²⁺	Calcium ion
Ccl5	Chemokine (C-C motif) ligand 5
CD28	Cluster of Differentiation 28
CD40	Cluster of Differentiation 40
Cdc42	Cell division cycle 42
cDNA	Complementary deoxyribonucleic acid
CMRI	Westmead Children Medical Research Institute
CSII	Continuous subcutaneous insulin infusion
Cxcl10	chemokine (C-X-C motif) ligand 10
Cxcl11	chemokine (C-X-C motif) ligand 11
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
Ddx60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58
DNA	Deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein

ER	Endoplasmic reticulum
FAH	Fumarylacetoacetate hydroxylase
FDR	False detection rate
FFO	Full flow occlusion
Foxa2	Forkhead box A2
G6pc	Glucose-6-phosphatase catalytic
Gbp3	Guanylate binding protein 3
Gbp6	Guanylate binding protein 6
GDM	Gestational diabetes mellitus
GK	Glucokinase
GLUT2 (Slc2a2)	Glucose transporter 2
GLUT4 (Slc2a4)	Glucose transporter 4
Gm4951(Isg15)	predicted gene 4951
GPCRs	G protein coupled receptors
Gyg	Glucogenin
Gys2	Glycogen synthase 2
h	Hour
H4IIE/ND	Rat liver cell line (H4IIE) transfected with NeuroD1 gene
H4IIEins/ND	Rat liver cell line (H4IIE) transfected with INSFUR and NeuroD1 genes
HDAd	Helper-dependent adenoviral vector
HK3	Hexokinase3
HIV	Human immunodeficiency virus
HLAs	High-risk human leukocyte antigens
HMD/INSFUR	Lentiviral vector carrying INSFUR gene
HMGA2	AT-hook2 gene
HNF1	Hepatocyte Nuclear Factor 1
HNF6	Hepatocyte Nuclear Factor 6
HSV	Herpes simplex virus
IDF	International Diabetes Federation
Ifi44	Interferon-induced protein 44
Ifit1	Interferon-induced protein with tetratricopeptide repeats 1

Ifit3	Interferon-induced protein with tetratricopeptide repeats 3
IFN- γ	Interferon γ
Ikbke	Inhibitor of nuclear factor kappa B kinase subunit epsilon
Ikbkg	Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma
IL-1 β	Interleukin 1 β
<i>INS-FUR</i>	<i>Furin-cleavable insulin</i>
<i>i.p</i>	intraperitoneal
IPA	Ingenuity Pathway Analysis
IPCs	Insulin-producing-cells
IRF	Interferon regulator factor
Irf7	Interferon regulatory factor 7
Isg15	ISG15 ubiquitin-like modifier; Predicted gene 9706
ITRs	Inverted terminal repeats
kb	Kilobase
kg	Kilogram
Keap1-Nrf2	Kelch-like ECH-associated protein 1-nuclear factor (erythroid-derived 2)-like 2
LSP	Apolipoprotein E (ApoE) enhancer and the human α 1-antitrypsin (hAAT) promoter
LTRs	Long terminal repeats
Ly6a	Lymphocyte antigen 6 complex, locus A
Ly6c1	lymphocyte antigen 6 complex, locus C1; lymphocyte antigen 6 complex, locus C2
<i>MafA</i>	<i>V-maf musculoaponeurotic fibrosarcoma oncogene homolog A</i>
<i>MafB</i>	<i>V-maf musculoaponeurotic fibrosarcoma oncogene homolog B</i>
Map3k7	Mitogen-activated protein kinase 7
min	Minute
mRNA	Messenger ribonucleic acid
mmol	Millimole
MSCV	Mouse stem cell virus
Mx1	MX dynamin-like GTPase 1

Mx2	MX dynamin-like GTPase 2
<i>NeuroD1</i>	<i>Neuronal differentiation 1</i>
ng	nanogram
<i>Ngn3</i>	<i>Neurogenin3</i>
<i>Nkx2.2</i>	<i>Nierenberg and Kim 2 homeobox 2</i>
<i>Nkx6.1</i>	<i>Nierenberg and Kim 6 homeobox 1</i>
Nlrc5	NLR family, CARD domain containing 5
NOD	Non-obese diabetic
NOD/scid	Non-obese-diabetic/ severe combined immunodeficiency
NTBC	2-(2-nitro-4-trifluoro-methylbenzoyl) 1,3-cyclohexedione
Oas1a	2'-5' oligoadenylate synthetase 1A
Oas1l	2'-5' oligoadenylate synthetase-like 1
Oas12	2'-5' oligoadenylate synthetase-like 2
<i>Pax4</i>	<i>Paired box 4</i>
<i>Pax6</i>	<i>Paired box 6</i>
<i>PC1</i>	<i>Processing endopeptidase 1</i>
<i>PC2</i>	<i>Processing endopeptidase 2</i>
PCA	Principal component analysis
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic gene
PCR	Polymerase chain reaction
<i>Pdx1</i>	<i>Pancreatic duodenal homeobox1</i>
PKR	Protein Kinase R
Pklr	Pyruvate kinase liver and red blood cell gene
Ptf1	Pancreatic specific transcription factor 1
Ptf1a (or P48)	Pancreatic specific transcription factor 1a
Rsad2	Radical S-adenosyl methionine domain containing 2
Rtp4	Receptor transporter protein 4
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
SAP	Sensor augmented pump
<i>s.c</i>	Subcutaneous
SCID	Severe combined immunodeficiency

sec	Second
Sp100	Nuclear antigen Sp100
SPINK 2	Serine protease inhibitor Kazal-type 2
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAC	Transcriptome Analysis Console
Tgtp1	T cell specific GTPase 1
Tgtp2	T cell specific GTPase 2
Th1	T helper1
Th2	T helper 2
Tlr3	Toll-like receptor 3
Tu	Transduction unit
Usp18	Ubiquitin specific peptidase 18
VCN	Vector copy number
vg	Viral genome
VSV-G	Vesicular stomatitis virus envelope glycoprotein G
Wnt	Wingless-related integration site
Xaf1	XIAP associated factor 1
Zbp1	Z-DNA binding protein 1
α -cell	Alpha cell
β -cell	Beta-cell
δ -cells	Delta-cell

Abstract

Type I diabetes mellitus (T1D) is a chronic metabolic disorder resulting from the autoimmune attack and destruction of the pancreatic β -cells, leading to insulin deficiency and hyperglycaemia. The condition is currently managed by insulin therapy, which delays but does not fully prevent the long-term complications of the disease. The only cure for T1D is pancreas or islet transplantation; however, due to the lack of available organs and the complications from immunosuppression, transplantation is not widely applied. Gene therapy is one treatment being considered to treat and/ or cure T1D.

In earlier studies, our group delivered a lentiviral vector (HMD) carrying the furin-cleavable human insulin gene (*INS-FUR*) to streptozotocin (STZ)-induced diabetic rats, non-obese-diabetic mice, pancreatectomised diabetic pigs and humanized FRG mice using intervallic infusion of the vectors in full flow occlusion (FFO), a surgical technique that isolated the liver from the circulation. Reversal of diabetes with normal glucose tolerance and the expression of key β -cell transcription factors in the liver tissue of the transduced animals were achieved.

The main aim of the current study was to explore the possibility of reproducing the results of the HMD/*INS-FUR* transduction using less invasive transduction techniques. Traditional AAV8 vectors that have a higher tropism toward the hepatocytes were used to deliver the transgene *INS-FUR*, the β -cell transcription factors *Pdx1* or *NeuroD1*. The liver specific promoter (LSP) was also incorporated into the vector system to limit the expression of the transgene(s) to the livers and allow the vectors to be delivered by a simple intraperitoneal injection.

The expression of *Pdx1* or *NeuroD1* in the livers of the STZ-induced diabetic non-obese-diabetic and severe immune incompetent (NOD/scid) mice by the AAV8-LSP system did not cure diabetes. The delivery of the *INS-FUR* gene by AAV8-LSP led to hypoglycaemia in both STZ-induced diabetic NOD/scid mice and autoimmune non-obese-diabetic (NOD) mice. The transcriptome analysis of the livers of the NOD mice transduced by

AAV8-*INS-FUR* alone showed that severe hypoglycaemia that was observed in the animals may have been caused by an activation of the glycolysis pathway and an inhibition of the gluconeogenesis pathway. Using the same AAV8-LSP vector system to deliver *INS-FUR* with β -cell transcription factors (*Pdx1* and/ or *NeuroD1*) caused hypoglycaemia in diabetic NOD/scid mice, while the combination of *INS-FUR* and *Pdx1* was ineffective in treating diabetes in NOD mice. The combination of the AAV8-*INS-FUR* and FFO surgery or the empty HMD vector could not reverse diabetes in NOD mice either. In addition, the transcriptome analysis showed that the AAV8 transduction could also generate an antiviral immune reaction, but the AAV8 transduction with or without the FFO surgery may not cause any liver disease.

In this project the hybrid AAV8/*piggyBac* that could facilitate the integration of the transgene into the host genome was also used to deliver the *INS-FUR* gene. The hybrid system had both the transposon and *transposase* constructs of the *piggyBac* system incorporated into the AAV8-LSP vectors (AAV8/*piggyBac-INS-FUR*). The delivery of the *INS-FUR* gene by the AAV8/*piggyBac* could not normalise the random blood glucose levels in the NOD mice, but normal glucose tolerance was achieved. By comparison NOD mice that received FFO surgery after the AAV8/*piggyBac-INS-FUR* transduction had normal blood glucose levels, normal glucose tolerance and possibly glucose-responsive insulin secretion without the expression of any endogenous β -cell transcription factors. It was thought that the stable blood glucose levels were achieved because the FFO surgery may have helped to reduce the number of *transposases* and allowed a more stable integration of the *INS-FUR* gene to the host genome. In addition, the results suggested that the clinical desirable outcomes (normal blood glucose levels and glucose tolerance) could be achieved without the expression of β -cell transcription factors. This is the first *in vivo* study using the hybrid AAV8/*piggyBac* system to treat T1D. The study suggested that AAV8/*piggyBac* system may be further developed to become an alternative therapy for the disease.

Chapter 1:

Introduction

1.1 Introduction

Diabetes is a chronic multi-aetiology metabolic disorder [1, 2]. The condition is characterised by chronic hyperglycaemia caused by defects in insulin secretion, and/or in the insulin response [1]. Worldwide, in 2019 there were 463 million people living with diabetes and the disease incurred a cost of about 760 billion USD [3]. In Australia, 1.2 million Australian adults aged over 18 years had diabetes in 2017-18 [4]. It was estimated that \$2.5 billion was attributed to the Australian health care system as the expenditure for the disease in 2015-2016 [4].

According to the International Diabetes Federation (IDF) (IDF, 2019), the diagnostic criteria for diabetes are: (1) plasma glucose level of 7 mmol/L or higher after 8 h fasting, or (2) plasma glucose level of 11.1 mmol/L or higher 2 h after a 75g oral glucose load, or (3) blood HbA1c levels of 48 mmol/mol or higher, or (4) random plasma glucose of 11.1 mmol/L or higher. Diabetes can lead to acute complications such as ketoacidosis and hyperglycaemic hyperosmolar syndrome or chronic complications including, neuropathy, retinopathy, renal failure, infection, delayed wound healing and cardiovascular disease [1].

Diabetes is classified into three main types: type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes (GD) [5]. T1D is the result of an absence of insulin secretion due to autoimmune related pancreatic beta-cell (β -cell) dysfunction [1]. The onset of T1D is usually during childhood or early adulthood and T1D patients rely on life-long insulin therapy for the control of blood glucose levels (BGL) [5]. T2D is characterised by a deficiency of, or defects in insulin secretion, either with or without insulin resistance of body tissues [1]. T2D is less likely to be diagnosed early as the symptoms progress more slowly and are commonly less severe than those of T1D. Thus, by the time patients are diagnosed, many have developed some of the severe complications associated with the disease [5]. GD is characterised by hyperglycaemia which occurs during pregnancy as a result of carbohydrate intolerance [1]. GD can increase the risk of many other adverse events during pregnancy and is a risk factor for later onset of T2D in both mothers and children [5]. T2D is the most prevalent of the 3 types and, according to the IDF (2019), 90% of people living with diabetes have T2D, with less than 10% having T1D. There is

no current data capturing the prevalence of T1D in all age groups, but in 2019, an estimated 1.1 million children aged 0-19 were suffering from T1D worldwide [3]. In Australia, over 6500 children aged 0-14 had T1D in 2017 [4].

Despite the lower prevalence, managing T1D is more complicated than managing T2D. While many T2D patients can be managed by oral medications and/or lifestyle and dietary modifications [6], T1D patients depend on insulin pumps or several insulin injections daily to control their BGL [7]. Insulin therapy can delay the onset of chronic complications (such as neuropathy, retinopathy and renal failure), but it cannot restore the function of pancreatic β -cells or eliminate all the complications. As T1D is more likely to develop in children and young adults, patients with T1D are more likely to begin to develop chronic complications earlier and therefore suffer from them for a longer period of time. Together with the cost of life-long insulin therapy, managing chronic complications of T1D can become a significant psychosocial and financial burden, not only to the patient, but also to society [5]. It is therefore of high importance to find a cure for T1D.

Currently, the most effective treatment for T1D is to restore the ability of the body to produce insulin by either pancreas or pancreatic islet transplantation; however this therapy is hampered by insufficient numbers of donor organs, and the need for life-long immunosuppression of patients after transplantation [8]. Generating insulin-producing cells (IPCs) by stem cell therapy and gene therapy are being considered as potential cures. Our increasing knowledge of the mechanism of insulin synthesis and release, as well as pancreatic organogenesis, has contributed to the progress of these new therapies. Furthermore, recent progress in viral vector development, gene therapy in general and liver-directed gene therapy in particular, has shown great potential. This thesis is focused on T1D and examines the possibility of curing the disease using gene therapy.

1.2 Overview of insulin synthesis, storage, release and mechanism of action

Insulin is produced, stored and secreted by the β -cells located in the islets of Langerhans in the pancreas [9]. Initially the mRNA of the human insulin gene is translated to produce preproinsulin, which contains the A chain, B chain, C-peptide and the signal polypeptide at the N-terminus of the molecule [10] (Figure 1.1). Proinsulin is then formed in the endoplasmic reticulum of the pancreatic β -cells by cleavage of the signal peptide of preproinsulin [9]. Proinsulin, containing the C-peptide between the A and B chains, is transported to the Golgi apparatus and packaged into secretory granules, which also contain proinsulin processing endopeptidases (PC1 and PC2) and carboxypeptidase H [9]. These enzymes cleave the C-peptide at the B-C junction and the C-A junctions to produce bioactive insulin comprised of the A and B chains linked by disulphide bonds [11-13]. Both insulin and the C-peptide are stored in secretory granules [9].

β -cells secrete insulin by two mechanisms: (1) a background or a continuous low level of secretion, which occurs independently of BGL, and (2) a rapid secretion from secretory granules in response to a rise in BGL or other signals from hormones and neurotransmitters [14]. Glucose transporter 2 (GLUT2) and glucokinase (Gck) are two important components of the glucose sensing mechanism of β -cells [9]. In the presence of high concentrations of extracellular glucose, GLUT2 transports glucose into the pancreatic β -cells [9]. Gck phosphorylates glucose to form glucose-6-phosphate, which then undergoes glycolysis and generates adenosine triphosphate (ATP) [9]. The elevation of ATP levels leads to a blockage of ATP-sensitive potassium channels and depolarisation of the cell membrane [9]. This leads to an influx of Ca^{2+} triggering the secretion of insulin from secretory granules [9] (Figure 1.2).

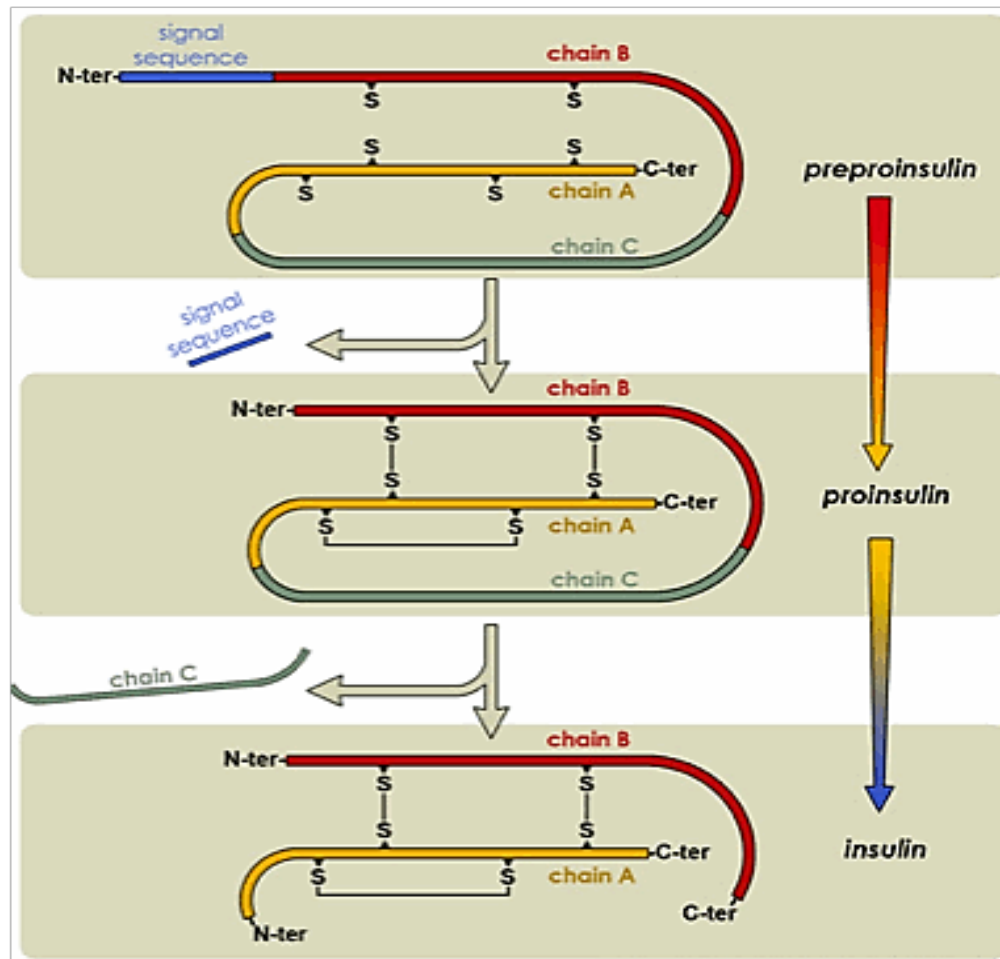


Figure 1.1: Processing of bioactive insulin from its inactive precursor

Preproinsulin contains the A chain, B chain, C-peptide and the signal polypeptide at the N-terminus. The signal peptide is cleaved from preproinsulin to form proinsulin. The enzymes PC1 and PC2 then cleave the C-peptide from proinsulin to produce bioactive insulin comprising the A and B chains linked by disulphide bonds (from Jones and Persaud, 2010).

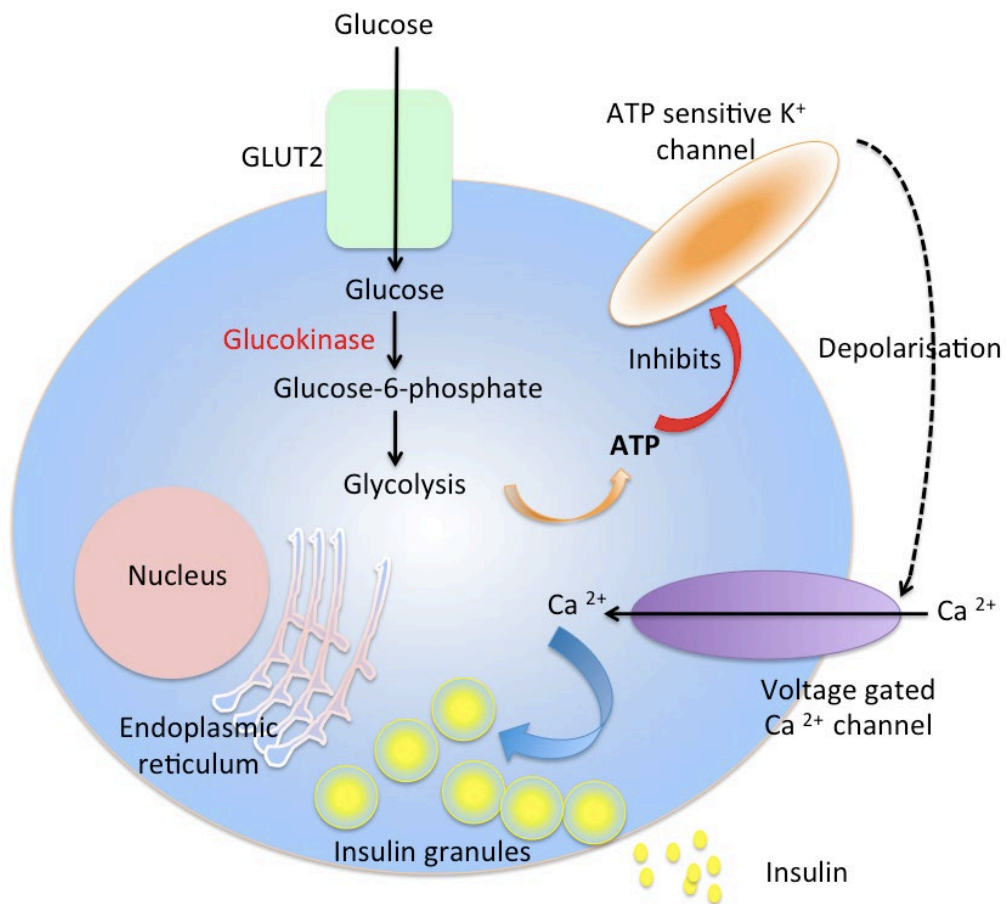


Figure 1.2: The mechanism of insulin secretion

GLUT2 transports glucose into the pancreatic β -cells. GCK phosphorylates glucose to allow glycolysis and generation of ATP. The elevation of ATP leads to a blockage of ATP-sensitive potassium channels, an influx of Ca^{2+} , and secretion of insulin from secretory granules.

Insulin increases the transport of glucose into adipocytes and myocytes by stimulating the translocation of the intracellular glucose transporter 4 (GLUT4) to the cell membranes [15] (Figure 1.3). Although the exact mechanism is not fully understood, it is believed that insulin binds to the extracellular component of the tyrosine kinase receptor [16]. The receptor consists of 4 subunits: 2 α -subunits and 2 β -subunits, and the α -subunits inhibit the kinase activity of the β -subunits [16]. By binding to the α -subunits, insulin triggers the dissociation of the α -subunits from the β -subunits, and terminates the repressive activity of the α -subunits [16]. This process leads to the phosphorylation of the β -subunits

and an induction of a conformational change that increases the kinase activity to trigger a number of intracellular cascades [16]. The function of these signalling cascades includes, but is not limited to, mitogenesis, protein synthesis, glycogen genesis and glucose transportation [15]. One of the most important outcomes of these signalling pathways is the translocation of the GLUT4 transporter from intracellular vesicles to the cell membrane, which allows the transportation of a large amount of glucose into the cells [15].

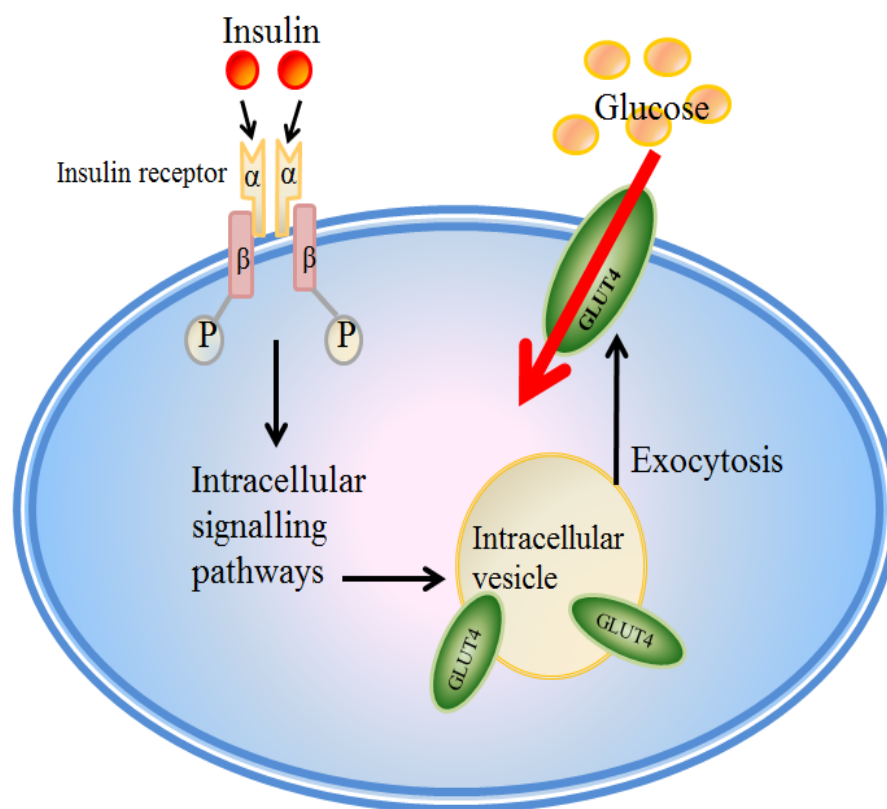


Figure 1.3: Insulin induces cellular glucose uptake

Insulin binds to its receptor to activate the kinase activity of the β -subunit, which triggers other intracellular signalling pathways leading to the exocytosis of intracellular vesicles containing the GLUT4 glucose transporter and insertion of the transporter into the cell membrane.

1.3 The roles of pancreatic transcription factors in the development of pancreatic β -cells

During embryogenesis, the pancreas develops from the endoderm, which is one of the three primary germ layers [17]. The differentiation of endodermal cells into pancreatic β -cells is known to be regulated by various TFs. Different combinations of pancreatic transcription factors are expressed at different stages of β -cell development (Figure 1.4). The transcription factors have multiple roles, and are involved in multiple pathways [18]. Due to the complexity of the interactions, the exact mechanism of the process of β -cell differentiation is not fully understood. The roles of important pancreatic transcription factors are summarised in Table 1.1. The different ways by which the transcription factors are thought to regulate the process of β -cell differentiation are shown in Figure 1.5.

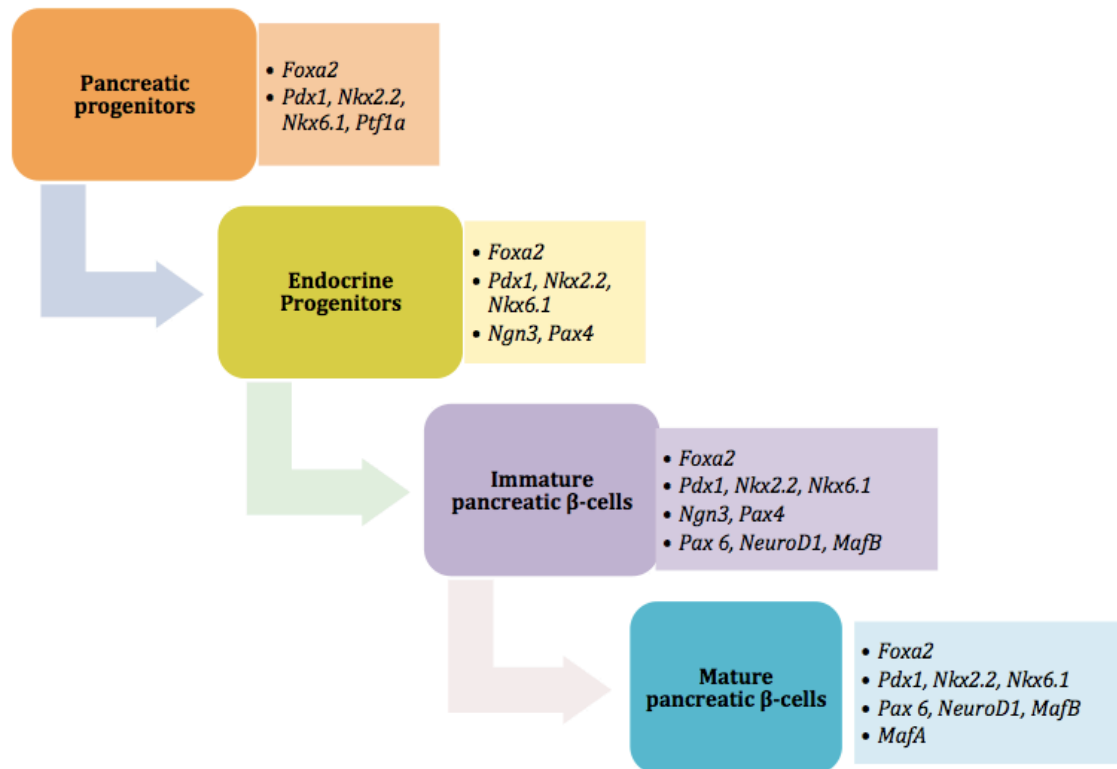


Figure 1.4: The expression of pancreatic transcription factors at various stages of pancreatic β-cell development.

At different stages of β-cell development different combinations of transcription factors are expressed. **Key:** Forkhead box A2 (*Foxa2*); Pancreatic specific transcription factor 1a (*Ptf1a* or *P48*); Pancreatic duodenal homeobox1 (*Pdx1*); Neuronal differentiation 1 (*NeuroD1*); Neurogenin 3 (*Ngn3*); Pair box 4 (*Pax4*); Nierenberg and Kim 2 homeobox 2 (*Nkx2.2*); Nierenberg and Kim 6 homeobox 1 (*Nkx6.1*); V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*); V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MafB*).

Table 1.1: Roles of pancreatic transcription factors in β -cell development

Transcription factor	Role(s)	Consequence(s) of homozygous (-/-) knockout
<i>Foxa2</i>	Involved in endodermal formation [18]	Death from multiple defects [19, 20]
<i>Ptf1 (P48)</i>	- Directs endodermal progenitor cells to early pancreatic progenitors, which can differentiate into all pancreatic cell types [21-23] - Directs exocrine differentiation in later stages [22]	Absence of acinar cells [24]
<i>Pdx1</i>	- Required for the growth of all pancreatic lineages [18] - Maintains function of mature β-cells [18] - Regulator of <i>insulin</i>, <i>GLUT2</i> and <i>GCK</i> gene expression [25-27]	Pancreas agenesis [28, 29]
<i>NeuroD1</i>	Marker of endocrine differentiation in pancreatic lineages [18, 30]	- Small disorganised islets [31] - Hyperglycaemia at birth [32]
<i>Nerogenin3</i>	Endocrine differentiation in pancreatic lineages [33]	Absence of endocrine cells in both the intestine and pancreas in mice [34]
<i>Pax4</i>	Initiates β-cell differentiation [35-38]	Reduction in β-cell number [37]

Table 1.1 (Continued): Roles of pancreatic transcription factors in β -cell development

Transcription factor	Role(s)	Consequence(s) of homozygous (-/-) knockout
<i>Pax6</i>	• Participates in β-cell development and α-cell formation [39]	• Islet disorganisation [39, 40] • Lack of α-cells [41]
<i>Nkx6.1</i>	• Transcriptional activator and repressor [42]	• Reduced islet size [43] • Significant reduction in β-cell number [43]
<i>Nkx2.2</i>	• Transcriptional activator and repressor [17, 18] • Initiates β-cell differentiation[44, 45]	• Absence of insulin producing cells [46, 47] • Hyperglycaemia at birth [46] • Death shortly after birth [46]

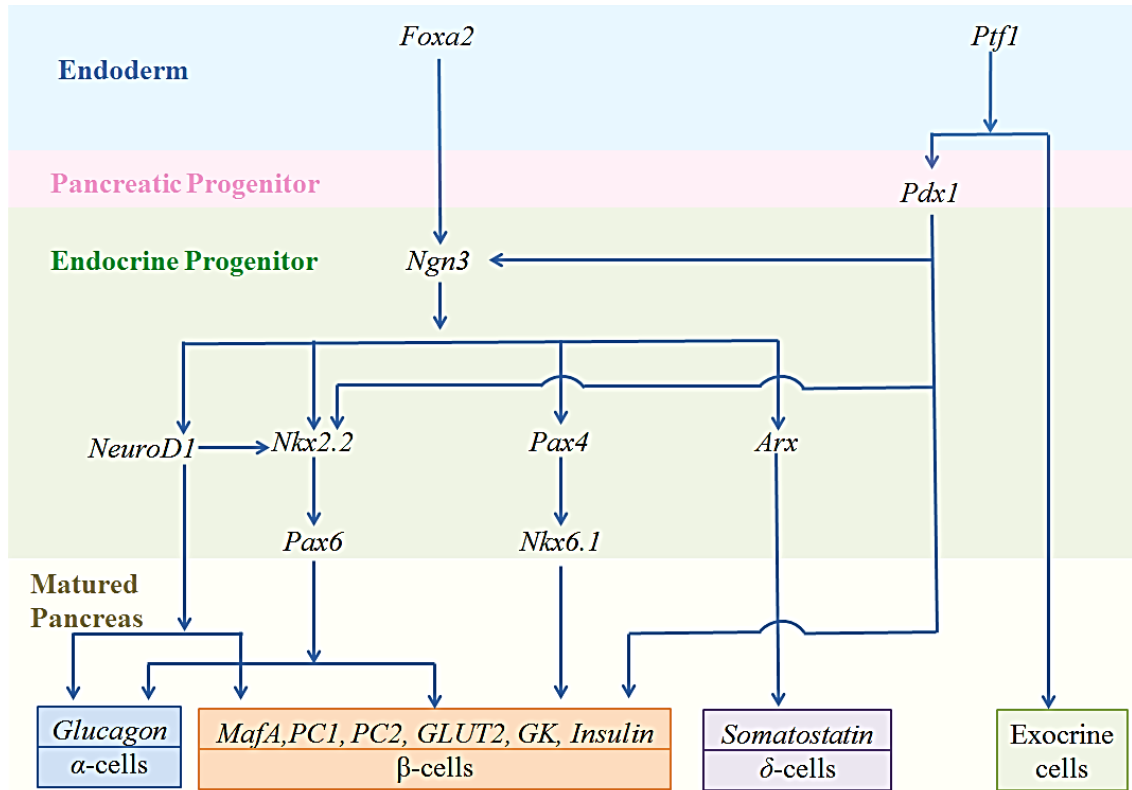


Figure 1.5: Pancreatic β -cell differentiation pathways

Foxa2 signals the formation of the endodermal layer. Endodermal cells expressing *Ptf1* adapt to the pancreatic progenitor fate. *Ptf1* up-regulates *Pdx1*, which signals pancreatic genesis. *Foxa2* upregulates *Ngn3* to direct pancreatic progenitor cells towards the endocrine progenitor cell fate. Endocrine progenitor cells that express *NeuroD1* adapt to either a β -cell or an α -cell fate, while cells that do not express *NeuroD1*, but express *Arx*, differentiate to δ -cells. *Pdx1*, *NeuroD1* and *Ngn3* work in synergy to maintain the expression of *Nkx2.2*, which is needed for the expression of *Pax6*. Cells that express both *Pax4* and *Pax6* differentiate to β -cells.

1.3.1 Forkhead box A2 (*Foxa2*)

The formation of the endoderm layer is signalled by several transcription factors that were initially described in mature liver and include hepatic nuclear factor (HNF) 1b, 3a, 3b, 4a and 6. *HNF3b* is also known as *Foxa2* [48]. The *Foxa2* gene is expressed at all the developmental stages of pancreatic β -cell generation [18]. *Foxa2*-null (or *HNF3b*-null) mice have multiple defects and die in the early embryonic stage [19, 20]. In mature

animals, the disruption of *Foxa2* expression alone does not lead to the deregulation of all other downstream transcription factors in all pancreatic cells [49, 50]. These observations demonstrate that the roles of *Foxa2* can change depending on the stage of pancreatic β -cell development.

1.3.2 Pancreatic specific transcription factor 1 (*Ptf1*)

Ptf1 is a heterooligomeric transcription factor composed of 4 subunits: the basic helix-loop-helix (bHLH), P48 (or *Ptf1a*), P64 and P75 subunits [18]. *Ptf1* is expressed in all the prospective pancreatic endodermal and pancreatic progenitor cells that differentiate to pancreatic endocrine, exocrine and ductal cells [24]. *Ptf1* expression is essential in early pancreas formation, because the endodermal cells adapt to the intestinal fate when *Ptf1* is absent [24]. *Ptf1* up-regulates the expression of the *duodenal homeobox1* (*Pdx1*) gene in early pancreatic progenitor cells [21]. Through interaction with the Notch signalling pathway, the P48 subunit initiates the differentiation of these cells into exocrine cells [22]. The Notch signalling pathway is a gene regulatory pathway that controls multiple cell differentiation processes [23]. Notch proteins generally span the cell membrane and have intracellular and extracellular domains [23]. When ligands bind to the extracellular domain of the Notch proteins, the intracellular domain is released and recruits other pathways to modify gene expression in the nucleus [23].

1.3.3 Pancreatic duodenal homeobox1 (*Pdx1*)

Unlike *Ptf1*, which is not expressed in mature pancreatic β -cells, *Pdx1* is expressed at all stages of pancreatic β -cell development [18]. In both mouse and human, *Pdx1* knockout (-/-) leads to the formation of pancreatic rudiment buds in the pancreatic endodermal region, but the pancreas fails to develop [28, 29]. As *Pdx1* was not activated in *Foxa2*-null mice, *Foxa2* is thought to be the upstream activator of *Pdx1* in pre-pancreatic endoderm and pancreatic buds [51]. *Ptf1* was found to upregulate the expression of *Pdx1* by binding to its promoter [21]. During early pancreatic development, *Pdx1* is widely expressed because it is needed for the development of the full spectrum of pancreatic lineages, however, in mature pancreas, high expression of *Pdx1* is limited to β -cells [52]. Interestingly, the expression of *Pdx1* in later stages of pancreatic development, and in

mature β -cells, is regulated by many other downstream transcription factors as well as hormones and nutritional status [53].

In mature β -cells, *Pdx1* regulates the expression of several genes that are involved in glucose sensing and metabolism, including *insulin*, *GLUT2* and *Gck* [25-27]. At low glucose concentrations, *Pdx1* localises at the nuclear periphery [54]. When the glucose concentration is elevated, *Pdx1* translocates to the nucleoplasm and binds to the promoters of the *insulin*, *GLUT2* and *Gck* genes [55]. *Pdx1* regulates the expression of the *insulin* gene by binding to the A-element within the insulin promoter and mediating its histone modification [56].

1.3.4 Neurogenin3 (*Ngn3*)

Transient expression of *Ngn3* signals the differentiation of endodermal cells into early pancreatic endocrine progenitor cells [57]. The expression of *Ngn3* is thought to be independent of *Pdx1* and *Ptf1*, because *Ngn3* positive cells have been detected in the pre-pancreatic endoderm in the absence of either *Pdx1* or *Ptf1* [58]. However, *Pdx1* expression is needed for the maintenance of *Ngn3* expression in the later stages of development, as *Pdx1* knockout mice do not have *Ngn3* positive pancreatic endocrine progenitor cells [58]. *Ngn3* is regulated by hepatic nuclear factors that are widely expressed in the endoderm and the pancreatic buds, including *Hepatocyte Nuclear Factor 1* (*HNF1*), *Foxa* and *Hepatocyte Nuclear Factor 6* (*HNF6*) [48]. These transcription factors have binding sites within the *Ngn3* gene promoter [48]. Mice lacking *HNF6* do not have normal islets at birth, with severe reduction in the number of endocrine cells and *Ngn3* expression [34]. In a mouse model in which *Ngn3* was disrupted, both intestinal and pancreatic endocrine cells failed to develop [33]. In contrast, uniform expression of *Ngn3* throughout the pancreatic epithelium leads to a premature differentiation of the pancreas into endocrine cells [59].

Lineage and morphology studies have indicated that *Ngn3* positive cells are endocrine progenitor cells [60]. The expression of *Ngn3* gradually declines in the later stages of pancreas development [33] and the detection of *Ngn3* is very rare in the mature pancreas [33, 60]. Upregulation of Notch signalling has been found to be associated with a down-

regulation of *Ngn3* and a decrease in the formation of endocrine progenitor cells [57]. Pancreas progenitor cells with suppressed *Ngn3* expression, due to Notch signalling, but still expressing *P48*, can differentiate into exocrine cells [48]. The mechanism of action of *Ngn3* is poorly understood [18]. Several other transcription factors found to be targets of *Ngn3* are *neuronal differentiation 1 (NeuroD1 or BETA2)*, *Nierenberg and Kim 2 homeobox 2*, and *paired box 4* [57].

1.3.5 *Neuronal differentiation 1 (NeuroD1)*

The loss of *NeuroD1* expression in *Ngn3*-null embryos suggests that, in the hierarchy of transcription factors governing pancreas and β -cell development, *NeuroD1* is expressed downstream of *Ngn3* [59]. *NeuroD1* is expressed in all pancreatic endocrine progenitor cells and its expression is maintained in all later stages of β -cell development [18]. *NeuroD1* has been found to be both an activator and repressor of the expression of other transcription factors [30]. In mature islets, *NeuroD1* coexists with insulin and glucagon but not with somatostatin [30]. It can activate insulin gene expression by binding to the E-box within the insulin gene promoter [61]. At a normal glucose concentration, *NeuroD1* is mainly found in the cytoplasm, however, upon exposure to high glucose concentrations, it translocates to the nucleus [62, 63]. The transcription factor is found to regulate the expression of several ion channels and other genes involved in the exocytosis of insulin [56]. Despite the important roles of *NeuroD1* in regulating insulin action, deletion of *NeuroD1* does not lead to an absence of insulin secretion [32]. Even though *NeuroD1*^{-/-} mice are hyperglycaemic at birth, their pancreases still generate insulin producing cells [31]. However, many of these cells eventually undergo apoptosis leading to the formation of abnormal islets with a significant reduction in the number of β -cells [31, 32].

1.3.6 *Paired box 4 (Pax4)*

Pax4 is another transcription factor found to be activated by *Ngn3* and to participate in directing the endocrine precursor cells towards β and δ cell fates [17]. *Pax4* is exclusively expressed during pancreas formation and little or no *Pax4* can be detected in the mature pancreas [35, 36]. Immediately upon activation by *Ngn3*, cells that express *Pax4* also express *Aristaless* related homeobox (*Arx*) transcription factor [37]. This population of cells eventually separate into 2 cell types that express either *Pax4* or *Arx* [38]. Cells that

express *Pax4* subsequently develop into β -cells, while those that express *Arx* become δ -cells [37]. The expression of *Pax4* also represses the expression of *glucagon* [37].

Pax4-null mice develop diabetes at birth as they have significantly lower numbers of β cells [37]. They also have a higher number of α cells, and fail to develop pancreatic δ cells [37]. There is also evidence that *Pax4* is required for the maintenance of the expression of *Nierenberg and Kim 6 homeobox 1* (*Nkx6.1*) and *Pdx1* [46]. Even though *Pax4* is essential for β -cell differentiation, *Pax4* alone is not sufficient to drive the differentiation of *Ng3*-positive progenitor cells into β or δ -cells [46].

1.3.7 *Nierenberg and Kim 2 homeobox 2* (*Nkx2.2*)

Both *Nkx2.2* and *Nkx6.1* are expressed at all stages of β -cell development [18]. They also have binding sites on the insulin gene promoter and may therefore also act as activators of insulin gene expression [17]. *Nkx2.2* is expressed in β -cells and other pancreatic cells, but is absent from δ -cells [47]. *Nkx2.2* gene expression is maintained in mature endocrine cells by the synergistic action of both *Pdx1* and *NeuroD1* [30]. Knocking out *Nkx2.2* leads to a reduction of *Pax6* expression and absence of insulin producing cells [46, 47]. *Nkx2.2* is thought to be essential for the differentiation of endocrine precursors into pancreatic β -cells as *Nkx2.2* knockout (-/-) mice develop hyperglycaemia and die shortly after birth [46]. *Nkx2.2* has also been shown to stabilise β -cell fate by repressing *Arx* [45]. A mutation in the *Nkx2.2* gene has been shown to lead to ectopic expression of *Arx* and transdifferentiation of the β -cells to α -cells [45]. Deletion of *Pax4* has similar effects to the deletion of *Nkx2.2* [46]. Therefore, it is possible that *Nkx2.2* and *Pax4* control similar regulatory pathways to initiate β -cell differentiation.

1.3.8 *Nierenberg and Kim 6 homeobox 1* (*Nkx6.1*)

Nkx6.1 is widely expressed in the early pancreatic epithelium, but is restricted to the β -cell lineage in the later stages of pancreatic development [17, 43]. In *Pax4*-null mice, the expression of *Nkx6.1* occurs in the early embryonic stages but is not maintained in the mature pancreas [46]. Thus the maintenance of *Nkx6.1* requires *Pax4* [46]. *Nkx6.1* knockout mouse embryos still have a normal number of endocrine precursor cells, but neogenesis of insulin producing cells is significantly reduced [43]. This leads to a

significantly lower number of mature β -cells and smaller islets [43]. By repressing *Arx* *Nkx6.1* can also inhibit α -cell specification [64]. In mature β -cells, conditional inactivation of *Nkx6.1* leads to: (1) insulin deficiency, (2) smaller insulin containing secretory granules, (3) a reduction in the expression of *GLUT2*, *PC1* and *MafA*, (4) a reduction in the ability of β -cells to respond to a glucose challenge, (5) *Ngn3* expression and (6) appearance of cells that expresses both somatostatin and insulin [42].

1.3.9 Paired box 6 (*Pax6*)

Pax6 is an essential transcription factor for the differentiation of α -cells and for maintaining the normal morphology and function of the islets [39]. *Pax6* (-/-) mice do not have glucagon producing cells and the number of all endocrine cells is reduced [39, 40]. *Pax6*-nullizygous mice also have a significant reduction in *Pdx1* and in the number of insulin expressing cells [46]. St-Onge et al, (1997) suggested that endocrine progenitor cells that express both *Pax4* and *Pax6* become β -cells, and those expressing only *Pax6* become α -cells. The *Pax6* transcription factor regulates the expression of *glucagon*, *insulin*, *somatostatin*, *PC2* and *NeuroD1* [40, 41]. In mature β -cells, *Pax6* also up-regulates the transcription of *Pdx1* [65].

1.3.10 *V-maf musculoaponeurotic fibrosarcoma oncogene homolog A* (*MafA*)

MafA is a transcription factor that acts as a potent activator of the *insulin* gene by binding to the C1 element of the insulin gene promoter [57, 66]. *MafA* is expressed in the later stages of β -cell development [67]. In mature β -cells, the transcription of *MafA* requires a high glucose concentration [56]. At low glucose concentrations the expression of *MafA* is very low, but exposure to high glucose concentrations leads to a dramatic increase in *MafA* expression [56].

MafA knockout mice have a normal islet structure at birth but eventually develop diabetes due to a deficiency in the transcription of *mouse insulin 1*, *mouse insulin 2*, *Pdx1* and *GLUT2*, leading to a severe impairment in glucose-stimulated insulin secretion [68]. *FoxA2*, *Nkx2.2* and *Pdx1* have been found to regulate the expression of *MafA* by binding directly to the *MafA* promoter [69]. *NeuroD1* can also bind to the *MafA* promoter.

Interestingly both *MafA* and *Pax6* can regulate the expression of *Pdx1*, which appears much earlier in pancreatic genesis [57].

1.4 Overview of T1D

1.4.1 Aetiology and pathogenesis

T1D is characterised by the selective autoimmune destruction of the pancreatic β -cells leading to a severe insulin deficiency [1]. The mechanism of this process is still not fully understood, but a few contributing factors have been identified [70]. The concordance rate of the disease among monozygotic twins is between 30 to 50% [71]. This suggests that apart from genetic factors, there may also be an association between T1D and a number of environmental factors including: viral infection, dietary factors, high birth weight and growth rate, psychological stress and exposure to toxins [70]. The link between T1D and autoimmune dysfunction was established after the discovery that a number of high-risk human leukocyte antigens (HLAs) can facilitate the activation of T lymphocytes, which can infiltrate the pancreatic islets and selectively destroy pancreatic β -cells [70]. While the high-risk HLA gene represents approximately 50% of the familial risk of T1D, at least 47 other genetic factors are also thought to contribute [70].

The major component of pathogenesis in T1D is β -cell apoptosis [72]. The autoimmune process leading to β -cell dysfunction is evidenced by the detection of antibodies against islet cells in most T1D patients [70]. β -cells secrete antigens that can be detected by antigen presenting cells, which then process and present pancreatic self-antigens to autoreactive T cells [72]. After activation, the T cells undergo clonal expansion and migrate to the islet, where they secrete cytokines such as interleukin 1 β (IL-1 β) and interferon γ (IFN- γ) [72, 73].

The accumulation of unfolded and misfolded proteins in the endoplasmic reticulum (ER), which is also known as ER stress, has been shown to be a major trigger of β -cell apoptosis [72]. IFN- γ has been found to be directly involved in pathways that cause ER stress [72]. On the other hand, IL-1 β can trigger intracellular pathways leading to apoptosis with or without causing ER stress [72]. IL-1 β is involved in pathways that lead to the release of

mitochondrial proteins to activate apoptosis [72]. The impaired mitochondrial function can cause a disruption in calcium homeostasis of the ER leading to ER stress and apoptosis [72]. The apoptotic β -cells release more β -cell antigens and further exacerbate the cascade (Figure 1.6).

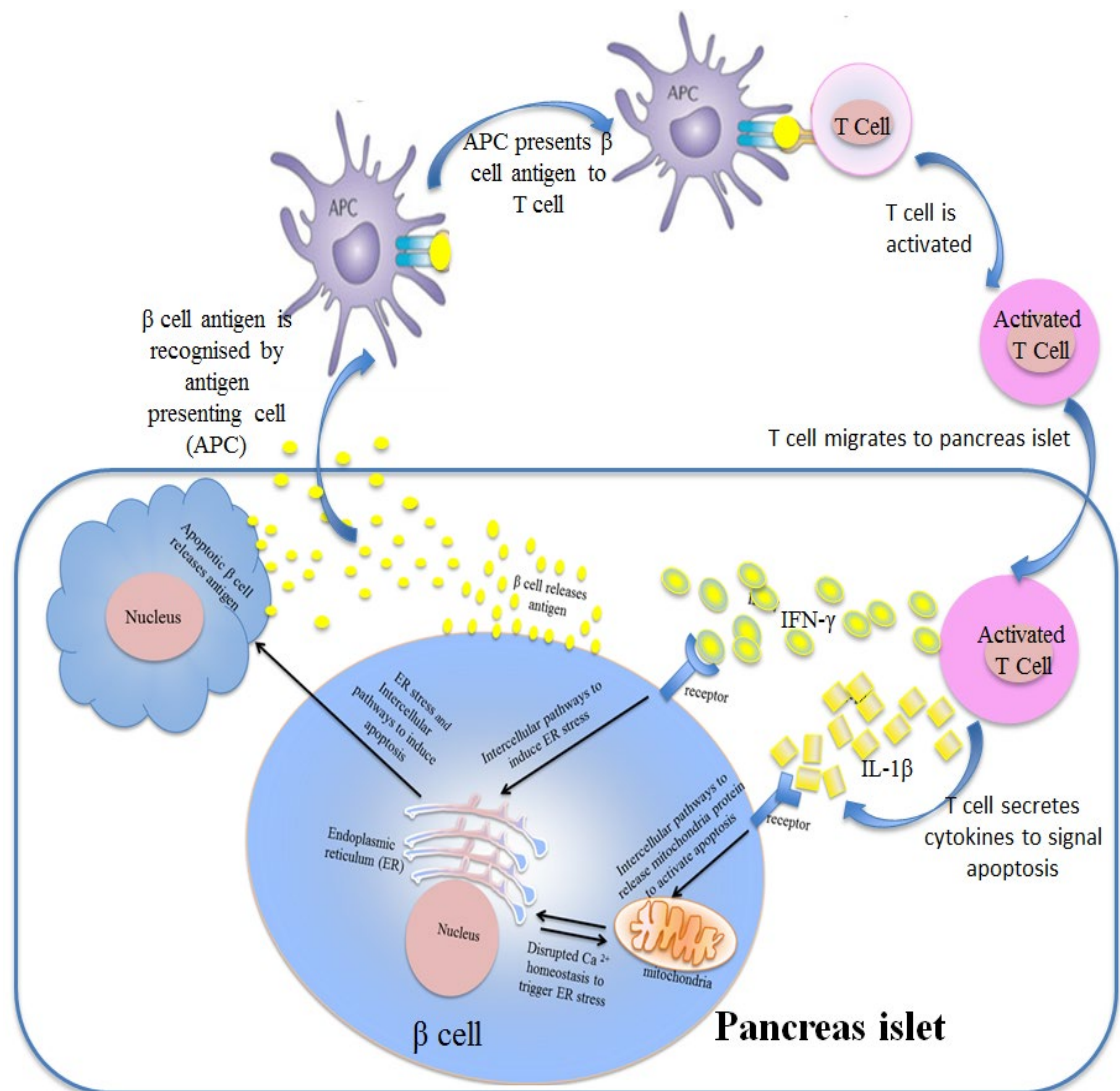


Figure 1.6: Immunological processes leading to pancreatic β -cell dysfunction

Antigens secreted by β -cells are detected by antigen presenting cells (APC) and presented to T-cells. After being activated, T-cells migrate to the pancreatic islets, where they secrete cytokines (IL-1 β and IFN- γ). The cytokines bind to receptors on the outer membrane of β -cells and trigger intracellular pathways to induce ER stress and activate apoptosis. The apoptotic β -cells release more antigens that exacerbate the process.

1.4.2 Clinical features and complications of T1D

Insulin deficiency impairs cellular glucose uptake and results in hyperglycaemia [1]. The decrease in glucose supply to the cells manifests as fatigue, constant hunger, weight loss and delayed wound healing [7]. In addition, insulin deficiency also promotes energy generation from lipolysis and ketogenesis leading to an accumulation of ketone bodies and ketoacidosis [74]. Due to the elevation in BGLs, patients can experience symptoms such as dry mouth, abnormal thirst, polyuria, recurrent infections and blurred vision [7]. Chronic hyperglycaemia leads to many complications such as renal failure, neuropathy, retinopathy, and vascular diseases, which increase the mortality rate for those with concurrent cardiovascular and cerebral vascular diseases [7].

1.4.3 Current treatments for T1D

1.4.3.1 Insulin therapy

Patients with T1D are managed with insulin therapy, which mimics the physiological release of insulin from pancreatic β -cells [14]. Combinations of long acting, short acting and rapid acting insulin may be administered according to the timing and the amount of carbohydrate intake [14]. One of the main limitations of insulin therapy is that it relies heavily on regular glycaemic and dietary monitoring to prevent hypoglycaemia, which is a recurrent cause of morbidity and can be fatal for patients with T1D [75].

The invention of the continuous subcutaneous insulin infusion (CSII) with a pump has reduced the need for regular injections and has led to better glycaemic control, but patients still need to regularly check their BGLs [14]. In adults with T1D, CSII was shown to be more effective in reducing the HbA1c level compared to multiple daily injections of insulin [76]. In order to avoid the necessity to regularly check their BGLs, the sensor augmented pump (SAP) system including the use of insulin pumps in conjunction with real-time continuous glucose monitoring was developed [77]. However, patients using the SAP system still have to manually modify the insulin infusion rate according to the BGLs [77]. Compared to the CSII, the SAP treatment has been shown to decrease the duration of hypoglycaemia and HbA1C levels [77], reduce the incident of diabetes-related complications [78] and be more cost effective [79].

Artificial pancreas, also referred to as close loop glucose control, is a new therapy combining an insulin pump, continuous glucose monitoring and a control algorithm to deliver insulin in a glucose responsive manner (single-hormone artificial pancreas system) [80]. In a dual-hormone artificial pancreas system, both insulin and glucagon can be delivered in a similar glucose responsive fashion [81]. In comparison to the CSII or the SAP system, both single and dual hormone artificial pancreas systems were shown to increase the percentage of time that the BGLs remained in the near normal glycaemic range (3.9 – 10 mmol/L), especially at night time when hypoglycaemia unawareness is more likely to occur [81, 82]. Compared to the single-hormone system, the dual hormone system has been associated with a greater improvement in the percentage of time that the BGLs were in the target range [82]. Despite the sophisticated algorithm to titrate the amount of insulin and glucagon infusion according to the real-time BGLs, the major limitation of the artificial pancreas is that the systems still require the patients to monitor their BGLs before eating and adjust the pre-meal insulin boluses according to the carbon hydrate intake [80].

1.4.3.2 Pancreas and islet transplantation

Pancreas and islet transplantation has been clinically applied for the treatment of T1D, however, transplantation therapy has several limitations including the lack of donor organs, the need for life-long immunosuppressive therapy after transplantation and the decline of graft function over time [8, 83]. Despite the application of immunosuppression and the refinement of the islet isolation protocols, the 3-year success rate of both pancreas and islet transplantation are 70% [84], and only 10% of the patients who receive islet grafts are insulin-independent for longer than 5 years [83].

To avoid the use of immunosuppressive therapy, islets have been encapsulated in various types of capsules for transplantation. The capsules allow the exchange of oxygen, insulin and nutrients between the graft and the surrounding tissue, but shelter the islet from the host immune surveillance [85]. However, compared to normal islets, the encapsulated islets have a slower response to a glucose challenge, and, to date, no clinical trial has shown permanent reversal of diabetes either with or without the use of immunosuppressive therapy [86].

1.5. Potential treatments for T1D

1.5.1 Stem cell therapy

In order to overcome the lack of donor pancreata, stem cell therapy has been widely studied to engineer insulin-producing-cells (IPCs). Owing to their ability to regenerate, differentiate into specialised cell types, and modulate immunological responses, mesenchymal stroma cells, embryonic stem cells, and induced pluripotent stem cells have been considered as targets for engineering IPCs [87, 88]. These cells can be transduced by various pancreatic genes or their growth conditions can be manipulated to trigger their differentiation into IPCs that can be transplanted [87]. This therapy still has many limitations, including: (1) insufficient *in vitro* differentiation, (2) the possibility of culture contamination, (3) inadequate *in vivo* insulin secretion, (4) the lack of response of IPCs to a glucose challenge, (5) teratogenic and tumorigenic potential, and (6) an immunological response to the transplanted IPCs leading to a reduction in the graft function [87, 88].

Many protocols to induce the differentiation of embryonic stem cells and mesenchymal stem cells into IPCs have been described, but none of the IPCs can function in the same manner as β -cells. For instance, intravenous injection of bone marrow derived mesenchymal stem cells into streptozotocin (STZ) induced diabetic mice has led to a reduction in BGL, but euglycaemia was not restored [89]. Many of the stem-cell-derived IPCs were partially differentiated. They have characteristics of the pancreatic progenitor cells that secrete multiple hormones (insulin, somatostatin and glucagon) and cannot secrete insulin in response to glucose [90]. In most differentiation protocols, the presence of β -cell markers such as *MafA*, *PC1*, *PC2*, *Gck*, and *GLUT 2* is only observed sporadically in the IPCs [90].

More recent studies have shown that after transplantation, the *in vivo* microenvironment allows the partially differentiated stem cells to further differentiate into a cell type that more closely resembles β -cells [89, 91]. The transplantation of partially differentiated embryonic stem cells has led to glucose responsive insulin secretion and prevented STZ-induced hyperglycaemia in rats [91]. However, the *in vivo* differentiation process takes months to complete, and the carryover of undifferentiated cells may lead to the formation

of teratomas [90]. Therefore, there is an increasing interest in encapsulating stem-cell-derived IPCs to protect them from immunological attack and prevent the spread of teratomas [86]. The most recent significant progress in stem cell therapy is the clinical trial (phase I/II) for transplanting encapsulated partially differentiated embryonic stem cells (VC-01TM device) developed by ViaCyte Inc (USA) [92].

1.5.2 Gene therapy

A number of adult cells can be re-programed to form IPCs that can secrete insulin in response to glucose by transfecting the chosen cells with either one or a combination of genes that are normally expressed in β -cells [93]. The extent of the transdifferentiation processes are widely varied depending on the chosen transgenes, methods of delivering the transgenes, the use of different promoters and the characteristics of the target cells chosen for the therapy. Even though many clinical trials have been approved and completed for gene therapies treating a number of pathological conditions such as: cancer, cardiovascular disease, and inherited monogenic diseases, a clinically applicable therapy for T1D has not been achieved. With the increasing knowledge in the fields of virology and viral vector engineering, gene therapy is a promising approach to treat T1D.

1.6 Vectors for gene therapy

There are many ways of delivering transgenes into cells. Some examples of non-viral delivery systems include: direct injection of DNA into tissues or cells, lipofection and modified bacteria or yeast. However, the levels of transgene expression achieved by all these methods are lower than those achieved with viral vectors [94]. The 5 main classes of vectors that can be clinically applicable are derived from oncoretro-virus, lentivirus, adenovirus (Ad), adeno-associated virus (AAV) and herpes simplex virus (HSV) [95]. There is not a single vector system that has been shown to be superior to the others. To choose an appropriate vector for a target cell type, several variables must be considered, which include vector tropism, stability of transgene expression and safety and immunogenicity of the vectors [95]. Characteristics of the main vectors used in gene therapy are summarised in table 1.2.

Table 1.2: Characteristics of viral vectors used for gene therapy

Viral vectors	Advantages	Disadvantages
Retroviral	• Can transduce dividing cells only [95] • Allows transgene integration into the host genome [96] • Stable transgene expression [96] • Low immunological response [95]	• Low <i>in vivo</i> transduction efficiency [97] • Random transgene integration into the host genome may induce oncogenic transformation [98, 99] • Cannot carry large transgene [95]
Lentiviral	• Can transduce both dividing & quiescent cells [96] • Broad host range [97] • Stable transgene expression [97] • Low immunological response [96]	
Herpes simplex viral	• Can transduce both dividing & quiescent cells [100] • Broad host range [101, 102] • Can transduce neurons [100, 103] • High transgene capacity [100]	• Can induce antiviral immune response [95] • Episomal transgene expression leads to transient expression [104]

Table 1.2 (Continue): Characteristics of viral vectors used for gene therapy

Viral vectors	Advantages	Disadvantages
Adenoviral	• Can transduce both dividing & quiescent cells [105, 106] • High titres of vector stocks • Broad host range [95]	• Can initiate strong immunological response [107-109] • Episomal transgene expression leads to transient expression [105, 106]
Recombinant adeno-associated viral	• Can transduce both dividing & quiescent cells [110] • Transgene can either remain episomal or integrate into the host genome [111-115] • Stable transgene expression [116] • High titres of vector stocks [110] • Broad host range [116]	• Low transgene size capacity [95] • Can trigger pre-existing antiviral immune response leading to a reduction in transduction efficiency [117, 118]

1.6.1 Retroviral and lentiviral vectors

Retroviral and lentiviral vectors are derived from oncoretro viruses [97]. Most retroviral vectors are derived from the mouse Moloney retrovirus [119], while the majority of lentiviral vectors are based on modification of the HIV-1 virus [97]. Both lentiviral and retroviral vectors are suitable for therapies requiring long-term expression of transgenes, because they allow integration of transgenes into the host genome [96]. One limitation of retroviral vectors is that they can only transduce dividing cells [95]. This reduces the *in vivo* transduction efficacy as the target cells may be in a quiescent state. Different from

retroviral vectors, lentiviral vectors can transduce quiescent cells without generating any significant immunological response [96]. Together with their ability to induce long-term transgene expression, lentiviral vectors are considered to be suitable candidates for delivering genes to treat T1D.

One concern about the use of retroviral and lentiviral vectors is the risk of generation and co-infection of pathogenic replication-competent virus [120]. Lentiviral vectors were engineered to address this issue. The wild type HIV-1 virus consists of two copies of a single stranded RNA genome encoding the following: (1) *Gag*, *Pol* and *Env* genes encoding viral structural proteins, (2) regulatory or accessory genes that can inactivate the host antiviral response and (3) *Rev* and *Tat* regions encoding proteins that regulate viral gene transcription and nuclear export of viral transcripts [97]. The viral genome is flanked by long terminal repeats (LTRs) that are required for reverse transcription and integration [97]. The viral genome dimerisation and the packaging signal are located between the long terminal repeat and the *Gag* gene [97]. To reduce the risk of producing replication competent virus, the latest generations of lentiviral vector systems are separated into 4 plasmids. These are: (1) a packaging construct containing *Gag* and *Pol* without the viral accessory genes and *Tat*, (2) a plasmid expressing *vesicular stomatitis virus envelope glycoprotein G* (*VSV-G*) instead of *Env*, (3) a plasmid containing *Rev* and (4) a transfer vector construct containing the transgene, the LTRs and the packaging signal [97]. The first 3 plasmids were carefully engineered without the packaging signal and the LTRs, so that they would not be packaged into the capsids [97]. The replacement of the *Env* gene by *VSV-G* significantly improved the tropism of the vector toward a wider range of cells [97]. As a result, the lentiviral vectors are made from a *VSV-G* capsid containing the transgene with all the essential elements that are required for the reverse transcription and integration without expressing any HIV protein [97].

Another concern about integrating vector systems (including the lentiviral and especially retroviral vectors) is that they may induce oncogene expression when the residual viral genome is integrated close to the location of an oncogene region in the host genome. This concern came from the reports of T cell leukaemia development in 5 out of 20 severe combined immunodeficiency (SCID) patients treated with retroviral vectors [98, 99]. Due

to this incident, the number of incident trials and preclinical studies using retroviral vectors have significantly decreased and researchers are also very cautious about the use of lentiviral vectors [94].

Approaches have been taken to engineer safer integrating vector systems. As the LTRs of the lentiviral vectors have enhancer and promoter regions that can bind to host transcription factors, the proximal integration of the LTRs to the location of oncogenes can potentially activate the genes [97]. To address this issue, in the third generation lentiviral vectors, the U3 component of the LTRs, which can act as the viral gene enhancer or promoter, has been deleted [97]. Nevertheless, the true degree of safety of lentiviral vectors is still uncertain. A clinical trial using a lentiviral vector expressing β -globin cDNA to treat β -thalassemia-based anaemia showed that about 3% of the transduced cells had the transgenes integrated into a high mobility group *AT-hook2* gene (*HMGA2*) encoding chromatin-modifying protein [121]. The dysregulation of the *HMGA2* gene has been linked to cellular de-differentiation and oncogenic transformation [119]. However, this integration has not resulted in malignancy [119]. An *in vitro* study has shown that the random integration of the transgene delivered by the lentiviral vectors induced alternative splicing and generated chimeric or aberrant transcripts in more than 50% of the target cells, but the accumulation of the transcripts was low due to nonsense-mediated mRNA degradation [122].

1.6.2 Herpes simplex viral vectors

Different from retroviral and lentiviral vectors, HSV vectors do not allow integration of the transgene into the host genome [104]. One advantage of this vector system is that it has the largest transgene packaging capacity of up to 40kb of DNA [100]. Another advantage is the ability of HSV vectors to transduce a number of human cell types, especially neurons [101, 102]. Similar to the wild type HSV-1 virus that can establish persistent latent infection, the HSV vectors allow transgenes to be persistently present in neurons without integration [100, 103]. This characteristic makes HSV vectors attractive for gene therapy for neuropathological disorders [95]. On the other hand, as the prevalence of wild type HSV-1 exposure in the human population is high, the viral vector can trigger inflammatory and immunological reactions that may cause negative

consequences to the host [95]. In addition, the lack of understanding of the mechanisms by which HSV vectors attach to and enter the target cells has limited studies of these vectors for gene therapy for nervous system disorders [95].

1.6.3 Adenoviral vectors and helper-dependent-adenoviral vectors

Adenoviral vectors (Ad) have also been extensively studied. There are about 57 wild type adenovirus serotypes that can infect human cells, but most of the vectors are based on Ad2 and Ad5 [123]. Adenoviruses have an icosahedral protein capsid containing linear DNA encoding approximately 35 viral proteins [123]. In replication-incompetent adenoviral vectors, 3 genes (E1A, E3 and E4) encoding proteins that control viral DNA replication in the early stage of infection are usually deleted or replaced by transgenes [123]. Ad vectors can transfect many cell types with high transduction efficiency, but transgene expression is only transient due to the lack of integration of the transgenes into the host genome [105, 106]. The transient transgene expression makes adenoviral vectors unsuitable for gene replacement therapy, where long-term expression of the transgene is required.

Another type of replication-incompetent adenoviral vector is helper-dependent adenoviral vector (HDAd), which has most of the viral genome deleted, except for the packaging signal and the 2 inverted terminal repeats (ITRs) that flank the two ends of the transgene [123]. This approach allows a larger transgene packaging capacity (up to 37 kb) [123]. The production of HDAd requires the presence of replication competent helper Ad vectors, which provide viral proteins that are needed for replication [124]. The helper Ad normally has LoxP sites flanking the packaging genes and the packaging cell line conditionally expresses Cre recombinase which excises the LoxP sites to disable the packaging of helper Ad [125]. The residual Ad is separated from HDAd by ultracentrifugation on cesium chloride density gradients, by anion exchange and by size exclusion chromatography [123].

In the human population, the prevalence of exposure to the wild type adenoviruses is very high [123]. Most people have been infected with at least one serotype leading to life-long immunity [123]. The pre-existing immunity to adenovirus can reduce the transduction

efficiency of the vectors [123]. It was also noted that the innate immune response to adenoviruses is very strong. Adenoviral vectors have also been known to cause immune reactions and hepatic lesions when delivered systemically [107, 108]. The most severe case reported was the death of a patient with ornithine transcarbamylase deficiency, who received a high vector dose (6×10^{11} /kg of body weight) injected into the right hepatic artery [107]. The strong innate immune response to the vectors led to multiple organ failure [109].

1.6.4 Recombinant adeno-associated viral vectors

Recombinant adeno-associated viral (AAV) vectors can also be suitable for T1D gene therapy. The wild type adeno-associated virus is a single stranded DNA virus that relies on a helper virus such as adenovirus, or herpes virus, to replicate [126]. Before being replicated by DNA polymerase of the host cells, the single stranded DNA of AAV must be hybridised with the DNA of the helper virus to form the double stranded DNA [110]. In the presence of adenovirus or herpes simplex virus, adeno-associated viruses undergo the lytic life-cycle [110]. In the absence of the helper virus, adeno-associated viruses establish latency infection by either remaining episomal or by integration into the host genome at a specific site of human chromosome 19 (AAV1 site) [114, 115]. A recent study found the integration of wild type AAV2 in the hepatocytes of 11 of 193 patients with hepatocellular carcinoma [127]. This has led to concern about the insertional mutagenesis potential of AAV vectors.

The single stranded DNA genome of wild type AAV contains the Rep and Cap genes flanked by 2 ITRs [126]. Through the use of 2 different promoters and alternative splicing, the open reading frame of the virus encodes proteins needed for DNA replication and integration (Rep 78, Rep 86, Rep 52 and Rep 48) [110]. The integration ability of the wild type virus is enabled by Rep 78 and Rep 68 proteins [110]. Three capsid proteins (VP1, VP2 and VP3) are generated from the Cap gene by a single promoter (p40) through alternative splicing [110]. AAV vectors are engineered without the Rep genes to prevent the vectors undergoing a lytic cycle. This leads to a significant reduction in the integration ability of the vectors [110]. However, a low level of random integration has been seen both *in vivo* and *in vitro* at a frequency of 10^{-3} to 10^{-4} [110]. The AAV2 vector has been

found to integrate into transcriptionally active chromatin regions in hepatocytes [111]. The possibility of cancer-related genes targeted by AAV vector integration is quite low (3.5%) [112]. Despite the significantly lower integration frequency compared to the retroviral and lentiviral vectors, AAV vectors have been found to promote long-term (few years) transgene expression in both small and large animal models [116]. This makes the vector system relatively safe and sufficient for gene therapy. There are currently a number of clinical trials using AAV to transduce several tissues types, including skeletal muscle, cardiac muscle, the central nervous system, synovial joints, eyes, lungs and liver [128].

Another limitation of AAV vectors is that they cannot carry large gene inserts [95]. Whilst the DNA packaging capacity of HSV, and Ad vectors is up to 40 and 37 kb respectively, AAV vectors can only package up to 4.7 kb [110]. This carrying capacity is also a lot lower than the retroviral vectors, which can carry up to 8 kb sequences [110].

Initially, it was thought that systemic delivery (such as through intravenous injection) of vectors should be avoided, because the vectors could be taken up by non-target cells [117]. This may lead to a reduction in transduction efficiency, and systemic immunological responses [117]. In addition, the majority of the human population have been exposed to AAV and have developed neutralising antibodies specific to the viral capsids [128]. AAV capsid epitopes can also be cross-presented on the major histocompatibility complex leading to the elimination of transduced cells by capsid specific cytotoxic T lymphocytes [128]. As a result, the expression of the transgene is significantly reduced. Such immune responses have been observed in clinical trials that delivered AAV carrying factor IX intravenously or through the hepatic vein of patients with severe haemophilia B [118].

Recent studies using directed evolution strategies have shown that the AAV vector capsids can be modified to significantly improve the tropism and transduction efficacy toward the target organs [128]. There are many methods to generate large libraries of new AAV capsids, such as: (1) DNA shuffling to combine fragments of genes encoding several wild type capsids, (2) error prone polymerase chain reaction (PCR) to introduce point mutations into a wild type capsid, and (3) insertion of DNA sequences encoding

random peptides into the open reading frame of the capsid genes [128]. The libraries of new serotypes are then placed under several rounds of *in vivo* and/ or *in vitro* selection, such as affinity columns, transduction of cultured targeted cells with or without the presence of antibodies, or transduction of animal models [128]. By applying such selection pressures, new serotypes that are less likely to be neutralised by antibodies, and have higher tropism to the target cells, can be generated. These new vectors may allow higher transduction efficiency and improve transgene expression.

As a suitable vector system for gene therapy to treat chronic conditions such as T1D, the vectors should allow stable and long-term transgene expression. Retroviral, lentiviral and AAV vector systems are good candidates as they all allow long-term transgene expression with less immunotoxicity than the HSV-1 and the Ad vectors. In comparison to retroviral and lentiviral vectors, AAV vectors may be the safer choice due to their lower risk of insertional mutagenesis.

1.7 Target cells for T1D gene therapy

The ideal IPCs should be able to regulate glucose homeostasis in a similar manner to pancreatic β -cells. Therefore, IPCs would ideally be able to store insulin in granules and have a glucose sensing and processing machinery similar to what is normally present in the pancreatic β -cells, including *GLUT2*, *Gck*, *PC1* and *PC2* [93]. Pituitary cells, gut K cells, muscle, liver, and pancreas exocrine cells, which have some features in common with pancreatic β -cells, have been considered as potential target cells for T1D gene therapy.

Pituitary cells are thought to be good target cells because they have an exocytosis system as well as proinsulin-processing enzymes [129]. The transfection of insulin, together with *GLUT2* and *Gck* genes, into these cells resulted in both constitutive and glucose-responsive release of insulin [129]. However, the co-release of adrenocorticotrophic hormone inhibited insulin function [129].

Gut K cells are enteroendocrine cells secreting gastric inhibitory peptide and incretin in the intestine [130]. They possess *GLUT2*, *Gck*, *PC1* and *PC2*, allowing them to sense glucose levels and process proinsulin [131]. Diabetic transgenic mice expressing the insulin gene under the control of the insulinotropic peptide (GIP) promoter produced insulin in K cells [131]. They were able to maintain normal BGL in response to glucose challenges [131]. On the other hand, transgene expression in mature K cells is unlikely to be a lasting treatment as the gut cells are continuously replaced [131].

Exocrine pancreatic cells could be a good candidate for generating IPCs because they have the same pancreatic endodermal origin as β -cells [60]. *In vitro* experiments have shown that dissociated pancreatic exocrine cells cultured in epidermal growth factor and leukemia inhibitory factor can transdifferentiate into IPCs [132]. *In vivo* experiments revealed that transducing the pancreas of streptozotocin (STZ)-induced diabetes mice with Ad vectors carrying *Pdx1*, *Ngn3* and *Mafa* led to the transdifferentiation of the pancreatic exocrine cells into IPCs [133]. These IPCs expressed a number of endogenous β -cell genes such as: *GLUT2*, *Gck*, *PC1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1* without expressing other hormones such as glucagon, somatostatin or pancreatic polypeptide [133]. Several exocrine genes (such as: *P48*, *amylase*, *Ck19*, *nestin* or *vimentin*) were also down regulated in these cells [133]. The BGLs of the mice were also improved significantly, but normal BGLs were not achieved [133].

Both muscle and liver cells have been the target of gene therapy for T1D as they are organs that can sense the changes in the BGL. Muscle cells have the insulin-stimulated glucose transporter GLUT4 and hexokinase II, which can function as a glucose sensing system [134]. Similar to pancreatic β -cells, hepatocytes have GLUT2 and Gck. On the other hand, neither muscle nor liver cells have an exocytosis system, or proinsulin processing endopeptidases. To overcome the lack of insulin processing enzymes in these target cells, the furin-cleavable insulin gene (*INS-FUR*) and single chain insulin gene analogs have been engineered [135, 136]. As furin is abundant in both liver and muscle, *INS-FUR* can be processed into its bioactive form [135].

Transplantation of smooth muscle cells expressing *INS-FUR* under the regulation of a glucose-regulatable-promoter resulted in an improvement in BGL in rats [137]. Double transgenic mice expressing both *INS-FUR* and Gck proteins in muscle cells were able to withstand diabetic hyperglycaemia induced by STZ [134]. The *in vivo* transduction of skeletal muscle with AAV1 vectors carrying *INS-FUR* and *GCK* in STZ and alloxan induced diabetic dogs led to reversal of diabetes [138]. The euglycaemic state was maintained for up to 1600 days, and hypoglycaemia was not observed during exercise [138]. The animals also demonstrated glucose-responsive insulin release, however the amounts of insulin released and the BGLs during the oral glucose tolerance test were significantly different from that of the normal healthy controls [138].

The liver and liver cell lines are good targets for T1D gene therapy. Compared to the pituitary cells and gut K cells, muscle and liver cells may be better candidates for T1D gene therapy because it is easier to deliver transgenes to these organs. Compared to the liver, the efficiency of secretion of insulin from muscle fibres into the circulation is a lot lower, and administration of the vectors to the muscle is associated with a higher risk of triggering an anti-transgene immune response [116]. Similar to pancreatic β -cells, hepatocytes also have a glucose sensing system comprised of GLUT2 and liver specific Gck enzyme [139]. In addition, both the liver and the pancreas develop from the same endodermal origin, which can potentiate the liver-to-pancreas transdifferentiation [139]. These advantages make the liver an attractive target organ for gene therapy. Various degrees of liver-to-pancreas-transdifferentiation have been observed in the livers and liver cell lines depending on the types of transgene and vector systems employed in the therapy.

1.8 Liver-directed gene therapy

The possibility of generating IPCs from hepatocytes has been demonstrated in earlier studies from our laboratory. Transfecting a human liver cell line (HEP G2) with a mammalian expression vector carrying the full-length human insulin complementary DNA (cDNA) (HEP G2ins) led to regulated release of insulin, however the cells were not responsive to glucose, due to the absence of GLUT2 in this cell line [140]. The subsequent expression of *GLUT2* in the HEP G2ins cells (HEP G2ins/g) resulted in the formation of

insulin-secretory granules and a glucose-stimulated insulin release *in vitro* [141]. This was the first remarkable *in vitro* study that showed the possibility of inducing a liver-to-pancreas transdifferentiation using gene therapy. However, following transplantation into immunoincompetent mice, these cells lost the ability to regulate insulin secretion due to shut down of *GLUT 2* expression *in vivo* [142].

The earliest remarkable report of *in vivo* liver-to-pancreas-transdifferentiation was from Ferber et al. [143]. They were able to deliver *Pdx1* to the liver by systemic injection of a first generation Ad vector carrying the *Pdx1* gene to STZ-induced diabetic mice [143]. This resulted in the expression of *mouse insulin 2*, *glucagon*, *somatostatin* and a number of downstream pancreatic β -cell transcription factors, as well as a reduction in BGL [106, 143]. However, the amount of insulin detected in the livers of the treated mice was 100 times lower than that of normal pancreas, and exocrine transdifferentiation into trypsin producing cells led to hepatitis in the treated animals [106, 143]. Another limitation for applying this therapy in the clinical setting is the potential immune reactions to the Ad vectors that have been demonstrated in both *Rhesus macaque* and humans [107, 109, 144].

One of the main limitations of many liver directed gene therapies is the transient nature of insulin expression. Ferber et al. (2003) demonstrated that delivering *Pdx1* under either a universal promoter or a liver specific promoter, by HDAd, to STZ-induced-diabetic mice resulted in a reduction in BGL for about one week only. *In vivo* transduction of parenchymal hepatocytes by an AAV vector carrying the *Ngn3* gene resulted in transient insulin secretion [145]. The transient insulin expression may be a result of the lack of integration of the transgene into the host genome due to the choice of vectors for the therapies.

To prolong the transgene expression without using vectors that allow DNA integration, transcription factor genes were co-delivered with *betacellulin*. Kojima et al. [146] co-delivered the *NeuroD1* and *betacellulin* genes to the livers of STZ mice by HDAd vectors. This therapy improved the BGLs of the treated mice [146]. Processing endopeptidases as well as other upstream and downstream pancreatic transcription factors were detected in

the mouse livers [146]. The mice that received the combination of *NeuroD1* and *betacellulin* responded to a glucose challenge, but the response was not the same level as the healthy controls [146].

When the livers of STZ-induced diabetic mice were co-transfected with *Ngn3* and *betacellulin* by HDAd vectors, normal BGLs were restored and insulin was detected in the liver using immunofluorescence [145]. The livers also expressed mRNA of many pancreatic hormones and transcription factors such as: *Glucagon*, *somatostatin*, *pancreatic polypeptide*, *Pdx1*, *NeuroD1*, *Pax4*, *Pax6*, *Nkx2.2* and *Nkx6.1*. The hepatic insulin release was also found to be glucose responsive, but the amount of insulin released was lower than that of the pancreas [145]. The euglycaemic state was maintained for 6 months despite the fact that insulin could no longer be detected in the hepatocytes of the transduced animals by immunofluorescence [145]. The authors discovered that a stable long-term insulin expression was achieved as *Ngn3* triggered islet neogenesis in the endoderm-derived oval cells of the periportal region [145]. It should be emphasised at this point that *betacellulin* would not be suitable for use in humans as it is a mitogen.

Work in our laboratory has previously shown that transduction of the rat liver cell line H4IIE with a retroviral vector carrying *NeuroD1* (H4IIE/ND) led to the expression of rat insulin as well as a range of rat pancreatic transcription factors [147]. The H4IIE/ND cells could also store and secrete insulin, but did not have glucose-responsive insulin release [147]. By comparison, the co-transfection of H4IIE cells with *INS-FUR* and *NeuroD1* (H4IIEins/ND) significantly increased the amount of both human and rat insulin storage and secretion, and induced a glucose-responsive insulin release [147]. The subcutaneous transplantation of H4IIEins/ND cells into diabetic immunoincompetent mice led to the reversal of the diabetic state within 14 days [147]. During the intraperitoneal glucose challenge test, the glucose dose response curves of the transplanted mice peaked at significantly lower BGLs compared to healthy control mice [147].

Different from the studies involving the transfection of pancreatic transcription factors, our laboratory has achieved a higher level of insulin secretion that is responsive to the fluctuation of BGLs. The transduction of hepatocytes *in vitro* with the lentiviral vector

(HMD) carrying *INS-FUR* driven the hybrid HIV-mouse stem cell virus promoter (MSCV) (HMD/*INS-FUR*) resulted in insulin production with the formation of insulin secretory granules [148, 149]. Pancreatic transdifferentiation was observed after *in vivo* transduction of the livers by a surgical procedure involving full flow occlusion (FFO) of the hepatic circulation and intervallic infusion of HMD/*INS-FUR* [148]. The livers of the transduced rats spontaneously expressed several pancreatic β -cell transcription factors [148]. The transduced rat hepatocytes also had insulin granules which allowed insulin to be secreted in response to a glucose challenge [148]. Human *INS-FUR* was expressed in the transduced animals, but rat insulin could not be detected [148]. Diabetes in STZ-diabetic rats was reversed for up to 500 days (the end point of the experiment) [148]. This therapy has been repeated in spontaneously diabetic, non-obese diabetic (NOD) mice, and a pancreatectomised pig model with similar results were achieved [150-152].

To study the effect of the HMD/*INS-FUR* vector system on human hepatocytes in an *in vivo* setting, this therapy was also applied to a chimeric mouse-human liver model (humanised FRG mouse model). The FRG mice carry 3 gene deletions: *fumarylacetoacetate hydroxylase* gene (*FAH*), *recombination-activation-2*, and *Interleukin 2 receptor subunit gamma* genes [153]. The mice are severely immunodeficient such that they can accept the engraftment of human hepatocytes [153]. The lack of *FAH* leads to impairment of tyrosine catabolism, in which the toxic metabolite fumarylacetoacetate cannot be eliminated [153]. Accumulation of this toxin damages the murine hepatocytes [153]. Mice can be treated with 2-(2-nitro-4-trifluoro-methylbenzoyl) 1,3-cyclohexedione (NTBC) to stop the conversion of tyrosine into fumarylacetoacetate and nullify the liver damage [153]. To enhance the engraftment of human hepatocytes, prior to transplantation, FRG mice are treated with a urokinase expressing adenovirus, which causes hepatotoxicity and consequent destruction of murine hepatocytes [153, 154]. After human hepatocyte engraftment, cessation of NTBC treatment leads to further damage of murine hepatocytes, but allows the transplanted human hepatocytes to proliferate in the liver of the mice [153]. After the human hepatocytes have successfully repopulated in the mouse livers, the chimeric organs were transduced by the HMD/*INS-FUR* vector using the FFO technique [152]. Diabetes was reversed in these chimeric animals with partial liver to pancreas transdifferentiation of both human and murine

hepatocytes [152]. The expression of both mouse and human pancreatic transcription factors, mouse insulin, human insulin and human c-peptide were also detected in the chimeric livers [152].

There are several limitations to the use of the HMD/*INS-FUR* vector and the FFO surgical technique in a clinical setting. Firstly, there are concerns about the possibility of insertional mutagenesis and the activation of oncogenes due to the random integration of the transgene into the host genome [99, 119]. Secondly, the FFO surgery must be performed to improve the transduction efficiency of the lentiviral vectors. FFO surgery cannot be easily applied in the clinical setting, as it is invasive. Thirdly, the cause of the observed liver-to-pancreas transdifferentiation induced by the therapy developed by our laboratory is not well understood.

1.9 Aims of the research project

Due to the limitations of the lentiviral vector system, the current project aimed to develop a clinically applicable gene therapy system to treat T1DM using a safer vector system and a less invasive technique. The AAV8 vector was considered a good candidate for the therapy because it (i) has high transduction efficiency toward the mouse hepatocytes [155, 156], (ii) allows long-term expression of the transgenes of interest [113], (iii) has lower risk of insertional mutagenesis due to a low integration rate of the transgenes [112], (iv) does not cause severe host immune responses [118], and (v) has not been found to be pathogenic or to exhibit hepatotoxicity [110, 157]. It has been shown that high transduction efficiency can be achieved by incorporation of the AAV8 vector and the liver-specific enhancer-promoter element composed of the apolipoprotein E (ApoE) enhancer and the human α 1-antitrypsin (hAAT) promoter, which is referred to as a LSP promoter [156, 158]. In adult mice, 1 week after AAV8-LSP transduction, 50-100% of hepatocytes expressed the transgene with little vector detected outside the livers [157]. The episomal AAV8 and the transgene could still be detected in the livers of both male and female mice 12 months post-transduction [158].

Previous studies conducted in our laboratory and others have demonstrated that the transfection of an insulin gene or a transcription factor gene alone can induce a partial liver-to-pancreas transdifferentiation and ameliorate diabetes, but does not result in normal glucose-responsive insulin release [138, 145-147]. Therefore, in this project, the co-transduction of *INS-FUR* and pancreatic transcription factors, using the AAV8-LSP vector system with high tropism towards mouse hepatocytes was considered.

It was hypothesised that the *in vivo* co-transduction of *INS-FUR*, *Pdx1* and/or *NeuroD1*, mediated by the non-integrating AAV8-LSP vector system with high tropism toward mouse hepatocytes will sufficiently induce the liver-to-pancreas transdifferentiation of these cells without the use of the FFO surgical procedure. To validate this hypothesis, individual genes (*INS-FUR*, *Pdx1* and *NeuroD1*), or combinations of *INS-FUR*, *Pdx1* and *NeuroD1* were delivered to STZ-induced non-obese-diabetic/severe combined immunodeficiency (NOD/scid) mice using the AAV8 vectors.

It has been previously observed that, in the liver cells, the expression of pancreatic transcription factors can occur as a result of an insult, such as growth in high glucose and exposure to a toxin [159-161]. Liver-to-pancreas transdifferentiation in response to gene therapy was also enhanced by partial hepatectomy [162]. The application of the FFO surgical procedure to isolate the liver from the blood circulation may temporarily induce hypoxia, which could be perceived as an insult by the liver [150, 151]. However the infusion of empty HMD vectors under FFO surgery in NOD mice did not induce the expression of any pancreatic transcription factor other than *Pdx1* [150]. Hence, it was thought that certain components or characteristic of the HMD/*INS-FUR* vector (such as the HMD viral capsid, the MSCV promoter and the ability of the vector to integrate the transgenes into the host genome) may also have contributed to the reversal of diabetes and the partial liver-to-pancreas transdifferentiation process. Therefore, in the current project, the effectiveness of various combinations of the AAV8-LSP vectors, the FFO surgical technique and the empty HMD vectors in treating T1D were also evaluated.

The most significant difference between the AAV8 vector and the HMD vector is that the AAV8 vector does not allow the integration of the transgene into the host genome at high

frequency [110]. Studies of AAV vectors have reported a low level of random integration has been seen both *in vivo* and *in vitro* at a frequency of 10^{-3} to 10^{-4} [110]. The high tendency of the AAV in allowing the episomal expression of the transgene could create a challenge in maintaining a high level of the gene expression in growing animals or in organs that can regenerate, because the non-integrated genes could be lost during cell replication [157]. In order to overcome this limitation, the hybrid non-viral/viral vector system AAV8/*piggyBac* was created [163]. The *piggyBac* transposition system isolated from the cabbage looper moth *Trichoplusia ni* can mediate the transposition of the transgenes into the host genome [44]. The *piggyBac* system consists of 2 different components: (1) transposon and (2) *transposase* that work as a “cut and paste” mechanism, in which the transposase enzyme cut the transgene out of the transposon and inserted it into the host chromosomes [44]. It has been shown that the hybrid AAV8/*piggyBac* can allow the persistent expression of the transgenes in the growing murine liver by increasing the integration frequency of the transgene [163]. In this project, in order to mimic the HMD/*INS-FUR* therapy and to study the effect of the integration of the transgene, we also delivered the *INS-FUR* gene driven by the LSP to the liver using the AAV8/*piggyBac* system.

In summary, the main aims of the project were:

- To examine the possibility of inducing the liver-to pancreas transdifferentiation and revert the STZ-induced diabetes in non-obese-diabetic/ severe combined immunodeficiency (NOD/scid) mice by using the AAV8-LSP vector system to deliver various combinations of transgene *INS-FUR*, *Pdx1* and *NeuroD1*
- To examine the possibility of curing diabetes and inducing the liver-to pancreas transdifferentiation in non-obese-diabetic (NOD) mice by using various combinations of: transgenes (*Pdx1* and *INS-FUR*), vectors (AAV8-LSP, AAV8/*piggyBac*-LSP and the empty HMD containing the MSCV promoter) $\pm$ FFO surgery

Chapter 2:

General Materials and Methods

2.1 Vector constructs

2.1.1 HMD (lentiviral) vector

The HMD lentiviral vectors carrying the enhanced green fluorescent protein (eGFP) reporter gene driven by the MSCV promoter were produced by Dr Binhai Ren (University of Technology Sydney) using previously published protocols [147, 148, 150].

2.1.2 AAV8 and AAV8/piggyBac vector systems

The adeno-associated viral (AAV8) vectors and vector constructs were produced by Ms Neeta Khandekar, Dr Grant Logan and Dr Sharon Cunningham at the Gene Therapy Research Unit, Children's Medical Research Institute (CMRI), Westmead. To visualise the transduced cells by microscopy, the fluorophore reporter genes venus, mCherry and cerulean were incorporated into the AAV8 vector constructs carrying either the *INS-FUR* or *NeuroD1* transgenes. The *INS-FUR* and *NeuroD1* transgenes were codon optimised during the synthesis process to increase the efficiency of protein production. The *Pdx1* transgene used was not codon optimised and did not carry any reporter gene. AAV8 vectors carrying only the reporter genes were included in the experiments to identify the effect(s) of the capsids on the transduced cells or tissues. The expression of all transgenes was driven by the LSPs [164]. To ensure that the vector dosages were the same across the experiments, every new batch of vector synthesised was titred and the titre of both the new and previous batches of the vectors were checked at the same time using qPCR.

In order to study the effects of the integrating transgene expression, the AAV8/piggyBac system carrying the *INS-FUR* construct driven by the LSP (AAV8/piggyBac-*INS-FUR*) was created. The AAV8/piggyBac-*INS-FUR* integrating vector system consisted of 2 different vectors: (1) a transposase vector carrying a gene sequence encoding the transposase enzyme and (2) a transposon vector carrying the *INS-FUR* gene and the reporter gene mCherry. The LSPs was incorporated into both vectors. The list of all the vector systems and the schematic presentation of the AAV8 construct maps that were studied are shown in Table 2.1 and Figure 2.1

Table 2.1: List of the vector systems used in this research work

Vector	Cloned transgene	Reporter gene	Abbreviation for the vector construct
AAV8	<i>INS-FUR</i>	venus or mCherry	AAV8- <i>INS-FUR</i>
AAV8	<i>Pdx1</i>		AAV8- <i>Pdx1</i>
AAV8	<i>NeuroD1</i>	cerulean	AAV8- <i>NeuroD1</i>
AAV8		venus	AAV8-venus
AAV8		mCherry	AAV8-mCherry
AAV8		cerulean	AAV8-cerulean
AAV8/ <i>piggyBac</i>	<i>INS-FUR</i>	mCherry	AAV8/ <i>piggyBac</i> - <i>INS-FUR</i>
HMD		eGFP	HMD

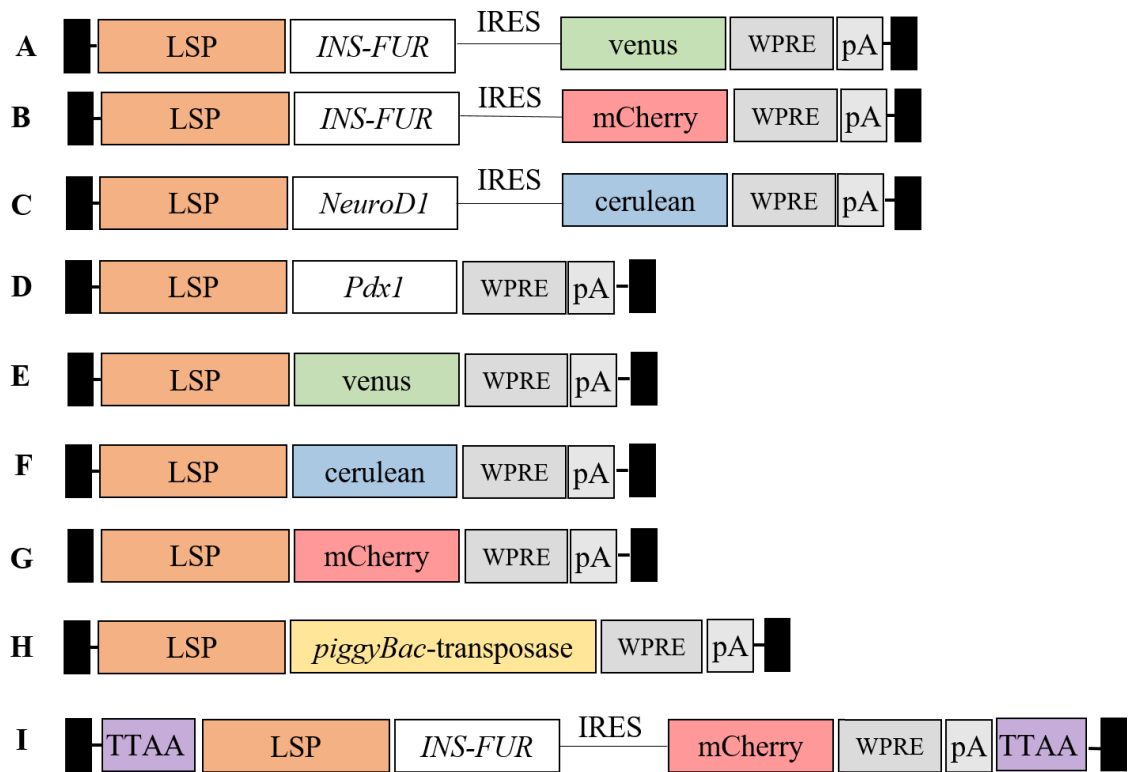


Figure 2.1: Schematic representation of the AAV8 construct maps.

A: AAV8-*INS-FUR* with the venus reporter fluorophore, B: AAV8-*INS-FUR* with mCherry reporter fluorophore; C: AAV8-*NeuroD1*, D: AAV8-*Pdx1*, E: AAV8-venus; F: AAV8-cerulean, G: AAV8-mCherry, H: The transposase construct of the AAV8/*piggyBac-INS-FUR* system and I: The transposon construct of the AAV8/*piggyBac-INS-FUR* system

Key: Black filled rectangles represent the inverted terminal repeat sequences; LSP represents the liver specific promoter; the transgenes used were either *INS-FUR* or murine *Pdx1*, or codon optimised murine *NeuroD1*; IRES represents the internal ribosome entry site; the fluorophores used were either venus (green), mCherry (red) or cerulean (blue); WPRE represents a Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element; pA represents a polyadenylation signal and TTAA represent the transposable marker sites for the transposase enzyme.

2.2 Animal housing conditions, monitoring and care

In vivo experiments were conducted at the University of Technology Sydney (UTS). The project was approved by the UTS Animal Care and Ethics Committee (Approval number 2014-721 and ETH17-1559) and the UTS Institutional Biosafety Committee (Approval number 2014-14-R-G).

2.2.1 Housing conditions

Male NOD/scid mice (6-8 weeks) and female NOD mice (8-12 weeks) were obtained from the Animal Resources Centre (WA, Australia). They were housed within the Ernst Facility at UTS in individual ventilated cages, with the room temperature maintained at 18-24°C and humidity at 40%-70%. Corncob bedding, irradiated mouse feed and reverse osmosis water were provided. All the cages, drink bottles and bedding were autoclaved prior to use. After arriving at the facility, a maximum of 5 mice were housed per cage and BGL monitoring or experiments commenced after at least 7 days to allow for acclimatisation. Cages and drink bottles were routinely changed every 7 days or when they were soiled.

2.2.2 Animal monitoring and care

Weight, BGLs and the mouse grimace scale (MGS) [165] (Appendix 1 and 2) were monitored daily during the experimental period. The daily monitoring record is shown in Appendix 2. Small drops of blood were collected by pricking the tails using 27G needles (BD Precision Glide™) and the BGLs were analysed using an ACCU-CHECK Performa blood glucose meter and test strips (Roche, USA). The detection range of the instrument was 0.6-33.3 mmol/L. When the BGLs of the mice were either under or over the detection limits of the instrument, the values were recorded as 0.6 mmol/L or 33.3 mmol/L, respectively. The mice were considered to be hypoglycaemic when random BGLs were <3 mmol/L and hyperglycaemic when BGLs were > 8 mmol/L. The mice were euthanised when hypoglycaemia occurred whilst the mice were on a normal diet or when the mice lost more than 20% of their body weight.

2.2.3 Intraperitoneal injection of AAV vectors

A solution of 1X phosphate buffered saline (PBS) was prepared by diluting 10X PBS tablets (Amresco, Ohio, USA) in 1 L of dH₂O. The solution was then filtered through a 0.2 µm Minisart® syringe filter (Merck-Millipore ©) and autoclaved. The solution was stored in 15 mL aliquots at -20°C.

On the day of AAV injection, 1X PBS was thawed and filtered through a 0.2 µm Minisart® syringe filter (Merck-Millipore©). AAV8 vectors were added into the 1X PBS solution to the required concentration and injected into the mouse peritoneum using a 30G BD-Ultrafine Insulin Syringe. Each mouse received a single dose of vector and volumes of injections varied between 150-500 µL.

2.2.4 Intraperitoneal glucose tolerance test (IPGTT)

The mice were fasted with an unlimited supply of reverse osmosis water for 6 h prior to conducting the IPGTT. Blood samples were collected at 0,5, 15, 30, 60, 90 and 120 min timepoints. The tests were conducted under anaesthesia by delivering, via inhalation, Isoflurane (VCA, Australia) and oxygen using a Stinger Research Anaesthetic Machine (Advance Anaesthesia Specialist, Sydney, Australia). Before the induction of anaesthesia, the mice were placed into the anaesthetic cage and received pre-operative oxygenation for 5-10 min with the oxygen flow at the rate of 2-3 L/min delivered by the Stinger Research Anaesthetic Gas Machine (Advance Anaesthesia Specialists, Sydney, Australia). Anaesthesia was induced by 2% isoflurane (Attane™, USA) in oxygen (2 L/min) delivered by a vaporiser and breathing system (Advance Anaesthesia Specialists, Sydney, Australia). After the animals lost their pedal and tail reflexes, they were placed on heat mat with their heads in head cones of the Stinger Research Anaesthetic Gas Machine (Advance Anaesthesia Specialists, Sydney, Australia) that delivered 0.5%-1% isoflurane in 2 L of oxygen per min. Respiratory rate and pulse were monitored while the animals were under anaesthesia. A solution of 50% w/v Glucose (Baxter™, Australia) was injected intraperitoneal using a 30G BD-Ultrafine Insulin Syringe (Baxter™, Australia). The animals were kept under sedation for the first 30 min of the test to allow blood collection at the 5, 15, and 30 min time points. The anaesthesia was then ceased and the animals were transferred to recovery cages, where they were allowed to recover

and drink to minimise dehydration. Fifty-five min after the glucose injection, the anaesthesia induction was restarted and maintained using the same amounts of isoflurane as described above to obtain the blood samples at 60 min time point. As soon as the blood collection was completed, anaesthesia was ceased and the animals were allowed to be recovered and rehydration. Another 2 cycles of anaesthesia induction, maintenance and recovery were implemented for the blood collection at 90- and 120-min time points.

2.2.5 Pre-operative care, anaesthesia and monitoring

FFO surgery and portal vein infusion of the HMD vectors were performed under general anaesthesia. The pre-operative, intra-operative and post-operative anaesthesia, analgesia and monitoring protocols were developed based on the current literature following consultation with the institutional veterinary surgeon of the Ernst Facility (UTS), Dr Neroli Sunderland. The anaesthesia, operative monitoring and care plans are shown in Appendix 3 and 4.

Prior to the procedures, the baseline weights, BGLs and MGS of the animals were recorded. The mice received subcutaneous (s.c.) injections of 1.5 µg Buprenorphine (Reckitt Benckiser, UK) in isotonic saline (0.9% NaCl) (Baxter™, Australia) and 0.7 mL of Hartmann solution (compound sodium lactate) (Baxter™, Australia) as pre-operative analgesia and hydration [166]. The abdominal area was shaved and cleaned with 80% (v/v) ethanol.

Before induction of anaesthesia, the mice were placed into the anaesthetic cage and received pre-operative oxygenation for 5-10 min with the oxygen flow at the rate of 2-3 L/min delivered by the Stinger Research Anaesthetic Gas Machine (Advance Anaesthesia Specialists, Sydney, Australia). Anaesthesia was induced by 2-4% isoflurane (Attane™, USA) in oxygen (2-3 L/min) delivered by a vaporiser and breathing system (Advance Anaesthesia Specialists, Sydney, Australia) until the surgical anaesthesia stage was achieved. The surgical anaesthesia stage was determined as the stage when the animal lost pedal, tail and eye reflexes, with a respiratory rate of 40-80 breaths/min [166-168]. After the surgical anaesthesia stage was achieved, the vital signs of the animals

(respiratory rate, heart rate, blood oxygen saturation and temperature) were monitored at 5-min-intervals.

After anaesthesia had been achieved, Viscotears (Novartis, France) was applied to the eyes to prevent abrasion and the animals were transferred to a preheated surgical heat mat (PhysioSuite, Kent Scientific, USA) with the temperature maintained at 34-37 °C. The animal was placed in a supine position with its head in the head cone of the Stinger Research Anaesthetic Gas Machine (Advance Anaesthesia Specialists, Sydney, Australia) that delivered 0.5%-2% isoflurane in 2 L of oxygen per min. The mice were secured to the heat mat by Micropore tape and were appropriately positioned under a surgical microscope (Leica™, Australia). A thermometer (PhysioSuite, Kent Scientific, USA) was placed between the animal and the heat mat to measure the peripheral temperature. A second thermometer was applied to the rectum to measure the core temperature of the animal. A pulsoximetry probe (PhysioSuite, Kent Scientific, USA) was applied to the tail of the mouse to measure the heart rate and the oxygen saturation of the blood. Local analgesia with Bupivacaine (Marcain®, AstraZeneca, Australia) at a dose of 6 mg/Kg was injected into the intradermal region of the abdomen. The area was cleaned with 80% ethanol and surgical drapes were applied.

2.2.6 Intra-operative care, anaesthesia and monitoring

Surgical anaesthesia was maintained by delivering 1%-2% isoflurane in 1-2 L of oxygen per min via a head cone of the Stinger Research Anaesthetic Gas Machine (Advance Anaesthesia Specialists, Sydney, Australia). The level of isoflurane was titrated to maintain the surgical anaesthesia stage with a respiratory rate of 40-70 breaths per min, a heart rate of 350- 450 beats per min and blood oxygen saturation of 95-100% [169].

Laparotomy, followed by the FFO surgery, either with or without the portal vein infusion of the HMD vector, were performed by Dr Binhai Ren (UTS) under a surgical microscope as described in previous publications [147, 148, 150, 152]. Immediately after the laparotomy, 50-100 µL of warmed isotonic saline (Baxter™, Australia) was dripped into the abdominal cavity. During the FFO procedure, the adrenal vein, infrahepatic vena cava, portal vein, suprahepatic vena cava and hepatic artery were clamped for 1 min. The

transduction of the liver by the HMD vector was performed by injecting 5×10^6 TU of the vector (diluted in 50 μ L of PBS) into the portal vein. Bleeding from the portal vein puncture site was controlled by an Ethicon Surgicel Original Absorbable Hemostat (Ethicon, Johnson & Johnson, USA). After the FFO surgery, or the portal vein infusion, 100 μ L of isotonic saline (BaxterTM, Australia) was dripped into the abdominal cavity before the muscle and the skin of the cavity was closed using dissolvable surgical sutures (Ethicon, Johnson & Johnson, USA).

2.2.7 Post-operative care

After the closure of the abdominal cavity, isoflurane was ceased while the oxygen flow was maintained at 2 L/min. Povidone iodine (Betadine, France) (10% w/v) was applied to the wound and 20 mg of Ampicillin (Sandoz, France) was given intramuscularly to prevent any surgery-related infections. Isotonic saline (BaxterTM, Australia) (0.6 mL), as an s.c. injection. Weights and BGLs of the mice were recorded. The mice were kept on the surgical heat mat with oxygen supplied via the head cone at 2 L/min until the animals were fully awake, regained righting reflex and could move with 4 limbs [166].

After regaining consciousness, the mice were transferred to heated cages with soft bedding. The food and water bowls placed on the bedding. The weight and BGLs were monitored for 4 h before returning the animals to the regular housing facility. Isotonic saline (Baxter, Australia), Hartmann's solution (Compound Sodium Lactate, Baxter, Australia) or glucose were given s.c. to correct post-operative hypoglycaemia or excessive fluid loss. Four hours after the surgery, s.c. injections of 1.5 μ g buprenorphine (Reckitt Benckiser, UK) in isotonic saline (Baxter, USA) and 0.6 mL Hartmann's solution (Baxter, USA) were given for analgesia and hydration.

During the first 2 days after surgery the mice were monitored at least 3 times per day for the appearance of the wound, weights, BGLs, oral intake and signs of pain (MGS, posture, condition of the coat and level of activity). Ampicillin (Sandoz, Germany) (20 mg) s.c. was given 1-2 times per day to prevent any surgery-related infections. Various dosages of Buprenorphine (Reckitt Benckiser, UK) in isotonic saline (Baxter, Australia), and Hartmann's solution (Baxter, Australia) s.c. injections were provided according to the observations to minimise pain and maintain body weight. From day 3 after surgery, the

animals were monitored and received post-operative care (heat, antibiotics, hydration fluid and analgesia injections) at least two times per day. The frequencies of the observation and post-operative care were gradually reduced until the animals resumed a normal level of activity and oral intake, MGS of 2 or less and the laparotomy wounds were completely healed.

2.3 Tissue collection and storage

At the experimental endpoint, the animals were euthanised by cardiac bleed under anaesthesia using isoflurane. Blood serum and other organs were collected for subsequent analysis. The livers of the transduced animals were analysed for the levels of transgene expression and the degree of liver-to-pancreas transdifferentiation by reverse transcriptase polymerase chain reaction (RT-PCR), quantitative RT-PCR (RT-qPCR) and immunohistochemistry to detect the expression of various pancreatic β -cell hormones and transcription factors. The untransduced, non-diabetic mouse livers were used as the negative control, and the pancreas from the mice that had normal BGL were used as the positive control tissue.

Once collected, the organs were washed in sterile isotonic saline (0.9%) (Baxter, USA). The livers were snap frozen in dry ice for DNA and RNA extraction and all the samples were stored at -80°C until required. Due to the higher rate of degradation, the pancreases were flash frozen in dry ice and RNA was extracted immediately following organ collection. For the immunohistological studies, the livers and pancreas were embedded in optimal cutting temperature (OCT) compound (VWR International BVBA, Belgium) and frozen in dry ice. All samples collected were stored at -80°C.

2. 4 DNA extraction from mouse livers

2. 4.1 DNA extraction

Liver DNA was isolated using the Maxwell[®] RSC Tissue DNA Kit and the Maxwell[®] RSC instrument (Promega, USA). The Maxwell[®] RSC Tissue DNA Kit contained TE buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA), elution buffer and cartridges that were

preloaded with lysis buffer and other reagents. Each 50 mg of liver tissue was homogenised in 80 µL of TE buffer and 20 µL of RNase A solution (4 mg/mL) (Promega, USA). The homogenates were then loaded into the designated wells of the Maxwell[®] RSC Tissue DNA cartridges containing lysis buffer. The homogenised tissue samples were mixed with the lysis buffer by pipetting 10 times. The DNA extraction was performed by the instrument according to the pre-set program of the manufacturer. The DNA samples were eluted in elution buffer and stored at -80°C until required for quantitative PCR to measure AAV8 vector copy number.

2.4.2 Quality and quantity assessment of isolated DNA

The concentration and purity of the DNA samples was assessed using the NanoDrop[™] 2000 spectrophotometer (Thermo Fisher Scientific, USA). Measuring the ratio of sample absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) gave an estimation of the sample purity. The samples with A_{260}/A_{280} ratio of 1.8 – 2.0 were considered to be of satisfactory quality. Samples with A_{260}/A_{280} ratio less than 1.8 were not used for analysis.

2. 5 RNA extraction from mouse tissues

2. 5.1 RNA extraction

RNA was isolated using the Maxwell[®] RSC simply RNA Tissue Kit and Maxwell[®] RSC instrument (Promega, USA) according to the protocol published by the manufacturer with a few modifications. The Maxwell[®] RSC simply RNA Tissue Kit contained nuclease-free water, homogenisation solution, 1-Thioglycerol, lysis buffer, DNase I and Maxwell[®] RSC cartridges, which contained preloaded reagents. To prevent contamination by exogenous ribonucleases (RNase), all the surfaces were washed with 80% (v/v) ethanol and treated with RNase Away[®] (Thermo Fisher Scientific, USA). All plastic ware was autoclaved before use.

Each 15 mg tissue sample was homogenised in 0.2 mL homogenisation solution containing 1-Thioglycerol (0.004% v/v). Proteinase K (20mg/mL) (35 µL) was added to the homogenate to increase homogenisation of the tissue. In the case of pancreas tissue, 80 U of RNase inhibitor (Promega, USA) was also added to the homogenised tissue to

prevent RNA degradation. The homogenates were then mixed with 0.2 mL lysis buffer and vortexed to mix. The mixture (0.4 mL) was then added into each cartridge. To remove genomic DNA contamination, DNase I was added into the appropriate well of the cartridge. The isolation process was performed by the instrument according to the pre-set program of the manufacturer. The RNA samples were eluted in nuclease-free water and stored at -80°C until required for complementary DNA (cDNA) synthesis.

2.5.2 Quality and quantity assessment of isolated RNA

The quality of the isolated RNA was assessed by agarose gel electrophoresis. A 2 µL aliquot of each RNA sample was mixed with an equal volume of 5X gel loading dye (Bioline) and 6 µL of sterile water. The samples were loaded into 1% (w/v) agarose gels containing 0.005% (v/v) Gelred® (Biotium). Electrophoresis was carried out at 100 Volts (V) in 1X TBE buffer (90 mM Tris-Borate, 0.5 mM EDTA, pH 8.3). Gels were visualised under UV light and images were captured using the InGenius3® system and processed using Syngene® software. RNA samples were considered to be of satisfactory quality if the 28S and 18S rRNA bands could be visualised clearly.

The concentration and purity of the RNA samples was assessed using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). Measuring the ratio of sample absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) gave an estimation of the sample purity. The samples with A_{260}/A_{280} ratio of 1.8-2.0 were considered to be of satisfactory quality. Samples with A_{260}/A_{280} ratio less than 1.8 were not used for analysis.

2.6 Removal of genomic DNA from RNA extracts

Despite the fact that the RNA isolation procedure using the Maxwell RSC instrument included a DNase digestion step, the samples were further treated with DNase I (Amplification Grade, Invitrogen, USA) to remove any residual genomic DNA. The DNase I reaction constituents are shown in Table 2.2. DNase I digestion was carried out for 15 min at room temperature, after which 1 µL of EDTA (25 mM) was added to each reaction and the DNase enzyme was inactivated by heating (65°C, 10 min).

Table 2.2: Reaction constituents for removal of contaminating genomic DNA from RNA samples by DNase digestion

Reaction component	Volume (μL)
RNA sample (1 μg)	Variable (dependent on the RNA concentration)
10X DNase I Reaction Buffer	1
DNase I (1 U/ μL)	1
DEPC-treated water	Variable (depending on total reaction volume)
Total reaction volume	10

2.7 Synthesis of complementary DNA (cDNA)

The synthesis of cDNA from the RNA samples was performed using the Omniscript[®] Reverse Transcriptase Kit (Qiagen) or the Tetro cDNA Synthesis kit (Bioline) according to the manufacturers' protocol. The reaction mixtures (Table 2.3 and 2.4) were initially set up on ice to avoid premature cDNA synthesis and RNA degradation [170]. cDNA synthesis using the Omniscript[®] Reverse Transcriptase Kit, was carried out at 37°C for 60 min. When using the Tetro cDNA Synthesis Kit, the mixtures were incubated at 25°C for 10 min, followed by 45°C for 30 min. The reaction was terminated by heating the samples to 85°C for 5 min and chilling on ice. Once the reaction was complete, all cDNA samples were stored at -20°C.

Table 2.3: Components of the Omniscript[®] Reverse Transcriptase reaction mixture

Reaction component	Volume (μL)
10X RT Buffer	2
dNTP Mix	2
Random hexadeoxynucleotide primers	2
RNase inhibitor (10 U/μL)	1
Omniscript Reverse Transcriptase	1
RNA mixture (1 μg of DNase1 treated RNA)	10
RNase-free water	2
Total reaction volume	20

Table 2.4: Components of the Tetro cDNA Synthesis Reverse Transcriptase reaction mixture

Reaction component	Volume (μL)
5X RT Buffer	4
10 mM dNTP Mix	1
Random hexadeoxynucleotide primers	1
RiboSafe RNase inhibitor	1
Tetro Reverse Transcriptase (200 U/μL)	1
RNA mixture (1 μg of DNase1 treated RNA)	10
DEPC-treated water	2
Total reaction volume	20

2.8 Preparation of DNA standards for quantitative polymerase chain reaction

AAV8 vector copy number (VCN) was quantitated using real-time PCR targeting the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) that was incorporated into the AAV8 vectors. AAV8 vector copy number was normalised against the copy number of the endogenous *Glyceraldehyde 3-phosphate dehydrogenase*

(*GAPDH*) gene. In order to construct the standard curves for measuring the WPRE and GAPDH copy numbers, AAV8-eGFP (empty AAV8 plasmid) containing the WPRE sequence and the pGEMTEZ-GAPDH containing the GAPDH cDNA sequence were propagated and digested to produce the standards. Both plasmids were provided by Dr Sharon Cunningham from the CMRI.

2.8.1 Preparing media for transformation and propagation of plasmids

Luria Broth (LB) (25 g), containing 10 g of peptone, 5 g of yeast extract and 10 g of NaCl (Life Technologies™, USA) was added to 1 L of ultra-pure water. The pH of the solution was adjusted to 7.5 and then autoclaved.

Media for bacterial transformation (S.O.C media) was prepared by adding 1 mL of 2 M Mg^{2+} (containing $MgCl_2 \cdot 6H_2O$ and $MgSO_4$), 1 mL of 2 M glucose and 0.25 mL of 1 M KCl into 98 mL of LB broth.

Both the AAV8-eGFP and the pGEMTEZ-GAPDH plasmids were selected based on ampicillin resistance. For the purposes of selection, LB broth for growth of these plasmids contained ampicillin at a concentration of 100 μ g/mL. Bacterial culture plates were also prepared by adding 3 g of agar into 200 mL of LB broth and heating the solution until the Agar was completely dissolved. The solution was then cooled to 50°C and ampicillin was added before the mixture was poured into petri dishes.

2.8.2 Propagation of the AAV8-eGFP and pGEMTEZ-GAPDH plasmids

2.8.2.1 Bacterial transformation of the AAV8-eGFP plasmid

The AAV8-eGFP plasmid was propagated using SUR2 supercompetent cells (Agilent, USA). After thawing on ice, 100 μ L of SUR2 cells were transferred to chilled 15 mL Falcon polypropylene round bottomed tubes and 2 μ L of β -mercaptoethanol (1.22 M) was added. The cell mixture was swirled gently and incubated on ice for 10 min. Then 50 ng of the AAV8-eGFP plasmid was added and the mixture swirled gently and incubated on ice for 30 min. The cells were then incubated at 42°C for 30 sec and then placed on ice for 2 min. S.O.C medium (0.9mL) was heated to 42°C and added to the cell mixture. The cells were incubated at 37°C with shaking at 225-250 rpm for 1 h. The bacterial culture

was then spread on selective LB agar plates containing ampicillin and incubated at 37°C overnight.

2.8.2.2 Bacterial transformation of pGEMTEZ-GAPDH plasmids

The pGEMTEZ-GAPDH plasmid was propagated in JM109 cells (Promega, USA). After the cells had been thawed on ice, 50 ng of pGEMTEZ-GAPDH plasmid was added and the mixture was mixed by flicking the tube. The cells were immediately returned to ice for 30 min. The mixture was then incubated at 42°C for 15-20 sec followed by a further incubation of 2 min on ice. S.O.C medium (450 µL) was added to the cell mixture, which was then incubated for 1 h at 37°C with shaking at 225 rpm. Serial dilutions (1:10 and 1:100) of the transformed JM109 cells were made by diluting the original cell mixture with the LB broth containing ampicillin. Aliquots (100 µL) of the undiluted, 1:10 and 1:100 diluted bacterial cultures were subsequently spread on the selective LB agar plates containing ampicillin and incubated at 37°C overnight.

2.8.2.3 Generation of the AAV8-eGFP and the pGEMTEZ-GAPDH plasmid clones

An AAV8-LSP-eGFP transformed SUR2 colony and a pGEMTEZ-GAPDH transformed JM109 colony were picked from the selective LB agar plates. Each colony was inoculated into 3 mL of LB medium containing ampicillin in a 15 mL Falcon tube. The cultures were grown overnight (12-16 h) at 37°C with shaking at 225 rpm.

2.8.2.4 Plasmid preparation

After overnight growth, the bacterial cultures were centrifuged at 6800g for 3 min at room temperature. The supernatant was removed and the plasmid DNA was isolated from the transformed bacteria using the QIAprep[®] Spin Miniprep Kit (Qiagen) as per the manufacturer's protocol. The plasmids were eluted in EB buffer provided with the kit and were stored in Lo-Bind Eppendorf tubes. The concentration of the plasmid DNA was determined using the NanoDrop[™] 2000 spectrophotometer (Thermo Fisher Scientific[™]).

2.8.2.5 Plasmid digestion and purification

Both plasmids were cut at a single restriction point to form linear DNA molecules. The AAV8-eGFP construct was digested at the *SacI* restriction site and the pGEMTEZ-

GAPDH construct was digested at the *StuI* restriction site. Both restriction reactions were carried out overnight at 37 °C using restriction enzymes purchased from New England Biolabs. The components of the reactions are listed in Table 2.5 and 2.6.

Table 2.5: Restriction digest reaction components for digestion of the AAV8-LSP-eGFP plasmid

Reaction component	Volume (μL)
AAV8-eGFP plasmid (10 μg)	Variable (dependent on plasmid concentration)
<i>SacI</i> enzyme (20 U/uL)	10
1.1 Buffer (10X NE Buffer)	5
Nuclease-free water	Variable (depending on final reaction volume)
Total reaction volume	50

Table 2.6: Components of the pGEMTEZ-GAPDH plasmid restriction reaction mixture

Reaction components	Volume (μL)
pGEMTEZ-GAPDH (10 μg)	Variable (dependent on plasmid concentration)
<i>StuI</i> enzyme (10 U/uL)	10
Cut Smart Buffer (10X NE Buffer)	5
Nuclease-free water	Variable (depending on final reaction volume)
Total reaction volume	50

After the restriction digestion, plasmids were purified using the Wizard SV Gel and PCR Clean Up System (Promega, USA) as per the manufacturer's instructions. The purified DNA was eluted in nuclease-free water and stored in Lo-Bind Eppendorf tubes. The concentration of the DNA was measured using the NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, USA) and the number of DNA molecules were calculated according to the molecular weight of each plasmid. The restricted plasmid DNA was diluted with TE buffer to a concentration of 10⁹ molecules/5 μL. Serial dilutions were

performed to achieve the following concentrations: 10^8 molecules/5 μ L, 10^7 molecules/5 μ L, 10^6 molecules/5 μ L, 10^5 molecules/5 μ L, 10^4 molecules/5 μ L and 10^3 molecules/5 μ L.

2.9 Polymerase chain reaction

2.9.1 Oligonucleotide primers

Oligonucleotide primers for the detection, and subsequent quantitation of expression of the genes of interest were purchased from Invitrogen (Thermo Fisher Scientific) and delivered as a lyophilised powder. Primers were reconstituted in TE buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA) at a stock concentration of 200 μ M. All primer stocks were stored at -20 °C until required. All primers were 18-22 bases in length, and all PCR products were 100-220 base pairs (bp) in length. Primers for RT-PCR were designed to cross intron and exon boundaries to prevent amplification of any contaminating genomic DNA if present. All primer sequences are shown in Table 2.7. Primer sequences for the reference gene β -actin and the endogenous target genes *Neuro D1*, *Pdx1*, *P48*, *GLUT2*, *Glucokinase* (*GCK*), *glucagon*, *mouse insulin 1*, *mouse insulin 2*, and *synaptophysin* have been previously described [150]. Primer sequences for the target genes *Neurogenin3*, *Pax4*, *Nkx2.2*, *Nkx6.1*, *Mafa*, *Mafb*, *Proconvertase 1(PC1)* and *2 (PC2)*, *somatosatin*, *Pancreatic polypeptide* and *Pax6* were obtained from searches of qPrimer Depot (www.mouseprimerdepot.nci.nih.gov), a public database of the National Cancer Institute and National Institute of Health (NCBI) [171] providing pre-used primer sequences for quantitative real time PCR. The primer sequences for the codon-optimised transgenes, *INS-FUR* and *NeuroDI*, were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Table 2.7: Sequences of primers used to amplify and quantitate the expression of the target genes of interest.

Gene	Forward Primer Sequence	Reverse Primer Sequence	PCR Product length (bp)
<i>β-actin</i>	ATGGAGGGGAATACA GCCC	TTCTTTGCAGCTCCTTC GTT	149
<i>Pdx1</i>	CCAGTGGGCAGGAGGT GCTTACA	GGGGCCGGGAGATGTA TTTGTTA	128
<i>Nkx2.2</i>	ACTTGAGCTCGAGTC TTGG	AGCCCTTTCTACGACA GCAG	121
<i>Nkx6.1</i>	GAAGTCCCCTTGAGCC TCTC	TATTCTCTGGGGATGA CGGA	140
<i>NeuroD1</i>	GCTCCAGGGTTATGAG ATCG	CTCTGCATTCATGGCTT CAA	205
<i>MafA</i>	CTCTGGAGCTGGCACT TCTC	AGGAGGTCATCCGACT GAAA	124
<i>P48</i>	GAAGGTTATCATCTGC CATCG	GGGTGGTTCGTTCTCT ATGTT	211
<i>PC1</i>	TTTCCAACCTGGGTTA CTGG	GGCTGCTGGTATCTTT GCTC	121
<i>PC2</i>	AAATCCTTCTTGTTGC AGCG	TTGCAGAAGGCCTGTA TCACT	128
<i>GLUT2</i>	TCTTCACGGCTGTCTCT GTGCT	AGCAGCACAAGTCCCA CCGAC	118
<i>GCK</i>	GTCGCAGGTGGAGAGC GACTC	GCGCACAATGTGCGAG TCGG	109
<i>Glucagon</i>	ACCCAGATCATTTCCC AGCTTCCC	AATCTTGGGCACGGCG GGAG	135
<i>Somatostatin</i>	CCAGGGCATCATTTCTC TGTC	CCCAGACTCCGTCAGT TTCT	121

Table 2.7 (Continued): Sequences of primers used to amplify and quantitate the expression of the target genes of interest.

<i>Gene</i>	Forward Primer Sequence	Reverse Primer Sequence	PCR Product length (bp)
<i>Mouse insulin 1</i>	TGGCTTCTTCTACACA CCCAAG	ACAATGCCACGCTTCT GC	132
<i>Mouse insulin 2</i>	GAGTCCCACCCACCC AG	TCCACTTCACGGCGGG ACA	125
<i>Synaptophysin</i>	ACCTCGGTGGTGTGTTG GCTTCC	CCTGGAGGTGCGCGCA TGAA	107
<i>Ppy</i>	GCATAGTCGCCTGGGT ACAT	CCAACACTCACTAGCT CAGCA	149
<i>Pax6</i>	CTTCATCCGAGTCTTCT CCG	AGTTGGTGTCTCTCCC CCT	107
<i>Transgene NeuroDI</i>	AGAAGAGGCCCAAG AAAAA	TTGCTCAGCTTCTGGG TCTT	171
<i>INS-FUR (A)</i>	GAGCGGGGCTTCTTC TACAC	TGCAGCACTGTTCCA CGAT	157
<i>INS-FUR (B)</i>	CGCTTTCGTGAACCA GCAC	AGTTCCAACCTGTCC GACCT	135
<i>INS-FUR (C)</i>	GCTTTCGTGAACCAG CACC	GGTCCTCGGCCTCTC TCTT	112
<i>GAPDH</i>	ACGGCAAATTCAAC GGCAC	TAGTGGGGTCTCGCT CCTGG	93
<i>WPRE</i>	CCGTTGTCAGGCAAC GTG	AGCTGACAGGTGGT GGCAAT	85

The lengths of the amplified sequences are expressed as base pairs (bp). Different sets of primer pairs for the same gene are indicated by the labels (A), (B) and (C).

2.9.2 Detection of target gene expression by reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR reactions were performed at the optimised annealing temperature with the reaction mixtures outlined in Table 2.8. The genes of interest were amplified from cDNA derived from either: (1) transduced mouse livers (treated livers), (2) the non-diabetic normal mouse livers (untreated livers) (i.e. negative control), or (3) the normal mouse pancreases (i.e. positive control). PCRs were also performed with RNA samples that had undergone reverse transcription in the absence of reverse transcriptase (no RT control) to test for the presence of any residual contaminating genomic DNA. A PCR reaction without a cDNA template (no template control) was also included in all PCR runs to identify the possibility of contamination in the reaction components. RT-PCR reactions were performed using the Go Taq[®] Green 2X PCR Master Mix (Promega) containing *Taq* Polymerase, dNTPs, MgCl₂ and reaction buffer (pH 8.5). All reagents within the master mix were provided at optimal concentrations for efficient template amplification [172]. PCR reactions were set up on ice to avoid non-specific amplification and primer dimer formation (Table 2.9). After PCR amplification (Table 2.10), the RT-PCR products were loaded onto 2% (w/v) agarose gels containing 0.005% (v/v) GelRed[®] and electrophoresis was carried out at 110 V for 60 min in 1X TBE buffer. The gels were then visualised under ultraviolet (UV) light and images were captured using the InGenius3[®] system and processed using Syngene[®] software.

Table 2.8: Typical reaction constituents for PCR amplification reactions

Reaction component	Volume (μL)
2X Go Taq [®] Green Master Mix	10
Forward primer (20 pmol/μL)	0.3-0.5
Reverse primer (20 pmol/μL)	0.3-0.5
cDNA template (50 ng/μL)	1
Nuclease-Free water	Variable (depending on final reaction volume)
Total reaction volume	20

Table 2.9: RT-PCR amplification parameters

Stage	Steps	Temperature	Duration
Denaturation	Initial cDNA denaturation	95°C	3 min
PCR amplification cycles (repeated 34 times)	Denaturation	95°C	30 sec
	Primer annealing	60°C – 61°C	30 sec
	Extension of annealed primers	72°C	30 sec
Long Extension	Final extension of amplified products	72°C	5 min
Hold	Hold	4°C	Indefinitely

2.9.3 RT-qPCR using SYBR Green

SYBR Green based real time quantitative PCR was carried out on the QuantStudio™ 6 Flex Real Time PCR instrument (Thermo Fisher Scientific, USA). The relative quantification technique requires a reference gene, to which target gene expression was compared. In this project *β-actin* was chosen as the reference gene. This gene was expressed at similar levels in all tissues used in this study. The comparison was possible when the reaction efficiencies of the target genes and the endogenous control gene were similar [173].

RT-qPCR reactions were carried out using the PowerUp™ SYBRGreen® Master Mix (Applied Biosystems, Life Technologies, USA), which contained Uracil-N-glycosylase (UNG) to degrade any previously amplified PCR products that could possibly contaminate the mixture. The reaction components of the PCR mixture are outlined in Table 2.10 and the qPCR amplification parameters are outlined in Table 2.11. Melting curve analysis was incorporated into the qPCR protocols to identify the target products and any potential primer dimers or non-specific products if present. When required, peaks appearing in the melting curves were correlated with the target sequence amplification by electrophoresis of an aliquot of each PCR product on 2% (w/v) agarose.

Table 2.10: Components of the RT-qPCR reactions using the PowerUp™ SYBRGreen® Master Mix qPCR reaction mixture

Reaction component	Volume (μL)
PowerUp™ SYBRGreen® Master Mix (2X)	10
Nuclease-Free water	7
cDNA template (50 ng/μL) (or nuclease-free water for the no-template control)	1
Forward primer (optimal concentration)	1
Reverse primer (optimal concentration)	1
Total reaction volume	20

Table 2.11: Fast RT-qPCR parameters for amplification using the PowerUp™ SYBRGreen® Master Mix

Stage	Steps	Temperature	Duration
Activation	UNG activation	50°C	2 min
	AmpliTaq Fast DNA Polymerase activation	95°C	2 min
PCR (Repeat for 40 cycles)	Template denaturation	95°C	1 sec
	Primer annealing, extension and detection of fluorescence emission	60°C	30 sec
Melting curve analysis	Denaturation	95°C	15 sec
	Annealing	60°C	1 min
	Fluorescence detection and melting curve analysis	60°C to 95°C	
	Denaturation	95°C	15 sec
Hold	Hold	4°C	Indefinitely

2.9.4 RT-qPCR using TaqMan assays

Pre-optimised TaqMan® Gene expression Assays (Applied Biosystems by Life Technologies, USA) were used for the reference gene *β-actin*, and the genes of interest. The expression assays contained TaqMan® probes and the forward and reverse primers. The probe was Fluorescein labelled at one end and had a quencher molecule at the other [173]. The reactions were performed with TaqMan® Fast Advanced Master Mix (Applied Biosystems, USA) according to the manufacturer's instructions. The master mix also contained UNG. The constituents of the reactions are summarised in Table 2.12. The amount of cDNA recommended by the manufacturer was 1 pg to 100 ng. In this project 100 ng of cDNA was added to each reaction as the target genes were expected to be present at low levels. The reactions were performed using the QuantStudio™ 6 Flex Real Time PCR System (Thermo Fisher Scientific, USA) and the method is summarised in Table 2.13.

Table 2.12: Reaction components of RT-qPCR using the TaqMan® Fast Advanced Master Mix qPCR reaction mixture

Reaction component	Volume (μL)
2X TaqMan® Fast Advanced Master Mix	10
Nuclease-Free water	8
cDNA template (50 ng/μL) (or Nuclease-Free water for no-template control)	2
TaqMan® Gene expression Assays	1
Total reaction volume	20

Table 2.13: RT-qPCR parameters for gene expression quantitation using the TaqMan® Fast Advanced Master Mix

Stage	Steps	Temperature	Duration
Activation	UNG activation	50°C	2 min
	AmpliTaq Fast DNA Polymerase activation	95°C	20 sec
PCR	Template denaturation	95°C	1 sec
Repeat for 45 cycles	Primer annealing, extension and detection of fluorescence emission	60°C	20 sec

2.9.5 AAV8 vector copy number (VCN) quantitation by qPCR using SYBR Green

The AAV8 VCNs were quantitated using real-time PCR targeting the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) that was incorporated into the AAV8 vectors. The VCN was normalised against the copy number of the endogenous *GAPDH*. SYBR Green based real time quantitative PCR was carried out on the QuantStudio™ 6 Flex Real Time PCR instrument (Thermo Fisher Scientific, USA). The PCR protocol and the constituents of the reactions are shown in Table 2.11 and 2.14.

In order to construct the standard curves for measuring the numbers of WPRE and GAPDH copies, qPCR was performed for a set of serial dilutions of the linearised AAV8-eGFP (containing the WPRE sequence) and the pGEMTEZ-GAPDH (containing the GAPDH cDNA sequence) plasmids. (The serial dilution of the linearised plasmids was formed at the following concentrations: 10^8 molecules/ 5 μ L, 10^7 molecules/ 5 μ L, 10^6 molecules/ 5 μ L, 10^5 molecules/ 5 μ L, 10^4 molecules/ 5 μ L and 10^3 molecules/ 5 μ L). qPCR for the standards and the DNA extracted from the liver of each animal were performed in triplicate and the experiments were repeated 3 times. The copy numbers of WPRE and GAPDH in the livers were read directly from the standard curves. The AAV8 VCN was reported as either the number of WPRE per 50 ng of total DNA or the number of WPRE per GAPDH.

Table 2.14: Components of the RT-qPCR reactions using the PowerUp™ SYBRGreen® Master Mix qPCR reaction mixture

Reaction component	Volume (µL)
PowerUp™ SYBRGreen® Master Mix (2X)	10 µL
Nuclease-Free water	4.4 µL
DNA standards or liver DNA (50 ng) (or nuclease-free water for the no-template control)	5 µL
Forward primer (optimal concentration)	0.3 µL
Reverse primer (optimal concentration)	0.3 µL
Total reaction volume	20

2.10 Detection of the Reporter Fluorophores

2.10.1 Slide fixation

The tissues embedded in the OCT compound were cut into 8-10 µm thick sections using the Leica CM1950 Cryostat (Leica, USA). The sections were placed onto Poly-L-Lysine coated glass microscope slides (StarFrost®, Germany) and dried overnight at room temperature. The slides were then submerged in acetone at 4°C for 20 min and dried overnight at room temperature. VECTASHIELD® Antifade Mounting medium or VECTASHIELD® Antifade Mounting medium with DAPI was applied (VECTOR Labs, USA) to the slides. The slides were visualised under the microscope or stored at 4°C.

2.10.2 Microscopic detection of fluorescent reporter fluorophores

The slides were visualised using the BX51 upright Fluorescent Microscope (Olympus) and the images were photographed using the DP70 Colour Camera (Olympus).

2.11 Statistical analysis

Statistical analyses were performed using the GraphPad Prism 8 program (version 8.0.1) and Microsoft Excel 2016 (Microsoft Office). A more detailed explanation is contained in each chapter.

Chapter 3:

Studying the effects of AAV8 vectors transduction in the STZ-induced diabetic NOD/scid mouse model

3.1 Introduction

During early pancreatic development, *Pdx1* is essential for the development of the full spectrum of pancreatic lineages [52]. In mature β -cells, *Pdx1* regulates the expression of several genes that are involved in glucose sensing and metabolism including insulin, *GLUT2* and *Gck* [25-27]. It has been shown that the overexpression of *Pdx1* in the livers of STZ-induced diabetic mice by adenoviral (Ad) vectors resulted in the expression of mouse insulin 2, glucagon, somatostatin and a number of downstream pancreatic β -cell transcription factors, as well as a reduction in BGL [106, 143]. The limitation of applying this therapy in the clinical setting is the potential for immune reactions to the Ad vectors that have been demonstrated in both *Rhesus macaque* and human cells [107, 109, 144]. More recently it has been shown that intrasplenic injection of the AAV2 vector carrying the *Pdx1* gene resulted in the reduction of BGL and the expression of insulin in the humanised livers of the STZ-induced diabetic TK-NOG mice [174].

NeuroD1 is expressed in all pancreatic endocrine progenitor cells and its expression is maintained at all later stages of β -cell development [18]. *NeuroD1* has been found to be both an activator and repressor of the expression of other transcription factors [30]. It can activate expression of the insulin gene by binding to the insulin gene promoter [61]. *NeuroD1* is found to regulate the expression of several ion channels and other genes involved in the exocytosis of insulin [56]. It has been shown that co-delivery of *NeuroD1* and *betacellulin* genes to the livers by HDAd vectors could improve BGL of the STZ-induced diabetic mice [146]. Other upstream and downstream pancreatic transcription factors were detected in the mouse livers [146].

Even though overexpressing the β -cell transcription factor could induce the partial pancreatic transdifferentiation of the livers, the main limitations of these studies were the insufficient insulin production from the transduced hepatocytes (BGL was improved, but was not normalised), and the unregulated insulin secretion leading to abnormal glucose tolerance tests. To overcome this limitation, it was hypothesised that the co-overexpression of β -cell transcription factor and *INS-FUR* may increase insulin production and could possibly regulate insulin secretion. Our research group have previously transduced the rat liver cell line, H4IIE, with a combination of *INS-FUR* and

NeuroD1 genes to create the H4IIEins/ND cell line expressing several pancreatic transcription factors such as *Pdx1*, *Nkx2.2*, *Nkx6.1*, *insulin*, *insulin2*, *somatostatin*, *glucagon*, *PC1*, *PC2* and *pancreatic polypeptide* [147]. The H4IIEins/ND cell line also stored insulin in vesicles and exhibited regulated insulin secretion in response to glucose [147]. The BGLs of the STZ-induced diabetic NOD/scid mice were normalised after receiving s.c. transplantation of the H4IIEins/ND cells, but a normal glucose tolerance test could not be achieved [147].

In order to further stimulate the liver-to-pancreas transdifferentiation, in this project, we attempted to deliver various combinations of *INS-FUR*, *NeuroD1* and *Pdx1* to the livers of STZ-induced diabetic NOD/scid mice. The transgenes were delivered by the AAV8-LSP vector system with the AAV8 capsid that had high transduction efficiency to the murine hepatocytes and the LSP that could limit the expression of the transgenes to the liver.

Our laboratory previously cured diabetes in multiple animal models by the intervallic infusion of the HMD/*INS-FUR* vector into the portal vein in full flow occlusion (FFO) of hepatic circulation [148, 150-152]. It was hypothesised that the FFO surgery may have contributed to the partial liver-to-pancreas transdifferentiation. With the FFO, the transduced animals had normal BGLs, normal intravenous glucose tolerance, and their livers expressed several endogenous pancreatic genes (such as: *Pdx1*, *NeuroD1*, *Ngn3*, *Nkx2.2*, *Pax4*, *MafA*, *mouse insulin1*, *somatostatin*, *glucagon* and *synaptophysin*) [150]. Without the surgery, constitutive insulin secretion was seen and pancreatic transcription factors could not be detected in the livers [150]. It has been reported that the liver-to-pancreas transdifferentiation could be enhanced by inducing the liver regeneration together with gene therapy [162]. Insults to the liver, such as partial hepatectomy, can enhance the liver-to-pancreas transdifferentiation when *Pdx1* was delivered by a recombinant adenoviral vector [162]. Many studies have also shown that injuries to the liver, including those caused by hypoxia, played important roles in the regeneration of the livers [175-177]. By briefly stopping the blood supply, the FFO procedure could transiently reduce the oxygen supply to the liver and induced an insult to the liver. To study the possibility of replicating the results of the previous experiment with the

HMD/*INS-FUR* vector, we injected (i.p.) the STZ-induced diabetic NOD/scid mice with AAV8-*INS-FUR* and performed the FFO surgery 2 days later.

3.2 Specific aims

- To determine if expression of the individual genes, *INS-FUR*, *NeuroD1* and *Pdx1*, or combinations thereof, delivered intraperitoneal (i.p.) by the AAV8-LSP vector system, would normalise blood glucose in the STZ- diabetic NOD/scid mice.
- To determine if the combination of AAV8-*INS-FUR* and FFO surgery would normalise blood glucose levels of STZ-diabetic NOD/scid mice.

3.3 Materials and methods

3.3.1 Inducing diabetes in NOD/scid mice using STZ

3.3.1.1 Preparation of STZ solution for injection

All the experiments using the NOD/scid mice were performed with Dr. Binhai Ren (UTS), who also developed the protocol for inducing diabetes in mice using STZ.

Acetate buffer was prepared by diluting 17.4 M glacial acetic acid to 0.1 M (pH 5.2) with dH₂O to a final volume of 100 mL. Sodium acetate anhydrous (166 mg) was dissolved in 0.1 M (pH 5.2) glacial acetic acid and filtered through a 0.2 µm Minisart® syringe filter (Merck-Millipore®). Acetate buffer was stored in 5 mL aliquots at -20°C. On the day of diabetes induction, 100 mg of STZ (Alexis® Biochemicals) was added into the thawed 5 mL aliquot of acetate buffer (final concentration was 20 mg/mL). The solution was mixed thoroughly by vortexing and was filtered through a 0.2 µm Minisart® syringe filter (Merck-Millipore®).

3.3.1.2 Inducing diabetes in NOD/scid mice by STZ injections

Prior to STZ injection, 8-12 week old male NOD/scid mice had their body weight and BGL recorded. The STZ dosage was 170 mg/Kg of body weight and the STZ solution was administered by i.p. injection using a 0.5 mL Ultrafine Insulin syringe (BD Medical®). The volume of STZ solution for injection was calculated according to the

weight of the mice. After receiving the STZ injection, weight and BGL of the mice were monitored daily. Animals with random BGL of 10 mmol/L or higher, for 3 consecutive days, were considered diabetic and were utilised for the *in vivo* experiments. An extra dose of STZ (85 mg/Kg of body weight) was given to any mouse that did not develop diabetes (BGL < 8 mmol/L) within 1 week after the initial injection.

3.3.2 *In vivo* transduction

Male STZ-induced diabetic NOD/scid mice were transduced when their random BGL was 10 mmol/L or higher on 3 consecutive days. To determine the effects of *INS-FUR* and the β -cell transcription factors *Pdx1* and *NeuroD1* in reversing diabetes, NOD/scid mice were injected with different combinations of AAV8 vectors carrying the transgenes. The positive controls were the non-diabetic NOD/scid mice. The negative controls were the diabetic mice treated with AAV8 vectors carrying only the reporter fluorophores, venus or cerulean. A total of 10 groups of NOD/scid mice were treated with the vectors at the dosages outlined in Table 3.1. The vector doses were chosen after consultation with Dr Binhai Ren and Prof Ann Simpson (UTS) and Dr Grant Logan (CMRI). The dosages were chosen based on the previous preliminary and published studies at CMRI regarding the transduction efficiency of the vectors [156-158, 163, 178, 179]

The weights and BGLs of the animals was monitored daily. The expected endpoint of the experiments was 28 days after the AAV8 injection, with the exception of the mice that became hypoglycaemic (BGL < 3mmol/L) or those animals the general health of which deteriorated. At the endpoint of the experiments, the livers were collected for RT-PCR, VCN analysis and detection of the reporter fluorophores as described in sections 2.4 - 2.8.

Table 3.1: Combinations of AAV8 vectors and dosages used in the liver transduction experiments

Vector	Dose (viral genome (vg))	Number of mice (n)
AAV8-venus	5×10^{10}	3
AAV8-cerulean	5×10^{11}	3
AAV8- <i>INS-FUR</i>	1.5×10^{10} (Low dose)	5
AAV8- <i>INS-FUR</i>	5×10^{10} (High dose)	6
AAV8- <i>Pdx1</i>	5×10^{11}	5
AAV8- <i>NeuroD1</i>	5×10^{11}	5
AAV8- <i>INS-FUR</i> AAV8- <i>Pdx1</i>	5×10^{10} 1.7×10^{11}	7
AAV8- <i>INS-FUR</i> AAV8- <i>NeuroD1</i>	5×10^{10} 5×10^{11}	4
AAV8- <i>INS-FUR</i> AAV8- <i>Pdx1</i> AAV8- <i>NeuroD1</i>	5×10^{10} 1×10^{11} 5×10^{10}	4
AAV8- <i>INS-FUR</i> +FFO	5×10^{10}	6

3.3.2 Quantitative PCR (qPCR) analysis of AAV8 vector copy number after transduction

The qPCR analysis of the vector copy number was analysed by our collaborators at the CMRI. For mice that received a single transgene (either *INS-FUR* or *Pdx1* or *NeuroD1*), the AAV8 VCNs were analysed by qPCR measuring the numbers of WPRE and *GAPDH* sequences using a previously published protocol [178]. For mice that were transduced with combinations of *INS-FUR*, *Pdx1* and *NeuroD1*, qPCR was performed in 2 steps. Firstly, to determine the total VCN, the copy number of the internal ribosome re-entry site (IRES) sequence was determined. In the next step, the copy numbers of *GAPDH*, *INS-FUR*, *NeuroD1* and *Pdx1* were analysed separately using the respective primers.

3.3.3 Statistical analysis

The analysis was performed using the GraphPad Prism 8 program (version 8.0.1). A two-way ANOVA was performed followed by Tukey's or Sidak's multiple comparison tests to test for the differences in the daily weights and BGLs of the mice. The differences in the mean BGLs or weights on each day were considered to be significant when the P value was less than 0.05. For mice that received combinations of 2 or 3 different AAV8 vectors, a paired T-test was used to compare the VCNs of each vector. When comparing the VCNs between different groups of mice, Mann Whitney tests were performed. The differences in the mean VCNs were considered to be significant when the P value was less than 0.05.

3.4 Results

3.4.1 The transduction of STZ-induced diabetic NOD/scid mice with AAV8-INS-FUR

In order to study the effect of *INS-FUR* transduction by AAV8, two groups of 5 NOD/scid mice received i.p. injections of AAV8-*INS-FUR* at doses of either 5×10^{10} vg (high dose) or 1.5×10^{10} vg (low dose). After transduction, the reporter fluorophore venus was detected in the livers of the transduced mice indicating that the livers had been transduced with the vector (Figure 3.1).

The STZ-induced diabetic NOD/scid mice that received the low dose of AAV8-*INS-FUR* (1.5×10^{10} vg) remained hyperglycaemic for the whole 14 day period of the experiment (Figure 3.2A) and their BGLs were not statistically different from the diabetic mice transduced by AAV8-venus (negative controls). Mice treated with the low dose of AAV8-*INS-FUR* also had significantly higher BGLs compared to the normal mice for the entire duration of the experiment (Figure 3.2A).

After receiving the high dose of AAV8-*INS-FUR* (5×10^{10} vg), all the mice became hypoglycaemic (random BGL < 3mmol/L) and the experiment was ended on day 14 (Figure 3.2A). At the end of the experiment, the livers of the mice treated with the high dose of AAV8-*INS-FUR* were analysed for AAV8 vector copy number. The mean AAV8 copy number per 50 ng of total DNA was $(6.39 \pm 0.62) \times 10^4$. The minimum VCN was 5.16

$\times 10^4$ and the maximum VCN was 7.63×10^4 copies per 50 ng of total DNA. The VCNs in the livers of each animal, and the median VCN are shown in Figure 3.3

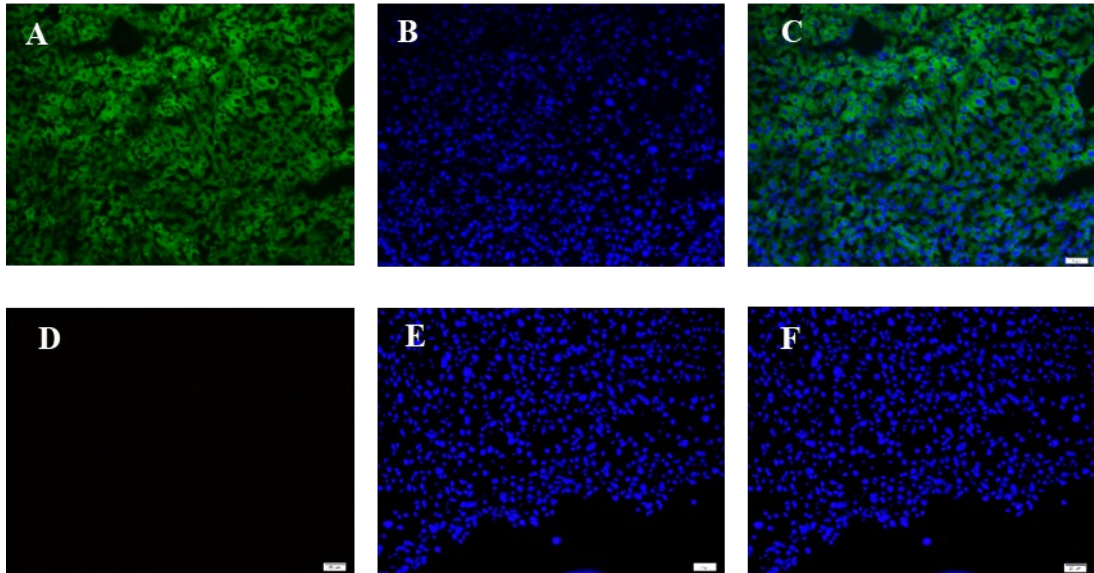
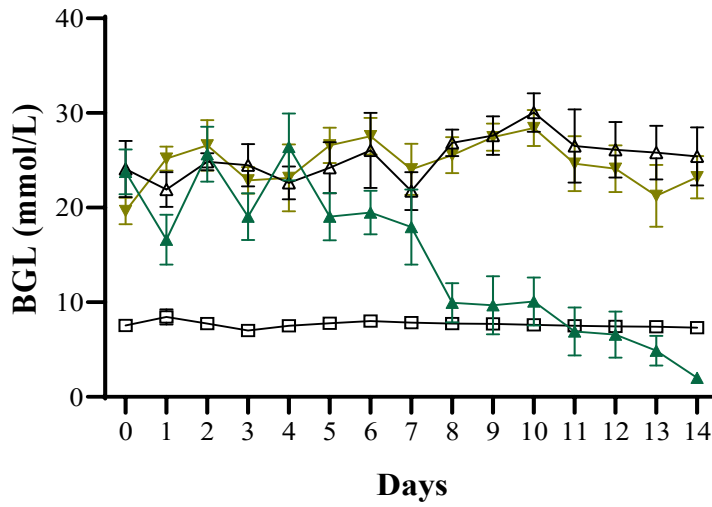


Figure 3.1 Immunofluorescent detection of the marker gene venus in a liver of a representative diabetic NOD/scid mouse transduced by AAV8-*INS-FUR*

Frozen sections of normal livers and the AAV8-*INS-FUR* transduced liver were prepared for fluorescence microscopy. The animals were transduced by the i.p. injections of AAV8-*INS-FUR* (5×10^{10} vg). The reporter fluorophore for the AAV8-*INS-FUR* vector was venus (green). Representative images from a liver of a transduced mouse and a liver of a normal mouse are shown. (A) venus fluorescence in the livers of the transduced mice, (B) DAPI stained nuclei of A and (C) is a merged image of A and B. (D) Normal liver section showing no venus fluorescence, (E) is an image of DAPI-stained nuclei of (D) and (F) is a merged image of (D) and (E). Bar=20 μ m.

(A)



(B)

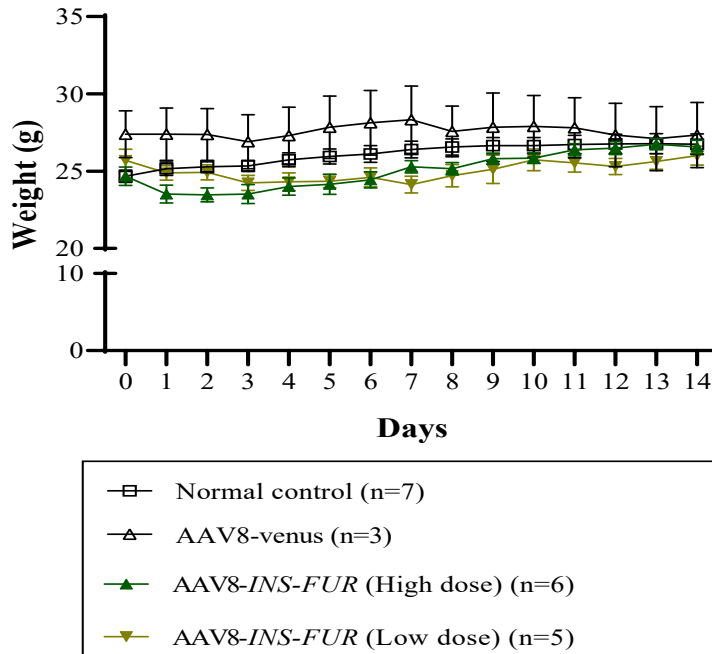


Figure 3.2: BGLs and body weights of diabetic NOD/scid mice transduced by AAV8-INS-FUR or AAV8-venus

The STZ-induced diabetic NOD/scid mice received i.p. injections of AAV8-INS-FUR at a dose of 5×10^{10} vg (High dose) or 1.5×10^{10} vg (Low dose) or AAV8-venus (5×10^{10}) on day 0. BGLs (A) and weights (B) were recorded daily for 14 days. The data are shown as mean $\pm$ SEMs.

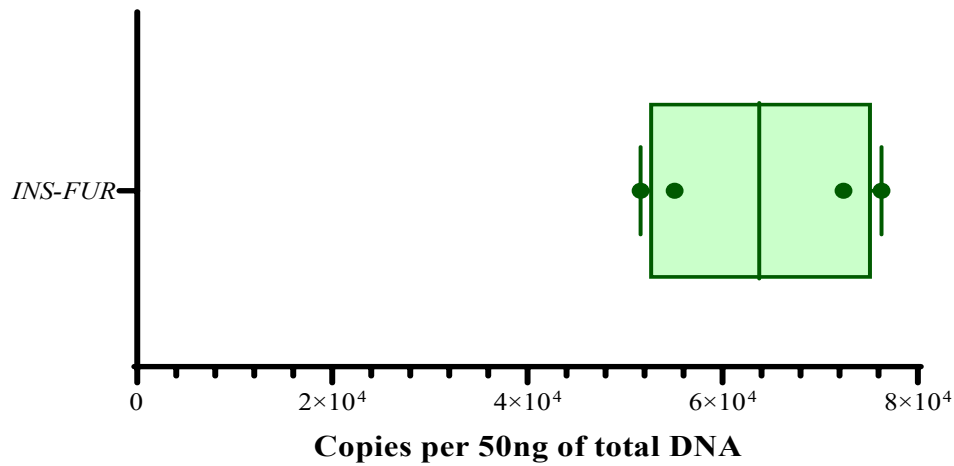


Figure 3.3: AAV8 copy numbers in the livers of the diabetic NOD/scid mice transduced by AAV8-*INS-FUR*

Total DNA was extracted from the livers of 4 mice transduced by i.p. injection of AAV8-*INS-FUR* (5×10^{10} vg). The total DNA from each liver was analysed in duplicates by qPCR to determine the AAV8 VCNs. The dots on the boxplot show the average VCN of each mouse liver. The middle line of the plot represents the median VCN of all the samples.

3.4.2 The transduction of STZ-induced diabetic NOD/scid mice with AAV8-*Pdx1*

In order to study the possibility of inducing the liver-to-pancreas transdifferentiation by overexpressing the pancreatic transcription factor *Pdx1*, five diabetic NOD/scid mice were injected (i.p.) with AAV8-*Pdx1* (5×10^{11} vg). After transduction, the livers of the transduced mice were analysed for AAV8 VCN by qPCR. The WPRE sequence detected by qPCR indicated that the livers had been transduced. The mean AAV8 VCN (per 50 ng of total DNA) in the livers of all the animals was $(5.79 \pm 0.62) \times 10^4$. The minimum, maximum and median VCN in the livers of each of the animals are shown in Figure 3.4. Despite the fact that the AAV8 dose used to transduce the mice with AAV8-*Pdx1* (5×10^{11} vg) was 10 times higher than that used to transduce the mice with AAV8-*INS-FUR* (5×10^{10} vg), the AAV8 VCNs of the two groups were not significantly different from each other (Figure 3.3 and Figure 3.4).

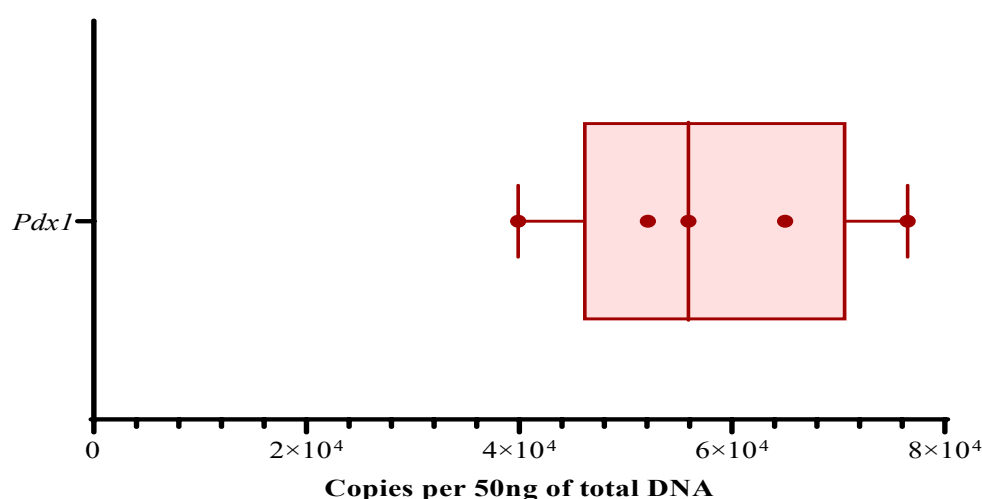
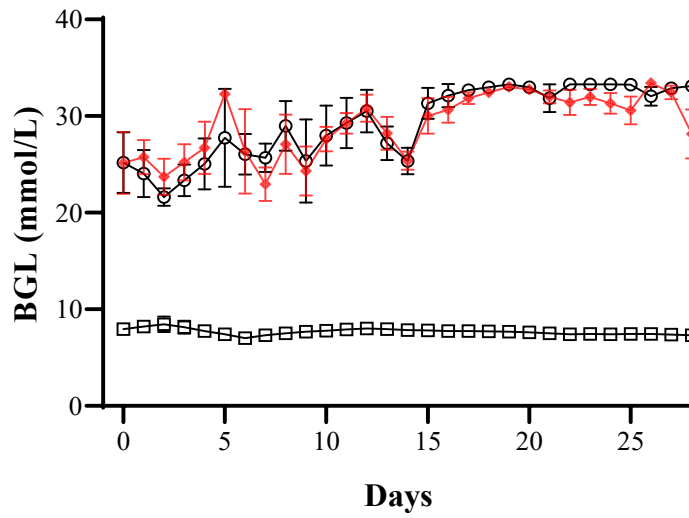


Figure 3.4: AAV8 copy numbers in the livers of diabetic NOD/scid mice transduced by AAV8-*Pdx1*

Total DNA was extracted from the livers of the 5 STZ-induced diabetic NOD/scid mice transduced by the i.p. injections of AAV8-*Pdx1* (5×10^{11} vg). The total DNA from each liver was analysed in duplicate using qPCR to determine the AAV8 VCNs. The dots on the boxplot show the average VCN of each mouse liver. The middle line of the plot represents the median VCN of all the samples.

During the 28 day period of the experiment, the transduced mice remained hyperglycaemic and their BGLs were not significantly different from the diabetic controls (Figure 3.5A). A two-way ANOVA followed by Tukey's multiple comparison test showed that the BGLs of the transduced mice were significantly higher than the normal mice. The two-way ANOVA also showed that there were significant differences in the daily weights among the 3 groups of mice. The Tukey's multiple comparison tests indicated that at the beginning of the experiment, the weights of all the mice were not significantly different from each other, but from day 6 after receiving the AAV8-*Pdx1* injections, the weights of the transduced mice were significantly lower than the normal controls (Figure 3.5B). Similar to the diabetic controls, the *Pdx1*-transduced mice also exhibited symptoms such as polyuria and polydipsia. The weights of the two groups of mice were not significantly different from each other (Figure 3.5B). The high BGLs and low body weight, together with the symptoms associated with diabetes, suggested a poor control of the disease in the transduced animals.

(A)



(B)

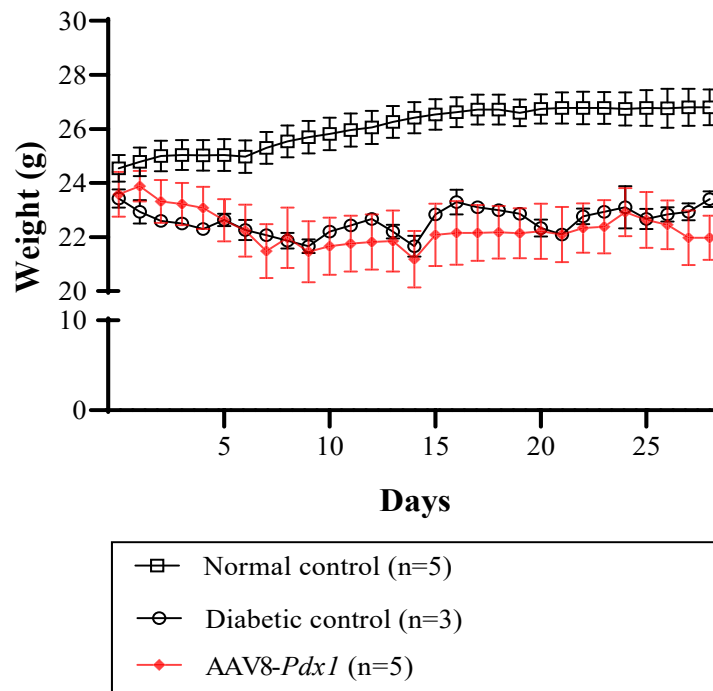


Figure 3.5: BGLs and body weights of diabetic NOD/scid mice transduced by AAV8-*Pdx1*

Diabetic NOD/scid mice received i.p. injections of AAV8-*Pdx1* (5×10^{11} vg) on day 0. BGLs and weights were recorded daily for 28 days. The data are shown as mean $\pm$ SEMs.

(A): Daily BGLs; (B): Daily weights

3.4.3 The transduction of STZ-induced diabetic NOD/scid mice with AAV-*NeuroD1*

In order to study the possibility of inducing the liver-to-pancreas transdifferentiation by overexpressing *NeuroD1*, five diabetic mice were injected with AAV8-*NeuroD1* (5×10^{11} vg) (experimental group). Another group of 3 diabetic mice injected with AAV8-cerulean (5×10^{11} vg) served as the negative controls for the experiment. Despite the fact that the cerulean reporter fluorophore was detected in the livers of the experimental group, indicating that the organs were transduced (Figure 3.6), all the mice were hyperglycaemic and exhibited symptoms of diabetes such as polyuria and hyperglycaemia. The BGLs of the experimental group were significantly higher than the normal controls but were not significantly different from the negative controls on most days (Figure 3.7A). For the duration of the experiment, the weights of all the groups of animals were not significantly different from each other (Figure 3.7B). All the livers of the mice in the experimental group were subsequently analysed for AAV8 VCNs by qPCR. The mean AAV8 VCN of the experimental group was $(21.58 \pm 2.42) \times 10^4$ copies per 50 ng of total DNA. The median VCN and the average VCN in the liver of each animal are shown in Figure 3.8. The minimum, maximum, and median VCN per 50 ng of total DNA of the mice transduced by *NeuroD1* were also shown in Figure 3.9.

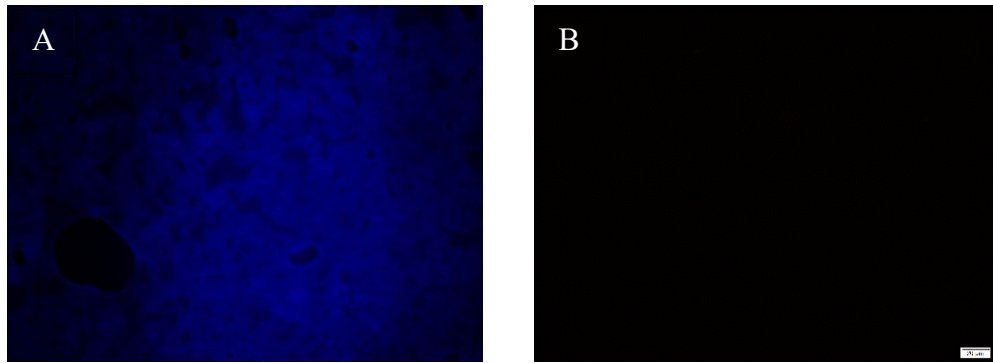
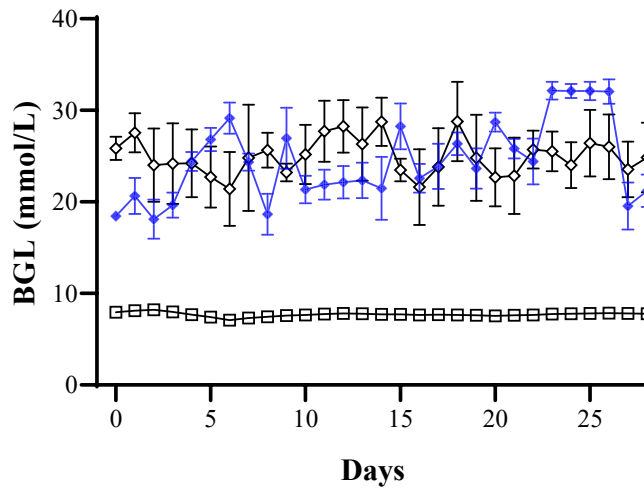


Figure 3.6: Expression of the marker gene cerulean in a liver of a representative diabetic NOD/scid mouse transduced by AAV8-*NeuroD1*

Frozen sections of normal livers and the AAV8-*NeuroD1* transduced livers were prepared for fluorescence microscopy. The animals was transduced by the i.p. injection of AAV8-*NeuroD1* (5×10^{11} vg). The reporter fluorophore for the AAV8-*NeuroD* vector was cerulean (blue). Representative images from a liver of a transduced mouse and a liver of a normal mouse are shown. (A) Cerulean fluorescence in the livers of the transduced mouse, (B) Normal liver section showing no venus fluorescence. Bar=20 μ m.

(A)



(B)

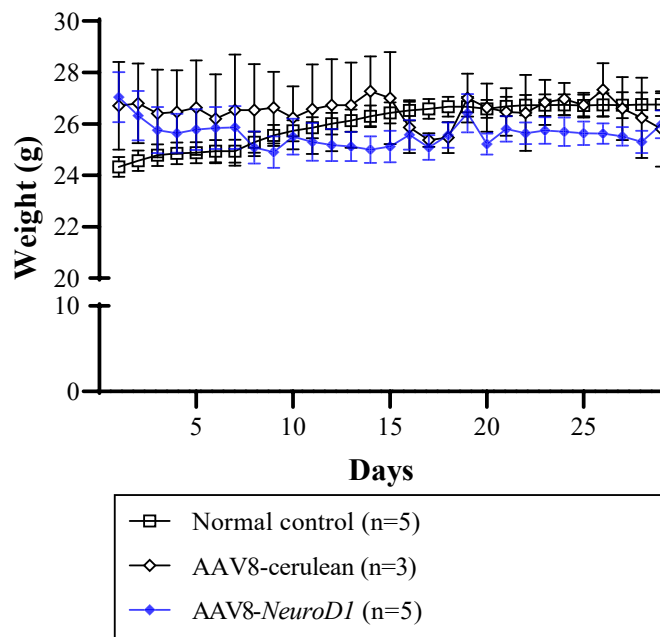


Figure 3.7: BGLs and body weights of diabetic NOD/scid mice transduced by AAV8-*NeuroD1* or AAV8-cerulean

The mice received i.p. injections of AAV8-*NeuroD1* (5×10^{11} vg) or AAV8-cerulean (5×10^{11} vg) on day 0 and BGLs and body weights were recorded daily for 28 days. The data are shown as mean $\pm$ SEMs. (A): Daily BGLs; (B): Daily weight

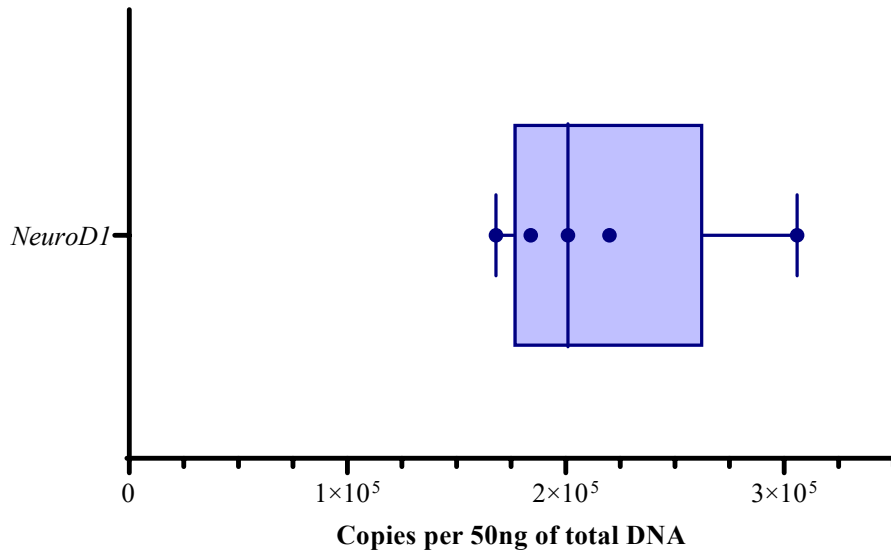


Figure 3.8: AAV8 copy numbers in the livers of diabetic NOD/scid mice transduced by AAV8-*NeuroD1*

Total DNA was extracted from the livers of the 5 STZ-induced diabetic NOD/scid mice transduced by the i.p. injections of AAV8-*NeuroD1* (5×10^{11} vg). The total DNA from each liver was analysed in duplicate using qPCR to determine the AAV8 VCNs. The dots on the boxplot show the average VCN of each mouse liver. The middle line of the plot represents the median VCN of all the samples.

3.4.4 The transduction of STZ-induced diabetic NOD/scid mice with the combination of AAV8-*INS-FUR* and AAV8-*Pdx1*

For the purpose of examining the possibility of curing diabetes and inducing liver-to-pancreas transdifferentiation, 7 diabetic NOD/scid mice were injected (i.p.) with a combination of AAV8-*INS-FUR* (5×10^{10} vg) and AAV8-*Pdx1* (1.7×10^{11} vg). The weights and BGLs of the transduced animals were monitored for 28 days. At the end of the experiment, the livers of the mice were analysed for *Pdx1* and *INS-FUR* transgene copy numbers. The mean *INS-FUR* copy number was $(7.87 \pm 3.02) \times 10^4$ per 50 ng of total DNA and the mean *Pdx1* copy number was $(2.57 \pm 1.45) \times 10^4$ per 50 ng of total DNA. The median and the individual VCN values are shown in Figure 3.9. The detection of both *INS-FUR* and *Pdx1* transgenes in the livers indicated that the livers were transduced. Despite the fact that the dose of AAV8-*Pdx1* was 3.4 times higher than the dose of AAV8-

INS-FUR, the paired t-test showed that the copy number of *Pdx1* was significantly lower than the *INS-FUR* copy numbers (Figure 3.9).

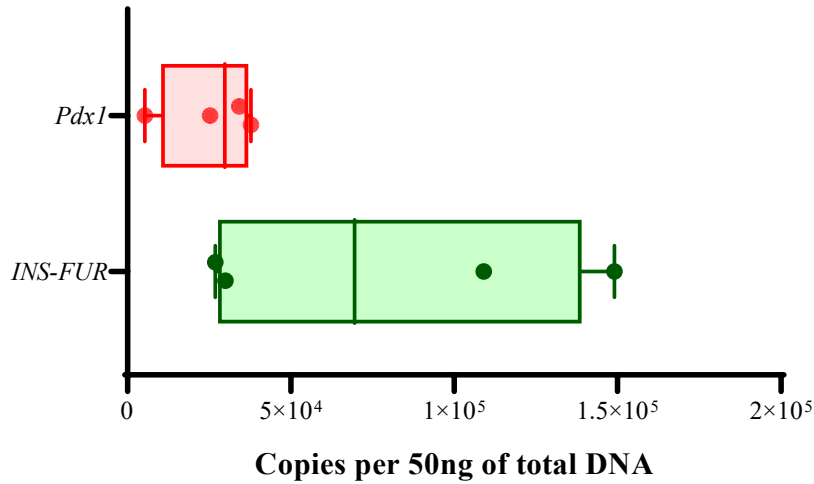
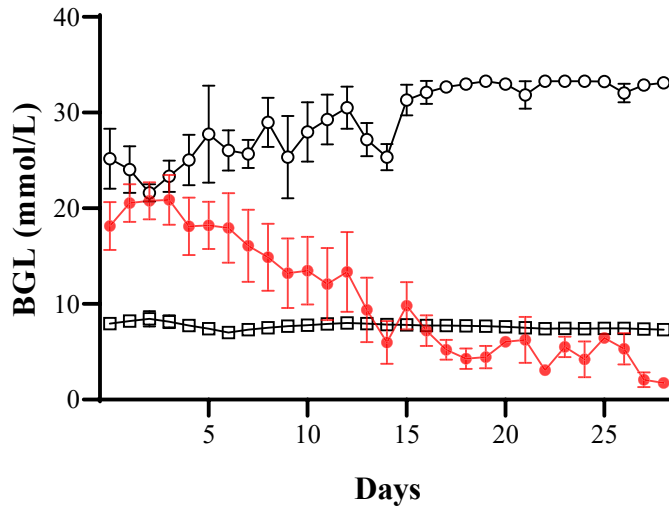


Figure 3.9: Transgene copy numbers in the livers of the diabetic NOD/scid mice transduced by the combination of AAV8-*INS-FUR* and AAV8-*Pdx1*

Total DNA was extracted from the livers of 4 STZ-induced diabetic NOD/scid mice transduced by AAV8-*INS-FUR* (5×10^{10} vg) and AAV8-*Pdx1* (1.7×10^{11} vg). The total DNA from each liver was analysed in duplicate for the *INS-FUR* and *Pdx1* transgene copy numbers using qPCR. The dots on the boxplots show the average transgene copy numbers for each liver. The middle vertical lines in the plots represent the median copy numbers of all the analysed samples.

Two-way ANOVA analysis followed by Sidak's multiple comparison test also showed that BGLs of the transduced mice were significantly higher than the normal control group from day 0 to day 8 (Figure 3.10A). After day 8, the BGLs of all the treated mice progressively decreased and the mice eventually became hypoglycaemic (Figure 3.10A). The daily weights of the treated mice were not significantly different from the normal controls throughout the 28 day-period of the experiment (Figure 3.10B). From day 6 to the end of the experiment, the body weights of the transduced mice were significantly higher than the diabetic controls. (Figure 3.10B).

(A)



(B)

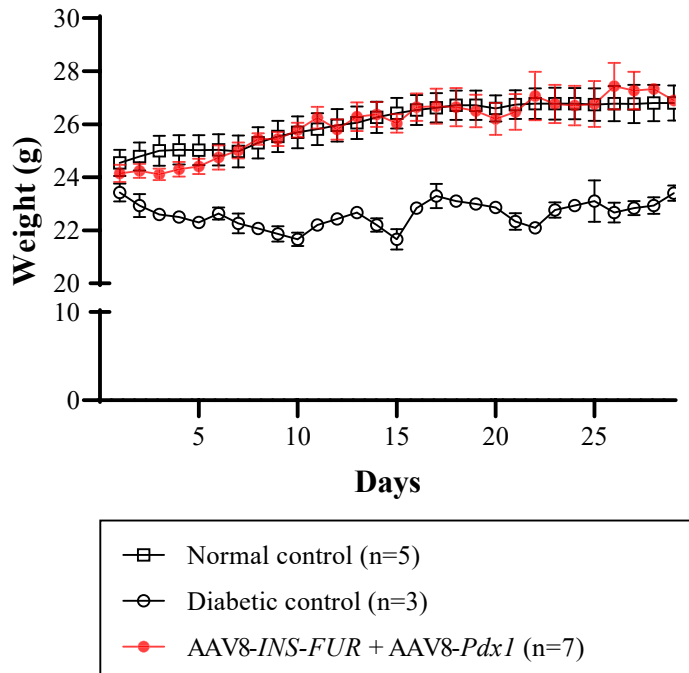


Figure 3.10: BGLs and body weights of diabetic NOD/scid mice transduced by the combination of AAV8-INS-FUR and AAV8-Pdx1

The mice received i.p. injections of AAV8-INS-FUR (5×10^{10} vg) and AAV8-Pdx1 (1.7×10^{11} vg) on day 0. The weights (A) and BGLs (B) were recorded daily for 28 days or until the mice became hypoglycaemic. The data are shown as mean $\pm$ SEMs.

3.4.5 The transduction of STZ-induced diabetic NOD/scid mice with the combination of AAV8-*INS-FUR* and AAV8-*NeuroDI*

In order to examine the possibility of curing diabetes and inducing the liver-to-pancreas transdifferentiation, the combination of *INS-FUR* and *NeuroDI* was used. For these experiments, 4 diabetic NOD/scid mice were injected with a combination of AAV8-*INS-FUR* (5×10^{10} vg) and AAV8-*NeuroDI* (5×10^{11} vg). At the experimental endpoint, the livers of all the animals were analysed for *INS-FUR* and *NeuroDI* transgene copy number. The detection of both the *INS-FUR* and *NeuroDI* transgenes indicated that the livers had been transduced with the vectors. The mean copy numbers of *INS-FUR* and *NeuroDI* (per 50 ng of total DNA) were $(3.47 \pm 1.55) \times 10^4$ and $(39.10 \pm 15.59) \times 10^4$ respectively. The copy numbers of the *INS-FUR* and *NeuroDI* transgenes in each liver are shown in Figure 3.11. As expected, the paired t-test showed a significantly higher copy number of *NeuroDI* in comparison to *INS-FUR* (Figure 3.11).

When comparing the treated mice to the normal controls, the two way ANOVA analysis indicated differences in the daily BGLs of the two groups. The mean BGL of the treated mice was significantly higher compared to that of the normal control group from day 0 to day 6 (Figure 3.12A). On day 8 after the AAV8 i.p. injections, a mouse became severely hypoglycaemic and was euthanised. The experiment was also terminated on day 14 due to hypoglycaemia in all the remaining mice. The two-way ANOVA and follow up Sidak's multiple comparison test did not show any significant differences when comparing the daily weights of the transduced mice to the normal mice (Figure 3.12B). The body weights of the diabetic controls were significantly different from both the transduced and the normal controls from day 7 to the end of the experiment (Figure 3.12B)

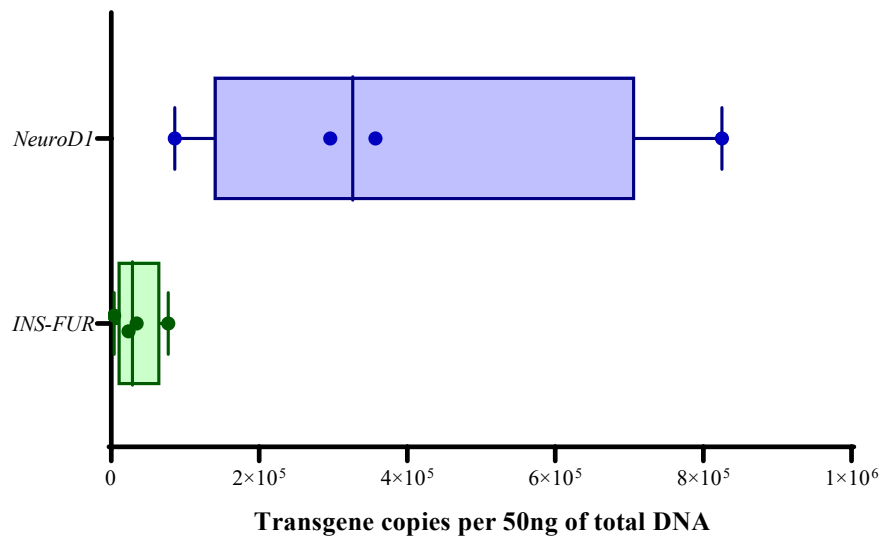
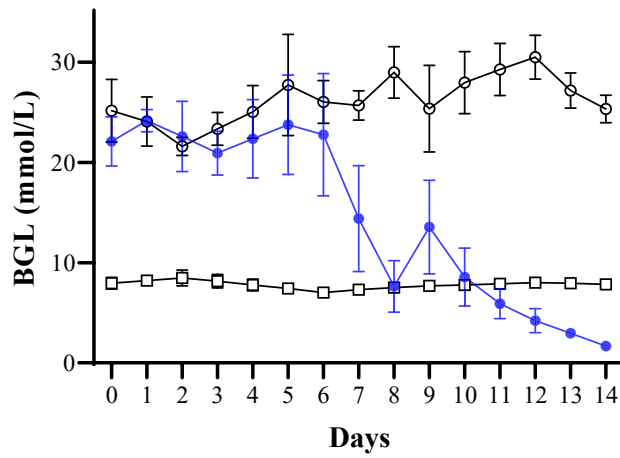


Figure 3.11: *Transgene* copy numbers in the livers of the diabetic NOD/scid mice transduced by the combination of AAV8-*INS-FUR* and AAV8-*NeuroD1*

Total DNA was extracted from the livers of 4 STZ-induced diabetic NOD/scid mice transduced by AAV8-*INS-FUR* (5×10^{10} vg) and AAV8-*NeuroD1* (5×10^{11} vg). The total DNA from each liver was analysed in duplicate for the *INS-FUR* and *NeuroD1* transgene copy numbers using qPCR. The dots on the boxplots show the average transgene copy numbers for each liver. The middle vertical lines in the plots represent the median copy numbers of all the analysed samples.

(A)



(B)

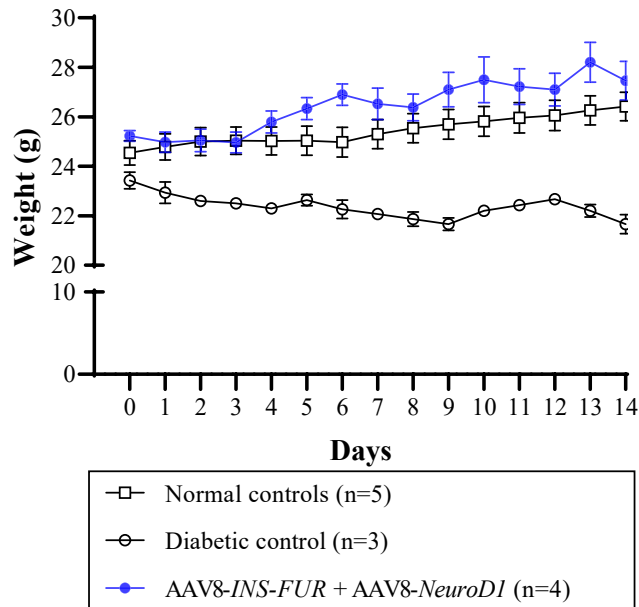


Figure 3.12: Daily BGLs and weights of diabetic NOD/scid mice transduced by the combination of AAV8-INS-FUR and AAV8-NeuroD1

Mice received i.p. injections of AAV8-INS-FUR (5×10^{10} vg) and AAV8-NeuroD1 (5×10^{11} vg) on day 0. BGLs (A) and weights (B) were recorded daily until all the treated mice became hypoglycaemic (14 days). The data are shown as mean $\pm$ SEMs.

3.4.6 Transduction of STZ-induced diabetic NOD/scid mice with the combination of AAV8-INS-FUR, AAV8-Pdx1 and AAV8-NeuroD1

To examine the possibility of curing diabetes using a combination of *INS-FUR* and β -cell transcription factors, 4 diabetic mice received i.p. injections of AAV8-*INS-FUR* (5×10^{10} vg), AAV8-*Pdx1* (10^{11} vg) and AAV8-*NeuroD1* (5×10^{10} vg). The subsequent detection of all the transgenes, *INS-FUR*, *Pdx1* and *NeuroD1*, by qPCR indicated that the livers had been transduced by all 3 AAV8 vectors. In the experimental group, the mean *INS-FUR*, *Pdx1* and *NeuroD1* transgene copy numbers (per 50ng of total DNA) were $(1.52 \pm 0.75) \times 10^4$, $(4.02 \pm 2.15) \times 10^3$ and $(1.29 \pm 0.63) \times 10^3$ respectively. The median copy number of each transgene and the copy number of each gene in each liver are shown in Figure 3.13.

The experiment was ended on day 26 after AAV8 injections due to the occurrence of severe hypoglycaemia in all the transduced animals. Analysis by two-way ANOVA followed by Sidak's multiple comparison tests showed differences in the daily BGLs of the transduced mice relative to the controls. The BGLs of the transduced animals were significantly higher than the normal mice from days 1-7 and days 9-11 (Figure 3.14A). On day 8 and from days 12-26, despite the fact that all the transduced mice progressively became hypoglycaemic while the normal controls continued to be euglycaemic, the mean BGLs of the two groups were not statistically different from each other (Figure 3.14A). In comparison to the diabetic controls, the BGLs of the transduced mice were significantly lower during day 4 to day 8 and day 10 to day 26 (Figure 3.14A). The daily weights of the transduced and normal mice were not significantly different from each other (Figure 3.14B).

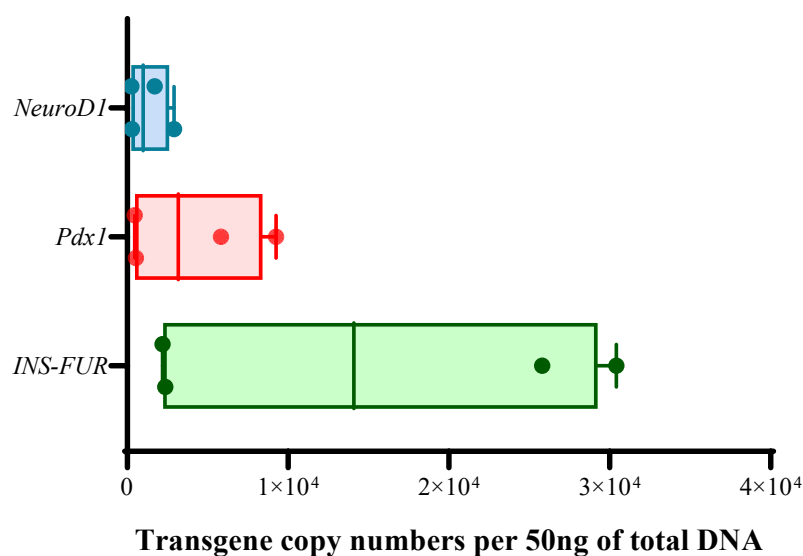
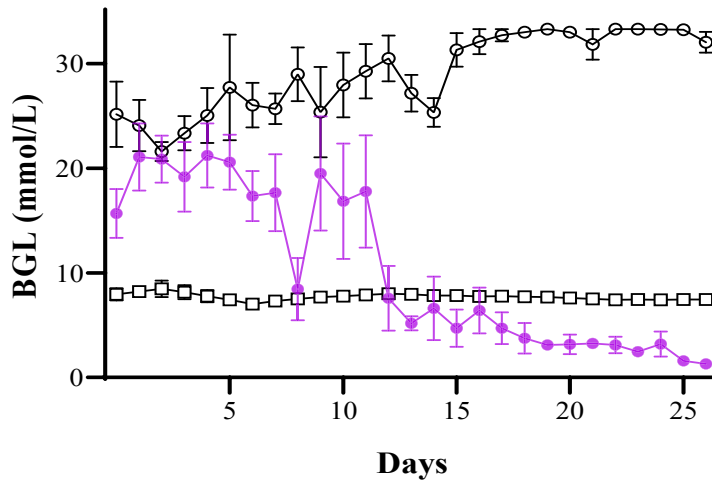


Figure 3.13 Transgene copy numbers in the livers of diabetic NOD/scid mice transduced by the combination of AAV8-*INS-FUR*, AAV8-*Pdx1* and AAV8-*NeuroD1*

Total DNA was extracted from the livers of 4 STZ-induced diabetic NOD/scid mice transduced by AAV8-*INS-FUR* (5×10^{10} vg), AAV8-*Pdx1* (10^{11} vg) and AAV8-*NeuroD1*-cerulean (5×10^{10} vg). The total DNA from each liver was analysed in duplicate for the *INS-FUR*, *Pdx1* and *NeuroD1* transgene copy numbers using qPCR. The dots on the boxplots show the average transgene copy numbers for each liver. The middle vertical lines in the plots represent the median copy numbers of all the analysed samples.

(A)



(B)

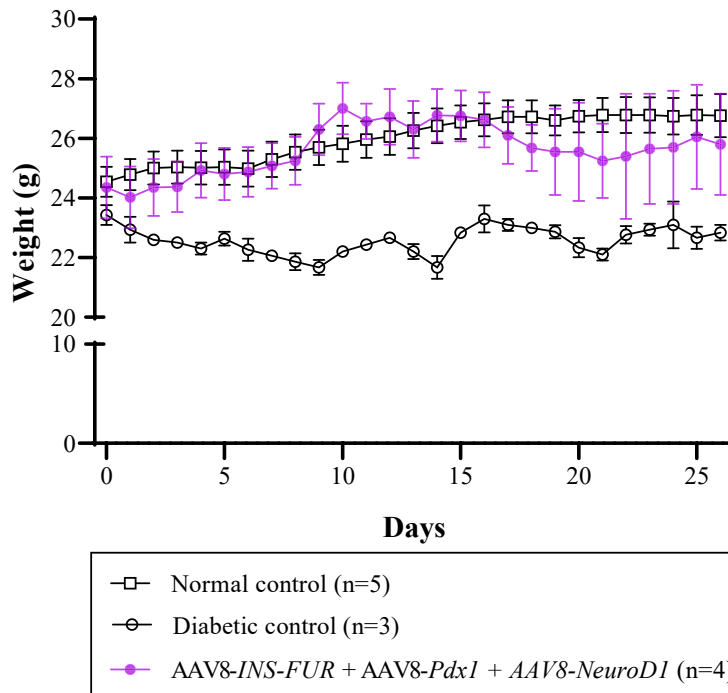


Figure 3.14: BGLs and body weights of diabetic NOD/scid mice transduced by combinations of AAV8-INS-FUR, AAV8-Pdx1 and AAV8-NeuroD1

Mice received i.p. injections of a combination of AAV8-INS-FUR (5×10^{10} vg), AAV8-Pdx1 (10^{11} vg) and AAV8-LSP-NeuroD1 (5×10^{10} vg) on day 0. BGLs (A) and weights (B) were recorded daily until all the transduced mice became hypoglycaemic. The data are shown as means $\pm$ SEMs.

3.4.7 RT-PCR analysis of pancreatic gene expression in the transduced livers of the STZ-induced diabetic NOD/scid mice: Determining evidence of transdifferentiation

Total RNA was extracted from the livers of all the experimental groups for RT-PCR detection of the transgenes *INS-FUR*, *NeuroD1*, *Pdx1* and other pancreatic genes. *Beta-Actin* was used as the endogenous control (section 2.9.1 and 2.9.2). All livers of the transduced mice expressed the corresponding transgenes indicating that the livers were transduced and that all the transgenes were translated to mRNA. The mice transduced by *INS-FUR* also expressed *mouse insulin 2* in their livers (Figure 3.15, lane 2). Mice transduced by *Pdx1* or *NeuroD1*, or the combination of *INS-FUR* and *Pdx1*, expressed *mouse insulin 2* and *somatostatin* (Figure 3.15, lanes 3-5). The endogenous *Nkx6.1*, *MafA*, *mouse insulin 2* and *somatostatin* were detected in the livers of the mice that received the combination of *INS-FUR* and *NeuroD1* (Figure 3.15, lane 6). Even though all the transgenes were detected in the mice that had the combination of *INS-FUR*, *Pdx1* and *NeuroD1*, other endogenous pancreatic genes could not be detected (Figure 3.15, lane 7). The pancreas expressed all the endogenous pancreatic genes (Figure 3.15, lane 8) as expected.

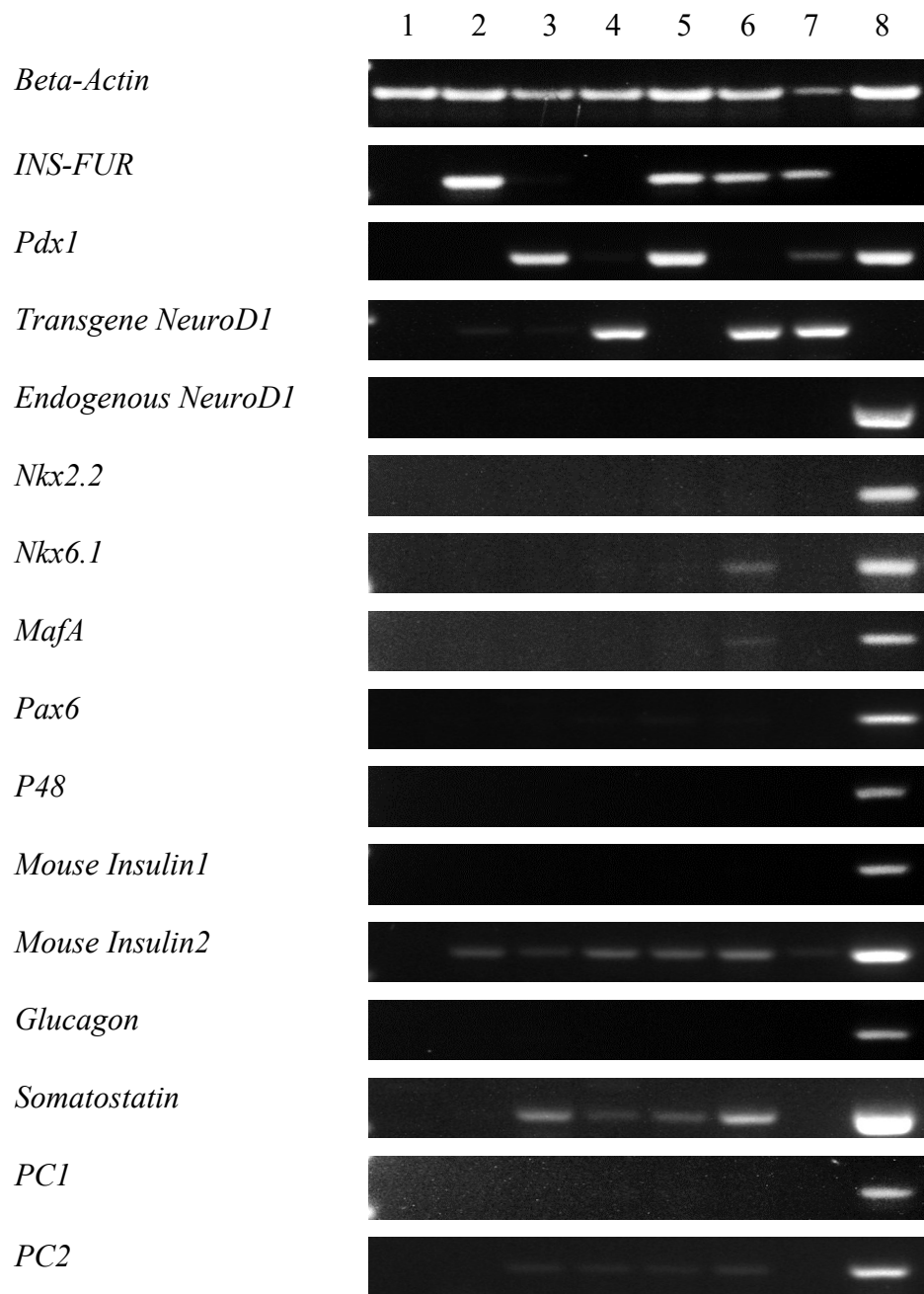
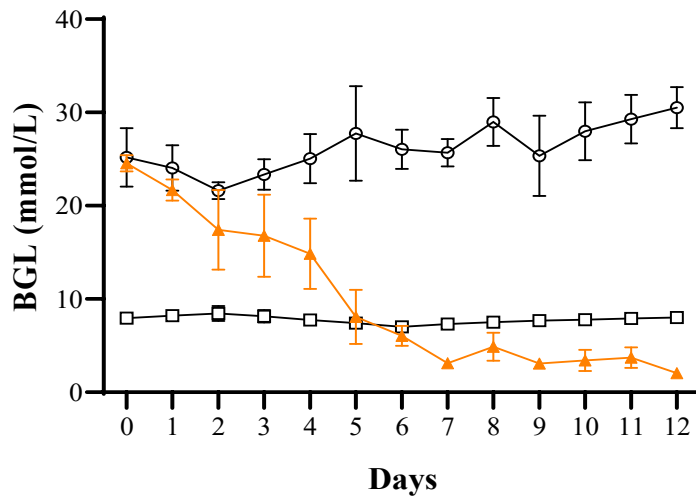


Figure 3.15: RT-PCR detection of transgene and other pancreatic gene expression in the AAV8 transduced diabetic NOD/scid mouse livers. Lane 1: Normal NOD/scid mouse liver, Lanes 2-7: Diabetic NOD/scid mouse livers transduced by: *INS-FUR* (lane 2), *Pdx1* (lane 3), *NeuroD1* (lane 4), *INS-FUR* and *Pdx1* (lane 5), *INS-FUR* and *NeuroD1* (lane 6), *INS-FUR*, *Pdx1* and *NeuroD1* (lane 7) and lane 8: Normal mouse pancreas.

3.4.8 The transduction of STZ-induced diabetic NOD/scid mice with the combination of AAV8-INS-FUR and FFO surgery

To determine if the FFO surgery, used in previous successful transductions with the HMD lentiviral vector, had any effect on the liver-to-pancreas transdifferentiation, 6 male STZ-induced diabetic NOD/scid mice were transduced by i.p. injection of AAV8-INS-FUR at the dose of 5×10^{10} vg and FFO surgery was performed 2 days after the injections. Post transduction, the BGLs reduced to normal levels on day 5, but all the mice gradually became hypoglycaemic and the experiment was ended on day 12 (Figure 3.16A). As a result, the experiment was terminated and IPGTT was abandoned. RT-PCR analysis could not detect the expression of any pancreatic transcription factors in the liver (data not shown).

A



B

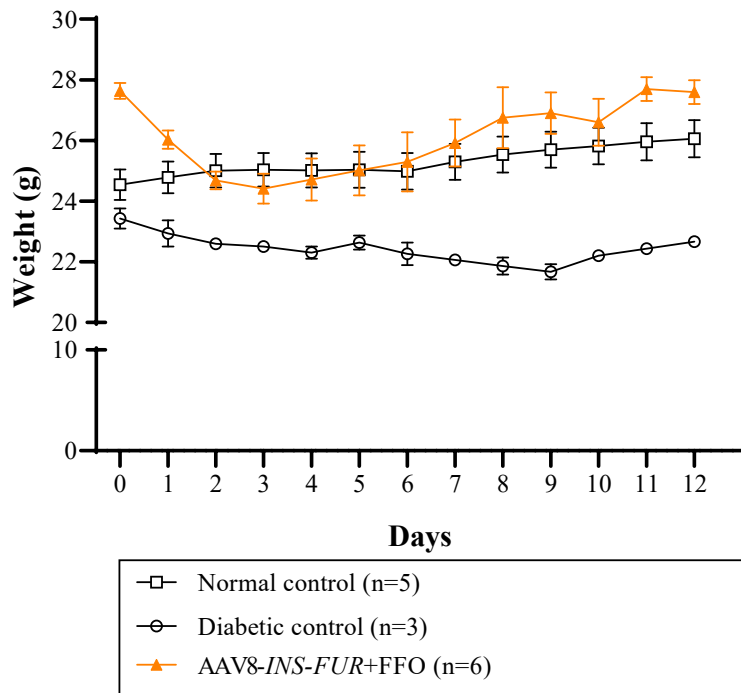


Figure 3.16: weekly BGLs and weights of diabetic NOD/scid mice transduced by AAV8-INS-FUR and followed with FFO surgery

The mice received an i.p. injection of AAV8-INS-FUR (5×10^{10} vg) on day 0 and FFO surgery was performed 2 days later. Weights and BGLs were monitored until all the mice became hypoglycaemic. Graph (A) shows the daily BGLs and graph (B) shows the daily weights. The data are shown as means $\pm$ SEMs.

3.5 Discussion

In this project the AAV8 vector was chosen to deliver *INS-FUR*, *Pdx1* and *NeuroD1* because AAV8 vectors allow long-term episomal expression of the transgenes [116]. This characteristic was considered an advantage because the lack of integration of the transgenes can reduce the risk of insertional mutagenesis. In addition, the AAV8 capsid has been shown to have high transduction efficiency in mouse liver cells [180]. When used in conjunction with the LSP, it was possible to deliver the vectors by an i.p. injection [181].

Previous studies from our laboratory showed that the lentiviral transduction of *INS-FUR* (HMD/*INS-FUR*) using the FFO surgical technique led to a normalisation in BGLs as well as the expression of several endogenous pancreatic genes in the livers of several diabetic animal models [148, 150-152]. In contrast, when *INS-FUR* was delivered by portal injection to diabetic NOD mice [1], without the FFO technique, BGLs were reduced but not to normal levels and no pancreatic transcription factors were seen in the transduced livers. Similarly, Elsner *et.al* (2012) reported that the third generation lentiviral vector delivery of *INS-FUR* to the liver via portal vein injection resulted in a reduction of BGLs, but could not induce the expression of the endogenous pancreatic transcription factors in the livers of both the STZ-induced and autoimmune diabetic rats. Such observations suggested that the FFO surgery may have contributed to the transdifferentiation. The current project showed that the over-expression of the *INS-FUR* by the AAV8 vectors at the high dose (5×10^{10} vg) caused hypoglycaemia in the STZ-induced diabetic NOD/scid mice (Figure 3.2A) and the therapy could not induce the expression of any pancreatic transcription factor in the livers (Figure 3.15, lane 2). In contrast, mice transduced by the i.p. injections of AAV8-*INS-FUR* at the low dose of 1.5×10^{10} vg remained hyperglycaemic (Figure 3.2A). The combination of AAV8-*INS-FUR* (5×10^{10} vg) and FFO surgery also caused severe hypoglycaemia (Figure 3.16A). It was very likely that the expression of *INS-FUR* by AAV8 was not regulated, but dependent on the vector dose. Compared to the HMD vectors, the AAV8 vectors could not induce the transdifferentiation.

It has been demonstrated that the ectopic expression of *Pdx1* by adenoviral (Ad) vectors could reduce the BGLs and induce the expression of both pancreatic endocrine and exocrine marker genes in the livers of the STZ-induced diabetic C57BL/6 [106, 143]. Similar results were also observed when HDAd vectors were used to deliver the *Pdx1* gene [146, 160]. In contrast, the current project showed that the overexpression of the β -cell transcription factor *Pdx1* by AAV8 could not normalise the BGLs (Figure 3.5A). Endogenous *mouse insulin 2* mRNA expression was detected in the transduced livers, but other pancreatic genes were not detected (Figure 3.15, lane 3). The result suggested that, the AAV8 vectors lacked certain characteristics that allowed the Ad and the HDAd vectors to induce liver-to-pancreas transdifferentiation by *Pdx1* gene therapy.

Kojima et.al (2003) demonstrated a partial reversal of diabetes in STZ-induced diabetic C57/BL6 mice after the HDAd vector delivery of the *NeuroD1* gene at the dose of 3×10^{11} particles per mouse. The BGLs of the animals in the study were reduced, but not normalised and they had abnormal glucose tolerance [146]. The RT-PCR showed the expression of many pancreatic genes such as: *mouse insulin 1*, *mouse insulin 2*, *glucagon*, *somatostatin*, *pancreatic polypeptide*, *Neurogenin 3*, *Pax4*, *Pax6*, *Nkx2.2*, *Nkx6.1*, *PC1* and *PC2* in the liver [146]. In the current study, the *NeuroD1* gene was also utilised, but the AAV8 delivery of the gene did not have any effect on the BGLs or the liver-to-pancreas transdifferentiation. The diabetic NOD/scid mice transduced by *NeuroD1* alone expressed the endogenous *mouse insulin 2* and *somatostatin* (Figure 3.15, lane 4), but they were hyperglycaemic (Figure 3.7A). The result suggested that the AAV8 vectors lacked the ability to induce pancreatic transdifferentiation seen when adenoviral vectors or HDAd vectors were used, at least in the NOD/scid model.

The AAV8 co-delivery of *INS-FUR* and *Pdx1* or *INS-FUR*, *Pdx1* and *NeuroD1* led to hypoglycaemia (Figure 3.10A & 3.14A), without the expression of the pancreatic transcription factors (Figure 3.15, lane 5 & 7). The mice transduced by the combination of *INS-FUR* and *NeuroD1* expressed several pancreatic genes such as *Nkx6.1*, *MafA*, *mouse insulin 2* and *somatostatin* (Figure 3.16, lane 6), but they were hypoglycaemic indicating that insulin was not released in a glucose-responsive manner (Figure 3.12A). It has been shown that *insulin* and other pancreatic genes could be expressed in the livers,

adipose tissue and bone marrow of: (1) nondiabetic C57/BL6 mice that were exposed to hyperglycaemia, (2) STZ-induced diabetic mice and rats, (3) obese diabetic mice and (4) mice fed a high-fat diet [146, 161]. Similarly, the random detection of pancreatic gene expression in the current study could also be a result of STZ-induced hyperglycaemia and such expression did not have any effect on the liver-to-pancreas transdifferentiation.

Similar to the current project, it was demonstrated that the AAV8 delivery of the *Pdx1* gene did not have any effect on the liver-to-pancreas transdifferentiation in STZ-induced diabetic C57BL/6 mice [183]. Interestingly, the co-delivery of the plasmids carrying the *Pdx1* gene and the Ad vectors carrying an unrelated gene (the *human coagulant factor IX* gene) could reverse diabetes in the C57BL/6 mice, but was ineffective in an immunocompromised mouse model [183]. The authors suggested that certain immune reactions to the Ad vector may have contributed to the transdifferentiation [183]. By comparison to previous work from our laboratory [150], a major difference in this study was that the NOD/scid mouse model has a deficiency in mature B- and T-cells [184] and the previous animal models used had a functioning immune system. Therefore, we hypothesised that the lack of a response to the AAV8 vectors expressing insulin $\pm$ the beta cell transcription factors, may be attributed to the immune deficiency of the NOD/scid model. Another major difference between the AAV8 and the HMD vectors was that the HMD allowed the stable integration of the transgenes into the host genome while the AAV8 mostly allowed episomal expression [97-99, 111, 113-115]. The inability of the AAV8 system to stably incorporate the genes may lead to the loss of the transgene expression and reduce the efficacy of the therapy. In the next chapters the AAV8 system will be tested in the autoimmune NOD mouse model that has been used successfully to reverse diabetes using the HMD/*INS-FUR* system [150] to determine if the lack of an immune system was the issue. Secondly, the hybrid AAVV/*piggyBac* system that allows the integration of the transgenes will be examined.

Chapter 4:

**Studying the effects of AAV8 vector
transduction in autoimmune non-
obese-diabetic (NOD) mouse model
using**

4.1 Introduction

Work presented in the previous chapter showed that the AAV8 delivery of *INS-FUR*, either alone or in combination with β -cell transcription factors (*Pdx1* and *NeuroD1*) and FFO surgery, could not induce liver-to-pancreas transdifferentiation that were previously observed in the HMD/*INS-FUR* therapy [148, 150]. Our laboratory has previously cured diabetes in NOD mice, STZ-induced diabetic rats, pancreatectomised pigs and the humanised FRG mice by the intervallic infusion of the HMD/*INS-FUR* vector into the portal vein with FFO surgery [148, 150-152]. Therefore, the main aim of this chapter was to reverse diabetes by modifying the protocol to the use of a clinically relevant AAV8 vector system to mimic the HMD/*INS-FUR* therapy.

It was demonstrated that the degree of transdifferentiation may be dependent upon many factors, such as: (i) the type of vector used, (ii) the vector delivery technique used, (iii) the promoter used to drive transgene expression and (iv) the diabetic animal model used in the study. To verify the hypothesis that different diabetic mouse models could have different responses to the AAV8 therapy, in this chapter, we also transduced the livers of female autoimmune diabetic NOD mice with either *INS-FUR* alone or in combinations with *Pdx1* using the AAV8-LSP vector system. In order to mimic the HMD/*INS-FUR* system, we co-transduced the NOD mice by AAV8-*INS-FUR* and the HMD vectors carrying the eGFP marker gene driven by the MSCV promoter (empty HMD vectors). The hybrid AAV8/*piggyBac*-LSP system that allowed the incorporation of the transgene was also utilised to study the effect of the integration of the *INS-FUR* gene on the BGLs.

A peer-reviewed journal publication describing the experiments addressing the main aims of the chapter was published. This published paper, entitled “Use of a Hybrid Adeno-Associated Viral Vector Transposon System to Deliver the Insulin Gene to Diabetic NOD Mice” is included as the main part of this chapter. The article is shown below and a signed copy of the author contribution declaration is shown in the beginning of this thesis (page iv-vi).

Article

Use of a Hybrid Adeno-Associated Viral Vector Transposon System to Deliver the Insulin Gene to Diabetic NOD Mice

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Abstract: Previously, we used a lentiviral vector to deliver furin-cleavable human insulin (*INS-FUR*) to the livers in several animal models of diabetes using intervallic infusion in full flow occlusion (FFO), with resultant reversal of diabetes, restoration of glucose tolerance and pancreatic transdifferentiation (PT), due to the expression of beta (β)-cell transcription factors (β -TFs). The present study aimed to determine whether we could similarly reverse diabetes in the non-obese diabetic (NOD) mouse using an adeno-associated viral vector (AAV) to deliver *INS-FUR* $\pm$ the β -TF *Pdx1* to the livers of diabetic mice. The traditional AAV8, which provides episomal expression, and the hybrid AAV8/*piggyBac* that results in transgene integration were used. Diabetic mice that received AAV8-*INS-FUR* became hypoglycaemic with abnormal intraperitoneal glucose tolerance tests (IPGTTs). Expression of β -TFs was not detected in the livers. Reversal of diabetes was not achieved in mice that received AAV8-*INS-FUR* and AAV8-*Pdx1* and IPGTTs were abnormal. Normoglycaemia and glucose tolerance were achieved in mice that received AAV8/*piggyBac*-*INS-FUR*/FFO. Definitive evidence of PT was not observed. This is the first in vivo study using the hybrid AAV8/*piggyBac* system to treat Type 1 diabetes (T1D). However, further development is required before the system can be used for gene therapy of T1D.

Keywords: diabetes; liver; adeno-associated viral vector; transposon; gene therapy

1. Introduction

Type 1 diabetes (T1D) is characterised by the autoimmune destruction of pancreatic beta (β) cells, resulting in a lack of insulin secretion and hyperglycaemia [1]. Currently, a patient's blood glucose levels are controlled by multiple daily injections of insulin or by insulin pumps [2] and the development of fast and long-acting insulin analogues has provided more physiological control than older insulins [3]. However, this approach also results in susceptibility to severe hypoglycaemia,

insulin resistance, and mild obesity and does not eliminate complications such as nephropathy, retinopathy, cardiovascular disorders and various neurological problems, which increase morbidity and mortality [4]. Continuous glucose sensors and insulin pumps have led to the development of the artificial pancreas, which can provide better glycaemic control [5]. However, issues such as the high costs of the systems, scar tissue associated with microneedle insertion and sensor failure limit their current usefulness [6]. Transplantation therapy of whole pancreas, human islets or combinations of islets and mesenchymal stem cells [7] are other alternatives to exogenous insulin treatment. However, the limitations of lack of donors, complications of immunosuppressive therapy and issues such as blood-mediated inflammatory reactions [8] underscore the need for alternative treatment approaches.

Gene therapy offers an alternative approach to the treatment/cure of T1D, whereby an “artificial β cell” that is capable of synthesising insulin in response to the normal metabolic signals is genetically engineered from the patient’s own cells. This approach would avoid the problem of rejection seen with both allogeneic transplantation of islets and pancreas and would release T1D patients from daily insulin injections, the risk of hypo- and hyperglycaemia episodes and the long-term chronic complications that lower quality of life and cost the community millions of dollars in patient care. As the patient’s own cells will become insulin-secreting cells, adverse allogeneic immune responses will be avoided. Additionally, there will be no requirement for the ex vivo manipulation of a patient’s liver, and subsequent adoptive transfer of artificial β cells. Hepatocytes have been shown to be suitable candidates for the generation of artificial β cells [9–25]. In most cases, hyperglycaemia has been ameliorated by the expression of various β -cell transcription factors in liver cells, delivered most commonly by adenoviral vectors [13,16,17,19]. However, a number of problems have been encountered using adenoviral vectors. These include the development of exocrine cells in the liver [13,16] and resultant hepatitis following the delivery of *Pdx1* [17]. Other attempts to deliver combinations of transcription factors, such as *Pdx1* and *Neurogenin 3* (*Ngn3*), improved the blood glucose levels of diabetic mice, but could not fully cure diabetes [18,19].

Our laboratory has established that the dual expression of insulin and specific β -cell transcription factors in liver cell lines and primary hepatocytes has a synergistic effect causing pancreatic transdifferentiation, storage of insulin in granules, regulated insulin secretion to glucose and other β -cell secretagogues, and, most importantly, the ability to permanently reverse diabetes [9,10,20,25]. In order to obtain consistently high transduction rates and transgene isolation to the liver in diabetic animals, we developed a novel microsurgical procedure of intervallic infusion in full flow occlusion (FFO) to deliver a lentiviral vector. This technique involved clamping the major veins and arteries to stop blood flow to the liver whilst the vector was injected into the portal circulation. This allowed the vector, containing the insulin gene, to remain isolated to the liver with resultant high transduction rates: 60% in streptozotocin (STZ)-diabetic rats [9] and 42% in spontaneously diabetic non obese diabetic (NOD) mice [10]. These results are likely attributable to the FFO technique, eliminating significant quantities of blood that would have rapidly inactivated the vector by a complement-mediated mechanism. In a world first, we have also observed the expression of human β -cell transcription factors in primary human hepatocytes engrafted into the liver of the humanised FRG mouse [25], in which chimeric human/mouse livers can be generated [26,27].

However, this approach of using the second-generation lentiviral vector in combination with the FFO technique has limitations due to clinical concerns, such as susceptibility to complement inactivation [28] and the invasiveness of the surgical procedure. Ultimately, a more clinically applicable vector system to deliver insulin is required for the translation of this technology. Therefore, in the present study, we endeavoured to design a protocol for the reversal of diabetes using liver-directed gene therapy without the requirement of invasive surgery. For this, we initially used a non-integrating recombinant adeno-associated vector (rAAV). These vectors are attractive candidates for gene therapy as they show long-term gene expression and lack both pathogenicity and immunogenicity. The rAAVs can transduce liver cells with high efficiency and are showing promise in clinical trials [29,30]. In this project we utilised the highly murine liver tropic type 8 capsid [31], with the incorporation of a liver

specific promoter (LSP) [32] to the AAV8 vector, which allowed the systemic delivery by intraperitoneal (i.p.) injection to mouse livers.

We transduced the livers of diabetic NOD mice via i.p. injections of AAV8-*INS-FUR* and AAV8-containing the β -cell transcription factor, *Pdx1* (AAV8-*Pdx1*), with the intention of inducing pancreatic transdifferentiation in the livers. Diabetic mice that received i.p. injections of AAV8-*INS-FUR* became hypoglycaemic with abnormal responses to (i.p.) glucose tolerance tests (IPGTTs). In addition, expression of β -cell transcription factors was not detected in the livers, indicating that this approach was not able to induce β -cell transdifferentiation as anticipated.

In previous successful studies, the lentiviral vector system stably incorporated the *INS-FUR* gene into liver cells [9–11,25]. In an attempt to investigate whether it was the episomal expression of *INS-FUR* using the AAV8 system that resulted in the absence of pancreatic transdifferentiation and persistence of abnormal glucose tolerance, we also employed the AAV8/*piggyBac* system, which can mediate the transposition of transgenes into the host genome [33–35]. As the *piggyBac* system has shown sustained gene expression in adult mice [35], we hypothesised that the AAV8-*INS-FUR-piggyBac* system would result in somatic integration and long-term gene expression, thereby reversing their diabetes in a similar manner to the lentiviral system [9,10,25]. Expression of *INS-FUR* resulted in euglycaemia and normal IPGTTs in the mice that received AAV8/*piggyBac-INS-FUR* and these were subsequently subjected to FFO surgery. The *INS-FUR* gene and the pancreatic hormones, somatostatin and pancreatic polypeptide, were detected in the livers. However, wider evidence of pancreatic transdifferentiation was not seen. This is the first in vivo study using the hybrid AAV8/*piggyBac* system in an attempt to cure autoimmune T1D. However, whilst integration of the AAV8/*piggyBac* vector produced superior results compared to the episomal AAV8 system, it is apparent that the lentiviral system possesses a certain factor(s) that enables widespread pancreatic transdifferentiation to occur in the animal livers that was not seen with either AAV vector.

2. Materials and Methods

2.1. Vector Construction and Production

2.1.1. AAV Vectors

AAV vector constructs (Figure S1) were prepared using a previously reported construct [36], where a powerful liver-specific promoter drives transgene expression. New constructs were built using an In-Fusion cloning kit (Takara-bio, Scientifix Pty Ltd., Clayton, Australia), where the GFP transgene was replaced by sequences encoding *INS-FUR* or murine *Pdx1* with a downstream IRES. AAV vector stocks were produced by triple transfection of HEK 293 cells, as previously described [36]. The titre was acquired using real-time quantitative PCR (qPCR) (Table S1) [37]. The vectors were diluted with phosphate-buffered saline to the required concentration for injection. When combinations of vectors were used, the vectors were mixed and delivered in a single (i.p.) injection.

2.1.2. HIV/MSCV Lentiviral Vector

The HIV/MSCV (HMD) lentiviral vector, which expresses the enhanced green fluorescent protein (EGFP), has a HIV/murine stem cell virus (MSCV) hybrid long-terminal repeat as the promoter [38]. The vector was produced by calcium phosphate precipitation in 293T cells using conditioned medium, as previously described [9]. The culture medium was harvested 48 h after transfection and subjected to syringe and tangential flow filtration, followed by centrifugation to pellet the vector (50,000 $\times$ g, 2 h). Virus titre was determined by transducing 293T cells (5×10^5) with serially diluted vector stocks and quantifying numbers of EGFP-positive cells by flow cytometry, as previously described [2]. Viral replication-competency was also assessed by RT-PCR [9].

2.2. Transduction of Liver Tissue

Female NOD mice were obtained from the Animal Resources Centre, Perth, Australia and were housed at the Ernst Facility, University of Technology Sydney, Sydney, Australia). The housing and experimental conditions complied with the Australian Code for the Care and Use of Animals for Scientific Purposes. Experiments were approved by the Animal Care and Ethics Committee, University of Technology Sydney (ETH17-1559). The mice received treatments after they had spontaneously developed diabetes (blood glucose levels ≥ 10 mmol/L for at least 3 consecutive days).

The vector dose used for a mouse was 5×10^{10} vector genomes (vg). To study the effect of the AAV8-LSP system, the mice were divided into groups of seven and injected i.p. with AAV8 vectors expressing appropriate marker genes: AAV8-*INS-FUR-mCherry* or a combination of AAV8-*INS-FUR-venus* and AAV8-*Pdx1* at equivalent doses. Untreated female diabetic and non-diabetic NOD mice were used as controls. To determine whether the FFO technique had a stimulatory effect on pancreatic transdifferentiation of the livers, the surgery was performed 7 days after the mice received i.p. injections of AAV8-*INS-FUR-venus* in order to allow expression from AAV8-*INS-FUR* prior to performing the FFO technique.

To determine whether the lentiviral capsid/promoter combination was capable of stimulating pancreatic transdifferentiation in the livers expressing AAV8-*INS-FUR-mCherry*, a further group of diabetic NOD mice ($n = 6$) received 5×10^6 transduction units (TU) of HMD/MSCV-eGFP infusion via the portal vein during FFO surgery 7 days after i.p. injections of AAV in order to enable expression from the AAV vector to develop prior to injecting the lentiviral vector.

The AAV8/*piggyBac* vector system is comprised of two different vectors: a transposon vector carrying the *INS-FUR-mCherry* construct and a transposase vector, which works by a ‘cut and paste’ mechanism [33]. To determine whether FFO surgery could induce pancreatic transdifferentiation, FFO surgery was carried out 7 days after the diabetic NOD mice received i.p. injections of the AAV8/*piggyBac-INS-FUR-mCherry* (transposon and transposase dose was 3×10^{10} vg and 3.5×10^{10} vg, respectively). It was anticipated that the mild injury induced by the FFO surgery [9–11,25] would stimulate hepatocyte regeneration thereby clearing the transposase to avoid the continuous excision and insertion of the transgene on the chromosomes.

2.3. Functional Analysis

Mouse body weights and blood glucose levels (BGLs) were monitored daily after the AAV8 treatments. IPGTTs were performed under anaesthesia after fasting the mice for 6 h with water ad libitum. For the IPGTTs, glucose was injected i.p. at a dose of 2 g/kg body weight. Blood was collected and glucose levels were measured at 0, 5, 15, 30, 60, 90 and 120 min after i.p. glucose injection. Human insulin in sera was quantitated using an Invitron Insulin ELISA Kit (IV2-102E, Invitron Ltd., Monmouth, UK), according to the manufacturer’s protocol.

2.4. Microscopic Analysis

Serial frozen sections (15 μ m) of the livers were prepared and fixed using acetone. Mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) was applied to the fixed sections to visualise nuclei. Images were acquired using a fluorescent microscope and camera (Olympus BX60, Olympus Imaging, Macquarie Park, Australia). The excitation and emission ranges of the marker genes were as follows: 400–550 and 500–650 nm for venus, and 540–590 and 550–650 nm for mCherry, respectively.

2.5. Vector Copy Number Analysis

Livers were collected for analysis of vector copy number (VCN) at the end of the experiment. Finely diced tissue pieces (25 mg) were homogenized in 500 μ L of lysis buffer (10 mM Tris-Cl (pH 8), 0.1 M EDTA (pH 8), 20 mg/mL RNase A) and incubated for 1 h at 37 °C. Proteinase K (Sigma-Aldrich,

North Ryde, Australia) was then added at a final concentration of 100 µg/mL, and the digestion was continued overnight at 55 °C. DNA was extracted by adding phenol/chloroform/isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, Macquarie Park, Australia). The mixture was then centrifuged (16,000× g, 5 min), after which the aqueous phase containing the DNA was collected. This extraction process was performed twice and was followed by two extractions with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with ice-cold 100% ethanol containing 1.7 M ammonium acetate and was washed with 70% ethanol. The DNA was dissolved in 10% TE buffer. The amount of DNA extracted from each of the samples was quantified using the Nanodrop spectrophotometer (Thermo Fisher, Macquarie Park, Australia).

The VCN for the mice that received AAV-*INS-FUR*-mCherry was quantified using primers and probes specific to the woodchuck hepatitis post-transcriptional regulatory element (WPRE) [39]. Quantitative PCR was carried out using Platinum® *Taq* DNA polymerase enzyme (Invitrogen/ Thermo Fisher Scientific, Macquarie Park, Australia), as per the manufacturer's instructions. WPRE primers and probe concentrations were 0.8 and 0.2 µM, respectively (Table S1). The initial denaturation was carried out at 95 °C for 10 min (1 cycle), followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Fluorescence was acquired at 60 °C and all the analyses were performed on the Rotorgene 8000 system (Qiagen, Chadstone Centre, Australia). The VCN in all samples was normalised against a qPCR specific for mouse GAPDH [34]. All standards consisted of linearised plasmids. The details of the primer and probe (Sigma-Aldrich, North Ryde, Australia) sequences are presented in Table S1.

The VCNs for the mice that received the combination of AAV-*INS-FUR*-venus and AAV8-*Pdx1* were analysed in two steps. Firstly, to determine the total VCN, the copy number of the internal ribosome re-entry site (IRES) sequence was determined using the SYBR®Premix Ex Taq™ system (Takara Bio, Scientifix, Clayton, Australia). Each of the samples was analysed in duplicate. The reaction (25 µL) contained 0.4 µM final concentrations of each primer, 50 ng of DNA template and 12.5 µL of the 2x SYBR premix. The reactions were carried out at 95 °C for 30 s (1 cycle), followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s. Fluorescence was acquired at 72 °C. In the next step, the copy numbers of *INS-FUR* and *Pdx1* were analysed separately using the respective primers. The VCN for the transposon vectors in the mouse livers was determined by real-time qPCR. The VCN was expressed as vector copies/50 ng DNA.

2.6. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

For RT-PCR analysis, liver and pancreas were collected at experimental end points and frozen in dry ice. Control pancreas and liver tissues were obtained from NOD mice that did not develop diabetes. Total RNA was extracted using the MaxWell®RSC instrument and the MaxWell®RSC Simply RNA Tissue Kit (Promega, Madison, WI, USA). RNA samples were treated with DNase I (Applied BioSystems, Thermo Fisher, Macquarie Park, Australia), according to the manufacturer's protocol. Reverse transcription was performed using the Tetro cDNA Synthesis Kit (Bioline, Everleigh, Australia) and random primers, as per the manufacturer's protocol. PCRs were performed using GoTaq Green PCR® Master Mix (Promega, Madison, WI, USA) with PCR parameters optimised for the amplification of the following genes: *Beta-Actin*, *INS-FUR*, *Pdx1*, *NeuroD1*, *Nkx2.2*, *Nkx6.1*, *MafA*, *Pax6*, *P48*, Mouse Insulin 1 and Insulin 2, *Glut 2*, pancreatic polypeptide and somatostatin (Table S2). Primers used were designed to cross intron exon boundaries to avoid amplification of any residual genomic DNA.

2.7. Statistical Analysis

Data were analysed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Two-way ANOVA followed by Tukey's multiple comparison tests were performed to compare the BGLs during the IPGTTs and the weekly random BGLs of the treated mice with that of the control groups. The Mann–Whitney test was applied when comparing the vector dosages and the VCN between the experimental groups. For the mice that were transduced by a combination of *INS-FUR*

and *Pdx1*, a paired t-test was applied when comparing the AAV8-*INS-FUR*-venus and AAV8-*Pdx1* copy numbers. The differences were considered significant when $p < 0.05$.

3. Results

3.1. Microscopic Analysis

Immunofluorescent expression of the fluorphores, mCherry and venus was examined in frozen sections of the transduced livers to examine transduction efficiency. Figure 1A shows expression of the mCherry marker gene (AAV8-*INS-FUR*-mCherry), Figure 1B,C show DAPI-stained nuclei and a merged image, respectively, 9 weeks after initial transduction with the non-integrating AAV8 vector. Figure 1D–F shows images of the venus marker gene (AAV8-*INS-FUR*-venus) and DAPI-stained nuclei at the experimental end point of 9 weeks after initial transduction with the non-integrating AAV8 vector. Figure 1A–F indicates widespread hepatocyte transduction as seen in our previous studies [39]. By comparison, normal liver tissue showed no expression of venus or mCherry (Figure 1G–I), with only the DAPI-stained nuclei evident. Likewise, expression of the AAV8/*piggyBac*-*INS-FUR*-mCherry transposon/transposase system was also extensive in the liver tissue (Figure 1J–L) at 15 weeks post-transduction.

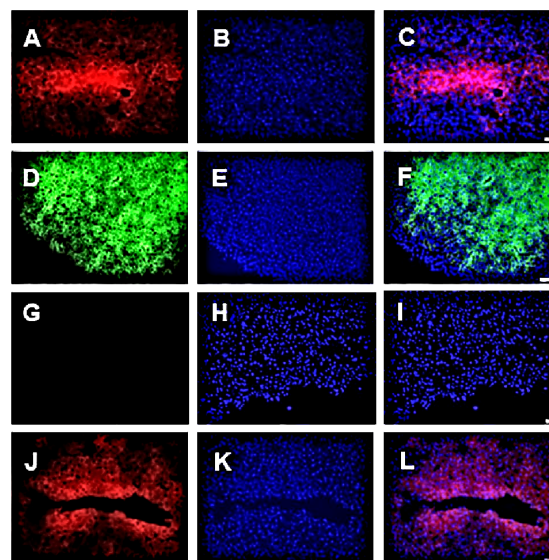


Figure 1. Immunofluorescence detection of marker gene expression in NOD mouse livers following transduction with AAV vectors. Frozen sections of mouse liver were prepared for immunofluorescence detection and visualised by fluorescence microscopy. (A) m-Cherry-*INS-FUR* immunofluorescence 9 weeks after initial transduction, (B) DAPI-stained nuclei of A and (C) is a merged image of A and B. (D) Venus-*INS-FUR* immunofluorescence 9 weeks after *INS-FUR* and *Pdx1* were delivered together. (E) shows the DAPI-stained nuclei of D and (F) is a merged image of D and E. (G) is a normal liver section showing no immunofluorescence for either venus or mCherry and (H) is an image of DAPI-stained nuclei of (G) and (I) is a merged image of (G) and (H). (J) *piggyBac*-*INS-FUR*-mCherry immunofluorescence 15 weeks after initial transduction, (K) is the DAPI-stained nuclei of (J) and (L) is a merged image of (J) and (K). Bar = 20 μ m.

3.2. Delivery of AAV8 Expressing *INS-FUR* $\pm$ *Pdx1* Fails to Reverse Diabetes

In order to determine whether expression of *INS-FUR* alone would reverse hyperglycaemia in the diabetic NOD mice and establish normal glucose tolerance, the animals received an i.p. injection of the AAV8-*INS-FUR*-mCherry vector. The animals exhibited normalisation of BGLs on week 3,

but became hypoglycaemic on week 5 (Figure 2A). The copy number of AAV8-*INS-FUR*-mCherry in the livers of these mice was $3.33 \pm 0.18 \times 10^5$ copies per 50 ng of DNA (Figure 2C). At all the time points during the IPGTTs, the BGLs of the mice which received AAV8-*INS-FUR*-mCherry were lower than those of the diabetic mice ($p < 0.05$) (Figure 2B). During IPGTTs, the BGLs of the mice which received AAV8-*INS-FUR*-mCherry were also lower than those of the non-diabetic control mice at 0, 5, 15, 30 and 120 min (i.e., all time points sampled excluding 60 and 90 min; $p < 0.05$) (Figure 2B).

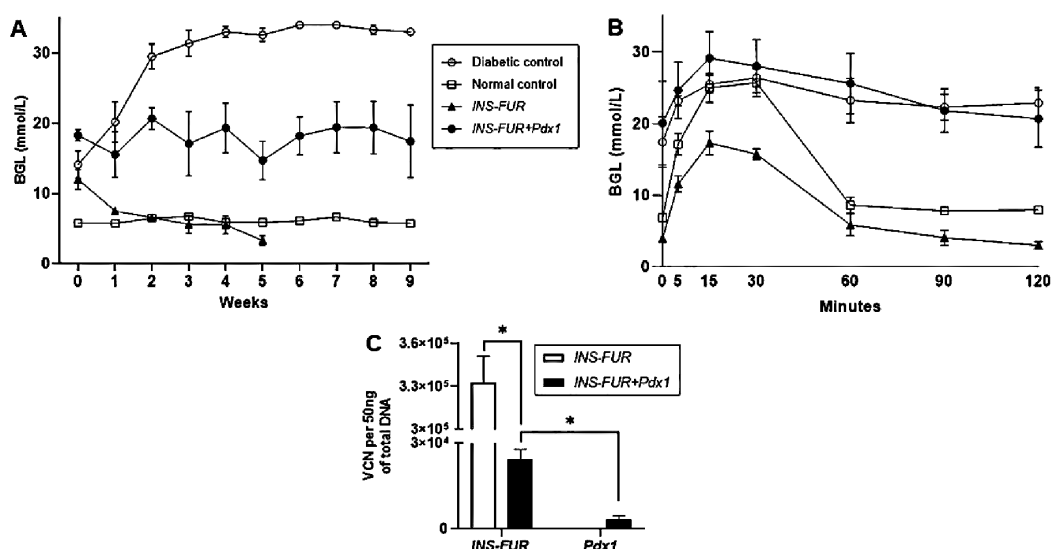


Figure 2. Blood glucose levels, IPGTTs and AAV8 VCNs of NOD mice after expression of *INS-FUR* alone, or with *Pdx1*, using the AAV8 vector. Transduction of NOD mice with AAV8-*INS-FUR* or AAV8-*INS-FUR* + AAV8-*Pdx1* did not reverse hyperglycaemia in diabetic mice. (A) The mean weekly blood glucose levels of diabetic ($n = 7$), normal (non-diabetic) ($n = 7$) and treated diabetic mice that received i.p. injections of either AAV8-*INS-FUR*-mCherry ($n = 4$), or AAV8-*INS-FUR*-venus + AAV8-*Pdx1* ($n = 5$) are shown. (B) Blood glucose levels following an IPGTT of diabetic ($n = 3$), non-diabetic ($n = 7$) and treated diabetic mice that received i.p. injections of either AAV8-*INS-FUR*-mCherry ($n = 3$) or AAV8-*INS-FUR*-venus + AAV8-*Pdx1* ($n = 4$). (C) AAV8 vector copy numbers of the diabetic mice transduced by AAV8-*INS-FUR*-mCherry ($n = 4$) or AAV8-*INS-FUR*-venus + AAV8-*Pdx1* ($n = 5$). Results are expressed as the means $\pm$ SEMs. * indicates a significant difference of $p < 0.05$ when comparing the VCNs.

In an attempt to force pancreatic transdifferentiation, the β -cell transcription factor *Pdx1* [13] (AAV8-*Pdx1*) was expressed in the livers together with the *INS-FUR* gene (AAV8-*INS-FUR*-venus). Mice that received the combination of AAV8-*INS-FUR*-venus and AAV8-*Pdx1* remained hyperglycaemic (Figure 2A). At all the time points (0–120 min) during the IPGTTs, the BGLs of the mice that received the combination of *INS-FUR* and *Pdx1* were not significantly different from the diabetic mice (Figure 2B). At the end point of the experiment, the mean AAV8-*INS-FUR*-venus copy numbers in the livers were $2.41 \pm 0.37 \times 10^4$ copies per 50 ng of DNA, and the mean AAV8-*Pdx1* copy numbers were $3.41 \pm 1.12 \times 10^3$ copies per 50 ng of DNA (Figure 2C). The paired t-test showed that the AAV8-*INS-FUR*-venus copy number was significantly higher than the AAV8-*Pdx1* copy number ($p < 0.05$) in the livers of the animals.

We have previously hypothesised that since liver and pancreas are from the same endodermal origin, it is likely that the FFO procedure represents an insult to the liver that stimulates differentiation of the hepatocytes to an immature phenotype. It is this differentiation process that causes expression of the β -cell transcription factors, allowing pancreatic transdifferentiation to occur in the presence of insulin and a hyperglycaemic environment [9–11,25]. Given the previous efficacy of using the FFO

surgical technique and the lentiviral delivery of *INS-FUR* alone to reverse diabetes, we attempted to induce pancreatic transdifferentiation and normalise BGLs of diabetic mice by performing this procedure subsequent to the i.p. injection of AAV8-*INS-FUR*-venus. Unfortunately, BGLs were not normalised (Figure 3A). This group of mice had significantly lower VCNs ($5.12 \pm 1.06 \times 10^4$ per 50 ng DNA) (Figure 3C) compared to the mice that received i.p. injections of AAV8-*INS-FUR*-mCherry ($3.33 \pm 0.18 \times 10^5$ copies per 50 ng DNA) ($p < 0.001$) (Figure 2C). The higher BGLs and the lower AAV8 VCNs of the mice that had AAV8-*INS-FUR*-venus (i.p.) and FFO surgery, as compared to the mice that only received an i.p. injection of AAV8-*INS-FUR*-mCherry supported the hypothesis that the FFO surgery may have induced tissue damage, leading to the regeneration of hepatocytes and, therefore, the reduction in AAV8 VCNs. Despite having high BGLs, during the IPGTTs, the BGLs of the mice that received AAV8-*INS-FUR*-venus and FFO surgery were not significantly different from those of the normal controls (Figure 3B).

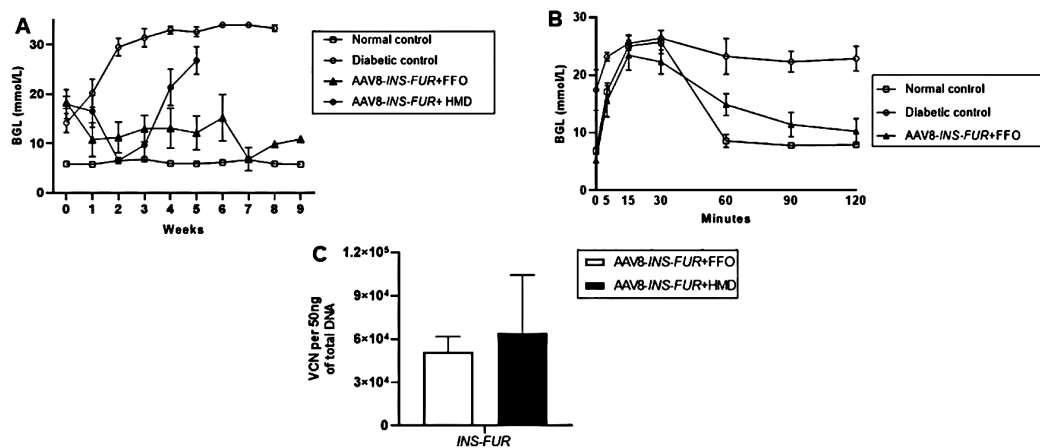


Figure 3. Blood glucose levels, IPGTT and AAV8 vector copy numbers of NOD mice after AAV8-mediated expression of *INS-FUR* in combination with the FFO surgical procedure, with or without dual expression of the lentiviral HMD vector. NOD mice were transduced with AAV8-*INS-FUR* in combination with the FFO surgery, as well as with dual expression of HMD. Blood glucose levels and results of subsequent IPGTT tests of the mice are shown. (A) Mean weekly blood glucose levels of the diabetic ($n = 7$), normal, non-diabetic ($n = 7$), and diabetic NOD mice that received i.p. injections of AAV8-*INS-FUR*-venus + FFO surgery ($n = 6$) and diabetic NOD mice that received AAV8-*INS-FUR*-mCherry + HMD-EGFP ($n = 6$). (B) Blood glucose levels following an IPGTT of diabetic ($n = 3$), non-diabetic ($n = 7$) and diabetic mice that received i.p. injections of AAV8-*INS-FUR*-mCherry ($n = 3$). The symbols for each group are indicated in the accompanying legend. Results are expressed as the means $\pm$ SEMs at each time point. (C) AAV8 vector copy numbers of the diabetic mice that received i.p. injections of AAV8-*INS-FUR*-venus + FFO surgery ($n = 6$) and diabetic NOD mice that received AAV8-*INS-FUR*-mCherry + HMD-EGFP ($n = 5$). Results are expressed as the means $\pm$ SEMs.

To determine whether the lentiviral capsid/promoter combination was capable of inducing pancreatic transdifferentiation in the livers expressing *INS-FUR*, NOD mice received HMD/MSCV-eGFP as an infusion via the portal vein during FFO surgery 7 days after having i.p. injections of AAV *INS-FUR*-mCherry. The BGLs of the mice were normalised on week 2, but they became hyperglycaemic from week 4 onwards (Figure 3A). The general health of the mice also deteriorated and symptoms of chronic hyperglycaemia, such as polyuria and polydipsia, persisted, leading to termination of the experiment before IPGTTs were performed.

3.3. Reversal of Autoimmune Diabetes Using the AAV8/piggyBac-LSP-INS-FUR Vector System and FFO Surgery

The *piggyBac* transposition system, which allows for the stable expression of transgenes over time [33], was employed to determine whether the episomal (non-integrating) expression provided by the traditional AAV8 system was insufficient to stimulate pancreatic transdifferentiation in the mouse livers. Firstly, we examined if the hyperglycaemia of the diabetic NOD mice could be normalised by injection of *INS-FUR* alone. The BGLs of the mice that received an i.p. injection of the AAV8/*piggyBac-INS-FUR-mCherry* without FFO surgery were reduced, but normoglycaemia was not reached (Figure 4A). The transposon and transposase copy numbers in the livers of the mice that received i.p. injections of the AAV8/*piggyBac-INS-FUR-mCherry* vector system were 1.89 ± 0.05 and $1.57 \pm 0.05 \times 10^5$ copies per 50 ng DNA, respectively, and were not significantly different (Figure 4B).

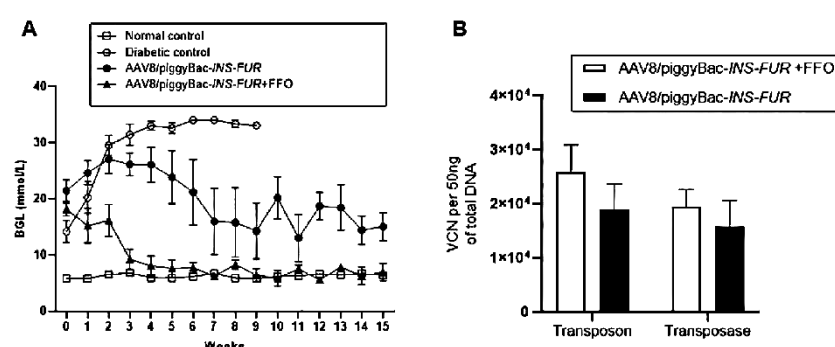


Figure 4. Blood glucose levels and AAV8 vector copy numbers of NOD mice after *piggyBac*/AAV8-mediated expression of *INS-FUR* and *INS-FUR* combined with the FFO surgical procedure. Diabetic NOD mice were transduced with either *INS-FUR* or *INS-FUR* in combination with the FFO surgical procedure at 7 days after transduction. (A) The mean weekly blood glucose levels of untreated diabetic ($n = 7$), normal, non-diabetic ($n = 7$) and treated diabetic NOD mice that received either i.p. injections of AAV8-*piggyBac*/INS-FUR-mCherry ($n = 7$) alone, or AAV8-*piggyBac*/INS-FUR-mCherry with FFO surgery ($n = 7$). (B) Transposon and transposase copy numbers of diabetic NOD mice that received i.p. injections of AAV8/*piggyBac-INS-FUR* or i.p. injection of AAV8/*piggyBac-INS-FUR* and FFO surgery. The results are expressed as the means $\pm$ SEMs.

Interestingly, despite abnormal BGLs, the IPGTT results for the animals that received an i.p. injection of the AAV8/*piggyBac-INS-FUR-mCherry* without FFO surgery were not significantly different from those for the controls (Figure 5A). This was possibly related to this vector favouring integrated expression of *INS-FUR*. Alternatively, the constitutive expression of insulin may have reached a balanced level in response to rising glucose levels in these animals as normal IPGTTs were also seen when the non-integrating AAV8 was used to deliver *INS-FUR* (Figure 3B).

In order to determine whether the FFO procedure had a stimulatory effect on pancreatic transdifferentiation of the livers and correction of hyperglycemia, diabetic mice received the AAV8/*piggyBac-INS-FUR-mCherry* vector and FFO surgery 7 days later. These animals showed a reduction in BGLs at three weeks post-treatment that was then maintained at concentrations not significantly different to normal controls (experimental end point, week 15) (Figure 4A). Additionally, for animals that reverted to normoglycaemia, the BGLs during IPGTTs were not statistically different from values observed for the control mice (Figure 5B). Analysis of human insulin concentrations in sera obtained during the IPGTTs showed that the levels of human insulin for mice that had received AAV8/*piggyBac-INS-FUR-mCherry* and the FFO procedure peaked 15 min after glucose delivery and returned to baseline levels by 60 min (Figure 5C). These results indicated that the AAV8/*piggyBac-INS-FUR-mCherry* and the FFO procedure normalised BGLs for a significant period of time with normal glucose tolerance on IPGTT and human insulin peaked at levels seen in normal

animals [40]. In the livers of the mice that received the AAV/*piggyBac*-*INS-FUR*-mCherry system and FFO surgery, the copy numbers of the transposon ($2.6 \pm 0.05 \times 10^5$ copies per 50 ng DNA) and transposase ($1.9 \pm 0.03 \times 10^5$ copies per 50 ng DNA), were not significantly different (Figure 4B).

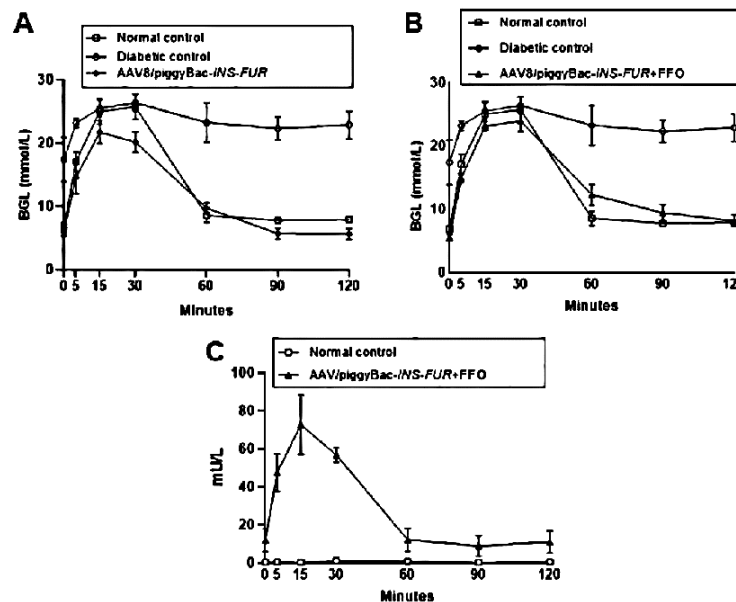


Figure 5. Normalisation of glucose tolerance of diabetic NOD mice after expression of *INS-FUR* by the integrating *piggyBac*/AAV8 vector combined with the FFO surgical procedure. Diabetic NOD mice were transduced with *INS-FUR*, or *INS-FUR* in combination with the FFO surgical procedure at 7 days after transduction, using the integrating *piggyBac*/AAV8. (A) Blood glucose levels following an IPGTT of diabetic ($n = 3$), non-diabetic ($n = 7$) and diabetic NOD mice that received i.p. injections of *INS-FUR* alone as AAV8-*piggyBac*/*INS-FUR*-mCherry ($n = 7$). (B) Blood glucose levels following an IPGTT of diabetic ($n = 3$), non-diabetic ($n = 7$) and diabetic NOD mice that received i.p. injections AAV8-*piggyBac*/*INS-FUR*-mCherry combined with the FFO surgery ($n = 6$). (C) Serum concentration of human insulin following IPGTT of the mice represented in (B), which received i.p. injections of AAV8-*piggyBac*/*INS-FUR*-mCherry combined with FFO surgery. Results are expressed as the means $\pm$ SEMs.

3.4. RT-PCR Analysis

It can be seen from Figure 6 that expression of β -cell transcription factors was inconsistent across the experimental groups, indicative of the absence of induction of reproducible β -cell transdifferentiation in any of the treatment groups. *INS-FUR* was expressed in all samples transduced with the transgene and *Glut2* was expressed in all tissues. As expected, normal mouse pancreas expressed all genes with the exception of *INS-FUR*. The livers of mice that received AAV8-*INS-FUR*-mCherry expressed *NeuroD1*, *somatostatin* and *pancreatic polypeptide*. However, the dual transduction of *INS-FUR* and *Pdx1* resulted in expression of only *INS-FUR* and *Pdx1* and the dual transduction of AAV8-*INS-FUR*-venus + HMD/MSCV-EGFP, resulted in the expression of *INS-FUR* alone. Expression of other transcription factors indicative of β -cell transdifferentiation (*Nkx2.2*, *Nkx6.1*, *MafA*, *Pax6*, or mouse *insulin 1*) was not observed. The exocrine marker *p48* was also not expressed (data not shown). Similarly, no evidence of β -cell transdifferentiation was observed when the *piggyBac* system was used to express *INS-FUR*. In this instance, only the *INS-FUR* was expressed. Use of the *piggyBac*-*INS-FUR* system in combination with FFO surgery only resulted in expression of *somatostatin* and *pancreatic polypeptide*.

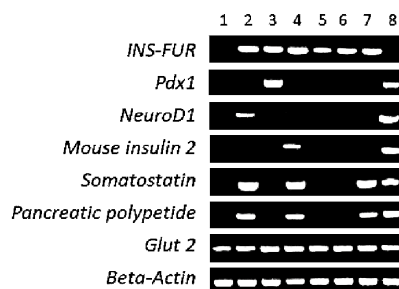


Figure 6. Expression of β -cell transcription factors and pancreatic hormones in the livers of transduced NOD mice. Standard end point (non-quantitative RT-PCR) analysis was conducted from RNA derived from liver tissues of the NOD mice to detect the expression of *INS-FUR*, *Pdx1*, *NeuroD1*, *mouse insulin 2*, *mouse somatostatin*, *pancreatic polypeptide*, *Glut 2*, and *beta-actin* (positive control) in normal mouse liver (lane 1), liver tissue from diabetic NOD mice expressing *INS-FUR* via i.p. injection of AAV8-*INS-FUR*-mCherry (lane 2), *INS-FUR* and *Pdx1* via i.p. injection of AAV8-*INS-FUR*-venus + AAV8-*Pdx1* (lane 3), AAV8-*INS-FUR*-venus + FFO surgery (lane 4), via i.p. injection of AAV8-*INS-FUR*-venus + HMD/MSCV-EGFP via the portal vein (lane 5), AAV8/*piggyBac*-*INS-FUR*-mCherry (lane 6), AAV8/*piggyBac*-*INS-FUR*-mCherry + FFO surgery (lane 7) and normal mouse pancreas (lane 8).

4. Discussion

Whilst treatment options for T1D are numerous, they are all limited in their long-term effectiveness [8] and, as a result, the search for more innovative and efficacious ways to treat/cure T1D is urgently required. Both insulin gene therapy and the reprogramming of liver cells to a β -cell phenotype have been studied by many groups as potential options [41]. The liver is considered an appropriate choice for these studies, as the liver and pancreas share a close developmental origin and the liver has great regenerative capacity. These studies have largely centred on the delivery of insulin and insulin analogues and/or β -cell transcription factors to liver cells using viral vectors, which suffer from varying multiple drawbacks. The most commonly used viral vectors are adenoviral vectors which cannot provide long-term expression of genes and are immunogenic [42]. Retroviral vectors are limited by their inability to transduce non-dividing cells and insertional mutagenesis has been problematic in a clinical trial of a severe-combined immunodeficiency patient [43]. Lentiviral vectors demonstrate long-term transgene expression but, as an integrating vector, may suffer from issues of insertional mutagenesis, although third-generation vectors have a much improved safety profile [44]. Non-integrating adeno-associated vectors show long-term expression and lack pathogenicity and immunogenicity, together with the ability to transduce liver tissues with high efficiency [31]. The AAV/*piggyBac* system is known to confer stable integration, and studies with the AAV2/*piggyBac* in our laboratory have shown less frequent integrations in intragenic regions in comparison to lentiviral vectors [34] and more importantly, the integrations were not found in the loci of genes associated with hepatocellular carcinoma [36].

The β -cell transcription factor *Pdx1* has been shown to induce pancreatic transdifferentiation of liver tissue when delivered using adenoviral vectors [13,16] and some improvement in hyperglycaemia when delivered to a humanized mouse model using an AAV2 vector [22]. However, in the current study, delivery of *INS-FUR* alone (AAV8-*INS-FUR*-mCherry) or *INS-FUR* together with *Pdx1* (AAV8-*INS-FUR*-venus + AAV8-*Pdx1*), using the non-integrating AAV8 vector did not reverse hyperglycaemia and there was no evidence of expression of β -cell transcription factors that lead to pancreatic transdifferentiation. As noted in the methods, the mice received equal doses of AAV8-*INS-FUR*-mCherry, AAV8-*INS-FUR*-venus and AAV8-*Pdx1*, but the VCN of the *INS-FUR*-mCherry was significantly higher than the *INS-FUR*-venus at the conclusion of the experiments and the *INS-FUR*-venus was significantly higher than the *Pdx1*. We have much experience

in quantifying the VCN by quantitative RT-PCR and are thus confident in the values presented. However, the differences in the VCN of the constructs cannot be attributed to the composition of the vectors (Figure S1) and, therefore, a definitive explanation is not possible for this observation. The 10-fold difference in the insulin vectors may be explained by the age of the mice. The NOD mice used in these experiments spontaneously developed diabetes from 12 to 26 weeks of age and it is thus not possible to isolate a diabetic cohort that is of exactly the same age. The mice used in the early experiment with the *INS-FUR-mCherry* vector that recorded VCNs 10-fold higher than those of the *INS-FUR-venus* vector averaged 16 weeks of age, whereas the second group averaged 21 weeks of age, and there is evidence that the vectors may transduce young animals more efficiently [45]. The lower transduction efficiency of the *Pdx1* vector may be due to some associated toxicity with the *Pdx1* vector, where *Pdx1*-expressing cells are lost after vector transduction. Another possible scenario may involve immune reactions against the vector. It has been recently reported that significant barriers to effective AAV2/8-insulin gene therapy in NOD mice were caused by reactivation of anti-insulin autoimmune responses as well as immune reactivity against vector components [24]. The researchers found that the efficacy of AAV-gene therapy in the NOD mouse was improved with anti-CD4 antibody treatment, indicating that T-helper subsets occurred. Future studies in NOD mice should look more closely at the immunogenicity of the vector, which may also be age dependent and consideration should be given to inducing diabetes with multiple low doses of streptozotocin (STZ) so all experimental cohorts are a similar age.

This is the first study to utilise the AAV8/*piggyBac* system to deliver human insulin to diabetic NOD mice. We showed that i.p. delivery of the AAV8/*piggyBac-INS-FUR* vector, significantly reduced the BGLs of spontaneously diabetic NOD mice, but did not completely reverse hyperglycaemia. By comparison, i.p. delivery of this vector, followed 7 days later by a surgical procedure that isolates the liver from the circulation (FFO), resulted in reversal of diabetes from week 3 to 15 (experimental end point), without induction of hypoglycaemia and with restoration of normal glucose tolerance. Interestingly, in both circumstances delivery of the AAV8/*piggyBac-INS-FUR* vector resulted in normal glucose tolerance following a 6 h fast. These results occurred without the expression of β -cell transcription factors, and, therefore, pancreatic transdifferentiation. These observations suggested that the integration of the *INS-FUR* gene alone was beneficial for the regulation of BGLs only if the FFO procedure was also used. This result was likely attributable to efficient integration of the *INS-FUR* construct (due to removal of a proportion of the transposase because of cell division) resulting in higher insulin production and reversal of hyperglycaemia. However, the integration of the *INS-FUR* gene induced by the AAV8/*piggyBac* system was insufficient to stimulate the liver-to-pancreas transdifferentiation seen with the use of the lentiviral system because the necessary pancreatic transcription factors were not also expressed. This observation suggested that the FFO surgery and the presence of a certain element(s) in the HMD vector, which were not present in the AAV8 vector, were required to induce the transdifferentiation process when *INS-FUR* was delivered.

Pancreatic transdifferentiation that results in insulin storage and regulated secretion from storage granules is one gene therapy strategy under investigation to cure T1D. It is likely that for this to occur, a “pancreatic switch” must be activated [46]. This switch may involve expression of β -cell transcription factors [9–11,25], transient destruction of some liver tissue by the FFO delivery technique [9–11,25], and/or factors present in the second generation lentiviral vector [38]. In our previous studies, the lentiviral vector likely induced pancreatic transdifferentiation in certain lineage(s) of hepatic cells that displayed plasticity, such as oval cells or stem cells, and/or took advantage of their propensity to transdifferentiate into different cell types when stressed [47]. A study by Wang et al. [19] using STZ-diabetic mice indicated that the forced liver-to-pancreas transdifferentiation was not possible utilising AAV8 vector expression of *Pdx1* and *NeuroD1*. The additional insult of an adenoviral vector that induced immune responses was required for pancreatic transdifferentiation, and some amelioration of the diabetic hyperglycaemia. Likewise, a study by Cerad-Esteban et al. [48] reported that the TALE homeoprotein, TGIF2, acts as a developmental regulator of pancreas versus liver fate in cell lines and

primary rodent hepatocytes. The AAV-mediated delivery of TGIF2 first represses hepatic identity and initiates a ‘switch’ that turns on a pancreatic cell identity. We saw a similar pattern in our earlier study in NOD mice using the lentiviral vector to deliver *INS-FUR*, where there was significant upregulation of key β -cell transcription factors (*Pdx1*, *NeuroD1* and *Neurog3*), and significant down regulation of hepatic markers (*C/EBP- β* , *G6 PC*, *AAT* and *GLUT1*) at 7 and 10 days post-transduction of the livers, which was maintained until the experimental end point (150 days) [10].

Based on our work, it would appear that for AAV vectors to induce liver-to-pancreas transdifferentiation an additional factor(s), such as concomitant immune responses, a minor insult, or a developmental regulator, is required. A combination of the AAV8/*piggyBac* system and a cocktail of β -cell transcription factors may warrant future investigation [49]. The current study suggests that, with further development of the AAV vector system and a better understanding of the pancreatic transdifferentiation process, the integrating AAV8/*piggyBac* system may be useful to at least satisfy basal insulin requirements, and pancreatic transdifferentiation may not be required to achieve some advantageous clinical outcomes. Such outcomes may also be achieved with the use of inducible promoter systems such as the Tet-off system that has been shown to regulate insulin delivered by an AAV8 system in diabetic NOD.cg-Prkdcscidll2 rgtm1 Wjl/szJ mice [23]. Non-viral delivery mechanisms such as insulin constructs in minicircle DNA [21] which resulted in glucose-regulated insulin production from rat livers is a promising system that avoids possible complications of viral vectors. Haematopoietic stem cell-mediated gene therapy can produce a tolerogenic environment for islets and prevent destruction on transplantation, by halting antigen-specific memory T-cell responses [50]. This is one of many other possible gene therapy technologies being examined to treat/cure T1D.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4409/9/10/2227/s1>, Figure S1: AAV vector maps; Table S1: Primer and probe sequences used for the quantitation of vector and transcript copy number; Table S2: Primer sequences for the detection of target transcripts by RT-PCR.

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4.2 Chapter conclusion

The results of the experiments presented in the published article show that the liver-to-pancreas transdifferentiation could not be induced in the NOD mice by the delivery of the *INS-FUR* gene alone or the combination of the *INS-FUR* gene and the transcription factor *Pdx1* by the AAV8 vector. In addition, the inclusion of the VSV-G envelope and hybrid HIV/MSCV promoter of the HMD vector system did not cause the liver-to-pancreas transdifferentiation. The FFO surgery in conjunction with the HMD/*INS-FUR* vector was more effective in inducing the liver-to-pancreas transdifferentiation than the combination of FFO and the hybrid AAV8/*piggyBac* system. The NOD mice transduced by the HMD/*INS-FUR* under FFO surgery had normal BGLs, normal glucose tolerance test, insulin excretion in glucose-responsive manner, and the expression of the endogenous pancreatic genes in their livers [150]. By comparison, the NOD mice that had FFO surgery after AAV8/*piggyBac-INS-FUR* transduction also had normal BGLs and normal glucose tolerance, but the expression of the endogenous pancreatic transcription factors could not be detected in the livers. The current study suggested that using the AAV8/*piggyBac-INS-FUR* system, pancreatic transdifferentiation may not be required to achieve clinically advantageous outcomes. The integrating AAV8/*piggyBac-INS-FUR* system was useful to satisfy both the basal and glucose-responsive insulin secretion.

Even though T1D could not be reversed by the gene therapy utilising the traditional AAV8 vectors, the system may still be valuable in treating other diseases because it is non-pathological and does not generate any strong immune response. It was apparent that the FFO surgery in conjunction with the HMD/*INS-FUR* vectors was much more effective in inducing the liver-to-pancreas transdifferentiation than the combination of FFO and the AAV8-*INS-FUR* vectors. It was thought that the insult from the surgery after the AAV8 transduction was not enough to enhance a large scale liver regeneration that was required for the transdifferentiation. However, this speculation is yet to be validated. In order to identify any other effect of the AAV8-*INS-FUR* system and the FFO surgery to the livers, transcriptome analysis was performed and the results of this analysis will be reported in the following chapter.

Chapter 5:

Transcriptome analysis of the NOD mouse livers transduced by AAV8- *INS-FUR*

5.1 Introduction

In chapter 4, it was shown that NOD mice that had FFO surgery after the AAV8-*INS-FUR* transduction demonstrated a slight reduction in the BGLs, while the transduced mice that did not have FFO surgery became hypoglycaemic (Chapter 4). The differences in the BGLs of the mice could be the result of the differences in the metabolic pathways that affect the metabolism of glucose, such as: glycolysis, gluconeogenesis, glycogen synthesis and degradation. Therefore, the first aim of this chapter was to identify the link between the differences in the BGLs and the differences in the glucose metabolism of the mice that were transduced by AAV8-*INS-FUR* with and without FFO surgery. A microarray based transcriptomic analysis was performed to compare the diabetic NOD mice transduced by AAV8-*INS-FUR* with or without FFO to the NOD mice that had normal BGLs

In previous experiments, diabetic NOD mice transduced by portal vein injections of the lentiviral HMD/*INS-FUR* vectors had normal BGLs, but abnormal glucose tolerance and did not express any β -cell transcription factors [150]. By comparison, the diabetic NOD mice transduced by the portal vein infusion of the HMD/*INS-FUR* vector using the FFO procedure had normal BGLs, and normal glucose tolerance [150]. The microarray and RT-qPCR analysis of NOD mouse livers transduced by the HMD/*INS-FUR* vector using the FFO procedure showed a down-regulation of liver markers (CEBP-beta, CEBP-alpha, G6pc and AAT) and up-regulation of β -cell transcription factors and other pancreatic genes (*Pdx1*, *NeuroD1*, *Ngn3*, *Mouse insulin 1*, *mouse insulin 2*, *Gck*, *glucagon*, and *synaptophysin*) [150]. These observations suggested that the FFO procedure may help to induce the pancreatic transdifferentiation in the presence of the HMD lentiviral vectors. In contrast, mice transduced by AAV8-*INS-FUR* with FFO surgery had abnormal BGLs but did not express any of the major β -cell transcription factors. These observations led us to speculate that the AAV8 vector had inherent differences that did not induce the cascade of liver-to-pancreas transdifferentiation seen with the lentiviral vector. As the vectors are derived from markedly different viruses, the reasons could be due to the interaction of many and varied factors. It was also hypothesised in our experiments with the lentiviral system that the FFO procedure induced an insult to the liver leading to liver cell death and stimulation of the pancreatic transdifferentiation, as liver-to-pancreas

differentiation has been seen in other studies after an insult to the liver [150]. In the current study it was hypothesised that this insult provided by the FFO procedure, could lead to cell death and subsequent reduction of the *INS-FUR* transgene expression. The VCN analysis revealed that the AAV8 VCNs of the diabetic NOD mice that had the surgery after AAV8-*INS-FUR* transduction were significantly lower than the mice that did not have the surgery despite the fact that both groups received the same vector dose (5×10^{10} vg) (Chapter 4). It was apparent that the lower numbers of the *INS-FUR* transgene in the mice that received the FFO surgery may have led to the higher BGLs in comparison to the transduced mice that did not have the surgery. However, the hypothesis that the FFO procedure would reduce the *INS-FUR* genes expression by causing liver cell death was yet to be validated. One of the possible ways to speculate if cell death had occurred was performing the transcriptomic analysis to identify the activation or inhibition of the pathways, diseases, toxicity and bio functions that may associate with or directly cause liver injury and liver cell death. Therefore, the **second aim** of the chapter was to identify any possible impacts of the AAV8-*INS-FUR* vectors and the FFO surgery on the livers by performing a microarray-based transcriptome analysis of the livers transduced by AAV8-*INS-FUR* with and without FFO surgery.

5.2 Materials and Methods

Livers were collected from 3 non diabetic NOD mice (normal group), 3 diabetic NOD mice transduced by i.p injection of AAV8-*INS-FUR* (5×10^{10} vg) (AAV group) and 3 diabetic NOD mice that had FFO surgery 7 days after the AAV8-*INS-FUR* (5×10^{10} vg) transduction (AAV+FFO group). The 3 mice in the normal group were euthanised and their livers were collected at 28 weeks old. They had normal random BGLs (3.5 - 7 mmol/L) for their entire lifespan. The BGLs of the mice in the AAV group normalised 1 week after the AAV8 transduction, but progressively reduced to hypoglycaemic levels (< 3 mmol/L); therefore, the experiment had to be terminated on week 5, when the livers were collected (Chapter 4). The BGLs of the AAV+FFO were reduced, but were not completely normalised and the experiment was terminated 8 weeks after the FFO surgery (equivalent to 9 weeks after the AAV8-*INS-FUR* transduction) (Chapter 4).

RNA was extracted from livers following the protocol in section 2.5. The purity, quality and quantity of the RNA samples were assessed using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) and an Agilent Bio-Analyser with an Agilent Eukaryote Total RNA Nano Kit (Agilent, USA). The samples were considered suitable for microarray-based transcriptome analysis if the RNA integrity number (RIN) was 8 or higher and both the A_{260}/A_{280} and the A_{260}/A_{230} ratios were in the range of 1.8-2.0. RNA samples from the normal group and the AAV groups were pooled and analysed in triplicate. As there was a variation in the daily random BGLs of the 3 mice in the AAV+FFO group, RNA samples from each mouse were analysed in duplicate to allow for comparison between individual animals if necessary. The entire microarray transcriptomics analysis was performed by the Ramaciotti Centre for Genomics (University of New South Wales, Australia) using the GeneChip® 3' IVT Reagent Kit and the GeneChip® HT-MG-430 PM 16 Array plate (Applied Biosystems, USA).

The data obtained were analysed using the Transcriptome Analysis Console (TAC) (Applied Biosystems, USA) to determine the differences in gene expression levels between the three groups of mice. The expression level differences were considered to be significant when: (i) the P-values were less than 0.05, (ii) the fold-changes in gene expression were higher than 2 or lower than -2 and (iii) the P-values for the false detection rates (FDR P-value) were less than 0.05. The TAC program can also compute the Principal component analysis (PCA) graph illustrating the distribution in the gene expression profiles of the 3 groups of mice.

The pathway analyses were performed using the TAC software, the Reactome database [185-188] and the Ingenuity Pathway Analysis (IPA) software (Qiagen, Victoria, Australia). The TAC software mapped the differentially expressed genes into corresponding pathways based on the WikiPathways database and ranked them according to the significance using the P-value estimated by Fisher's Exact Test (two sided). Pathways that had $-\log(P\text{-value})$ higher than the threshold of 1.3 (equivalent to $P\text{-value} < 0.05$) were considered relevant. The pathways involved in the metabolism of glucose were identified using the TAC software. Among the genes involved in glucose

metabolism, the fold-change of the genes of interest were further verified by RT-qPCR using pre-optimised TaqMan assays (Applied Biosystems, USA).

One of the limitations in performing pathway analysis using the TAC system was that a number of interactions between the genes or proteins in the pathways were not fully described. Therefore, the differentially expressed genes in the particular pathways of interest identified by TAC were subsequently subjected to further analyses using the Reactome database, which also mapped the genes of interest to biological functions and pathways and illustrated the relevance according to the P-value and false detection rate (FDR) [185-188]. When the Reactome database was used, the identified pathways were considered as relevant when P-value <0.05 and FDR<0.05. The second limitation of the TAC program was that the program did not provide any statistics indicating whether the activity of the identified pathways was increased or decreased. This limitation was overcome by further analysis using the IPA program.

The IPA software could algorithmically map the genes of interest into pathways, networks, toxicity functions, diseases and bio functions as well as identifying the upstream regulating molecules of the genes of interest. In the current project, in order to identify any possible effects of the AAV8-*INS-FUR* vectors and FFO surgery to the livers, 3 different analyses were performed for each set of the differentially expressed genes to identify their association with: (i) canonical pathways, (ii) diseases and bio functions and (iii) hepatotoxicity functions. The association between a gene set and a particular target (e.g. a pathway, a toxicity function, a disease or a bio function) was demonstrated by 2 parameters: (i) ratio and (ii) $-\log$ (P-value). The ratio was calculated in 2 steps. Firstly the IPA program identified the number of genes from the input dataset that could be mapped to a particular target in the database (Ingenuity[®] Knowledge Base) curated from millions of published research articles. This number was then divided by the total number of genes associated with the target. The ratio was subject to bias because it was dependent on the total number of genes that were known to be associated with the particular target. Therefore, the ratio value was not reported in this thesis.

Similar to the TAC program, the IPA program calculated the P-value using Fischer's exact test (right-tailed) determining the probability that the association between the genes of interest and the target was due to chance alone. The associations that had $-\log(\text{P-value}) > 1.3$ (equivalent to $\text{P-value} < 0.05$) were considered relevant. The $-\log(\text{P-value})$ was, therefore, the criteria for determining significance of the association between the various gene sets and the targets. As the number of the identified canonical pathways was large (over 50), the Benjamini-Hochberg correction for multiple testing was also implemented to further filter for the most significant ones; hence the Benjamini-Hochberg-P-value (B-H-p-value) was reported. The pathways were identified as statistically significant when the $-\log(\text{B-H-p-value}) > 1.3$ (equivalent to $\text{B-H-p-value} < 0.05$).

The IPA program also allowed the analysis to identify the activity patterns of the targets of interest (pathways, or diseases and bio function, or hepatotoxicity functions) based on a comparison of the observed changes in the expression levels of the genes of interest with records from the previously published database. The dimension and direction of the activation was statistically computed and presented as an activation z-score. A positive z-score was indicative of an "increase" or an activation and a negative z-score indicated a "decrease" or inhibition in the disease(s), function(s) or pathway(s). The targets of interest were considered as significantly activated when $\text{z-score} > 2$ or inhibited when $\text{z-score} < -2$.

5.3 Results and Discussion

5.3.1 Summary of the microarray results

The transcriptome analysis was performed to compare the following 3 groups of NOD mice: (1) normal (non-diabetic) NOD mice (will be referred as the normal group), (2) diabetic NOD mice transduced by AAV8-*INS-FUR* (will be referred as the AAV group) and (3) diabetic NOD mice that had FFO surgery after AAV8-*INS-FUR* transduction (will be referred as the AAV+FFO group). The PCA graph shows the distinctive differences in the gene expression profiles of the 3 groups of mice (Figure 5.1).

The numbers of differentially expressed genes, as well as the fold-changes and FDR P-values are summarised in Figure 5.2 – 5.5. There were 1,528 genes differentially

expressed when comparing the transduced mice with and without FFO to the normal controls. By comparison to the normal group, the AAV group had 1366 up-regulated and 597 down-regulated genes, which brought the total number of differentially expressed genes to 1,963. Similarly, the AAV+FFO group had 1,503 genes that were differentially expressed including 1,068 up-regulated and 435 down-regulated genes. When comparing the AAV group to the AAV +FFO group, 332 genes were found to be differentially expressed, with 169 being up-regulated and 163 being down-regulated.

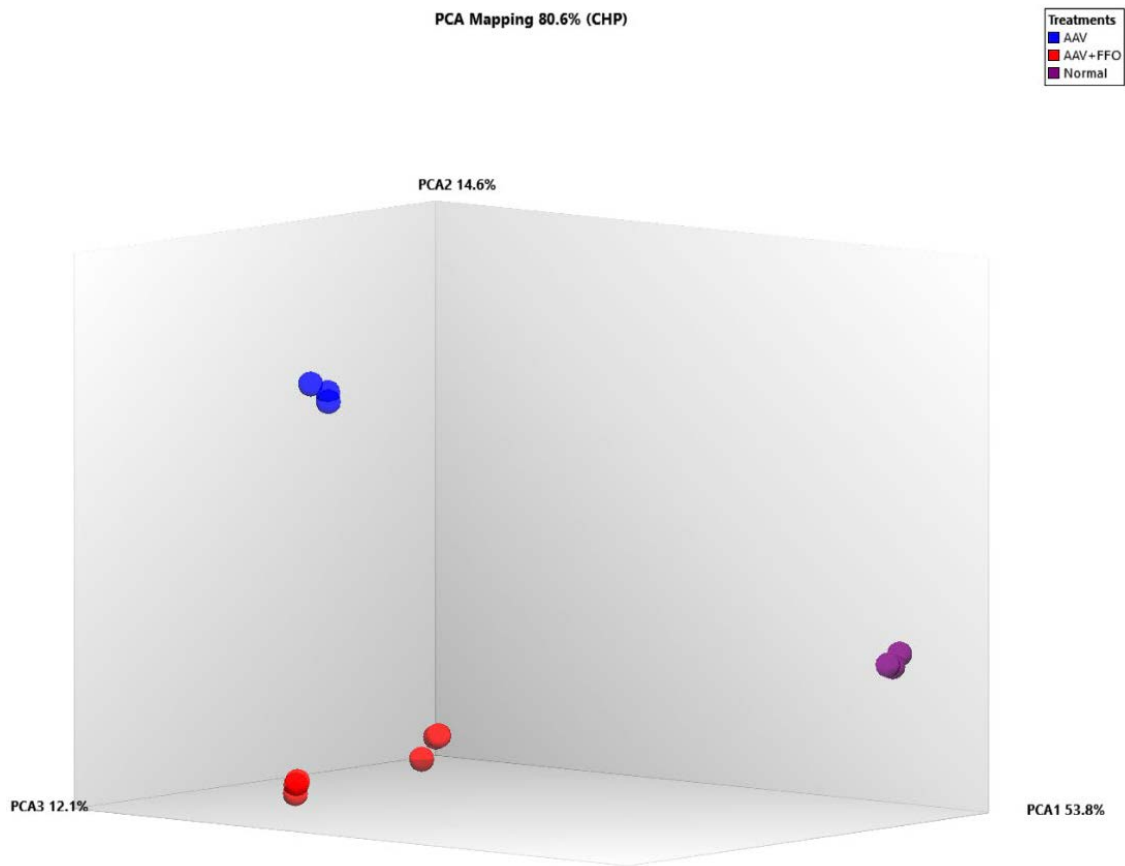


Figure 5.1: PCA mapping of the microarray datasets from the AAV8-*INS-FUR* transduced and the normal livers of the NOD mice. The PCA map indicates distinctive differences in the gene expression profiles among the three different mouse treatment groups. **Key:** The purple spheres represent the normal NOD mice, the blue spheres represent the diabetic NOD mice transduced by AAV8-*INS-FUR* and the red spheres represent the diabetic NOD mice that had FFO surgery after AAV8-*INS-FUR* transduction.

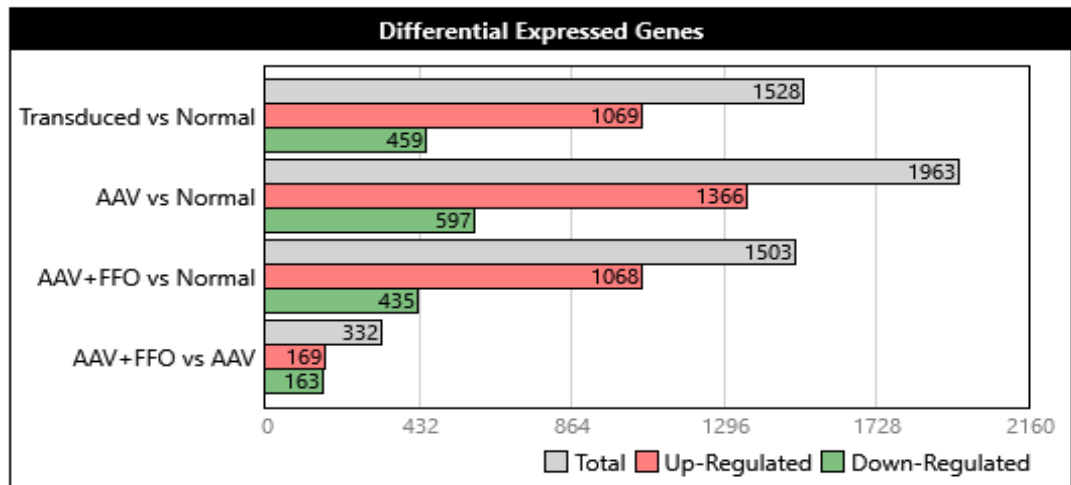


Figure 5.2: Differential gene expression in the livers of the AAV-*INS-FUR* transduced NOD mice

Key: Transduced vs Normal: comparing all the mice transduced by AAV8-*INS-FUR*, both with and without FFO surgery, to the normal controls. AAV vs Normal: comparing the AAV8-*INS-FUR* transduced without FFO surgery mice (AAV group) to the normal controls (normal group). AAV+FFO vs Normal: comparing the mice that had FFO surgery after AAV8-*INS-FUR* transduction (AAV+FFO group) to the normal controls (normal group). AAV+FFO vs AAV: comparing the AAV8-*INS-FUR* transduced diabetic NOD mice (AAV group) to those that had FFO surgery after the AAV8-*INS-FUR* transduction (AAV+FFO group).

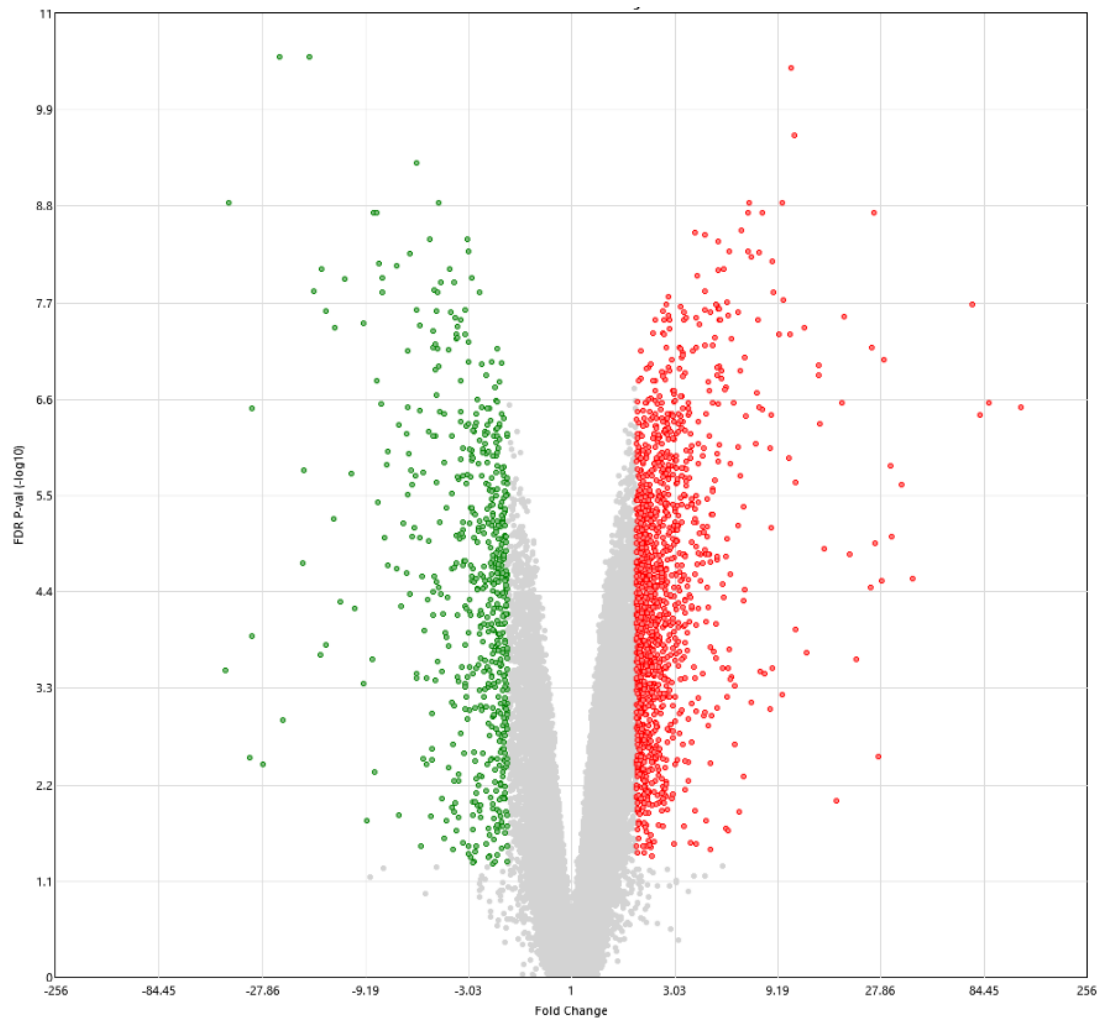


Figure 5.3: The differential gene expression of the AAV group in comparison to the normal group

The volcano plot showing the distribution of the differentially expressed genes according to their fold-changes and FDR P-value. The red dots represented the up-regulated genes and the green dots represented down-regulated genes.

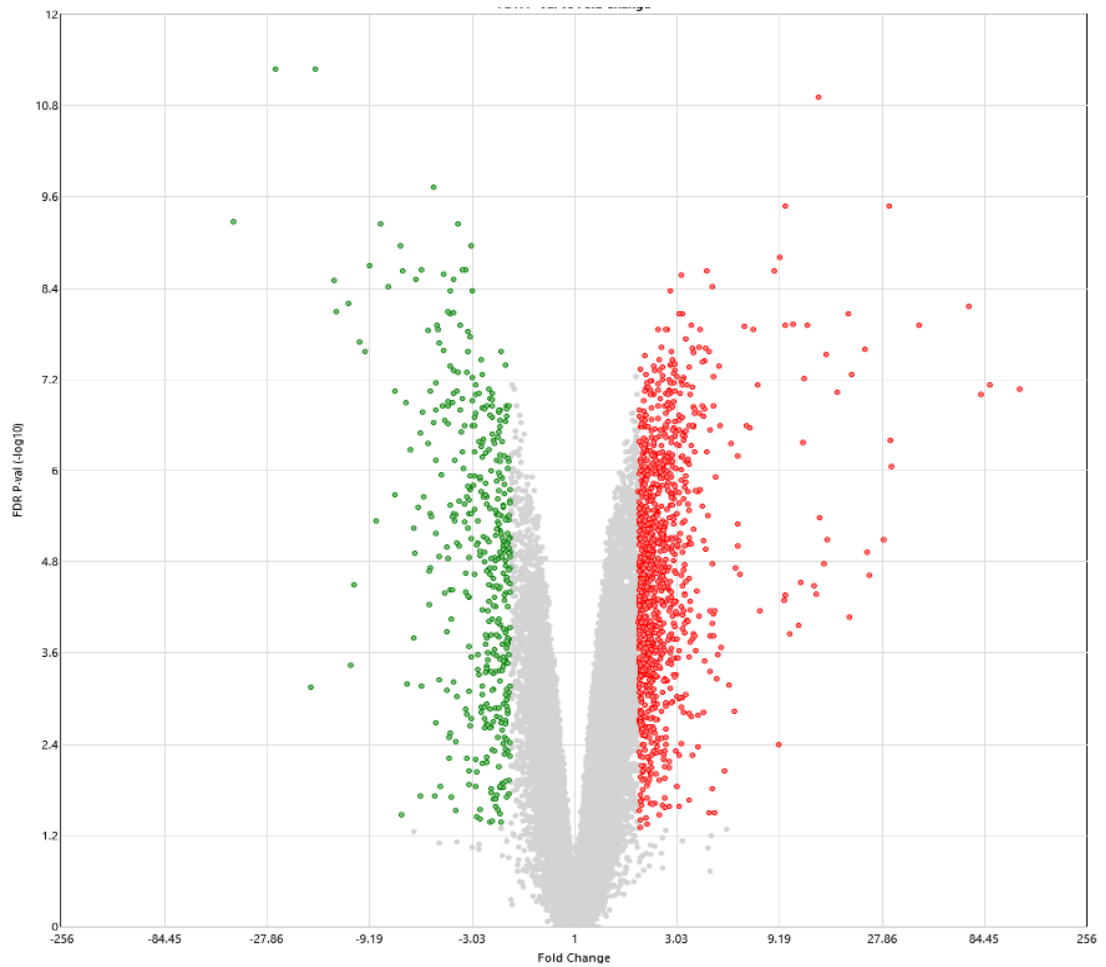


Figure 5.4: The differential gene expression of the AAV+FFO group in comparison to the normal group

The volcano plot shows the distribution of the differentially expressed genes according to their fold-changes and FDR P-value. The red dots represent the up-regulated genes and the green dots represent the down-regulated genes.

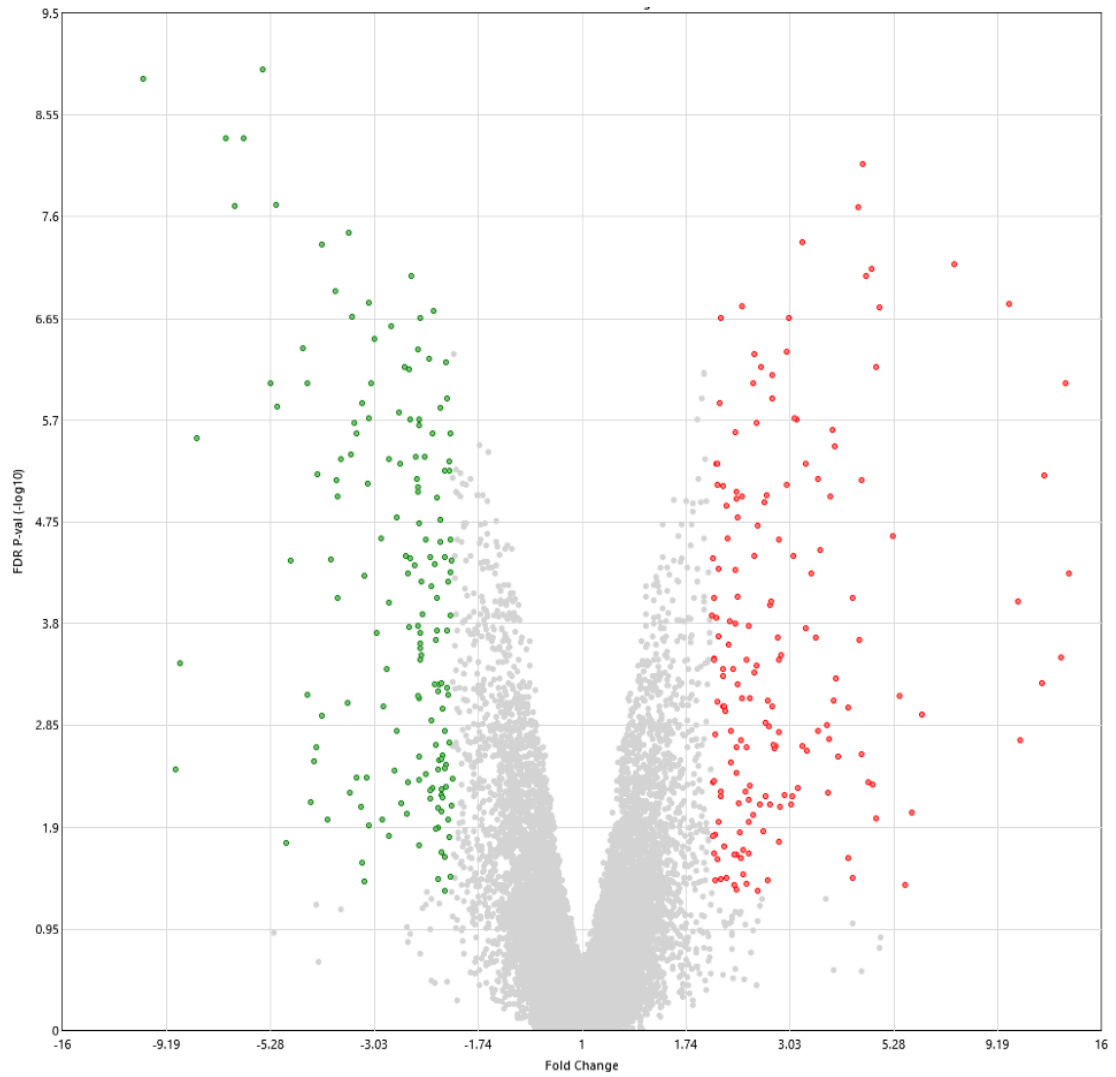


Figure 5.5: The differential gene expression of the AAV+FFO group in comparison to the AAV group

The volcano plot shows the distribution of the differentially expressed genes according to their fold-changes and FDR P-value. The red dots represent the up-regulated genes and the green dots represent the down-regulated genes.

5.3.2 Pathways, diseases and biological functions and hepatotoxicity functions associated with the genes that were differentially expressed

5.3.2.1 Comparing the AAV group to the normal group

When the AAV group was compared to the normal controls, the analysis by the TAC program showed 44 pathways that were relevant to all the 1,963 differentially expressed genes. A majority of the top 20 pathways (Table 5.1) associated with immunological functions (e. g.: Double-stranded RNA-specific adenosine deaminase 1 (Adar1) editing deficiency immune response, type II interferon signalling (IFNG), microglia pathogen phagocytosis, toll-like receptor signalling, B cell receptor signalling, IL-7 signalling, TYRO protein kinase-binding protein (TYROBP) causal network, Immune response in Tg26 glomeruli, IL-5 signalling, apoptosis, T cell receptor signalling pathway, and macrophage marker). The detection of these pathways highlights either the auto immune response of the NOD mouse model or the possibility of an immune response to the AAV8 vectors.

As the TAC program did not provide statistical details for extrapolating the activities of the pathways, the differentially expressed genes were further analysed using the IPA program, which found 55 enriched pathways (Figure 5.6). A majority of the pathways identified by the IPA program did not have any activity pattern. Of the 55 pathways identified, 30 had a positive z-score and 3 had a negative z-core. The top 10 pathways that were activated (had positive z-scores) and all the pathways that were inhibited (had negative z-scores) are listed in Table 5.2 and 5.3 respectively.

Similar to the TAC program, the IPA program also detected a number of pathways related to immunological functions. B-cell receptor signalling and T-helper cell signalling pathways were identified by both programs as highly relevant to the differentially expressed genes. This observation suggested that immunological functions of the AAV group were significantly different from the normal controls. The differences may be attributed to the response to the AAV8 vectors because pathways related to antiviral functions (e.g. the role of protein kinase R (PKR) in interferon induction and antiviral response, Adar1 editing deficiency immune response) were identified as activated. As

immunology is not the focus of the current project, further analysis for immunological pathways was not performed.

Table 5.1: The top 20 pathways identified by the TAC program as being associated with the differential gene expression in the comparison between the AAV group and the normal group

Pathway name	Ranking
Adar1 editing deficiency immune response	1
Type II interferon signalling (IFNG)	2
Odorant G-protein-coupled receptors (GPCRs)	3
Microglia pathogen phagocytosis pathway	4
Toll-like receptor signalling pathway	5
B cell receptor signalling pathway	6
Adipogenesis genes	7
White fat cell differentiation	8
GPCRs, others	9
Exercise-induced circadian regulation	10
IL-7 signalling pathway	11
TYRO protein kinase-binding protein (TYROBP) causal network	12
Omega-3/Omega-6 fatty acid synthesis	13
XPodNet-protein-protein interaction in the expanded STRING	14
Immune response in Tg26 glomeruli	15
IL-5 signalling pathway	16
Apoptosis	17
T cell receptor signalling pathway	18
Omega-9 FA synthesis	19
Macrophage marker	20

The pathways were ranked according to the significance (P-value). All pathways had P-value < 0.05.

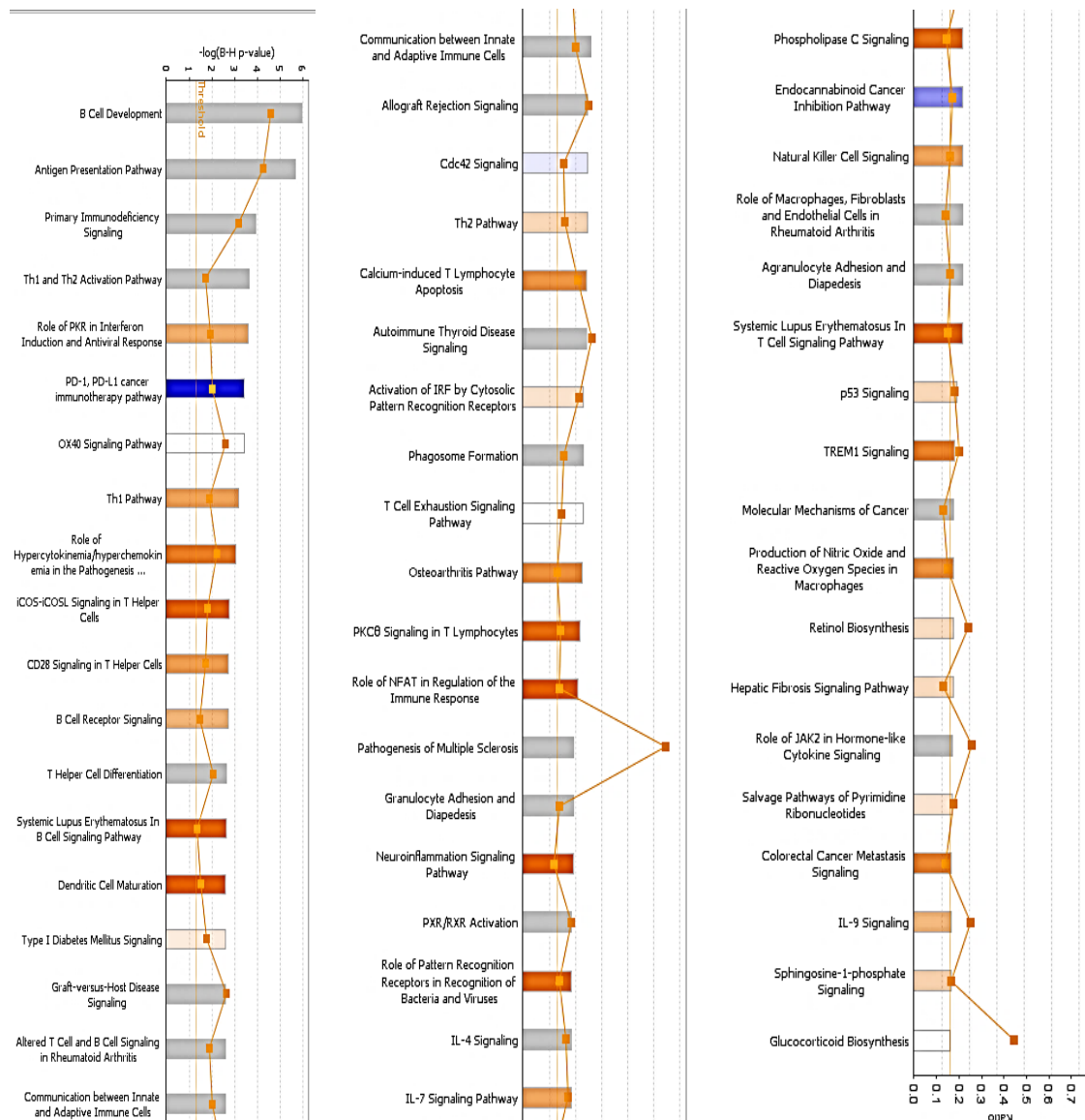


Figure 5.6: The canonical pathways associated with the differential gene expression of the AAV group relatively to normal group

The graph was produced using the IPA program. Each orange point represents the ratio of the number of genes differentially expressed over the total number of genes in the corresponding pathway. Each bar represents the $-\log(B-H-p\text{-value})$ indicating the significance levels of the pathway. The orange coloured bars represent the pathways that had positive z-scores. The blue coloured bars represent the pathways that had negative z-scores. The white bars represent pathways that had Z-score=0, and the grey colour bars represent pathways for which the activity patterns were not available.

Table 5.2: Top 10 canonical pathways with positive z-score associated with the differential gene expression of the AAV group relatively to normal group

Pathways with positive z-score	z-score	Ranked
Role of PKR in interferon induction and antiviral response	2.13*	5
T helper1 (Th1) pathway	2.36*	8
Role of hypercytokinemia/ hyper chemokineemia in the pathogenesis of influenza	3.15*	9
Inducible costimulator (Icos)- Icos ligand (iCOSL) signalling in T helper cells	3.64*	10
CD28 signalling T helper cells	2.50*	11
B cell receptor signalling	1.96	12
Systemic Lupus erythematosus in B cell signalling	3.68*	13
Dendritic cell maturation	3.78*	14
Type I diabetes mellitus signalling	0.56	15
T helper 2 (Th2) pathway	1.16	21
Calcium-induced lymphocyte apoptosis	2.67*	22

The list of the differentially expressed genes of the AAV group relatively to the normal group were analysed using the IPA program to identify the canonical pathways that had positive z-score. The pathways were ranked according their level of relevancy (-log (B-H-p-value)). * Activated pathways with the statistically significant z-score.

Table 5.3: Canonical pathways with negative z-score associated with the differential gene expression of the AAV group relatively to normal group

Pathways with negative z-score	z-score	Ranked
Programmed cell death 1 (PD-1), programmed cell death ligand (PD-L1) cancer immunotherapy pathway	-2.98*	6
Cell division cycle 42 (Cdc42) signalling	-0.33	21
Endocannabinoid cancer inhibition pathway	-1.46	39

The list of the differentially expressed genes of the AAV group relatively to the normal group were analysed using the IPA program to identify the canonical pathways that had negative z-score. The pathways were ranked according their level of relevancy (-log (B-H-p-value)). * Inhibited pathways with the statistically significant z-scores.

The IPA program ranked T1D signalling as the 15th most relevant pathway when comparing the AAV group to the normal controls (Figure 5.6) (P-value=6.77x10⁻⁵, B-H-p-value= 2.43x10⁻³ with 21 out of 98 genes in the pathway being found to be differentially expressed). The z-score of the pathway was negative, but did not meet the cut-off criteria of z-score < -2 and was, therefore, not considered to be significantly inhibited. This observation suggests that the transcriptome profile of the normal control mice still exhibited hallmarks of T1D as would be expected. Whilst their BGLs were normal they could be considered pre-diabetic. Further analysis for diseases and bio function also showed insulin-dependent diabetes mellitus, systemic autoimmune syndrome, diabetes mellitus and glucose metabolism disorder as the top diseases that were enriched in the dataset (Table 5.4). The z-score of these diseases were, however, not statistically significant. Therefore, it could not be confidently concluded that the AAV8-*INS-FUR* transduction had any effect on the autoimmune background of T1D.

Table 5.4: The top 5 diseases and bio functions associated with the differentially expressed genes of the AAV group relatively to normal group

Diseases and bio functions	P-value	z-score
Insulin dependent diabetes mellitus	2.84x10 ⁻³⁷	+0.16
Systemic autoimmune syndrome	8.70x10 ⁻³⁶	+1.09
Diabetes mellitus	3.93x10 ⁻³⁰	-0.50
Glucose metabolism disorder	1.97x10 ⁻²⁹	+1.15
Inflammation of absolute anatomical region	2.22x10 ⁻²³	+0.43

The analysis for diseases and bio functions associated with the differentially expressed genes of the AAV group relatively to the normal group were performed using the IPA program. The functions were listed according the level of relevancy (P-value).

From the pathway analysis, the hepatic fibrosis pathway was ranked 49th in terms of the level of significance or relevance ($P\text{-value}=1.3\times10^{-3}$, $B\text{-H-p-value}=3.73\times10^{-2}$) (Figure 5.6). There was not enough evidence to conclude that the AAV8-*INS-FUR* transduction would actually cause fibrosis because the z-score was not statistically significant (z-score = +1.07). Similarly, the analysis for identifying the relevant diseases and bio functions did not show liver disease as highly relevant to the differentially expressed genes. Further analysis for the relevant hepatotoxicity was performed using the IPA program. The analysis revealed that 15 hepatotoxicity functions (including hepatic steatosis, liver necrosis, cell death and apoptosis) to be relevant, but only the proliferation of the liver cells was found to be inhibited in the AAV group (z-score = -2.47 and $P\text{-value} = 4.44 \times 10^{-3}$) (Table 5.5).

Table 5.5: Comparison of hepatotoxicity functions between the AAV group and the normal group.

Toxicity function	P-value	z-score
Hepatic steatosis	3.50×10^{-8}	+0.53
Cell death of liver cell	9.00×10^{-6}	+1.38
Necrosis of liver	2.30×10^{-5}	+1.93
Apoptosis of liver cells	3.46×10^{-5}	+1.34
Apoptosis of hepatocyte	4.44×10^{-4}	+1.10
Hepatocellular carcinoma	1.44×10^{-3}	-0.46
Inflammation of the liver	1.93×10^{-3}	+0.43
Liver tumour	2.09×10^{-3}	-0.32
Liver cancer	3.07×10^{-3}	-0.12
Proliferation of liver cells	4.44×10^{-3}	-2.47*
Liver regeneration	6.56×10^{-3}	-0.56
Liver steatosis	1.67×10^{-2}	+0.00
Histopathological change of liver	2.07×10^{-2}	NA
Development of hepatocellular carcinoma	2.66×10^{-2}	-0.33
Proliferation of hepatocyte	3.38×10^{-2}	-1.75

The analysis for toxicity were performed by the IPA program. The functions were listed according the level of relevancy (P-value). NA: the activity patterns was not available. * Statistically significant z-score.

5.3.2.2 Comparing AAV+FFO group to the normal group

Comparing the AAV+FFO group to the normal controls, the top 20 pathways associated with the differentially expressed genes identified by the TAC program were shown in Table 5.6. The IPA program found 23 enriched pathways including 8 activated and 1 inhibited pathway (Figure 5.7 and Table 5.7). Pathways that were related to diabetes could not be found, but immunological pathways, were consistently detected. The B cell receptor signalling (z-score = +0.69) was again identified by both the TAC and the IPA programs as the highly relevant. Pathways related to antiviral functions (such as: role of pattern recognition receptors in recognition of bacteria and virus (z-score= +2.76), role

of hypercytokinemia/ hyperchemokineemia in the pathogenesis of influenza (z-score=+2.11) and role of protein kinase R (PKR) in interferon induction and antiviral response (z-score=+1.29)) were shown to be activated indicating an immune response of the livers toward the AAV8 vectors.

Table 5.6: The top 20 pathways identified by the TAC program as being associated with the differentially expressed genes of the AAV+FFO group relatively to the normal group

Pathway name	Ranking
PodNet-protein-protein interaction in the podocyte	1
Adipogenesis genes	2
Odorant GPCRs	3
XPodNet-protein-protein interaction in the expanded STRING	4
White fat cell differentiation	5
Adar1 editing deficiency immune response	6
mRNA processing	7
Immune response in Tg26 glomeruli	8
Chemokine signalling pathway	9
B cell receptor signalling pathway	10
Apoptosis	11
Omega-9 fatty acid synthesis	12
Keap1-Nrf2	13
Myometrial relaxation and contraction pathways	14
Microglia pathogen phagocytosis pathway	15
GPCRs, others	16
Type II interferon signalling (IFNG)	17
Wingless-related integration site (Wnt) signalling pathway and pluripotency	18
Toll-like receptor signalling pathway	19
IL-3 signalling pathway	20

The pathways were ranked according to the significance. All pathways had P-value < 0.05.

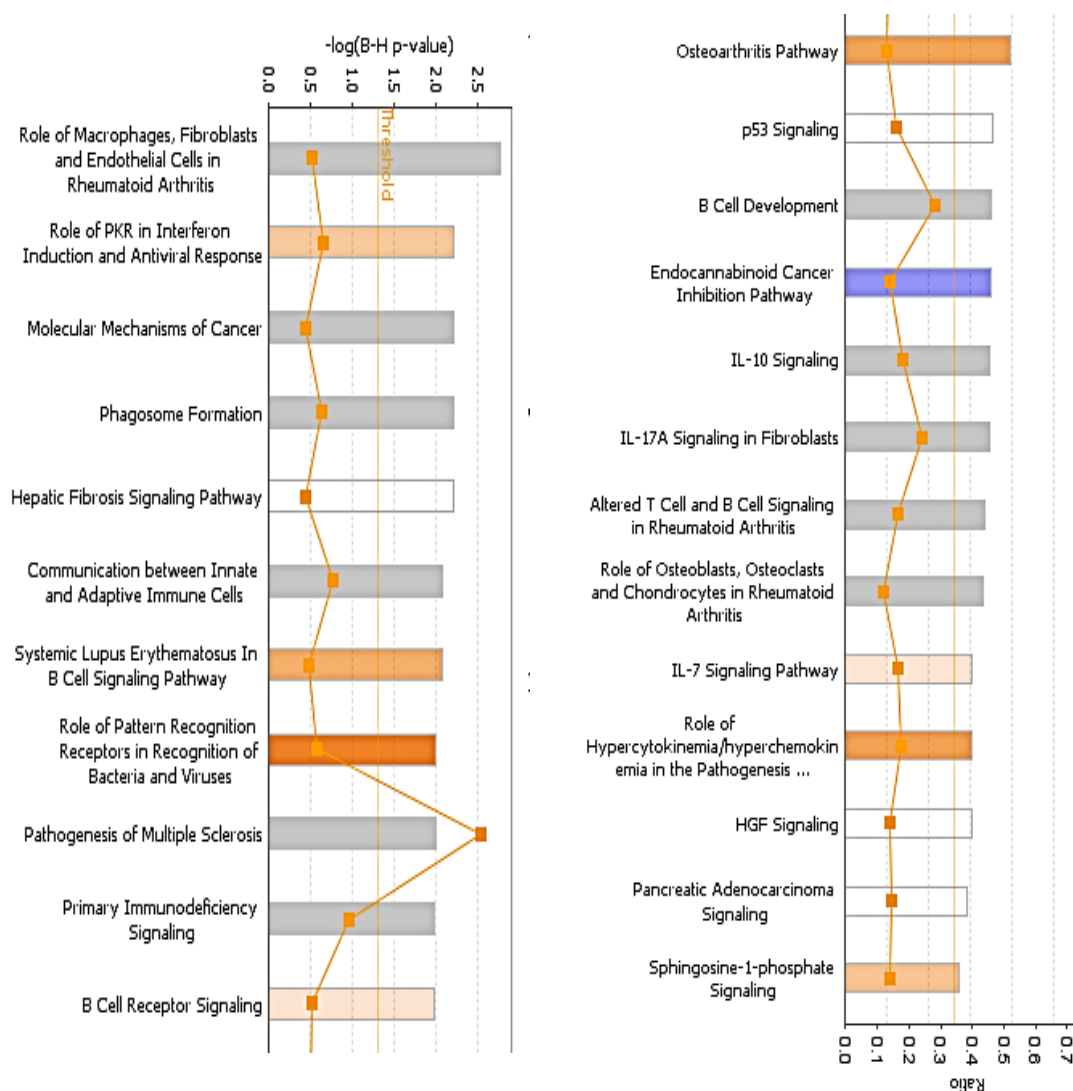


Figure 5.7: Canonical pathways associated with the differential genes of the AAV+FFO group relative to the normal group.

The graph was produced using the IPA program. Each orange point represents the ratio of the number of genes differentially expressed over the total number of genes in the corresponding pathway. Each bar represents the $-\log(B-H-p\text{-value})$ indicating the significance levels of the pathway. The orange coloured bars represent the pathways had positive z-scores. The blue coloured bars represent the pathways that had negative z-scores. The white bars represent pathways that had z-score=0, and the grey colour bars represent pathways for which the activity patterns were not available.

Table 5.7: Pathways with positive z-score identified by the IPA program as being associated with the differentially expressed genes of the AAV+FFO group relatively to the normal group.

Pathways with positive z-score	z-score	Ranked
Role of PKR in interferon induction and antiviral response	+1.29	2
Systemic Lupus erythematosus in B cell signalling	+1.83	7
Role of pattern recognition receptors in recognition of bacteria and virus	+2.67*	8
B cell receptor signalling	+0.69	11
Osteoarthritis pathway	+2.04*	12
IL-7 signalling pathway	+0.62	19
Role of hypercytokinemia/ hyper chemokineemia in the pathogenesis of influenza	+2.11*	20
Sphingosine-1-phosphate-signalling	+1.39	23

The analyses were performed by the IPA program. The pathways were ranked according their level of relevancy ((-log(B-H-p-value))). * Activated pathways with the statistically significant z-score.

When comparing the AAV+FFO to the normal group, the hepatic fibrosis signalling pathway was found to be relevant (ranked 5th with B-H-p-value= 6.23×10^{-3}) (Figure 5.7), but the z-score of 0 indicated that the activity level of the pathway remained unchanged. Similarly, hepatotoxicity function analysis also showed 18 functions as relevant (Table 5.8), but the activity patterns could not be extrapolated from the z-score that was not significant. Therefore, it cannot be concluded that the combination of AAV8 and FFO surgery caused any liver disease or toxicity.

Table 5.8: Comparison of hepatotoxicity functions between the AAV+FFO group and the normal group

Toxicity function	P-value	z-score
Hepatic steatosis	5.71×10^{-7}	+0.78
Cell death of liver cell	1.31×10^{-4}	+1.38
Liver tumour	1.49×10^{-4}	+0.50
Apoptosis of liver cells	1.66×10^{-4}	+0.56
Proliferation of liver cells	2.29×10^{-4}	-1.32
Necrosis of liver	3.14×10^{-4}	+0.72
Development of liver tumour	4.81×10^{-4}	+0.86
Liver regeneration	4.92×10^{-4}	-0.65
Hepatocellular carcinoma	6.35×10^{-4}	+0.22
Size of liver tumour	6.80×10^{-4}	NA
Proliferation of hepatocyte	9.05×10^{-4}	-0.94
Development of hepatocellular carcinoma	1.16×10^{-3}	+0.79
Liver cancer	1.31×10^{-3}	+0.63
Cell death of hepatocyte	1.35×10^{-3}	+0.04
Apoptosis of hepatocyte	2.67×10^{-3}	+0.42
Liver cholestasis	1.64×10^{-3}	+1.17
Hepatic injury	3.55×10^{-3}	+0.71
Quality of liver tumour	5.99×10^{-3}	+0.82

The analysis for toxicity was performed by the IPA program. The functions were listed according the level of relevancy (P-value). NA: the activity patterns was not available.

Analysis for disease and bio functions indicated that systemic autoimmune syndrome, glucose metabolism disorder and diabetes were among the top diseases that were relevant, but the z-scores were not statistically significant (Table 5.9). The detection of the diseases indicated that the transcriptome profiles of both the normal group and the AAV+FFO group exhibited the common autoimmune characteristic of T1D. Even though the BGLs of the AAV+FFO group were improved, the therapy did not have any effect on the autoimmune background of T1D.

Table 5.9: Top diseases and bio functions associated with the differentially expressed genes of the AAV+FFO in comparison to the normal group.

Diseases and bio functions	P-value	z-score
Systemic autoimmune syndrome	9.56×10^{-20}	+0.96
Glucose metabolism disorder	3.73×10^{-18}	+1.54
Insulin-dependent diabetes mellitus	2.81×10^{-15}	-0.19
Diabetes mellitus	5.30×10^{-15}	-1.44
Quantity of blood cell	7.26×10^{-15}	+1.84

The analysis for diseases and bio functions associated with the differentially expressed genes were performed by the IPA program. The functions were listed according the level of relevancy (P-value)

5.3.2.3 Comparing the AAV+FFO group to the AAV group

Pathway analysis was performed to identify the effects of FFO surgery post AAV8- *INS-FUR* transduction. The top 20 pathways identified by the TAC program were listed in Table 5.10. The Adar1 editing deficient immune response was ranked 1st (Table 5.10). Glycolysis and gluconeogenesis (was ranked 8th) were also found to be significantly relevant (Table 5.10) and were further analysed. The details of the analysis are shown in the later parts of this chapter.

When comparing the AAV+FFO group to the AAV group, the analysis by the IPA program showed that the 332 differentially expressed genes related to 8 different pathways (Figure 5.8). The inhibited pathways were: the role of hypercytokinemia/ hyper chemokinemia in the pathogenesis of influenza (z-score = -2.45), and the superpathway of cholesterol biosynthesis (z-score = -2.00). The activation of the interferon regulator factor (IRF) by cytosolic recognition receptors pathway (z-score = -1.63), and pyrimidine ribonucleotide *de novo* biosynthesis (z-score = -1.00) were relevant but did not meet the cut-off (z-score < -2). The Serine protease inhibitor Kazal-type 2 (SPINK 2) pancreatic cancer pathway was activated (z-score= 2.24).

Table 5.10: The top 20 pathways identified by the TAC program as being associated with the differentially expressed genes of the AAV+FFO group relatively to the AAV group.

Pathways	Ranking
Adar1 editing deficiency immune response	1
Cholesterol metabolism	2
G1 to S cell cycle control	3
Type II interferon signalling (IFNG)	4
Oxidation by cytochrome P450	5
Steroid biosynthesis	6
Omega-9 fatty acid synthesis	7
Glycolysis and gluconeogenesis	8
Cell cycle	9
Toll-like receptor signalling pathway	10
Glucocorticoid and mineralcorticois metabolism	11
Glycolysis	12
DNA replication	13
Omega-3/Omega-6 fatty acid synthesis	14
Eicosanoid metabolism via cytochrome P450 mono-oxygenase (CYP) pathway	15
Peroxisome proliferator- activated receptor (PPAR) signalling pathway	16
Leptin insulin overlap	17
Metapathway biotransformation	18
Spinal cord injury	19
Erythropoietin receptor signalling	20

The pathways were ranked according to the significance (P-value). All pathways had P-value <0.05.

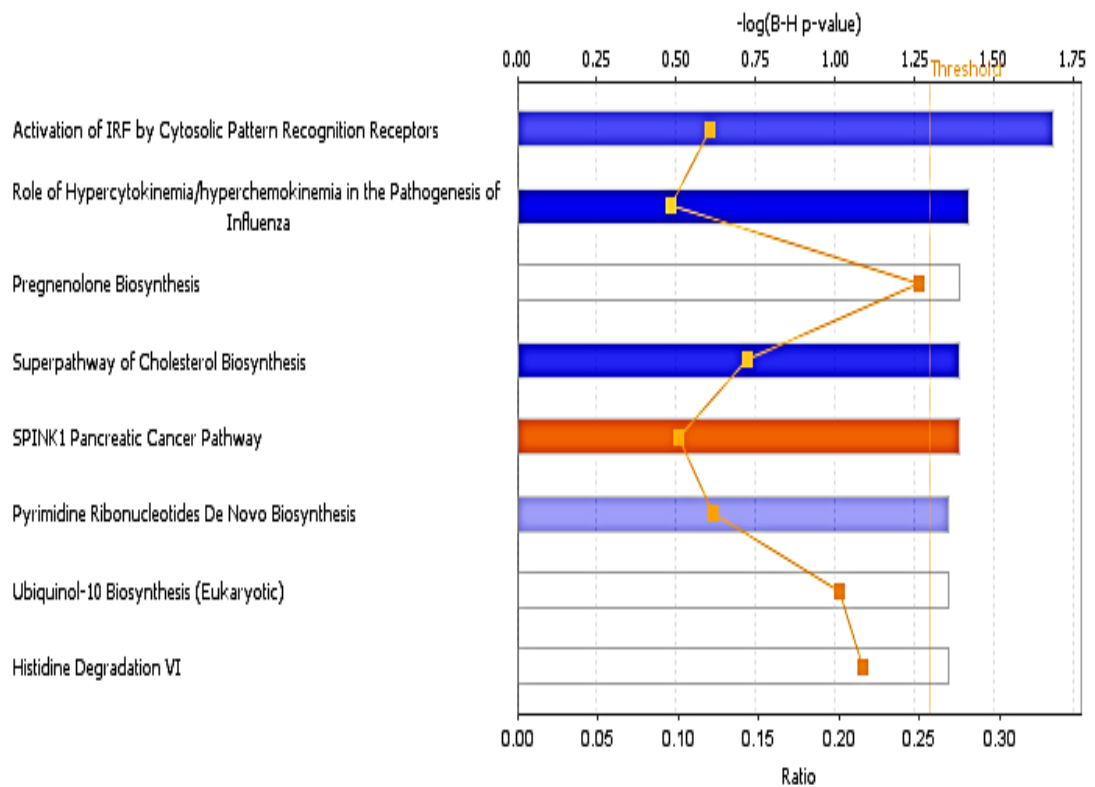


Figure 5.8: Canonical pathways associated with the differentially expressed genes of the AAV+FFO group relatively to the AAV group.

The graph was produced using the IPA program. Each orange point represents the ratio of the number of genes differentially expressed over the total number of genes in the corresponding pathway. Each bar represents the $-\log(B-H-p\text{-value})$ indicating the significance levels of the pathway. The orange coloured bars represent the pathways had positive z-scores. The blue coloured bars represent the pathways that had negative z-scores. The white bars represent pathways that had z-scores = 0.

Analysis for the top diseases and bio functions consistently showed diabetes, autoimmune as the most relevant disease as expected (Table 5.11). An antiviral response was also ranked 5th in the list. The consistent detection of pathways and functions related to the antiviral responses in the comparisons between the AAV+FFO group to either the normal controls (Figure 5.7) or the AAV group (Figure 5.8) implicated long-lasing (at least 9 weeks) antiviral reactions toward the AAV8 viral vector.

Table 5.11: The top 5 diseases and bio functions and bio functions associated with the differentially expressed genes of the AAV+FFO group relatively to the AAV group

Diseases and bio functions	P-value	z-score
Diabetes mellitus	1.07×10^{-18}	NA
Insulin-dependent diabetes mellitus	3.77×10^{-16}	NA
Glucose metabolism disorder	2.45×10^{-15}	-0.205
Systemic autoimmune syndrome	2.77×10^{-13}	NA
Antiviral response	2.31×10^{-9}	NA

The analysis for diseases and bio functions associated with the differentially expressed genes were performed by the IPA program. The functions were listed according the level of relevancy (P-value).

No canonical pathway relating to the any liver disease, dysfunction or damage were found (Figure 5.8). In order to identify any other possible impact of FFO surgery to the liver, analysis of the top hepatotoxicity functions was also performed. Fifteen hepatotoxicity functions were found to be relevant, but their activity patterns could not be determined (Table 5.12). The result suggested that the effect of the FFO surgery on the liver may have been milder than expected and that the surgery may not be directly linked to liver damage. Therefore, the hypothesis that the FFO technique induces liver damage and in turn reduces the number of the *INS-FUR* transgenes, could not be supported, at least in this group of experiments. The fact that the experimental endpoint of the AAV group was 5 weeks post transduction due to hypoglycaemia while the endpoint of the AAV+FFO

group was 9 weeks post transduction, may have influenced the outcome. The 4 weeks difference in the length of the experiments may have allowed the livers to recover from the insult induced by the surgery; hence the evidence of the liver damage or cell death could not be found.

Table 5.12: Comparison of hepatotoxicity functions between the AAV+FFO group and the AAV group.

Toxicity function	P-value	z-score
Hepatic steatosis	3.88×10^{-5}	-0.127
Nonalcoholic fatty liver disease	6.66×10^{-4}	NA
Microvesicular hepatic steatosis	4.74×10^{-3}	NA
Liver tumour	8.56×10^{-3}	-0.766
Size of liver tumour	1.33×10^{-2}	NA
Damage of liver tissue	1.38×10^{-2}	NA
Proliferation of liver cancer cells	1.38×10^{-2}	NA
Quantity of liver tumour	1.42×10^{-2}	NA
Liver cancer	1.68×10^{-2}	NA
Focal necrosis of liver	2.24×10^{-2}	NA
Liver cholestasis	2.74×10^{-2}	NA
Focal necrosis of liver cell	4.08×10^{-2}	NA
Liver dysplasia	4.08×10^{-2}	NA
Hepatocellular carcinoma	4.22×10^{-2}	NA
Liver damage	5.01×10^{-2}	-0.946

The analysis for toxicity were performed by the IPA program. The functions were listed according the level of relevancy (-p-value). NA: the activity patterns was not available.

5.3.3 Comparing the pathways of interest across the three groups of mice

Based on the gene expression patterns, the TAC program suggested a number of pathways that were differentially affected among the 3 groups of mice (AAV, AAV+FFO and normal). The most significantly altered pathways, along with the metabolic pathways that potentially contribute to the minute-to-minute regulation of BGLs were further analysed.

5.3.3.1 The Adar1 editing deficiency immune response pathway

The Adar1 editing deficient immune response was ranked 1st in both comparisons of the AAV group to the normal group (Table 5.1) and the AAV+FFO group to the AAV group (Table 5.10). The pathway was ranked 6th in the comparison between AAV+FFO to the normal group (Table 5.6). This observation suggests that the Adar1 editing deficient immune response was a highly relevant pathway that was further analysed.

The *adenosine deaminase acting on RNA (Adar)* gene is a component of the innate immune system that can effect both autoimmune reactions and responses to viral infection [189, 190]. Through alternative splicing, the *Adar* gene can be transcribed into 3 different isoforms, Adar1, 2 and 3 [191-193]. Adar1 is the enzyme that converts adenosine to inosine leading to the unwinding of dsRNA [194, 195].

The Adar1 editing deficiency immune response was ranked as the most significant pathway in the comparison between the AAV group and the normal group. The *Adar* gene (fold-change = +2.7) and 36 other genes in the pathway were uniformly up-regulated (Table 5.13 and Figure 5.9). The genes shown to be up-regulated by Adar1 were further analysed using the Reactome database to identify other relevant immunological pathways. The top 5 pathways found to be highly prevalent were: (1) interferon alpha/beta signalling, (2) interferon signalling, (3) cytokine signalling in the immune system, (4) Isg15 antiviral mechanism and (5) antiviral mechanism by IFN-stimulated genes (Figure 5.9). The detection of the pathways related to the antiviral functions was consistent with the pathway analysis using the IPA program.

Table 5.13: Fold-changes in the expression levels of genes in the Adar1 editing deficiency immune response pathway.

Genes	Description	Fold-changes		
		AAV vs normal	AAV+FFO vs normal	AAV+FFO vs AAV
<i>Adar</i>	adenosine deaminase, RNA-specific	+2.70	NSD (+1.84)	NSD (-1.47)
<i>Tlr3</i>	toll-like receptor 3	+3.43	+2.71	NSD
<i>Dhx58</i>	DEXH (Asp-Glu-X-His) box polypeptide 58	+4.23	NSD	-2.50
<i>Ddx58</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	+2.14, +2.58	NSD	NSD
<i>Ikbkg</i>	inhibitor of kappaB kinase gamma	+2.43, +2.09	+2.33, +2.32	NSD
<i>Ikbke</i>	inhibitor of kappaB kinase gamma	NSD	NSD	-2.41
<i>Irf7</i>	interferon regulatory factor 7	+2.16	NSD	-2.81
<i>Map3k7</i>	mitogen-activated protein kinase 7	+2.07	NSD	NSD
<i>*Ifi44</i>	interferon-induced protein 44	+5.32	NSD	-3.83
<i>*Apol9a</i>	apolipoprotein L 9a	+4.19	NSD	-2.77
<i>*Tgtp1</i>	T cell specific GTPase 1	+9.59	NSD	-5.13
<i>*Tgtp2</i>	T cell specific GTPase 2	+9.59	NSD	-5.13
<i>*Apol9b</i>	apolipoprotein L 9b	+4.19	NSD	-2.77
<i>*Ifit1</i>	interferon-induced protein with tetratricopeptide repeats 1	+8.47	NSD	-7.82
<i>*Isgl15</i>	predicted gene 9706; ISG15 ubiquitin-like modifier	+5.40	NSD	-6.39
<i>*Zbp1</i>	Z-DNA binding protein 1	+5.51	+2.34	-2.36, -2.35
<i>*Oas1l</i>	2'-5' oligoadenylate synthetase-like 1	+5.12	+3.02	-2.53

Table 5.13 (Continued): Fold-changes in the expression levels of genes in the Adar1 editing deficiency immune response pathway.

Genes	Description	Fold-changes		
		AAV vs normal	AAV+FFO vs normal	AAV +FFO vs AAV
<i>*9330175E14Rik</i>	RIKEN cDNA 9330175E14 gene	+2.47	+2.13	NSD
<i>*Ifit3</i>	interferon-induced protein with tetratricopeptide repeats 3	+5.02	NSD	-3.37
<i>*Rsad2</i>	radical S-adenosyl methionine domain containing 2	+2.74, +4.5, +3.38	NSD	-4.01, -4.10, -5.11
<i>*Mx2</i>	MX dynamin-like GTPase 2	+2.07	NSD	-2.59
<i>*Nlrc5</i>	NLR family, CARD domain containing 5	+3.42	+2.98	NSD
<i>*Mx1</i>	MX dynamin-like GTPase 1	+3.30	+2.11	NSD
<i>*Oasl2</i>	2'-5' oligoadenylate synthetase-like 2	+4.28	NSD	-2.52
<i>*Gm4951(Isg15)</i>	predicted gene 4951	+4.66	+3.46	NSD
<i>*Cxcl11</i>	chemokine (C-X-C motif) ligand 11	+4.19	+3.01	NSD
<i>*Oasl1a</i>	2'-5' oligoadenylate synthetase 1A	+4.30	+2.62	NSD
<i>*Ddx60</i>	DEAD (Asp-Glu-Ala- Asp) box polypeptide 60	+4.00	NSD	-2.07
<i>Usp18</i>	ubiquitin specific peptidase 18	+4.13	NSD	-4.0

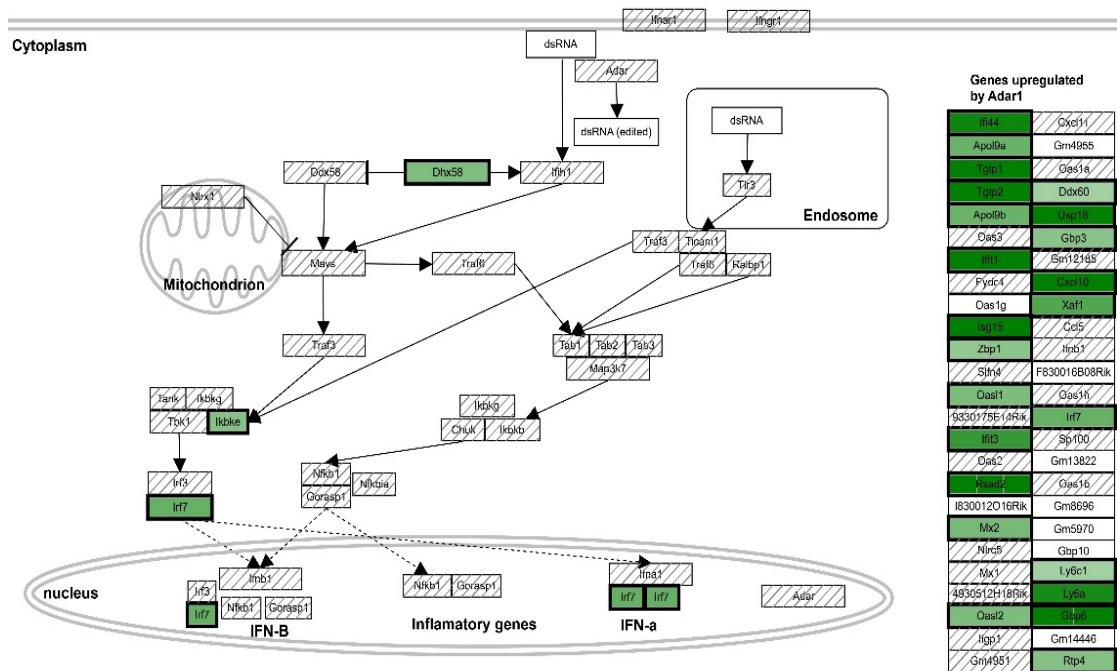
Table 5.13 (Continued): Fold-changes in the expression levels of genes in the *Adar1* editing deficiency immune response pathway.

Genes	Description	Fold-changes		
		AAV vs normal	AAV+FFO vs normal	AAV +FFO vs AAV
* <i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10	+10.32	+2.17	-4.75
* <i>Xaf1</i>	XIAP associated factor 1	+3.97	NSD	-3.04
* <i>Ccl5</i>	chemokine (C-C motif) ligand 5	+5.02	+3.16	NSD
* <i>Sp100</i>	nuclear antigen Sp100	+3.13	+2.42	NSD
* <i>Ly6c1</i>	lymphocyte antigen 6 complex, locus C1; lymphocyte antigen 6 complex, locus C2	+2.06	NSD	-2.07
* <i>Ly6a</i>	lymphocyte antigen 6 complex, locus A	+4.85	NSD	-3.73
* <i>Gbp6</i>	guanylate binding protein 6	+10.58	NSD	-4.45, -5.51
* <i>Rtp4</i>	receptor transporter protein 4	+3.49	NSD	-2.42

As multiple probe sets were used to detect a gene, multiple fold-changes were reported.

Key: * Genes up-regulated by *Adar1*, NSD not significantly different. Red coloured cells represent the up-regulation and green coloured cells represent the down-regulation of the genes.

When comparing the AAV+FFO group to the AAV group, *Adar1* was not significantly down-regulated (fold-change = -1.47, P-value < 0.05, FDR P-value < 0.05), but the *Adar1* editing deficiency immune response was ranked as the most significant pathway. None of the genes in the pathway was up-regulated and 25 genes were down-regulated (Figure 5.11 and Table 5.13). The results suggested that the pathway was activated in the transduced mice, but the activation level was lower in the AAV+FFO group in comparison to the AAV group. The down-regulation of the genes indicated a lower immune reaction in the AAV+FFO group. The analysis using the Reactome database showed 5 highly relevant pathways: (1) interferon alpha/beta signalling, (2) interferon signalling, (3) cytokine signalling in immune system, (4) Isg15 antiviral mechanism and (5) antiviral mechanism by IFN-stimulated genes. With the experimental endpoint of the AAV+FFO group was 4 weeks after that of the AAV group, the reaction to the viral vectors would have been subsided leading to the down regulations of the genes in the *Adar1* editing deficiency immune response and other related antiviral pathways.



Related immunological pathway identified by the Reactome database	Total number of genes in the pathway	Differentially expressed gene up-regulated by Adar1
Interferon alpha/beta signalling	191	<i>Irf7</i> , <i>Adar</i> , <i>Rsad2</i> , <i>Mx2</i> , <i>Ifit1</i> , <i>Ifit3</i> , <i>Usp18</i> , <i>Xaf1</i> , <i>Isg15</i>
Interferon signalling	401	<i>Irf7</i> , <i>Gbp3</i> , <i>Adar</i> , <i>Rsad2</i> , <i>Mx2</i> , <i>Ifit1</i> , <i>Gbp6</i> , <i>Ifit3</i> , <i>Usp18</i> , <i>Xaf1</i> , <i>Isg15</i>
Cytokine signalling in immune system	1108	<i>Irf7</i> , <i>Gbp3</i> , <i>Adar</i> , <i>Cxcl10</i> , <i>Rsad2</i> , <i>Mx2</i> , <i>Ifit1</i> , <i>Gbp6</i> , <i>Ifit3</i> , <i>Usp18</i> , <i>Xaf1</i> , <i>Isg15</i>
Antiviral mechanism by IFN-stimulated genes	37	<i>Isg15</i> , <i>Mx2</i> , <i>Oas11</i> , <i>Usp18</i>
Isg15 antiviral mechanism	33	<i>Isg15</i> , <i>Mx2</i> , <i>Ifit1</i> , <i>Usp18</i>

Figure 5.11: Differential expression of the genes in the Adar1 editing deficiency immune response pathway of the AAV+FFO group relatively to the AAV group. Genes highlighted in the green shading were down-regulated (fold-change < -2). The darker shades indicate the more prominent fold-changes. A single gene can be coloured in different shades of green to illustrate the different fold-changes detected by different probe sets. The genes up-regulated by Adar1 were further analysed to identify the top 5 related immunological pathways using the Reactome database.

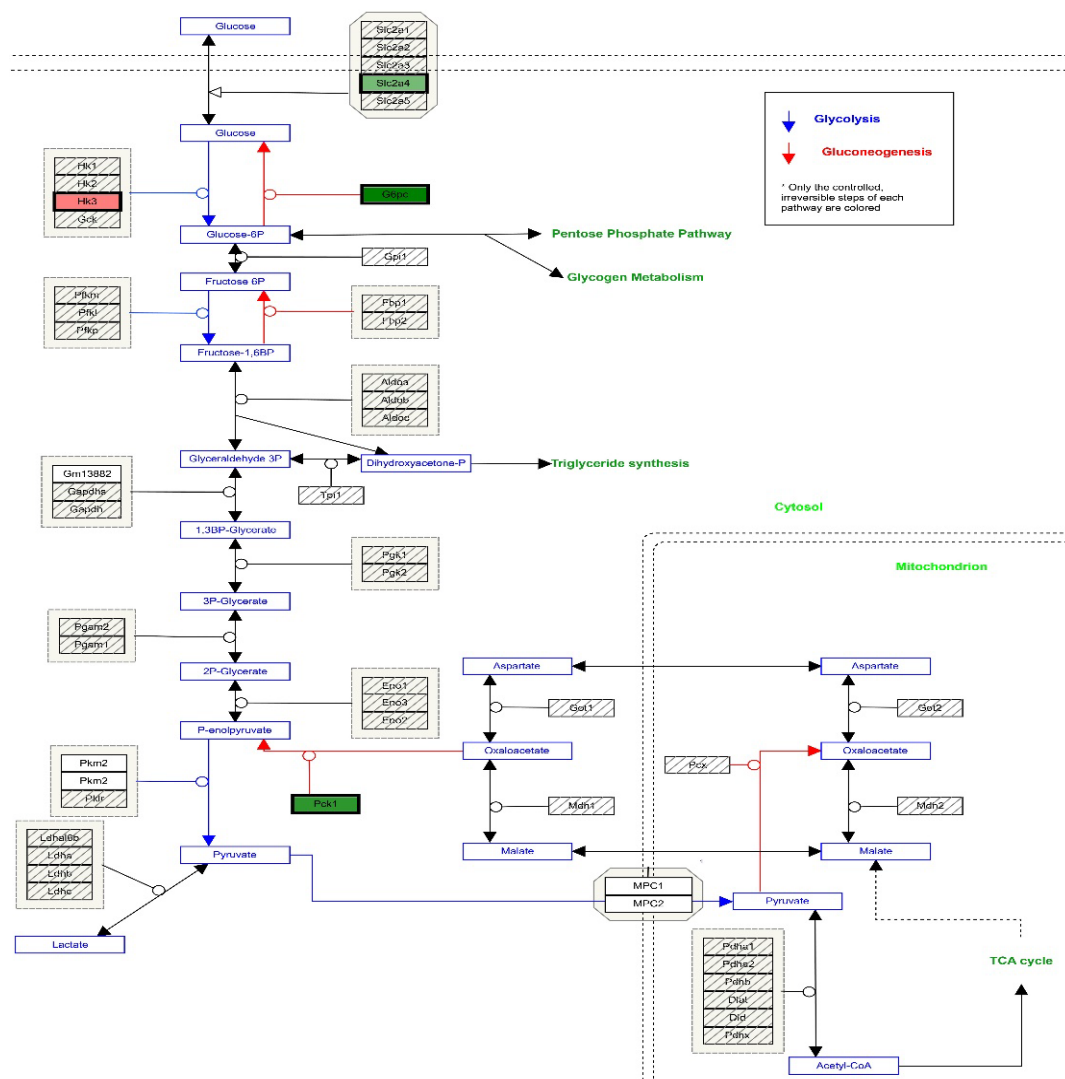
5.3.3.2 Glycolysis and gluconeogenesis

It was observed that the AAV+FFO group was hyperglycaemic, but their BGLs were reduced and the AAV group was hypoglycaemic (Chapter 4). Therefore, we also attempted to find the link between the metabolism and the differences in the BGLs between the two groups of mice by studying the metabolic pathways such as the glycolysis and gluconeogenesis. The liver is known to be able to regulate glucose homeostasis via the metabolism of glycogen [196]. Hence, the glycogen synthesis and degradation pathways were also studied in detail.

In comparison to the normal controls, mice of the AAV group favoured glycolysis over gluconeogenesis (Figure 5.12). In the muscle cells and adipocytes, insulin is known to regulate the BGLs by increasing the translocation and exocytosis of Glut4 glucose transporters (Slc2a4) from their storage vesicles to the cell membrane and the down regulation of the gene was observed to be associated with insulin resistance [197, 198]. In the liver, Glut4 is known to be expressed in stellate cells at mRNA level, but is not expressed in hepatocytes [198]. Surprisingly, the over expression of *INS-FUR* by AAV8 did not increase the *Glut4* expression in the livers of the AAV group, but the gene was significantly down-regulated (micro array fold-change= -2.62 and RT-qPCR fold-change = -6.50) (Figure 5.12). Similar to *Gck*, the three hexokinase isoenzymes (hexokinase 1, 2 and 3) participated in glycolysis by catalysing the reaction converting glucose to glucose-6-phosphate [179, 199]. Different from *Gck*, which was mainly expressed in the livers and β -cells, hexokinase enzymes were expressed in many tissues. While *Gck* was only activated when the BGLs was high, the hexokinase can function at any glucose concentration to provide the cells with a consistent supply of glucose-6-phosphate [199]. In the livers of the AAV group, the *Hexokinase3 (HK3)* gene was up-regulated (micro array fold-change = +2.54 and RT-qPCR fold-change= +4.39) suggesting an activation of the glycolysis pathway (Figure 5.12).

In the fasting state, the livers produced glucose by gluconeogenesis with the conversion of Glucose-6 phosphate to glucose by the glucose-6-phosphatase catalytic (G6pc) enzyme [198, 200, 201]. The release of glucose was mediated by Glut 2 (Slc2a2), which was the major glucose transporter of hepatocytes [200, 201]. In the current study, the expression

of Glut 2 was not altered and the *G6pc* gene was down-regulated (micro array fold-change = -4.42 and RT-qPCR fold-change = -1.56) suggesting an inhibition of the gluconeogenesis pathway (Figure 5.12). The *phosphoenolpyruvate carboxykinase 1, cytosolic* gene (*Pck1*) involved in the early steps of gluconeogenesis was also down-regulated (micro array fold-change = -3.46) (Figure 5.12). The activation of glycolysis and inhibition of gluconeogenesis aligned with the severe hypoglycaemia observed in the AAV group.



Genes	Pathway	Fold-changes	
		Micro array results	RT-PCR results
<i>Hk3</i>	Glycolysis	+2.54	+4.39
<i>G6pc</i>	Gluconeogenesis	-4.42	-1.56
<i>Pck1</i>	Gluconeogenesis	-3.46	Not tested
<i>Slc2a2 (Glut2)</i>	Both pathways	No change	No change
<i>Slc2a4 (Glut4)</i>	Both pathways	-2.62	-6.50

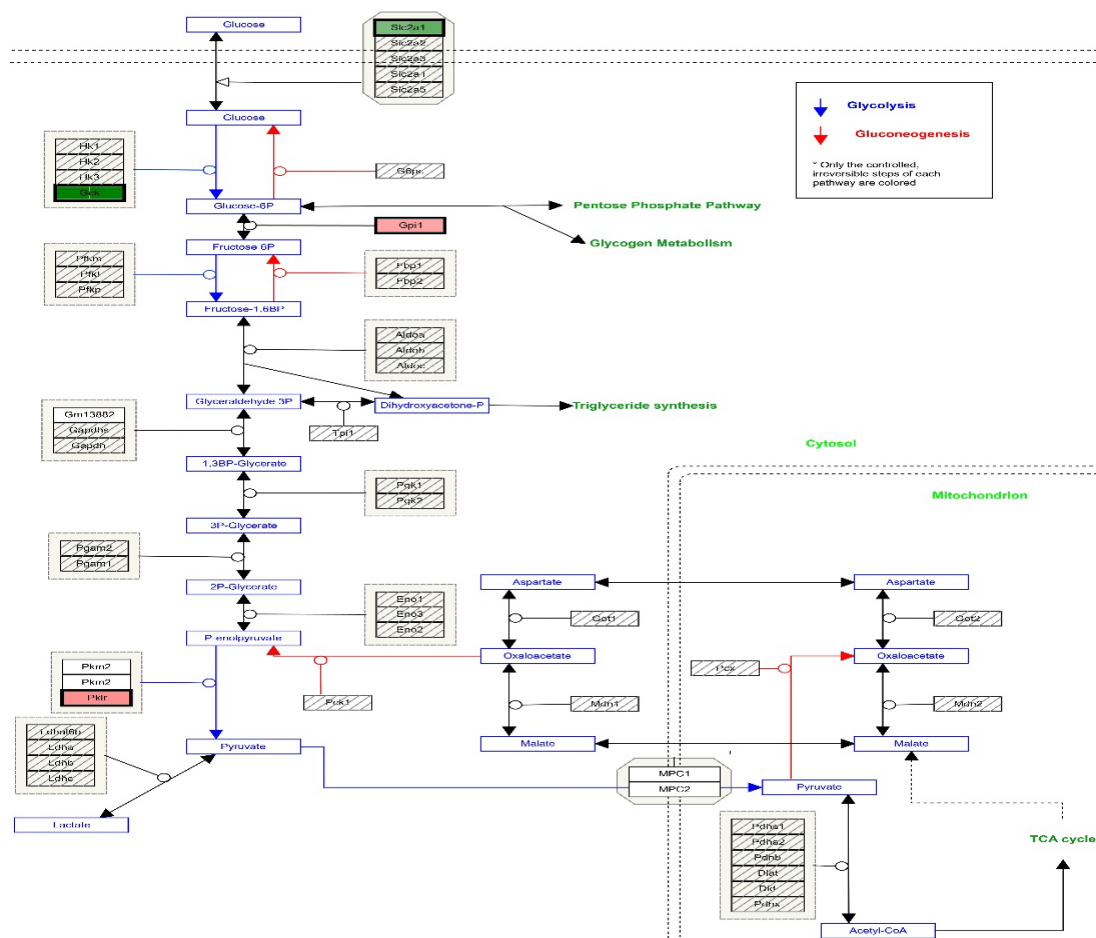
Figure 5.12: Differential expression of genes in the glycolysis and gluconeogenesis pathways in the AAV group relative to the normal group

Genes highlighted in green and red shading were down- and up regulated respectively. The darker shades indicate more prominent fold-changes in expression. The fold-changes of the glucose transporters of interest *Slc2a2 (Glut 2)* and *Slc2a4 (Glut4)* and the genes that exclusively regulate either glycolysis (blue arrows) or gluconeogenesis (red arrows) were further validated by RT-qPCR. All the reported fold-changes had P-value<0.05.

When comparing the AAV+FFO group to the normal group, there was no change in all the genes that are exclusively involved in gluconeogenesis (Figure 5.13). The BGLs of the AAV+FFO group were reduced, but not completely normalised (Chapter 4) suggesting that the glycolysis pathway was less active in the AAV+FFO. The *pyruvate kinase liver and red blood cell* gene (*Pklr*) were upregulated (micro array fold-change = +2.31) suggesting a possible increase in glycolysis (Figure 5.13). In contrast, *Gck* was down regulated (micro array fold-change = -6.70 and -6.11; RT-qPCR fold-change = -4.39) suggesting a reduction in glycolysis (Figure 5.13). As the *Pklr* gene involved in the glycolysis of both livers and red blood cells, it was not certain whether the up-regulation of the gene was detected from the liver tissue or from the residual blood in the hepatic vasculature. Hence, it was likely that the glycolytic activity was lower within the livers of the AAV+FFO group in comparison to the normal controls due to the down-regulation of the *Gck* gene alone.

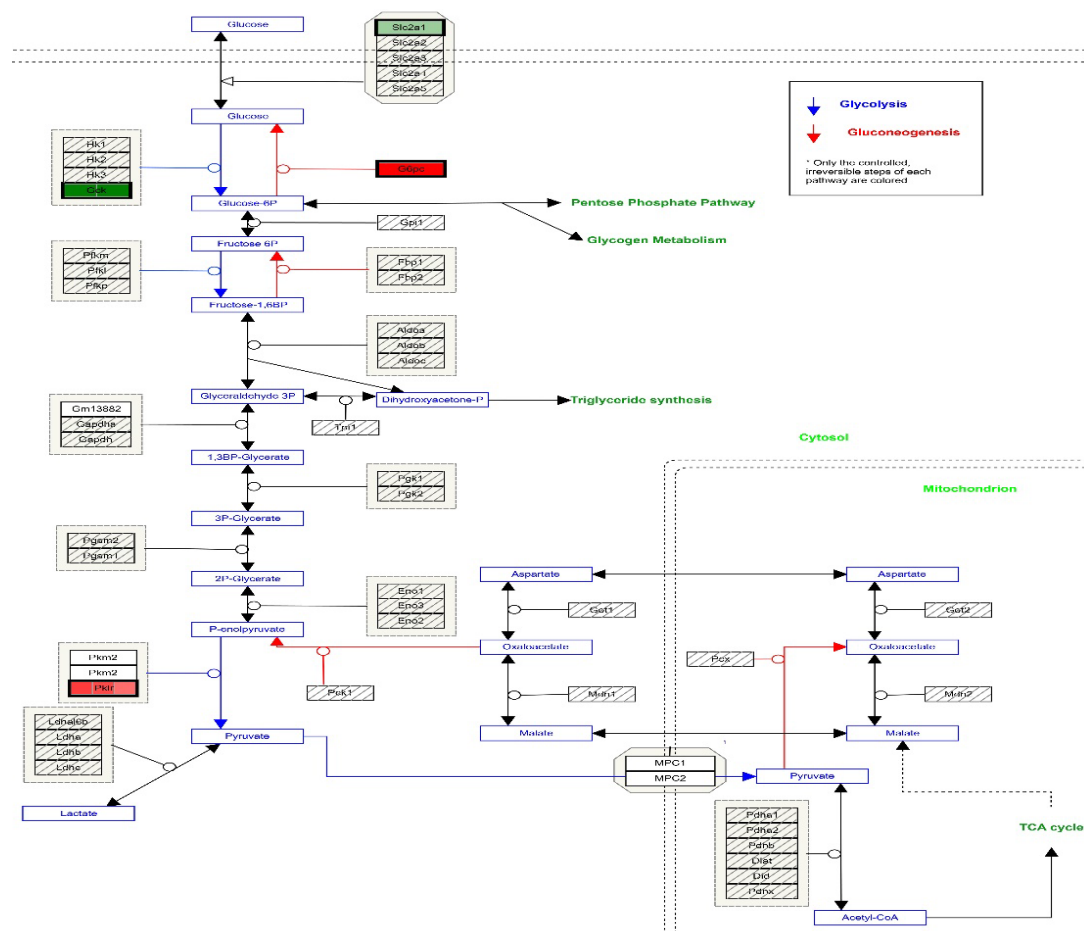
When comparing the AAV+FFO group to the AAV group, there was an increase in gluconeogenesis because *G6pc* was significantly up-regulated (micro array fold-change = +5.12, RT-qPCR fold-change= +10.73) (Figure 5.14). There could also be a reduction in glycolysis in the AAV+FFO group because *Gck* (micro array fold-change = -6.70 and -6.11; RT-qPCR fold-change= -5.67) was significantly down-regulated (Figure 5.14). It was observed that the AAV group were hypoglycaemic while the AAV+FFO group was still hyperglycaemic. This observation could be explained by the increase in gluconeogenesis and the decrease in glycolysis in the AAV+FFO group.

The *Glucose transporter Glut1 (Slc2a1)* gene was down-regulated in the AAV+FFO group in comparison to both the normal and the AAV groups (Figure 5.13 and 5.14). Glut1 regulates the basal transportation of hexose carbohydrate in many types of cells because its activity is insulin-independent [202]. During embryonic development the deletion of *Glut1* has led to severe malfunction of multiple organs and especially liver necrosis [203]. In adult animals, the up-regulation of the gene was observed to be associated with liver diseases [198], but the down-regulation of the gene has not been well studied.



Genes	Pathway	Fold-changes	
		Micro array results	RT-PCR results
<i>Gck</i>	Glycolysis	-8.19, -6.61	- 4.39
<i>Pklr</i>	Gluconeogenesis	+2.31	Not tested
<i>Gpi</i>	Both pathways	+2.06	Not tested
<i>Slc2a1 (Glut1)</i>	Both pathways	-2.85, -2.56	Not tested
<i>Slc2a2 (Glut2)</i>	Both pathways	No change	-2.53
<i>Slc2a4 (Glut4)</i>	Both pathways	No change	No change

Figure 5.13: Differential expression of the genes in the glycolysis and gluconeogenesis pathways in the AAV+FFO group relative to the normal group. Genes highlighted in green shading were down-regulated (fold-change <-2), and the genes highlighted in red were up-regulated (fold-change > 2). The darker colours indicate more prominent fold-changes. Multiple fold-changes in expression were reported for the genes that were detected by multiple probe sets. The fold-changes of the glucose transporters of interest *Slc2a2 (Glut 2)* and *Slc2a4 (Glut4)* and the genes that exclusively regulate either glycolysis (blue arrows) or gluconeogenesis (red arrows) were further validated by RT-qPCR. All the reported fold-changes had P-value<0.05.



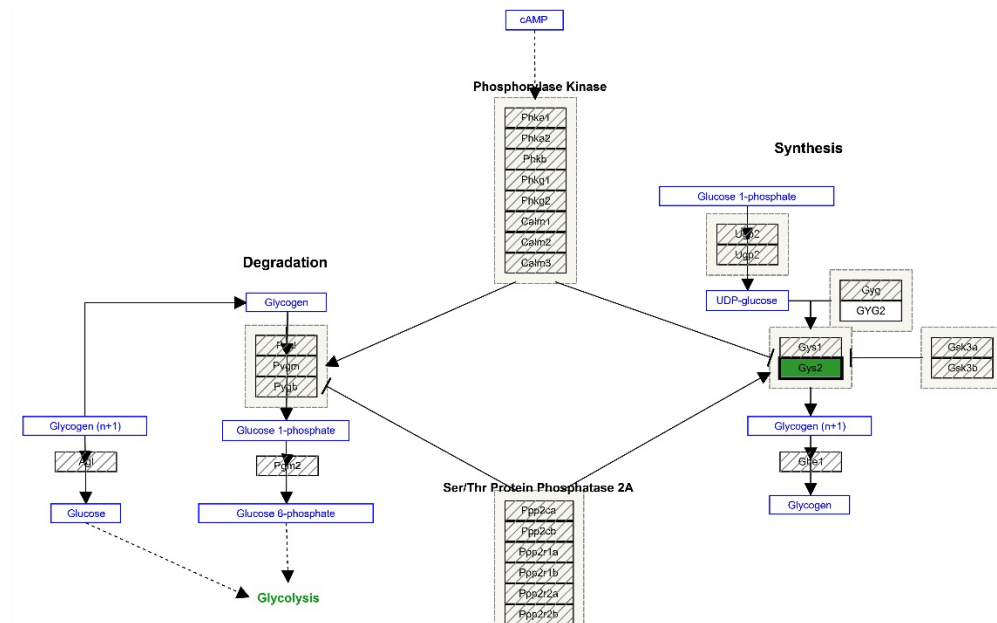
Genes	Pathway	Fold-changes	
		Micro array results	RT-PCR results
<i>Gck</i>	Glycolysis	-6.70; -6.11	-5.67
<i>Pfkfb1</i>	Glycolysis	+3.14; +3.30; +2.72	Not tested
<i>G6pc</i>	Gluconeogenesis	+5.23	+10.73
<i>Slc2a1 (Glut1)</i>	Both pathways	-2.15	Not tested
<i>Slc2a2 (Glut2)</i>	Both pathways	No change	No change
<i>Slc2a4 (Glut4)</i>	Both pathways	No change	No change

Figure 5.14: Differential expression of the genes in the glycolysis and gluconeogenesis pathways of the AAV+FFO group relatively to the AAV group

Genes highlighted in green shading were down-regulated (fold-change < -2), and genes highlighted in red were up-regulated (fold-change > 2). The darker colours indicate more prominent fold-changes. Multiple fold-changes were reported for genes that were detected by multiple probe sets. The fold-changes of the glucose transporters of interest, *Slc2a2 (Glut 2)* and *Slc2a4 (Glut4)*, and the genes that exclusively regulate either glycolysis (blue arrows) or gluconeogenesis (red arrows) were further validated by RT-qPCR. All the reported fold-changes had P-value < 0.05.

5.3.3.3 Glycogen metabolism pathway

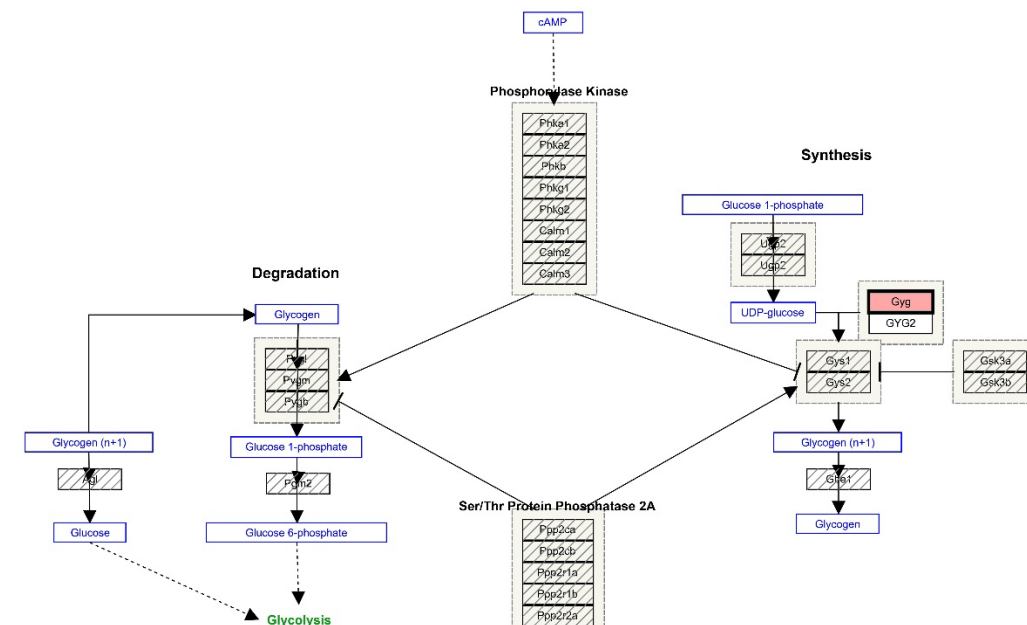
There was a reduction in glycogen synthesis in the AAV group in comparison to the normal controls, because *glycogen synthase 2* gene (*Gys2*) was significantly down-regulated (fold-change = -3.45) (Figure 5.15). The down-regulation of glycogen synthesis could be a result of the negative feedback from the low BGLs while the animals were hypoglycaemic. In contrast, glycogen synthesis was activated in the AAV+FFO in comparison to the normal controls because *glucogenin* (*Gyg*) was up-regulated (fold-change = 2.03) (Figure 5.16). Similarly, when comparing the AAV+FFO group to the AAV group, the up-regulation of *Gys2* (fold-change = 2.49) also suggested an increase in glycogen synthesis (Figure 5.17). This may be related to the fact that the AAV+FFO group were hyperglycaemic, which could provide a positive feedback for the livers to store glycogen. The expression levels of the genes involved in glycogen degradation of the 3 groups of mice were not significantly different from each other (Figure 5.15-5.17). The observation suggested that the AAV8-*INS-FUR* transduction did not alter the glycogen degradation pathway of the livers.



Gene	Fold-change (Microarray)	Fold-change (RT-qPCR)
<i>Gyst 2</i>	-3.45	No change

Figure 5.15: Differential expression of the genes involves in the glycogen metabolism pathways of the AAV group relatively to the normal group

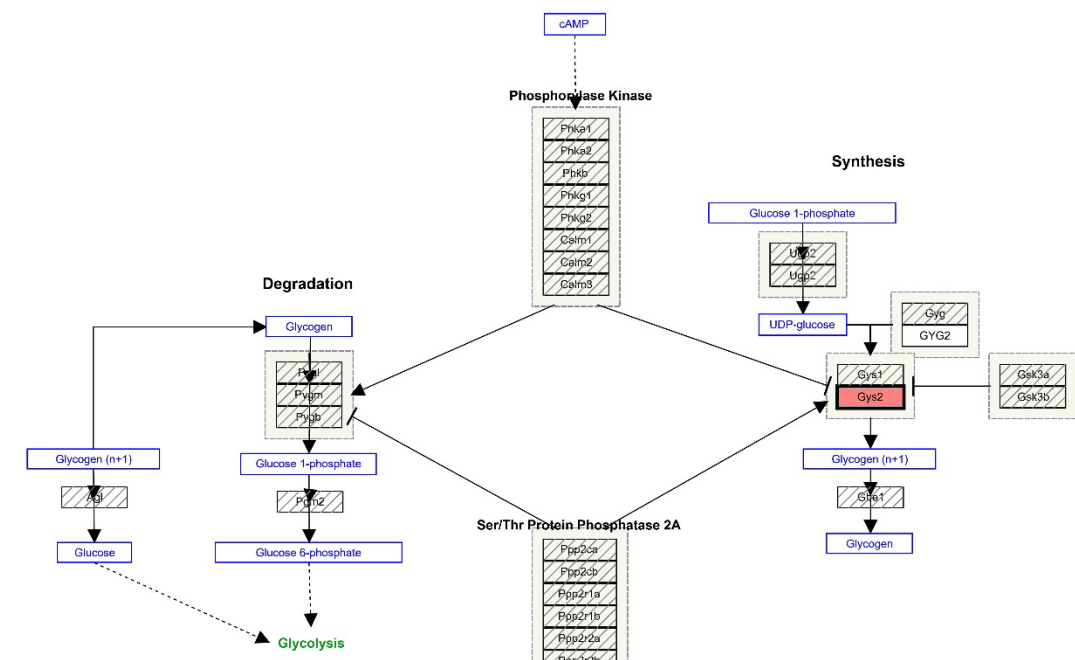
The gene highlighted in green shading was down-regulated.



Gene	Fold-change (Microarray)	Fold-change(RT-qPCR)
<i>Gyg</i>	+2.03	No change

Figure 5.16: Differential expression of the genes involves in the glycogen metabolism pathways of the AAV+FFO group relatively to the normal group

The gene highlighted in red shading was up-regulated.



Gene	Fold-change (Microarray)	Fold-change (RT-qPCR)
<i>Gys2</i>	2.49	7.05

Figure 5.17: Differential expression of the genes involves in the glycogen metabolism pathways of the AAV+FFO group relatively to the AAV group

The gene highlighted in red shading was up-regulated.

5.3.4 Comparing the expression levels of the pancreatic genes of interest across all the experiments

As pancreatic transdifferentiation was one of the focuses of the current study, the expression levels of the pancreatic genes of interest were also validated by the microarray. When the default statistical criteria were applied (P-value < 0.05, FDR P-value < 0.05 and fold-change > 2 or < -2), the only gene that showed differential expression was *Gck*. In order to detect the very small fold-changes of the pancreatic genes of interest, statistical filtering criteria for the FDR P-value < 0.05 and the fold-change > 2 or < -2 were removed from the analysis. This allowed to detect any gene that had the P-value of the fold-change of less than 0.05.

As expected, the fold-changes of most pancreatic genes of interest and the endogenous control genes (*β -Actin* and *GAPDH*) were between 1 and 2 or -1 and -2 (Table 5.14). A number of genes also had FDR P-values > 0.05, which indicated a lower degree of statistical significance (Table 5.14). When comparing the AAV group to the normal group, the endogenous control *β -Actin* gene was found to be slightly up-regulated, but the expression level of *GAPDH* gene of the AAV group was not changed (Table 5.14). When comparing the AAV+FFO group to the normal group or to the AAV group, *β -Actin* was down-regulated and *GAPDH* was up-regulated (Table 5.14). As the endogenous control genes were not uniformly up- or down-regulated, it was very likely that the small differences in the fold-changes were the common variances in the expression levels across the samples. Except for *Gck*, the fold-changes of most pancreatic genes were very similar to the fold-changes of either *β -Actin* or *GAPDH* (Table 5.14). Therefore, the microarray analysis further confirmed the absence of liver-to-pancreas transdifferentiation in both the AAV and the AAV+FFO groups.

Table 5.14: Fold-changes in the expression levels of pancreatic genes of interest and endogenous control genes

Gene	Fold-change in gene expression		
	AAV vs normal	AAV+FFO vs normal	AAV +FFO vs AAV
<i>Pdx1</i>	NSD	+1.19*, +1.18	+1.17
<i>Ptf1a (P48)</i>	-1.33*	-1.34*	NSD
<i>NeuroD1</i>	+1.66*, +1.31*	+1.30*, +1.41*	-1.18
<i>Ngn3</i>	NSD	NSD	NSD
<i>Pax4</i>	+1.89*	+1.90*	NSD
<i>Pax6</i>	NSD	+1.12*, +1.18	+1.15
<i>Nkx2.2</i>	NSD	+1.34*	NSD
<i>Nkx6.1</i>	+1.60*	+1.24	NSD
<i>MafA</i>	-1.40*	NSD	+1.58*
<i>Mouse Insulin1</i>	NSD	NSD	+1.18
<i>Mouse Insulin2</i>	NSD	+1.21*	NSD
<i>Glucagon</i>	NSD	-1.12	NSD
<i>Synaptophysin</i>	-1.31*	-1.26*	-1.16
<i>Somatostatin</i>	+1.22*, +1.20	+1.23*	-1.16
<i>Ppy</i>	NSD	NSD	NSD
<i>Pc1</i>	NSD	NSD	NSD
<i>Pc2</i>	+1.24	+1.25*	NSD
<i>Gck</i>	-1.22	-8.19*, -6.61*	-6.70*, -6.11*
<i>Glut2</i>	-1.47*	NSD	+1.67*
<i>β-Actin</i>	+1.32*	-1.12	-1.19
<i>GAPDH</i>	NSD	+1.49*, +1.29*	+1.46

As multiple probe sets were used to detect a gene, multiple fold-changes were reported. Genes were considered as differentially expressed when the P-value <0.05. Genes that had P-value >0.05 was consider as not significantly different (NSD). The differences were more significant when the FDR P-value < 0.05 (*).

5.4 Conclusion and future directions

The microarray analysis and the RT-PCR confirmed the direct link between the metabolic pathways and the observed BGLs of the transduced mice. Glycolysis was activated and gluconeogenesis was inhibited in the AAV group with the up-regulation of the *HK3* gene and down-regulation of *G6pc* and *Pck1*. The activation of glycolysis and inhibition of gluconeogenesis corresponded to the severe hypoglycaemia observed in the animal. In contrast, glycolysis was inhibited in the AAV+FFO group with the down-regulation of *Gck*. The reduction in glycolysis correlated to the moderate diabetes observed in the AAV+FFO group.

Even though the liver fibrosis signalling pathway as well as a number of hepatotoxicity functions were found to be relevant when comparing the AAV group to the normal group, their z-score was not significant. The only function that was found to be inhibited (z-score < -2) in the AAV group was the proliferation of the liver cells. Therefore, the link between the AAV8 transduction and liver diseases could not be confidently established.

The microarray, pathways, diseases and hepatotoxicity analysis did not support the hypothesis that the FFO procedure induced an insult to the liver leading to liver cell death and subsequently the reduction of the numbers of the *INS-FUR* transgene. Even though the liver fibrosis signalling pathway and a number of hepatotoxicity functions including hepatic injury and liver cell death were found to be relevant when comparing the AAV+FFO group to the normal group, their z-score was not statistically significant. Similarly, comparing the AAV+FFO group to the AAV group, liver damage was found to be a relevant hepatotoxicity function, but the z-score was not significant. Therefore, the direct link between the surgery and the liver injury or cell death could not be established.

A large number of pathways related to antiviral functions (e.g: the *Adar1* editing deficiency immune response, *Isg15* antiviral mechanism, Role of PKR in interferon induction and antiviral response, B cell receptor signalling pathway and Role of hypercytokinemia/ hyper chemokinemia in the pathogenesis of influenza) were consistently detected in the all the datasets. Therefore, it could be concluded that the

AAV8 could potentially generate an immune response in the transduced animals. As immunology was not the focus of this project, the antiviral reactions were not studied in detail, but they could be studied in future projects.

One of the main focuses of the whole project was to find any possible link between the AAV8 transduction and the liver-to-pancreas transdifferentiation. Noting that the BGLs of both the AAV group and the AAV+FFO group were not normalised, there was no evidence from RT-PCR analyses (Chapter 3 and 4) suggesting that pancreatic transdifferentiation had occurred due to AAV8 transduction. The microarray analysis was consistent with the RT-PCR result that was published showing that the pancreatic genes of interest were randomly expressed at low levels and that the AAV8-*INS-FUR* transduction with or without FFO surgery did not significantly alter the expression levels of the β -cell transcription factors or other pancreatic genes. It was illustrated that the FFO surgery played an important role in the transdifferentiation in the context of the lentiviral HMD/*INS-FUR* transduction [150], but was ineffective in the context of AAV8-*INS-FUR* transduction. Therefore, it was very likely that the combination of both FFO and the HMD lentiviral vector were essential for the transdifferentiation to occur. Perhaps future studies could utilise the 3rd generation lentiviral vector to deliver the *INS-FUR* construct in combination with β -cell transcription factors to replace FFO surgery.

Chapter 6:

General discussion

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6.1 The efficacy of the AAV8 vectors

6.1.1 AAV8 delivery of *INS-FUR* and pancreatic transcription factor genes could not induce the partial liver to pancreas transdifferentiation

Our laboratory has previously reversed diabetes by delivering the 2nd generation lentiviral vector (HMD/*INS-FUR*) to the livers of animals using the FFO surgical procedure to briefly isolate the liver from the circulation [148, 150-152], but the invasiveness of the surgical transduction procedure hinders the application of the therapy in a clinical setting. The surgery was shown to improve the transduction efficiency and partially contribute to the liver-to-pancreas transdifferentiation [150]. In the current project, the FFO surgery was replaced by delivering the *INS-FUR* gene and the pancreatic transcription factors *Pdx1* and *NeuroD1* using the clinically applicable AAV8-LSP vector system. The AAV8 vector was chosen because it had been shown to not trigger a strong immune response, allowed long-term episomal expression of the transgenes and had a significantly lower risk of mutagenesis in comparison to the integrating vectors [116]. In addition, the AAV8 capsid was shown to have high transduction efficiency toward mouse liver cells [180]. When used in conjunction with the LSP, which could limit the expression of the transgenes to the livers, the vector system (AAV8-LSP) allowed a simple systemic delivery via i.p. injection [181].

Ferber *et.al* (2000) showed that the transduction of the adenoviral vector carrying the *Pdx1* gene driven by the CMV promoter could reverse the BGLs in STZ-induced diabetic mice [143]. Kojima *et.al* (2003) demonstrated a partial reversal of diabetes in STZ-induced diabetic C57/BL6 mice after a helper-dependent-adenoviral vector delivery of the *NeuroD1* gene. The BGLs of the animals in the study were reduced, but not normalised and they had abnormal glucose tolerance [146]. From then on, the gene therapy that involve transducing the livers with β -cell transcription factors had been extensively studied [143, 160, 162, 174, 183, 204], but no study has demonstrated a complete long-term complete reversal of diabetes.

In the current project, the systemic delivery of the β -cell transcription factor *Pdx1* or *NeuroD1* by AAV8-LSP system (5×10^{11} vg) via i.p injection did not have any effect on the BGLs of the STZ-induce diabetic NOD/scid mice (Chapter 3). The overexpression of

the *INS-FUR* construct by the AAV8-LSP system to the livers of both STZ-induced diabetic NOD/scid mice and the autoimmune NOD mice was very efficient, but the insulin was not produced by the livers in a glucose responsive manner. Both NOD/Scid and NOD mice became severely hypoglycaemic after the transduction at the dose of 5×10^{10} vg. The IPGTT test also showed an abnormal response to a glucose challenge (Chapter 4 and Figure 6.1). The combination of *INS-FUR* and *Pdx1* or *INS-FUR* and *NeuroD1* or *INS-FUR*, *Pdx1* and *NeuroD1* delivered by AAV8-LSP vectors could not normalise blood glucose in either STZ-induced diabetic NOD/Scid or autoimmune NOD mice (Chapter 3, Chapter 4, and Figure 6.1). The RT-PCR showed that the transgenes were consistently detected, and the endogenous pancreatic transcription factor genes such as *NeuroD1*, *mouse insulin 2 somatostatin* and *pancreatic polypeptide* mRNA were randomly expressed and the mice that were either hyperglycaemic or hypoglycaemic. It had been shown that insulin and other pancreatic genes could randomly expressed at the mRNA level in the livers in response to metabolic disorders (such as hyperglycaemia, high-fat diet) or toxicity [146, 161]. Similarly, the random detection of pancreatic genes in the current study could be a result of hyperglycaemia. The random appearance of the transcription factors did not have any effect to the liver-to-pancreas transdifferentiation.

Similar to the current project, the ineffectiveness of the AAV delivery of *Pdx1* and *INS-FUR* in inducing the liver-to-pancreas transdifferentiation was reported [174, 183, 204, 205]. Wang *et.al* (2007) showed that glycaemic control could not be improved in STZ-induced diabetic C57/BL6 mice after the tail vein injections of AAV8 vector carrying *Pdx1* gene driven by transcriptional control of the elongation factor 1- α promoter that has high activity in the liver [183]. Tang *et.al* (2013) demonstrated that the AAV2 delivery of the super active form of *Pdx1* gene (*Pdx1-VP16*) by portal vein injection could induce the expression of insulin in the liver and reduce the BGLs in STZ-induced diabetic NOD/scid mice. The AAV2 delivery of *Pdx1* gene via intra splenic injection to the STZ-induced diabetic humanized NOG mice reduced the BGLs slightly for 120 days [174]. The transduction of the AAV8 vectors carrying the *INS-FUR* gene to the livers of NOD mice, and STZ-induced diabetic C57BL/6a and NOD/scid mice also led to the reduction of the BGLs, but could not completely stabilise the BGLs within the normal range [205]. All of the above studies indicated that the AAV delivery of the *INS-FUR* gene with or

without β -cell transcription factors could turn liver cells to insulin-producing cells, but the liver-to-pancreas transdifferentiation did not occur.

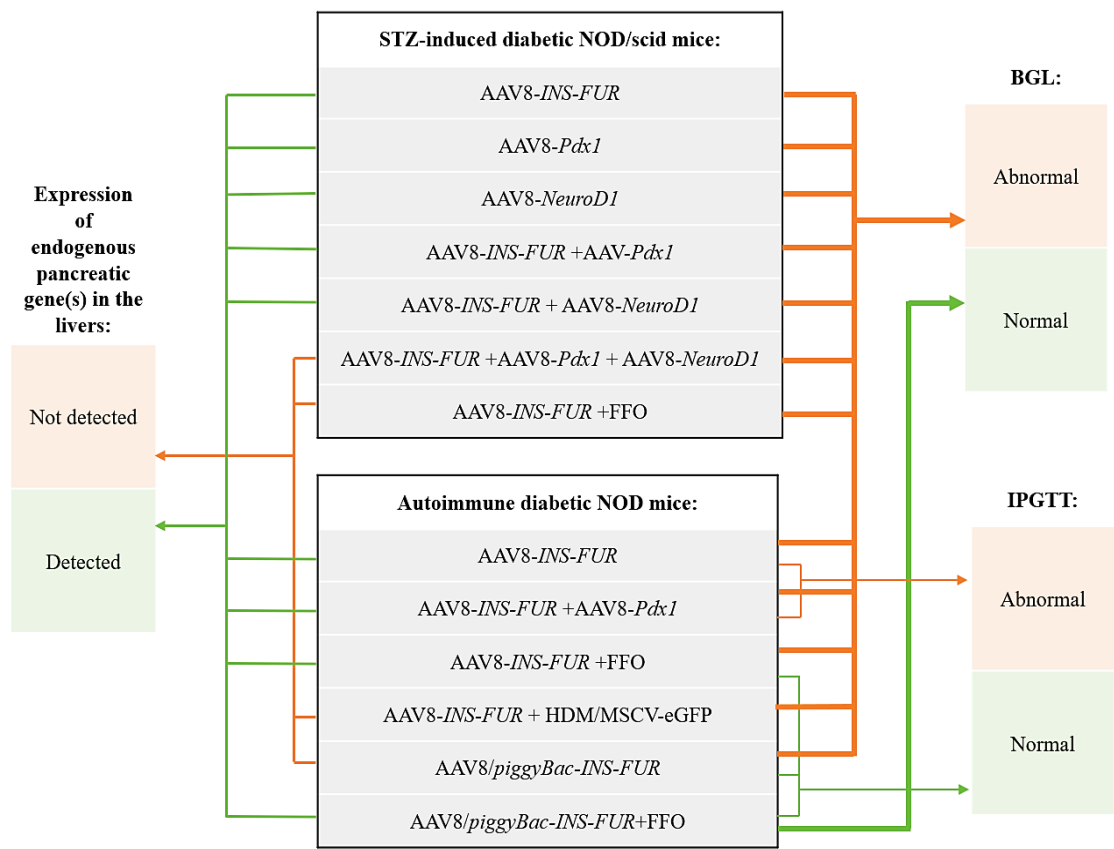


Figure 6.1: Summary of the BGLs, IPGTTs and the expression of endogenous β -cell transcription factor and other pancreatic genes in the livers of the NOD/scid and NOD mice transduced by AAV8 vectors

RT-PCRs were performed to detect the expression of the endogenous β -cell gene(s) of interest (*Pdx1*, *NeuroD1*, *Nkx2.2*, *Nkx6.1*, *MafA*, *Pax6*, *P48*, *mouse insulin1*, *mouse insulin2*, *glucagon*, *somatostatin*, *Ppy*, *PC1* and *PC2*) in the livers. The results were documented as “Detected”, when any of the genes of interest were detected after RT-PCR. The BGLs were considered to be abnormal when the mice were either hypo or hyperglycaemic at the endpoint of the experiments. The IPGTTs were considered to be abnormal when the BGLs at any one or more test time points were statistically different from the normal control mice.

6.1.2 Transduction efficiency of the AAV8 vectors

In order to understand the reasons for the ineffectiveness of the AAV8 vector in inducing the liver-to-pancreas transdifferentiation, the analysis for the transduction efficiency was performed. Even though the NOD/scid mice transduced by AAV8-*Pdx1* (5×10^{11} vg) had a 10-fold higher AAV8 dose in comparison to the mice transduced by AAV8-*INS-FUR* (5×10^{10} vg), the AAV8 copy numbers of the two groups were not significantly different from each other. The VCN analysis suggested that the transduction efficiency of the AAV8-*Pdx1* could be lower than the AAV8-*INS-FUR* vector. However, the differences in the transduction efficiency cannot be attributed to the composition of the vectors as both vectors utilised the same LSP promoter and the AAV8 capsid. It has been shown that the AAV8 VCN could reduce over time because the transgenes were not integrated [158]. As the *INS-FUR* transduced mice were euthanised 2 weeks earlier than the *Pdx1* transduced mice, the AAV8 VCN of the *INS-FUR* transduced mice could be higher than the *Pdx1* transduced mice.

In contrast, both the *NeuroD1* and the *Pdx1* transduced mice received the same AAV8 dose (5×10^{11} vg) and the experiments on both groups lasted for the same 28 days, but the mean AAV8 VCN of the *Pdx1* transduced mice was significantly lower than that of the *NeuroD1* transduced mice (Chapter 3). Analysis of the VCNs, suggested that the AAV8-*Pdx1* vectors may potentially have lower transduction efficiency than the AAV8-*NeuroD1* vectors, or the liver cells transduced by *Pdx1* could have been lost at the high rate after the transduction leading to the reduction of the transgene.

In the NOD and NOD/scid mice transduced by the combination of *INS-FUR* and *Pdx1*, the dosages of AAV8-*Pdx1* were either equal or 3.4 folds higher than the dose of AAV8-*INS-FUR* (Chapter 3 and Chapter 4). Interestingly, the copy numbers of *Pdx1* were significantly lower than the copy numbers of *INS-FUR* (Chapter 3 and Chapter 4). The discrepancy between the dosages and the transgene copy numbers in the double transduced mice aligned with the hypothesis that the liver cells transduced by *Pdx1* may have been lost at the higher rate than the cells transduced by *INS-FUR* alone leading to the reduction in the *Pdx1* copy numbers. Similarly, it was demonstrated in a previous study that murine livers transduced by pancreatic transcription factors delivered by AAV2 (via portal vein

injection) were susceptible to autoimmune destruction [204]. The author showed the appearance of insulin producing cells in the liver and a normalisation of the BGLs in the STZ-induced diabetic NOD/scid mice transduced by the combination of *Pdx1* and *Ngn3* delivered by AAV2 [204]. However, hyperglycaemia reoccurred after the transduced NOD/scid mice received i.p injections of splenocytes harvested from new-onset diabetic female NOD mice [204]. The authors also found T-lymphocyte and B-lymphocyte infiltration of the surrounding insulin producing cells suggesting that the transduced livers were susceptible to autoimmune destruction [204]. When the NOD mouse splenocytes were injected into the NOD/scid mice transduced by the AAV2 carrying the eGFP, such T-lymphocyte and B-lymphocyte infiltration was not observed, indicating that the immune reaction did not target the AAV2 components [204]. The authors suggested that after the *Pdx1* and *Ngn3* transduction, the hepatocytes could express antigens that resemble the β -cells; therefore, they were susceptible to the immune destruction mediated by the T-lymphocyte and B-lymphocyte of the NOD mouse model [204]. Similarly, in our project it was possible that the autoimmune reaction of the NOD mouse model was accelerated after *Pdx1* transduction leading to a fast clearance of cells transduced by either *Pdx1* alone or both *INS-FUR* and *Pdx1*.

6.2 FFO surgery was insufficient in inducing the liver-to-pancreas transdifferentiation after the AAV8 transduction

It had been shown that the HMD/*INS-FUR* transduction of the NOD mouse livers by portal vein infusion did not induce the expression of any pancreatic genes; however, when the vectors were delivered by FFO, the expression of *Pdx1*, *NeuroD1*, *Neurogenin3*, *Pax4*, *Nkx2.2*, *MafA*, mouse insulin 1, somatostatin, glucagon and synaptophysin were detected [150]. It was, therefore, hypothesised that the FFO surgery had an important role in the liver-to-pancreas transdifferentiation. The mechanism by which the FFO surgery enhanced the transdifferentiation was not fully understood. It was previously shown that hepatic regeneration induced by partial hepatectomy could accelerate the liver-to-pancreas transdifferentiation in response to *Pdx1* transduction by an adenoviral vector [162]. Similarly, it was thought that the FFO surgery may also have induced a mild injury to the liver and subsequently a regeneration of the liver cells.

Surprisingly, the current study with the AAV8 demonstrated that the FFO surgery did not contribute to the liver-to-pancreas transdifferentiation. The diabetic NOD and NOD/scid mice that received the FFO surgery after being transduced by the AAV8-*INS-FUR* did not express any major pancreatic transcription factors such as *Pdx1*, *NeuroD1*, *Neurogenin3*, *Pax4*, *Nkx2.2*, *Nkx6.1* and *MafA*. The BGLs of the NOD mice transduced by the combination of AAV8 –*INS-FUR* and FFO surgery were improved, but could not be normalised. Their VCNs were also significantly lower than the mice transduced by AAV8-*INS-FUR* without FFO surgery even though both group of mice received the same vector dose of 5×10^{10} vg (Chapter 4). The significant reduction in the VCNs of the mice that had FFO surgery suggested that the transgene was lost. The reduction of the VCNs appeared to be aligned with the speculation that the FFO surgery could cause liver cell death leading to the clearance of the non-integrated transgenes.

In contrast, the microarray comparison between the AAV8–*INS-FUR* transduced NOD mice with or without FFO did not show any significant different in term of liver cell death or regeneration (Chapter 5). It could be argued that the liver of the mice transduced by the combination of AAV8–*INS-FUR* and FFO surgery were collected at 8 weeks after the surgery; therefore, livers might have fully recovered from the injury induced by the surgery. As the result, the evidence of liver damage or regeneration could not be found by the microarray analysis. More importantly, when comparing the diabetic NOD mice treated by AAV8-*INS-FUR* and FFO to the normal controls, the microarray analysis for liver diseases and toxicity function did not show that liver damage or liver cell death were significantly activated, but the proliferation of the liver cells was slightly inhibited ($P = 2.29 \times 10^{-4}$, z-score = -1.32) (Chapter 5). Therefore, it was possible that the insult from the FFO surgery after AAV8 transduction was not sufficient to induce the liver regeneration or liver-to-pancreas transdifferentiation.

6.3 The roles of the VSV-G envelope and the hybrid HIV/MSCV promoters of the HMD vector in the transdifferentiation

It had been shown that the transduction of the HMD vector carrying the reporter gene eGFP driven by the hybrid HIV/MSCV promoter (empty HMD vectors) to the liver NOD

mice by intervallic infusion into the portal vein during FFO resulted in the expression of *Pdx1* [150] suggesting that the combination of the HMD capsids and FFO surgery may also be beneficial to the liver-to-pancreas transdifferentiation. In order to determine if the VSV-G envelope and the hybrid HIV/MSCV promoter of the HMD vector system could contribute to the liver-to-pancreas transdifferentiation, 5 NOD mice received portal vein infusion of the HMD/MSVC-eGFP (5×10^6 TU) 7 days after the AAV8-*INS-FUR*-mCherry (5×10^{10} vg) transduction by i.p. injections (Chapter 4). The BGLs of the transduced mice were normalised on week 2 and 3 after the AAV8 transduction, but they became hyperglycaemic again from week 4 onward (Chapter 4 and Figure 6.1). The high BGLs of the transduced animal correlated to the low AAV8 VCN. More importantly, the double transduction of AAV8 and HMD did not lead to the transdifferentiation because pancreatic genes could not be detected in the livers (Chapter 4 and Figure 6.1). The result demonstrated that the liver-to-pancreas transdifferentiation observed in our previous studies with the HMD vectors was not caused by the VSV-G envelope and the hybrid HIV/MSCV promoter.

6.4 The efficacy of the AAV8/piggyBac vectors system in delivering the *INS-FUR* gene

This was the first study utilising the AAV8/piggyBac system to deliver human insulin to diabetic NOD mice. We showed that i.p. delivery of the AAV8/piggyBac-*INS-FUR*-mCherry system followed by FFO surgery, resulted in the reversal of diabetes from week 3 to 15 (experimental end point), without induction of hypoglycaemia (Chapter 4 and Figure 6.1). The

NOD mice treated with the AAV8/piggyBac system had a lower *INS-FUR* dose than the mice transduced by the combination of AAV8-*INS-FUR* and AAV8-*Pdx1* or the combination of AAV8-*INS-FUR* and FFO (Chapter 4). Interestingly, the *INS-FUR* VCN of the AAV8/piggyBac transduced mice was not statistically different from the *INS-FUR* VCNs of the AAV8 transduced mice. The results showed that when using the AAV8/piggyBac system, lower *INS-FUR* dosages were required to achieve the same transduction efficiency as the traditional AAV8 vector system. More importantly, while the mice that had the combination of AAV8-*INS-FUR* and FFO or AAV8-*INS-FUR* and AAV8-*Pdx1* were hyperglycaemic, the BGLs of the diabetic mice transduced by the

AAV8/*piggyBac-INS-FUR* and FFO were normalised (Chapter 4 and Figure 6.1). One of the possible explanations was that the integration of the *INS-FUR* constructs mediated by the AAV8/*piggyBac* and FFO surgery allowed a higher and more stable expression of the transgene at the protein level.

In addition to the normal random BGLs and the IPGTTs, the mice treated with the AAV8/*piggyBac-INS-FUR* and FFO surgery may also had a glucose-responsive insulin secretion. The blood serum collected during the IPGTTs also showed a peak of human insulin secretion between 5 to 30 mins time points and the levels returned to baseline at 60 min time point of the test (Chapter 4 and Figure 6.1). The tightly regulated BGLs induced by the AAV8/*piggyBac-INS-FUR* system and FFO surgery occurred without the expression of β -cell transcription factors. The integration of the *INS-FUR* gene induced by the AAV8/*piggyBac* system was insufficient to stimulate the liver-to-pancreas transdifferentiation that was seen with the use of the lentiviral system, because the β -cell transcription factors were not expressed.

6.5. Conclusion and future recommendation

Pancreatic transdifferentiation that results in the regulated secretion of insulin is one gene therapy strategy under investigation to cure T1D. It was likely that for this to occur, a “pancreatic switch” must be activated [206]. This switch may involve the over expression of β -cell transcription factors [106, 143, 160], a transient destruction and regeneration of some liver tissue [148, 150-152], and/or factors present in the vector used in the therapy [149]. The transdifferentiation could not be induced by the over expression of the *INS-FUR* and /or *Pdx1* and /or *NeuroD1* using the AAV8 vector. It was possible that the engineered liver cells expressing the β -cell transcription factor(s) became the target of the autoimmune system and the transgenes were lost [204]. Therefore, the integration of the transgenes may be useful to maintain the transgene expression.

Performing the FFO surgery after the AAV8-*INS-FUR* transduction was ineffective as the insult from the surgery and the AAV vectors could not induce the liver regeneration. When used in combination with the AAV8-*INS-FUR* vectors, the VSV-G capsid and the

hybrid HIV/MSCV promoter of the HMD vector system could not cause the liver-to-pancreas transdifferentiation either.

The current study suggested that, using the AAV8/*piggyBac-INS-FUR* system, pancreatic transdifferentiation may not be required to achieve advantageous clinical outcomes such as normal BGLs and normal glucose tolerance. The integrating AAV8/*piggyBac-INS-FUR* system was useful to satisfy both the basal insulin and the glucose-responsive insulin secretion. The major hurdle to the use of the AAV8/*piggyBac* system will continue to be the instability of the transgene integration if the *transposase* cannot be cleared. While we demonstrated that the *INS-FUR* expression was sustained, it was apparent that the FFO surgery could not fully eliminate the *transposase*. Future studies could focus on finding a more effective way to reduce the expression of *transposase* without invasive surgical procedure or modification of the vector system.

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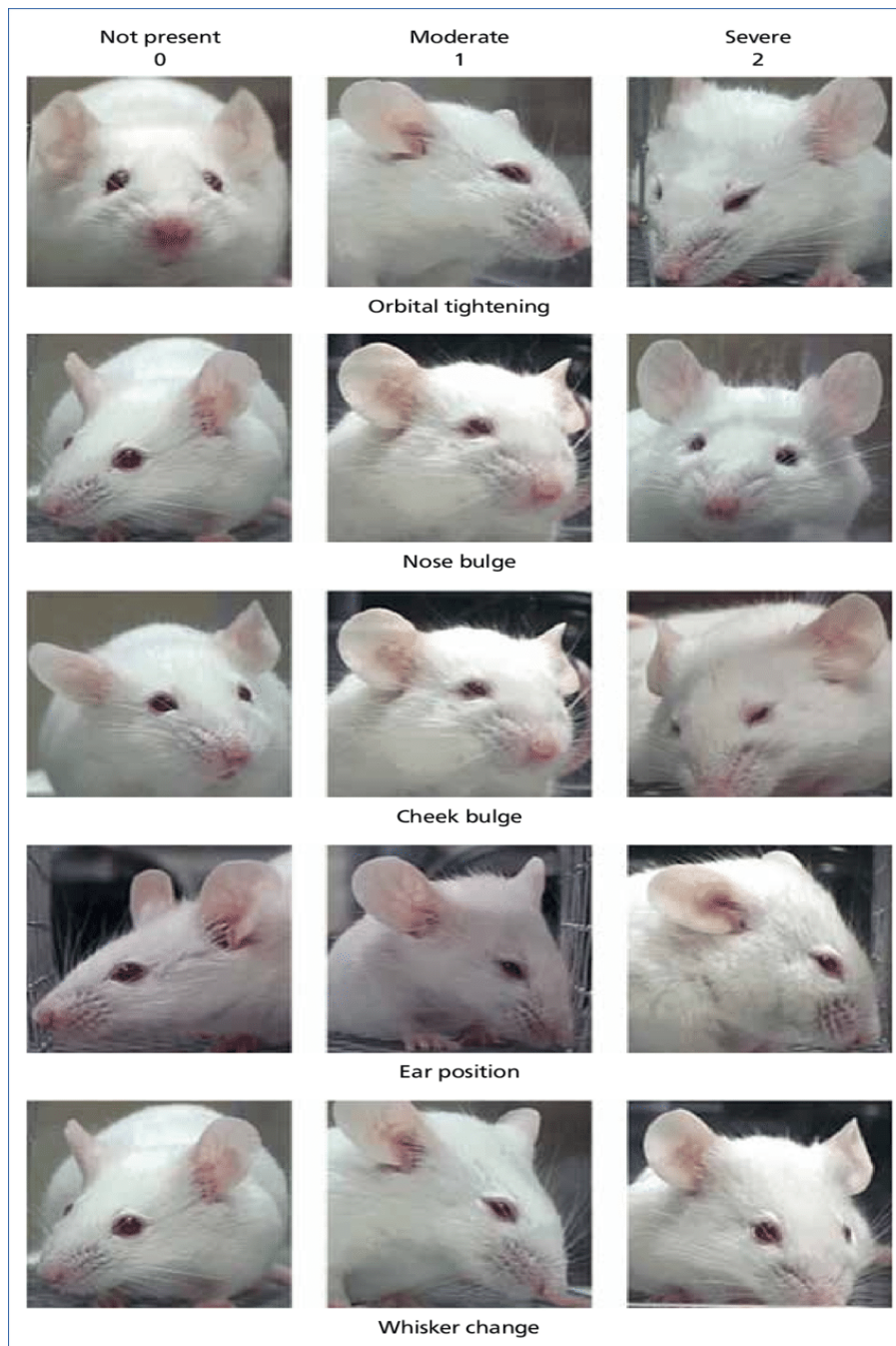
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Appendices



Appendix 1: Mouse grimace scale (MGS)

MGS utilised mouse facial expressions as criteria for pain and the effectiveness of analgesia in mice. The scale was composed of 5 different facial signs: orbital tightening, nose bulge, cheek bulge, ear position and whisker change. A score of 0-2 was assigned for each facial sign and the pain was recognised as moderate or severe when a total score of 2-5 or above 5 was assigned, respectively.

ANAESTHETIC AND OPERATIVE RECORD												
Animal ID:		Animal issued date:		ACEC No: ETH 17-1559		Procedure:						
Surgery date:			Chief investigator: Ann Simpson			Surgeon: Binhai Ren			Anaesthetist: Que La			
Species/strain/Sex : Mouse/NODIt/Female			Weight (g):			Blood Glucose Level (mmol/L):			Mouse Grimace Score:			
Pre-operative treatments:												
Eye lubricant: Viscotear			Analgesia: 50µl Buprenorphine (1:10 dilution of 300 µg/ml stock) s.c					Hydration: 500 µl of Hartmann s.c				
<i>**The following parameters must be recorded at every 5 mins from administration of pre-anaesthetic, throughout surgery, and continued until the animal has recovered its righting reflex.**</i>												
Time	Isoflurane %	Oxygen L/min	Heart rate	Respiratory rate	SpO2%	Eye reflex	Pedal reflex	Mucous membranes	Temp. °C	Hydration	Comments:	Sign
Pre-op:											Bupivacaine (6mg/Kg) (intradermal)	
Intra-op:												
Time of righting reflex (The animal is awake, alert and moves with 4 limbs):												
Time	Comments:											
Time	Post-operative medications and hydration: Ampicillin 100 µl (immediately post-op)											

Appendix 2: Anaesthetic and operative record

The record was completed during the FFO surgery and/or the infusion of the vectors into the portal vein.

Day 0 Post-Operative Care and Monitoring				
Animal ID:	Animal issued date:	Species/Strain/Sex: Mouse /NODIt/Female	Procedure date:	ACEC No. ETH 17-1559
Contact name 1: Binhai Ren	Phone #		After hours #	
Contact name 2: Que La	Phone #		After hours #	
Surgical procedure:	Temporary full flow occlusion of hepatic circulation (FFO procedure)			
Day 0 Post-operative monitoring and care plan: 1. Provide supplement Oxygen if needed 2. Provide soft food, cereal and water on the bedding. 3. Provide heat. 4. Check weight, BGL, MGS , level of activity, eye and pedal reflexes, mucoumembrane and wound imediatly post-op, half hourly for 2 hours, hourly for another 2 hours and then 4 hourly 5. Injection of 70 µl of Buprenorphine (1:10 dilution) (s.c) 4h post-op				

Time:	Oxygen/ room air	Weight	BGL	MGS	Eye reflex	Pedal reflex	Mucous membranes	Appearance of the incision	Medication, hydration and other comments

Appendix 3: Post-operative care and monitoring record of the operation day (day 0)

The care plan was performed immediately after the surgery.

Post-Operative Care and Monitoring (Day 1 onward)							
Animal ID:	Animal issued date:		Species/Strain/Sex: Mouse /NODIt/Female		Procedure date:	ACEC No. ETH 17-1559	
Contact name: Que La Binhai Ren	Phone #		After hours #		Chief investigator: Ann Simpson		
Surgical procedure:		Temporary full flow occlusion of hepatic circulation (FFO procedure)					
Post-op monitoring and care plan: On day 1 & 2 post-op: (1) Check weight, BGL, MGS and wound at least 3 times per day. (2) Provide soft food, cereal and water in cage. (3) Provide heat if needed. (4) Administer fluid and glucose replacement injections every 3-4 hours. (5) Administer analgesia 2 times per day. Suggested analgesia and fluid supplement: • Morning: 50-80µl Buprenorphine (1:10 dilution 300microgram/ml stock) + 500-700µl of isotonic saline or Hartman +/- glucose (s.c or i.p) +100µl of Ampicillin if needed (s.c) • Every 3-4 hours: 250-600µl of isotonic saline or Hartman +/- glucose (s.c or i.p) • At the end of the day: 50-80µl Buprenorphine (1:10 dilution of 300microgram/ml stock) + 500-700µl of isotonic saline or Hartman +/- glucose (s.c or i.p) From day 3 onward: (1) Check weight, BGL, MGS and wound 1 to 3 times per day. (2) Give fluid +/- glucose injections, analgesia, soft food and cereal as needed							
Date:	Time:	Weight:	BGL:	Grimace score:	General Observations: (e.g: coat condition, posture, gait, activities and oral intake)	Appearance of the incision:	Treatments and other comments:

Appendix 4: Post-operative care and monitoring record

The care plan and monitoring were performed from the next day after the surgery until the animals resumed normal behaviour, normal levels of activity and body weight