

Beta-cell therapy for diabetes: development of quality assured transport of human islets for transplantation at remote centres; investigation of augmentation of human islets *ex vivo*; and exploration of the potential for expansion and redifferentiation *in vitro*

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Abstract

Beta cell replacement is the only therapy currently able to restore insulin independence in C-peptide negative Type 1 diabetes. This can be achieved through either vascularised whole pancreas transplantation or isolated islet transplantation. Whole pancreas transplantation can deliver long-term normalisation of blood glucose but nevertheless is associated with significant peri-operative morbidity and mortality. Islet transplantation is a much less invasive procedure which can provide complete resolution of disabling hypoglycaemia with potential insulin independence. However, at the outset of this research, this procedure was restricted to UK centres with islet isolation facilities. Two main hurdles prevent wide application of this treatment modality. First, a limit to the number of isolation facilities that can be established. These are sophisticated and very expensive laboratories and require extensive staff training and commitment. Second, there is significantly limited supply of suitable donor pancreases meeting less than 1% of the potential clinical need.

The goal of this PhD project was to address, from the UK perspective, the limitations in transplantation sites and scarcity of suitable islets for clinical transplantation maintaining a clinically relevant focus through the study of primary human islets.

The overall aims were:

1. To demonstrate safe and effective islet transport despite relatively long journey time in the UK without need for complete revalidation / reparation for transplant at satellite site.
2. To evaluate potential for enhancing mass and function in intact human islets including the potential of *in vitro* incubation with pregnancy-related hormones
3. To evaluate potential for *in vitro* expansion of human pancreatic cells with determination of whether a functional beta-cell phenotype can be maintained over passage and whether putative pancreatic stem cells can be identified in culture.
4. To determine potential for redifferentiation of expanded human pancreatic cells towards a functional beta-cell phenotype by *in vitro* pseudo-islet formation.

A safe, practical and clinically viable islet transportation system was successfully established to suit the UK setting. This system enabled efficient utilisation of central islet isolation facilities for production of clinical grade high quality islets for transportation to several islet transplantation centres. Cooling a relatively small volume of high density islets in transport bags enabled transported islets to be transplanted at the satellite centres without any further manipulation and reparation. This work underpinned government funding of a national islet transplantation program and led to the first successful UK islet transplantation of transported islets. Validated standard operating procedures (SOPs) created in this project were adopted nationally by all islet receiving centres.

Augmentation of islet mass and function in intact primary human islets was attempted following treatment with pregnancy related hormones and a panel of other growth factors. In contrast to previously published reports on rodent islets or adherent human islets, these studies confirmed that growth factor treatment can only maintain as opposed to increasing islet mass or function in intact human islets. Nevertheless, prolactin induced a significant increase in insulin expression and potentiated insulin secretion in response to elevated glucose concentrations.

Proliferative cultures from pancreatic islets were established and characterised achieving high proliferative capacity with potential to produce transplantable mass sufficient for several recipients from a single donor. Despite maintenance of differentiated phenotypic markers in early passages there was an accelerated loss of β -cell function. Nevertheless expression of pluripotency-associated markers was identified for first time suggesting stem cells residing in adult pancreas.

Potential for enhanced β -cell function in islet proliferative culture with minimal manipulation was demonstrated through pseudo-islet formation. This was associated with down-regulation of pluripotency-associated markers and enhanced β -cell function *in vitro*

and limited, but confirmed activity, *in vivo*. However, currently this remains limited and insufficient for clinical impact.

In conclusion, validation of an effective and safe islet transport system was achieved underpinning unique National Health Service funding of a UK national clinical programme of islet transplantation and enabling the first successful UK transplantation of transported islets. Definitive studies to demonstrate functional enhancement in human islets in response to pregnancy related hormones and/or other factors would require toxic pre-conditioning. Significant islet mass expansion was attained *in vitro* with potential functional enhancement with pseudo-islet formation. However, further differentiation studies and validation of a practical large scale culture system remain an important requirement for any potential future clinical application.

Declaration

I hereby declare that all work presented in this thesis has been designed, carried out and analysed by myself except where otherwise acknowledged. In particular, islet transplantations at Newcastle were carried out, managed and followed up by the Newcastle Islet Transplantation Team, my part in which was in devising islet transport protocols and carrying out, or training other members of staff to carry out, quality assessment prior to transplantation. Assays carried out in the clinical program were not undertaken by myself.

All sources of information have been referenced and all collaborations were declared.

This work has not been presented to any other university for examination or degree. Any views expressed in this thesis are those of the author.

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Peer reviewed publications

1. A Aldibbiat, GC Huang, M Zhao, GN Holliman, L Ferguson, S Hughes, K Brigham, J Wardle, R Williams, A Dickinson, SA White, PRV Johnson, D Manas, SA Amiel, JAM Shaw. Validation of islet transport from a geographically distant isolation center enabling equitable access and National Health Service funding of a clinical islet transplant program for England. *Cell Medicine, Part B Cell Transplantation*. (2012) 21:1-100
2. M White, H Al-Turaifi, GN Holliman, A Aldibbiat, A Mahmoud, JAM Shaw. Pluripotency-associated stem cell marker expression in proliferative cell cultures derived from adult human pancreas. *J Endocrinol*. (2011) 211(2):169-76.
3. A Aldibbiat, CE Marriott¹, K T Scougall, S C Campbell, GC Huang, WM Macfarlane¹ and JAM Shaw. Inability to process and store proinsulin in transdifferentiated pancreatic acinar cells lacking the regulated secretory pathway. *Journal of Endocrinology* (2008) 196, 33-43
4. SC Campbell, A Aldibbiat, CE Marriott, C Landy, T Ali, WF Ferris, CS Butler, JAM Shaw, WM Macfarlane. Selenium stimulates pancreatic beta-cell gene expression and enhances islet function. *FEBS Lett*. (2008) 582(15):2333-7

Presentations to learned societies

1. A Aldibbiat, GN Holliman, H Al-Turaifi, SC Campbell, GC Huang, JAM Shaw. Generation of insulin-synthesising pseudo-islets from adult stem cell cultures derived from human pancreas. *Diabetologia*, 2008, Volume 51 suppl 1, P439, page S183
2. A Aldibbiat, GN Holliman, H Al-Turaifi, SC Campbell, GC Huang, JAM Shaw. Generation of insulin-synthesising pseudo-islets from adult stem cell cultures derived from human pancreas. *Biochemical Society (UK) poster presentation*, 3-4 December 2007

3. A Aldibbiat, GC Huang, M Zhao, MS Reddy, GN Holliman, J Dunn, AM Dickinson, DM Manas, SA Amiel, JAM Shaw. Confirmation of maintained sterility, viability and function in clinical-grade human islets following transport from an approved geographically-distant isolation facility. *Diabetic Medicine*. 2006. March 2006. Vol 23, supplement 2, p23, A86

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Table of abbreviations

~	Approximately
ACCORD	Action to Control Cardiovascular Risk in Diabetes
B2M	Beta-2-microglobulin
BR	Bioreactor
BSA	Bovine serum albumin
BTC	Betacellulin
CBC	Comparative Biology Centre
cDNA	Complementary DNA
CHOP	C/EBP homologous protein
CK19	Cytokeratin 19
DAPI	4,6-diamidino-2-phenylindole
DDIT3	DNA damage inducible protein 3
DKA	Diabetes ketoacidosis
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleotide triphosphates
DRI	Diabetes Research Institute
DTZ	Dithizone
ECL	Electrochemiluminescence
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMT	Endodermal mesenchymal transition
Esc	Embryonic stem cells
ET	Ethidium bromide
FCS	Foetal Calf Serum
FDA	Fluorescein Diacetate
GG	Gey and Gey
GIP	Gastric inhibitory polypeptide
GLP1	Glucagon-like peptide 1
GSIS	Glucose stimulated insulin secretion
GSIS	Glucose-stimulated insulin secretion
HBSS	Hank's Buffered Salt Solution

HD	Hanging drop
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HHS	Hyperosmolar hyperglycaemic state
HONK	Hyperosmolar nonketotic state
HSA	Human Serum Albumin
IA-2	Islet antibody 2
ICC	Immunocytochemistry
IEQ	Islet equivalent
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IP	Intra-peritoneal
IPGTT	Intra-peritoneal glucose tolerance test
ISC	Islet survivor cell
ITS	Insulin, Transferrin, Selenium
KCIIF	King's College Islet Isolation Facility
LDIS	Local donor for islets
MCP-1	Monocyte chemoattractant protein 1
MDA	Malondialdehyde
MM	Micromanipulator
MM1	Miami-defined medium 1
MREC	Multi Centre Research Ethics Committee
MSD	Meso-Scale Discovery
MW	Micro-well
OCDEM	Oxford Centre for Diabetes Endocrinology and Metabolism
OD	Optical density
OPTN	Open Procurement and Transplant Network
P/S	Penicillin / Streptomycin
PBS	Phosphate buffer saline
PCNA	Proliferative cell nuclear antigen
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PDX1	Pancreatic and duodenal homeobox 1
PES	Polyethersulfone
PFA	Paraformaldehyde

PFC	Perfluorocarbon
PI	Propidium Iodide
PL	Placental lactogen
PRL	Prolactin
PTA	Pancreas transplant alone
q-PCR	Quantitative PCR
RNA	Ribonucleic acid
RPM	Rotations per minute
RT	Reverse transcription
RVI	Royal Victoria Infirmary
SCID	Severe combined immunodeficiency
SOP	Standard operating procedure
SOX2	(Sex determining region Y)-box 2
SPK	Simultaneous pancreas and kidney transplantation
STZ	Streptozotocin
TF	Tissue factor
TLM	Two layer method
TPA	Tripropylamine
UWS	University of Wisconsin Solution
ZnT8	Zinc Transporter 8

Chapter 1. Introduction

1.1 Introduction to diabetes

1.1.1 Definition

Diabetes mellitus (DM) comprises a group of metabolic disorders that share the phenotype of hyperglycaemia. Several distinct types of diabetes mellitus exist and are caused by a complex interaction of genetics, environmental factors and life-style choices. Current World Health Organisation (WHO) diagnostic criteria for diabetes includes fasting plasma glucose of ≥ 7 mmol/l, on two occasions in people without symptoms, or 2 hour plasma glucose after 75 g of glucose intake of ≥ 11.1 mmol/l (WHO Publications, 2006). More recently utilisation of Glycosylated haemoglobin A1c (HbA1c) for diagnosis of diabetes was approved with HbA1c $\geq 6.5\%$ (47.5 mmol/mol) given that there are no conditions to affect assay accuracy such as impaired erythropoiesis, altered haemoglobin or glycation interference (WHO and IDF, 2011).

The metabolic dysregulation associated with diabetes mellitus causes secondary pathophysiological changes in multiple organ systems that impose a tremendous burden on the individual with diabetes and on the health care system (WHO and IDF, 2004). Currently, 346 million people worldwide have diabetes with an estimate of 3.4 million deaths in 2004 because of hyperglycaemia, a number that is expected to double by 2030 (WHO Publications, 2011).

1.1.2 Classification and aetiology of diabetes mellitus

According to aetiology, diabetes has been classified into four main groups (WHO Publications, 1999) with several sub-groups. These groups comprise Type 1 diabetes (T1D), Type 2 diabetes (T2D), Gestational Diabetes and other specific types of diabetes (Table 1.1).

Type of diabetes	Aetiology
Type 1	<ul style="list-style-type: none"> • Beta-cell destruction: autoimmune or idiopathic
Type 2	<ul style="list-style-type: none"> • Insulin resistance with relative insulin deficiency
Gestational	<ul style="list-style-type: none"> • Hyperglycaemia with onset or first recognition during pregnancy
Other specific types	<ul style="list-style-type: none"> • Genetic defect in β-cell function or insulin action • Diseases of the exocrine pancreas • Endocrinopathies • Drug or chemically induced • Infections • Others

Table 1 Diabetes aetiological classification (WHO Publications, 1999)

1.1.2.1 Type 1 diabetes mellitus

This accounts for approximately 10% of total DM cases (Deshpande et al., 2008). It is characterised by selective loss of β -cells from pancreatic islets of Langerhans through autoimmune or idiopathic destruction resulting ultimately in complete β -cell loss and absolute insulin deficiency.

The autoimmune process may precede the clinical signs and presentation. Islet autoimmunity is usually detected through the presence of antibodies including non-specific islet cell autoantibodies (ICAs) and antibodies against specific antigens including glutamic acid decarboxylase 65 (GAD), islet antigen-2 (IA-2), zinc transporter-8 (ZnT8) and insulin autoantibodies (IAAs) (Bingley, 2010). The underlying pathophysiology is characterised by islet infiltration with activated T lymphocytes (mostly cytotoxic and suppressor) resulting in what is termed 'insulinitis' (reviewed in (In't Veld, 2011)).

It has been hypothesised for many years that a viral-induced immune response may play a role in the pathological processes leading to development of Type 1 diabetes. Recently evidence has been provided by close correlation between detection of enteroviral capsid protein vp1 and double-stranded RNA activated protein kinase R (PKR) in islet post mortem sections in those with newly diagnosed Type 1 diabetes and Type 2 diabetes; consistent with persistent viral infection of the islet, compared to healthy controls (Richardson et al., 2009).

In a relatively small sub-group, particularly in non-Caucasians, no autoimmune activity is detected and these are designated as idiopathic T1D (Daneman, 2006; WHO Publications, 1999).

1.1.2.2 Type 2 diabetes mellitus

This is the most common type of diabetes accounting for 85-90% of cases. It is characterised by variable degrees of three different abnormalities (Monnier et al., 2008; WHO Publications, 1999): a defect in β -cell function which tends to progress over time (Levy et al., 1998); decreased disposal of glucose in peripheral tissue with insulin resistance (Yki-Jarvinen, 1995) occurring in the three major target tissues (liver, muscle and adipose tissue); and increased hepatic glucose production (Radziuk and Pye, 2002). Overt Type 2 diabetes is usually preceded by impaired fasting plasma glucose (IFG) (6.1-6.9 mmol/l) and impaired glucose tolerance (IGT) with 2 hour plasma glucose after 75 g oral glucose load of 7.8-11.1 mmol/l (WHO Publications, 2006).

Genetic predisposition plays an important role in T2D development. Genetic analysis has revealed susceptibility genes including the Pro12Ala variant in the peroxisome proliferator-activated receptor gamma (PPARG) gene (Altshuler et al., 2000) and the Glu23Lys variant in the potassium inwardly rectifying channel, subfamily J, member 11 (KCNJ11) gene (Gloyn et al., 2003). More susceptibility genes are being discovered through genome wide association studies (GWAS) (Prokopenko et al., 2008).

1.1.2.3 Gestational diabetes

This is characterised by hyperglycaemia first discovered during pregnancy. It affects 1-14% of pregnancies (ADA, 2006). Incidence rate has been increasing over recent decades reflecting the increase in obesity and Type 2 diabetes (Mokdad et al., 2003). Gestational diabetes is associated with adverse pregnancy outcomes including macrosomia, together with increased need for Caesarean section, in addition to significantly increased long-term risk of development of Type 2 diabetes in the

mother (16-63%) within 5-16 years post pregnancy (Casey et al., 1997; Kim et al., 2002; Metzger, 2007). There are several measures which can reduce risk of development of Type 2 diabetes in women with previous gestational diabetes including life-style modifications comprising healthy diet and physical exercise (Knowler et al., 2002), breast feeding (Kjos et al., 1993) and preventative pharmacotherapy with thiazolidinedione (Xiang et al., 2006) or metformin (Ratner, 2007).

1.1.2.4 Other specific types of diabetes

These are less common and comprise a wide range of aetiologies which can be sub-classified into several sub-groups including: genetic defects in β -cell function (e.g. Maturity-Onset Diabetes of the Young (MODY)), genetic defects in insulin action (e.g. Type A insulin resistance), disease of the exocrine pancreas (e.g. pancreatitis), endocrinopathies (e.g. Cushing's Syndrome), drug or chemically induced diabetes (e.g. glucocorticoids), infections (e.g. congenital rubella), uncommon forms of immune-mediated diabetes (e.g. insulin autoimmune syndrome) and other genetic syndromes (e.g. Down's syndrome). A full list is summarised in Table 1.2 (WHO Publications, 1999).

1.1.3 Complications of diabetes mellitus

1.1.3.1 Acute complications

1.1.3.1.1 *Diabetic ketoacidosis*

Diabetes ketoacidosis (DKA) is a medical emergency of uncontrolled diabetes and remains one of the most common causes of acute hospital admissions in people with Type 1 diabetes (Siafarikas and O'Connell, 2010). It is a serious condition that can be potentially fatal if not urgently and adequately treated. DKA is characterised by

hyperglycaemia and hyperketonaemia in the complete or relative absence of insulin. DKA is often the presenting state of newly diagnosed Type 1 diabetes patients (Siafarikas and O'Connell, 2010). Also it can be a result of poor compliance with insulin therapy or severe stress such as major infections when insulin demand increases significantly. Absence of insulin results in overproduction of endogenous glucose and lipolysis. Endogenous glucose accumulation occurs due to lack of its utilisation in the absence of insulin. Moreover, lack of insulin leads to ketogenesis, a process through which ketone bodies are produced in the mitochondria of hepatocytes from free fatty acids (Miles and Gerich, 1983). Management strategy includes stringent fluid, electrolyte and insulin replacement until effective clearance of ketones in addition to treating any underlying trigger (Savage et al., 2011).

1.1.3.1.2 *Hyperosmolar hyperglycaemic state*

Hyperosmolar hyperglycaemic state (HHS), also known as hyperosmolar non-ketotic (HONK) state, occurs in uncontrolled Type 2 diabetes. HHS is characterised by marked hyperglycaemia, high plasma osmolality and dehydration in the absence of any significant ketosis (Kitabchi et al., 2001). Ketogenesis is usually suppressed by presence of endogenous insulin, but the relative insulin deficiency secondary to insulin resistance and state of stress, e.g. infection, leads to progressive hyperglycaemia, dehydration, electrolyte imbalance, and elevated osmolality (Kitabchi et al., 2001). There is also a state of hypercoagulopathy predisposing to risk of thromboembolic events. This is a severe clinical emergency associated with high morbidity and mortality and requires urgent management. Management of HHS involves insulin infusion, rehydration and anti-coagulation with close electrolyte and glucose monitoring in addition to treating any underlying trigger (Kitabchi et al., 2001).

Other causes of diabetes	Examples
Genetic defects in beta cell function	<ul style="list-style-type: none"> • HNF4α- MODY1 • Glucokinase-MODY2 • HNF1α-MODY3 • PDX1-MODY4 • HNF1β • Others
Genetic defects in insulin action	<ul style="list-style-type: none"> • Typ1 A insulin resistance • Leprechaunism • Lipotrophic diabetes • Others
Disease of the exocrine pancreas	<ul style="list-style-type: none"> • Cystic fibrosis • Pancreatitis • Trauma / pancreatectomy • Neoplasia • Others
Endocrinopathies	<ul style="list-style-type: none"> • Cushing's syndrome • Acromegaly • Pheochromocytoma • Glucagonoma • Hyperthyroidism • Others
Drug induced diabetes	<ul style="list-style-type: none"> • Nicotinic acid • Glucocorticoids • Thyroid hormone • Alpha-adrenergic agonist • Beta-adrenergic agonist • Thiazide • Interferon-α therapy • Others
Infections	<ul style="list-style-type: none"> • Congenital rubella • Cytomegalovirus • Others
Uncommon forms of immune-mediated Diabetes	<ul style="list-style-type: none"> • Insulin auto-immune syndrome • Anti-insulin receptor antibodies • Stiff Man syndrome • Others
Other genetic syndromes	<ul style="list-style-type: none"> • Down's Syndrome • Friedreich's Ataxia • Huntington's Chorea • Klinefelter's syndrome • Lawrence-Moon-Biedel • Turner's Syndrome • Others

Table 2 Other Causes of diabetes (WHO Publications, 1999)

1.1.3.1.3 Hypoglycaemia

Hypoglycaemia is a state of low blood glucose of below 4 mmol/l (Diabetes UK, 2011). In the context of diabetes this classically occurs due to relative excess of circulating insulin either with exogenous insulin administration or intake of insulin secretagogues with relative deficit of carbohydrate intake and/or increased energy expenditure. This is a serious complication which can be fatal and represents a significant burden for people with diabetes and their families (Cryer et al., 2003). Hypoglycaemia triggers autonomic responses (e.g. palpitations, sweating, hunger and tremor) and awareness of low glucose. Severe cases of hypoglycaemia can cause seizures and collapse requiring third party intervention. Hypoglycaemic awareness becomes blunted over time when hypoglycaemia becomes frequent resulting in attenuated sympathoadrenal response. This leads to a state of hypoglycaemia unawareness (ADA hypoglycaemia workgroup, 2005). Hypoglycaemia can become disabling and may lead to significant psychological morbidity (ADA hypoglycaemia workgroup, 2005; Cryer *et al.*, 2003).

1.1.3.1.4 Lactic acidosis

This is a result of rapid elevation of serum lactate levels due to either overproduction in cases of tissue hypoxia; or underutilisation when liver and/or kidney fail to remove it from the system (Luft, 2001). In Type 2 diabetes, biguanides e.g. phenformin (not in clinical use anymore) and to much less extent metformin, can potentially lead to lactic acidosis in the presence of renal impairment, a risk that can be minimised with careful prescribing and close monitoring (Luft, 2001; Strack, 2008).

1.1.3.2 Chronic complications

The discovery of insulin by Banting and Best in 1922 was a historical breakthrough in the management of Type 1 diabetes in particular and diabetes in general (Banting and

Best, 1990). This immediately reduced early ketoacidosis-related mortality but unmasked the chronic potential impact of hyperglycaemia on almost all systems of the body resulting in a very wide range of potential complications.

1.1.3.2.1 *Microvascular complications*

Glucose dependent endothelial damage occurs with prolonged hyperglycaemia in both Type 1 and Type 2 diabetes leading to a wide range of complications including retinopathy, nephropathy and neuropathy (Brownlee, 2001; Vithian and Hurel, 2010).

1.1.3.2.1.1 *Diabetic retinopathy*

This is a common diabetic microvascular complication affecting more than one third of people with diabetes and remains the most common cause of preventable blindness in working-age people (Cheung et al., 2010). Severity of diabetic eye disease depends on the stage of retinopathy ranging between background retinopathy with microaneurysms, dot/blot haemorrhages and hard exudates; pre-proliferative retinopathy characterised by cottonwool spots and venous beading; proliferative retinopathy characterised by formation of new vessels; advanced eye disease and maculopathy (Vithian and Hurel, 2010). Pregnancy can aggravate diabetic retinopathy so does rapid improvement in glycaemic control necessitating careful diabetes management and retinal screening. Careful retinal screening and early referral to specialist ophthalmology services for consideration of laser photocoagulation therapy is key to prevent progression of diabetic eye disease and blindness (Vithian and Hurel, 2010). Newer treatment modalities which have less side effects compared to laser therapy are emerging including intra-ocular steroid delivery and anti-angiogenic factors look promising (Cheung et al., 2010).

1.1.3.2.1.2 Diabetic nephropathy

This is another common microvascular complication which affects nearly one third of patients with diabetes (Ritz and Orth, 1999). Both chronic hyperglycaemia and uncontrolled hypertension lead to glomerular damage, particularly affecting the microvasculature causing thickening of basement membrane, atherosclerosis and fibrosis. Microalbuminuria is an early manifestation of diabetic nephropathy which progresses over time to overt proteinuria in about 20% of patients (Vithian and Hurel, 2010).

This necessitates aggressive hypertension treatment and effective control of diabetes to slow progression of the disease (DCCT, 1993; UKPDS, 1998). Referral to the renal team is important when renal function continues to deteriorate despite appropriate management.

1.1.3.2.1.3 Neuropathy

Nearly all nerves in the body can be affected with varying degrees of disease manifestations and symptom heterogeneity. The most common form is chronic sensorimotor peripheral diabetic neuropathy which can be symptomatic in almost 50% of the cases. Other manifestations include cranial nerve palsies, mononeuropathies and autonomic dysfunction. This could potentially lead to gastroparesis, cardiac arrhythmias and sexual dysfunction, ulcer formation and / or Charcot foot deformity (Boulton et al., 2005). Risk can be reduced with good glycaemic control and management is usually supportive (DCCT, 1993; Boulton et al., 2005).

1.1.3.2.2 Macrovascular complications

In addition to hypertension, hyperlipidaemia and smoking, hyperglycaemia in both Type 1 and Type 2 diabetes is a major risk factor for atherosclerotic macrovascular

disease. This can affect coronary arteries leading to ischaemic heart disease which remains the most common macrovascular complication in people with diabetes (Laing et al., 2003; Mahgoub and Abd-Elfattah, 1998). These atherosclerotic changes can also result in cerebrovascular disease with a significant increase in stroke risk (Ergul et al., 2009). Moreover, similar atherosclerotic changes can affect peripheral arteries leading to much higher risk of limb ischaemia especially in presence of kidney failure (Wang et al., 2007; Knight et al., 2004), and remain the most common cause of non-traumatic amputations in man.

1.1.3.3 Benefits and potential problems of glycaemic control

Seminal studies have demonstrated clear benefit of tight glycaemic control especially on reducing complications of microvascular origin in both Type 1 and Type 2 diabetes. In the Diabetes Control and Complications Trial (DCCT) people with Type 1 diabetes who used an intensive insulin regimen for a relatively long period (6.5 years) achieved average HbA1C of 7% and demonstrated significant reduction of retinopathy, nephropathy and neuropathy (DCCT, 1993). These benefits were still observed after 5 years (DCCT-EDIC, 2002), 10 years and even longer (White et al., 2008; de Boer et al., 2011) follow up with reduced retinopathy and regression of nephropathy in the intensive treatment group. Even after 30 years of diabetes the intensive arm subjects had significantly lower cumulative incidences of retinopathy, nephropathy and cardiovascular disease (Nathan et al., 2009).

The United Kingdom Prospective Diabetes Study (UKPDS) (UKPDS, 1998) demonstrated parallel results in terms of microvascular benefits in people with Type 2 diabetes who were intensively treated for tight glycaemic control. Interestingly, in a similar pattern to the DCCT follow up outcome, the 10 year follow up report after original UKPDS study completion demonstrated continued microvascular benefit in

the original intensive control subjects despite early loss of glycaemic control difference from the conventional treatment group post randomised control trial study completion. Moreover, there was evidence of reduced myocardial infarction rates and all-cause mortality in the intensive control group follow up (Holman et al., 2008a). A similar benefit was not seen when blood pressure control was lost after completion of the study (Holman et al., 2008b).

On the other hand, tight glycaemic control was associated with 2 to 3 fold increase in risk of severe hypoglycaemia as shown in both DCCT and UKPDS (DCCT, 1993; UKPDS, 1998).

The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study was conducted to investigate potential benefit of tight glycaemic control in those with longer duration of Type 2 diabetes with a target of HbA1C <6.0% with intensive multi-agent treatment. The study was terminated prematurely at 3.5 years follow up as a significant increase in all-cause mortality and cardiovascular mortality in the intensive control group as compared to standard treatment group became evident (Gerstein et al., 2008). However analysis of the data showed a significant reduction in non-fatal myocardial infarctions in the intensive treatment arm compared to standard treatment arm.

1.1.4 Conventional management of diabetes

Over recent years there have been dramatic changes in the management protocols for both Type 1 and Type 2 diabetes with the advent of new classes of oral and injectable agents in addition to advances in insulin analogues and delivery methods. However, life style modifications with increased physical activity and healthy diet remain the cornerstone of Type 2 optimal self-management.

1.1.4.1 Management of Type 1 diabetes

1.1.4.1.1 Animal insulin

Since its discovery (Banting and Best, 1922) insulin continues to be the main and essential glucose lowering agent in Type 1 diabetes. Initial insulin preparations were unmodified soluble insulin extracted from animal pancreata including swine and bovine preparations. These preparations were life-saving but necessitated multiple daily injections and produced problems with erratic blood glucose profile associated with insulin peaks and troughs. Longer action preparations were formulated in the 1930s with protamination of insulin, i.e. creating complexes of insulin-protamine (a fish protein) and further addition of zinc, also known as neutral protamine Hagedorn (NPH) (Hagedorn *et al.*, 1984; Chevalier, 1950). Biphasic insulin mixtures were successfully generated and used clinically (Oakley *et al.*, 1966).

1.1.4.1.2 Human insulin

The advent of recombinant protein technology enabled for the first time production of pure soluble human insulin in the late 1970s (Cohn, 1978). Similarly, biphasic human insulin mixtures were used to enable reduction in the number of insulin injections and provide cover for post-prandial glucose levels. However, variability in action in addition to different peaks and troughs made optimal glycaemic control in the absence of dangerous hypoglycaemia extremely difficult to achieve (DCCT, 1993). Moreover, the DCCT and UKPDS demonstrated clearly that the long term benefits of achieving optimal glycaemia are maximised by more daily injections as opposed to reduction in injection numbers (DCCT, 1993; Sheldon *et al.*, 2009; UKPDS, 1998). Therefore, mimicking physiological insulin profile has become the goal of modern treatment modalities.

1.1.4.1.3 Insulin analogues

Genetic modification of the human insulin gene enabled generation of new insulin-like molecules with different pharmacokinetic and pharmacodynamics profiles, termed insulin analogues. Insulin analogues became available for clinical use in the mid-1990s with a range of rapid acting and basal insulins (Vajo et al., 2001). Insulin analogues have revolutionised diabetes management and made therapeutic options much more effective and flexible (Owens, 2002; Rossetti et al., 2008; Sheldon et al., 2009). Insulin analogues have been shown to have a consistent, less variable and more predictable mode of action. They can be classified in two major classes: fast acting or rapid analogues and long acting or basal analogues. There are three clinically used fast acting insulin analogues, namely aspart, lispro and glulisine. Modifications were in the form of amino acid(s) substitutions to disable or weaken formation of hexamers and aggregation thus leading to more rapid disaggregation and mobilisation into the blood stream following subcutaneous injection, effectively mimicking the physiological post-prandial peak in circulating insulin following injection immediately before eating (Figure 1.1). All three share similar profiles and pharmacokinetics with an onset of action at about 10-15 minutes after subcutaneous injection reaching maximum effect at 30 minutes which then starts to diminish at 2 hours. The two long-acting insulin analogues used clinically are insulin glargine and insulin detemir. Two different strategies were followed to enable long and less variable action of insulin. In insulin glargine, asparagine was replaced by glycine at position A21 of human insulin and two arginine molecules were added at position B30 (Figure 1.1) resulting in a shift of the isoelectric point from pH 5.4 to pH 6.7. Insulin glargine is stored as an acidic solution to keep eluted and upon delivery subcutaneously it precipitates into crystals, followed by slow delivery to the blood stream (Owens, 2002). The biological effect of insulin glargine starts approximately 2

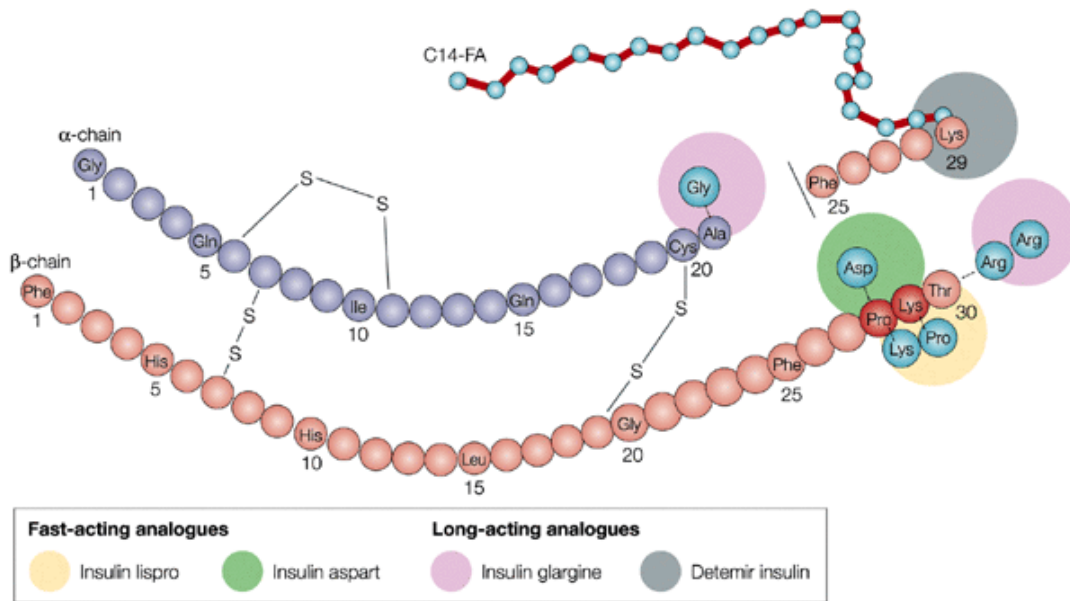


Figure 1.1 Insulin analogues showing modifications to human insulin (Owens 2002).

hours after injection and reaches its maximum effect at 8-10 hours starting to wane after approximately 16 hours. In the case of insulin detemir, threonine at B30 in human insulin was removed and lysine at B29 was acylated with a fatty acid residue (Figure 1.1) to promote increased self-association and enable reversible albumin binding. Insulin detemir pharmacodynamics are comparable to those of insulin glargine but with possibly shorter duration and less variable profile (Heise and Pieber 2007; Sheldon et al. 2009).

1.1.4.1.4 Continuous insulin infusion systems (CSII)

Optimal glycaemic control without disabling hypoglycaemia is not achievable in all people with diabetes using conventional insulin injections (Bruttomesso et al., 2009). Continuous subcutaneous insulin infusion (CSII) utilising insulin pump was developed to enable more consistent and adequately controlled insulin delivery, providing a utility for closely mimicking the physiological secretory profile of endogenous insulin. Insulin pumps utilise rapid acting insulin analogues due to favourable quick action and shorter half-life. Both multiple daily insulin injections and CSII were used in the intensive glucose control group in the DCCT (DCCT, 1993). Since then more evidence has become available from randomised control trials confirming improved glycaemic control and reduction in severe hypoglycaemia in comparison to non-analogue insulin multiple daily injections (Hirsch et al., 2005; Pickup and Keen, 2002).

Reduction in hypoglycaemia however was comparable between CSII and multiple insulin injections using analogue insulins (Pickup and Renard, 2008; Thomas et al., 2007).

Thomas in our group showed potential for restoration of hypoglycaemia awareness with both CSII and multiple analogue insulin injections (Thomas et al., 2007).

Most CSII systems offer useful and practical utilities to calculate more accurate insulin bolus doses in light of before-meal blood glucose reading, amount of carbohydrates in food and estimated remaining insulin ‘on board’ in the subcutaneous depot. Some newer CSII systems offer real-time glucose monitoring utility that can be used to enhance self-management. Evidence for prevention of severe hypoglycaemia is currently lacking, however (Mastrototaro et al., 2008; Peyrot and Rubin, 2009).

1.1.4.2 Management of Type 2 diabetes

This is largely dependent on disease progression, health status and personal choices. When life style modifications fail to achieve glycaemic control, addition of metformin, particularly in the majority who are overweight, is recommended if tolerated without problematic gastrointestinal side effects (NICE, 2008). Insulin secretagogues i.e. sulphonylureas are effective in lowering blood glucose but can cause hypoglycaemia especially in those with severely impaired kidney function in addition to causing weight gain (NICE, 2008). Sulphonylurea efficacy is dependent on at least residual β -cell function and the effect of these agents diminishes as β -cell function deteriorates; while metformin and thiazolidinediones (TZDs) are likely to maintain their effect over time (Kahn et al., 2006).

Thiazolidinediones (TZDs), which are peroxisome proliferator-activated gamma (PPAR γ) receptor agonists, are particularly useful in improving insulin sensitivity but can result in fluid retention and weight gain. In addition the possible increase in cardiovascular disease with these drugs has caused considerable recent controversy and indeed the withdrawal of one agent, rosiglitazone in Europe (Home et al., 2007; Nissen and Wolski, 2007).

Incretin hormones are insulinotropic polypeptides that are synthesised and secreted from the enteroendocrine cells in the intestines. The most studied are glucagon-like

peptide 1 (GLP1), secreted from L cells mainly in the ileum and colon; and gastric inhibitory polypeptide (GIP), secreted from K cells in the duodenum and proximal jejunum (Drucker, 1998; Ahren, 2003). These hormones are secreted in response to oral carbohydrate ingestion. Numerous effects of these hormones have been identified that impact positively on glucose metabolism and diabetes. These include enhancing insulin release in response to glucose stimulation (Drucker, 1998), suppression of glucagon secretion (Suzuki et al., 1989), delaying gastric emptying (Wettergren et al., 1993), inhibiting hepatic gluconeogenesis and improving satiety (Drucker, 1998; Unger, 2010). Incretin based-therapies are focused either on augmentation of endogenous GLP1 levels through inhibition of dipeptidyl peptidase (DPP) IV, a ubiquitous enzyme responsible for the short *in vivo* half-life of GLP1 (2-5 minutes) through cleavage of proline at amino acid 2 of the active form of GLP1 (7-36) (Mentlein et al., 1993); or administration of pharmacological doses of exogenous GLP1 analogues / homologues.

GLP1-based therapies have been the focus of many studies and therapeutic approaches recently. Clinical trials have demonstrated an average of 0.8% reduction in HbA1C with DPP IV inhibitors e.g. Sitagliptin and Vildagliptin (Goldstein et al., 2007; Pi-Sunyer et al., 2007). Exenatide is an injectable GLP1 homologue (derived from the saliva of the Gila lizard) resistant to DPP IV deactivation, extending mean half-life from minutes to 4 hours (Chia and Egan, 2008). Subjects treated with Exenatide have maintained an average 1.1% reduction in HbA1C and weight reduction of 4 kg after 2 years therapy (Buse et al., 2007). Liraglutide is another injectable GLP1 analogue resistant to DPP IV with longer half-life of 12 hours (Chia and Egan, 2008). Similar initial clinical outcomes have been observed with

Liraglutide treatment (Vilsboll et al., 2007). Long term safety and efficacy of GLP1 analogues remain to be confirmed given relatively limited clinical experience to date. Insulin therapy in Type 2 diabetes is required when endogenous beta-cell function is insufficient and all other tolerated agents fail to achieve optimal glycaemic control. Basal insulin formulations such as insulin glargine, are usually sufficient to achieve satisfactory fasting blood glucose (Riddle et al., 2003). However; as β -cell stress and failure progress there will be an additional need for fast-acting insulin e.g. insulin aspart or insulin lispro, to cover prandial needs (Skamagas et al., 2008).

1.1.5 Need for alternative therapeutic strategies

The discovery of insulin was life saving for those with Type 1 diabetes mellitus revolutionising their treatment. More recently, insulin has increasingly been used in the management of those with Type 2 diabetes. Randomized, prospective clinical trials involving large numbers of individuals with Type 1 or Type 2 diabetes mellitus have conclusively demonstrated that tight glycaemic control can prevent or reduce the incidence microvascular complications but this was associated with a three-fold increase in severe hypoglycaemia (DCCT, 1993; UKPDS, 1998). This is possibly the most feared complication of insulin treatment as it can lead to collapse without warning and poses a burden on both people with diabetes and their families (Smith et al., 2009; Barnard et al., 2010). Moreover, tight control of patients' blood glucose requires considerable ongoing effort and is still far from the simple practical therapy that patients desire. This in itself can negatively affect quality of life. Diabetes mellitus is a major health problem for all countries in the world taking into consideration the chronic nature of the disease, the fact that up to 4% of the population are affected and the huge cost of treating the disease and its complications.

These alarming facts make the search for new potentially curative therapeutic strategies for this lifelong disease of vital importance.

1.2 Alternative therapeutic strategies for Type 1 diabetes

A number of alternative approaches aiming at restoration of endogenous insulin production, storage and minute by minute regulated secretion have been sought. These include prevention or reversal of early Type 1 diabetes; cell replacement therapies employing whole pancreas transplantation, islet transplantation or stem cells; non-endocrine adult somatic-cell transdifferentiation; and gene therapy.

1.2.1 Type 1 diabetes prevention/ reversal approach

One strategy is generation of vaccines that may halt the autoimmune insulinitis process that leads to β -cells destruction. This is being researched employing diabetes associated antigen based vaccines e.g. DiaPep277, an immunogenic peptide from heat shock protein (Eldor et al., 2009); and Diamyd, a GAD65 antigen (Ludvigsson, 2009). Safety of DiaPep277 was demonstrated in phase I trials. However, despite preservation of residual C-peptide in adults there was no meaningful change in daily insulin requirement (Huurman et al., 2007). Moreover, a Phase II trial in children showed no meaningful benefit as C-peptide loss could not be halted or slowed (Schloot et al., 2007). GAD65 antigen vaccine trials demonstrated safety and efficacy with less overall drop in C-peptide levels compared to placebo controls, in both treated adults at 5 years follow up post treatment (Agardh et al., 2009), and adolescent subjects at 4 years follow up post treatment (Ludvigsson et al., 2011).

Trials to induce immune tolerance to insulin via nasal insulin in children with HLA-DQB1 susceptibility alleles for Type 1 diabetes and presence of autoantibodies failed

to prevent development of diabetes and the trial was terminated early (Nanto-Salonen et al., 2008).

In a phase III randomised trial teplizumab, an anti CD-3 antibody, was used in newly diagnosed Type 1 diabetes subjects with a primary outcome of use of <0.5 unit/kg insulin. At 1 year there was no difference in primary outcome between subjects who were treated with teplizumab (different dose and different treatment durations) and placebo. However 5% of patients who were treated with teplizumab were still not using insulin compared to control group (p= 0.03) (Sherry et al., 2011).

1.2.2 Cell replacement therapy

1.2.2.1 Vascularised whole pancreas transplantation

Vascularised whole pancreas transplantation was performed for the first time in 1966 (Kelly et al., 1967). In combination with a deceased donor kidney transplant, it has become the standard therapy for people with Type 1 (or even Type 2 diabetes without significant insulin resistance) and end-stage renal failure (Robertson et al., 2003). It offers freedom from insulin and associated acute metabolic complications: hypoglycaemia, hyperglycaemia and ketoacidosis. It necessitates major surgery and involves complicated organ retrieval, preservation and transplantation procedures. Early procedures were associated with high morbidity, poor survival and high graft failure rates. Surgical complications include thrombosis, haemorrhage, anastomotic leak, sepsis, fistula and graft pancreatitis (White et al., 2009). Significant advances were achieved in donor and recipient selection criteria, together with surgical techniques for both retrieval and transplantation, and optimised immunosuppressant therapy leading to improved graft success rates (75.5% pancreas transplant alone (PTA) and 80.0% simultaneous pancreas and kidney (SPK)) and recipient survival (97.8% PTA and 97% SPK) at 1 year post transplant. However there is still

considerable decline in both graft function (51.5% PTA and 53.4% SPK) and recipient survival (88.7% PTA and 84.5% SPK) at 5 years post-transplant (OPTN-SRTR, 2009). The current most common immunosuppressant therapy includes Tacrolimus and Mycophenolate Mofetil (MMF) with a trend to avoid steroids due to their adverse effects including dysglycaemia, dyslipidaemia and decreased bone mass (Meloche, 2007). Whole pancreas transplantation remains a major surgical procedure with associated peri-operative morbidity and mortality. Moreover, there is a significant shortage of donors limiting availability of this therapeutic approach to less than 1% of potential recipients.

1.2.2.2 Islet transplantation

Significant advancements have occurred in the field of islet transplantation in the past two decades (Shapiro et al., 2000). This minimally invasive procedure can offer resolution of problematic hypoglycaemia and hypoglycaemia unawareness with a potential period of insulin independence (Ricordi and Strom, 2004). It is associated with less morbidity and mortality compared to vascularised whole pancreas transplantation. However, availability of suitable donor tissue remains a major challenge for wide application of this treatment modality.

Islet transplantation will be discussed in detail in Section 1.3.

1.2.2.3 Stem cells

1.2.2.3.1 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from *in vitro* isolation of cells from the inner cell mass of a blastocyst. These cells exhibit pluripotency to differentiate to cells from all germ layers of the adult organism. Embryonic development of the pancreas has been extensively studied and the stages of development and the critical transcription factors involved have been largely, but not completely, revealed (Figure

1.2 based on multiple sources (Murtaugh, 2007; Zaret, 2008; Zaret and Grompe, 2008)). Trials on controlling the differentiation of the ESCs to mature islet cells including β -cells were limited initially by lack of efficiency and heterogeneity of the end product. A major breakthrough in the field was achieved when protocols for creating definitive endoderm (DE) were realised (D'Amour et al., 2005; Kubo et al., 2004). Several research groups have focused efforts on creating functional β -cells from ESCs following different differentiation protocols with varying success (Cho et al., 2008; D'Amour et al., 2005; Jiang et al., 2007; Kroon et al., 2008; Phillips et al., 2007; Shim et al., 2007).

Many issues have hindered successful clinical use of the cells including mixed phenotype of the end product and presence of undifferentiated cells which increases risk of teratoma development; uncertainties regarding true insulin expression and processing (Rajagopal et al., 2003) and imperfect glucose responsiveness of the putative β -cells (D'Amour et al., 2006). Kroon et al., have demonstrated, in a protocol that mimics physiological embryonic development of the pancreas (Figure 1.3), the possibility of generating insulin producing β -cells from human ESCs which exhibit glucose responsiveness following *in vivo* transplantation (Kroon et al., 2008). This is very promising and certainly a significant step forward. However there still remain issues concerning safety (particularly given the need for final differentiation post-transplant of incompletely differentiated precursors), as yet untested functionality in man and need for immunobarrier encapsulation and / or immunosuppressive therapy.

1.2.2.3.2 *Adult stem/progenitor cells*

Replacing β -cells from multi-potent adult stem cells is very attractive and offers several potential advantages including abolishing the need for immunosuppressive therapy when recipient-derived autologous stem cells are employed; although

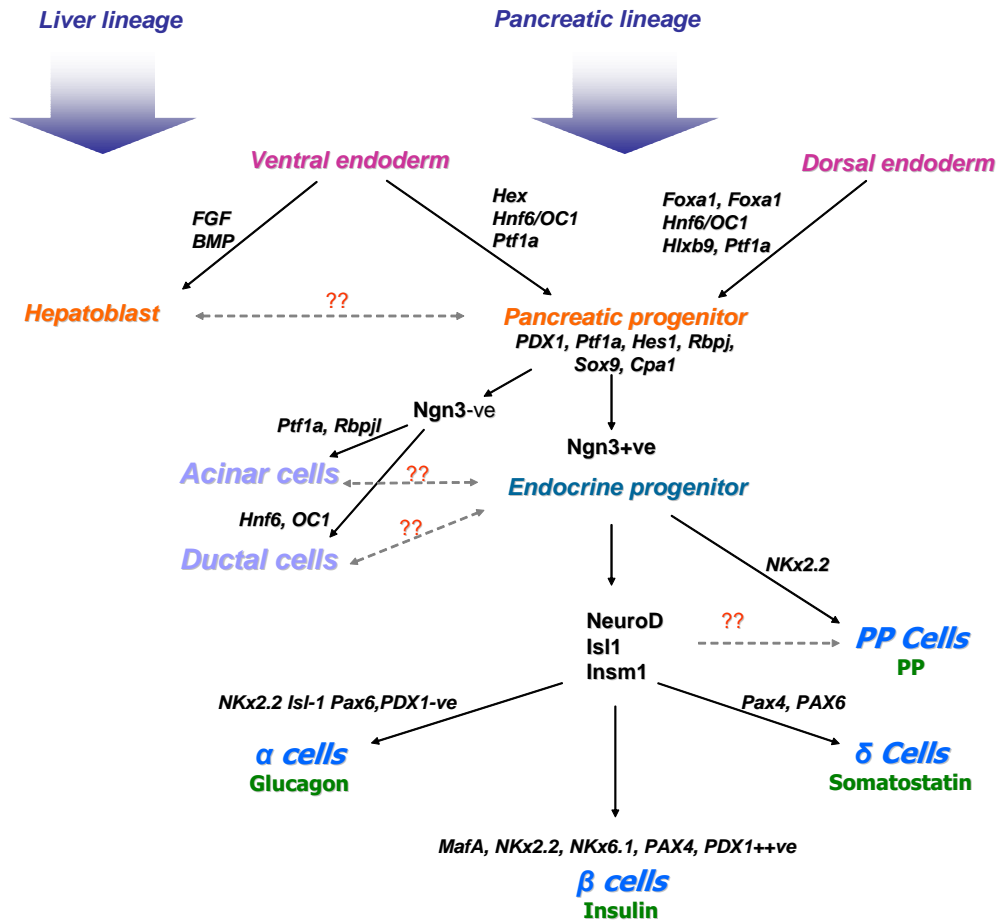


Figure.1.2 Embryonic developmental pathways of the pancreas [(Zaret 2008; Murtaugh 2007; Zaret and Grompe 2008) and others].

Stage 1		Stage 2		Stage 3	Stage 4
Definitive endoderm		Primitive gut tube		Posterior foregut	Pancreatic endoderm and endocrine precursors
ActA + Wnt	ActA	KGF	RA + Cyc + Nog	No factors	No factors
RPMI + 0% FBS	RPMI + 0.2% FBS	RPMI + 2% FBS	DMEM + 1% B27	DMEM + 1% B27	DMEM + 1% B27
1 day	2 days	3 days	3 days		3 days
ES	ME	DE	PG	PF	PE
OCT4 NANOG SOX2 ECAD	BRA FGF4 WNT3 NCAD	SOX17 CER FOXA2 CXCR4	HNF1B HNF4A	PDX1 HNF6 PROX1 SOX9	NKX6-1 PTF1A NGN3 NKX2-2

Figure 1.3 Novocell protocol for β -cell differentiation from human ESCs (Kroon et al. 2008).

recurrent autoimmunity would still have to be addressed (Street et al., 2004). Cell replacement is less complicated with lower risk in comparison to major organ transplant procedures. Adult stem cells can be manipulated and expanded *ex vivo* potentially offering unlimited transplant material and there are fewer ethical issues than in the case of human ESCs. Potential stem cells from different sources have been studied including cord blood (Koblas et al., 2005; Yoshida et al., 2005), haematopoietic cells (Moriscot et al., 2005; Ianus et al., 2003), skin (Guo et al., 2009), liver (Zalzman et al., 2003) and pancreas.

1.2.2.3.2.1 Umbilical cord blood cells

Pessina has demonstrated a sub-population of cord blood stem cells that express important developmental markers of the endocrine pancreas including Ngn3, Pax4 and PDX1 (Pessina et al., 2004). Sun developed a differentiation protocol which enabled cord blood cells to differentiate to a β -cell-like phenotype which is capable of producing insulin and C-peptide (Sun et al., 2007). Yoshida reported *in vivo* differentiation of cord blood derived cells to islet cells when transplanted into NOD mice (Yoshida et al., 2005). Thus cord blood cells remain a promising source for future β -cell replacement therapies.

1.2.2.3.2.2 Haematopoietic cells

These cells are particularly exciting due to their ease of retrieval and proven plasticity. The potential of adult bone marrow cell differentiation to resolve hyperglycaemia has been reported by several groups (Zhao et al., 2008; Tang et al., 2004; Lee et al., 2006b). Couri reported successful complete independence from insulin therapy in 12 out of 22 patients with newly diagnosed diabetes for a period of more than 31 months following transplantation with autologous hematopoietic stem cells without

myeloablation (Couri et al., 2009). However, it is still debated whether this effect is due to mesenchymal stem cell differentiation to insulin producing cells or whether this is a result of providing a regeneration-enhancing environment or niche which promotes endogenous β -cell maintenance / proliferation (Hasegawa et al., 2007; Hess et al., 2003). There are conflicting reports regarding the direct differentiation hypothesis (Kang et al., 2005; Lavazais et al., 2007); with more evidence in support of environmental modulation, possibly through immune-modulation (Kang et al., 2005; Steptoe et al., 2003; Voltarelli et al., 2008; Zorina et al., 2003).

1.2.2.3.2.3 Skin stem cells

Plasticity of skin derived precursors (SKPs) has been reported by several groups (Joannides et al., 2004; Toma et al., 2001). SKPs are an attractive source for cell replacement therapies due to their abundance and ease of access and isolation. Guo reported a protocol that enabled differentiation of SKPs into a β -cell-like phenotype through culture manipulation. Differentiated cells expressed several β -cell developmental and mature genes, however functionality was not fully validated (Guo et al., 2009). Further research is required before SKPs become a realistic potential candidate for cell replacement therapy for diabetes.

1.2.2.3.2.4 Liver stem cells

Multipotency of liver stem cells has been confirmed (Suzuki et al., 2002). Differentiation of murine hepatic oval cells to insulin producing cells with ability to reverse hyperglycaemia in STZ diabetic animal models has been reported (Yang, 2006; Yang et al., 2002). Zalzman attempted to genetically manipulate hepatocytes by PDX1 gene transfer which resulted in a change in phenotype but did not yield true β -cells. However, the manipulated cells enabled restoration of normoglycaemia post-transplantation (Zalzman et al., 2003).

1.2.2.3.2.5 Pancreas stem cells

Presence of true stem cells in the pancreas is contentious. Several groups have reported actively proliferative populations that can be derived from the pancreas (Zhang et al., 2005; Seaberg et al., 2004; Lin et al., 2006; Huang and Tang, 2003; Hao et al., 2006). Ramiya demonstrated generation of islet-like structures derived from pre-diabetic mice through long term culture and manipulation. These islet-like structures were able to reverse insulin-dependent diabetes in NOD mice (Ramiya et al., 2000). Others have reported similar findings in pseudo-islets derived from rat neonatal ductal cells (Ogata et al., 2004). Hao successfully isolated pancreatic epithelial cells with stem/progenitor cell properties from the non-endocrine fraction of the pancreas digestion through selective culture protocols with G418. The resulting cells showed limited insulin secretory capacity *in vitro*. However, further differentiation was observed following sub-kidney capsule transplantation in SCID mice (Hao et al., 2006). More recently, Zhao reported expression of classical stem cell markers in the human pancreas (Zhao et al., 2007). Similar findings were observed in both murine and human pancreases in our group (Holliman, 2008). Isolation and further characterisation together with differentiation potential has yet to be reported.

1.2.2.4 Non-endocrine cell transdifferentiation

Transdifferentiation is a process through which a specific cell changes its phenotype to another one (Tosh and Slack, 2002). During this process, expression of some specific genes is lost and new genes are expressed resulting in deactivation and activation of different pathways giving the cell its new identity.

Ductal cells of the pancreas have been suggested to exhibit the potential to transdifferentiate towards an endocrine and more specifically β -cell-like phenotype. Bonner-Weir et al., have manipulated human duct-rich pancreatic tissue in tissue

culture conditions and generated islet-like structures (Bonner-Weir et al., 2000). Similar findings have been confirmed by other groups with resolution of hyperglycaemia following transplantation in STZ-treated animal models of diabetes (Ogata et al., 2004). In animal models of endocrine damage, Wang showed histological changes in the tail of the pancreas following ligation resulting in conversion of acinar tissue to new ductal complexes and new islet growth (Wang et al., 1995). Highly purified liver oval cells have been shown to transdifferentiate towards insulin producing cells upon culture in a high glucose environment and were able to reverse diabetes in SCID mice (Yang et al., 2002). Hepatocytes have shown ability to express insulin in addition to other β -cell genes upon transduction with PDX-1 or Btc (Kojima et al., 2003).

1.2.2.5 Beta-cell neogenesis / replication

Beta-cell turnover and the source of new β -cells have been, and still are, the subject of considerable controversy. Several animal model studies have shown proliferative activity within islets and this has been linked to pancreas damage (Movassat et al., 1997), autoimmunity (Sherry et al., 2006) or pregnancy (Karnik et al., 2007). Dor demonstrated in a pulse-chase *in vivo* study, in which existing β -cells were labelled and then followed, that new β -cells originated from existing labelled cells and not from other sources, as the number of insulin positive-labelled cells did not dilute over time (Dor et al., 2004). This was further supported by the work of Lee where they demonstrated regeneration of the islets of 50% pancreatectomized mice without activation of NGN3, the key transcription factor in embryonic development of the endocrine compartment of the pancreas (Lee et al., 2006a). Nir further demonstrated in a lineage tracing study, recovery from β -cell damage induced via β -cell specific expression of a tetracycline-dependent diphtheria toxin when tetracycline exposure

was withheld showing strong evidence of recovery through β -cell proliferation (Nir et al., 2007). In autopsy studies on human pancreases carried out by Meier, β -cell replication was concluded to be the primary source of β -cell mass expansion (Meier et al., 2008). Further studies of β -cell replication suggest key roles for CDK4 and D2 in driving the cell to G1/S stages of the cell cycle (Cozar-Castellano et al., 2006). It has also been proposed that both the PI3K-AKT (Fatrai et al., 2006) and the calcineurin/NFAT signalling pathway (Heit et al., 2006) play roles in β -cell proliferation. On the other hand it has been shown by several groups that neogenesis of β -cells does occur with suggestions that new β -cells come from the ductal tree (Bonner-Weir et al., 2004), acinar cells (Minami et al., 2005), spleen (Kodama et al., 2003), biliary duct epithelium (Eberhard et al., 2008) and possible other sources. The strong evidence behind both proliferative and neogenesis suggests possible co-existence, although factors triggering each mechanism are still to be fully revealed and understood (Khalaileh et al., 2008).

1.2.3 Gene therapy

Utilising transfer of exogenous genes to target cells has been proposed as a gene therapy approach to achieve improved glycaemic control. Several genes have been employed in pre-clinical studies including insulin, insulin transcription factors and incretins.

1.2.3.1 Insulin gene therapy

Several groups have studied insulin gene therapy for diabetes. Liver targeted insulin gene therapy has been reviewed by Nett (Nett et al., 2003) and muscle targeted insulin gene therapy has been reviewed by Ratanamart and Shaw (Ratanamart and Shaw, 2006). Within our group, wild type human preproinsulin (hppI1) and a mutated human preproinsulin (hppI4) cDNA were used for these studies. In hppI4,

prohormone convertase 1/3 and 2 cleavage sites were mutated to form tetrabasic consensus furin-recognition cleavage sites and thus enable processing of proinsulin to mature insulin by furin, an enzyme ubiquitously expressed in the trans-Golgi network of all eukaryotic cells. Strategies for restoring basal and controlled expression / release of insulin strategies have been explored. Basal insulin secretion is achieved by transfer of an insulin gene driven by a constitutively expressed CMV promoter. This mimics the background basal insulin secretion from pancreatic β -cells but does not cover the increased insulin requirements following meals and cannot be stopped without removal of the exogenous gene-expressing cells (Shaw *et al.*, 2002; Aldibbiat *et al.*, 2004). Systems with controlled insulin secretion can offer more flexible insulin expression / release to meet changing glycaemic burden. Suggested systems include tetracycline-regulated insulin expression and the RPD aggregation regulated insulin secretion system (Ratanamart and Shaw, 2006).

In the tetracycline regulated system, tetracycline is utilised to induce or repress expression of the insulin transgene with newly synthesised insulin then secreted through the constitutive pathway (Scougall and Shaw, 2003; Wilson *et al.*, 2005).

The RPD aggregation system utilises several copies of a self-dimerizing mutant form of the protein FKBP (FM) fused to the target transgene, forming large aggregates trapping the fusion protein in the endoplasmic reticulum. De-dimerization occurs in the presence of a non-immunosuppressant rapamycin-like ligand (Rivera *et al.*, 2000).

The RPD-regulated insulin expression system consists of furin-cleavable proinsulin-4FM fusion protein. Administration of the specific ligand induces dissociation of the aggregates which results in the fusion protein moving to the trans-Golgi apparatus where furin cleaves proinsulin-4FM fusion protein into 4FM and proinsulin and then proinsulin to mature insulin and C-peptide (Mahmoud, 2010). However, these

systems lack the physiological sensing of glucose and the corresponding finely regulated insulin secretion and thus cannot offer minute-to-minute control of blood glucose.

1.2.3.2 Transcription factor gene therapy

Transcription factor gene transfer is employed to induce a change in the expression pattern of otherwise silent genes in the cellular genome. Beta-cell transcription factor gene transfer to induce insulin gene expression has been used with considerable success (Miyatsuka et al., 2008). Examples include pancreatic duodenal homeobox-1 (PDX1) (Ferber et al., 2000), NeuroD, betacellulin (Btc) (Kojima et al., 2003), MafA (Matsuoka et al., 2007) and paired box protein (Pax-4) (Tang et al., 2004) gene transfer or a cocktail of transcription factors (Kaneto et al., 2005). PDX-1 gene transfer to liver cells resulted in insulin expression in hepatocytes (Ferber et al., 2000); however, this was accompanied by fulminant hepatitis which suggested inappropriate pancreatic exocrine enzyme expression (Kojima et al., 2003). Similar observations were reported but without hepatitis when a NeuroD construct was transferred to hepatocytes of diabetic mice using a helper dependent adenovirus (HDAD) gene transfer method together with betacellulin administration (Kojima et al., 2003).

1.2.3.3 Incretin gene therapy

Incretins have been extensively studied in the course of diabetes and recently GLP1 analogues and DPP-IV inhibitors have been incorporated in the treatment algorithms of T2D (1.1.4.1) and some pre-clinical experimental models of T1D (Dupre et al., 2004; Sheffield et al., 2007). Strategies involving muscle-targeted GLP1 gene therapy are being explored by several groups (Choi et al., 2005; Lee et al., 2008b). Within our group both constitutive and regulated expression and secretion of GLP1 are under

investigation. The former would provide increased background GLP1 availability, while the latter could enable mimicking of physiological post-prandial GLP1 peaks (Mahmoud et al., 2008). This approach is promising especially for the treatment of T2D. However, issues of efficiency, safety and practicality necessitate further studies.

1.3 Pancreatic islet transplantation

1.3.1 What is islet transplantation?

Islet transplantation is a minor surgical procedure performed under local anaesthesia in which purified islets isolated from the pancreas of a deceased donor are infused, most commonly, into the portal vein (Figure 1.4 (Merani and Shapiro, 2006)). Despite comparable need for life-long immunosuppressant therapy to whole organ transplantation, the procedure of islet transplant is associated with considerably less peri-operative morbidity and no transplant-related mortality. It requires much shorter hospital stay in addition to potentially reducing overall allo-antigen load.

1.3.2 History of islet transplantation

Initial trials date back to the 19th century when Dr Watson-Williams and Mr Harsant attempted a xenograft transplantation of 3 small pieces of sheep pancreas in a 15 year old boy with problematic ketoacidosis in Bristol, UK. Despite rejection and failure of the grafts an initial mild symptomatic improvement was noted (Williams, 1894). Allograft pancreatic tissue transplantation attempts were as early 1916 when Mr Fredrick Pybus in Newcastle upon Tyne carried out transplantation of fragments of diseased human pancreas into two patients, one of whom benefited from a transient symptomatic improvement (Pybus, 1924). Discovery of insulin by Banting and Best (Banting and Best, 1990) and the experience of rapid graft failure of transplanted pancreas fragments discouraged further attempts of transplantation for a few decades.

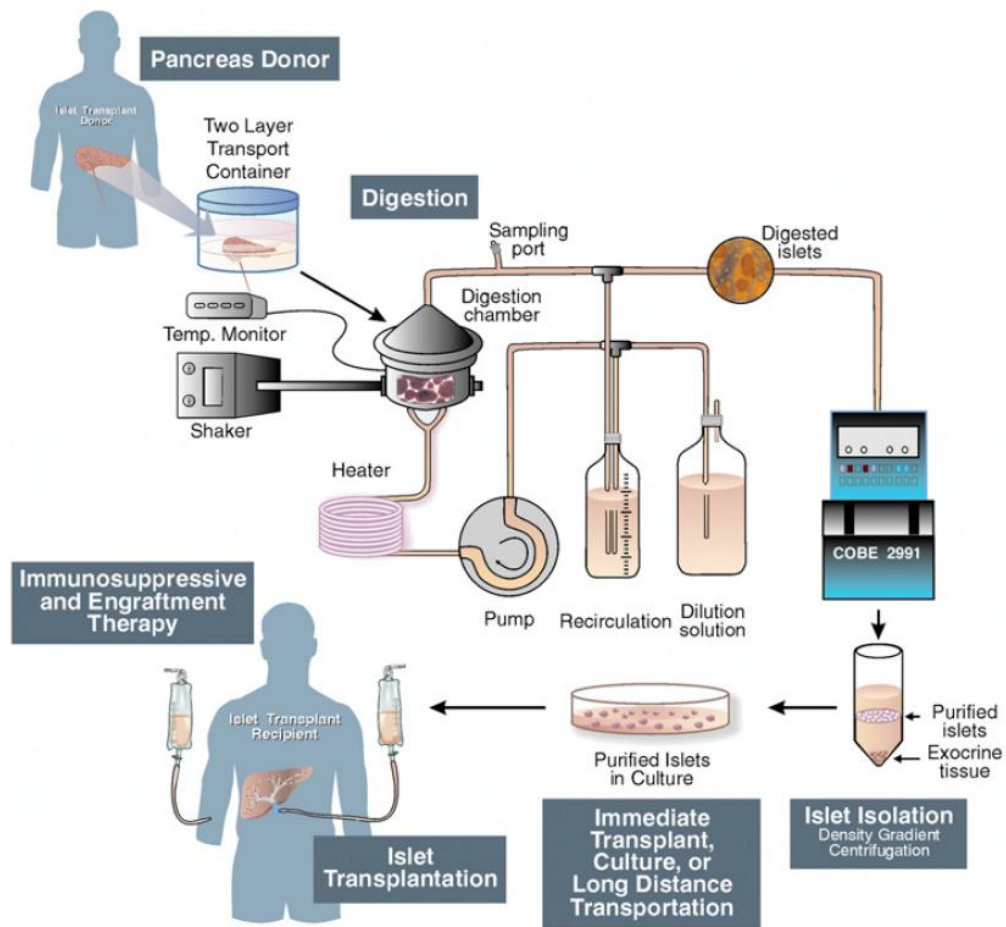


Figure 1.4 Summary of islet isolation and transplantation procedures (Merani and Shapiro 2006).

Later it became evident that life-saving treatment of insulin was not a “cure” for diabetes with numerous diabetes and insulin related complications arising. Renewed interest in endocrine cell-replacement therapy was driven by need for improved treatment / cure for diabetes associated with fewer complications. Moskalewski’s attempts to liberate islets of Langerhans from surrounding tissue by means of enzymatic digestion of the pancreas using collagenase derived from *Clostridium histolyticum* made the potential for large scale production of the islets possible (Moskalewski, 1965). This was further modified by Lacy and his team by distending the pancreas with balanced salt solutions injected via the common duct which enabled greater islet yields. They also utilised a sucrose density gradient for islet purification (Lacy and Kostianovsky, 1967). This provides important proof of concept which enabled production of meaningful numbers of islets but the osmotic damage to islets caused by the sucrose density gradient was not acceptable. This problem was later resolved by the introduction of Ficoll, sucrose high molecular weight polymers (Lindall et al., 1969; Scharp et al., 1973). These advances enabled successful islet transplantation in rodent models (Reckard et al., 1973) but were still not efficient enough for human pancreases due to its overall size and different content of fibrous tissue. Successful consistent digestion of large animal pancreas including human was achieved following introduction of a series of improvements to the isolation method including intra-ductal collagenase solution delivery either via direct injection (Gray et al., 1984) or continuous perfusion (Rajotte and Thomson, 1987), more efficient temperature and pressure control during the digestion process (Warnock et al., 1988), invention of the semi-automated dissociation chamber by Ricordi (Ricordi et al., 1988), utilisation of the COBE 2991 for high throughput optimal purification of pancreatic digestion products (Lake et al., 1989), and introduction of purified blends

of collagenase enzyme with more defined composition of collagenase I and II and neutral proteases in addition to depletion of endotoxin contamination (Gill et al., 1995; Lakey et al., 1998; Linetsky et al., 1995; Linetsky et al., 1997).

Clinical transplantation of human islets was attempted by several groups with varying but progressive success. One of the first series of transplantations was reported by Najarian, which showed some success limited to transient improved glycaemic control but never insulin independence (Najarian et al., 1977). The first reported insulin independence following islet transplantation was in 1978 following a single donor un-purified islet transplantation infused in the spleen of the recipient with simultaneous kidney transplantation (Largiader et al., 1980). However unacceptable risk of complications related to intra-splenic transplantation including rupture and infarction (Gores et al., 1994; White et al., 2000) made spleen an unfavourable site for transplantation (Shapiro, 2007). Scharp reported first successful islet transplantation that led to insulin independence via portal vein islet infusion in a patient with stable renal transplant achieving insulin independence (Scharp et al., 1990). Over the following years significant progress was attained in both islet isolation techniques, yielding higher counts and purer and more viable islets, and in islet transplantation protocols benefitting from collective and pioneering experience in both autograft (Pyzdrowski et al., 1992) and allograft (Brendel et al., 1998) transplantations culminating in the seminal publication by the Edmonton group reporting insulin independence in a series of 7 allotransplant recipients when adequate islet mass was infused and a glucocorticoid-free immunosuppression was used (Shapiro et al., 2000).

1.3.3 Pancreas procurement criteria

The criteria for accepting candidate donor pancreases for islet transplantation have been developed and refined over several years to ensure safety and success, with

further refinements being introduced as the field continues to progress. Exclusion criteria for organs include: age extremes (>65 years, <20 years), donor diabetes, confirmed pancreatitis (amylase or lipase >5 times reference), positive serology for hepatitis B, hepatitis C, HIV, treponema pallidum, high risk social history (intravenous drug abuse, non-professional tattoo), and prolonged cold ischaemic time (CIT). The quality of the pancreas contributes greatly to the quality of the isolated islets and graft function post-transplantation. O'Gorman devised a 'donor variables assessment form' based on extensive analysis of the Edmonton Clinical Islet Transplant Program data (O'Gorman et al., 2005) (Figure 1.5). The form gives donor points (DP) to each donor variable including age, CIT, BMI, cause of death, hospital stay, evidence of pancreatitis, use of vasopressors / inotropes, evidence of hyperglycaemia, and experience of retrieval team in addition to other factors summarised in Figure 1.5.

1.3.4 Pancreas preservation and transport

Following surgical retrieval, the pancreas undergoes gradual hypoxia and depletion of energy stores resulting in progressive damage. Excitement over possible organ resuscitation and preservation during transport using the two layer method (TLM) was clearly shown in several publications (Hiraoka et al., 2001; Kuroda et al., 1996; Tsujimura et al., 2004; Zhang et al., 2006). The two layer method consists of a lower layer of oxygen-charged perfluorodecalin, a member of the perfluorocarbon (PFC) family of reagents known for their high affinity for oxygen; and an upper layer of University of Wisconsin solution (UWS) - a system that provides both oxygen and nutrients. Tsujimura reported significant improvement in the metabolic state of the pancreas with increased levels of adenosine triphosphate (ATP) and decreased

Donor Information						
Age (yrs old)	Allocated Points	< 25	25 – 35	35 – 55	55 - 65	> 65
	20	5	10	20	10	5
CIT (hrs)	Allocated Points	0 – 3	3 – 8	8 – 12	> 12	
	15	10	15	7.5	4	
BMI	Allocated Points	< 20	20 – 24.9	25 – 30	30 - 40	> 40
	11	2	7	11	5	0
Cause of Death	Allocated Points	Suicide	Anoxia	Traumatic with Injury	Traumatic Without Injury	Cerebral Vascular
	18	0	3	8	14	18
Hospital Stay (days)	Allocated Points	0 – 4	4 - 7	7 – 14	> 14	
	5	5	3	2	0	
Amylase / Lipase (U/L)	Allocated Points	Normal	1.5 – 2x Normal	2 – 5x Normal	> 5x Normal	
	5	5	2	1		
Vasopressors	Allocated Points	Low	Moderate	High		
	4	4	2	0		
Blood Glucose	Allocated Points	Normal	High	Normal Treated	High Treated	
	4	4	2	3	1	
Procuring Team	Allocated Points	Edmonton	Distant			
	9	9	2			
Social History	Allocated Points	Drug Abuse	Promiscuous Behavior	Jail Time	Other	
	4	-1	-1	-1	-1	
Medical History	Allocated Points	Hypertension	Alcohol Abuse	Arrests > 5 min	Transfusions	Other
	5	-2	3	-5	-1	-1
Subtotal A						/ 100

Pancreas Physical Properties						
Size	Allocated Points	Very Small	Small	Average	Large	Very Large
	-3	-3	-2	0	0	-1
Quality of Flush	Allocated Points	Very Poor	Poor	Adequate	Good	Excellent
	-3	-3	-2	-1	0	0
Pancreas Consistency	Allocated Points	Very Soft	Soft	Average	Firm	Very Firm
	-5	-5	-4	0	-1	-3
Quality of Procurement	Allocated Points	Very Poor	Poor	Adequate	Good	Excellent
	-3	-3	-2	-1	0	0
Damage	Allocated Points	None	Acceptable	Unacceptable		
	-3	0	-1	-2		
Quality of Packaging	Allocated Points	Very Poor	Poor	Adequate	Good	Excellent
	-3	-3	-2	-1	0	0
Fat Content	Allocated Points	Very Lean	Lean	Average	Fatty	Very Fatty
	-5	-5	-3	0	0	-2
Subtotal B						
Subtotal A _____						
Subtotal B _____						
Sum of Subtotals _____						

Figure 1.5 Donor variables assessment form formulated by O’Gorman (O’Gorman 2005).

malondialdehyde (MDA), a marker of oxidative stress, when resuscitated in TLM in comparison to UWS alone following prolonged CIT (>11 hours). Moreover, this resulted in improved islet recovery and *in vitro* function (Tsujiura et al., 2004). However, recent reports have questioned the true benefit of the TLM, demonstrating that the benefit of oxygenation is limited to the 1 millimetre of tissue at the organ surface with no detectable enhanced oxygen partial pressure at 1 cm depth (Papadimitriou et al., 2005). Kin from the Edmonton Clinical Transplantation Program reported no evidence of benefit of TLM over UWS alone preservation protocols on islet transplant outcome following careful analysis of the centre's database (Kin et al., 2006). Scott reported significant improvement in pancreas quality with the oxygen persufflation storage method compared to TLM confirmed by increased energy stores as were evaluated using ³¹P-Nuclear Magnetic Resonance (NMR) spectroscopy (Scott, III et al., 2010).

1.3.5 Islet isolation

This is performed through enzymatic digestion of the pancreas employing the enzyme collagenase (Johnson et al., 1996). Lot to lot enzyme activity variation, excessive endotoxin contamination (Vargas et al., 1998) and instability often resulted in unsuccessful pancreas digestion and organ loss. Improved understanding of collagen content in the pancreas (Hughes et al., 2005) and development of purified collagenase blends such as Liberase HI from Roche and Collagenase NB from Serva, in which known concentrations of collagenase I and II are optimised for pancreas digestion and islet release in addition to reduced levels of endotoxin, resulted in significant improvement in islet yield, viability and post-transplant function (Brandhorst et al., 2005; Linetsky et al., 1997).

The isolation procedure starts with organ cleaning and careful removal of the attached C-loop of the duodenum, remaining spleen portion and surrounding fatty tissue. This is followed by decontamination and then cannulation of the common pancreatic duct to enable infusion of collagenase dissolved in Hanks balanced salt solution (HBSS) which results in pancreas distension. Following distension, the pancreas is sliced into small pieces and placed inside a Ricordi chamber where digestion takes place (Ricordi et al., 1988). The Ricordi chamber is connected to a heating loop to enable control of the digestion temperature at 37 °C. Motion within the digestion set is controlled by a peristaltic pump. Digestion is monitored by dithizone (DTZ) staining every 5 minutes to avoid over-digestion and islet fragmentation. Once satisfactory islet separation from surrounding tissue is achieved the digestion reaction is stopped with the addition of human serum albumin and reducing temperature to 4 °C through dilution with ice cold medium (Mirbolooki et al., 2007). Digestion products are concentrated through centrifugation and the islet / non-endocrine mixture is then separated using a continuous density gradient employing Ficoll in a COBE 2991 cell processing centrifuge (Huang et al., 2004; Lake et al., 1989). This enables fractionation of the digestion products into islet-rich fractions and islet-depleted fractions. Islet rich fractions are then further assessed for sterility, viability, and function (Merani and Shapiro, 2006; Yamamoto et al., 2009).

1.3.6 Islet quality assessment

This is carried out to confirm safety and suitability of isolated islets for clinical transplantation.

1.3.6.1 Purity, integrity and islet equivalent count

Purity of islets is evaluated following staining with the zinc indicator, dithizone (DTZ), which labels insulin-containing cells red. Non-islet tissue does not take-up the

stain. Estimation of islet purity is important as preparations of purity lower than 50% are not usually accepted for transplantation. Lower purities can contribute to increased tissue loading and thus increased risk of portal venous pressure elevation. Moreover; this may promote the inflammatory response leading to deleterious effects on the graft in addition to possibly increasing graft immunogenicity (Inoue and Miyamoto, 2000; Secchi et al., 1997).

Due to the large variability in the size of purified islets (diameters can vary between 50 and 500 microns), a method of counting has been devised that uses the concept of an 'islet equivalent (IEQ)' rather than simply total islet numbers. An IEQ is a standard islet measuring 150 microns in diameter, and the counting method adjusts islet counts against this standard. Islets of different sizes are multiplied by a specific correction factor. In this method large islets contribute significantly to the overall IEQ count although controversy surrounds the destiny of these large islets. Due to the disruption of the microvasculature of islets during isolation, cells in the inner core of big islets have less access to nutrients and oxygen compared to cells located on or close to the surface. This potentially increases risk of necrosis leading to whole islet fragmentation. Recently reported insulin release capacity from IEQs of different islet sizes demonstrated higher insulin release from the same IEQs comprising smaller islets compared to IEQs comprising bigger islets (Fujita et al., 2011).

1.3.6.2 Assessment of viability

Following islet isolation and purification, assessment of islets is undertaken to decide on their suitability for clinical transplantation. The commonly used technique for viability assessment employs a viability stain: fluorescein diacetate (FDA), and necrosis stain: propidium iodide (PI) or Ethidium bromide (EtBr) (Gray and Morris, 1987; London et al., 1990). FDA is a non-fluorescent cell-permeant molecule which is

actively converted in living cells into fluorescein, a UV-excitabile green fluorescent dye. PI is a non-cell permeant nucleic acid dye which enters nuclei of cells with compromised membrane (necrotic cells) and fluoresces red under UV light excitation. Due to perceived variability of results and lack of reproducibility by some groups, other methods have been evaluated including SYTO-13 / Ethidium bromide and Calcein AM / Ethidium Bromide in comparison with the FDA / PI method with varying results (Barnett et al., 2004). Ichii developed a comprehensive but resource demanding method for assessment of islet graft viability. This involved islet digestion into single cells employing accutase followed by analysis of the single cell suspension using fluorescent activated cell sorting (FACS) studying mitochondrial membrane activity employing tetramethylrhodamine, ethyl ester TMRE (Scaduto, Jr. and Grotyohann, 1999) which binds to the mitochondrial membrane of viable and metabolically active cells. Newport green (NG), a fluorescent zinc indicator, is used to differentiate β -cells from other cell types in the preparation. To study other islet markers, cells in suspension are transferred onto microscope slides and stained with different islet markers enabling assessment of cell composition of the islets. Ichii reported that conventional viability assessment methods are not accurate and do not correlate with *in vivo* islet assessment when transplanted in diabetic SCID mice (Ichii et al., 2005). However; correlation with clinical outcome remains to be confirmed with this novel assay. Another proposed method for estimating islet cell viability is measurement of ATP/ADP ratio which has been shown to correlate with murine *in vivo* transplants (Goto et al., 2006). Others have suggested assessment of ATP/DNA ratio instead of ADP/ATP ratio to compensate for dead cells in the culture which are otherwise missed with the ADP/ATP ratio (Suszynski et al., 2008).

1.3.6.3 Assessment of islet function

Standard *in vitro* techniques adopted by most centres worldwide to assess islet function focus on insulin secretion in response to glucose challenges (Wiesli et al., 2004) either in static incubations or in dynamic perfusion experiments. However this has been limited in enabling any firm prediction of graft function post-transplantation, since secretory defects incurred during and after the traumatic isolation process might recover following transplantation (Bertuzzi and Ricordi, 2007). Correction of STZ-induced hyperglycaemia in the severe combined immuno-deficient (SCID) mouse model following sub-renal capsule transplantation of islets remains the most reliable bioassay correlating with clinical transplant outcome (Zhao et al., 2002). However, this *in vivo* bioassay cannot practically be completed before clinical transplantation. In addition; it requires a significant number of already limited islets. Better *in vitro* and relatively quick assessment methods that can safely predict engraftment and function post-transplantation are thus needed.

1.3.7 Islet recipient selection

Despite being a relatively minor intervention, islet transplantation is associated with a range of possible side effects related directly to the procedure (e.g. risk of bleeding, portal vein thrombosis, raised portal pressure) (Owen, 2007) or to the immunosuppressant therapy (e.g. mouth ulceration, blood pressure disturbances, dyslipidaemia, infection and tumorigenicity) (Faradji et al., 2007). Within the current setting, it is necessary to carefully weigh risk-benefit in each individual candidate. The primary current indication for islet transplantation is unstable glycaemic control with recurrent disabling severe hypoglycaemia (Senior, 2007). Definition of impaired awareness of hypoglycaemia and severe hypoglycaemia can vary between centres and therefore several scoring systems have been proposed for objective evaluation

including Clarke score (Clarke et al., 1995), Gold score (Gold et al., 1997) and Ryan score (Ryan et al., 2004). Similarly, objective assessment of glycaemic lability is currently being considered utilising several tools including M-value (Schlichtkrull et al., 1965), mean amplitude of glycaemic excursion (MAGE) (Service et al., 1970) and lability index (LI) (Ryan et al., 2004). Exclusion criteria for islet transplantation include uncontrolled diabetes with HbA1C >10%; active proliferative retinopathy; obesity and evidence of insulin resistance; active cancer; uncontrolled elevated blood pressure; impaired renal function; hepatic disorders e.g. portal hypertension, haemangioma; advanced cardiac disease and in most current cases or future pregnancy intentions (Merani and Shapiro, 2006; Senior, 2007).

1.3.8 Islet release criteria

Quality standards for designating suitability of islets for clinical transplantation have been adopted following several years of experimentation. Despite controversy over classical assessment methods and their correlation with clinical outcome (Eckhard et al., 2004), current most consistently agreed minimal islet release criteria for transplantable graft include: negative Gram stain; viability $\geq 70\%$ as per FDA/PI or FDA/EB staining assessment; purity $\geq 50\%$ with a packed cell volume ≤ 10 ml; yield ≥ 4000 IEQs/Kg of recipient weight; and an endotoxin level < 5 units/Kg of recipient weight (Ichii et al., 2007b), although local release criteria can vary between different centres.

1.3.9 Islet transplantation procedure

Islets are packaged in a blood transfusion bag in transplant medium and sent to the theatre where the transplantation procedure is carried out. Islets are infused into the portal vein following ultrasound scan and X-ray guided cannulation under local anaesthesia with regular portal pressure monitoring (Owen, 2007). This procedure is

carried out by a skilled and experienced interventional radiologist. The procedure is usually terminated in the event of uncontrolled bleeding or portal pressure elevation. Despite trials of transplantation in other sites including spleen (Largiader et al., 1980), sub-renal capsule (Jindal et al., 1998), intramuscular (Rafael et al., 2008), and intra-thymic (Arias-Diaz et al., 1996); portal infusion remains the preferred implantation site by the majority of centres. Other potential sites have been explored by many groups in animal models including subcutaneous implantation (Juang et al., 2005), intra-pancreatic (Stagner et al., 2007), and intra-peritoneal (Fritschy et al., 1991) in addition to trials on both micro-encapsulation (Teramura et al., 2007) and macro-encapsulation (Pileggi et al., 2006).

1.3.10 Islet culture

Islet culture is becoming standard practice in many centres offering several advantages including islet recovery following a relatively traumatic isolation process, removal of apoptotic cells from the graft in addition to reserving a precious gap of time for recipient call-in, tissue typing and optimal immunosuppression induction / diabetes stabilisation (Ichii et al., 2007a). Islets are usually cultured in serum-free CMRL based medium with addition of nicotinamide and vitamin E (Ichii et al., 2007a).

1.3.11 Islet transport

Islet isolation facilities are sophisticated laboratories with very high level sterility requirements and are very expensive to establish. They require highly skilled and well-trained staff with 24 hour availability (Johnson, 2007). Isolation of quality islets is usually only achieved after several years of practice (Ichii et al., 2007b). On the other hand, islet transplantation can be carried out in standard interventional radiology theatres. Transportation of isolated islets enables establishment of an islet

transplantation program at several centres utilising a central islet isolation facility (Johnson, 2007; Merani and Shapiro, 2006). Islet transportation has been attempted in several centres including the Groupe Rhin-Rhone-Alpes-Geneve pour la Transplantation d'Ilots de Langerhans (GRAGIL) network (Kempf et al., 2005) the NORDIC network (Rydgard et al., 2001) and the Miami-Houston collaboration (Ichii et al., 2007b) following different methods.

1.3.12 Immunosuppressive therapy for islet transplantation

The Edmonton protocol revolutionised islet transplantation through introduction of a steroid-free immunosuppression regimen consisting of induction with dacluzimab (an anti-CD25 mAb) followed by maintenance with tacrolimus and sirolimus (Shapiro et al., 2000). There have been several modifications and alterations to the Edmonton protocol since it was published. Alternative induction methods have been attempted including addition of anti-thymocyte globulin (ATG) which depletes T-cells to dacluzimab or use of ATG alone especially in recipients with high percentage panel reactive antibody or who are at high risk of sensitization (CITR, 2008; Faradji et al., 2007; Merani and Shapiro, 2006). T-cell depleting induction therapy with Alemtuzumab (Campath- 1H), an anti-CD52 antibody and hOKT3 γ 1 (Ala-Ala) has also been used in several centres with encouraging results (CITR, 2008; Hering et al., 2004). Variations of maintenance immunosuppression protocols include sirolimus, tacrolimus, mycophenolate mofetil (MMF) and cyclosporine; singly or in different combinations with the most recent widely adopted protocol comprising tacrolimus and MMF. Side-effects and long term complications of immunosuppressants remain a considerable negative aspect of all transplants including islet transplantation. Side effects related to sirolimus and tacrolimus include mouth ulceration, nausea, diarrhoea and constipation, fatigue, anaemia, neutropenia, hypertension, dyslipidaemia, risk of

infections and possible tumour formation with higher doses of tacrolimus increasing potential risk of nephrotoxicity as described in other organ transplants (Faradji et al., 2007).

1.3.13 Persistent limitations and unmet needs

Despite all the concerted efforts and advances to improve islet survival post-transplant, it has not yet proved possible to prevent the inexorable loss of graft function with until recently only 10% of islet allo-transplant recipients maintaining insulin independence at 5 years post-transplant (Ryan et al., 2005). Several reasons have been implicated including:

- Possible detrimental effects of collagenase used for isolation on islet integrity, survival and function (Balamurugan et al., 2005; Cross et al., 2008).
- Instant blood mediated inflammatory reaction (IBMIR) which results in significant loss of islet graft mass within a very short period of time post-transplant (Bennet *et al.*, 2000; Nilsson *et al.*, 2011).
- Low oxygen tension in the portal vein which impairs islet function (Galindo, 1965).
- Imperfect immunosuppression protocols which fail to prevent long term gradual rejection of the islet graft in addition to potential toxic effects of many immunosuppression agents on islet cells, especially calcineurin inhibitors and corticosteroids, taking into account the higher intraportal drug levels compared to systemic levels post enteral intake (Bell et al., 2003; Faradji et al., 2007; Heisel et al., 2004; Kaestner, 2007).
- Impaired islet cell regeneration (Berney and Secchi, 2009; Kaestner, 2007).
- Reactivation of the auto-immune process against transplanted β -cells (Harlan et al., 2009).

Most of the above issues are still difficult to address and require further research.

1.3.13.1 Need for improved islet transplant outcome

Improving islet transplant outcome is dependent on many factors including improvement in the islet isolation process to enhance pancreas digestion without detrimental effects on isolated islets in addition to improving the purification process to recover most of the endocrine compartment of the digested pancreas. However, the isolation process is necessarily traumatic in nature and optimised recovery culture might be particularly worthy of further study and optimisation to improve islet activity and survival post-transplant.

1.3.13.2 Need for sufficient suitable tissue for beta-cell replacement

Limited availability of suitable donor tissue is a big hurdle facing primary β -cell replacement therapy. Islet recipients usually require more than one donor for successful transplantation with sustained function and the available suitable tissue is sufficient for less than 1% of potential islet recipients. Production of β -like cells from alternative sources such as adult or embryonic stem cells may offer a solution.

1.4 Aims and objectives

The overall aim of my PhD project was to investigate possible strategies to address geographical and overall shortage of suitable islet tissue for clinical transplantation.

Specific objectives were:

- To establish and validate feasible protocols for safe and practical transportation of clinical grade human islets for transplantation at a remote transplantation centre within the United Kingdom setting.
- To investigate the potential for enhancement of mass and function of transported human islets *in vitro*.

- To establish and maintain proliferative adherent cultures from human islets over several passages with characterisation of the evolving phenotype and to identify stem / progenitor cell-associated markers in the proliferating culture.
- To form pseudo-islets from proliferating cells established from primary human islets and to evaluate β -cell functional phenotype in comparison to primary human islets.

Chapter 2. Materials and Methods

2.1 Ethical approval and informed consent

Ethical approval for this work was obtained from both national (Multi-centre Research Ethics Committee (MREC)) and local (Newcastle Hospitals Trust Ethics Committee) authorities. Informed consent was obtained in all cases from donor relatives by transplant co-ordinators before pancreas was retrieved for research by the surgical team.

2.2 Collaborations and shared experience

During this project several academic links were established following personal visits to other laboratories which enabled training, transfer of knowledge, protocols and experience in addition to collaborative research. These included Dr GC Huang at King's College Hospital, London who carried out islet isolation and shared assessment techniques with our group in Newcastle; Prof PM Jones and Prof SJ Persaud at King's College London who shared their islet perfusion techniques; Prof C Ricordi and Dr H Ichii from Miami Diabetes Institute who shared their Standard Operating Procedures (SOPs) for assessment of islet quality and for islet transport; Dr P Senior, Prof AMJ Shapiro and Dr T Kin from Edmonton Diabetes Research Institute who shared their SOPs for assessment of islets and for clinical transplantation; Prof GS Korbitt from Edmonton Diabetes Research Institute for sharing his group's *in vivo* transplantation techniques and equipment; and Dr M Ungrin from University of Toronto with whom a collaboration was established to evaluate effect of his novel micro-wells on pseudo-islet formation from survivor cells derived from human islets.

2.3 Materials and reagents for pancreas transport

A Nalgene plastic container with the following specifications: volume: 1 L; opening diameter: 117 mm; height: 145 mm (Fisher Scientific Ltd, Leicestershire, UK. cat. no. JAR-660-110F) was adopted as the standard pancreas transport chamber. Perfluorodecalin is a perfluorocarbon (PFC) (F2 Chemicals, Lancashire, UK), an inert fluorinated carbon liquid with very low toxicity, lack of biological activity, short bioavailability and high ability to dissolve and retain oxygen for a relatively long period of time at low temperatures. Oxygenated PFC was used in selected transported pancreases to enrich the transport solution with oxygen. Filtering units with 0.2 µm polyethersulfone (PES) membrane (Helena Biosciences, Gateshead, UK. TPP: cat. no. 99500T) were used to filter-sterilise PFC. University of Wisconsin Solution (UWS) (ViaSpan, DuPont Pharma Ltd, Herts, UK) was provided by the retrieving surgical team. In the initial 5 runs temperature was monitored using a minimum-maximum temperature thermometer (VWR International Ltd, Leicestershire, UK. Cat. No. 620-2042). For all subsequent runs a Hobo pendant data-logger (Tempcon Instrumentation Ltd, West Sussex, UK) was used for continuous temperature monitoring during transport of both pancreas and islets. The data-logger was launched and recorded data were retrieved and managed using HOBOWare Lite 2.7.1 software (Onset, Pocasset, MA, USA). However, in the clinical programme this was changed to Hanna data-logger (VWR International Ltd, Leicestershire, UK. Cat. No. HANNHI140A) due to substantially longer battery life, estimated to last average of 3 years. An appropriate cool-box with secure-closure mechanism (MGD cooler, Staffordshire, UK) was sourced and used for safe and secure transport to and from the islet isolation facility.

2.4 Validation of temperature control

To validate ability of the transport box to maintain temperature and prevent fluctuations during transport, two cold packs (VWR International Ltd, Leicestershire, UK. Cat. No. 216-0192) chilled in the fridge to 4 °C were placed inside the box. The Hobo data-logger was placed inside the tray unit provided inside the box, which was closed and placed in the general laboratory (temperature 22±3 °C) for 5 hours.

2.5 Transport chamber preparation

Transport chambers were autoclaved at 126 °C for 30 minutes. Perfluorodecalin was filter-sterilized beforehand and was oxygenated in the surgical theatre inside the oxygenation kit using medical grade oxygen for 30-45 minutes at room temperature during the pancreas retrieval procedure. Oxygenated PFC was then poured inside the transport chamber and kept on ice until the pancreas was retrieved and ready for packaging.

2.5.1 Oxygenation chamber set up

This was designed as part of this PhD project to enable sterile, safe, flexible and simple oxygenation utility of PFC at the site of pancreas retrieval. This ensures maximum oxygenation of PFC before use and avoids need for recurrent batch oxygenation of PFC requiring storage at 4 °C. The oxygenation chamber was constructed from autoclavable material to enable sterilisation after each use. It consisted of a 2 L polypropylene nalgene bottle with a diameter of 119 mm and height of 260 mm (VWR International Ltd, Leicestershire, UK. Cat. No. 215-7712) capped with a filling/venting cap (VWR, Cat. No. 216-8911). The inlet was provided with a 0.2 µm filter unit (VWR, Cat. No. 514-4108) connected with autoclavable silicon

tubing (VWR, Cat. No. 228-0717). A schematic diagram of the unit is provided below (Fig 2.1). The unit was autoclaved at 120 °C for 10 minutes after each use.

2.5.2 PFC oxygenation

PFC was filter sterilised using 0.2 µm filtering units (2.2) and was stored in 300 ml aliquots in the dark at room temperature. When needed, PFC was transferred to the retrieval site and was oxygenated in the oxygenation kit using the medical oxygen supply at the hospital for 30-45 minutes at room temperature. Naked flames were avoided in the vicinity of the oxygenation chamber according to local hospital safety guidelines to prevent fire hazard.

2.6 Pancreas procurement

Informed consent was obtained from relatives of the potential donors (Table 2.1) by the transplant coordinator on call. Optimal retrieval of the pancreas by the transplant team was carried out using the same methodology adopted for retrievals for whole vascularised pancreas transplantation. The following method was kindly provided by Mr MS Reddy from the Freeman Hospital surgical retrieval team at Newcastle upon Tyne as the standard protocol for pancreas retrieval based on local and international (Brockmann et al., 2006) experiences. Briefly, the duodenal lumen was filled with a mixture of 50 ml of 10% betadine and 30 ml of 100,000 unit/ml nystatin (provided by the hospital pharmacy) through the NG tube to maintain sterility. Midline vertical incision from sternal notch to symphysis pubis was performed followed by exploratory laparotomy to rule out intra-abdominal malignancy. Gastro-hepatic omentum was dissected and common hepatic artery identified. The gastroduodenal artery was ligated distal to its origin from the common hepatic artery. Splenic artery was dissected from its origin at the coeliac axis. Duodenum and head of pancreas

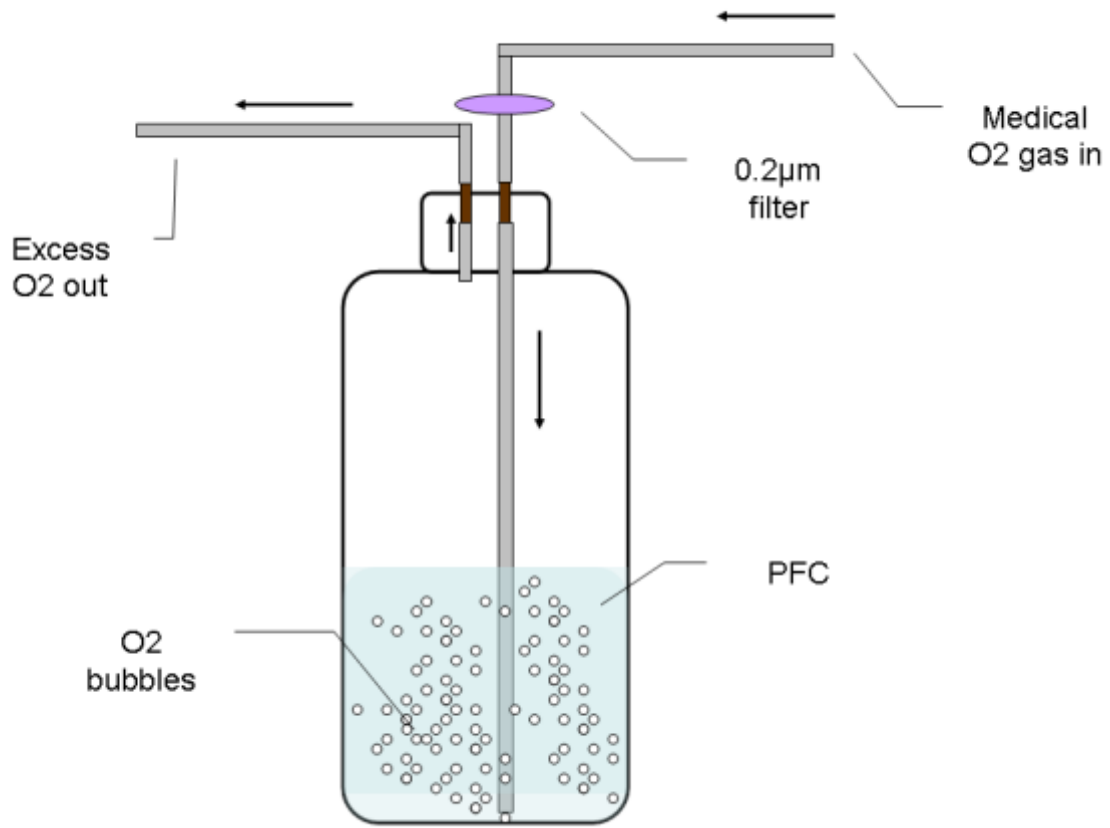


Figure 2.1 Oxygenation chamber set-up schematic diagram. Oxygenation was carried out in the surgical theatre while retrieval was being carried out. Care was taken to exclude any flame sources from the area as per local hospitals' guidelines to prevent fire.

Criteria	Accepted value
Age	20-60 years
BMI	Any
CMV	-ve and +ve
Hep B	-ve
Hep C	-ve
VRDL	-ve
HIV	-ve
Creatinine	<150 µmol/l
Consent	Confirmed
History of pancreatitis	-ve
History of drug abuse	-ve
History of malignancy	-ve

Table 2.1 Pancreas donor criteria. Fulfilment of these points is a prerequisite for accepting the organ into the islet program.

were kocherised to expose the ampulla of the common bile duct and gastrocolic omentum was divided to open the lesser sac. Transverse colon and the hepatic / splenic flexures were mobilised. The gastrosplenic ligament was divided by carefully ligating the short gastric branches. Spleen was mobilised and used as a handle to mobilise the pancreas from the retroperitoneum medially to the left margin of the aorta. The superior mesenteric artery (SMA) was identified at its origin from the abdominal aorta, dissected and looped. Once the aorta was cross-clamped and cold aortic perfusion with UW solution (ViaSpan, DuPont Pharma Ltd, Herts, UK) commenced, the abdominal cavity was packed with ice slush. Ice was also packed in the lesser sac and behind the mobilised pancreas for uniform cooling thus preventing any warm ischemic time. The liver was retrieved first, followed by the pancreas. The duodenum was stapled and divided at D3/D4 junction. The proximal duodenum was stapled and cut at the pyloro-duodenal junction. The root of the mesentery was stapled and cut. Dissection around the head region was completed and the pancreas removed. The organ was then placed in crushed ice in a wash tub and washed with UWS to remove blood traces and possibly remove any surplus tissue still attached to the pancreas. When ready the pancreas was placed inside the transport chamber floating on 250 ml oxygenated PFC or partially immersed in the PFC under a retention frame and then the chamber was filled with 300 ml UWS (Fig. 2.2 A). Alternatively 600-650 ml UWS only was used in later studies. The chamber was closed and double wrapped with sterile plastic bags and then placed in the transport box surrounded with ice. The Hobo pendant data-logger was placed in the transport box beside the transport chamber. The box was closed and secured using the self embedded locking device.

2.7 Pancreas transport

The packaged pancreas secured inside the transport box was collected by the driver of Lifeline, a specialised private organ transport courier (Lifeline, Blyth, Northumberland, UK). The transport box was delivered to the islet isolation facility at King's College Hospital, Denmark Hill, London. This courier uses dedicated organ transport vans to deliver the organ using the motorway. Emergency blue lights were used only occasionally where delay may otherwise have led to unacceptable cold ischaemic time (Fig. 2.2 B).

2.8 Islet isolation

Islet isolation was performed by Dr. Guo Cai Huang and Dr. Min Zhao at the cGMP grade cell processing facility of King's College Hospital (Fig. 2.2 C). The isolation protocol is summarised in brief as follows: when the transport chamber was transferred into the clean room through the room hatch, the pancreas was removed and placed on a cooled preparation tray inside a class II cabinet where it was cleaned from the surrounding fatty tissue and the attached duodenal C-loop and spleen portion were removed. The pancreas was then decontaminated in decontamination solution (Decom. HBSS, cat. No.: BESP250L150, BioWhittaker Europe). The common pancreatic duct was identified and working strength Liberase HI (Roche Diagnostics, East Sussex, UK) (used in the isolations in the pre-clinical programs) or Collagenase NB 6 GMP Grade (SERVA Electrophoresis GmbH, Heidelberg, Germany) (used in all isolations in the clinical program) was injected through the duct using a syringe with blunted needle to achieve organ distension. The switch from Liberase HI to Serva NB 6 was a global move in early 2007 for all clinical preparations, after



Figure 2.2 Pancreas transport system. A: transport chamber showing the two layer method: lower PFC / upper UWS and the pancreas in the middle. A retention frame helped maintain the pancreas at the PFC / UWS interface. B: Lifeline organ transport vehicle. C: cGMP accredited Islet isolation facility at King's College Hospital. D: cGMP accredited stem and cell processing unit at Newcastle University.



Figure 2.3 Ricordi Chamber. Pancreas pieces are inside the Ricordi chamber. Dr. Huang is assembling the chamber.

concerns were raised regarding the bovine brain products being used in Liberase HI products, to minimise risk of prion disease transmission (Kin et al., 2009; Szot et al., 2009). Following enzyme infusion, the pancreas was cut into small slices and transferred into the Ricordi chamber where digestion takes place (Fig. 2.3). The Ricordi chamber was connected to a heat controlling loop and feed-in / collection tubing (Fig. 2.4). The digestion reaction was monitored every 5 minutes by direct staining of digestion mixture with 0.05% Dithizone (DTZ) (Sigma-Aldrich, Pools, UK) (W/V in PBS), a chemical with high affinity for zinc which stains zinc-rich insulin-containing β -cells red. Digestion time varied depending on enzyme activity and pancreas variables including size, age and fat content with an average digestion time on most occasions between 15 and 30 minutes. When DTZ staining confirmed efficient liberation of islets, the digest mixture was cooled with 4 °C MEM medium (Lonza Biologics plc, Berkshire, UK) and collected on ice. Digestion products were centrifuged at 1000 rpm (32.5 G) at 4 °C for 1 minute. Concentrated digest mixture was separated into islet-rich and islet depleted fractions by continuous density gradient (between 1.065 to 1.095 g/mL) separation in a COBE 2991 cell processor (Gambro-BCT, USA). The gradient was prepared with linear dilution of Euro-Ficoll–sodium diatrizoate with UWS to create linear density gradients as per the published method of Dr GC Huang (Huang et al., 2004). Purified islet fractions were collected in 50 ml aliquots and then pooled according to purity of each tube. Pooled islets were assessed for total Islet Equivalent (IEQ) number, viability using FDA/PI (2.10) and assessment of cellular structure and secretory granules by electron microscopy (EM) (2.15). Islets were resuspended in 1 litre of complete transport medium (Table 2.2) and packaged in either blood transfusion bags (Baxter, Berkshire, UK, cat. no. R8003) or gas-permeable Permalife bags (Origen, Sweden, UK, cat. no. PL325).

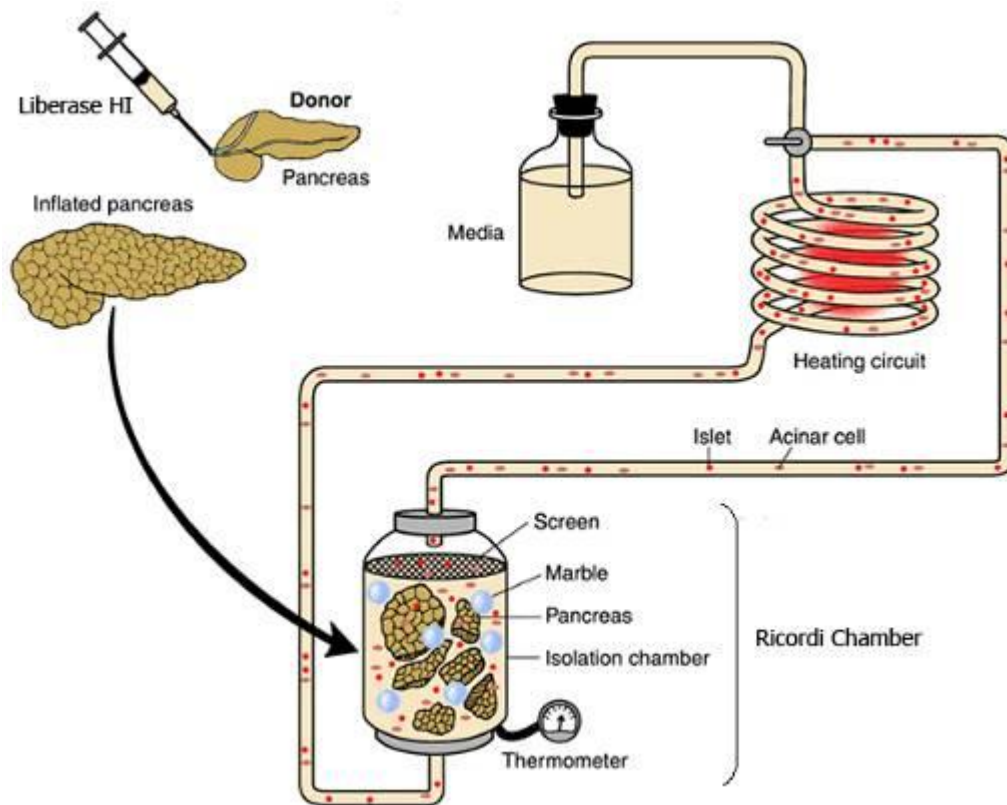


Figure 2.4 Islet isolation schematic. Pancreas is injected with Liberase HI and then cut into pieces and placed inside Ricordi chamber where enzymatic and mechanical digestion takes place. The heating loop maintains the required temperature.

Medium	Basal Medium 500ml	FCS	HSA 30%	HSA 20%	P/S 100x	Vit E 26.5mM	Nicotinamide 2.5M	Na- HCO ₃	ITS 100x	HEPES 1M
Transport Medium	CMRL-supple or CMRL NCL1		10 ml		5 ml	100 ml	2 ml			2 ml for CMRL- supple
Transplant Medium	CMRL 1066		40 ml	50ml						2 ml
Adherent culture medium	CMRL NCL1	100 ml			5 ml	100 ml	2 ml		10 ml	
Suspension Culture Medium (serum-free)	CMRL-supple or CMRL NCL1		20 ml		5 ml	100 ml	2 ml	1.1 gr		
Protein-free medium	CMRL NCL1				5 ml					

Table 2.2 Media formulations for human islet culture per 500 ml bottle.
Formulation of each basal medium is summarised in Tables 2.5 and 2.6.

2.9 Islet equivalent count and integrity scoring

Assessment of islet equivalents was carried out to standardise islet yield while correcting for the differences in size between islets. One islet equivalent was considered to be 150 μm in diameter. Integrity scoring was carried out to assess quality of the preparation and degree of islet damage during isolation. Dithizone (DTZ) was pre-diluted in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Pools, UK) (5mg in 5ml) and was further diluted in 100 ml 1x HBSS on a magnetic stirrer for 15 minutes. DTZ solution was cleared of the non-dissolved DTZ crystals by filtration through a 0.45 μm filter disc. Following purification and pooling of the whole islet preparation into a 50 ml volume, a 100 μl sample was removed. A volume of 900 μl PBS was added to the islet sample followed by 1 ml of working strength DTZ and the sample was incubated at room temperature for 5 minutes. Stained islets were then centrifuged at 14,000 rpm (16,000 G) for 30 seconds and 1800 μl of supernatant was discarded. Islets then were resuspended in 400 μl PBS and distributed in 10 drops on a cover of a 12 well plate. Size of stained islets in each drop was scored against a graticule (Figure 2.5) and documented. Final IEQ count in the sample was calculated by multiplying number of islets counted in each size group by a correction factor (Table 2.3 (Mirbolooki et al., 2007)). Final IEQ yield in the overall preparation was determined by multiplying total IEQs in the 100 μl sample by 500. Scoring of integrity was carried out according to border continuity in DTZ-stained islets. When continuity of the border was maintained, the islet was scored as intact, and when border continuity was broken then the islet was scored as damaged. Scoring integrity was carried out at the same time as performing IEQ count.

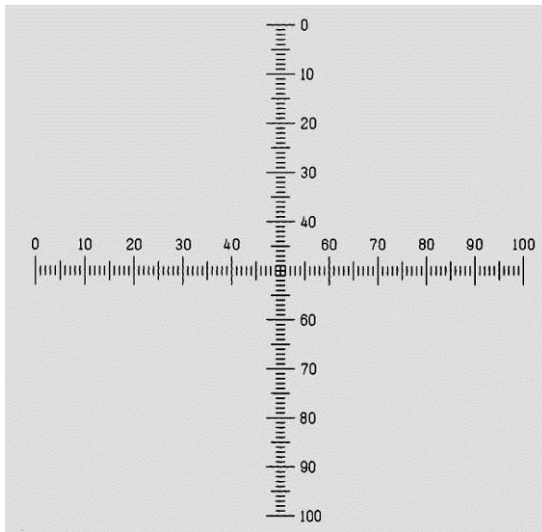


Figure 2.5 Eye-piece graticule for estimation of islet equivalent. Each 10 on the graticule is equivalent to 100 μm when the x10 objective is used.

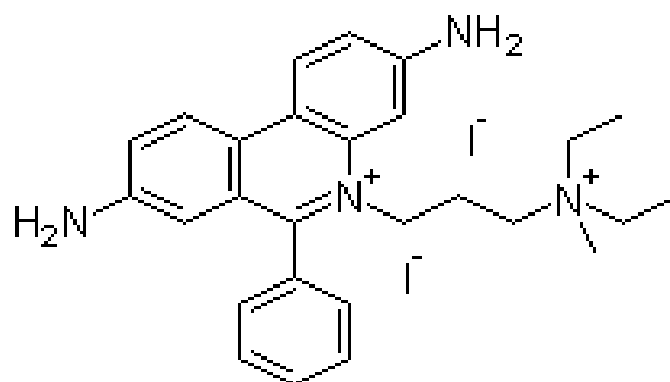
Islet size in μm	<50	50-100	100-150	150-200	200-250	250-300	300-350	350-400	>400
Number of islets									
Correction factor	0	x0.167	x0.65	x1.7	x3.5	x6.3	x10.4	x15.8	x22.8
Number of IEQs									

Table 2.3 Islet equivalent calculation. Each size group is corrected by multiplying number of islets in the group by the correction factor (Mirbolooki et al., 2007).

2.10 Viability staining

Propidium iodide (PI) (Figure 2.6 A) (Fluka, Sigma-Aldrich, Dorset, UK, Cat. No. 81845) is a non-cell permeant nucleic acid dye. It is able to label dead, membrane-compromised cells red under UV excitation leaving cells with intact membrane unstained. An original stock solution is prepared at a concentration of 0.5 mg/ml in distilled water. Fluorescein diacetate (FDA) (Figure 2.6 B) (Fluka, Sigma-Aldrich, Dorset, UK, cat. no. 31545) is a non-polar, non-fluorescent fluorescein analogue which can permeate the cell membrane of living cells entering the cytoplasm where intracellular esterases cleave off the diacetate group producing the UV excitable green dye fluorescein. Fluorescein thus accumulates in living cells with intact membranes demonstrating viability with green fluorescence under UV light. Cells with compromised membrane or severely impaired function do not accumulate fluorescein. FDA is prepared as a 10 mg/ml stock in acetone. Since acetone is the solvent used for FDA preparation, there is a danger of false positive PI staining as a result of acetone direct fixative effect of acetone on the cell membrane. Therefore a washing step was added to the standard protocol to minimise this. 100 μ l islet solution was placed in a 2 ml U-shaped bottom microcentrifuge tube (SARSTEDT Ltd., Leicester, UK. Cat. No. 72.695) and 880 μ l PBS (PAA Laboratories Ltd, Somerset, UK) added. A volume of 10 μ l of PI (0.5 mg/ml) and of 10 μ l FDA (10mg/ml) were added and mixed immediately. The tube was incubated for 2-3 minutes and then 1 ml fresh PBS added with islets centrifuged at 14,000 rpm (16,000 G) in a tabletop microcentrifuge for 30 seconds. Supernatant was then discarded and 1ml fresh PBS added. Islets were then pipetted into a well in a 12 well plate and visualised under inverted UV microscope. Scoring criteria for each islet is summarised below (Table 2.4). A total of 50 cell-

A



B

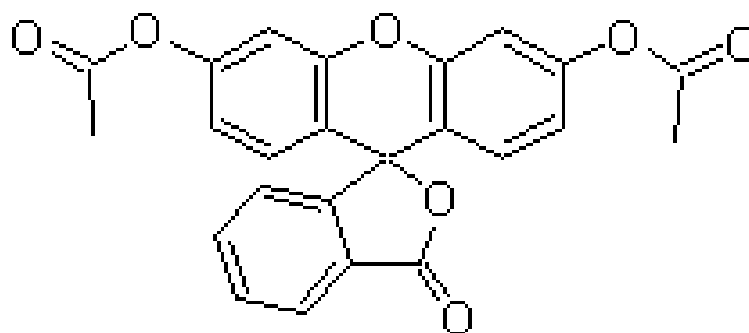


Figure 2.6 Molecular structure of (A) Propidium iodide (PI) and (B) Fluorescein diacetate.

aggregates were scored under fluorescence inverted microscopy. Each clump was scored individually. Viability score of the preparation was calculated as the mean viability score for all 50 aggregates.

2.11 Islet transport to Newcastle

Islets were packed in transport medium with final volume adjusted according to islet yield aiming for an average 300 ml per 100,000 IEQs. After islet packaging and labelling, bags were placed in the shelf compartment of the transport box with a HOBO pendant data-logger. The bottom of the box was filled with an average of 8-10 cold packs (pre-chilled to 4 °C). The box was closed and secured with the embedded locking device and handed to the Lifeline organ courier driver. The organ courier transported the islet box back to Newcastle University. Temperature control targets were set as within 5-22 °C.

2.12 Islet culture

2.12.1 Reagents and plastic-ware

All tissue culture plastic-ware was purchased from Greiner (Greiner, Gloucestershire, UK) apart from non-adherent flasks which were purchased from Sarstedt (Sarstedt, Leicester, UK) (T175 flasks) and Nunc (Thermo Scientific, Leicestershire, UK) (T25 and T75 flasks). CMRL 1066 medium (Tables 2.5) and its variations (Table 2.6) were purchased as outlined in the table. ITS-A supplement (Table 2.7) and TrypLe enzyme were purchased from Invitrogen (Paisley, UK). Thirty per cent human serum albumin (HSA) (non-clinical grade) was purchased from First Link (First Link, Birmingham, UK) and 25% HSA from Seralab Laboratories International (West Sussex, UK). Twenty per cent HSA medical grade was purchased from Bio Products Laboratories

Cluster size	Number of PI (red dot) positive cells				
	0 cells	<5 cells	5-15 cells	16-25 cells	>25 cells
50-150 μm	100%	90%	60%	30%	0%
150-250 μm	100%	95%	70%	40%	10%
250-350 μm	100%	100%	75%	50%	20%
350-450 μm	100%	100%	80%	60%	30%

Table 2.4 Methodology for viability scoring. Every cell cluster is scored according to PI positive cells and then average viability of 50 scored clustered is calculated. The calculated average indicates viability of the islet preparation.

COMPONENTS	Molecular Weight	Concentration (mg/L)	Molarity (mM)
Amino Acids			
Glycine	75	50	0.667
Hydroxy L-proline	131	10	0.0763
L-Alanine	89	25	0.281
L-Arginine hydrochloride	211	70	0.332
L-Aspartic acid	133	30	0.226
L-Cysteine	121	199.88	1.65
L-Cystine	240	20	0.0833
L-Glutamic Acid	147	75	0.51
L-Histidine hydrochloride-H ₂ O	210	20	0.0952
L-Isoleucine	131	20	0.153
L-Leucine	131	60	0.458
L-Lysine hydrochloride	183	70	0.383
L-Methionine	149	15	0.101
L-Phenylalanine	165	25	0.152
L-Proline	115	40	0.348
L-Serine	105	25	0.238
L-Threonine	119	30	0.252
L-Tryptophan	204	10	0.049
L-Tyrosine	181	40	0.221
L-Valine	117	25	0.214
Vitamins			
Ascorbic Acid	176	50	0.284
Biotin	244	0.01	0.000041
Cholesterol	387	0.2	0.000517
Choline chloride	140	0.5	0.00357

D-Calcium pantothenate	477	0.01	0.000021
Folic Acid	441	0.01	0.0000227
i-Inositol	180	0.05	0.000278
Niacinamide	122	0.025	0.000205
Nicotinic acid (Niacin)	123	0.025	0.000203
Para-Aminobenzoic Acid	137	0.05	0.000365
Pyridoxal hydrochloride	204	0.025	0.000123
Pyridoxine hydrochloride	206	0.025	0.000121
Riboflavin	376	0.01	0.0000266
Thiamine hydrochloride	337	0.01	0.0000297
Inorganic Salts			
Calcium Chloride (CaCl ₂ -2H ₂ O)	147	264	1.8
Magnesium Sulfate (MgSO ₄ -7H ₂ O)	246	200	0.813
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate (NaHCO ₃)	84	2200	26.19
Sodium Chloride (NaCl)	58	6799	117.22
Sodium Phosphate monobasic (NaH ₂ PO ₄ -2H ₂ O)	156	158	1.01
Other Components			
2'Deoxyadenosine	251	10	0.0398
2'Deoxycytidine	227	10	0.0441
2'Deoxyguanosine	267	10	0.0375
5-Methyl-deoxycytidine	225	0.1	0.000444
Co-carboxylase	461	1	0.00217
Coenzyme A	768	2.5	0.00326
D-Glucose (Dextrose)	180	1000	5.56
Diphosphopyridine nucleotide (NAD)	663	7	0.0106
FAD (flavin adenine dinucleotide)	786	1	0.00127
Glutathione (reduced)	307	10	0.0326
Sodium acetate-3H ₂ O	136	83	0.61
Sodium glucuronate-H ₂ O	236	4.2	0.0178
Thymidine	242	10	0.0413
Triphosphopyridine Nucleotide (NADP)	743	1	0.00135
Tween 80®		5	
Uridine 5'- triphosphate	484	1	0.00207

Table 2.5 Components of CMRL 1066 base medium.

Medium variation	Formulation	Supplier
CMRL (Invitrogen)	CMRL 1066 base medium + Phenol red 20 mg/L	Invitrogen, Paisly, UK
CMRL NCL1	CMRL 1066 base medium + 2mM HEPES	PAA Laboraroties, Somerset, UK
CMRL supplemented	CMRL 1066 base medium + other protected components	Cellgro, Mediatech, Herndon, USA

Table 2.6 CMRL media variations

Component	Concentration (g/L)
Sodium Selenite (anhydrous)	0.00067
Sodium Pyruvate	11
Insulin	1
Transferrin	0.55

Table 2.7 Insulin-Transferrin-Selenium-A Supplement (100X).

(ZENLAB20: Bio Products laboratories, Herts, UK). Heat inactivated foetal calf serum (FCS) Gold grade, phosphate buffer saline (PBS), Hanks balanced salt solution (HBSS), accutase enzyme, 100x Penicillin (10,000 units/ml) and streptomycin (10 mg/ml) solution were purchased from PAA Laboratories (Somerset, UK).

2.12.2 Evaluation post-transport

Upon arrival, a 5 ml sample of the bag content was removed aseptically in a class II safety cabinet according to validated SOP. Following standing vertically to allow islets to settle, 100-200 µl of the (islet enriched) sample was dispensed in a 2 ml U-bottom microcentrifuge tube, 1.5-2 ml was dispensed into a sterile 30 ml tube for Gram staining and the remaining ~3 ml of the sample was injected into a BacT/ALERT[®] PF bottle (bioMérieux UK Limited, Hampshire, UK) (provided by hospital pharmacy in accordance with Department of Microbiology at Freeman Hospital protocols) for bacterial culture and sent to the hospital clinical microbiology laboratory for microscopy and culture. Islet-enriched sample was used for viability staining (2.10).

Following this 'side-arm' sample collection, a reduction spin at 1000 rpm (32.5 G) for 1 minute was carried out with resuspension in a final volume of 50 ml. A representative 100 µl sample of homogeneous islet solution was removed for dithizone (DTZ) staining and estimation of total IEQs, integrity and islet purity. Another representative sample was removed for assessment of viability. Remaining islets were resuspended in the appropriate culture medium according to their planned use.

2.12.3 Islet culture in suspension

Serum was avoided in all suspension cultures of islets which were maintained in CMRL supplemented with HSA (Table 2.2). Islets were resuspended in suspension culture medium at a concentration of 500 IEQs/ml in 40 ml (~20,000 IEQs) per T175 non-adherent cap-vented flasks. Flasks were incubated at 37 °C and 5% CO₂ in tissue culture incubators. Medium was changed every 48 hours.

2.12.4 Islet adherent culture

In this type of culture, islets were resuspended in adherent culture medium (Table 2.2) at a concentration of 50-100 IEQs/ml. Fifteen ml of islet suspension (~1,000 IEQs) was seeded in non-adherent cap-vented T75 flasks (Nunc Brand purchased from Fisher Scientific Ltd, Leicestershire, UK. TKT-300-020G) or 8ml in T25 non-adherent cap-vented flasks (Nunc, TKT-300-010J). Use of standard tissue culture treated vessels resulted in very strong adherence to the culture surface requiring excessive and prolonged treatment with TrypLE express reagent (Invitrogen, Paisley, UK. SKU: 12563-029) to detach the cells from the culture flask. Medium was changed first at day 5 and then every 72 hours. By day five most islets were adherent and showing outgrowth of proliferating cells as a monolayer. Cells in the monolayer were termed islet survivor cells (ISCs). ISCs were passaged at 90-100% confluence. For passaging, medium was discarded from each flask and flasks were washed with fresh PBS followed by incubation with 5 ml (for T75 flask) or 3 ml (for T25 flask) TrypLE express reagent for 10-15 minutes at 37 °C. Ten ml of complete medium was added to each flask to stop the reaction and detached cells in suspension were recovered and centrifuged for 3 minutes at 1500 rpm (73.1 G). Supernatant was discarded and cells were resuspended in 5 ml medium. Cell number was quantified in a Neubauer haemocytometer chamber using the standard method used for differential

cell quantification of blood samples (Burechailo and Cunningham 1974) and cells were seeded in new flasks at 400,000-500,000 cells per T75 flask and 150,000-200,000 cells per T25 flask.

2.12.5 Formation of pseudo-islets (PIs)

Pseudo-islets were generated using two different methods. The ‘hanging drop’ method was used for proof of principle studies and the ‘micro-well’ (MW) method for high throughput production. A micro-gravity bioreactor was also investigated for pseudo-islet formation and maintenance culture. ISC preparation for these methods comprised passaging as above (2.12.3) including washing the pellet twice with PBS. Pellets were then resuspended in serum-free suspension medium (hanging-drop and MWs) or protein-free medium (bioreactor) (Table 2.2).

2.12.5.1 Hanging drop method

This technique is widely used for generation of 3 dimensional cellular structures for different purposes including islet re-aggregation (Cavallari et al., 2007) and different models of stem cell differentiation (Kurosawa et al., 2003; Yoon et al., 2006). Techniques for hanging drop culture vary significantly in the literature and the method followed in this project was devised and optimised in-house. ISCs were prepared as above (2.12.4) and made to a final concentration of 100,000 cells / ml. Cell suspension was gently agitated manually to maintain homogenous cell density and drops of 35 µl cell suspension were dispensed on the bottom of a 90 mm non-coated standard petri-dish with average total of 30 drops per dish. The lid of the Petri-dish was placed upside-down and 1 ml PBS was dispensed onto it to provide humidified environment in the dish and prevent evaporation in the hanging drops. The Petri-dish with the drops on was carefully and swiftly flipped upside-down and placed

on the lid (Figure 2.7). The dish was transferred carefully to a 5% CO₂ incubator and cells were incubated at 37 °C for 24 hours.

2.12.5.2 Micro-well method

Micro-wells (MW) were invented by Dr Mark Ungrin from University of Toronto for high throughput production of consistent embryoid bodies (Ungrin et al., 2008). A collaboration was established with Dr Ungrin to explore novel utilisation of MWs in potential production of pseudo-islets. Wells were made of Polydimethylsiloxane (PDMS), an inert organosilicon polymer. The MWs are produced as inserts for 6 and 24 well plates. The diameter of each MW was 800 µm with each insert providing 300 wells in the 24-well format insert or 1200 wells in 6-well format insert. All inserts were incubated overnight in 70% ethanol for sterilisation, followed by immersion in 5% w/v filter sterilised Pluronic F-127 (Sigma-Aldrich, cat. no P2443), made up in PBS, for non-stick coating to prevent adherence. Pluronic coating was carried out on wells without inserts also, to control for any direct pluronic effect. Wells without pluronic coating were also included as a control. Before seeding, plates with insert were centrifuged at 1500 rpm (73.1 G) for 3 minutes to eliminate any air residing in the MWs. ISCs were prepared in suspension as above (2.12.4) in serum-free medium at 1 million cells per ml. Cell seeding was carried out with 0.5 ml of cell suspension (0.5 million cells) per well in the 24/well plates and 2 ml (2 million cells) in the 6 well plates. All plates (plates with MWs inserts and coated with pluronic, plates with wells without MWs inserts, and plates with no MWs inserts and not coated with pluronic) were included with at least 3 repeats of each. Plates were incubated for 24 hours in a humidified 5% CO₂ incubator at 37 °C.

2.12.5.3 Bioreactor culture

Culture of intact islets in a micro-gravity bioreactor has been explored by other

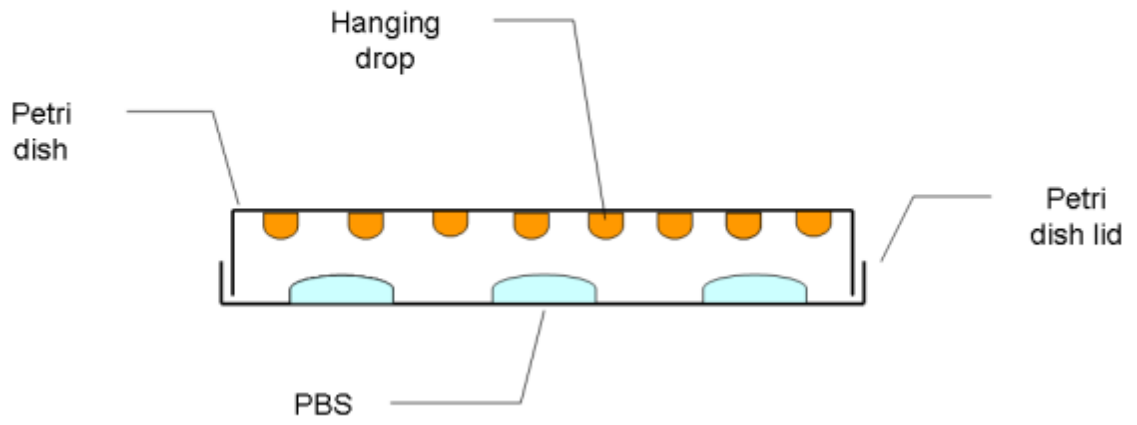


Figure 2.7 Hanging drop method for generation of pseudo-islets.

groups with evidence of improved islet function compared to standard culture (Rutzky et al., 2002). One disadvantage of hanging drop culture and micro-well culture was the short maximal culture duration at 24 hours due to limited nutrients in this high cell density culture. This short culture might not provide sufficient time for cells to differentiate. A Synthecon Bioreactor system (Cellon S.A., Bereldange, Luxembourg) was utilised for its potential of prolonged culture times of pseudo-islets and to explore the possibility of direct generation of pseudo-islets from single cell suspension. High Aspect Ratio Vessels (HARV) with a capacity of 10 ml were utilised for this purpose.

2.12.5.3.1 Culture of pseudo-islets

Pseudo-islets were harvested from MWs following 24 hours culture by brief plate agitation and aspiration of the pseudo-islet solution. MWs from each well of a 6 well plate were washed with 3 ml of PBS twice, to retrieve any remaining pseudo-islets in the well. All pseudo-islets from each well were pooled and spun for 1 minute at 1200 rpm (46.8 G). Supernatant was carefully removed and pseudo-islet pellet was resuspended in 2 ml protein-free medium (Table 2.2). FCS or HSA were avoided at this stage to prevent foaming during the seeding process. Three HARVs were prepared as per manufacturer's instructions. In brief, each vessel was washed with deionised water and then incubated in 70% ethanol overnight. All manipulations after that were carried out inside a class II safety cabinet. Ethanol was removed and the vessel was washed in sterile PBS twice. Finally, PBS was removed and the two ports of the vessel were fitted with a three way tap to enable sterile manipulation in the incubator using a syringe. Seeding with pseudo-islets was carried out in the safety cabinet. Each vessel was filled with 7 ml protein free medium and was then seeded with the 2 ml pseudo-islet suspension (pseudo-islets pooled from one well in a 6 well plate). All the inlets to the vessel were closed. Using a syringe, 0.4 ml of 30% HSA

and 0.6 ml protein free medium were infused inside the vessel via one inlet port while removing any trapped air through the second inlet port with another syringe. No air was left inside to prevent cellular lysis and foam formation. All ports were closed and capped to ensure maintained sterility. Vessels were attached to the rotating base and the unit was placed inside a 5% CO₂ incubator at 37 °C. An image of the system is shown below (Figure 2.8). Rotating speed was set at 20 rotations per minute. A sample from each vessel was collected every 24 hours through the three-way tap and the culture was terminated at either 24, 48 or 72 hours.

2.12.5.3.2 Generation of pseudo-islets in the bioreactor

ISCs were passaged as above (2.12.4) and resuspended in protein free medium at a final density of 500,000 cell /ml. The vessels were prepared as above and 9 ml of ISC suspension was seeded in each vessel followed by 0.4 ml of 30% HSA and 0.6 ml protein free medium using a syringe via the inlet ports while removing remaining trapped air. Vessels containing ISCs were attached to the rotating base inside the CO₂ incubator using the same settings as above. Samples were collected every 24 hours.

2.13 Rodent islet isolation

Three hundred gram Wistar rats were purchased from Charles River Ltd. (Charles River, UK). Each animal was humanely killed according to Home Office Schedule 1 (neck dislocation method). Immediately after confirmation of death the animal was placed on absorbent paper on a clean surface. It was sprayed with copious 70% ethanol and laparotomy performed through a vertical incision from xiphisternum to pubis. Intestines and liver were moved to expose the common bile duct. The pancreatic duct was clamped towards the ampulla of Vater to prevent collagenase leakage into the intestine. A small incision was made in the common duct and 10 ml

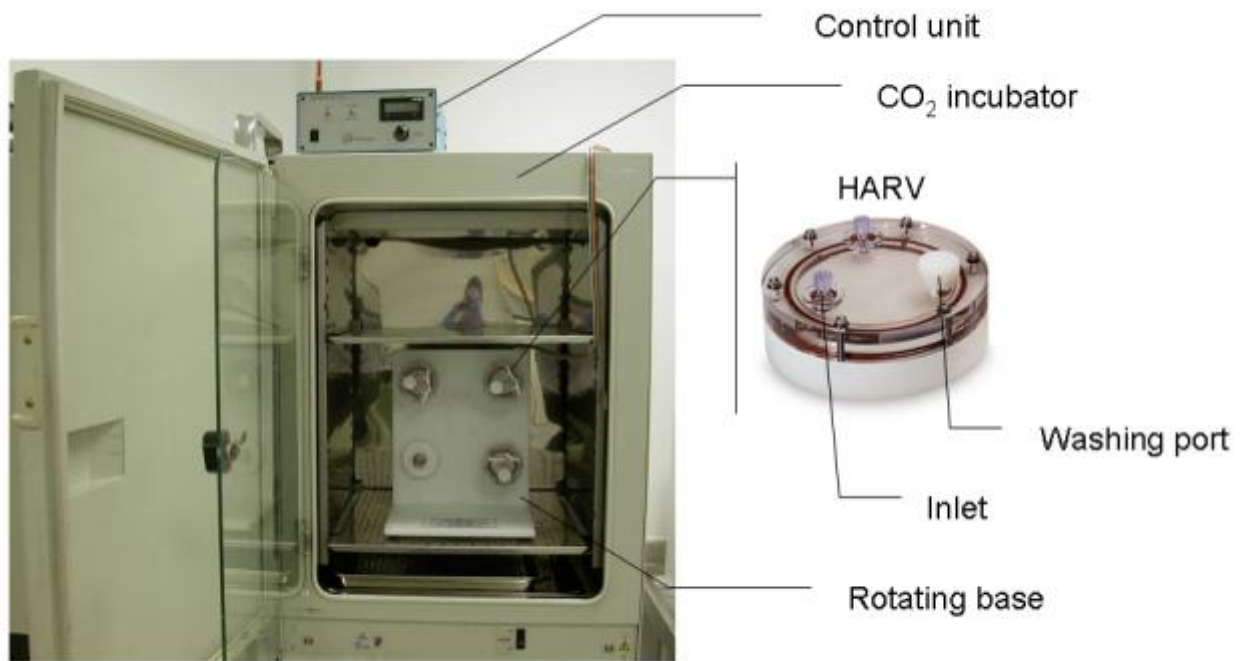


Figure 2.8 Bio-reactor setup and HARV vessel.

collagenase-XI 1 mg/ml (Sigma-Aldrich, Dorset, UK, cat. no.C7657) or collagenase P (Roche Diagnostics Ltd, West Sussex, UK. cat. No. 11213865001) was injected slowly while observing pancreatic distension. Fully distended pancreas was dissected from intestine and spleen and placed in a 20 ml universal tube either on ice, while distending other pancreases, or directly in a 37 °C water bath. Following 10 minutes incubation, the tube was hand shaken for 10-15 seconds and topped up to 20 ml using ice cold Hank's balanced salt solution (HBSS) and left to settle on ice for 10 minutes. Supernatant was discarded and 20 ml ice-cold HBSS was added to the tube and left to settle on ice for another 10 minutes. Supernatant was discarded and a final 20 ml ice-cold HBSS with 2% FCS was added and islets were hand-picked under a stereoscopic dissecting microscope (Prior Scientific Instruments, Cambs, UK Prior James Swift: S111ZA/S115). Collected islets were maintained in CMRL 1066 medium containing 2x ITS, 20% FCS and 1% P/S in a sterile 30 ml universal tube at room temperature. Islets were centrifuged at 1000 rpm (32.5 G) for 3 minutes. Supernatant was discarded and islets were resuspended in fresh 15 ml complete medium and cultured in non-adherent 60 mm Petri dishes at 37 °C in a 5% CO₂ incubator. Medium was changed every 48 hours.

2.14 Functional islet imaging

2.14.1 Fluorescent-tagged sulphonylurea uptake

Sulphonylureas are a group of oral hypoglycaemic drugs that bind selectively to sulphonylurea receptors (SUR) on the cell membrane resulting in closure of ATP-sensitive K⁺ channels, membrane depolarisation and opening of voltage-gated Ca²⁺ channels. High affinity SURs are found in the brain, cardiac muscle and pancreatic β -cells. The isoform expressed in β -cells is SUR1. Glibenclamide-BODIPY® FL

(Green Molecular Probes, Invitrogen, Paisley, UK. E34251 ER-Tracker™, (which will be referred to as glibenclamide-FL for short) is a sulphonylurea (SU) tagged with a green fluorescent molecule. It binds to SUR1 on β -cells and fluoresces green when excited with UV light. Original stock was prepared using DMSO to dissolve the powder to a concentration of 10 mM and was aliquoted in small 5 μ l aliquots which were stored at -20 °C. Re-freezing was avoided.

2.14.1.1 Sulphonylurea uptake optimisation

Proof of principle for β -cell labelling was carried out on human intact islets and utilised later for ISC characterisation. Human islets were washed in PBS and resuspended in serum-free medium. Aliquots of 500 μ l of islet solution (each containing 20-40 islets) were dispensed into wells of a 24 well plate. Glibenclamide-FL was added to each well to final concentrations of 0 nM, 50 nM, 100 nM, 500 nM and 1 μ M with at least 3 repeats in each. Islet plates were incubated for 15 minutes or 30 minutes at 37 °C in a tissue culture incubator. Islets were then visualised under inverted fluorescent microscope. Wells without glibenclamide-FL served as negative control. ISCs were stained in a similar protocol using 100 nM glibenclamide-FL for 15 minutes at 37 °C.

2.14.2 Newport green (NPG) labelling

NPG is a green fluorescent molecule. It is an excitable zinc indicator that naturally accumulates in zinc-rich cells resulting in fluorescent labelling (Lukowiak et al., 2001). NPG was utilised for labelling live β -cells in intact islets and in ISCs. NPG was purchased from Invitrogen (Invitrogen, Paisley, UK, cat. No. N-7991). NPG was reconstituted in DMSO to 5 mM and stored in 5 μ l aliquots at -20 °C. Re-freezing was avoided.

2.14.2.1 NPG labelling optimisation

Staining with NPG was carried out on ISCs following a similar approach to SU treatment above. Islets were washed in PBS and resuspended in culture medium without FCS. Aliquots of 500 μ l of islet solution (each containing 20-40 islets) were dispensed into wells of a 24 well plate. NPG was added to final concentrations of 0 nM, 1 nM, 5 nM, 10 nM, 100 nM, 1 μ M with at least 3 repeats of each. Islet plates were incubated for 15 minutes or 30 minutes at 37 $^{\circ}$ C in a tissue culture incubator. Islets were then visualised under inverted fluorescent microscopy. Wells with no NPG served as negative control. ISCs were stained in a similar protocol using 10 nM NPG for 15 minutes at 37 $^{\circ}$ C.

2.14.3 Transfections

Transfections with reporter gene plasmids were utilised for further characterisation of cells. These included a PDX1 reporter plasmid containing a β -cell-specific fragment of the PDX1 promoter upstream of eGFP (Campbell and Macfarlane 2002) (Figure 2.7 A). This plasmid was provided as a kind gift by Dr S Campbell within our group. For determination of transfection efficiency, constitutive expression reporter plasmids were included. These were pIRES-eGFP (Figure 2.7 B) and pCAG-DsRed (Figure 2.7 C). In pIRES-eGFP (available within the group), eGFP is expressed downstream of a CMV promoter to ensure constitutive expression. pCAG-DsRed was a kind gift from Dr Cepko at Harvard Medical School through the Addgene website (Plasmid 11151, www.addgene.org, Cambridge, USA). The red fluorescent protein DsRed cDNA is downstream of the CAG promoter (a combination of chicken beta-actin promoter and cytomegalovirus immediate-early enhancer) (Matsuda and Cepko, 2004).

ISCs were established in culture in 12 well plates. At 70-90% confluence, the transfection procedure was carried out using jetPEI transfection reagent (Source

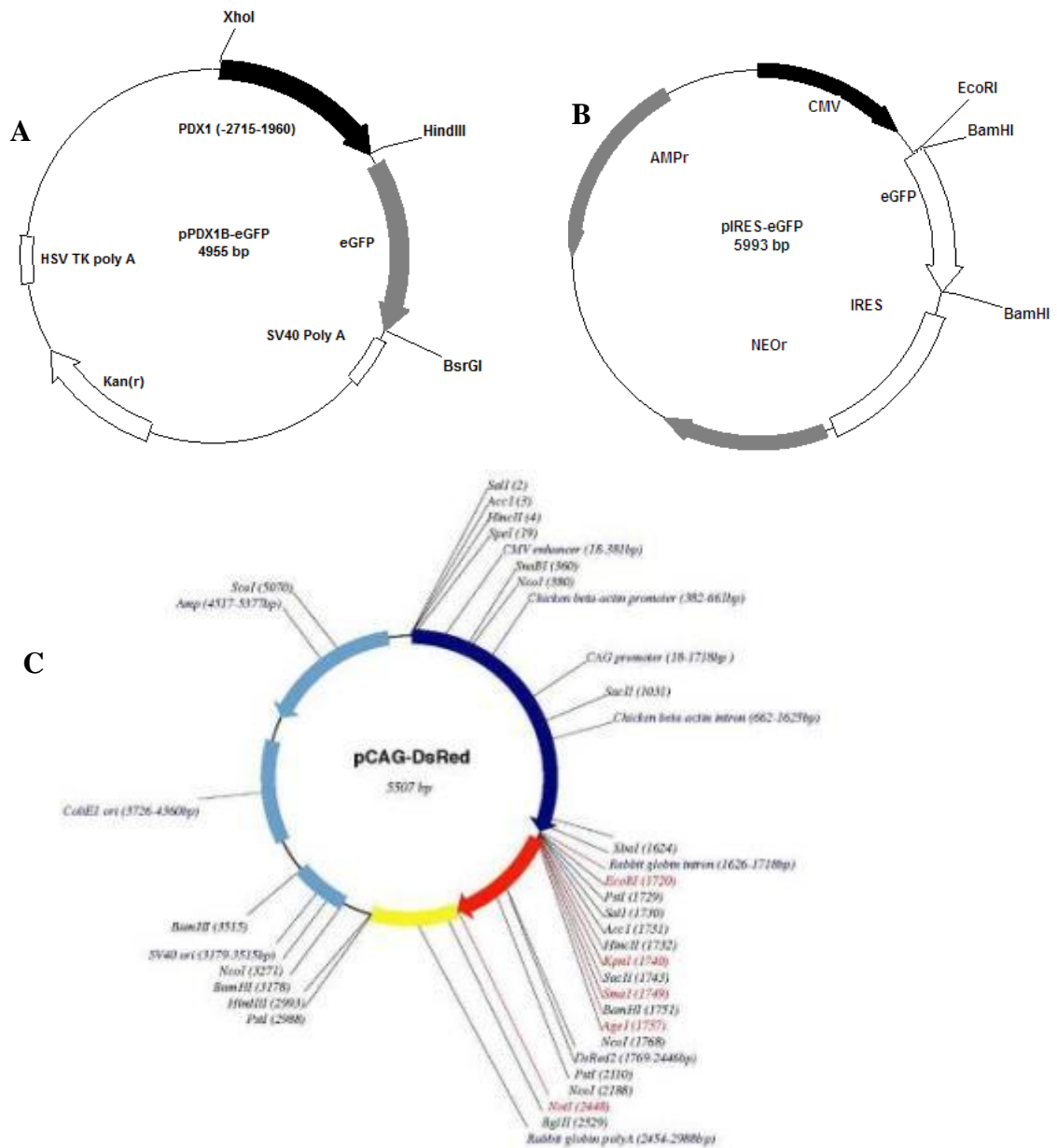


Figure 2.9 Plasmids employed for reporter gene studies. Maps denoting structure of three reporter gene plasmids utilised in transfection studies. These included pPDX1B-eGFP (A) in which eGFP is downstream of a β -cell specific fragment of the PDX1 promoter, pIRES-eGFP (B) in which eGFP is downstream of CMV promoter to ensure constitutive expression, and pCAG-DsRed (C) in which the red fluorescent protein DsRed is downstream of CAG promoter (= a chimera of chicken beta-actin promoter and cytomegalovirus immediate-early enhancer). Maps A and B were generated within the group and based on backbone maps provided by the manufacturers. Map C is courtesy of Dr Cepko.

BioScience Autogen, Nottingham, UK, cat. No. 101-10N). The transfection mixture was prepared for each well as follows: one tube with 50 μ l of 150 mM NaCl + 2 μ l jetPEI and one tube with 50 μ l of 150 mM NaCl + 1 μ g DNA. Tubes were left for 5 minutes at room temperature and then the contents of the jetPEI tube were added to the DNA tube with immediate mixing and tubes were left for 20 minutes at room temperature. ISC wells were washed during the incubation with PBS. When incubation of the transfection mixture was completed all solutions were removed from the wells and the transfection mixture was then added directly followed by addition of 400 μ l complete adherent medium. At 48 hours, cells were washed with PBS and incubated with 10 μ g/ml Hoechst solution for 3 minutes to counter-stain the nuclei. Fresh medium was dispensed and cells were visualised under fluorescent inverted microscopy.

2.15 Functional assessment

Islet function was assessed by glucose stimulated insulin secretion (GSIS). This was performed either in static incubations or in dynamic perfusion studies. Moreover, processing capacity of proinsulin to insulin was tested in static incubations.

2.15.1 Static incubations

An optimal static glucose stimulated insulin secretion (GSIS) protocol was developed after a series of protocol validations and modifications. Islets were incubated overnight in suspension culture medium (glucose 5.5 mM) (Table 2.2) in a tissue culture incubator (37 $^{\circ}$ C, 5% CO₂). The following morning, islets were centrifuged at 1200 rpm (46.8 G) for 2 minutes. Supernatant was discarded and islets were resuspended in 2 mM glucose DMEM (Glucose free DMEM, Gibco, Invitrogen, Paisley, UK, cat. No. 11966-025, adjusted to a final glucose concentration of 2 mM

using filter-sterile-grade 1 M D-glucose) at a density of 2,000 islets/ml. Initially, islets were incubated in 2 mM glucose to rest for 1 hour at room temperature. In the meantime a new 12 well plate was prepared with at least 3 wells filled with 2 ml glucose-free DMEM supplemented with 2 mM D-glucose; and a matching number of wells filled with 2 ml glucose-free DMEM supplemented with 20 mM D-glucose. When the 1 hour incubation was over, islets were mixed to ensure homogeneity and aliquots of 10 μ l were collected and dispensed in each well of the 12 well plate. The plate was cultured at room temperature for 2 hours. After incubation, samples were collected in 2 ml U-bottom tubes (Sarstedt Ltd, Leicester, UK. Cat. No. 72.695) and centrifuged at 14000 rpm (16,000 G) for 5 minutes. Supernatant was collected and stored at -20 $^{\circ}$ C for future analysis of insulin secretion. In some experiments the islet pellet was recovered and either lysed immediately or stored at -20 $^{\circ}$ C for future lysis and study of intracellular (pro)insulin levels. To lyse the islet pellet, it was resuspended in 1 ml PBS and cycled 3 times between snap freezing in liquid nitrogen, immediate thawing at 37 $^{\circ}$ C in a water bath and 15 seconds of vortexing. The lysate was finally centrifuged at 14,000 rpm (16,000 G) for 5 minutes to precipitate cell debris and was stored at -20 $^{\circ}$ C.

Due to absence of reproducible response in studies at room temperature, studies were repeated with incubation at 30 $^{\circ}$ C but results were still sub-optimal. Experiments were then repeated at 37 $^{\circ}$ C within a tissue culture incubator. This improved glucose response but variability between wells prevented confirmation of significant differences between groups. Ultimately, experiments were repeated at 37 $^{\circ}$ C and the number of repeats was increased to six in each condition. In two experiments with three repeats, proinsulin and insulin levels were assessed at 2 mM and 20 mM D-

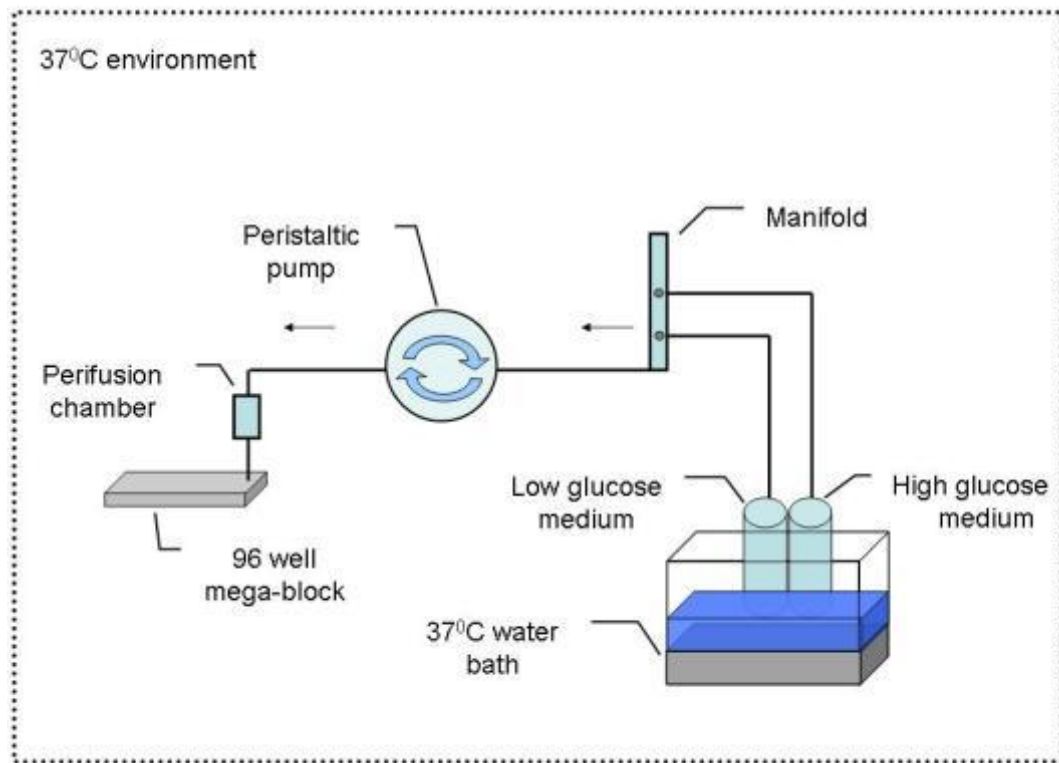
glucose in both lysates and medium to enable assessment of proinsulin to insulin processing capacity using differential ELISA assays (2.17).

2.15.2 Perifusion

2.15.2.1 Equipment setup

Perifusion experiments were conducted in purpose-constructed apparatus built as part of this project based on a validated system established at Prof P Jones' and Prof S Persaud's laboratory (King's College, London). An overall schematic view is shown below (Figure 2.10). All perifusion experiments were conducted in a 37 °C room and all solutions used were kept in a 37 °C water-bath for maintenance of temperature throughout the experiment. Different solutions feeding the perifusion circuit were withdrawn from their bottles via silicone tubes connected to a multiport manifold. The manifold outlet was connected via a peristaltic pump (Jencons (now part of VWR international Ltd), Leicestershire, UK. Model: Watson & Marlow SCI 32) to the perifusion chamber (Millipore UK Ltd, Livingston, UK. Swinnex. cat. No. SX0001300) (Figure 2.10 A). The perifusion chamber consisted of two compartments separated by a perforated plate on which a disposable 1 µm nylon filter disc was placed, onto which perifused islets were dispensed. The inlet of the upper compartment was connected to the tube feeding-in perifusion medium from the peristaltic pump while the lower compartment was connected to the collection tube of perifused medium (Figure 2.10 B). Perifusate was collected in a 96-well mega-block, with one row for each chamber. This system has multiple perifusion units with a maximum of 8 simultaneous perifusion chambers. Holders of perifusion chambers and frame were purchased from World Precisions International (World Precision Instruments Ltd, Stevenage, UK) and were assembled in house.

A



B

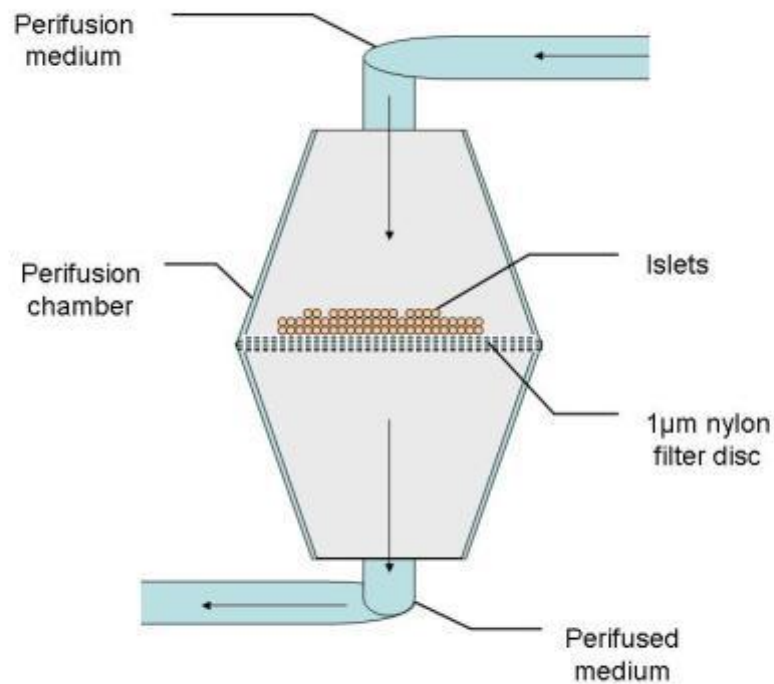


Figure 2.10 Schematic illustration of perfusion system components and set up. A: full system, B: perfusion chamber demonstrating the perfusion constant unidirectional flow enabling collection and thus assessment of hormone secretion minute by minute. Four chambers for each condition were included.

2.15.2.2 Solutions and islet preparation

Gey and Gey (GG) buffer (0.18 M KCl, 1 mM MgCl₂, and 10 mM NaHCO₃) was persufflated with 95% CO₂ and 5% O₂ gas mixture for 15-20 minutes to equilibrate pH at 7.4. This was followed by addition of bovine serum albumin (BSA) to a final concentration of 0.5 mg/ml. Different glucose concentrations were added to aliquots of the buffer according to study design. Standard glucose challenge studies were carried out using 2 mM and 20 mM D-glucose. Minimal glucose challenge studies were carried out using 5.6 mM and 11 mM D-glucose. Islets were washed with PBS and resuspended in fresh perfusion solution at a concentration of 1000 islets/ml. All perfusion experiments were carried out in a 37 °C warm room. In definitive studies, replicates of four chambers per condition were included.

2.15.2.3 Perfusion studies

The perfusion system was tested at the beginning of each study with PBS solution for 10 minutes to expel air from the circuit, confirm patency and exclude leakage. The inlet of each chamber was disconnected briefly and 100 µl of solution was removed and replaced with 100 µl of a homogeneous islet stock (~ 100 islets). Any remaining space in each chamber was completely filled with PBS to avoid any air bubbles. Islets were initially perfused with 2 mM D-glucose GG buffer for maximal challenge experiments or 5.6 mM D-glucose GG buffer for minimal challenges at a rate of 0.5 ml/minute for 60 minutes to wash-out any excess insulin and enable islets to adjust and equilibrate to the new environment. Sample collection was started soon after the washing period was completed. Perfusate was collected constantly at 2 minute intervals. Samples were collected in 96 well megablock units (SARSTEDT Ltd., Leicester, UK. Megablock 96 Well, 2.2 ml). Basal glucose medium (2 mM D-glucose) perfusion was continued for 20-30 minutes when the manifold was rapidly

switched to allow 20 mM D-glucose GG buffer (maximal challenge) or 11 mM D-glucose (minimal challenge). Sample collection was continued as before for 20-30 minutes. A switch back to basal glucose buffer was carried out in some experiments while sample collection was continued. Collected samples were stored at -20 °C for future insulin estimation. When sample collection was completed, filter discs were removed from each chamber / discarded and perfusion was continued for a further 15 minutes using distilled water to wash all circuit components and prevent remaining salts from blocking the circuit.

2.16 Growth hormones

Several growth factors were included in growth factor treatment studies including pregnancy related hormones (Table 2.8). All growth factors used were native purified recombinant proteins except gastrin which was a leucine-15 gastrin analogue peptide. In this gastrin isoform Leu-15 replaces Met-15 which is readily oxidised. This substitution results in biological stability while maintaining the same activity (Zhao et al, 1998). Concentrations used were guided by both published literature and manufacturers' recommendations. All growth factors were reconstituted according to manufacturers' recommendations and aliquots were stored at -20 °C. Treatments were carried out over 72 hours except with acute treatment experiments when islets were exposed to the hormone of interest over a short period of time.

2.17 Insulin, proinsulin and C-peptide analysis

Intracellular and secreted insulin in human cells was assayed employing human insulin ELISA kits (Human insulin ELISA: DakoCytomation, Cambridgeshire, UK, cat. No. K6219). Rat insulin was assayed using rat insulin ELISA kits (High Range Rat insulin ELISA kit (MercoDia through Diagenics, Melton Keynes, UK, cat. No. 10-

Growth factor	Abbreviation	Supplier	cat. No.	Concentration used
Prolactin	PRL	PeperoTech	100-07	80 nM
Placental Lactogen	PL	ABD Serotec	PHP157G	100 nM
Growth Hormone	GH	ABD Serotec	PHP153	4 nM
Glucagon-Like Peptide1 (7-36)	GLP1	TCRIS Biosciences	2082	100 nM
Activin A	Act A	PeperoTech	120-14	2 nM
Hepatocyte Growth Factor	HGF	PeperoTech	100-39	500 pM
Betacellulin	BTC	PeperoTech	100-50	100 pM
Placenta Growth Factor-1	PLGF-1	PeperoTech	100-06	1.7 nM
Transforming Growth Factor Alpha	TGF- α	PeperoTech	100-16A	3 nM
Insulin-Like Growth Factor 1	IGF-1	PeperoTech	100-11	13 nM
Insulin-Like Growth Factor 2	IGF-2	PeperoTech	100-12	13 nM
Epidermal Growth Factor	EGF	PeperoTech	AF-100-15	80 nM
[Leu15]-Gastrin I (human)	Gastrin	Sigma-Alrich	G9145	500 nM
Control vehicle (PBS)	C	PAA	H15-002	0.05 μ M

Table 2.8 Growth hormones used in growth factor treatment studies.

1145-01). Insulin in perfusion experiment samples was estimated using insulin hTRF assay (Cisbio Bioassays, France). Total proinsulin in human samples was estimated using human proinsulin ELISA kits (Merckodia through Diagenics, Melton Keynes, UK, cat. 10-1118-01). Human C-peptide was measured using standard C-peptide ELISA (Merckodia through Diagenics, Melton Keynes, UK, cat. No.10-1136-01) or ultrasensitive C-peptide ELISA (Merckodia through Diagenics, Melton Keynes, UK, cat. No. 10-1141-01). Quality assurance was carried out by each manufacturer and was confirmed for each batch. Assay limit of detection and precision are summarised in Table 2.9.

2.17.1 ELISA procedures

Frozen samples were thawed to room temperature. All ELISA kits were brought to room temperature before being used. Assay procedures were carried out according to each manufacturer protocol. For each condition there was at least one technical repeat and three biological repeats. All calibrators were run in duplicate. Sample and reagent dispensing and all washing steps were done manually. Plates were read using Dynatch MR7000 plate reader using double read mode at 450 nm (actual signal) and at 595 nm (background noise signal). Standard curve values were fitted using the quadratic fit model and graph formula was generated using CurveExpert 1.38 software (Daniel G. Hyams, Hixon, TN. USA). Hormone concentration for each sample was determined by converting the optical density (OD) of each sample (well) with the standard curve formula. OD values outside the standard curve were excluded and repeated with further dilutions.

2.17.2 hTRF insulin assay

2.17.2.1 Assay principle

Homogenous time resolved fluorescence (hTRF) assay is based on fluorescence

Assay	Species	Manufacturer and Cat. No.	Detection limits	Intra-assay CV	Inter-assay CV
Insulin ELISA	Human	Dako, K6219	3 pmol/l	7.5%	9.3%
Ultra-sensitive insulin ELISA	Human	Mercodia, 10-1132-01	0.42 pmol/l	<6%	<4%
Total proinsulin ELISA	Human	Mercodia, 10-1118-01	0.5 pmol/l	<5%	<6%
C-peptide ELISA	Human	Mercodia, 10-1136-01	15 pmol/l	<5%	<5%
Ultrasensitive C-peptide ELISA	Human	Mercodia, 10-1141-01	1.5 pmol/l	<6%	6%
Insulin- High range ELISA	Rat, mouse	Mercodia, 10-1145-01	263 pmol/l	<5%	<5%
Insulin hTRF	Human, mouse, rat	Cisbio, 62INSPEC	34.4 pmol/l	<3%	

Table 2.9 Immunoassay limit of detection and sensitivity.

resonance energy transfer (FRET) with donor-acceptor excitation-emission chemistry. Excitation of the donor results in light emission at a certain wavelength. The emitted light can, if within certain proximity of the acceptor, excite the acceptor which then emits light after a defined time delay. The insulin hTRF assay employs two anti-insulin antibodies, one labelled with Europium Cryptate (Cryptate for short) and one labelled with XL665 (Figure 2.11). Both Cryptate and XL665 molecules are excitable at 337 nm and the emission waves are recorded. The light emitted by XL665 by the 337 nm wavelength excitation has low intensity and short life. On the other hand, Cryptate emits light at 620 nm in response to the 337 nm wavelength excitation. If both Cryptate and XL665 are in close proximity then energy emitted by Cryptate is transmitted to XL665 resulting in excitation and emission of 665 nm light that is both high in intensity and longer in life which is then recorded.

2.17.2.2 Assay setup

All kit reagents were prepared according to the insert of the 20,000 tests kit. Cryptate, XL665 and insulin standards were stored at -80°C . Original stocks of Cryptate and XL665 were diluted 1:20 in reconstitution buffer (50 mM Phosphate buffer, pH 7.0, 0.8 M potassium fluoride (KF), 0.2 % BSA). Assay calibrators were prepared using the insulin standard provided in the kit with serial dilutions in equivalent fresh medium to that used for unknown samples. Fresh medium alone served as a negative control reference. All samples were run in duplicate. Ten microliter of each sample or calibrator was dispensed in each well of the black 384-well low-well plate (Greiner Bio-one, 784076). Five microliters working strength of Cryptate labelled antibody and 5 μl XL665 labelled antibody were added to each well. The assay plate was covered by another blank plate to prevent evaporation and incubated in the dark at room temperature for a minimum of two hours. The plate was read by SpectraMax5^e plate

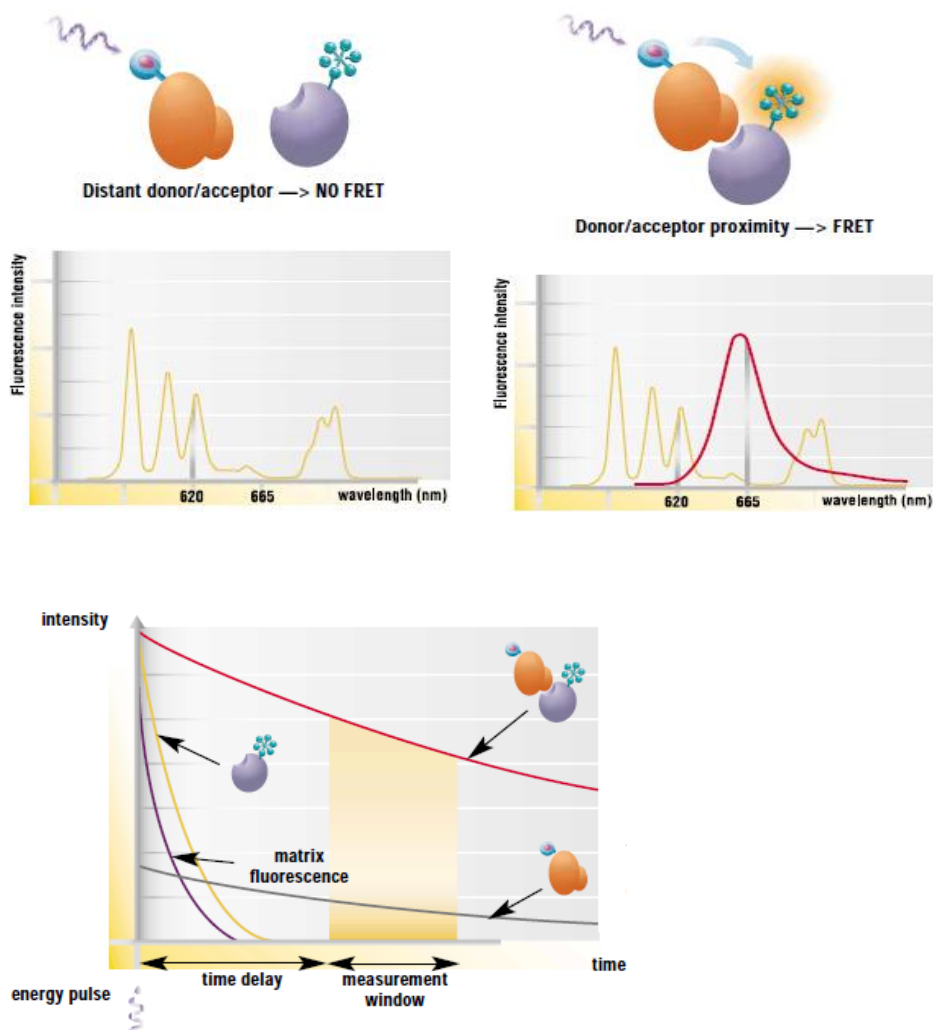


Figure 2.11 hTRF assay principle. When Cryptate and XL665 are not in close proximity no FRET occurs (upper left panel) and only decaying fluorescence of both probes is detected at 665 nm (lower panel). When both probes are in close proximity FRET then occurs with energy transfer from the donor (Cryptate) to the acceptor (XL665) resulting in detectable long-lived high fluorescence emitted from XL665 (upper right and lower panels). Images obtained from the manufacturer website (May 2011): <http://www.htrf.com/resources/>

reader (Molecular Devices Ltd., Berkshire. UK) and data were captured by SoftMax Pro 5.2 (Molecular Devices).

2.17.2.3 Calculations

Raw data were imported to MS-excel 2003. Ratio (R) between emission intensity at 665 nm and at 625 nm multiplied by 10,000 was calculated for all samples. The ratio of the negative control was subtracted from the ratio of each sample and the resulting figure was termed delta-R (d-R). Each d-R was divided by the ratio of the negative control and multiplied by 100 to give delta-F (d-F). A standard curve was constructed using CurveExpert 1.3 software using the best fit command and corresponding formula was generated. The generated formula was used to calculate the insulin value of each sample using its corresponding d-F value.

2.18 Protein assay

Total protein content of lysed cells was estimated using the Bradford protein assay (Bradford 1976). Briefly, 10 μ l sample or control (BSA) was mixed with 20 μ l working strength Bradford reagent (diluted 1:5 in PBS) (Biorad, Hertfordshire, UK, cat. No.500-0006) in wells of a transparent 96 well plate and read at 595nm wavelength to obtain optical densities (OD). All samples were run in duplicate. OD values of BSA standards at 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, 100 μ g/ml, 200 μ g/ml and 400 μ g/ml were used to construct a standard curve using Curve Expert 1.3 software with quadratic fit. Protein concentration of each sample was calculated from the OD using the formula generated.

2.18.1 Normalisation to protein content

Due to unavoidable variability in total cell content in each sample in the islet hormone studies correction to total protein content of each sample was carried out. This was

calculated as the ratio of total hormone content per sample divided by total protein per sample (in milligrams).

2.19 Proinflammatory profile assessment

Assessment of proinflammatory cytokine release into the transport medium was evaluated using Meso-Scale Discovery (MSD) proinflammatory-4 I assay (INF γ , IL6, IL1 β , TNF α) (Meso Scale Discovery, Gaithersburg, Maryland, US). The assay was carried out following the manufacturer's specific protocol. The detection plate was read using the specific Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, Maryland, US). The MSD technology is based on electrochemiluminescence (ECL) chemistry. MSD assay kits can enable detection of multiple proteins utilising the concept of sandwich ELISA on multi-spot wells (Figure 2.12 A). Each spot represents an electrode coated with specific capture antibody which immobilises the corresponding antigen in the test medium. A secondary antibody tagged with tris(2,2'-bipyridyl) ruthenium (Ru(bpy)) is then applied which then binds to the immobilised protein. In the presence of tripropylamine (TPA) a localised luminescence emission at 620 nm is produced on the application of an electrical current through the electrode (Figure 2.12 B (MSD website, 2009; Wilson, 2000)). Automated signal acquisition from all spots in each well was carried out using SECTOR Imager 2400 and data was analysed using MSD® DISCOVERY WORKBENCH® Software (Meso Scale Discovery, Gaithersburg, Maryland, US).

2.20 Immunostaining

Fluorescence immunocytochemistry was employed for phenotype characterisation.

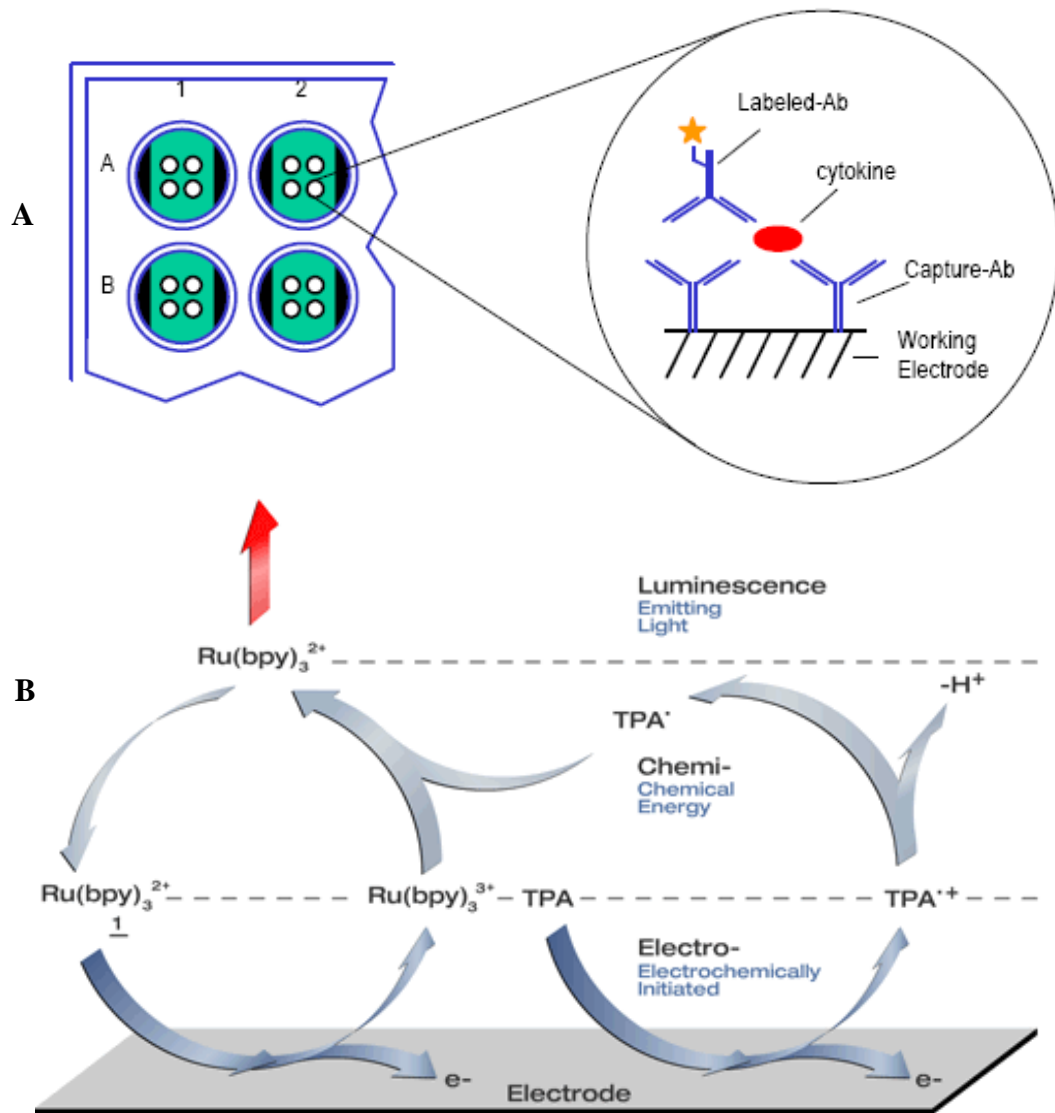


Figure 2.12 MSD assay. A: demonstration of plate layout. Each well has specific active spots. A specific capture antibody is attached to each spot to enable capture, and therefore detection, of the protein of interest. B: demonstrates chemistry of MSD assay. When an electrical current is applied to the electrode tripropylamine (TPA) locally changes to TPA⁺ which then releases chemical energy that facilitates activation of the tagged-ruthenium on the secondary antibody producing luminescence with a fluorescent wave of 617-620nm. These changes occur only at the electrode spot with specific local signal acquisition avoiding background noise (Wilson et al., 2000; MSD website, 2009).

2.20.1 Staining adherent cells

Cells were cultured in wells of 8-well chamber slides (Nunc Brand from Fisher Scientific Ltd, Leicestershire, UK. TKTT-210-490Q) at 3000-5000 cells per well in 300 µl medium. In some experiments 5000-8000 cells in 500 µl were established in each well of a 24-well plate in which 13 mm sterile round cover-slips had been placed (VWR International Ltd, Leicestershire, UK. Cat. No. 631-0149). Following 24 hours culture all wells were washed with fresh PBS and then fixed using 100 µl of 4% paraformaldehyde (PFA) per well for 20 minutes at room temperature. PFA was then discarded and PBS was applied for washing. This was followed by a permeabilization step with 100 µl of 0.5% Triton X-100 (V/V in PBS) for 20 minutes at room temperature. Triton solution was removed and 100 µl of working strength blocking buffer (Roche Applied Sciences, East Sussex, UK, cat. no. 11096176001) was applied for 1 hour at room temperature or over-night at 4 °C. In the meantime, working strength primary antibody solutions were prepared using 1x blocking buffer as diluent as outlined in the table below (Table 2.10). A total of 100 µl or 150 µl staining solution was prepared for each well in a chamber slide or 24 well plate respectively. Substituting primary antibody with vehicle or serum of the same species that the primary antibody was raised in (diluted to the same concentration as primary antibody) was used for negative control. Upon completion of the blocking step, blocking buffer was removed and working strength primary antibody mix was applied directly without any washing step. Incubation with primary antibody was either for 1 hour at room temperature or overnight at 4 °C. After primary antibody incubation all wells were washed 4 times with wash buffer (working strength blocking buffer diluted 1:5 in PBS) each for 10-15 minutes. Working strength secondary antibody solutions (Table 2.11) were prepared similar to primary antibody solutions. Upon completion of

Antibody	Species raised in	Clonality	Raised against	Confirmed Specificity	Dilution in ICC	Dilution in IHC	Supplier and cat. no.
Insulin	GP	Polyclonal	Swine	H, M, R	1:150	1:100	Dako. A0564
Ki67	Mouse	Monoclonal	H	H, M, R	1:100	1:50	Novocastra. NCL-L-Ki67-MM1
Glucagon	Rabbit	Polyclonal	H	H, M, R	1:100	1:50	Serotec. AHP534
Somatostatin	Rabbit	Polyclonal	H	H, M, R	1:100	1:50	Serotec. AHP533
Pancreatic polypeptide	Rabbit	polyclonal	H	H, M, R	1:100	1:50	Abcam. Ab16003-250
Nestin	Mouse	Monoclonal	Mouse	M, R	1:50	1:50	Abcam. Ab6142-50
CK19	Rabbit	Polyclonal	Human	H, M, R	1:50	1:50	Abcam. Ab15463-1
A-caspase3	Rabbit	Polyclonal	Human	H, M,	1:50	1:50	Abcam. Ab2302
C-peptide	Rat	Monoclonal	Human	H	1:50	1:50	DSHB*. GN-ID4
Amylase	Rabbit	Polyclonal	Human	H	1:100	1:100	Sigma. A-8273
Vimentin	Goat	Polyclonal	Human	H, M, R	1:50	1:50	Sigma. V4630
Albumin	Mouse	Monoclonal	Human	H	1:50	1:50	Abcam. ab10241
Nanog	Rabbit	Polyclonal	Human	H, M	1:50	1:50	Millipore. AB9220
Oct4	Rabbit	Polyclonal	Human	H, M, R	1:50	1:50	Santa Cruz. sc-25401
Sox2	Mouse	Monoclonal	Human	H, M	1:50	1:50	Millipore. MAB4343
CFTR	Mouse	Monoclonal	Human	H, M	1:50	1:50	Abcam. ab2784
Endoglin	Mouse	Monoclonal	Human	H	1:50	1:50	DAKO. M3527
NGN3	Mouse	Monoclonal	Human	H	1:50	1:50	DSHB. F25A1B3
PAX6	Mouse	Monoclonal	Mouse	M, R, H	1:50	1:50	DSHB. PAX6
Nkx6.1	Mouse	Monoclonal	Mouse	M, R, H	1:50	1:50	DSHB. F55A12

Table 2.10 Primary antibodies used in immunostaining. Combinations were attempted for double staining while respecting clonality compatibility to enable detection with different secondary antibodies. * DSHB: Developmental Study Hybridoma Bank, H: human, R: rat, M: mouse,

Antibody against	Species raised in	Conjugation	Specificity	Dilution in ICC	Dilution in IHC	Supplier and cat. No.
Mouse	Goat	FITC	IgG	1:200	1:200	JIR: 115-095-005
Mouse	Donkey	Cy3	IgG	1:200	1:200	JIR: 715-165-150
GP	Rabbit	FITC	IgG	1:200	1:200	Sigma: F-7762
GP	Donkey	Cy3	IgG	1:200	1:200	JIR: 706-165-148
Goat	Donkey	FITC	IgG	1:200	1:200	JIR: 705-095-003
Goat	Donkey	Cy3	IgG	1:200	1:200	JIR: 705-165-003
Rabbit	Donkey	Cy3	IgG	1:200	1:200	JIR: 711-165-152
Rabbit	Goat	FITC	IgG	1:200	1:200	Sigma: F-0382
Rat	Donkey	FITC	IgG	1:200	1:200	JIR: 712-095-153

Table 2.11 Secondary antibodies used in immunostaining.

the washing steps secondary antibodies were applied to the respective wells and incubated for 1 hour in the dark at room temperature or in a dark 4 °C environment overnight. This was followed by further 4 washes with wash buffer each for 10-15 minutes. When washing was completed the chamber guard and gasket were removed and the slide was dried briefly in dark. Alternatively when a 24-well plate was used, cover slips were removed from stained wells and left to dry in room air in the dark. Slides or cover slips were then mounted with Vectashield mounting medium (Vector Laboratories, Ltd., Peterborough, UK) containing 4,6-diamidino-2-phenylindole (DAPI), a nuclear stain fluorescing blue under UV light. Blank cover slips were then applied on the stained slide or blank slides were applied to the stained cover slips. The edge of each cover slip was sealed with transparent nail varnish to prevent Vectashield drying. Slides were stored in dark at 4 °C if not visualised immediately. Visualisation, image capturing and editing was performed using a Nikon fluorescent microscope (NIKON Eclipse E-400) provided with Nikon digital Camera DXM1200 (Purchased from Jencons Ltd, (now part of VWR international Ltd), Leicestershire, UK) and Lucia DXM200 Version: 4.71 software) (Laboratory Imaging, s.r.o. Za Drahou, Czech Republic).

2.20.2 Immunostaining of non-adherent cells

This was carried out on fresh islet tissue following preparation of a single cell suspension. Islets were digested into single cells with accutase (PAA Laboratories Ltd, Somerset, UK, Cat. No. L11-007) as follows. An average of 1000 islets were washed with PBS and centrifuged in a 2 ml U-bottom centrifuge tube (SARSTEDT Ltd., Leicester, UK. Cat. No. 72.695). Supernatant was discarded and islet pellet was resuspended in 1.5 ml neat accutase. The tube was firmly closed and incubated for 15 minutes in a 37 °C water bath. Islets were then passed through a P1000 filter tip

pipette several times to help islet dispersion. A 10 μ l sample was removed and placed on a microscope slide and visualised under microscope to ensure satisfactory single cell suspension. Further dispersion was carried out with a P200 pipette to further help dispersion if required. Cells were then centrifuged at 14,000 rpm (16,000 G) for 5 minutes. Supernatant was discarded and cells were resuspended in PBS. Staining on slides was carried out for phenotyping while staining in suspension was carried out for β -cell count only. Working strength primary and secondary antibody preparations used in staining on slides were similar to those used for adherent cell staining (2.20.1) while double the concentrations of primary antibodies were used for staining in suspension.

2.20.2.1 Staining on slides

Cell concentration was determined using the Neubauer chamber count method (2.10.3). Cells were diluted to a final concentration of 20,000 cells per 100 μ l using PBS in preparation for cytopinning onto standard super-frost microscope slides (VWR international Ltd, Leicestershire, UK. Cat. No. 631-0107) using a Cytospin 4 centrifuge (Thermo Scientific, from Fisher Scientific Ltd, Leicestershire, UK). The spinning funnel set was assembled as per machine manual (Figure 2.13). Between 100-150 μ l cell suspension was dispensed in each funnel set and funnels were centrifuged at 1000 rpm for 3 minutes. Following centrifugation, the spinning set was dismantled carefully and slides were left to dry at room temperature. The cell area was encircled using Dako Delimiting Pen (Dako UK Ltd, Cambridgeshire, UK. Cat. No. S2002). Cells were then fixed using 100 μ l of 4% PFA for 20 minutes at room temperature followed by washing in PBS. Cells were then permeabilized using 100 μ l of 0.5% Triton X-100 (V/V in PBS) for 20 minutes at room temperature. Triton solution was removed and slides were washed once in PBS. This was followed by a

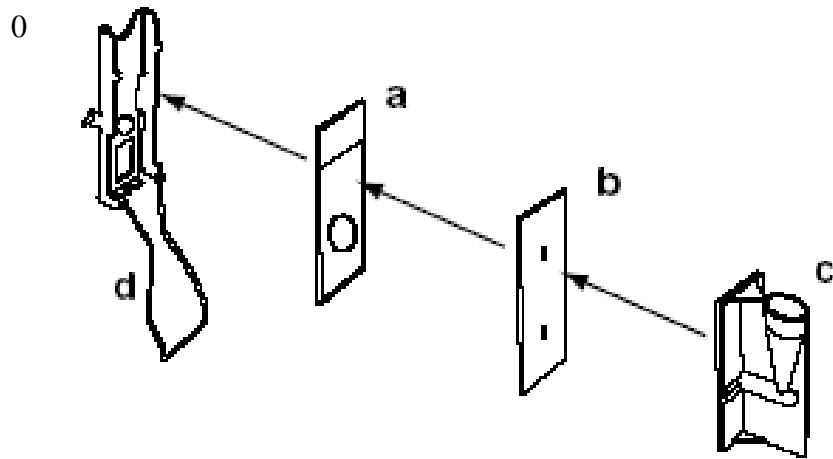


Figure 2.13 Cytospin funnel assembly diagram. a: polysin-coated slide. b: filter card. c: plastic funnel. d: is the metal clip. Once assembled cell solution is dispensed in c.

blocking step using 100 µl of working strength blocking buffer (Roche Applied Sciences, East Sussex, UK, cat. no. 11096176001) for 1 hour at room temperature or overnight at 4 °C. Blocking buffer was removed and slides were washed once in PBS. Working strength primary antibody solutions (100 µl per sample) were applied to the appropriate slide and incubated for 1 hour at room temperature or overnight at 4 °C followed by washing 4 times in wash buffer (working strength blocking buffer diluted 1:5 in PBS) each for 10-15 minutes. Secondary antibody mix (100 µl per sample) was then applied and incubated for 1 hour in the dark at room temperature or at 4 °C in a dark environment overnight. This was followed by further washes 4 times with wash buffer each for 10-15 minutes. A final wash was carried out in PBS alone for 1 minute and then slides were air dried in the dark. After drying, slides were mounted with Vectashield mounting medium (Vector Laboratories, Ltd., Peterborough, UK) containing DAPI. Blank cover slips were then applied on to the stained slide. The edge of each cover slip was sealed with transparent nail varnish to prevent Vectashield drying. Slides were stored at 4 °C if not visualised immediately. Visualisation, capturing and image editing was performed using Nikon fluorescent microscopy as above (2.20.1) In some cases cells were further visualised using a confocal microscope system (Leica TCS SP2 UV microscope with LCS 2.51 build 1537 software (Leica Microsystems GmbH Heidelberg Germany)).

2.20.2.2 Staining in suspension

This was employed for enumeration of β -cell number(s) in islet preparations. Following single cell digestion outlined above, cells were fixed in suspension using a final concentration of 1% PFA over 15 minutes at room temperature. The tube was topped up with PBS and cells were centrifuged at 14,000 rpm (16,000 G) for 3 minutes. The supernatant was discarded and cells were washed again with 1.5 ml PBS

and centrifuged. The cell pellet was resuspended in 500 μ l PBS. Total cell number in the 500 μ l cell solution was estimated by counting cells in a Neubauer chamber. Five hundred microliters of freshly prepared 1% Saponin (W/V PBS) was added for permeabilization over 30 minutes at room temperature. This was followed by centrifugation, removal of supernatant, resuspension in 500 μ l working strength blocking buffer and incubation for 30 minutes at room temperature. Cells were then distributed into separate tubes for different antibody stains. The appropriate primary antibody was added to each tube to the final required concentration (double that optimised for adherent cells) in a final volume of 300 μ l and incubated for 30 minutes. Tubes were filled with PBS to maximum capacity and centrifuged at 14,000 rpm (16,000 G) for 3 minutes. The supernatant was discarded and cells were washed again with PBS twice. Cells were then resuspended in 300 μ l blocking buffer and 1 μ l of the appropriate secondary antibody (1:300 final concentration) (Table 2.10) was added. Tubes were incubated at room temperature in the dark for 30 minutes. All tubes were then filled to maximum capacity with PBS and centrifuged at 14,000 rpm (16,000 G) for 3 minutes. Supernatant was discarded and the cell pellet was further washed twice in PBS twice. Finally, the pellet was resuspended in 150 μ l PBS. Tubes were then prepared for cytospinning onto standard super-frost microscope slides (VWR international Ltd, Leicestershire, UK. Cat. No. 631-0102) using Cytospin 4 centrifuge (Thermo Scientific, from Fisher Scientific Ltd, Leicestershire, UK). Cells were centrifuged at 1000 rpm for 3 minutes. When spinning was completed the spinning sets were dismantled carefully and slides were left to dry by air at room temperature in the dark. Cells were mounted with Vectashield containing DAPI and covered with a 13 mm circular cover slip. The edge of the cover slip was sealed in

place with transparent nail varnish and slides stored at 4 °C in the dark or visualised immediately with transmission fluorescence microscopy as above (2.201).

2.21 Electron microscopy

Electron microscopy (EM) was carried out to enable assessment of intra-cellular structural integrity and presence of secretory granules in endocrine cells including β -cells before and after transportation. Degranulation of β -cells is a sign of stress and impacts negatively on islet function (Pai et al., 1993). Electron microscopy was carried out by the EM services at both King's College and Newcastle University. A similar protocol was followed at both centres. An average of 1000 IEQs was removed from the overall islet solution stock and was centrifuged at 14000 rpm (16,000 G) for 30 seconds in a 2 ml tube. The supernatant was removed and 1 ml of fresh PBS was added to wash debris and remaining medium and was then centrifuged at 14,000 rpm (16,000 G) for 45 seconds. Supernatant was then discarded and 1 ml Sorenson's fixing buffer was added. Islets were then kept at 4 °C. Fixing buffer was changed after 24 hours. Fixed islets were then transferred to the Electron Microscopy Research Services Unit for processing as per the unit's established protocols (A Advani et al., 2007). Sections were visualised and images were captured using Philips CM100 transmission electron microscope (Biomedical EM unit, Newcastle University, UK).

2.22 Gene expression studies

Reverse transcription (RT) Polymerase chain reaction (PCR) using in either conventional or quantitative methods were employed in the studies of characterisation of gene expression profile in different cells cultures.

2.22.1 Ribonucleic acid extraction

Ribonucleic acid (RNA) was extracted from different cell samples including fresh islets and Islet Survivor Cells (ISCs). All samples were washed initially with PBS. RNA was extracted using GeneElute total mammalian RNA extraction kit (Sigma-Aldrich, Poole, UK, Cat. No. RNA350) following manufacturer's instructions. Total of 2 μ l of RNasin® Plus RNase Inhibitor (Promega UK Ltd, Southampton, UK, Cat. No. N2615) was added to each 50 μ l RNA sample to reduce future degradation of the RNA. All RNA samples were stored at -80 °C.

2.22.2 Reverse transcription

Complementary DNA (cDNA) was synthesised from RNA samples to enable PCR experiments. All reagents were purchased from Invitrogen (Invitrogen Ltd. Paisley, UK) except deoxynucleotide triphosphates (dNTPs) which were purchased from Promega (Promega UK Ltd, Southampton, UK). The procedure was conducted according to Invitrogen RT protocols with minimal modifications: 2 μ l of Oligo(dT)20 primer mix and 2 μ l of 10 mM dNTPs mixture was added to 16 μ l RNA and mixed. The mixture was then heated to 65 °C for 5 minutes followed by immediate transfer to ice. Samples were left on ice for at least 3 minutes. For each sample the following were added: 8 μ l of 5x first strand buffer, 2 μ l of 0.1 M DTT, 2 μ l SuperScript™ III RT (200 units/ μ l) (reverse transcriptase), 8 μ l RNase and DNase free DEPEC-treated H₂O. Samples were mixed and then heated in the thermal cycler at 50 °C for 1 hour. Generated cDNA samples were stored at -20 °C for future analysis.

2.22.3 Conventional PCR

All reagents used for conventional PCR were purchased from Sigma (Sigma-Aldrich, Poole, UK) unless specified otherwise. All oligonucleotide primers (Table 2.12) were

Target gene	Accession	Forward primer	Reverse primer	Product size
Insulin	NM_000207	TACCTAGTGTGCGG GGAACG	GTTCCACAATGCCAC GCTTC	168bp
PDX1	NM_000209	GATGAAGTCTACCA AAGCTCACG	GGCCGTGAGATGTAC TTGTTGAA	165bp
PCNA	NM_182649	CCACTCCACTCTCT TCAACG	AAGTGTCCCATATCC GCAATTT	93bp
Nanog	NM_024865	ACATTGGAAGGTTC CCAGTCG	GGCCTCAGCACCTAC CTACCC	89bp
Oct4	NM_203289.3	GAAACCCACACTG CAGATCA	CGGTTACAGAACCAC ACTCG	81bp
B2M	NM_004048.2	GCCTGCCGTGTGAA CCAT	TTACATGTCTCGATC CCACTTACCTATC	65bp

Table 2.12 Conventional PCR primers.

designed using the online-tool OligoPerfect™ Designer on the Invitrogen website (<http://tools.invitrogen.com/content.cfm?pageid=9716>) and were synthesized by Invitrogen Oligo-Synthesis services. PCR reactions were carried out as follows: 1 µl of cDNA template, 1 µl of 10 mM dNTPs, 1 µl of 0.25 nM forward primer, 1 µl of 0.25 nM reverse primer, 0.25 µl of 5 U/µl Taq polymerase, 2.5 µl of 10x PCR buffer, 18.25 µl PCR grade H₂O. Samples were mixed thoroughly and placed in the thermal cycler (Techne TC-512, Techne Ltd, USA). One universal PCR protocol was used for all primers, unless specified otherwise, and was as follows: 5 minutes at 95 °C; then 35 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 30 seconds. Final extension was carried out at 72 °C for 10 minutes. PCR products were resolved on 1% agarose gel if expected bands were more than 150 bp or 1.5% agarose gel when bands were expected to be less than 150 bp over 45 minutes at 110V. DNA ladder (Sigma-Alrich, Dorset, UK, Cat. No. D3937) was run alongside the samples for size determination. Agarose gels were visualised using UV transilluminator (UVItec complete system, UVItec Ltd, Cambridge, UK).

2.22.4 Quantitative PCR

Quantitative PCR (qPCR) was employed to measure change in gene expression profiles. The SYBR green method was used for detection and analysis of expression profile of proliferative cell nuclear antigen (PCNA) and C/EBP homologous protein (Chop), also known as DNA damage inducible protein 3 (DDIT3). The Taqman probe method was used for insulin and pancreatic and duodenal homeobox 1 (PDX1) expression.

2.22.4.1 SYBR green qPCR method

SYBR green is a double strand DNA fluorescent dye that is utilised in PCR reactions to detect DNA products. The primers employed in these studies (Table 2.13) were

Target gene	Accession	Forward primer	Reverse primer	Product size
Chop	NM_004083.4	AGCTGGAACCTGAG GAGAGA	TGGATCAGTCTGGA AAAGCA	92bp
PCNA	NM_182649	CCACTCCACTCTCTT CAAcG	AAGTGTCCCATATC CGCAATT	93bp
B2M	NM_004048.2	TTCTGGCCTGGAGGC TATC	TCAGGAAATTTGAC TTCCATTC	86bp

Table 2.13 SYBR green qPCR primers.

designed with the universal probe library online primer design tool (Roche 2009). Only DNA primers proven in conventional PCR were used for SYBR Q-PCR. SYBR green master mix for LightCycler480 real-time PCR machine was purchased from Roche Diagnostics Ltd (Roche Diagnostics Ltd, Burgess Hill, UK). PCR setup was carried out in a UV PCR cabinet. Each reaction was assembled as follows: 1 μ l cDNA, 1 μ l of 5 μ M forward primer, 1 μ l of 5 μ M reverse primer, 5 μ l 2x SYBR green master mix, 2 μ l PCR-grade H₂O. Samples were prepared in bulk without the cDNA and dispensed into the appropriate wells in the 96 well PCR plate and then cDNA was added to minimise risk of contaminating reagents with samples. All samples were run in triplicate. Beta-2-microglobulin (B2M) served as a reference gene. A standard curve was constructed with 1:10 serial dilutions of standard cDNA diluted in PCR grade H₂O. Results were analysed using the LightCycler480 software (Roche Diagnostics Ltd, Burgess Hill, UK).

2.22.4.2 Taqman method

This method was employed for the assessment of gene expression changes in insulin and PDX1 in comparison to B2M reference gene. Details of Taqman assays used are summarised below (Table 2.14). Each reaction was assembled as follows: 1 μ l cDNA, 0.5 μ l assay mix, 5 μ l Q-PCR master-mix, 3.5 μ l PCR-grade H₂O. Samples were prepared in bulk without the cDNA and dispensed into the appropriate wells in the 96 well PCR plate and then cDNA was added to minimise risk of contamination. All samples were run in triplicate. Standard curve was constructed with 1:10 serial dilution of standard cDNA in PCR grade H₂O. Results were analysed in the LightCycler480 software.

Target gene	Accession	Taqman assay ID	Amplicon length
PDX1	NM_000209.3	Hs00426216_m1	153bp
Insulin	NM_000207.2	Hs00355773_m1	126bp
B2M	NM_004048.2	Hs99999907_m1	75bp

Table 2.14 Taqman assays.

2.23 *In vivo* assessments

The severe combined immunodeficiency (SCID) mouse model was used as a biological assay to evaluate function and activity of different cells. Two main experiments were carried out, transplantation of primary islets under kidney capsule of streptozotocin (STZ)-treated mice and transplantation of pseudo-islets generated from ISCs in healthy mice. All *in vivo* studies were carried out under Home Office Project Licence: PPL:60/3668, and in accordance with the operating procedures established within the Comparative Biology Centre (CBC) at Newcastle University, in liaison with Prof Paul Flecknell.

2.23.1 Rodent islet transplantation experiments

2.23.1.1 Maintenance of SCID mice

SCID mice, strain: C.B-17/Icr were purchased from Charles River UK Ltd. Animals were housed in cages with a maximum of 4 animals per cage. All cages were maintained in isolator units under sterile conditions. Animals were fed with autoclaved standard chow and were given autoclaved water. Cages were cleaned every 24 hours by the CBC husbandry technician team. All animals in all experiments were incubated with no intervention in the first 7 days to ensure full acclimatisation.

2.23.1.2 Streptozotocin preparation

Streptozotocin (STZ) (Sigma-Aldrich, Dorset, UK, cat. no. S0130) was stored in the dark at 20 °C. STZ was reconstituted in citrate buffer pH 4.5 immediately before administration. Citrate buffer was prepared as follows: 0.19 g of citric acid was diluted in 10 ml H₂O (0.1M) and 1.47g Na Citrate was diluted in 50 ml H₂O (0.1M). Equal volumes were mixed and pH was adjusted to 4.5 with careful addition of HCl if required. The final buffer was sterilized through a 0.2 µm nylon filter. Citrate buffer

was stored at 4 °C overnight and was used strictly within 24 hours of preparation. STZ stock was freshly prepared at 30 mg/ml just before intraperitoneal administration. Dose was calculated according to the weight of each animal.

2.23.1.3 STZ injection method

Animals were fasted for 8 hours starting at midnight. All animals were weighed and their clinical score was documented (Table 2.14). Animals were labelled with tail bands to differentiate different groups. STZ injection volume was calculated according to animal's weight to deliver the required dose of STZ through the intraperitoneal route. An insulin syringe was used to inject STZ solution.

2.23.1.4 Glucose monitoring

Monitoring of animal blood glucose was carried out using an UltraSmart blood glucose meter (Lifescan Ltd, High Wycombe, UK). The blood sample was obtained from the tail vein. An average of 5 µl of blood was required for each test. Glucose readings were documented on a data collection sheet.

2.23.1.5 Islet preparation for transplantation

Islets cultured in suspension were removed from the incubator and transferred to a 50 ml conical tube with centrifugation for 2 minutes at 1000 rpm (32.5 G). Supernatant was discarded and the islet pellet was resuspended in 5 ml PBS. A sample of 100 µl was removed for islet count. Remaining islets were centrifuged as above and supernatant was discarded. The islet pellet was resuspended in transplant medium (Table 2.2). The islet suspension was made up to a final concentration of 5000 IEQs/ml (500 IEQs per 100µl). Islets were mixed thoroughly but gently before distributing aliquots of 100 µl to 1.5 ml sterile tube for each animal to be transplanted. Tubes were kept on ice and taken to the Comparative Biology Centre. Islets were left

to settle in the tube on ice. Approximately 50 μ l supernatant medium was removed using a P200 pipette. The remaining 50 μ l (containing the islets) was aspirated into a yellow tip and the end was blocked with Blu-Tak® (Staples, Doncaster, UK. Cat. No. 389533) and placed in a 2 ml tube and centrifuged just before transplantation to achieve an islet pellet. The islet pellet was transplanted under kidney capsule of the mouse using a P200 Gilson to deliver the pellet. Occasional leakage due to inefficient blu-tak seal was observed. On those occasions the sample was discarded and another one was prepared.

2.23.1.6 Transplantation protocol

The transplant procedure was carried out in a laminar flow safety cabinet to ensure sterility. Animals underwent general anaesthesia using 20-30% isoflurane in an anaesthesia chamber. The right flank of the abdomen was shaved and the whole of the abdomen was cleaned with 70% isopropyl alcohol and 0.5% chlorhexidine solution (Adams Healthcare; Leeds, UK). The body of the animal was covered with a sterile surgical cover giving access only to the right flank where a longitudinal incision was performed to enable right kidney externalisation. An incision in the lower part of the kidney capsule was made using a 23G needle tip. Islets or medium alone were injected under the kidney capsule very slowly to avoid kidney capsule rupture. Continuous gentle pressure for 2 minutes was applied to prevent leakage from the incision site while irrigation with 0.9% NaCl ensured avoiding organ dehydration. The abdominal wall was sutured and the transplanted animal was resuscitated and kept in a warm environment until full recovery. Animals of each group were housed back in one cage and transferred to the isolators. Upon completion of the study all animals were killed by neck dislocation according to Home Office Schedule One.

2.23.1.7 Intra-peritoneal glucose tolerance test

This was carried out to enable assessment of glucose tolerance and islet graft function in the transplanted animals. Glucose solution was prepared at 100 mg/ml in sterile water. Solution was further filter-sterilised using a 0.2 µm nylon filter. All animals were fasted overnight by removal of chow feed but maintained access to sterile water. In the morning animals were weighed and given an intra-peritoneal injection of D-glucose solution to deliver an equivalent of 1.5 g/kg. Injection time was considered to be 0 minutes. Blood glucose was measured at 0, 30, 60, 90 and 120 minutes.

2.23.2 Pseudo-islet transplantation experiment

2.23.2.1 Maintenance of SCID mice

SCID mice (strain: C.B-17/Icr) for this experiment were purchased from Harlan laboratories (Harlan Ltd., Bicester, UK). Two groups of 5 mice were housed in filter-cages and maintained in high standard cleanliness with autoclaved standard chow feed and access to sterile water. Cages were cleaned every 24 hours by the CBC husbandry technician team. All animals had a 7 day acclimatisation period before transplantation was performed.

2.23.2.2 Graft preparation

One day before the transplantation procedure ISCs were established in 6 wells in a 6 well plate provided with MW inserts for pseudo-islet generation as described before (2.12.4.2). At 24 hours an average of 1500 PIs from each well, containing an average of 2×10^6 cells, were retrieved and pooled into 200 µl CMRL NCL medium supplemented with 1% HSA and kept on ice ready for transplantation.

Human skin fibroblast cells (HDFn) were kindly provided by Dr Helen Swalwell from the Dermatology Department at Newcastle Medical School and were used as a negative control. HDFn cells were cultured in 6 wells in a 6 well plate at 1.5×10^6 cell

per well in 4 ml DMEM medium (PAA Laboratories Ltd, Somerset, UK) supplemented with 10% FCS and 1% P/S 24 hours before planned transplantation. At 24 hours post-culture cells were recovered following washing twice in PBS and TrypLE express treatment for 5 minutes at 37 °C. The cell pellet from each well was washed in PBS to remove TrypLE traces and finally resuspended in 200 µl CMRL NCL medium supplemented with 1% HSA and kept on ice ready for transplantation.

2.23.2.3 Equipment setup

Transplantation equipment was a kind gift from Professor Gregory S Korbitt from Alberta Diabetes Institute. This equipment was developed and constructed by Professor Korbitt and his team to enable efficient and high throughput transplantation in small animal models (Rayat et al., 2003). A green needle (Gauge 23) was attached to the micromanipulator (MM) and then 30 cm of PE50 tubing (Instech Laboratories, Inc.Plymouth Meeting, PA USA. cat. no. BPE-T50) was connected to the needle by sheathing over it. Graft solution was withdrawn into the PE50 tubing using the MM micro dial (Figure 2.14). The tip of the tubing was tied tightly to prevent leaking and the MM was then attached to the rotating motor and spun for 30 seconds at 3000 rpm. (362.9 G) This enabled graft separation from excessive medium (Figure 2.14). The tie was cut and the graft was then ready for sub-renal capsule delivery. Graft spinning was carried out by an assistant just before kidney externalisation.

2.23.2.4 Transplantation procedure

The transplant procedure was carried out in a laminar flow safety cabinet to ensure sterility. Animals underwent general anaesthesia using 20-30% isoflurane in an anaesthesia chamber. The left flank of the abdomen was shaved and the whole of the abdomen was cleansed with 70% isopropyl alcohol and 0.5% chlorhexidine solution (Adams Healthcare; Leeds, UK). The body of the animal was covered with a sterile

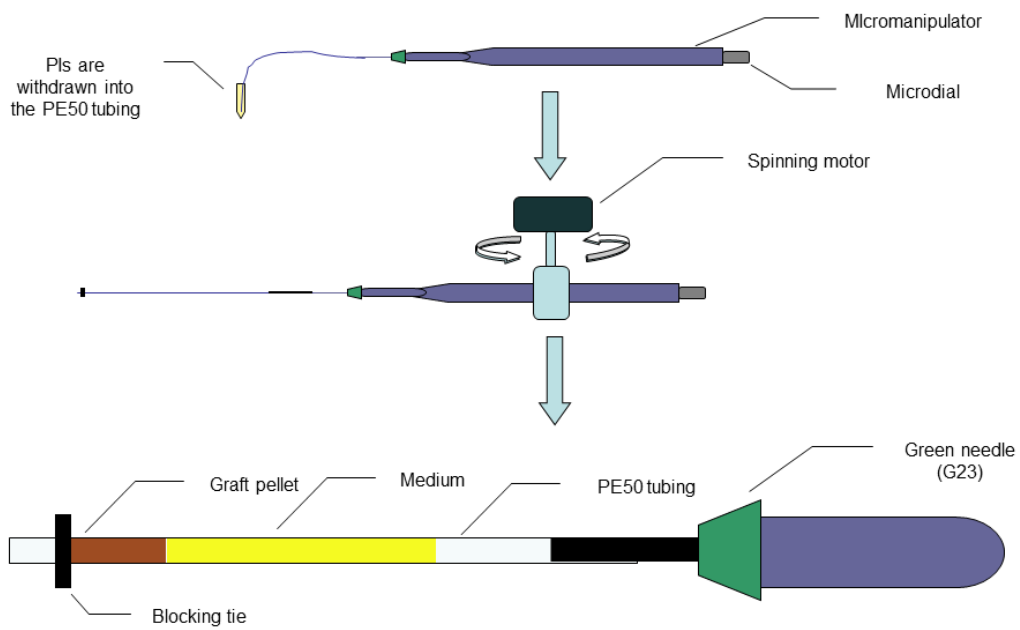


Figure 2.14 Micromanipulator and graft preparation. Cell solution is withdrawn into the PE50 tubing then distal end of the tubing is tied firmly. Micromanipulator is then attached to a spinning motor to enable centrifugation and separation of graft pellet from excess medium.

surgical drape giving access only to the left flank where a longitudinal incision was performed followed by left kidney externalisation and an incision in the lower part of the kidney capsule was made using a sterile glass rod (Figure 2.15 A). At this point the graft was prepared for transplantation as outlined above (2.23.2.3). PE50 tubing was inserted under the kidney capsule through the incision and the graft was slowly delivered using the micodial in the MM (Figure 2.15 B). Once delivery of the graft was completed the incision was cauterised (Figure 2.15 C). The kidney was then returned into the abdominal cavity and the abdominal wall was sutured. The transplanted animal was resuscitated and kept in a warm environment until full recovery. Control animals were transplanted with HDFn cells and test animals were transplanted with PIs. Animals were maintained for 17 days post-transplantation. Blood glucose and weight were checked on a regular basis. Before termination at day 17 an IPGTT was carried out. Upon completion of the study all animals were killed by neck dislocation according to Home Office Schedule One.

2.23.2.5 Intra-peritoneal glucose tolerance test

Animals were fasted overnight in preparation for IPGTT on day 17. All animals were injected intra-peritoneally with 1.5 mg/kg glucose at time 0. Blood samples were collected at 0 and 30 minutes from tail tip (75 µl) and a 60 minutes sample was collected from cardiac puncture following neck dislocation as outlined before (2.23.2.4). Blood samples were collected in heparinised tubes (SARSTEDT Ltd., Leicester, UK. Cat. No. 16.443) and kept on ice until the end of the procedure. Tubes were centrifuged at 14,000 rpm (16,000 G) to enable plasma separation. An average of 25 µl plasma sample was recovered from each tube and was frozen at -20 °C. Samples were later analysed for insulin content using ultrasensitive insulin ELISA (Merckodia via Diagenics, Melton Keynes, UK, cat. No10-1132-01).

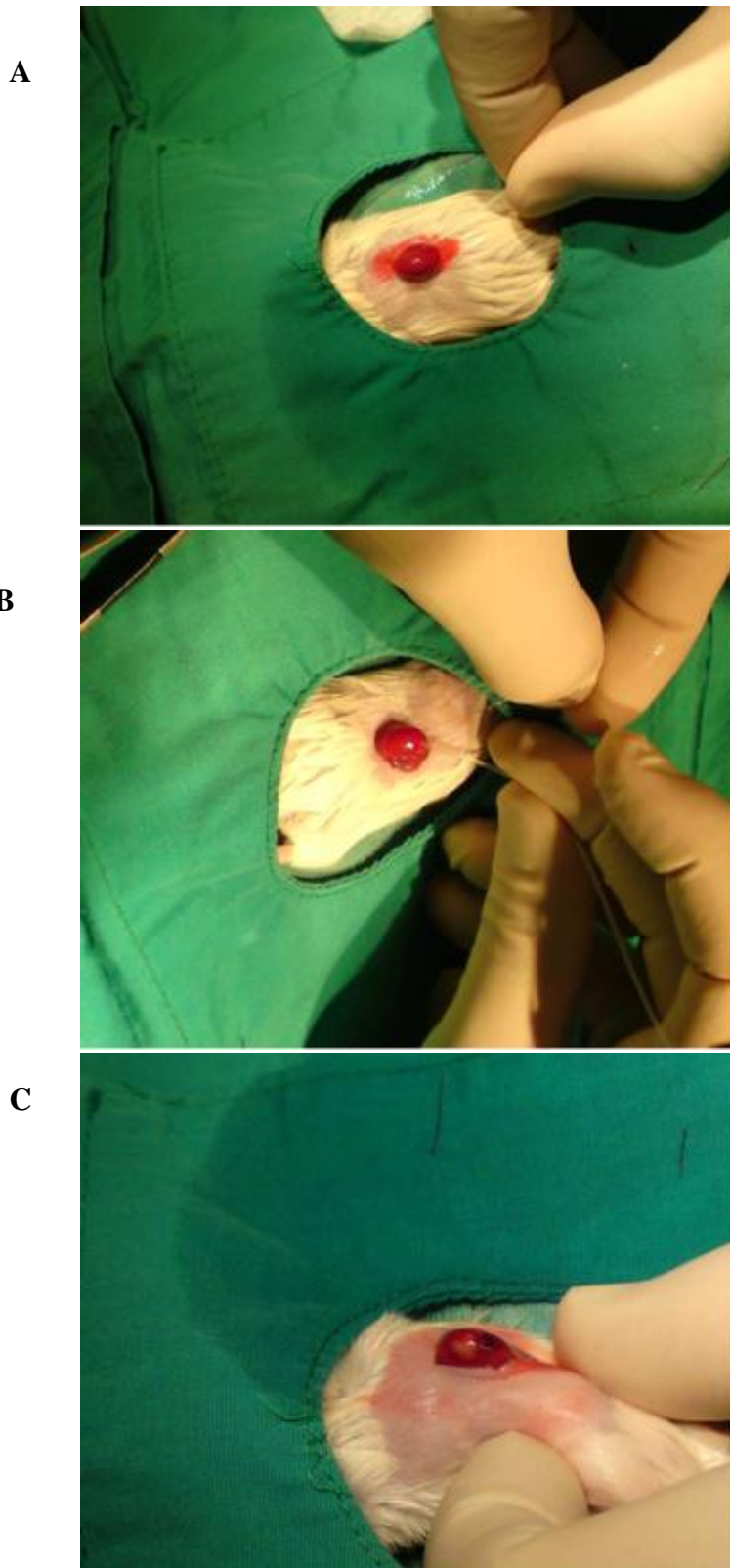


Figure 2.15 Sub-renal capsule transplantation. A: Left kidney externalisation and creation of an incision in the capsule to enable graft delivery. B: Delivery of graft under kidney capsule using PE50 tubing. C: Cauterised incision following completion of graft delivery.

**Chapter 3. Development and validation
of transport and pre-transplant quality
assurance protocols for clinical grade
human islets isolated at a remote islet
isolation facility**

3.1 Introduction

Viable islet transplantation programmes require a sustainable, efficient and effective supply of highest quality clinical-grade purified human islets. Production of such islet preparations is technically challenging and facilities that are able to produce such quality are usually long-established with experience gained over several years (Ault, 2003; Hering et al., 2004; Ricordi and Strom, 2004). Moreover, islet isolation facilities are sophisticated and expensive laboratories with an average cost of establishment of \$2 million (Kempf et al., 2005) and require highly trained personnel with 24 hour availability. Utilisation of a central islet isolation facility for processing of donor pancreases and production of clinical grade islets can enable clinical islet transport programmes in several geographically distant centres. This has been trialled in the mid-1990s with the collaboration between Portland and Minneapolis for auto-islet transplants which provided validation and proof of feasibility for successful clinical transplantation following islet transportation (Rabkin et al., 1999). Thereafter, several centres in the world adopted islet transport approaches for allografts, including Groupe Rhin-Rhone-Alpes-Geneve pour la Transplantation d'Ilots de Langerhans (GRAGIL) (Kessler et al., 2004; Kempf et al., 2005; Benhamou et al., 2001), the Nordic Network for Clinical Islet Transplantation (NNICT) (Rydgard et al., 2001), and the Miami-Houston transport program (Ichii et al., 2007b) with proven success. Protocols, when published, varied considerably between different centres. These variations were in most cases due to the specific setting of each centre and the lack of standardised published methods.

3.1.1 The GRAGIL experience

The GRAGIL group was established and became active in March 1999 as a French-Swiss collaboration for islet transplantation (Benhamou et al., 2001). Initially the

network included 5 centres in France and Switzerland with geographical proximity to one islet isolation facility, but later this was expanded to include more centres (Kempf et al., 2005). The network was designed to function as a single entity, having a common donor pool and a single waiting list in addition to congruent protocols in all transplant centres. Pancreas retrieval from deceased donors was carried out by the specialist team at each centre. Retrieved pancreases were transported to the Geneva islet isolation facility in University of Wisconsin Solution (UWS) on ice in an isothermic box by ambulance for islet isolation. Cold ischemic time was less than 8 hours in all cases. Islet isolation was carried out following standard protocols (Benhamou et al., 2001). Isolated islets were counted using the dithizone (DTZ) staining method and functionality was assessed by determination of glucose-stimulated insulin secretion in static incubation studies, 24 hours before transplantation. Viability was assessed by microscopic morphological assessment although no specific protocol was cited in the published work. In the first phase (between March 1999 and June 2001) 116 pancreases were offered with 56 (48.3%) accepted for islet isolation. Only 20 of these yielded transplantable islets. Isolated islets were cultured initially in CMRL 1066 medium supplemented with 10% FCS at 24 °C. Islets were transplanted within 24 hours when islet yield was >6000 IEQs/kg (transplantation threshold) or cultured for up to 7 days at 24 °C then transferred to 37 °C for a maximum of a further 7 days (14 days in total). The purpose of the prolonged culture was to enable pooling of preparations with lower yield to reach the transplantation threshold (6000 IEQs/kg). For transport, islets were prepared in 50 ml syringes in X-Vivo medium containing 4% human serum albumin (HSA) (Benhamou et al., 2001). Islet transport was carried out at room temperature by ambulance services and the journey times were ≤5 hours. A clinician from the isolation centre

accompanied the islets to the transplant site on all occasions to supervise the transplantation procedure. No assessment of transported islets was undertaken at the recipient centres and islet infusion was carried out directly with the transported material. Despite immediate function in all islet recipients there was loss of function (C-peptide <0.5ng/ml) at 10 months in 50%. However, 20% became insulin independent and maintained this over the following 12 months.

In the second phase (January 2002 to December 2003) 260 pancreases were offered to the network with 92 (35%) being isolated of which 44 (17%) were successful and 42 (16%) were transplanted. In phase 2 there were some changes in the culture and transport protocols with avoidance of FCS in all media and a change in culture medium to Miami-defined Medium (MM1) while reducing culture period to a maximum of 72 hours although culture temperature was unclear. For transport / transplant, medium was changed to CMRL 1066 and islets were packaged in transfer bags (locally-made (Baidal et al., 2003)) instead of syringes (Kempf et al., 2005).

The GRAGIL experience confirmed for the first time the feasibility of a multi-centre islet transplant program approach utilising a centralised isolation facility. The protocols adopted by this network have continued to evolve over time but details of changes have not been reported in any detail.

3.1.2 The Nordic Network for Clinical Islet Transplantation experience

The network was established in 2001 employing one central islet isolation facility serving 6 geographically distant transplantation centres. All transplantation centres contributed towards pancreas procurement and transplantation was carried out according to ABO compatibility on the waiting list. Immune suppression protocols were similar to Edmonton protocol. Isolated islets were initially transplanted without

a period of culture. However later islets were cultured for a period of 24-96 hours prior to transplantation. Detail of the transportation system used by the network is not published (Lundgren et al., 2004; Rydgard et al., 2001).

3.1.3 The Miami-Houston and Miami-Dallas experience

The Diabetes Research Institute (DRI) in Miami houses a very well established islet isolation facility and several islet research groups. The first collaborative islet transport program between DRI and the Methodist Hospital (TMH) in Houston was established in January 2002. Later on, the DRI collaboration expanded to include Baylor Regional Transplant Institute in Dallas, Fort Worth. Pancreases were retrieved by the satellite centres and transported by air to the DRI using either the two layer method (oxygenated-perfluorocarbon (PFC) / UWS) or UWS alone. Cold ischemic time was less than 7 hours (Barshes et al., 2004). Islet isolation was carried out according to standard DRI protocols. Islets were assessed at the DRI for sterility, viability, purity and IEQ count. Islets were either cultured for a short period (average 32 hours) or transported immediately to the satellite centre. Culture was carried out in Miami Medium 1 (MM1) containing 0.5% HSA in non-adherent vented T175 flasks at 37 °C in a humidified 5% CO₂ incubator. Islets were transported to the satellite centres in gas-permeable bags in MM1 medium without sodium bicarbonate at room temperature in a temperature-controlled container to prevent temperature changes during transport. Transportation was carried out using charter jet and transport journeys were less than 5 hours. Upon arrival islets were pooled for reassessment of sterility (endotoxin assay, Gram stain, bacterial culture), viability (FDA/PI), IEQ estimation (DTZ staining) and islet function (Static Stimulation index). All parameters assessed were comparable between the DRI and the satellite centres. The

DRI team concluded that gas-permeable bags were superior to standard packaging methods for transportation (Ichii et al., 2007b).

3.1.4 The UK status

Several centres in the UK have focused research on islet isolation and transplantation with considerable success. These include Leicester, King's College and Royal Free Hospitals in London and the Oxford Centre for Diabetes Endocrinology and Metabolism (OCDEM) in Oxford. Transplantation activity had been confined to each centre's locality with no islet transportation distally for transplantation being carried out before September 2005 (when this research was commenced). While this limited geographical access to transplantation, a further limiting factor was restricted access to sufficient donor material for patients on their waiting lists.

3.2 Aims

The overall aim of this chapter was to test the hypothesis that islet sterility, mass, viability and function can be maintained during the relatively long road transport distances within England to enable transplantation of quality-assured clinical grade transported islets from a central isolation facility. Specific objectives were to:

1. Develop and validate islet viability and functional assessment protocols in rodent islet models
2. Establish and validate Standard Operating Procedures (SOPs) for pancreas transport from the North of England to King's College islet isolation facility and for safe and effective islet transport back to Newcastle
3. Achieve successful clinical islet transplantation of transported islets

3.3 Ethical approval

Approval from both local (Newcastle upon Tyne Hospitals Foundation Trust) and national (Multi Centre Research Ethics Committee, MREC) was obtained before commencement of work. All pancreases were offered and accepted in the program solely when informed consent for use in this research was given by donor relatives.

3.4 Refinement and validation of viability and function assessment tools

3.4.1 Viability assessment protocol

Gray and Morris were first to describe use of Fluorescein diacetate (FDA) / ethidium bromide (ET) to evaluate islet viability (Gray and Morris, 1987). This method was widely adopted due to its simplicity and ease of use. However many centres substituted ET with Propidium iodide (PI) to avoid the potential carcinogenic risks associated with ethidium bromide. This method is however prone to false staining with ET or PI if scoring is not carried out within 5 minutes due to toxic effect of the solvent used to elute FDA leading to cell membrane compromise and thus ET or PI leak inside affected cells.

Validation of this technique was carried out in Newcastle initially employing digested rodent pancreatic tissue. Collagenase digested pancreatic cells were stained with FDA/PI following the same protocol used by King's College islet facility (Huang et al., 2004). This involved washing cells with PBS and then staining them with the concentrations of FDA/PI outlined in the Methods section (2.10). It was challenging to visualise and accurately score viability in stained islets within the 5 minutes thus avoiding false staining. This is an issue specifically when staining and scoring, is carried out by a single person.

Validation was undertaken to elucidate the potential benefit of an additional washing step. Preparation of rat islets were stained with FDA/PI as outlined before (2.10). Islets were visualised over time either with or without an additional PBS washing step straight after completion of FDA/PI staining (2.10). Both groups were maintained at room temperature and visualised under fluorescent microscope at 3, 10, 15, 30 minutes (Figure 3.1). In the existing protocol PI staining was noted to increase beyond 10 minutes in comparison to islets with additional washing step.in which consistent PI staining was noted even with late visualisation. Therefore the washing step was incorporated in all human islet staining studies.

3.4.2 Functional assessment by static *in vitro* glucose stimulated insulin secretion

Glucose stimulated insulin secretion (GSIS) is the main functional assay that is commonly used to assess functionality of islets. There are numerous protocols for assessing this with varying results between centres (Brandhorst et al., 1999; Lee et al., 2008a). Rat islets were employed in the present studies to optimise effective protocol for robust and reproducible evaluation of GSIS. An average of 20 islets in 2 ml medium was employed in each of 3 repeats included in either glucose treatment condition. Initial experiments carried out at room temperature and at 30 °C (2.15.1) showed no meaningful increase in insulin release in response to glucose challenges which was consistent with other groups' observations (Dr GC Huang and Professor P Jones at King's College, London, personal communications). All subsequent validations and experiments were carried out at 37 °C. Nevertheless, wide range of variability between samples in each condition could not be avoided (Table 3.1), making results difficult to interpret. This variability was attributed to differences in

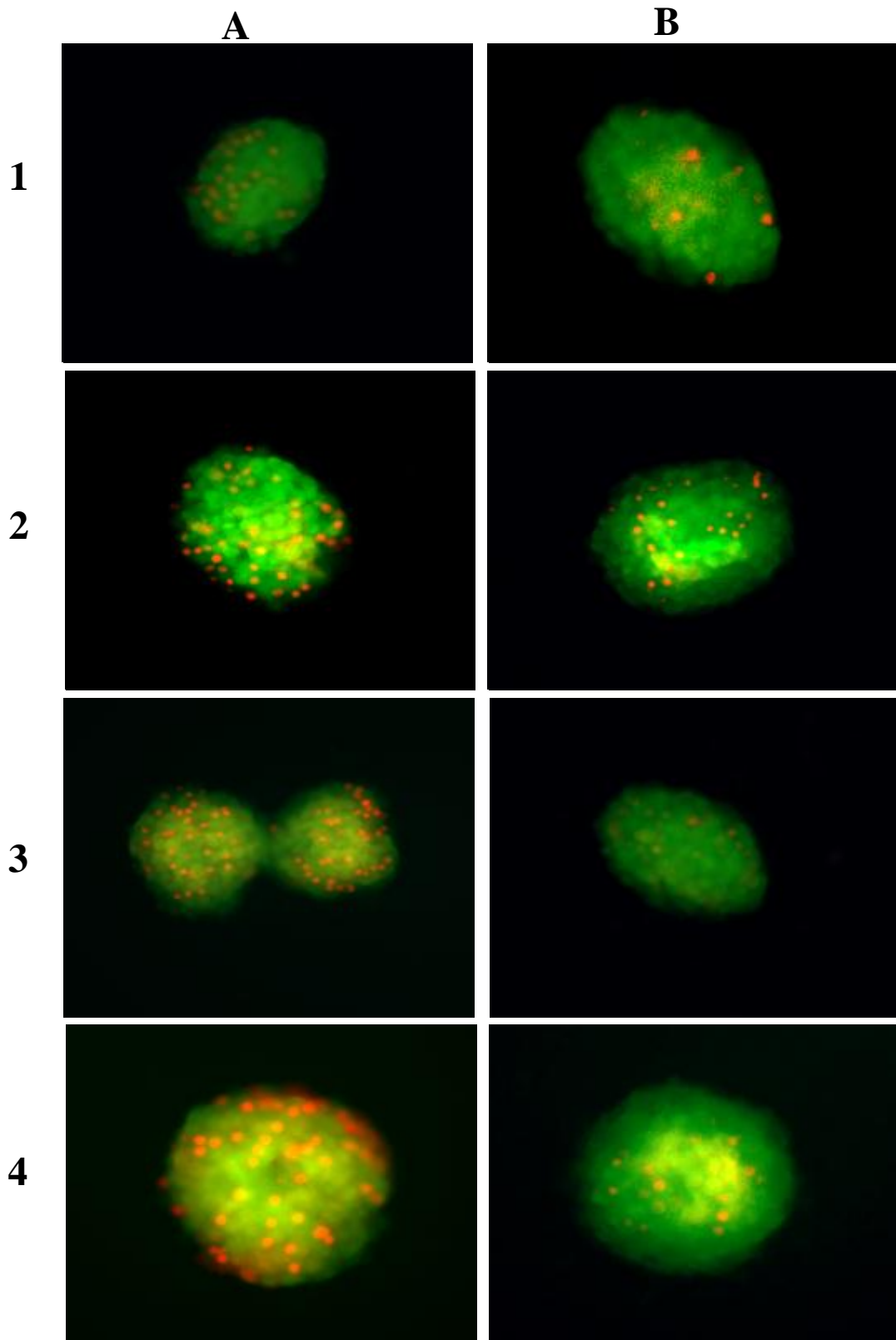


Figure 3.1 Viability staining validation in rodent pancreatic islets. Rat islets were stained with FDA/PI following pre-existing standard protocol (A) or with addition of a washing step (B). Viability was evaluated at 3 minute (1), 10 minutes (2), 15 minutes (3), 30 minutes (4). There was evident increase in PI staining over time in group A while similar consistent PI staining was seen in group B despite prolonged incubation.

Repeat	Insulin release in pmol/l	
	2mM	20mM
Rat islet repeat 1	421.04	312.63
Rat islet repeat 2	596.57	845.05
Rat Islet repeat 3	81.18	1430.59
Mean	366.26	862.76
SD	262.03	559.19
Fold change	2.4	
T test	0.24	

Table 3.1 Variability in static GSIS studies in rat islets. The glucose challenge (2 mM vs. 20 mM) on an average of 20 islets was successful in generating a mean 2.4 fold increase in insulin secretion. However, due to wide variability within each treatment group this did not reach statistical significance.

islet size and sample purity between replicates despite attempts to ensure homogeneity.

These experiments informed further refinement of the static GSIS protocol used in later experiments to include at least 6 repeats in each glucose condition (2 mM and 20 mM) while carrying out the entire experiment at 37 °C in a tissue culture incubator (2. 15). This enabled consistent and reproducible confirmation of statistically significant GSIS in primary rat islets (Figure 3.2). However, despite intensive efforts to refine and optimise the technique (trailing different islet numbers or different volumes, use of shaking 37 °C water bath) (2. 15) the fold change in insulin release remained relatively smaller than that reported in some of the literature (Burns et al., 1997; Hansson et al., 2004).

3.4.2.1 *In vivo* assessment of islet function

Islet transplantation in the sub-renal capsule of STZ-treated SCID-mice is considered the most accurate bio-assay for the assessment of how well islets will perform after clinical transplantation. Good quality islets with preserved activity can normalise hyperglycaemia in this model while stressed and apoptotic islets fail to do so. Protocol optimisation was carried out using rat islets.

No previous SCID mouse experiments had been carried out within our group thus this experimental model was established and optimised as part of this PhD project.

3.4.2.1.1 *Optimisation of STZ dose in SCID mice*

Streptozotocin (STZ) is a β -cell toxin that enters the cell through glucose transporter (Glut2) channels (Schnedl et al., 1994). The optimal dose is dependent on both animal (species and strain) and STZ preparation (brand and lot number). Local optimisation was thus required and an initial guiding dose of 185 mg/kg was used based on the experience of King's College Islet Isolation Facility (KCIIF). A series of experiments

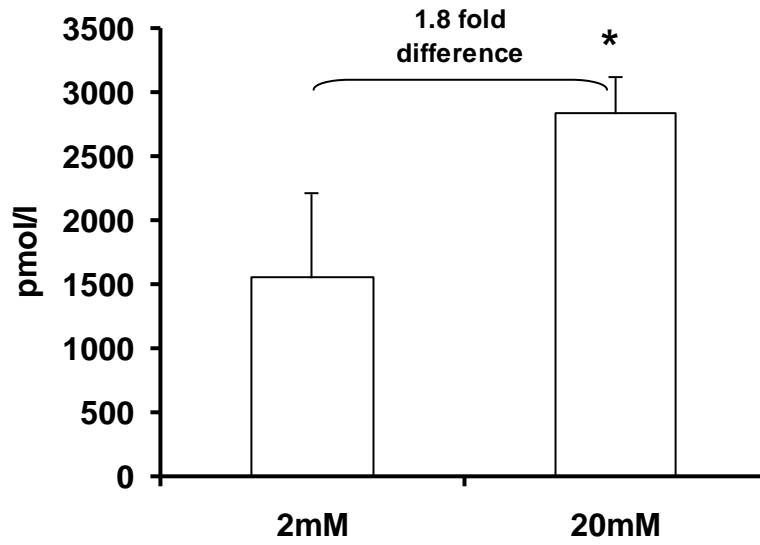


Figure 3.2 GSIS in rat islets. Equal aliquots of 25-30 rat islets were incubated in DMEM medium supplemented with either 2 mM glucose or 20 mM glucose for 2 hours in a tissue culture incubator at 37 °C. Insulin released into the medium was assayed by rat insulin ELISA. The experiment demonstrated a significant 1.8 fold increase in insulin release (n=6, mean ± SD, p=0.0013).

were undertaken. The first experiment included 6 SCID mice (20-25 g weight). Following acclimatization groups of two animals had intra-peritoneal (IP) injection of 180 mg/kg, 185 mg/kg or 190 mg/kg STZ. Blood glucose from a tail sample and weight were measured at baseline and at 3, 8, 11, 17 days. None of the animals developed hyperglycaemia.

Our group's previous experience with rat STZ treatment had demonstrated the importance of animal fasting to enable consistent and effective diabetes induction; therefore fasting was trialled in the subsequent experiment. Six animals were included, divided into two groups with 3 mice in each. The night before treatment one group was fasted for 9 hours overnight while the other group had free access to feed. Both groups were injected intravenously with STZ at 180 mg/kg. Blood glucose from tail sample was measured at baseline and 3, 6 and 10 days. Non-fasted animals did not develop hyperglycaemia and maintained their weight. One animal in the fasted group showed a marginal increase in blood glucose and one animal developed overt hyperglycaemia (Figure 3.3). All animals, apart from the diabetic animal, maintained their weight. The diabetic animal showed progressive weight loss during experiment follow up. This study confirmed a positive effect of fasting on potentiating the effect of STZ. However, the 180 mg/kg dose was not sufficient to induce consistent diabetes in all animals in the group.

In the following experiment only 3 animals were employed. One animal was fasted overnight for 9 hours. The following morning it was injected with 190 mg/kg STZ. Baseline blood glucose on day 3 and 7 were measured. This resulted in severe hyperglycaemia with significant weight loss requiring consideration of euthanasia (Figure 3.4 A).

On day 7 the other two animals were injected with 185 mg/kg STZ following overnight 9 hours fast. Weight and blood glucose levels were collected at baseline and over the following week. One animal developed overt hyperglycaemia while the other developed marginal hyperglycaemia (Figure 3.4 A).

Weight of all animals with blood glucose levels above 20 mM in this and the preceding study, decreased over the follow-up period confirming the metabolic defect (Figure 3.4 B).

To avoid severe hyperglycaemia with 190 mg/kg STZ and inconsistent induction of diabetes with 185mg/kg, we elected to adopt 187.5 mg/kg STZ with 9 hours fasting the night before diabetes induction for subsequent studies.

3.4.2.1.2 Validation of rat islet transplantation in STZ- treated SCID-mice

In this experiment 6 SCID mice were included. Following 7 days acclimatisation animals were fasted overnight. All animals were injected with 187.5mg/kg STZ (day 0). Hyperglycaemia was successfully achieved in all animals (Figure 3.5 A). On day 7, transplantation was performed (2.23.1.5 and 2.23.1.6) with one animal being successfully transplanted under the kidney capsule with 500 rat islets while the remaining 5 were transplanted with vehicle. Blood glucose and body weight were monitored over 28 days. Within 10 days post transplantation the islet-transplanted animal showed improved fasting glucose levels while control animals showed progressive hyperglycaemia. On day 23, intra-peritoneal glucose tolerance test (IPGTT) was performed following overnight fast (2.23.1.7). The transplanted-animal demonstrated ability to reduce blood glucose after 30 minutes of initial increase while control animals failed to do so (Figure 3.5 B). This confirmed capacity of transplanted islets to restore regulated glucose homeostasis in STZ-induced hyperglycaemia mouse model confirming validity of this protocol for assessment of islet function. This

Figure 3.3 STZ dose optimization-impact of fasting. All animal were injected with 180mg/kg STZ. One animal showed marginal increase in blood glucose and one had overt hyperglycaemia, both in the fasted group.

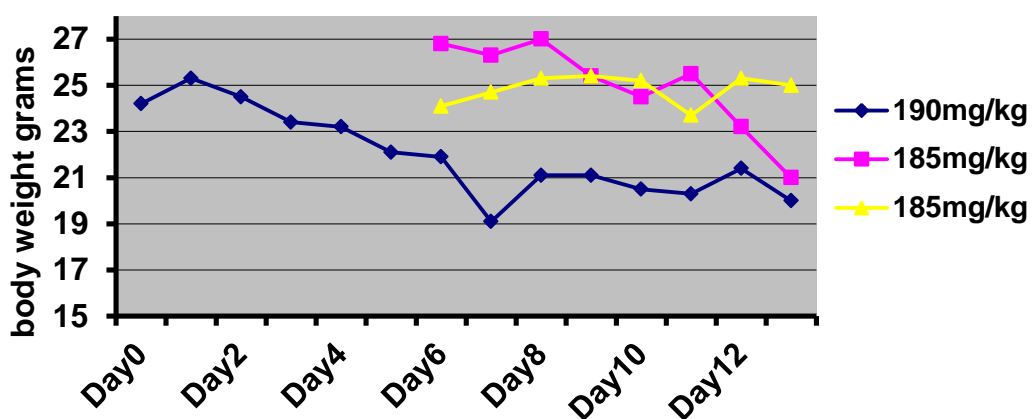
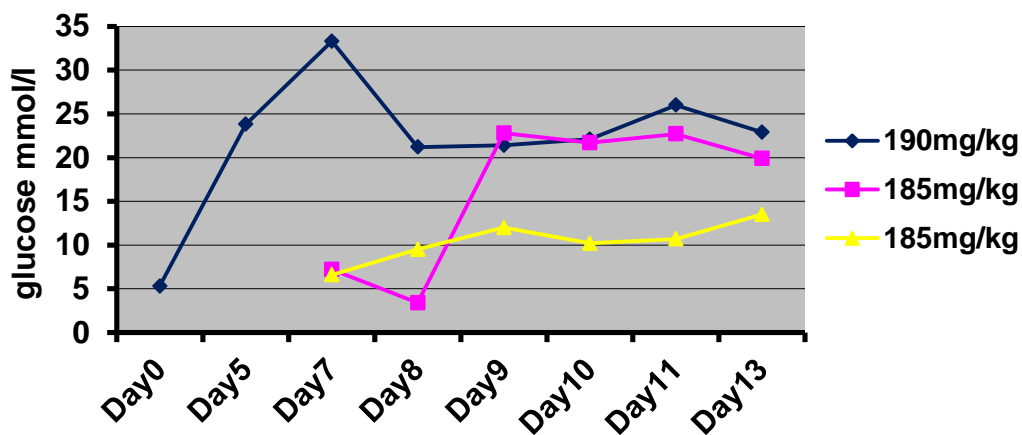


Figure 3.4 STZ dose titration. Animals were injected with STZ at 190mg/kg or 185mg/kg and their blood glucose and weight were monitored daily. 190mg/kg STZ resulted in severe hyperglycaemia (A) and significant weight loss (B). 185mg/kg STZ was not sufficient to reproducibly induce hyperglycaemia in all animals

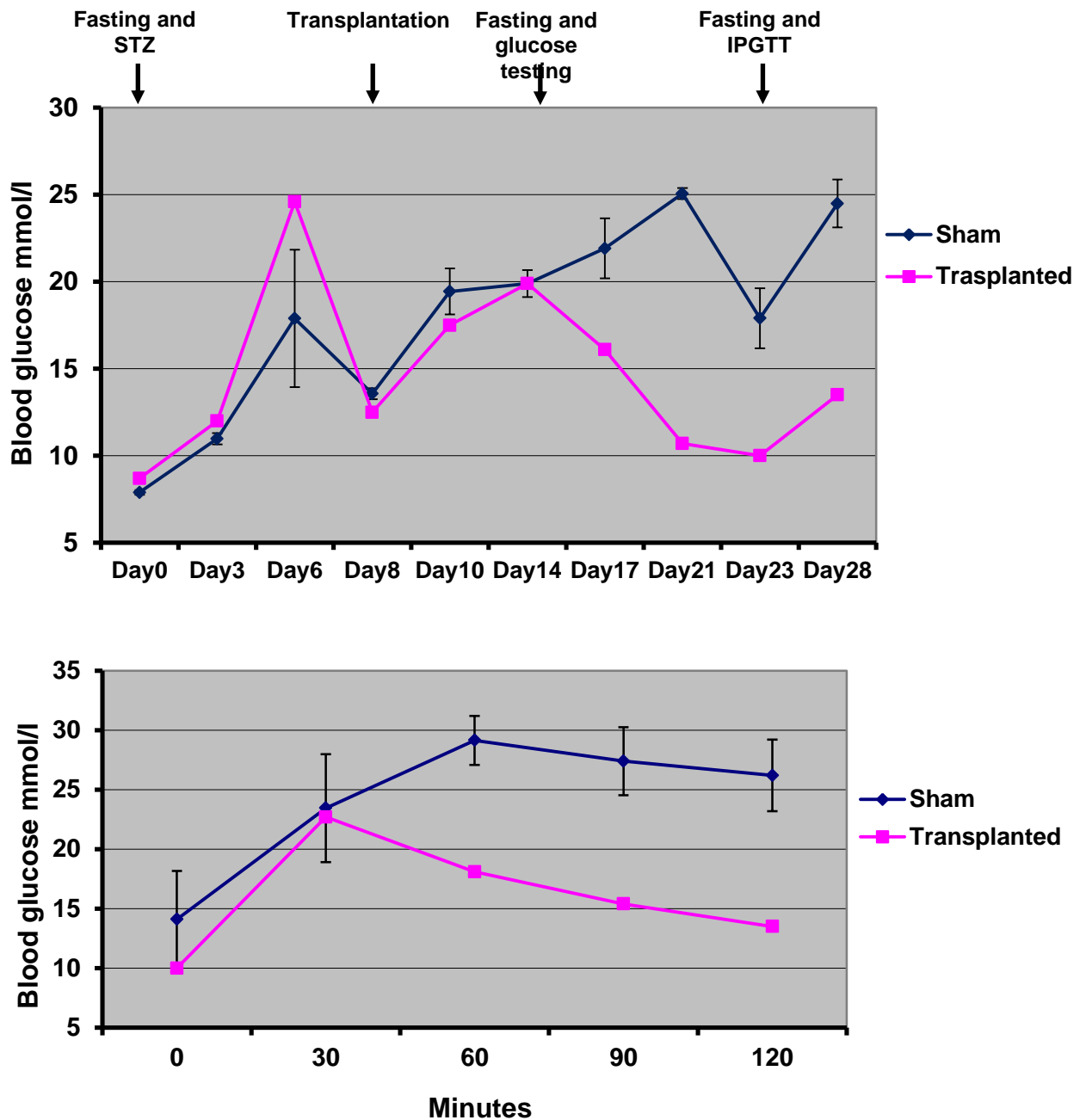


Figure 3.5 Rodent islet transplantation. A: Daily glucose monitoring in SCID mice demonstrating hyperglycaemia post STZ induction. Transplantation on day 8 resulted in gradual improvement in glucose levels in the islet recipient towards normal levels by day 21 while sham animals (n=5) maintained hyperglycemia. B: IPGTT, at day 23 blood glucose measured every 30 minutes. The islet recipient mouse was able to reduce blood glucose to near-normal levels at 120 minutes while sham animals had consistently high blood glucose throughout. Results of sham animals are plotted as mean of $5 \pm SD$.

protocol was subsequently adopted for human islet assessment.

3.5 Pancreas retrieval

Pancreas retrieval is a very well validated and adopted process nationally for solid organ transplantation and therefore no optimisation of this was carried out in rodent models.

3.5.1 Human pancreas offers

Pancreases from heart-beating deceased donors in the North of England were offered to the islet program when not used for whole organ transplantation. All pancreas offers with research consent were accepted in the absence of any exclusion criteria and with occasional acceptance of organs with longer than 12 hours CIT during the research phase (Table 3.2). Due to the activity of the local whole pancreas transplant program with vascularised transplantation having the priority, there were a very limited number of good quality organs offered to the islet program, as both programs shared similar inclusion criteria. Since all pancreases were retrieved from multi-organ donors, criteria were satisfied in most cases. Every accepted organ was given a chronological identification number starting with LDIS (Local Donor for Islets). All offered and accepted organs were transported and processed for islet isolation at King's College Islet Isolation Facility (KCIIF) (total of 10). The morphology of each pancreas was assessed by Dr Huang at KCIIF prior to commencement of islet isolation. Out of the 10 pancreases included in this study 4 (40%) were fatty, 1 (10%) had reduced peri-pancreatic fat and was noted to be oedematous, secondary to confirmed pancreatitis, 1 (10%) had abnormal vasculature, with the remaining 4 (40%) appearing macroscopically normal (Table 3.3).

Parameter	Value
Age	<20 years and >65years
Diabetes	Yes
Drug abuse	Yes
Alcohol abuse	Yes
Positive serology	Hep B, Hep C, Treponema, HIV
Chronic Kidney Disease	Yes
Malignancy	Yes
Recurrent pancreatitis	Yes
Hospital stay	>10 days
Pancreas abnormalities	Nodular, fibrotic, gross fat infiltration
Cold ischemic time	>12 hours
Warm ischemic time	>30 minutes

Table 3.2 Pancreas exclusion criteria. During the research phase organs with cold ischaemic time longer than 12 hours were occasionally considered.

Donor							Pancreas			Islet								
Code	Date	Sex	Age years	BMI kg/M ²	BG&Rh	Primary pathology	CIT hours	anatomy/pathology	Transport method	transport (hours)	viability		purity		integrity		IEQ count	
											KCIF	NCL	KCIF	NCL	KCIF	NCL	KCIF	NCL
LDIS001	12.09.05	M	40	22	A +	SAH	5	Normal	TLM	5	90%	90%	70%	70%	80%	80%	580	
LDIS003	23.10.05	F	51	23	O +	ICH	7.2	less fat, pancreatitis	TLM	6	90%	90%	80%	70%	70%	80%	420	
LDIS005	07.12.05	M	53	37	A +	SAH	8.4	Fatty	TLM	7.4	50%	50%	50%	40%	45%	50%	150	100
LDIS006	20.05.06	M	54	28	A -	R-MCA infarct	9.7	Fatty	TLM	5.7	30%	40%	70%	70%		70%	180	150
LDIS007	27.05.06	F	46	25	A -	ICH	6.7	Fatty	TLM	4.5	70%	80%	70%	70%	45%	30%	167	200
LDIS011	20.06.07	M	30	27	A +	SAH	7	Abnormal vessels	TLM	5.75	50%	70%	60%	70%		85%	300	200
Mean±SD (TLM)			46±9	27±5			7±2			6±1	63±24	70±21	65±16	65±12	60±18	66±22	172	163±48
LDIS009	14.12.06	F	42	31	B +	ICH	9	Normal	UWS	7.3	70%	70%	70%	70%	50%	50%	225	270
LDIS012	06.11.07	F	51	26	A +	ICH	19	Fatty	UWS	5	85%	85%	80%				350	
LDIS014	25.03.08	M	56	28	B +	ICH	4	Normal	UWS	12	90%	90%	90%	90%		90%	60	35
LDIS015	15.04.08	M	59	22	B +	ICH	7	Normal	UWS	14	90%	90%	60%	70%		70%	150	100
Mean±SD (UWS)			52±7	27±4			10±7			10±4	85±9	84±9	75±13	77±12	50±0	70±20	196±123	135±121
Mean±SD (TLM+UWS)			48±9	27±5			8±4			7±3	72±22	76±8	69±15	69±13	58±16	67±20	156	151±79
P value (TLM vs UWS)			0.3	0.9			0.5			0.2	0.1	0.2	0.3	0.2		0.8	0.3	0.7

Table 3.3 Pancreases accepted into the research program for islet isolation. This table summarises demographics of donors, pancreas characteristics and transport data, together with islet isolation outcome. Scores for transported islets are presented for islets transported in blood bags when both blood bags and Permalife bags were used. Islet count and quality outcome was compared according to pancreas transport method (TLM or UWS) using two tailed two-sample unequal variance t-test. LDIS002, LDIS004, LDIS008, LDIS010, LDIS013 were organs offered to the project but did not undergo islet isolation.

3.5.2 Pancreas packaging and transport

When an organ was accepted, the transport chamber, oxygenation kit, PFC and transport box were collected from the Newcastle University and taken to the surgical team transport ambulance. I accompanied the surgical team to the retrieval site on most occasions. Upon arrival at the retrieval site, PFC oxygenation was started while the surgical team commenced the multi-organ retrieval process. Time of cross-clamping of the aorta was documented as the start of cold ischemic time (CIT).

Upon removal of the pancreas-spleen block it was placed in a tub filled with crushed UWS ice and was then perfused with UWS through its vasculature for an average of 5 minutes to remove as much of the blood as possible. The majority of the spleen was removed before the pancreas was placed inside the transport chamber containing oxygenated PFC. The chamber was then filled with UWS and was firmly closed. The transport chamber was double-bagged using sterile organ transport bags to ensure avoidance of contamination and was then buried in crushed ice inside the transport on all initial runs. This was to enable development of good understanding of the practicalities of the transport process. Moreover, my attendance and assistance in all procedures and assessment protocols ensured that a congruent approach was adopted on repeat evaluation after transport to Newcastle and eventually helped me in the development of appropriate Standard Operating Procedures (SOPs). CIT was defined as time between aorta cross-clamp at retrieval centre and infusion of collagenase at KCIIF. Mean pancreatic CIT was 8 ± 4 hours. This included a single pancreas which had CIT of 19 hours. This long CIT was due to delay and an additional transport journey from a pancreas transplant centre where the pancreas was sent initially for whole organ transplantation but was then rejected by the transplant team who considered it too fatty. This organ was packaged in UWS only following already

established protocols for packaging pancreas for whole vascularised pancreas transplantation. The pancreas was then transported to KCIIF for islet isolation. Out of 10 processed pancreases 6 (60%) were transported using oxygenated PFC and UWS (TLM) and 4 (40%) transported in UWS alone. There was varying islet yield and quality post digestion and use of TLM or UWS alone yielded no significant advantage on shortening CIT ($p=0.5$), improving islet viability at KCIIF or Newcastle ($p=0.1$ and 0.2 respectively), islet purity at KCIIF or Newcastle ($p=0.3$, 0.2 respectively), islet integrity ($p=0.8$ at Newcastle) or IEQ count at KCIIF or Newcastle ($p=0.3$, 0.7 respectively) (Table 3.3). Even when the organ with 19 hour CIT was excluded no significant difference was noted. Moreover, islet yields from LDIS0014 and LDIS0015 were low despite the normal macroscopic appearance of the pancreas and acceptable CIT. This was thought to be secondary to problematic and incomplete enzyme digestion.

Although total number of organs was low in both groups due to limited organ availability it was difficult to demonstrate any clear superiority of TLM over UWS. Therefore, a pragmatic decision was made to mirror vascularised pancreas approach with packaging in UWS alone.

3.6 Islet isolation

Islet isolations were carried out according to the KCIIF established and optimised protocols (2.8). Full assessment of sterility (bacterial culture and gram stain), viability (FDA/PI method) and islet count (DTZ / graticule method) was carried out on each preparation. My attendance to all assessment procedures at KCIIF enabled learning these procedures and ensured that a congruent approach to evaluation was adopted on repeat evaluation after transport to Newcastle. In a limited number of preparations

electron microscopy was carried out at KCIIF. It was not possible to perform this on all preparations due to logistical and staff limitations.

3.6.1 Islet packaging and transport

3.6.1.1 Islet bag

In the first three preparations, islets were packaged using Permalife gas-permeable bags (Origen) and standard blood transfusion bags (Baxter) with equal loading to enable direct comparison of the effect of bag type on islet quality. Despite the fact that Permalife bags are designed to allow gas exchange with the surrounding atmosphere, no advantages were confirmed on simple *in vitro* islet assessment within this setting. Sterility was maintained in both bags and viability scores were equivalent (Table 3.4). However, more islet fragmentation was observed in islets transported in Permalife bags upon assessment at Newcastle (Table 3.4). On one occasion the Permalife bag ruptured spilling the islet solution. This was clearly particularly significant since this would jeopardise the quality of transported islets in particular and the feasibility of islet transport for clinical use in general. In light of these observations it was decided that subsequent islets should be packaged and transported in blood transfusion bags only.

3.6.1.2 Transport temperature

In the GRAGIL (3.1.1), Miami-Houston and Miami-Dallas (3.1.2) protocols, transportation was carried out at room temperature in a temperature controlled box. The transport journey in the GRAGIL group was carried out by road ambulance service after a period of culture at the isolation centre with journey duration 3-5 hours on average, followed by immediate transplantation at the receiving centre without manipulation. Transport journeys between Miami-Houston and Miami-Dallas were

	Viability (%)		Integrity (%)	
	Blood bag	Permalife bag	Blood bag	Permalife bag
LDIS001	90	90	80	60
LDIS003	90	90	80	60
LDIS005	50	55	50	40
Mean	76.7	78.3	71.7	53.3
SD	23.1	20.2	18.9	11.5
T test	0.2		0.02	

Table 3.4 Viability and integrity score comparison between different islet bags. There was no difference in viability between the two bags. A significantly lower integrity score was seen in Permalife bags compared to blood bags. Differences between blood bag and Permalife bag were evaluated using paired t-test.

carried out immediately post isolation or after a period of culture using air services in a temperature controlled box at room temperature followed by medium change and sterility and viability assessment at the receiving centres before transplantation. The transport model we proposed for the UK settings was similar to the GRAGIL approach with some differences. This initially included absence of resting culture post-isolation and relatively longer transport journeys (5-7 hours) due to geographic distance, with plans to carry out transplantation upon arrival following minimal manipulation to enable quality assurance reassessment. We aimed to maintain transport temperature between 5-22 °C in a temperature-proof cold box using cold packs. This was to reduce biological activity of islets during transport and thus to reduce metabolic demand and nutrient depletion in addition to minimising insulin accumulation in transport medium. Moreover, lower temperatures may suppress secretion of pro-inflammatory cytokines released post-isolation from transported cells.

3.6.1.2.1 Cold-box temperature control validation by HOBO data-logger

To confirm whether the cold box was able to maintain temperature during transportation; two cold packs cooled to 4 °C overnight in the fridge were laid inside the box and the Hobo data-logger was placed inside the shelf unit within the cold box (2.4). Despite the fluctuations in the laboratory temperatures, related to door openings and general activity of people and equipment (temperature 22 ± 3 °C) (mean \pm SD), data retrieved from the data-logger recorded over 5 hours showed a lag of about 30 minutes before temperature equilibrated which then levelled off with an average of 17.9 ± 0.2 °C (Figure 3.6). This confirmed ability of the box to maintain the temperature of its interior with no major fluctuations during a period of time equivalent to an islet transport journey from KCIIF to Newcastle, and thus suitability of this setup for this purpose.

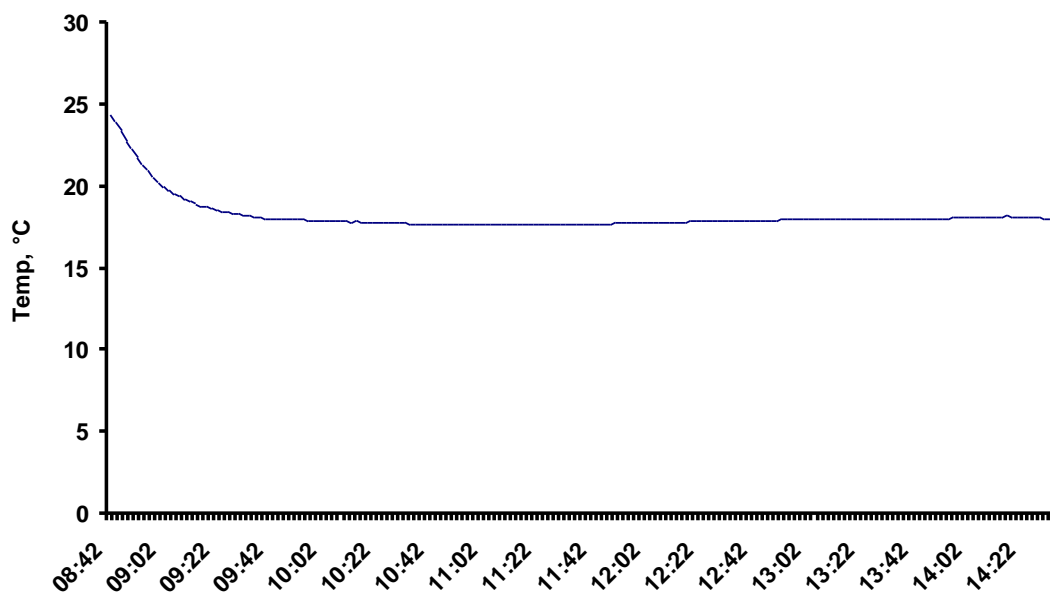


Figure 3.6 Validation of temperature control in the transport box. Two cold packs (4°C) were placed inside the box, which was closed and placed in the general lab (temperature $22\pm 3^{\circ}\text{C}$). Temperature was logged every 5 minutes. The temperature equilibrated at approximately 17°C within 30 minutes and then was maintained over the following 5 hours. Mean temperature was $17.9\pm 0.2^{\circ}\text{C}$ (mean \pm SD).

3.6.1.2.2 *Transport temperature monitoring*

To further reduce temperature below ambient, more cold packs were included by KCIIF for islet transport journeys. Four initial transport journeys were monitored by minimum-maximum temperature monitor (2.3). Maximum temperature figures reflected the temperature before starting the journey while minimum temperature reflected lowest temperature during the journey (Table 3.5 A). Average minimum temperature was 6.3 ± 1.4 °C which falls within temperature target for the journey. This system confirmed maintenance of temperature within target range in all tested journeys however it was not capable of detecting temperature fluctuations during the journey. Later, this system was replaced by HOBO data-logger to enable continuous real-time temperature recording during journeys and thus reveal any changes that might be occurring. Target temperature control of 5-22 °C (2.11) was maintained in all HOBO monitored journeys with an average transport temperature of 7.8 ± 1.0 °C (Table 3.5). Temperature fluctuations during journeys were limited with a mean standard deviation of transport temperatures of 1.8 °C (n=3) (Table 3.5). This confirmed ability of this transport system to maintain the temperature of islet environment. It was noted that HOBO data-logger required a battery change almost every month and on occasions the battery ran out during the journey or before commencing the transport journey. The battery change required specific tools and particular attention to the gasket to maintain water-resistance. Temperature of three transport journeys was not thus successfully monitored due to technical and logistical issues. Ultimately, the Hanna data-logger system was introduced for the clinical program with an average battery life of three years and availability of several loggers and data-management station at each isolation site.

A

Transport temp °C	LDIS001	LDIS003	LDIS005	LDIS006	Mean	SD
Minimum	8	4.5	6	6.5	6.3	1.4
Maximum	20	21	23	20.5	21.1	1.3

B

Transport temp °C	LDIS007	LDIS009	LDIS011	Mean	SD
Mean temperature	7.7	8.8	6.7	7.8	1.05
Standard deviation	0.9	1.2	3.3	1.8	

Table 3.5 Islet transport temperature monitoring. A: minimum and maximum temperatures. B: Hobo data-logger average temperatures of three transport journeys. Mean standard deviation of journeys' temperature profiles was 1.8 indicating robust temperature control with limited fluctuations within this transport system.

3.6.1.3 Transport medium

Different media have been studied and optimised for islet transport by several groups including the Miami group (Ichii et al., 2007b). In our setting without a wash step on arrival at the recipient centre, the selected islet medium would be employed for both transportation and transplantation and therefore we opted for the simplest medium formulation to serve these purposes. In the initial two (20%) runs, CMRL 1066 containing 20 mg/l phenol red (Table 2.6) was used and supplemented with 10% FCS due to limited HSA resource availability at the isolation centre and delay in sourcing phenol red-free medium. In subsequent runs, 350 ml of CMRL-supplemented medium (Table 2.6) was used in 3 (30%) preparations and 350 ml CMRL NCL1 (Table 2.6) was used in the remaining 5 (50%). Both media were supplemented in all journeys with 2% HSA. Both CMRL-supplemented and CMRL NCL1 media enabled maintenance of sterility post transport in all runs. Viability scores and counts of IEQs showed no significant change following transport using either media (Table 3.6).

CMRL NCL1 was a custom-made medium manufactured by PAA laboratories Ltd. at their cGMP accredited manufacturing plant in Austria. It was specifically mass produced for potential clinical use. Since no measurable difference was noted in the quality of transported islet in comparison to CMRL-supplemented, it was decided to use it for the clinical program.

3.6.2 Islet transport to Newcastle

The transport box was handed to the Life-Line ambulance driver to deliver to Newcastle. I accompanied transported islets when I had attended the isolation process at KCIIF. Transport journey by Life-Line from KCIIF to Newcastle University was 5.8 ± 1.3 hours (n=8; median \pm IQR). Two preparations (LDIS014, LDIS015) were

A

Donor Code	CMRL Medium	Viability				
		KCIIF	Mean±SD	NCL	Mean±SD	P value
LDIS005	supplemented	50%	50±20	50%	57±20.8	0.2
LDIS006	supplemented	30%		40%		
LDIS007	supplemented	70%		80%		
LDIS009	NCL1	70%	67±15.3	70%	81±10.3	0.3
LDIS011	NCL1	50%		70%		
LDIS012	NCL1	85%		85%		
LDIS014	NCL1	90%		90%		
LDIS015	NCL1	90%		90%		

B

Donor Code	CMRL Medium	Islet equivalents in x1000				
		KCIIF	Mean±SD	NCL	Mean±SD	P value
LDIS005	supplemented	150	166±15	100	150±50	0.6
LDIS006	supplemented	180		150		
LDIS007	supplemented	167		200		
LDIS009	NCL1	225	184±103	270	151±104	0.7
LDIS011	NCL1	300		200		
LDIS014	NCL1	60		35		
LDIS015	NCL1	150		100		

Table 3.6 Transport medium effect on islet viability and count. Viability (A) and islet equivalent count (B) were compared before and after transport when CMRL-supplemented medium or CMRL NCL1 medium were used using two-tail paired student t-test. No significant change in viability or IEQ count was seen with either medium.

sent overnight using commercial coach road services instead of Life-Line ambulance. Duration of the transport journey to Newcastle for these preparations was 12 and 14 hours.

3.6.3 Islet assessment in Newcastle

Upon arrival in Newcastle, samples were removed for assessment of sterility, islet count and integrity, islet viability, islet function and transport medium analysis.

3.6.3.1 Sampling

The first six (60%) preparations were sampled and handled in the general tissue culture laboratory. All subsequent runs (40%) were first sampled in the clean room at the stem cell CGMP laboratory. All tissue handling was carried out inside a class II safety cabinet.

3.6.3.1.1 Sampling in the general tissue culture laboratory

When carried out in the standard tissue culture laboratory a 5 ml sample was removed from the bag directly through the bag port using a sterile syringe for sterility testing. Remaining islets were pooled in 50 ml conical tubes and centrifuged at 1000 rpm (32.5 G) for 1 minute. Samples from the supernatant were removed for estimation of proinflammatory cytokine profiling and insulin content. Remaining supernatant was discarded.

Islets were pooled in one conical tube and made up to 50 ml in islet culture medium (Table 2.2). Two 100 µl samples were removed after gentle mixing to ensure homogeneity. One sample was used for IEQ and integrity estimation and one was used for viability testing. Remaining islets were cultured in T175 non-adherent flasks at a density of 30,000 IEQs per flask in 30 ml medium.

3.6.3.1.2 Sampling in the cGMP laboratory

When inside the clean room, bags were sprayed with 70% medical grade ethanol. A luer lock was inserted in the bag. The bag was mixed gently and a 4 ml sample was withdrawn using a sterile syringe. The syringe was closed using a syringe lock and was left vertical for 5 minutes inside a 30 ml tube to allow islets to settle by gravity. The lock was removed gently and 1 ml was dispensed into a universal tube to ensure all islets in the sample were recovered for subsequent assessment of integrity, count and viability. The remainder of the sample was used for the sterility test. These studies informed writing of an SOP for reception and sampling of islets for clinical use.

3.6.3.2 Sterility assessment

When carried out in the general tissue culture laboratory, the 5 ml recovered sample was divided into aliquots: 1 ml dispensed in a sterile 30 ml universal tube and 4ml injected inside a bacterial culture bottle. When sampling was carried out in the cGMP laboratory; the remaining 3 ml of the collected sample was divided into 0.5 ml dispensed in a sterile 30 ml universal tube and 2.5 ml was injected into a bacterial culture bottle. All tubes and bottles were labelled and sent to the Freeman Hospital Microbiology Laboratory for analysis.

Gram stain was negative in all samples at Newcastle. All bacterial culture bottles were reported as negative after 4 days culture.

3.6.3.3 Viability assessment

Initial validation of the KCIIF FDA/PI viability staining protocol demonstrated increased false positive staining when scoring was carried out beyond the 5 minutes post-staining window. A washing step was introduced to minimise this as outlined before (2.10 and 3.4.1). Viability staining at Newcastle was carried out using the new

modified protocol. The protocol was then formalised in an SOP (Appendix 1.4, ITSOP003 and ITSOP003 attachment I). Viability scores of all preparations were comparable between KCIIF and Newcastle (Table 3.3) with no statistical difference (KCIIF 72% vs NCL 76%, $p=0.6$) indicating absence of detrimental effects of the current transport system on islet viability. Interestingly; this was also observed after prolonged transport journeys when islets were transported overnight (LDIS0014 and LDIS0015).

3.6.3.4 Islet count, purity and integrity assessments

IEQ was estimated following the method adopted at the Diabetes Research Institute (DRI) at Miami (2.9). The method in principle is similar to all other methods adopted by different islet laboratories in terms of DTZ staining and IEQ correction factors but relies on measuring the diameter of each islet using a cross-shaped graticule (Figure 2.5). IEQ and purity was estimated at KCIIF for all preparations while islet integrity was reported for only 50%. In Newcastle, purity and islet integrity was estimated for all preparations except one (LDIS012) which was received and processed by my colleagues during my study leave at a conference overseas. IEQ was carried out for 7 preparations. When comparing KCIIF and Newcastle scores (Table 3.3) there was no statistical difference in IEQ count ($p=0.6$, $n=7$), purity ($p=0.8$, $n=9$) and integrity ($p=1$, $n=5$). Counts were comparable between KCIIF and Newcastle when recount was carried out following a volume reduction at Newcastle. However, a non-significant trend towards decreased IEQ number was observed on recounting on a sample retrieved via the side arm of the transport bag (Table 3.7). Sampling from side arm was therefore only used for qualitative reassessment and not for recounting as this may lead to underestimation.

3.6.3.5 Functional assessment

Functionality of transported islets was assessed by electron microscopy (EM) studies, glucose stimulated insulin secretion (GSIS), processing of proinsulin to insulin and correction of hyperglycaemia in STZ treated mice.

3.6.3.5.1 EM studies

EM was utilised for the assessment of possible negative effects of islet transport on cellular structures with specific appraisal of secretory granules. Sample preparation, sectioning and image capture were carried out on islets at KCIIF (Figure 3.7 A) and Newcastle (Figure 3.7 B) by the specialist EM units locally following similar methods (2.21). Direct enumeration of intracellular secretory granules was carried out on pre-captured images of islet cells performed at both sites. Number of secretory granules observed per cell was counted in 4 different cells. This was carried out on one preparation (LDIS001) at both KCIIF and Newcastle which showed no meaningful difference between the average of secretory granules from both sites (177 ± 62 at KCIIF, 206 ± 65 at NCL, $P= 0.5$) (Table 3.8). Having demonstrated maintenance of secretory granules post transportation and in view of technical and logistical challenges of performing these studies, no further EM studies were undertaken.

3.6.3.5.2 Glucose stimulated insulin secretion

GSIS was assessed either in static incubations or in perfusion studies.

3.6.3.5.2.1 Static GSIS

Initial protocols were established and validated using rodent islets (3.4.2). Experiments carried out on human islets were performed according to the optimised protocol (2.15.1).

Maintained function with a significant 1.3 ± 0.2 (mean \pm SD) fold higher secretion in

	KCIIF	NCL	<i>P</i> value	
A			0.63	
	LDIS005	150		100
	LDIS006	180		150
	LDIS007	167		200
	LDIS009	225		270
	Mean±SEM	181±16	180±36	
B			0.53	
	LDIS011	300		200
	LDIS014	60		35
	LDIS015	150		100
	Mean±SEM	170±70	112±48	

Table 3.7 Islet equivalent count following volume reduction (A) or side arm sampling (B). There was no significant difference in IEQ between KCIIF and NCL following both protocols however lower counts IEQ counts were noted more when counts were done on a side arm sample (B).

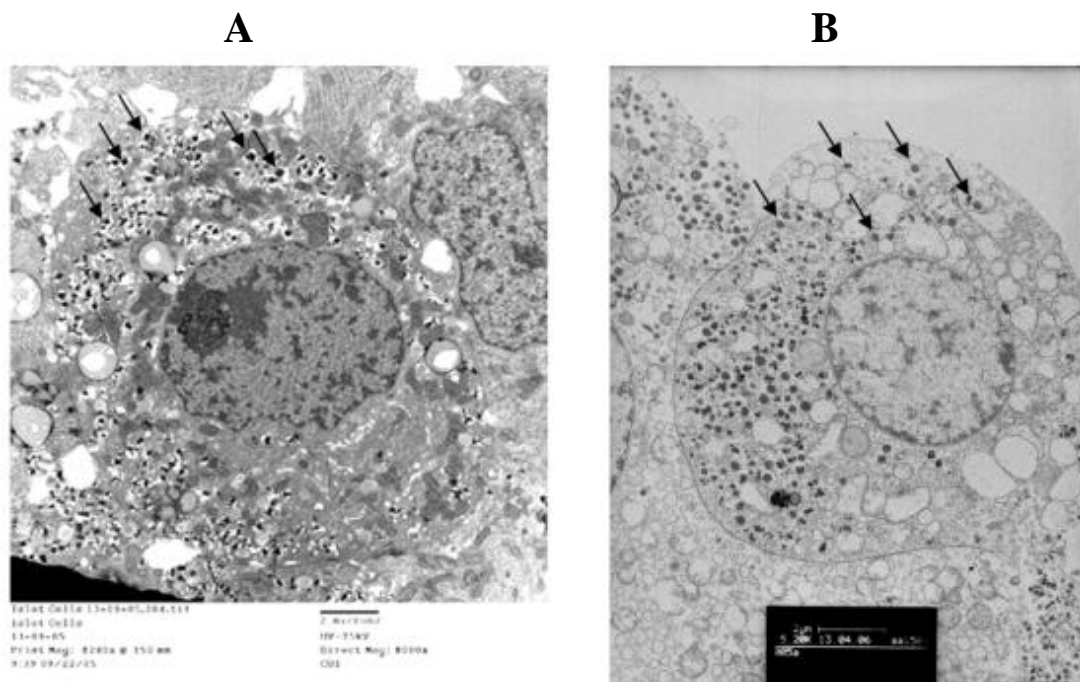


Figure 3.7 Electron microscopy studies on humans islets. Images of islets from LDIS001 at KCIIF (A) and after transport to Newcastle (B). Presence of secretory granules (arrows) was confirmed in both with no qualitative difference suggesting degranulation. Arrows indicate secretory granules.

EM images	KCIIF	NCL
Cell 1	237	265
Cell 2	156	149
Cell 3	217	151
Cell 4	99	262
Mean	177.3	206.8
SD	62.5	65.5
T test	0.5	

Table 3.8 Quantification of secretory granules in islet cells. This shows comparison of secretory granule count in four different islet cells at KCIIF and in Newcastle. There was no significant difference ($p=0.5$, $n=4$, two tailed two sample equal variance t-test).

high glucose ($p < 0.05$) was confirmed following islet transport (Table 3.9 A). Variability remained a major issue in all static GSIS experiments (Table 3.9 B).

One of the main limitations of static GSIS experiments (using the majority of established protocols) is the requirement for separate wells at low and high glucose (with inevitable variation between aliquots) as opposed to testing the response of each individual aliquot to different glucose concentrations. Therefore GSIS using a perfusion system was explored.

3.6.3.5.2.2 Perfusion GSIS

The equipment was built and validated in house based on the established setup at Prof Jones' and Prof Persaud's laboratory at King's College (2.15.2.1). Perfusion GSIS was employed in studying insulin secretion response in an individual islet sample to different glucose challenges. This minimises variability related to different sample volume and different β -cell content between samples and thus tests true change in insulin release in each sample in response to glucose changes. Both maximal (2 mM to 20 mM D-glucose) and physiological (5.6 mM to 11 mM) glucose challenges were carried out as outlined before (2.15.2.3). Stimulation index (SI) was calculated as the ratio between the average insulin release during the low glucose perfusion phase and the average insulin release during the high glucose perfusion phase in each individual chamber. SI for the islet preparation was calculated as mean SI between all channels (Table 3.10). Mean SI in maximal challenge experiments was 3.2 ± 1.5 (mean \pm SD, $p = 0.003$, $n = 4$ from a single preparation, LDIS015) (Figure 3.8 A). SI in minimal challenges was 1.9 ± 0.2 (mean \pm SD, $p = 0.008$, $n = 3$ in one preparation, LDIS014) (Figure 3.8 B). These experiments demonstrate and confirm dynamic glucose sensing and appropriate insulin response in transported islets.

A

Preparation	Experiments	Fold increase
LDIS001	Experiment 1	1.1
LDIS003	Experiment 2	1.6
LDIS005	Experiment 3	1.1
LDIS006	Experiment 4	1.2
LDIS006	Experiment 5	1.5
LDIS009	Experiment 6	1.3
	Mean	1.3
	SD	0.2
	P value	0.26

B

	Sample	Experiment 4			Experiment 5		
		2mM	20mM	Fold	2mM	20mM	Fold
Insulin pmol/l	well 1	44.5	67.5		54.1	88.9	
	well 2	161.6	57.5		49.3	87.6	
	well 3	84	145.5		56.4	96.6	
	well 4	73	138.6		68.3	148.2	
	well 5	63.8	171.6		127.9	153.4	
	well 6	103.5	77.5		120.9	144.1	
Mean		88.4	109.7	1.2	79.5	119.8	1.5
SD		40.9	48		35.4	31.8	
T test p=		0.4			0.07		

Table 3.9 Static GSIS. Islets consistently showed an increase in insulin secretion in response to glucose challenges from 2mM to 20mM with an average of 1.3 ± 0.2 fold increase (A). However high variability between samples was observed as demonstrated in these two representative experiments (B).

LDIS015	Insulin ng/ml		SI
	2mM	20mM	
Chamber 1	2.7	14.5	5.5
Chamber 2	4.5	13.8	3
Chamber 3	7.4	17.3	2.3
Chamber 4	5.6	11.4	2
Mean (SI)			3.2
SD			1.5
T-test p=			0.003

Table 3.10 Perifusion GSIS. Results of a single study of perifusion GSIS following maximal challenge protocol as an example of perifusion GSIS. The table shows SI in each channel. Mean SI of different channels is considered the overall SI of the islet preparation. (paired t-test).

3.6.3.5.3 Processing capacity of proinsulin to insulin

Efficient conversion of proinsulin to mature bioactive insulin is an important specialised function of the healthy β -cell. This takes place within the secretory granules through the action of pro-hormone convertases PC1/3 and PC2 (Goodge and Hutton, 2000; Steiner and James, 1992). The process becomes inefficient in stressed and exhausted β -cells such as some cases of Type 2 diabetes (Steiner and James, 1992). To evaluate whether transport has any detrimental effect on processing, insulin to (pro)insulin ratio was assessed on two transported islet preparations at low glucose (2 mM), when demand on insulin secretion is low, and high glucose (20mM), when demand on insulin secretion and therefore proinsulin processing is high, over 2 hour incubations. Each condition included 3 repeats (2.15).

Differential proinsulin and insulin ELISA assays demonstrated maintained processing capacity at more than 95% of secreted (pro)insulin at both low and high glucose (Figure 3.9). This result was further confirmed when intracellular (pro)insulin levels were analysed which showed an average of $70\pm 2\%$ (mean \pm SD) conversion at 2 mM; and slightly higher, but statistically significant, conversion ratio at 20mM glucose at $74\pm 3\%$ ($p=0.03$, $n=6$ on samples from two different experiments) (Figure 3.9). These results together demonstrated active proteolytic conversion within the secretory granules in addition to efficient glucose sensing in transported islets.

3.6.3.6 Safety assessment of transport medium for clinical infusion

Islets were transported at relatively low temperatures to minimise secretion and thus accumulation of different hormones and pro-inflammatory factors in transport/transplant medium.

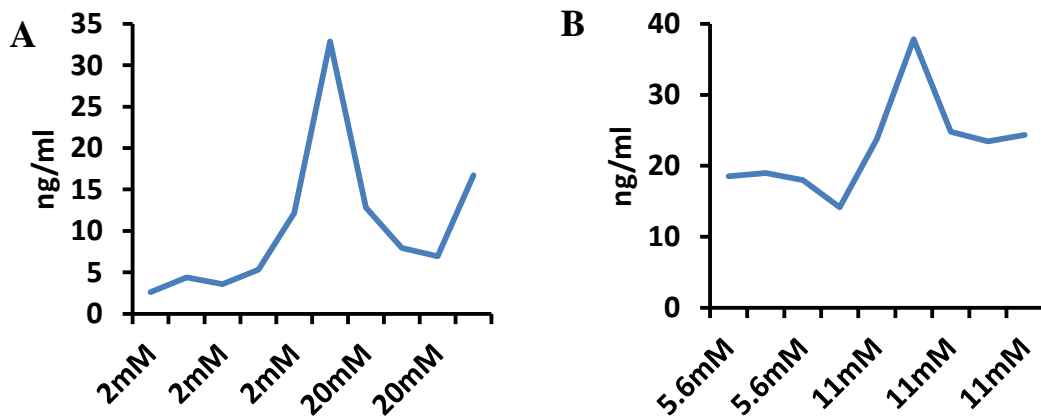


Figure 3.8 GSIS in perfusion studies. These are representative graphs of the dynamic changes of insulin-release in response to different glucose challenges. A: maximal glucose challenge in islets from LDIS015 with a shift between 2 mM and 20 mM. B: physiological glucose challenge in islets from LDIS014 with a shift between 5.6 mM and 11 mM. These are from two different islet preparations and insulin levels reflect IEQ load in each experiment.

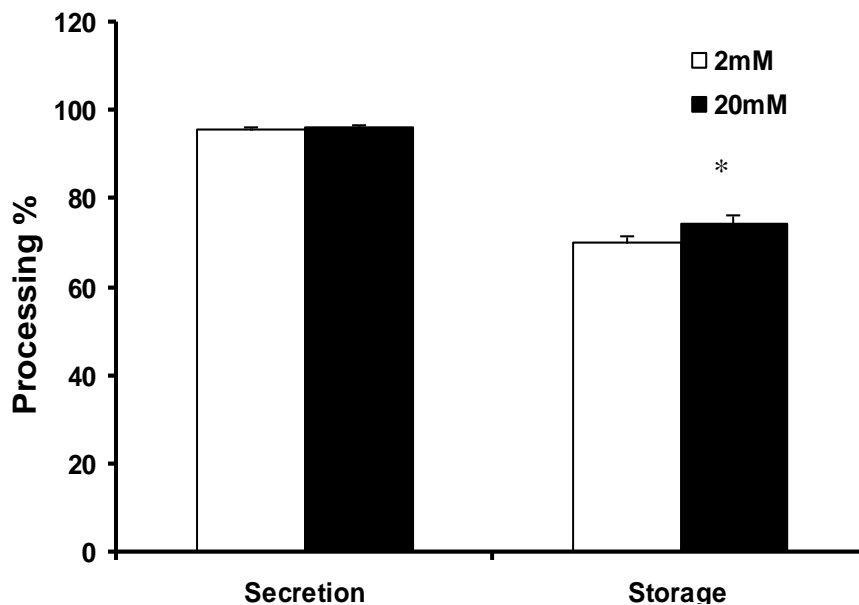


Figure 3.9 Proinsulin to insulin processing capacity. Results are expressed as mean of percentage insulin / (insulin + proinsulin). A total of 6 combined repeats from two different experiments were included and presented as mean \pm SD. (*p= 0.03).

To ensure safety of direct transplantation upon arrival without medium exchange, levels of insulin and proinflammatory cytokines were assessed in the transport medium upon arrival at Newcastle.

3.6.3.6.1 *Insulin content*

Insulin levels were evaluated in transport medium of three different bags upon arrival at Newcastle (Table 3.11). Average insulin release into transport medium was 6.4 ± 1.6 unit / million IEQs (mean \pm SD) with a mean duration of transport from KCIIF to Newcastle of 5.8 hours. This averages at 1.1 unit/hour/million IEQs. In practice, the majority of islet preparations comprise less than 0.5 million islets and transport journey to Newcastle was on average less than 6 hours. This translates to less than 3.3 units of insulin being released into the medium during transport. Despite being a limited amount of free insulin, this justifies extra vigilance and close glucose monitoring during islet infusion to prevent potential risk of hypoglycaemia.

3.6.3.6.2 *Proinflammatory cytokines content*

Levels of IL-1 β , INF γ , IL-6 and TNF α were evaluated in samples from 4 different bags of transport medium. The literature suggests absence of IFN- γ in healthy people or people with long standing diabetes but high levels (225 pg/ml) are seen in newly diagnosed T1D indicating a role during the active β -cell destruction phase (Hussain et al., 1996). Assessment of islet transport medium demonstrated modest INF- γ average levels at 32 ± 11 pg/ml (mean \pm SD). Minimal IL-1 β levels were detected in transport medium at 4.8 ± 0.5 pg/ml, although this cytokine is not usually detected in healthy people or even people with diabetes.

Circulating levels of IL-6 in healthy people are estimated at 1.4 pg/ml, while levels are higher in people with long standing diabetes and even higher in newly diagnosed

Preparations		Bag content	Volume	Transport duration	Insulin	Insulin
		IEQs	ml	Hours	Unit/bag	Unit/million IEQs
LDIS001	Bag1	125000	250	5	0.94	7.5
LDIS003	Bag2	125000	250	6	0.89	7.1
LDIS011	Bag3	285000	280	5.75	1.31	4.6
	Mean	178333	260	5.6	1	6.4
	SD	92376	17	0.5	0.2	1.6

Table 3.11 Insulin content in islet transport bag.

diabetes (1.75 and 2.05 pg/ml respectively) (Pfleger et al., 2008). In transport medium the average detected level was 4.9 ± 0.8 pg/ml. TNF- α is usually not detectable in healthy people. Newly diagnosed T1D people have mean serum concentration of 29 pg/ml while people with long standing T1D have average circulating levels of 17 pg/ml. In transport medium, the average detected level was 23.7 ± 4.6 pg/ml (Table 3.12).

Despite being higher than physiological levels, the overall load when transplanted would be considerably lower due to small volume of graft solution (200-300 ml). Moreover, use of T cell depleting agents such as ATG or Campath leads to significant cytokine release making the detected levels unlikely to cause any clinically significant reactions. Safety of these levels was demonstrated in 4 clinical transplantations with no immediate complications after transplant infusion. However; further *in vivo* studies are required to investigate whether a surge of cytokines could occur when bioactivity is resumed in the graft as it starts to warm to the recipient's core temperature.

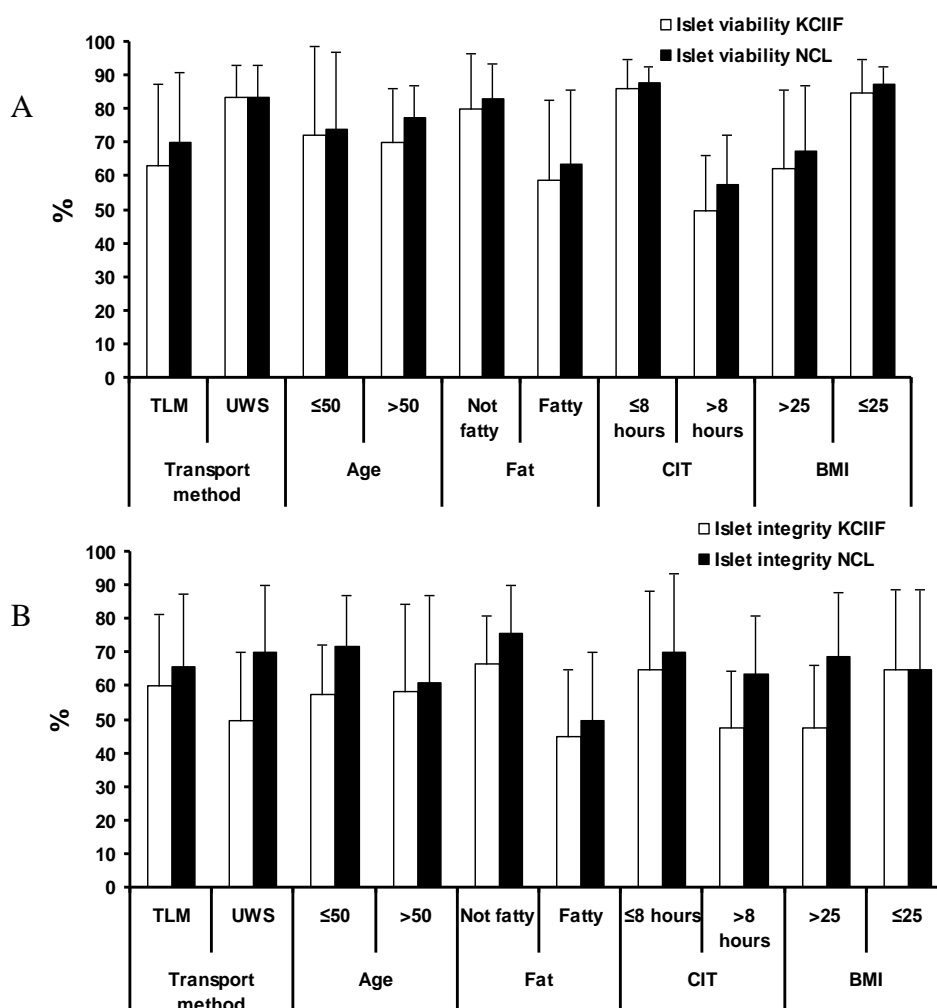
3.6.3.7 Assessment of effect of donor specific factors on islet quality post transport

Despite the low number of preparations included in this study an attempt to evaluate the potential effect of different donor factors on islet quality was carried out. Effect of age (6 > 50 years vs. 4 \leq 50 years), BMI (6 > 25 kg/m² vs. 4 \leq 25 kg/m²), pancreas fat content (6 normal vs. 4 fatty), CIT (6 > 8 hours vs. 4 \leq 8 hours) and pancreas transport method (6 TLM vs. 4 UWS) on scores of viability, integrity, purity and islet count before and after transport were analysed.

Within the available data from this study, no significant effect of these donor variables on quality of transported islets was confirmed (Figure 3. 10), although trends towards decreased viability, integrity, purity and yield with longer cold

Sample	IFN- γ pg/ml	IL-1 β pg/ml	IL-6 pg/ml	TNF- α pg/ml
LDIS001 Blood bag	39.49	5.27	5.42	28.79
LDIS001 PL bag	43.76	5.14	5.47	26.38
LDIS003	24.38	4.68	5.00	20.66
LDIS011	22.66	4.26	3.86	19.06
Mean \pm SD	32.57 \pm 10.6	4.84 \pm 0.5	4.94 \pm 0.8	23.73 \pm 4.6
Physiological range	0	0	1.4	0
Newly diagnosed T1D	225	0	2.05	29
Longstanding T1D	0	0	1.75	17

Table 3.12 Proinflammatory profile in transport medium. Levels of all tested cytokines were within safe limits. Comparative physiological / diabetes serum data for IL-6 were obtained from Pflieger (Pflieger et al. 2008). Data of IL-1 β , INF γ and TNF α were obtained from Hussain (Hussain et al. 1996).



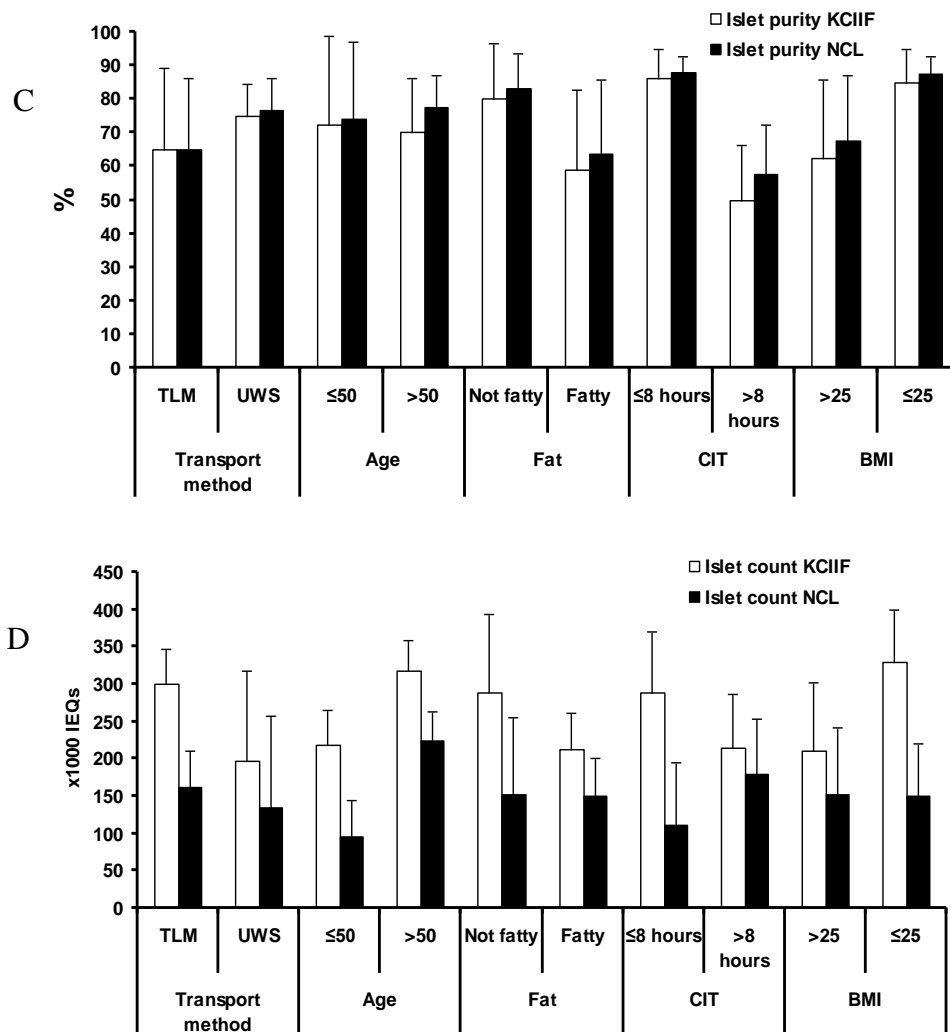


Figure 3.10 Evaluation of donor specific effect on islet quality after transport. Viability (A), integrity (B), purity (C), and islet count (D) were analysed at both KCIIF and Newcastle in groups divided according to transport method (6 TLM vs. 4 UWS), donor age (6 >50 years vs. 4 ≤ 50), pancreas fat (6 not fatty vs. 4 fatty), CIT (6 >8 hours vs. ≤ 8), and donor BMI (6 > 25 vs. 4 ≤ 25 kg/m²).

ischaemic time and macroscopically fatty pancreases were seen. There was clearly much variability between preparations with a number of potential outliers. Given the relatively small overall dataset, however, all preparations were included in this analysis which is, at best, hypothesis-generating.

3.6.3.8 *In vivo* human islet transplantation

Despite setting up and optimising sub-renal capsule transplantation of islets in the STZ treated SCID mouse model (3.4.2.11), it was not possible to carry out this with human islets. This was due to need for purchase, acclimatization and induction of hyperglycaemia before transplantation. This process takes an average of 3 weeks and animals will require euthanasia if not transplanted within a week of hyperglycaemia induction. This is not possible to achieve without ongoing induction of hyperglycaemia using an in-house bred SCID colony which was not available in Newcastle. Some groups have undertaken transplantation of human insulin expressing cells under the kidney capsule in SCID mice and have administered STZ treatment afterwards (D'Amour et al., 2006). Use of this sequence relies on the fact that expression of glucose transporter 2 (Glut2) is minimal in human β -cells in comparison to murine β -cells (De Vos et al., 1995; Hosokawa et al., 2001) and that toxicity of STZ is sustained following uptake of STZ through Glut2 transporter iso-enzyme. This approach remains controversial since human β -cells still express Glut2 and any human islet graft toxicity related to STZ use can jeopardise the validity of this bio-assay. Caiazzo tested the ability of human islets to engraft in normoglycaemic SCID mice thus avoiding toxicity of STZ altogether. Interestingly, human C-peptide levels detected in mice who had received 1% of the total clinical graft showed strong correlation with islet function in humans post transplantation (Caiazzo et al., 2008).

3.7 Standard operating procedures

The above studies enabled establishment, development and validation of standard operating procedures (SOPs) for transportation of clinical grade islets to remote centres, together with validation of islet quality and suitability for clinical transplantation.

These SOPs were worked up when relevant within the GMP Stem Cell facility at Newcastle University. Training, support and review were provided by the GMP team at the Department of Haematology at Royal Victoria Infirmary to ensure protocols are in-line with authority approved practices. A policy document was written to outline summary of work flow (Appendix 1.1). Details about islet packaging including bags, IEQ density, medium and volumes were outlined in the first SOP (Appendix 1.2 ITSOP001). The process of receiving transported islets at Newcastle and retrieving samples for QA assessments within the clean room was detailed in the second SOP (Appendix 1.3 ITSOP002 and attachment I and II). An SOP was written for each QA assessment including assessment of viability (Appendix 1.4 ITSOP003 and attachment I), assessment of IEQs and integrity (Appendix 1.5 ITSOP004 and attachments I, II and III), and download of temperature monitoring system (Appendix 1.6 ITSOP005). Another SOP outlining transport arrangements to the interventional radiology theatre at Freeman Hospital was formulated (Appendix 1.7 ITSOP006).

Successful implementation of these SOPs was confirmed when used in all transported preparations of clinical grade islets transplanted at Newcastle.

3.8 Clinical transplantation

Informed by the pre-clinical data generated in the present studies and including the Standard Operating Procedures developed / validated, the NHS National

Commissioning Group commenced dedicated funding of an integrated hub-and-spoke islet isolation / transport / transplant program in the UK on 1st April 2008. The clinical islet transplantation program was started in Newcastle as part of this government-funded national program. The model included central isolation facilities which operate on an on-call basis (OCDEM in Oxford, King's College and Royal Free hospitals in London) and six islet transplant centres (Bristol, King's, Manchester, Newcastle, Oxford, Royal Free) which receive transported islets from the central isolation facilities. Pancreases are offered to the program nationally and this is now managed by NHS Blood and Transplant (formally United Kingdom Transplant (UKT)).

Islets are offered according to a national waiting list with patients prioritised according to time on waiting list with maximum priority given to those awaiting a second transplant. The experience gained from the pre-clinical validation studies reported in this chapter was critical for designation, commissioning and implementation of the transport arm of the clinical program. The first transplant of transported islets in the UK took place in Newcastle with four clinical transplants being carried out in Newcastle to date.

3.8.1 Transported islets

During this project, islets from four donors were transported and transplanted in Newcastle (Table 3.12). Isolated islets were fully assessed and validated for suitability for clinical transplantation and were either packaged and transported to Newcastle directly (one preparation, 25%) or cultured overnight at the isolation centre (3 preparations, 75%). Islets were packaged using blood bags in 150ml CMRL NCL medium supplemented with 2% HSA. Transportation was carried out by the national organ transport service 'UK Transport for Transplant' (UK-TFT).

Average transport time, defined as time between islet packaging at the isolation centre for transplant and commencement of infusion in islet recipient, was 8.5 ± 0.9 hours (mean \pm SD). Viability and sterility assessment was carried out at Newcastle according to established and validated SOPs. There were no positive bacterial cultures or Gram stain at isolation centres or Newcastle.

3.8.2 Transplanted patients

A total of 4 transplants were carried out at Newcastle in 3 patients.

Recipient 1: This was a female aged 54 years, BMI 21 kg/m^2 , blood group O +ve, with a 42 year history of Type 1 diabetes. Pre-transplant insulin requirement was 38 units/24 hours. Indication for islet transplantation was problematic glycaemic control complicated by recurrent severe hypoglycaemia with impaired hypoglycaemia awareness despite trials of multiple daily insulin injection and continuous subcutaneous infusions with insulin pump therapy. The patient intentionally kept her blood glucose levels high to avoid dangerous hypoglycaemia and her HbA1c before transplantation was 10.1%. She received a single transplant with a dose of 5200 islets/kg (Table 3.12).

Immunosuppression protocol consisted of daclizumab for induction and tacrolimus / mycophenolate mofetil for maintenance. First pass main portal vein cannulation was achieved under local anaesthetic with no elevation in post-transplant portal venous pressure and no peri-operative complications. Initial graft function was confirmed by positive serum C-peptide and an initial small reduction in the insulin requirement to 32 units/24 hours. HbA1c was 7.6% at one month post-transplant. Glycaemic profile recorded by continuous glucose monitoring (CGMS) at one month post transplantation demonstrated marked improvement in time spent within target of 4-10 mmol/l and much lower time spent above 10 mmol/l with a moderate increase in time

	Donor					CIT	IEQs x1000	PCV	Purity	Viability at IC	Culture at IC	Transport time	Viability at NCL	Integrity at NCL
	IC	Sex	Age	BG	Rh									
ICP01	KC	M	42	O	+	4.1	300	2ml	70	90%	no	7.5	80%	60%
ICP02	KC	M	38	O	+	4.5	408	3ml	60	90%	yes	9.5	90%	90%
ICP03	Ox	F	39	O	+	8.5	600	3ml	70	90%	yes	9	90%	
ICP04	KC	F	54	O	+	3.0	503	3ml	75	90%	yes	8	85%	70%
Mean±SD			43.3±7.4	All O	All +	5±2.4	453±129	2.75±0.5	68.7±6.3%	90±0%	75% cultured	8.5±0.9	86.3±4.8	73±15%

Table3.13 Characteristics of donors and islet preparations in the Newcastle clinical program. IC: isolation centre, BG: blood group, Rh: rhesus, IEQ: islet equivalent, PCV: packed cell volume, KC: King's College, Ox: Oxford.

spent below 4 mmol/l probably due to improved confidence in insulin management with improved hypoglycaemic awareness (Figure 3.11 a). Hypoglycaemic episodes post transplantation were not severe clinically. This was further confirmed by small area under the curve for time spent less than 4 mmol/ l (Figure 3.11 b). Accelerated graft loss occurred and graft function was eventually lost altogether but with sustained improvement in glycaemic control with a total daily insulin requirement of 31 units/24 hour and an HbA1c of 8.9% at 6 month post transplantation. It was noted on the islet 'side arm' sample that was analysed in Newcastle that there was rapid islet fragmentation. It is not clear if this reflected an effect from the *in vitro* environment or whether islets were undergoing active fragmentation which might have led to the graft loss and recipient sensitisation against donor antigens. The patient is currently on the waiting list for second transplant. Although no deterioration in islets during transport had been seen in the pre-clinical validation studies, the possibility of potential fragmentation over the first 12-15 hours post-isolation led to a national recommendation that all preparations were cultured overnight (to reveal any potential deterioration) before transport for clinical transplantation.

Recipient 2: This was a female aged 45 years, BMI 31.6 kg/m², blood group O +, with a 39 years history of Type 1 diabetes. This patient was already taking tacrolimus / mycophenolate mofetil immunosuppression following a previous live-related renal graft. Pre-transplant insulin requirement was 38 units/24 hours. The indication for transplantation was problematic sub-optimal glycaemic control complicated by severe hypoglycaemia with impaired hypoglycaemic awareness.

Pre transplantation HbA1c was 8.6%. She received two islet preparations giving a total graft dose of 12,000 islets/kg (Table 3.12). Immunosuppression protocol consisted of

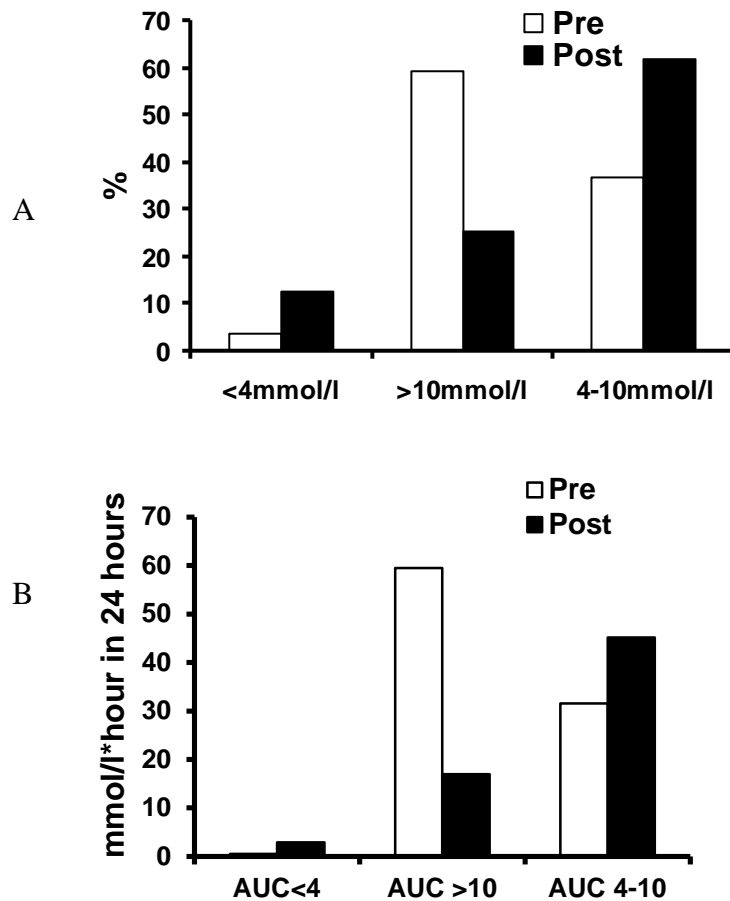


Figure 3.11 Continuous Glucose Monitoring System analysis for first Newcastle islet recipient pre-transplant and 1 month post-transplant. A: percentage of time spent within, above or less than target 4-10 mmol/l in 24 hours. B: Area under the curve of time spent within, blow or above target of 4-10 mmol/l. Despite apparent increase in time spent below 4 mmol/l (A), this was not associated with problematic hypoglycaemia, in keeping with relatively small area under the curve below 4 mmol/l (B).

Campath for induction (both transplants) and continued Tacrolimus (trough target serum level 8-10 ng/L) / mycophenolate mofetil for maintenance. There were no peri-operative complications. Initial and maintained graft function after first transplant was confirmed with reduction in daily insulin requirement to 25 units/24 hours and a reduction in HbA1c to 6.1% with positive serum C-peptide levels at 130 pmol/l 3 month post first transplant. A further reduction in HbA1c to 5.8% was achieved at one month after the second transplant. At 6 month post first transplant (3 month post second transplant) her daily insulin requirement fell to 20 units/24 hours with maintained detectable C-peptide and an HbA1c of 4.8%.

Recipient 3: This was a female aged 59 years, BMI 19.8 kg/m², blood group O +, with a 38 year history of Type 1 diabetes. Pre-transplant insulin requirement was 33 units/24 hours. Reason for transplantation was problematic glycaemic control complicated by severe hypoglycaemia with impaired hypoglycaemic awareness. Pre-transplantation HbA1c was 9.6%. She received one islet preparations with dose of 12,000 islets/kg (Table 3.12). Immunosuppression protocol consisted of Campath for induction and tacrolimus / mycophenolate mofetil for maintenance. There were no peri-operative complications. There was initial and maintained graft function resulting rapidly in complete insulin independence and confirmed 90 minute standardised meal tolerance test serum C-peptide levels at 340 pmol/l at 1 month with a reduction in HbA1c to 6.2% at one month post transplantation. At 6 month post transplantation she was still insulin independent with a C-peptide > 2000 pmol/l post standard 90 minute meal tolerance test and maintained HbA1c at 6.2%. CGMS study carried out 6 month post transplantation showed impressive glycaemic control with restoration of near physiological glucose levels (Figure 3.12). This has been maintained in addition to insulin independence for 27 months post-transplant (to date).

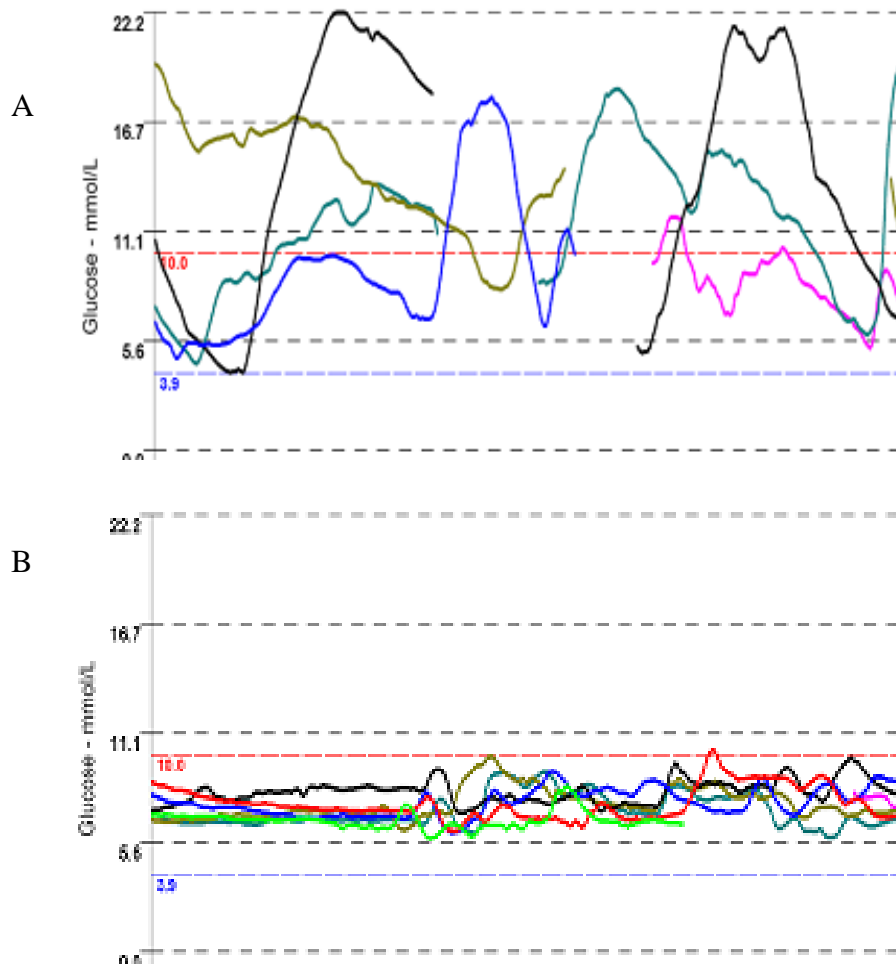


Figure 3.12 CGMS profiles for the third Newcastle islet recipient. A: CGMS profile before transplant outlining highly variable glucose levels and loss of specific pattern. B: CGMS profile after 6month of transplantation with very tight but safe glycaemic control.

3.9 Discussion

Transportation of human islets to remote transplant centres has been explored and followed at different centres and within different consortia. Protocols have varied between different programs to suit the specific setting of each centre (Barshes et al., 2004; Ichii et al., 2007b; Kempf et al., 2005). At the start of this project, no islet transport had been attempted in the UK for clinical use. The above studies were carried out between 2005 and 2008 to develop safe, simple and practical transportation protocols that suit the setting in the UK and thus enable clinical islet transplantation at remote centres utilising central isolation facilities.

3.9.1 Pancreas transport

There have been conflicting reports regarding the benefit of using the two layer method for pancreas transport (Zhang et al., 2006; Tsujimura et al., 2004; Papas et al., 2005). Within our program both TLM (Oxygenated-PFC/UWS) and UWS alone were evaluated. Islet outcome was not significantly improved with the use of TLM since both methods yielded both good quality and low quality islets in our relatively small dataset. Other factors may play more significant roles on islet isolation success and clinical outcome including quality of the pancreas and its donor (Mahler et al., 1999) in addition to the effect of isolation method and enzymes used for digestion (Sabek et al., 2008). The potential for resuscitation of the pancreas using the TLM post prolonged UWS-only preservation (more than 11 hours CIT) has been demonstrated to improve outcome (Tsujimura et al., 2004) and initiation of this following receipt of a pancreas on UWS is certainly an option for isolation centres to consider which can increase number of potential pancreases for islet isolation. The UWS-only transport method is well established, validated and routinely used for preservation of transported

pancreases for whole organ transplantation. Using a nationally co-ordinated offering system ensures delivery of the majority of organs for isolation in less than 8 hours. Despite the modifications explored in this project to facilitate use of TLM with a novel mobile oxygenation chamber, in view of lack of clear benefit in this setting it was decided that the UWS-only method would be adopted utilising techniques already in practice for whole organ packaging.

3.9.2 Islet assessment protocols

Assessment protocols for islet viability and function were successfully established and validated initially on rodent islets (3.4). This enabled definitive studies using validated approaches on all human islet preparations. Despite achieving satisfactory stimulation response with static GSIS on both rodent and human islets, variability remained significant (Papas et al., 2009). This led to building a perfusion system to enable more accurate testing of insulin secretory response to different glucose challenges in the same islet samples. Perfusion experiments demonstrated consistent and reliable dynamic glucose sensing and insulin secretion in transported islets at both physiological (5.6 mmol/l to 11 mmol/l) and extreme (2 mmol/l to 20 mmol/l) glucose challenges.

Sub-renal capsule transplantation in STZ-treated SCID mice remains the most reliable and truly predictive bio-assay for testing the quality of human islets and their ability to engraft and function (Sabek et al., 2005). Despite successful establishment of a STZ-treated SCID mouse sub-renal capsule transplantation model using rodent islets it was not possible to utilise this for human islets due to logistical challenges of establishing and maintaining an in-house bred SCID colony with regular STZ-induction. Moreover, STZ treatment post islet transplant was controversial due to potential toxic effects of STZ on human islets. Certainly avoidance of STZ altogether with islet transplantation

in normoglycaemic SCID mice has been shown to be a useful predictive tool for transplantation outcome (Caiazzo et al., 2008).

Establishment of this model has enabled further translational research programmes within the group focused on *in vivo* models of islet transplants. Future studies are planned which will include these models as the gold standard evaluation of further refinements in pancreas preservation, islet isolation, maintenance and transport towards optimised clinical islet transplant outcomes. Nevertheless, the complexities of employing these models underline the need for reliable and predictive *in vitro* assays that correlate with clinical outcome.

3.9.3 Islet transport conditions

There have been a number of previous approaches for packaging islets for transport. The GRAGIL group elected to package isolated islets ready for transplantation at the recipient centre, with islet product release for transplantation being given by the isolation centre. This involved culturing islets at the isolation site on most occasions before packaging. Their most recent published protocol reported packaging islets in blood transfusion bags in CMRL 1066 medium. Islets bags were transported at room temperature using road services and transport journey to transplant centres was in all occasions less than 5 hours (Kempf et al., 2005). It was not reported whether CMRL 1066 medium was supplemented with HSA or not, although this is probably the case since in our experience lack of protein in the medium has deleterious effects on islet viability resulting in cell lysis and islet clumping rendering them unsuitable for transplantation. On the other hand, the Miami-Houston/Dallas islet transport program elected to transport islets for further washing and full assessment repeated at the recipient centre before releasing islets for clinical transplantation (Barshes et al., 2004; Goss et al., 2002; Ichii et al., 2007b). Islets were transported in a large volume of

medium in several gas-permeable bags using Miami-defined medium (CMRL 1066 based medium) which contains 0.6% HSA at room temperature using air services. Transport journey was less than 5 hours. Islets were pooled and washed at the recipient centre and full assessment of IEQ count and quality was repeated by fully trained staff within a fully approved cGMP-grade clean room facility. Ichii found gas-permeable bags to be superior to non-gas permeable bags within this setting (Ichii et al., 2007b).

The model developed and validated in this project was to package islets in blood transfusion bags in 150 ml- 200 ml CMRL 1066 medium containing 2% HSA ready for transplantation without any additional washing steps at the receiving centre. In view of relatively longer journey for islet transport compared to Miami-Houston/Dallas (Ichii et al., 2007b) or GRAGIL (Kempf et al., 2005) (7.3 hours vs. <5 hours), and in order to minimise consumption of nutrients in transport medium and to limit the release of insulin and other hormones/ cytokines into the transport/transplant medium, a maintenance temperature lower than room temperature was chosen. Quality assessments of islets at Newcastle confirmed maintenance of sterility, viability and integrity. When IEQ counts were carried out at Newcastle following volume reduction, estimates were comparable with those of KCIIF. This indicates preserved islet count post transport following current protocols. However lower but not significant islet counts were calculated at Newcastle compared to KCIIF when counts were done on side arm sample reflecting the unavoidable lack of homogeneity. Based on this it was decided that a repeat of IEQ estimation at Newcastle for the clinical program would not be required.

Assessment of transport medium post transport revealed average insulin content at 1unit/bag or 6.4 units/1million IEQs. When corrected for transport time it was estimated that insulin release into the transport medium was on average 1.1

unit/hour/1million IEQs. Assessment of the pro-inflammatory cytokine profile in transport medium revealed minimal, but marginally higher than physiological, levels of IL-6, IL-1 β , TNF α and INF γ . Despite this, the absolute dose of each of these cytokines is considered trivial when given with the islet graft. It is not known whether more cytokines would be released once islets are transplanted and resume biological activity at 37 °C, and whether this would have any role in initiating/aggravating the instant blood mediated inflammatory response (IBMIR) which is believed to be responsible for initial islet loss (Bennet et al., 2000).

3.9.4 Standard operating procedures and the clinical program

Following consultations with experts in the field at Miami Diabetes Research Institute, Edmonton Islet Transplant Program, King's College Islet Isolation Facility in London and the Stem Cell laboratory at Newcastle University; SOPs that meet the needs and requirements of the UK were established. These SOPs were practically tested, modified and validated during this project and were made available to the UK Islet Transplant Consortium.

3.9.5 Conclusions

Islet transport for transplant is not a new concept. Several successful programs have confirmed both feasibility and benefit. Each program has its unique protocols that have commonalities and differences from other programs and continue to undergo development, optimisation and validation leading to continued success. The work undertaken in this project was an attempt to establish an islet transport model to specifically suit the UK settings.

Although there has inevitably been an empirical component with limited numbers of organs precluding detailed comparison of different approaches within all preparations (eg static vs perfusion GSIS), a rigorous scientific approach has been the goal

throughout. The studies in this project have successfully validated the feasibility of utilising a central islet isolation facility for delivering safe and quality-assured human islets for clinical transplantation at remote centres. They have underpinned a government funded national islet transplantation program in the UK with the data attained in this chapter now accepted for publication in Cell Medicine (Appendix 2.1). Four transplantations of transported islet preparations in three patients have taken place in Newcastle, representing the first clinical transplantation of transported islets in the UK.

Despite successful outcomes to date, there remains an unmet clinical need. There are a limited number of pancreases available for islet isolation and the majority of islet recipients require more than one donor to achieve sustained graft function. As the number of suitable pancreases cannot be significantly increased it would be very useful if islet survival post-transplantation could be augmented and maintained so that islets retrieved from one donor can become consistently sufficient for one recipient. One approach to this is exploring potential for augmenting islet mass and function *in vitro* together with minimising pro-inflammatory signalling prior to transplantation.

Chapter 4. Investigating the effect of pregnancy related hormones on mass and function of intact human islets *in vitro* in comparison to other growth factors

4.1 Introduction

Islet culture prior to transplantation offers several potential advantages. These include providing a valuable window of time to enable more quality testing, allowing the recipient enough time to reach the transplant centre and enabling initiation of time-dependent pre-transplant immunosuppressant induction. Moreover, islet culture might enable pooling of suboptimal islet yields, which are otherwise too small to transplant, in one optimal mass suitable for transplantation. However, several reports indicate possible loss of islet mass during culture (Bottino et al., 2002; Ichii et al., 2007a; Zhang et al., 2004). Suggested possible underlying factors associated with greater loss over time include longer cold ischemic time (CIT), use of the two layer method (TLM), lower purity and high islet index (ratio between IEQ and islet count) which indicates predominance of relatively large islets (Kin et al., 2008). Mita reported a negative effect of Ficoll gradient on islet survival in post-isolation culture with increased proinflammatory cytokine/chemokine production in comparison to OptiPrep-based preparations (Mita et al., 2009). However, islet loss associated with these factors might suggest early islet damage that leads to latent inevitable islet loss post-transplantation. Hence islet culture may enable this latent islet loss to become manifest. Moreover, cytokine release from disintegrating islets might trigger / potentiate the instant blood-mediated inflammatory reaction (IBMIR) (Moberg et al., 2003; Saito et al., 2010) leading to further graft loss. Culture, washing and resuspension prior to transplantation may enable dilution of these pro-inflammatory and pro-thrombogenic signals.

Culture conditions can play a role in whether islet mass and function is lost or maintained. CMRL 1066 and other CMRL 1066-based media are the most commonly used for islet culture (Ichii et al., 2007a; Kempf et al., 2005; Kin et al., 2008; Moberg

et al., 2003). Lee reported improved islet recovery from culture when CMRL 1066 medium was supplemented with human serum albumin (HSA) in comparison to human serum (Lee et al., 2008a). Barbaro reported possible reduction in caspase 3 activation with an increase in HSA concentration up to 5% in the medium (Barbaro et al., 2008). Moberg reported reduced TF and MCP-1 expression and reduction in an *in vitro* IBMIR model with nicotinamide treatment (Moberg et al., 2003). Similarly, Lund observed an anti-inflammatory effect of glucocorticoids on human islets following 48 hours exposure *in vitro* with reduction in mRNA expression of tissue factor, MCP-1, and IL-8 (Lund et al., 2008). These examples of manipulations of medium type, medium supplements and culture conditions suggest a potential important role of islet culture in maintaining islet mass and function pre-transplant. To date no optimal culture conditions have been universally agreed.

4.2 Growth factors

There have been several studies evaluating the effect of different growth factors on β -cell mass and function in human islets, rodent islets and a variety of cell-lines. Pregnancy-related hormones are of potential interest as a considerable physiological increase in β -cell mass and function during pregnancy is manifest to compensate for the increase in metabolic demand and insulin resistance (Sorenson and Brelje, 1997). These factors include prolactin (PRL), placental lactogen (PL) and growth hormone (GH). It has been shown that these hormones exhibit a positive effect on β -cell proliferation without negative effects on their ability to produce insulin (Brelje et al., 1993; Nielsen et al., 1999; Sorenson and Brelje, 1997). Over-expression of PL downstream of the rat insulin promoter (RIP) in murine β -cells resulted in hypoglycaemia and hyperinsulinaemia (Vasavada et al., 2000). Mice with PRL

receptor (PRL-R) deficiency demonstrated β -cell hypoplasia, reduction in insulin mRNA and impaired glucose stimulated insulin secretion (Freemark et al., 2002). These findings indicate an important role for the PRL-R and its activators on both β -cell mass and function. Similar findings were observed in mice with GH receptor (GH-R) deficiency (Liu et al., 2004).

Hepatocyte growth factor (HGF) has also been studied as a candidate for enhancing β -cell replication. Otonkoski reported a mitogenic effect of HGF on both rodent and human islets grown as a monolayer (Otonkoski et al., 1996). However it was not possible to replicate such an effect in β -cells in intact adult human islets (Lefebvre et al., 1998). Beattie has, however, confirmed a possible effect of HGF on β -cell replication in human islets when the 3D configuration of the islet was supported in culture with a fibrin matrix (Beattie et al., 2002). Gahr reported the need for low glucose concentration for HGF to have a clear mitogenic effect in INS-1 cells, a human β -cell line model (Gahr et al., 2002). Garcia-Ocana demonstrated a positive *in vivo* effect of HGF on β -cell number and insulin secretion in a transgenic mouse model with HGF being specifically expressed in β -cells driven by the rat insulin II promoter (Garcia-Ocana et al., 2000).

Insulin-like growth factors (IGF) 1 and 2 are expressed in many cells in the body including the pancreas. IGF-1 and IGF-2 are expressed at varying levels during embryonic development (Hill et al., 1999; Portela-Gomes and Hoog, 2000). A role of IGF-1 and IGF-2 in enhancing DNA synthesis and β -cell replication in a glucose independent manner in rodent models has been reported (Hogg et al., 1993). Overexpression of IGF-1 in β -cells driven by insulin promoter I in a transgenic mouse model resulted in a 3 fold increase in β -cell proliferation (George et al., 2002). Similar

results were observed with IGF-2 overexpression driven by the insulin I promoter in mice (Petrik et al., 1999).

Glucagon-like peptide 1 (GLP1) has been shown to induce β -cell proliferation in both intact rodent islets and insulinoma cell-lines *in vitro* (Buteau et al., 2003). However, this effect was dependent on the presence of betacellulin (BTC), a member of the epidermal growth factor family. Acute administration of exendin-4 (a naturally occurring long-acting GLP1 homologue) or GLP1 resulted in an increase in β -cell mass in normal (Edvell and Lindstrom, 1999), pre-diabetic (Tourrel et al., 2002) and diabetic rats (Farilla et al., 2003; Li et al., 2003). Moreover, GLP1 has been shown to reduce apoptosis and enhance glucose stimulated insulin secretion (GSIS) in freshly isolated human islets (Farilla et al., 2003).

Yamamoto demonstrated an increase in β -cell mass, reduction in body weight and improvement in glucose tolerance in a mouse model with glucose intolerance following 8 week treatment with human recombinant betacellulin (BTC) (Yamamoto et al., 2000). These findings were further confirmed by Li in rats when treated with Activin A (Act A) and BTC following STZ treatment (Li et al., 2004). This resulted in improved glucose tolerance, reduced hyperglycaemia and increased DNA synthesis in a number of pancreatic cell phenotypes including β -cells. Suarez-Pinzon demonstrated a positive effect on total β -cell number in cultures of human islets dissociated into single cells following dual treatment with gastrin and epidermal growth factor (EGF), a phenomenon attributed at least in part to enhancing β -cell neogenesis from the ductal cells present in the culture (Suarez-Pinzon et al., 2005).

These growth factors have been assessed in unrelated experiments employing a range of cell models and species including murine cells and have used differing culture

systems including adherent cells. Whether these effects are seen in comparative studies in intact human islets has not been established yet.

The goal of the current studies was to establish an experimental platform for undertaking studies focused on primary intact human islets. To obtain sufficient funding for these challenging studies, I was co-applicant on a successful Project Grant application to the Diabetes Research and Wellness Foundation entitled: 'Enhancement of β -cell mass and function in human islets by physiological factors upregulated in pregnancy'.

4.3 Aims

The overall aim of this chapter was to explore the effects of pregnancy related hormones on human islets in comparison to other growth factors. Specific objectives were to:

1. Evaluate gene expression profile of key functional, stress and proliferative markers in β -cell cultures following pregnancy-related hormones and other growth factor treatment
2. Investigate the effect of pregnancy-related hormone on human islet mass *in vitro* in comparison to other growth factors
3. Study (pro)insulin biosynthesis and processing activity in β -cells following pregnancy-related hormone and other growth factor treatment
4. Investigate the effect of acute exposure to pregnancy related hormones on islet function

4.4 Growth factors

Pregnancy related hormones including prolactin (PRL), placental lactogen (PL) and growth hormone (GH) were included in comparison to a group of other growth factors

that have been reported to have a positive effect on islet mass and / or function (Table 2.8). All growth factors were sourced as human recombinant proteins to minimise inconsistencies and possible bias related to unreported additional growth factors and other impurities within biological extracts.

4.5 Growth factor treatments

Human islets were incubated in the growth factor supplemented medium following overnight culture in suspension in serum-free islet medium. Treatment medium was prepared using CMRL NCL1 medium (Table 2.6) supplemented with 0.5% HSA and the growth factor of interest at optimal concentration obtained from the published literature (Table 2.8). Four separate growth factor (GF) experiments were carried out on different islet preparations. Each experiment included an internal control treated with vehicle (PBS) alone. The experiments were designated as GF1 (LDIS011), GF2 (LDIS012), GF3 (LDIS014), GF4 (LDIS015) (further preparation details are outlined in Table 3.3). In each experiment an average of 20 islets in 4 ml treatment medium were included in each well of 6 well plates with at least five repeats for each treatment hormone. Treatments were carried out over 72 hours in a humidified 37 °C incubator maintained with constant 5% CO₂. After 72 hours, medium and islets were separated by centrifugation at 14,000 rpm (16,000 G) for 5 minutes. All media samples were stored at -20 °C for later analysis. One islet pellet from each condition was used for RNA extraction and the remaining pellets underwent cellular lysis for assessment of protein and hormone content. Lysates were stored at -20 °C for later analysis.

4.5.1 Growth factor treatment and gene expression studies

Gene expression analysis was carried out on GF1, GF3 and GF4 studies. cDNA was synthesised from all RNA samples (2.22.2). Quantitative real-time PCR (q-PCR)

experiments (2.22.4) using the SYBR green method were carried out for insulin, proliferative cell nuclear antigen (PCNA) and C/EBP homologous protein (Chop) (also known as DNA damage inducible transcript 3 (DDIT3)) in addition to beta-2 microglobulin (B2M) which served as a reference gene.

4.5.1.1 q-PCR experiment setup and validation

Since all primer sets were custom designed, confirmation of validity and suitability for q-PCR was internally validated. Initially conventional PCR reactions for q-PCR B2M primers were set up for cDNA and DNase-treated RNA samples of each islet sample tested. This was to confirm elimination of genomic DNA and successful synthesis of cDNA. When a PCR product was generated from a DNase-treated RNA sample the corresponding cDNA was excluded and new RNA sample was DNase-treated and used to produce new cDNA produced which was then revalidated.

q-PCR reactions for target genes and reference gene were carried out for all validated cDNA samples in triplicate. A standard curve was formulated using a 1 in 10 dilutional series derived from control human islet cDNA. Standard curve samples and growth factor treatment samples were run on the same plate and a triplicate of H₂O only in place of cDNA was added as a negative control in addition to a duplicate of DNase-treated RNA of each sample to exclude genomic DNA contamination. In all SYBR Green I q-PCR reactions, a melting curve step was added at the end of the reaction to exclude non-specific product amplification. All analysis was carried out using the LightCycler480[®] 1.5.0 software. Melting curve was automatically generated for all reactions using the T_m Calling analysis tool. A single peak was seen in all melting curves of all primers indicating single product amplification (Figure 4.1). No product was seen in all H₂O alone samples indicating purity of all reagents.

The level of target gene expression in each sample was corrected to B2M expression to eliminate differences related to variations in sample size between different treatments. All treatment samples in each experiment were normalised to the control of the same experiment to determine relative change in gene expression. The mean of three experiments was calculated.

4.5.1.2 Effect of growth factors on insulin gene expression

Insulin is a key β -cell functional marker. Relative quantitative RT-PCR was employed to evaluate whether growth factor treatment of cultured islets had any effect on insulin gene expression. Gastrin treatment resulted in relative reduction in insulin gene expression although this did not reach significance. Prolactin and the other comparator treatments showed a trend towards increased insulin expression, but no significant differences in comparison to control were seen (Figure 4.2).

4.5.1.3 Effect of growth factors on Chop gene expression

When cells are exposed to endoplasmic reticulum (ER) stress a number of ER chaperone proteins are up-regulated including Chop. Prolonged exposure to ER stress results in cell apoptosis. Expression of Chop in cultured islets was investigated to evaluate degree of cellular stress and possible modulating effect of pregnancy related hormone treatment.

There was no meaningful change in Chop expression in response to any of the different treatments in comparison to control (Figure 4.3).

4.5.1.4 Effect of growth factor on PCNA gene expression

Proliferative cell nuclear antigen (PCNA) is a marker of proliferation in eukaryotic

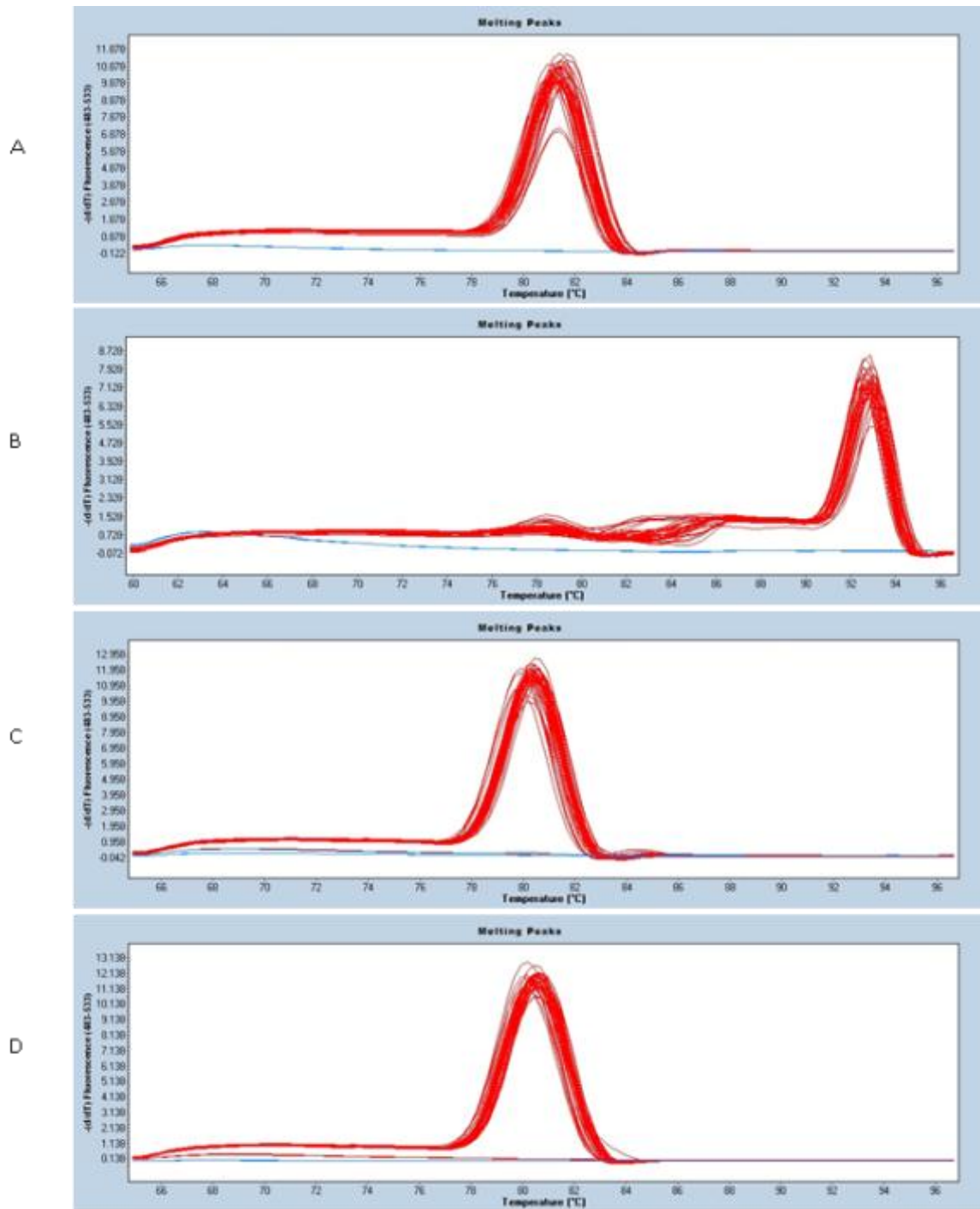


Figure 4.1 Melting curve analysis. A: B2M, B: Insulin, C: Chop, D: PCNA. In each graph there was a single peak indicating single PCR product and thus validity of the primer set and PCR reaction. Negative control (H₂O) did not produce a PCR product confirming purity of all reagents used.

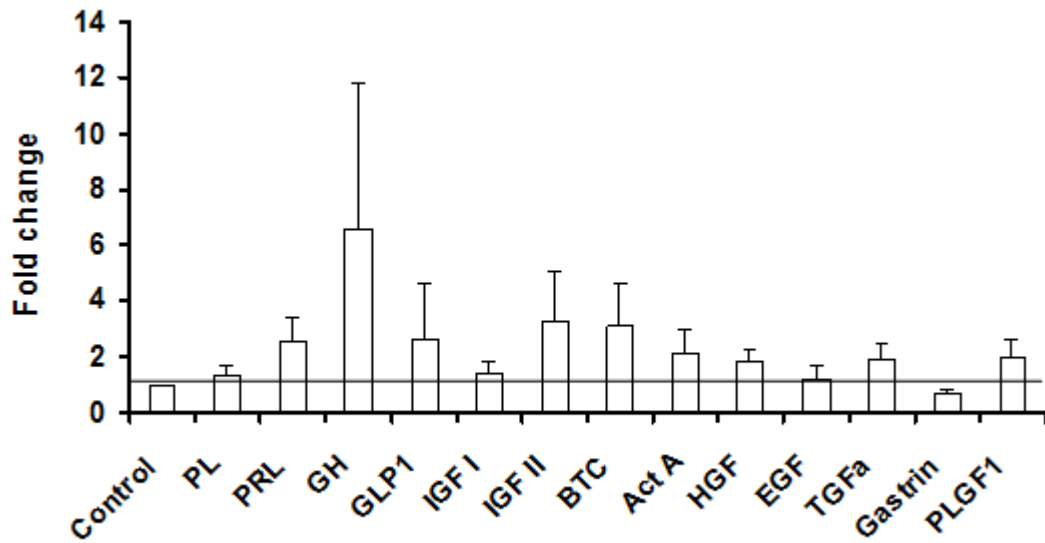


Figure 4.2 Insulin relative quantitative gene expression in growth factor treatment studies. Results represent mean of fold change in insulin expression (normalised for B2M expression) in comparison to control of three separate experiments. (n=3 ± SD).

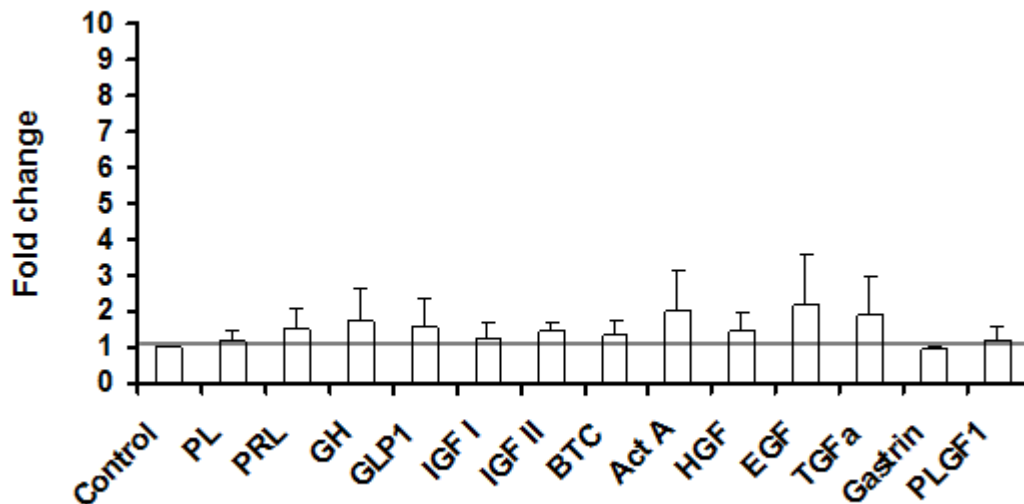


Figure 4.3 Chop relative quantitative gene expression in growth factor treatment studies. Results represent mean of fold change in Chop expression in comparison to control (normalised for B2M expression) of three separate experiments. (n=3 ± SD).

cells (Lee et al., 2005). To evaluate the effect of growth factor treatment on islet cell proliferation, PCNA expression was studied. Pregnancy-related hormones showed a trend towards increased PCNA expression in comparison to the control, although results when data were combined from three repeated studies did not reach statistical significance (Figure 4.4).

4.5.2 PRL treatment for gene expression analysis

Trends observed in treatments with growth factors did not reach statistical significance due to variability between the three repeated studies in different batches of human islets (even though each individual study had at least 5 biological repeats for each condition). This underlines the importance and complexities of including several batches of islets from different donors in undertaking screening studies of a range of growth factors and other potential modulators of mass and function in physiological human islets. In light of these results, a further study was designed to determine whether prolactin can mediate a positive enhancing effect on gene expression in human islets in culture. Six biological repeats were included in both PRL and vehicle groups. Each repeat contained an average of 50 IEQs (to optimise equal loading from a homogeneous islet solution). Each islet repeat was cultured in 4 ml CMRL NCL1 medium containing 1% HSA and 1% P/S in 6 well plates. The PRL group was supplemented with 80 nM PRL (equivalent concentration to preceding studies) while control islets were treated with vehicle (PBS). Treatment was for 72 hours at 37 °C in a 5% CO₂ incubator as before (4.5). Medium was then removed and islet pellet was processed for total RNA extraction and cDNA synthesis. Relative quantitative gene expression analysis for PDX1, insulin, PCNA and Chop was carried out. Due to problematic non-specific amplification seen in custom-made PDX1

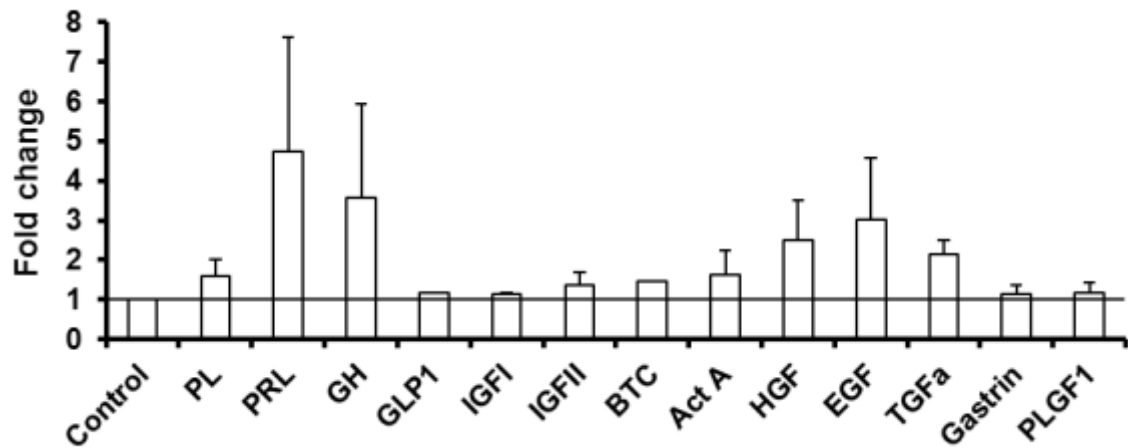


Figure 4.4 PCNA relative quantitative gene expression in growth factor treatment studies. Results represent mean of fold change in PCNA expression (corrected for B2M expression) in comparison to control of three separate experiments. (n=3 ± SD).

primers, relative quantitative PDX1 gene expression was assessed using a pre-validated Taqman probe q-PCR assay. The Taqman probe method was also used for analysis of insulin expression since PDX1 and insulin validation was carried out simultaneously on the same plate with B2M serving as a control for both (Table 2.13). Relative quantitative gene expression analysis for PCNA and Chop was carried out using the SYBR Green I method as before with B2M serving as reference gene (Tables 2.12).

4.5.2.1 PRL effect on insulin and PDX1 expression

A significant increase in insulin expression was seen in islets incubated in the presence of prolactin. (1.83 ± 0.3 fold (mean \pm SEM); $p=0.038$ vs. control; $n=6$) (Figure 4.5). No meaningful change in PDX1 expression was, however, seen in response to PRL treatment in comparison to control.

4.5.2.2 PRL effect on Chop and PCNA expression

PRL treated islets showed a trend towards reduced Chop expression but this did not reach statistical significance in intact human islets. No meaningful change in PCNA expression was observed (Figure 4.6).

4.5.3 Islet mass

Protein content was measured in all lysate samples (2.18). Mean of four biological repeats in each experiment was calculated. To elucidate any potential effect on overall islet mass, fold change in total protein concentration in comparison to control islets was calculated. The effect of growth factor treatment on mass was estimated as fold change in comparison to control within each experiment. Mean fold change of all four studies was calculated (Figure 4.7).

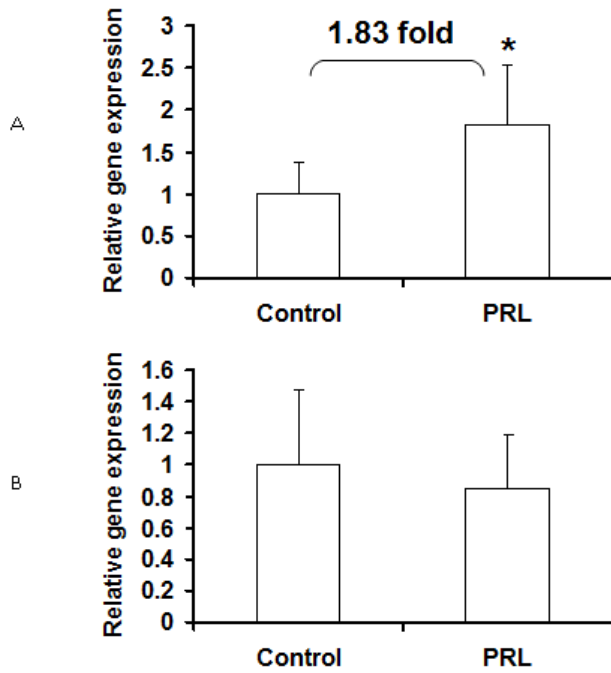


Figure 4.5 Insulin and PDX1 expression in islets treated with PRL. Results were obtained from a single human islet donor with mean of 6 biological repeats. There was a significant increase in insulin expression (A) but not PDX1 (B). ($p=0.038$ vs. control, $n=6$, \pm SEM).

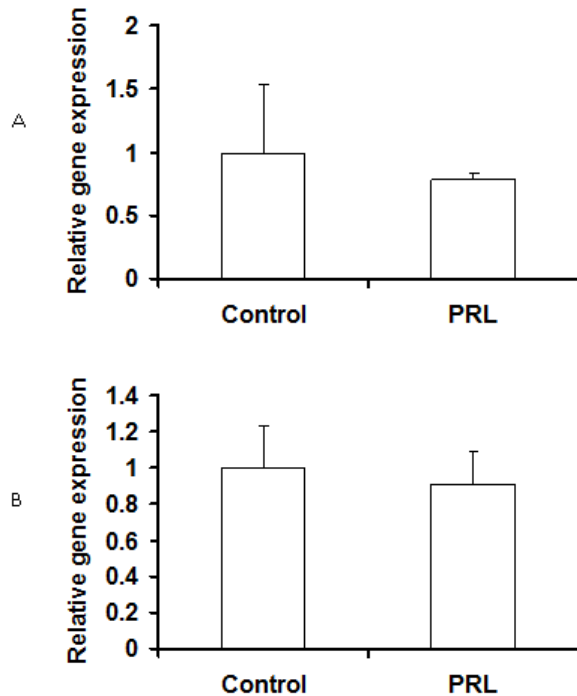


Figure 4.6 Chop and PCNA expression in islets treated with PRL. Results relate to one human islet donor with mean of 6 biological repeats. There was no statistical difference in Chop (A) or PCNA (B) expression in response to PRL treatment compared to control. ($n=6$, \pm SEM).

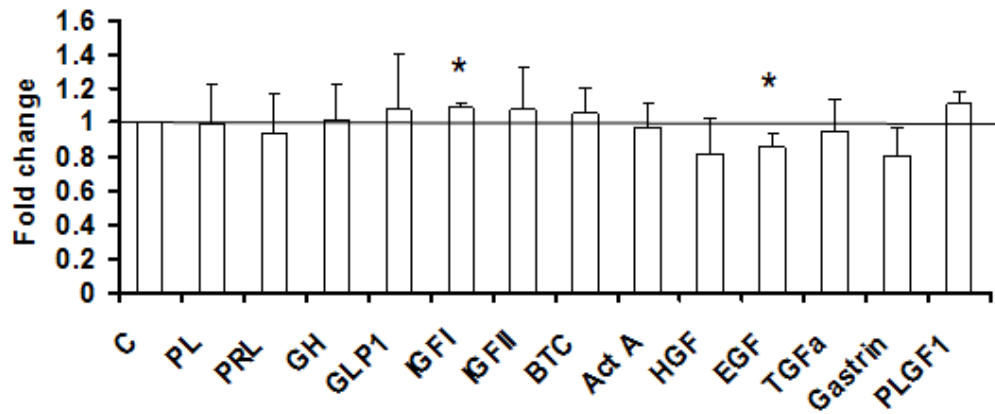


Figure 4.7 Growth factor effect on overall islet mass. Results represent fold change in protein content in islets treated with pregnancy related hormones and other growth factors (Table 2.8) for 72 hours. Results relate to four separate pancreas donor preparations ($n=4 \pm SD$, $p<0.05$).

There was a significant increase in mass in islets treated with IGF-1 (1.1 ± 0.02 (mean \pm SD), $p=0.003$) and a decrease in mass with EGF treatment (0.86 ± 0.08 , $p=0.009$, $n=4$).

4.5.4 Impact of growth factor treatment on (pro)insulin biosynthesis and processing

Biosynthesis of proinsulin and processing of proinsulin to insulin are key functional markers of β -cells. These functions were investigated in all growth factor treatment experiments to elucidate the potential effect of pregnancy-related hormones in comparison to other growth factors. GF1 is presented as a representative study of all GF experiments. Fold change in hormone levels in each study in comparison to control was calculated. Mean fold change including all GF studies was also calculated. Due to unavoidable variations in cellular content in each sample, all values were corrected for protein content in each sample.

4.5.4.1 Growth factor treatment effect on total insulin

Insulin biosynthesis is one of the primary functions of human β -cells. Total insulin levels (intracellular + secretion over a 72 hour period) in the presence / absence of pregnancy related hormones and other growth factors were assayed. In GF1, there was a trend towards increased total (secreted + stored) insulin level with pregnancy-related hormones and other growth factors. This was statistically significant with BTC treatment (Figure 4.8 A). However, calculated mean fold change in total insulin synthesis in 4 combined GF studies did not show any significant changes in response to different treatments despite continued trends towards increased insulin biosynthesis in presence of pregnancy-related hormones and betacellulin (Figure 4.10A).

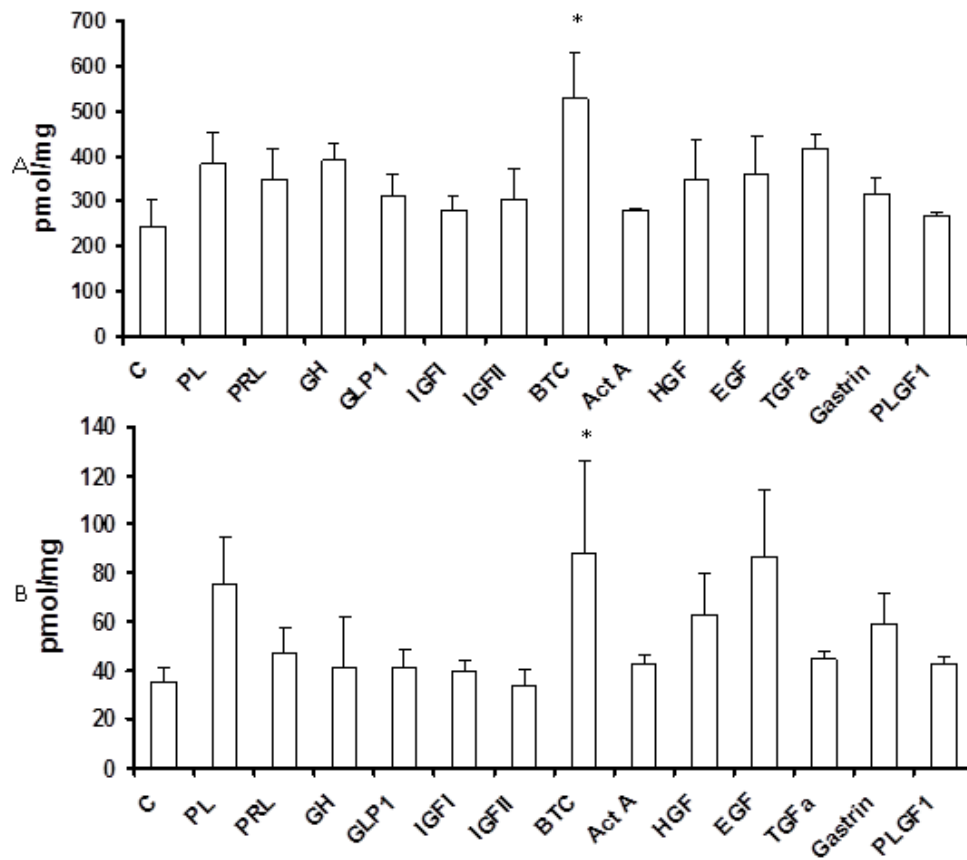


Figure 4.8 Insulin synthesis and storage in islets. Results represent mean of 3 biological repeats in the GF1 study (LDIS011). Pregnancy related hormones and other growth factors resulted in an overall trend towards increased total insulin (secreted over 72 hours + intracellular) (A) and increased insulin storage (intracellular alone) (B) corrected for total protein concentration compared to control. * $p < 0.05$, $n = 3$, \pm SEM.

4.5.4.2 Growth factor treatment effect on insulin storage

Hormone storage is an important function of endocrine cells. To elucidate whether treatment with pregnancy related hormones or other growth factors had any impact on storage capacity in β -cells, intracellular insulin levels were determined. In GF1, there was a trend towards increased insulin storage in islets treated with pregnancy related hormones and other factors. This was statistically significant with BTC treatment (Figure 4.8 B). However average fold change in insulin storage in comparison to control in 3 combined studies did not show any statistically significant differences.

4.5.4.3 Growth factor treatment effect on proinsulin levels

Healthy β -cells synthesise proinsulin which is then converted to insulin. The process is very efficient and only minimal amounts of proinsulin remain incompletely processed. Increased proinsulin release might indicate β -cell stress and exhaustion. Intracellular and extracellular proinsulin levels were calculated in all samples to elucidate possible effects of pregnancy related hormones and other factors on proinsulin synthesis efficiency of processing and control of secretion.

In GF1 there was a trend towards increased total proinsulin (Figure 4.9 A) with an increase in both intracellular (Figure 4.9 B) and extracellular (Figure 4.9 C) levels in response to pregnancy related hormones and other factors. However, mean fold change in comparison to control in 3 combined studies from different donors did not show any statistically significant differences from control in total proinsulin levels (Figure 4.10 B).

4.5.4.4 Growth factor treatment effect on proinsulin processing capacity

Processing of proinsulin to mature insulin is an essential function of healthy β -cells. It is believed that processing capacity becomes impaired under cellular stress and chronic insulin demand (Steiner and James, 1992). To investigate the possible role of

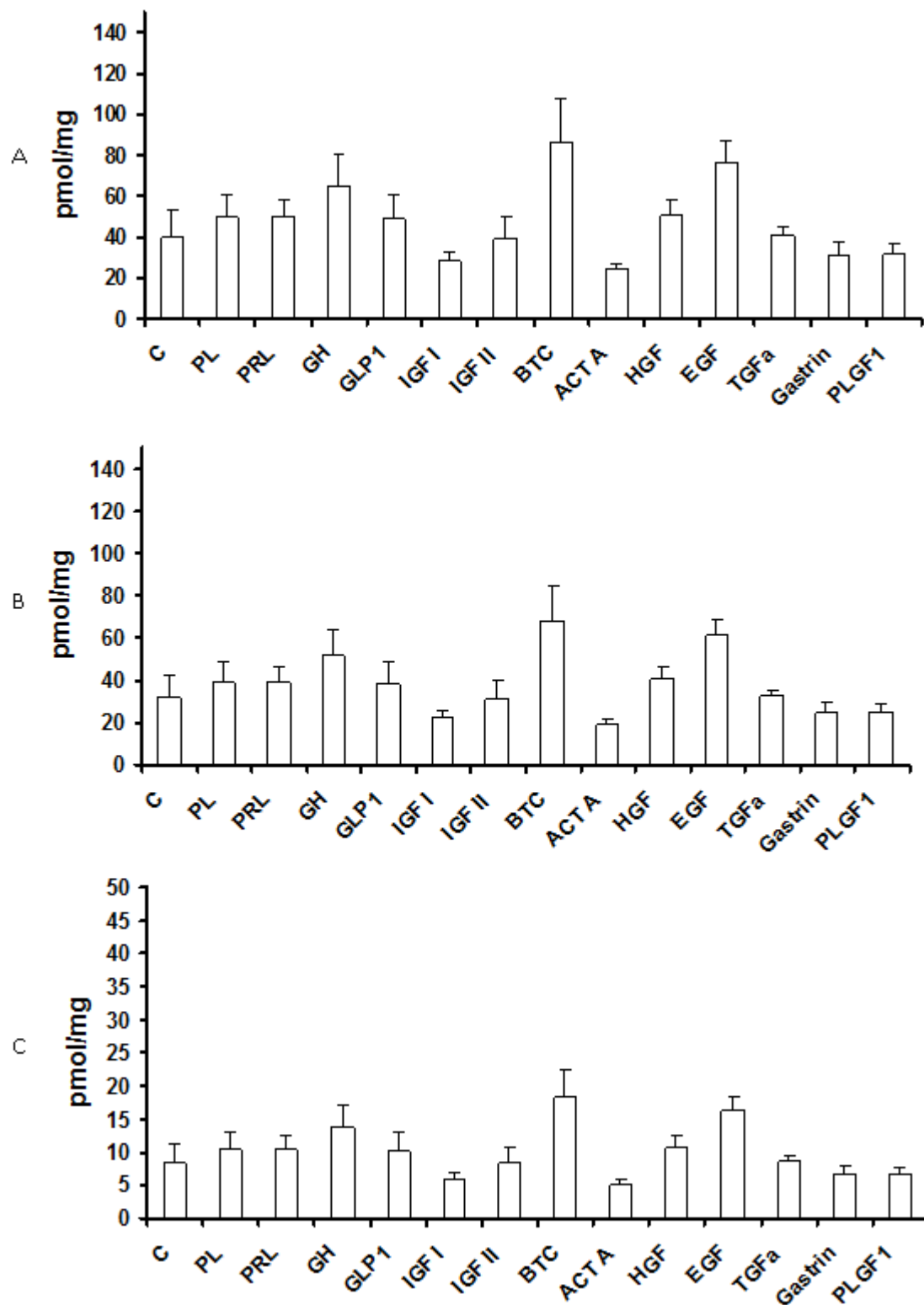


Figure 4.9 Proinsulin analysis in human islets. Results represent mean of three repeats in the GF1 (LDIS011) experiment demonstrating total proinsulin levels (intracellular + media following 72 hours incubation) (A) storage (B) and secretion (C). No significant change was seen in response to any specific treatment ($n=3 \pm SD$).

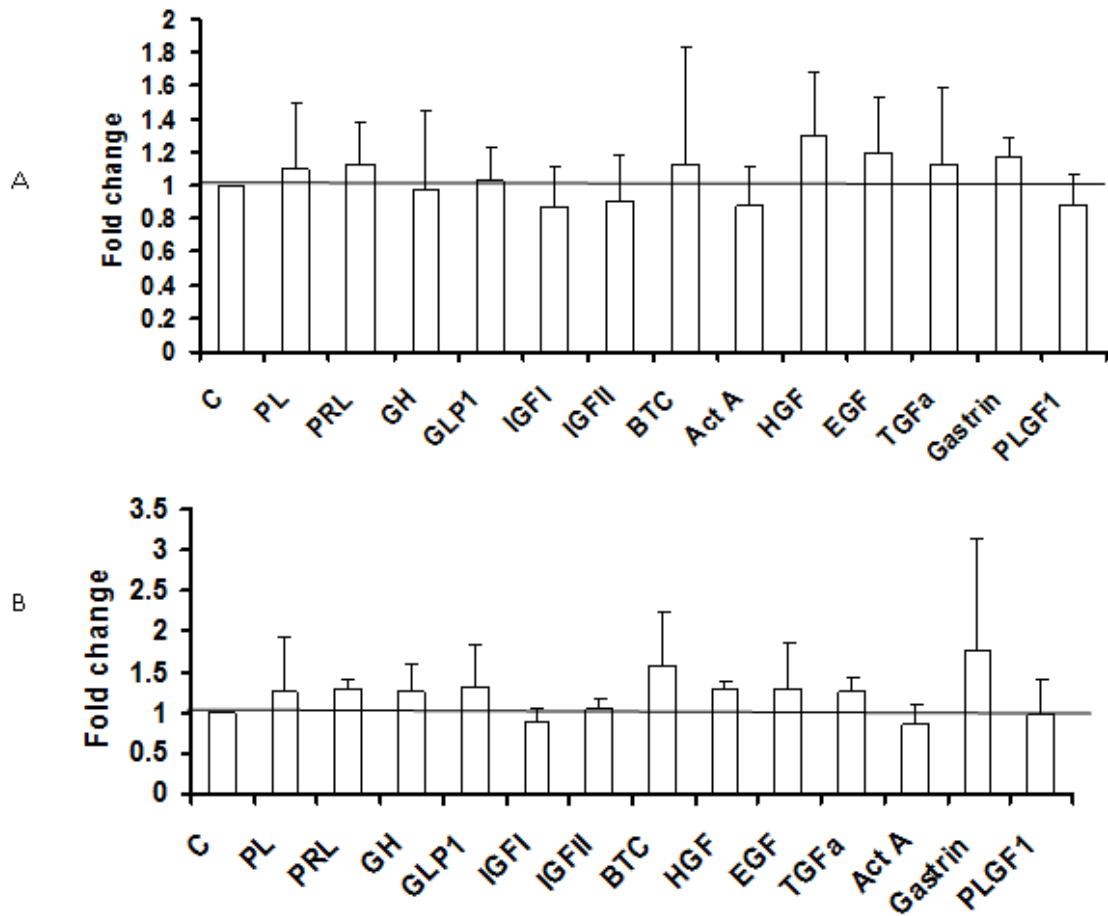


Figure 4.10 Fold change in overall (pro)insulin level. Results represent average fold change in total (intracellular + media after 72 hours incubation) insulin (A) and proinsulin (B) production over 72 hours corrected for total protein concentration in presence of different treatments. Results relate to four separate pancreas donor preparations (n=4, \pm SD).

pregnancy related hormones and other growth factors on β -cell processing capacity, ratio of insulin to (pro)insulin was calculated for intracellular and extracellular compartments in addition to total hormone levels in the above studies. Intracellular ratios were in all samples above 80% indicating efficient processing activity of internal proinsulin stores (Figure 4.11 A). In all samples, more than 97% of secreted (pro)insulin was fully processed to insulin (Figure 4.11 B). More than 93% of total (stored + secreted over 72 hours) (pro)insulin was processed to insulin (Figure 4.11 C). These results indicate that β -cell processing capacity is maintained in these culture conditions. It was not possible to elucidate whether any of the treatments can restore impaired processing capacity in these settings. Toxic conditions which impair processing ability of β -cells merit further study.

4.5.5 Acute prolactin treatment in perfusion studies

The acute effect of PRL on insulin release from human islets was investigated. Due to variability and inconsistency of results in static incubations for glucose stimulated insulin secretion (GSIS) experiments and to enable real-time study of islet responses to prolactin exposure and glucose changes in the environment this was evaluated in dynamic perfusion studies. Islets used in this study were kindly provided by Dr Huang from King's College following an overnight culture at KCIIF. Pre-transport viability was 90% with estimated purity at 70%. Post transport viability was 85% with maintained purity.

4.5.5.1 Acute PRL effect on human islets at physiological glucose

The purpose of this experiment was to evaluate insulin response in islets perfused with physiological glucose concentration after a pulse of PRL exposure. Equipment and islets preparation were set up as described before (2.15.2) using a four chamber

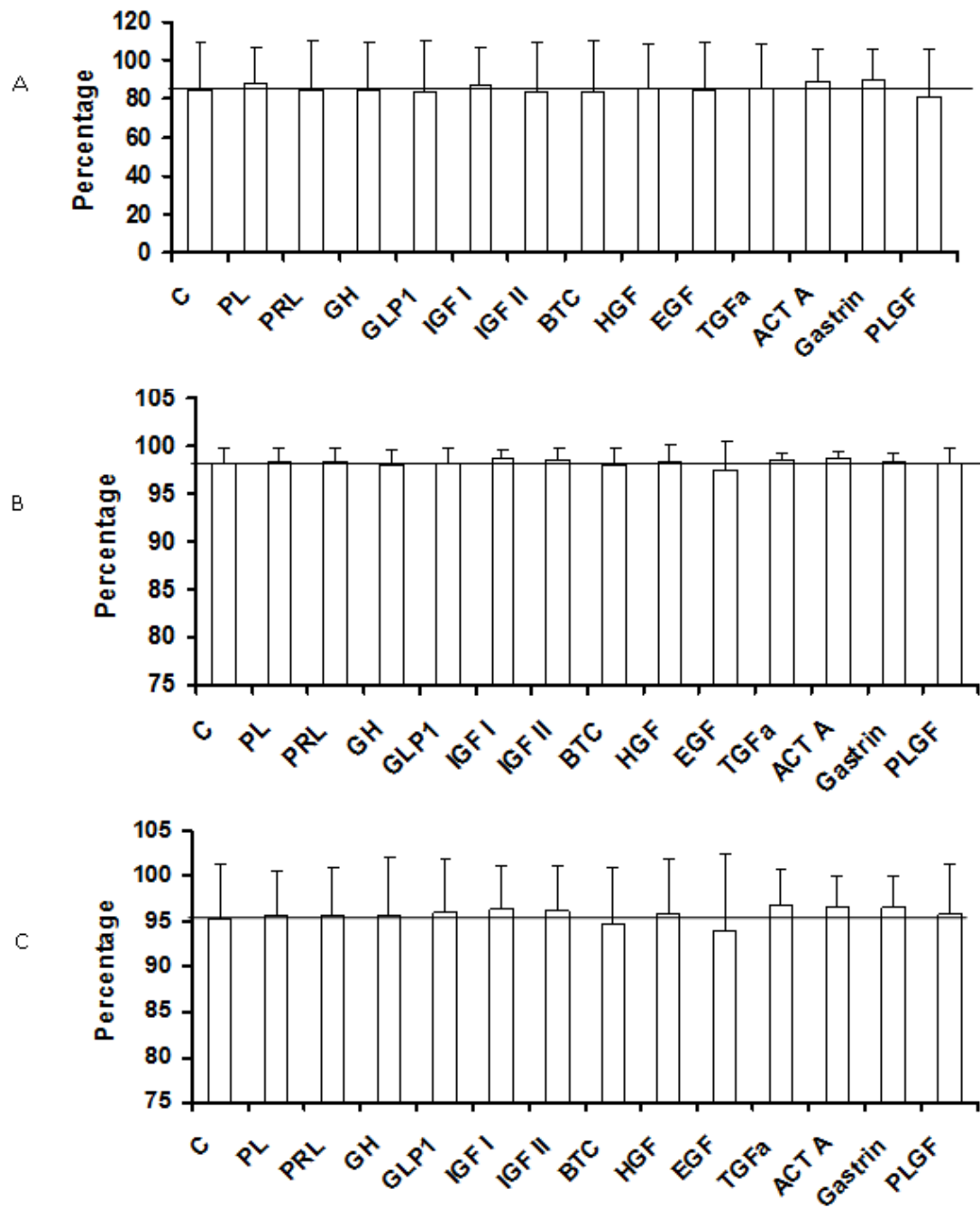


Figure 4.11 Effect of growth factors on proinsulin processing. All samples demonstrated effective processing of biosynthesised proinsulin to mature insulin. No significant differences were detected in response to growth factor treatment either in stored (A), secreted (B) or total insulin to overall (pro)insulin ratio (C). Results relate to three separate pancreas donor preparations (n=3, \pm SD)

setup. Islets were perfused with perfusion solution containing 5 mM glucose throughout the experiment with an end-pulse with 200 nM PRL during the last 20 minutes. Insulin was assayed in the perfusion samples as outlined before (2.17.2.2). An increase in insulin secretion in all chambers was observed (Table 4.1 and Figure 4.12) and a fold change in each chamber was calculated as the ratio between secreted insulin during the last 10 minutes before the switch to PRL treatment and secreted insulin during the first 10 minutes after the switch to PRL treatment. Mean fold change in all 4 chambers demonstrated a consistent and a borderline significant increase in insulin release by 1.6 ± 0.4 (mean \pm SD, $p=0.045$, $n=4$).

4.5.5.2 Effect of acute PRL exposure on insulin release in human islets during glucose changes

The purpose of this experiment was to investigate the acute effect of PRL on insulin release from human islets at different glucose challenges. The perfusion apparatus was set up as before (2.15.2) using two groups of 3 chambers. Group A (control group) was perfused with glucose-only supplemented solutions while group B was perfused with glucose and PRL (200 nM throughout) supplemented solutions. Initially all islets were perfused with 5 mM glucose-containing solution then switched to 11 mM 20 minutes before end of experiment in both groups. Perfusate was collected and insulin content was assayed as described before (2.17.2.2). Fold change in each chamber was calculated as the ratio between secreted insulin

Channel	Insulin level ng/ml		Fold change	Mean	SD	t-test
	5mM glucose	5mM glucose + 200nM PRL				
C1	9.93	20.78	2.09	1.6	0.4	0.045
C2	6.30	8.81	1.40			
C3	41.15	53.77	1.31			
C4	8.31	13.63	1.64			

Table 4.1 Effect of acute PRL exposure on islets at physiological glucose concentration. Results are mean insulin values measured over 10 minutes before or after PRL exposure.

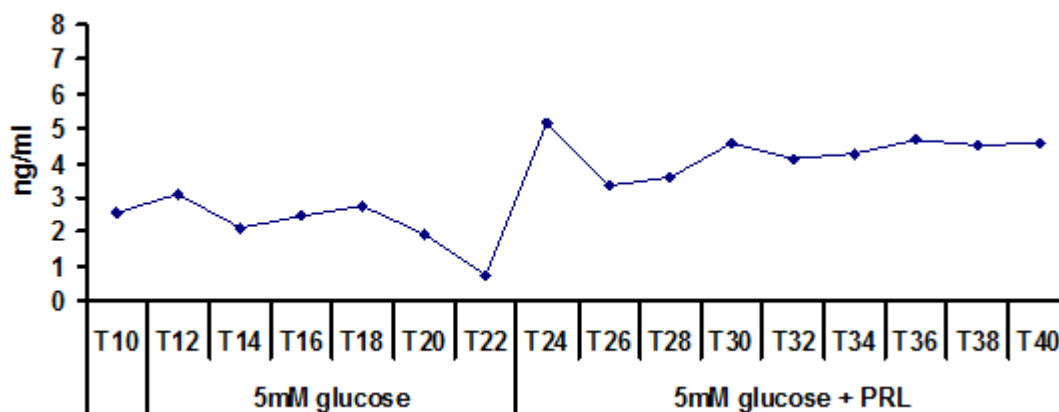


Table 4.12 PRL acute effect at physiological glucose concentration. This graph is a representative of one of 4 chambers included in the experiment.

during the last 10 minutes before the switch to 11 mM glucose and detected levels of secreted insulin during the first 10 minutes after the switch to 11 mM glucose.

There was a significant increase in insulin secretion in response to glucose increase from 5 mM to 11 mM glucose in both control (1.4 ± 0.2 , mean \pm SD, $p=0.02$, $n=3$) and PRL treated islets (2.1 ± 0.9 , $p=0.4$, $n=3$) (Table 4.2 and Figure 4. 13). These findings suggest a potentiating effect of PRL on glucose stimulated insulin secretion in human islets. However despite the trend towards enhanced GSIS following acute treatment with PRL this was not statistically significant when compared to control.

4.5.6 Discussion

Despite a number of potential benefits of islet culture pre-transplant there is still uncertainty over its impact on maintenance of islet mass and function. Optimised islet culture conditions are not yet fully defined or agreed upon. Several publications have reported potential positive effects on islet mass and function following growth factor treatment *in vitro* and/or *in vivo*. In this chapter a group of these growth factors has been screened for possible beneficial effects in primary human islets. Experiments discussed in this chapter included treatment of human islets with pregnancy related hormones including PRL, PL and GH in comparison to other growth factors for 72 hours. The purpose of these experiments was to evaluate the effect on islet mass and the expression of markers of function, proliferation and cellular stress, in addition to evaluating influence on (pro)insulin biosynthesis and processing capacity by β -cells. All results were compared to an internal control in which treatment was with vehicle only.

Despite initial studies showing a trend towards increased PCNA expression in islets treated with pregnancy related hormones and other factors, the more definitive gene

Channel	Insulin level ng/ml		Fold change	Mean	SD	t-test
	5 mM glucose	11 mM glucose				
C1	90.8	134.2	1.5	1.4	0	0.02
C2	122.3	143.4	1.2			
C3	101.8	147.1	1.4			

Channel	Insulin level ng/ml		Fold change	Mean	SD	t-test
	5 mM glucose + 200 nM PRL	11 mM glucose + 200 nM PRL				
C1	101.3	127.1	1.3	2.1	1	0.04
C2	64.1	130.2	2			
C3	43.3	128.7	3			

Table 4.2 PRL effect on insulin release in GSIS studies. Result for each channel represents mean collective insulin release over the 10 minutes before or after glucose switch. Both control (A) and PRL-treated (B) groups showed significant increase in insulin release in response to switch from 5 to 11 mM glucose. The higher stimulation index in the PRL group compared to control group was not significant.

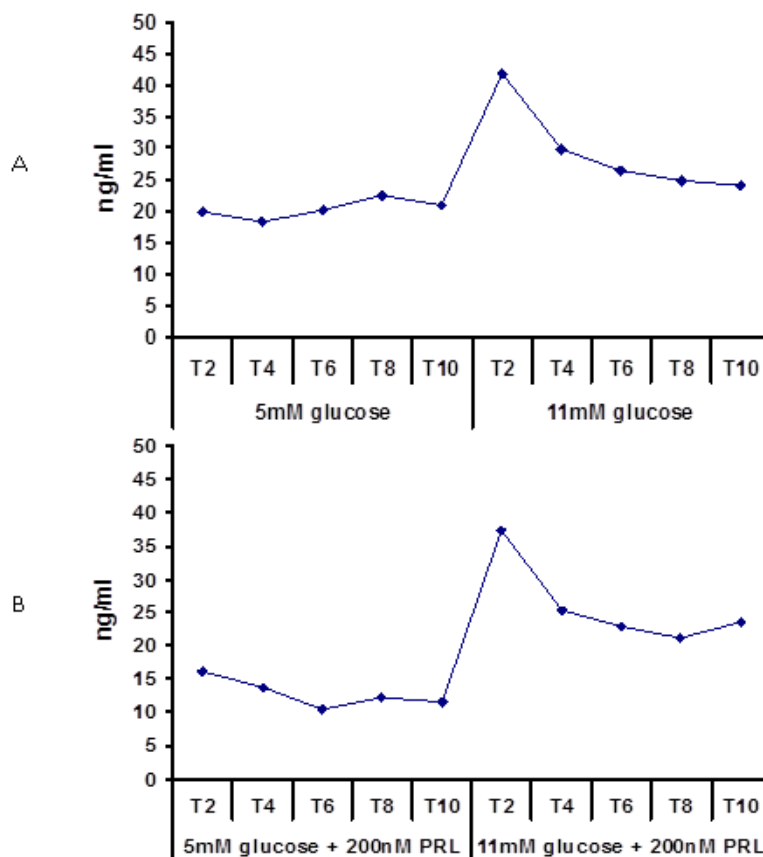


Figure 4.13 PRL effect on insulin release in perfusion studies. Figures are representative of control (A) and PRL (B) treated islets during GSIS perfusion experiment.

expression studies and direct protein mass evaluation showed maintenance rather than an increase in islet mass. Stress modulating effects of pregnancy related hormones and of the growth factors used were evaluated by studying variations in Chop expression, a marker of growth arrest and endoplasmic reticulum stress (Batchvarova et al., 1995; Barone et al., 1994). There was no significant change in Chop expression in response to any of the different treatments tested although a trend towards reduced expression was seen in response to PRL treatment (Figure 4.6 A). The marginal changes in Chop expression and the variations between experiments necessitate culturing islets in toxic and stressful conditions to enable more pronounced potential effects of pregnancy related hormones or other factors to ‘rescue’ islet health to be evaluated.

Studies of insulin gene expression (Figure 4.2) showed a positive trend towards increased expression in the presence of PRL. Study of PDX1, a key insulin transcription factor in β -cells, proved to be problematic due to primer design faults allowing non-specific amplification in PCR run. In the definitive PRL study (4.5.2) validated commercially available Taqman probe assays for both insulin and PDX1 were employed. These showed a statistically significant 1.8 fold increase in insulin expression (Figure 4.5 A) in comparison to control without any change in PDX1 expression (Figure 4.5 B).

When insulin hormone levels were studied there were considerable variations between different studies and despite significant increases in total insulin synthesis seen in individual studies (examples of GF1 (Figure 4.8)) the overall average of 4 separate experiments showed no meaningful difference from control (Figure 4.10).

Processing of proinsulin to insulin is one of the key functional properties of β -cells. This was assessed by studying proinsulin levels after 72 hours of islet culture in presence of different treatments. The findings suggest maintenance of efficient

processing capacity in all treatments including control (4.5.4.4). Although processing levels were efficient in all treatments, this did not enable elucidation of any potential positive effect of the studied factors. Therefore, further studies in toxic conditions which impair β -cell processing capacity will be required.

The acute effect of PRL on islet function was evaluated in perfusion studies. This demonstrated insulin-release enhancing effects of PRL at both basal physiological glucose perfusion state (5mM Glucose) (Table 4.1) and in response to glucose challenges in GSIS (Table 4.2). However, these findings were limited to a single study due to tissue and logistics availability. Further repeat experiments will be required to confirm these findings.

These findings are consistent in part with some of the published reports although do not fully replicate them. In our studies there was no meaningful change in islet mass in response to treatment with pregnancy related hormones or any other treatment despite numerous reports in this regard (Suarez-Pinzon et al., 2005; Sorenson and Brelje, 1997; Otonkoski et al., 1996; Nielsen et al., 1999; Li et al., 2004; George et al., 2002; Farilla et al., 2002; Edvell and Lindstrom, 1999; Brelje et al., 1993; Beattie et al., 1997). It should be noted, however, that these reports are not in intact adult human islets but were in rodent islets, or human islets in conditions enabling 2D adherence and proliferation.

In the current studies 72 hours treatment with PRL showed a significant effect on increasing insulin expression but not PDX1. Moreover, acute treatment with PRL resulted in enhanced insulin release at both physiological and high glucose levels. However 72 hours treatment with PRL and other markers did not result in increased insulin synthesis contrary to published reports (Sorenson and Brelje, 1997; Brelje et al., 1993). We hypothesise that prolactin may act by increasing PDX1 translocation to the

nucleus without any increase in PDX1 gene expression. This would then bind to the insulin promoter, directly upregulating insulin gene expression. Preliminary evidence for this has been generated by Dr Susan Campbell within the group in the MIN6 mouse β -cell line, demonstrating PDX1 translocation in the presence of prolactin (unpublished data). Further studies in human islets are planned to study underlying signalling pathways mediating PDX1 translocation. Robust demonstration of increased proinsulin biosynthesis in intact islets, necessitates a pulse-chase study design quantifying incorporation of label into newly synthesised peptide. The existing studies underline the difficulties in accurately determining this in β -cells which are dynamically synthesising / storing and secreting (pro)insulin which is then subject to metabolism and degradation in the surrounding medium.

No definitive reasons for discrepancies with published work were confirmed. However a number of factors were thought likely to contribute. First; the current studies were carried out on different islet preparations with different donors and islet quality. Despite attempts to standardise islet number and mass in each well, differences in volume, purity and constitution will inevitably have led to differences in absolute beta-cell numbers. Second; reported success in rodent islets or β -cell lines does not necessarily indicate the same effect on human islets due to cell-line or species specificity. Moreover, problems of variability between different human islet preparations are not usually present when studies are carried out on animal models or cell lines. Third; culture conditions used in these experiments ensured preservation of islet integrity. Indeed use of adherent cultures, specifically with addition of FCS, can significantly change islet phenotype and behaviour and provide uncontrolled growth factor supplementation contained in FCS (Chapter 5). Published literature indicates that although β -cell proliferation can clearly be induced in cell lines, which have been

transformed specifically to ensure proliferation, and indeed in intact rodent islets (derived from young growing animals), there is little evidence of beta-cell replication within human islets *in vitro* unless dissociation into adherent proliferative cells is permitted. Fourth; use of recombinant growth factors ensures purity of the protein and avoids indirect effects due to impurities with contaminating hormones usually seen in hormone extracts. Fifth; concentrations used for the screening experiments might have been sub-optimal. Indeed; more effects were evident when prolactin concentration was increased from 80 nM to 200 nM in the perfusion experiments. This higher concentration may be sufficient to induce stimulation through the placental lactogen pathway in addition to the prolactin pathway. Therefore dose response experiments would be of particular benefit as concentrations used were not necessarily optimal though were guided by published literature. Sixth; subtlety of growth factor treatment effect might become more prominent when islets are exposed to toxic conditions. This is important in particular in the stress modulating effect studies and in (pro)insulin processing studies. Moreover, creating a stressful environment can, to some extent, replicate the environment post transplantation. A different emphasis is recommended for future studies with focus on treatments which will reduce proinflammatory and pro-apoptotic signalling, enhancing engraftment and reducing early post-transplant loss of beta-cell mass, as opposed to attempting to increase function or mass *in vitro*, both of which remain fairly optimal despite the rigours of isolation and purification. Finally; *in vivo* studies to evaluate engraftment and function will ultimately provide robust evidence of whether growth factor treatment has induced and beneficial effects.

**Chapter 5. Establishment and
characterisation of human adult pancreatic
cells in proliferative adherent culture**

5.1 Introduction

Insufficient availability of suitable human islet tissue to meet clinical needs remains a major obstacle facing diabetes cellular therapy. Trials to enhance islet mass and function *in vitro* in our hands and internationally have been of limited success when carried out in intact human islets (Chapter 4). Many approaches to securing potential endocrine-like surrogate tissue suitable for diabetes cell replacement therapy depend on identification of pluri/multi-potent precursor cells, proliferative expansion *in vitro* and differentiation toward β -cell phenotype.

Several target cells have been studied for this purpose including embryonic stem cells (D'Amour et al., 2006; Assady et al., 2001), umbilical cord blood stem cells (Sun et al., 2007; Koblas et al., 2005), bone marrow mesenchymal stem cells (Zhao et al., 2008; Couri et al., 2009) and skin stem cells (Guo et al., 2009), in addition to different pancreatic cell phenotypes.

Knowledge about the extent of pancreas plasticity was limited until recently. Many publications have explored different aspects of this including cell expansion (Kayali et al., 2007; Bonner-Weir et al., 2000), transdifferentiation of pancreatic cells to different phenotypes (Tosh *et al.*, 2002; Horb *et al.*, 2003; Aldibbiat *et al.*, 2008), epithelial-mesenchymal transition (Gershengorn et al., 2004) and mesenchymal-epithelial transition (Teague et al., 2005).

Despite the strong evidence for β -cell regeneration through β -cell self-replication reported by Dor in rodent lineage tracing studies (Dor et al., 2004), it is not fully confirmed whether the self-duplication pathway accounts for all new β -cells (particularly in older mature animals / humans). The proliferative capacity of β -cells has been shown to be tightly controlled (Cozar-Castellano et al., 2006). There is evidence to suggest that new β -cells, at least in part, arise through β -cell neogenesis

from stem/progenitor cells (Abraham et al., 2002; Bouwens, 2004; Del Zotto et al., 2004). In fact it has been reported that the size of the pancreas is limited by the number of embryonic progenitor cells (Stagner et al., 2007). Culture of pancreatic islet cells has been shown to result in gradual loss of β -cell phenotype despite promising expansion (Beattie et al., 1999). Gershengorn reported epithelial to mesenchymal transition (EMT) of cultured β -cells as the mechanism of cell plasticity and expansion in culture (Gershengorn et al., 2004). The same group reported later in a genetic labelling experiment of rodent islets that the expanded cells were not a result of EMT (Morton et al., 2007). More recently the group confirmed the cycling state of islet precursor cells between the epithelial state and mesenchymal progenitor state through EMT (Davani et al., 2008). Despite confirmed plasticity of pancreas-derived cells it was not clear whether classical pluripotency-associated stem cell markers were expressed. Recently Zhao reported confirmed expression of Oct4 and Sox2 in human pancreas tissue with localisation to the ductal structures (Zhao et al., 2007). Moreover, expression of Oct4 in human and rodent pancreas sections has been confirmed in our group (White et al., 2011).

5.2 Aims

The overall aim of this chapter was to generate proliferative primary pancreatic cell cultures with high plasticity from human pancreas for further differentiation studies and utilisation in potential future cell replacement therapy for diabetes. Specific objectives were to:

1. To determine the proliferative potential of adherent cells cultured from islet-enriched human pancreatic digest.

2. To determine whether functional beta-cells can be expanded in proliferative adherent culture
3. To determine whether pluripotency-associated factors are expressed in expanded primary pancreatic cell culture

5.3 Establishment of ‘Islet Survivor Cells’

Human islets were transported to Newcastle from King's College Hospital, London in human serum albumin supplemented serum-free medium (Table 2.2). Human islets have an intrinsic tendency to adhere to culture vessels in the presence of foetal calf serum (FCS). Moreover, adherence was very strong when islets were cultured on standard tissue culture treated flasks, requiring prolonged treatment with TrypLE express agent at passaging and specifically at first passage (30-40 minutes). Use of ‘non-adherent’ tissue culture vessels enabled adherence, growth and proliferation with easier passaging (2.12.3).

Optimal growth of adherent and proliferative cultures of human islets was achieved in islet adherent medium. This medium consisted of CMRL 1066 NCL basal medium supplemented with 1% penicillin/streptomycin, 20% foetal calf serum (FCS) GOLD (a proprietary FCS preparation with standardised growth factor content), and 2x insulin-transferrin-selenium-sodium pyruvate (ITS-A) supplement (Table 2.2). Optimal active proliferative culture was achieved from islets initially incubated for five days before medium change. This was subsequently standardised for all preparations. Medium with any remaining non-adherent cells was removed and discarded and adherent islets cells, termed islet survivor cells (ISCs), were maintained in the same culture medium changed twice each week. At this stage, ISCs grow out of adherent islets resulting in gradual loss of islet structure (Figure 5.1 A, B). Passage

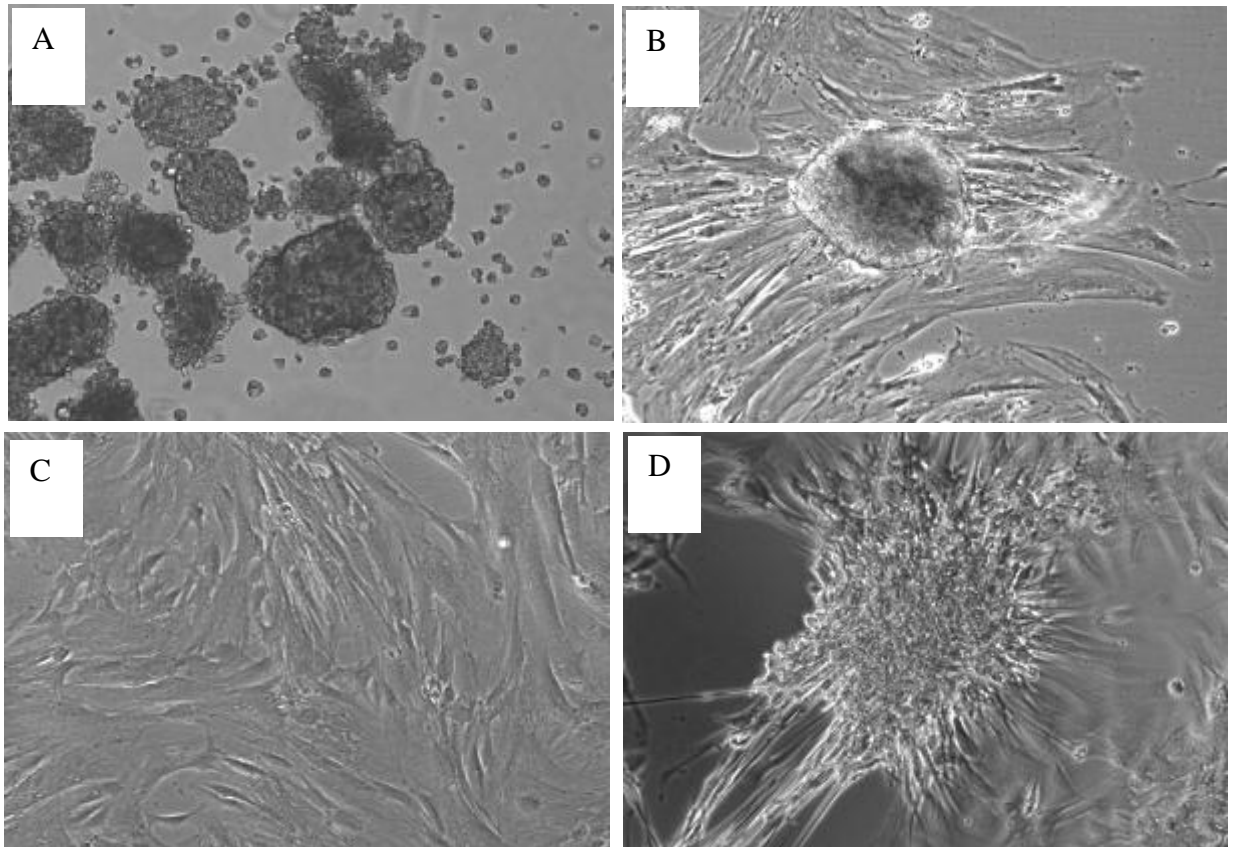


Figure 5.1 Establishment of human islet survivor cells in culture. When established in non-adherent flasks in adherent culture medium, transported islets (A) adhere to the flask (B) and gradually lose islet shape with out-growth of cells. Adherent cells form a monolayer, termed islet survivor cells (ISCs) (C). When cells approach confluence, the ISCs start forming sporadic cell clusters in the culture flask (D)

was carried out for the first time when cells were near confluence (3-4 weeks from establishment) with a splitting ratio of 1:2-1:3. As the cells grew in culture they formed a monolayer (Figure 5.1 C). However, spontaneous formation of new 3D cell clusters was observed when cells were approaching confluence (Figure 5.1 D).

5.4 Characterisation of ISCs

5.4.1 Growth characterisation

For the purpose of estimating doubling time and growth characteristics of ISCs, cells were passaged 1:2 with 50% being established in a new T75 flask and 50% divided equally between three T25 flasks. Cells were maintained in culture until confluence. Cells from the T25 flasks were harvested and enumerated and cells in the T75 were passaged following the same protocol of the initial passaging in this study giving 3 new T25 flasks and a new T75 flask. This was repeated over 4 passages (passage 6-9) after which growth arrest and senescence ensued (beyond passage 10).

Number of seeded cells, number of harvested cells and time required to reach confluence were documented for each passage. Doubling time was calculated as the ratio between mean fold increase in cell number in the T25 flask at each passage and number of days to reach confluence from passaging. Mean doubling time was 57.6 ± 14.4 hours (mean \pm SEM) with an average of 3.4 ± 0.72 doublings per passage. This growth rate was extrapolated to enable an estimate of potential total number of cells as the ISCs grow in culture. It was estimated that an average 110 doublings occur at passage 4 and 443 doublings at passage 6 (Figure 5.2). Active proliferation thus has been demonstrated with potential to provide copious amount to islet proliferative tissue. However, β -cell function in this proliferative culture need to be evaluated to enable assessment for any potential clinical application.

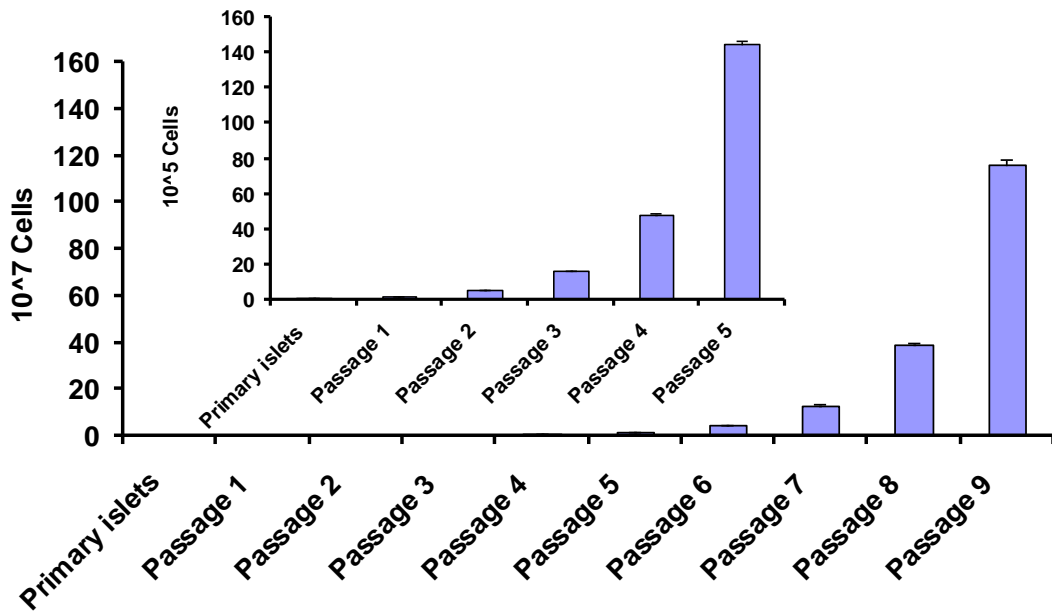


Figure 5.2 Estimated ISC growth rate. The graph demonstrates growth rate over several passages as estimated by actual growth rate extrapolation. Growth rate was counted over passages 6-9 in triplicates. Doubling time was estimated at 57.6 ± 14.4 hours (mean \pm SEM).

5.4.2 (Pro)insulin biosynthesis

5.4.2.1 Early passages

Storage and secretion of insulin, proinsulin and C-peptide were determined in ISCs at passage 4. Cells were established in 12 well plates with a minimum of 3 replicates in serum free medium. Medium and cell pellet were harvested after 24 hours. Insulin, proinsulin and C-peptide ELISA in addition to protein assays were carried out. Hormone levels were normalised to protein content of each sample for comparison purposes (2.18.1). There were detectable but low levels of all three ELISA assays confirming persistence of remaining β -cell phenotype within the proliferative culture. Averages of total insulin, proinsulin and C-peptide production in ISCs over 24 hours were 1514 ± 88 pmol/l (25.5 ± 5.9 pmol/mg protein), 15.5 ± 2.1 pmol/l (0.26 ± 0.06 pmol/mg protein) and 172.2 ± 14.9 pmol/l (2.8 ± 0.4 pmol/mg protein) respectively. This was much lower than that of primary human islets in which total insulin and proinsulin production over 24 hours were 40430 ± 33.82 pmol/l (105.5 ± 23.8 pmol/mg) and 24259 ± 389.5 (66.9 ± 22.7 pmol/mg) respectively. Moreover, storage capacity was impaired in ISC for insulin, proinsulin and C-peptide with only 2.7%, 29.3% and 33.5% respectively of total hormone stored (Figure 5.3 and Table 5.1). On the other hand, studies in primary human islets showed insulin storage at 33.8% of total insulin and proinsulin storage at 93.7% of total proinsulin

These findings indicate presence of β -cell phenotype in the ISCs culture at passage 4 but with significantly decreased hormone biosynthesis and impaired storage. Detection of relatively high insulin levels compared to proinsulin and C-peptide indicate possible contamination with insulin supplement to ISCs maintenance culture media although cells were washed thoroughly in PBS when passaged for this experiment.

	Storage		Secretion	
	Absolute pmol/l	Protein corrected pmol/mg	Absolute pmol/l	Protein corrected pmol/mg
Insulin	42.8±8.1	0.68±.015	1471.2±93.7	24.8±5.8
Proinsulin	4.5±0.53	0.07±0.015	10.9±1.6	0.18±0.4
C-peptide	59.5±9.5	112.7±9.6	0.94±0.1	1.86±0.37

Table 5.1 Insulin, proinsulin and C-peptide levels in ISCs at passage 4. Storage and secretion levels of insulin, proinsulin and C-peptide detected in ISCs culture (LDIS 011) at passage 4 following 24 hour culture in serum and ITS-A supplement free medium. n=3, mean±SEM

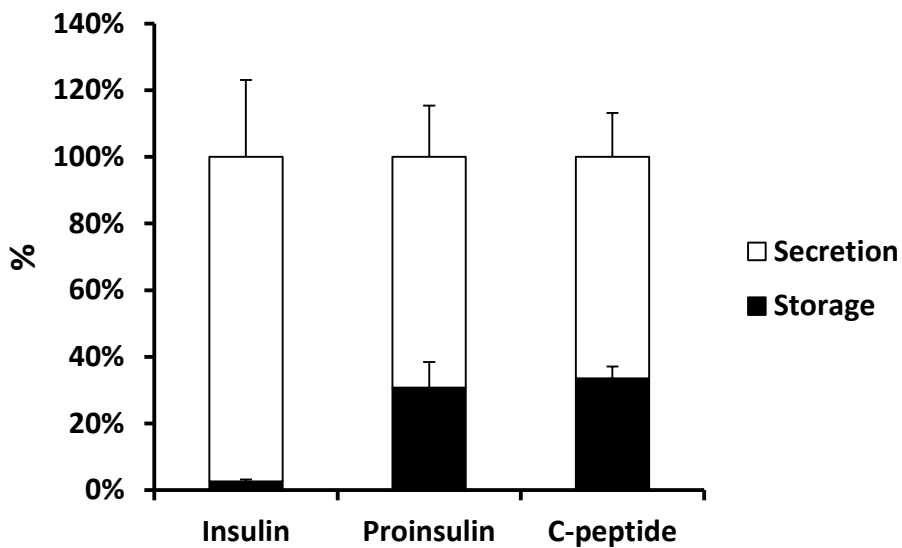


Figure 5.3 Percentage of storage and secretion of synthesised hormone. Graph represents mean percentage of storage and secretion of human insulin, proinsulin and C-peptide in ISCs (LDIS011) at passage 4 following 24 hour culture. n=3, mean±SEM

5.4.2.2 Late passages

Insulin and C-peptide profiles were studied in ISCs at passages 6-9. This was undertaken in parallel with growth characterisation studies (5.4.1). ISCs were established in 3 T25 flasks and at confluence, cells were washed twice in PBS and then incubated for 3 hours in serum-free and ITS-A-free medium after which both medium and cells were harvested. Initial insulin ELISA showed clearly detectable insulin levels despite attempts to prevent false-positivity (Figure 5.4 A). However standard C-peptide ELISA showed no detectable C-peptide levels. This was further confirmed by ultra-sensitive C-peptide ELISA (Figure 5.4 B). Absence of C-peptide clearly indicates loss of β -cell phenotype while false positive insulin detection is likely due to contamination from insulin supplement carryover from culture medium not being completely removed despite two PBS washes.

This demonstrates complete loss of β -cell phenotype in ISCs at late passages while maintaining active proliferative capacity.

5.4.3 Gene expression profiles

To further characterise the phenotype of cultured ISCs, conventional RT-PCR was carried out on different passages for a range of markers. This included β -cell markers: insulin, PDX1, PC1/3 glucokinase and Glut2; other islet endocrine markers including glucagon and pancreatic polypeptide; non-endocrine markers including CK19 / vimentin; and stem cell and developmental marks including PAX4, Oct4 and Nanog. At passage 4 all of these markers were detected by RT-PCR but with marked reduction in PDX1, PP and PC1/3 expression and an increase in PAX4 expression (Figure 5.5 A). Expression of CK19 and vimentin appeared to be enhanced (Figure 5.5 A). Expression of Oct4 and Nanog was confirmed and was compared with human embryonic stem cell expression (Figure 5.5 B). These results indicate continued

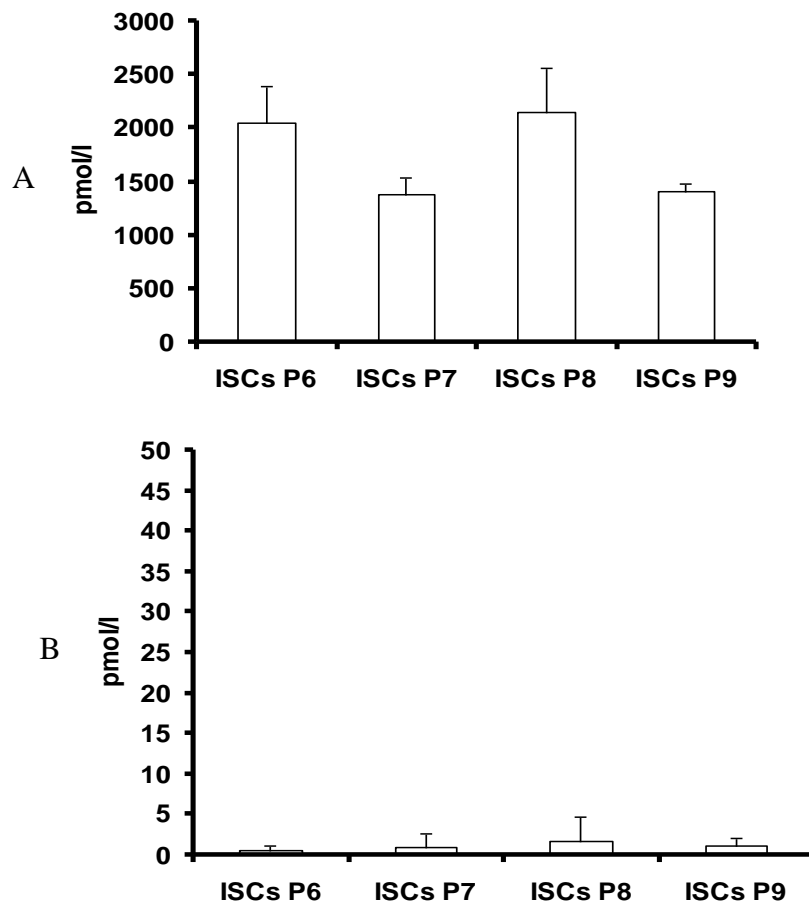


Figure 5.4 Insulin and C-peptide production in late passage ISCs. Insulin (A) and C-peptide (B) levels after 3 hour incubation of late passage ISCs. n=3, mean±SEM.

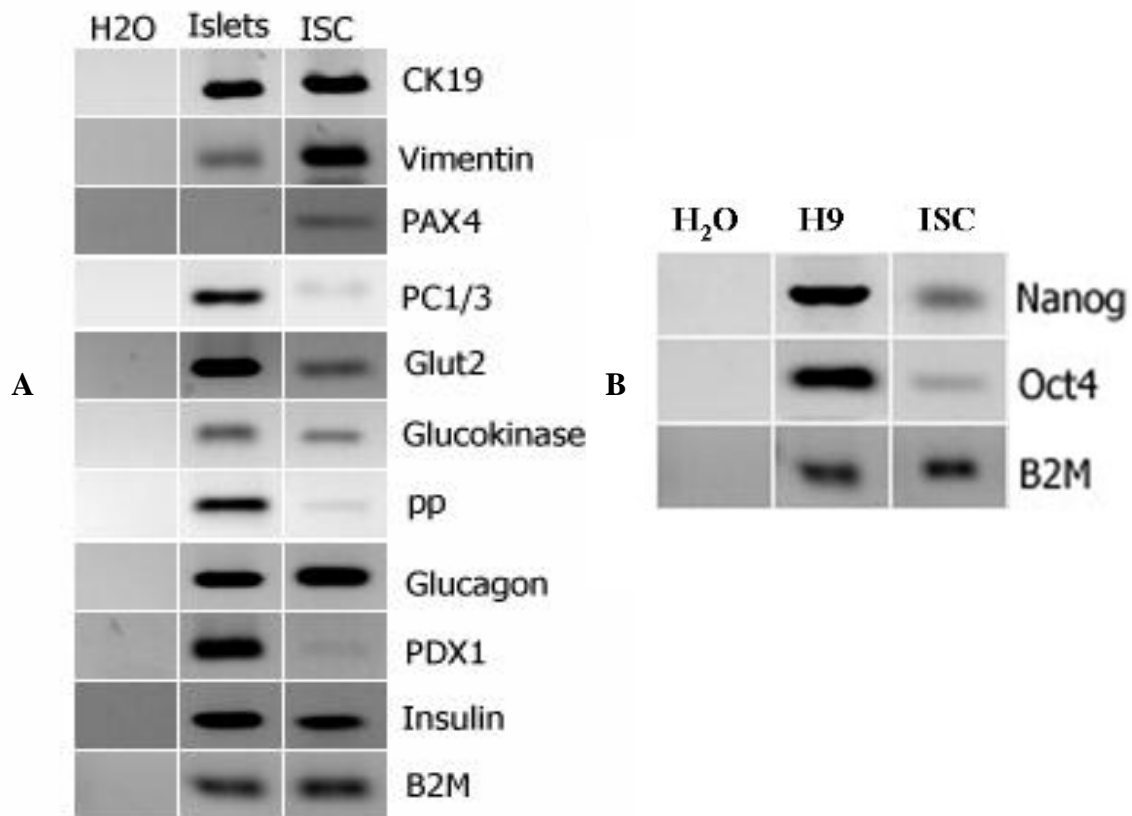


Figure 5.5 Gene expression profile in ISCs at passage 4. All islet markers were expressed but at markedly reduced levels except for glucagon and PAX4. CK19 and vimentin expressions appeared to be up-regulated (A). Expression of progenitor/stem cell markers including Nanog and Oct4 was observed in comparison to H9 human ES cells as a positive control (B).

expression of pancreas markers at this stage but with a change in the phenotype towards a less differentiated possibly progenitor-like state.

To elucidate the change in the phenotype further time-course studies were conducted by my colleague H.R.H. Al-Turaifi. Time-course analysis of expression profiles of insulin, PDX1 and glucagon by conventional RT-PCR demonstrated gradual loss of gene expression for all these markers (Figure 5.6). Time-course studies for stem cell markers Oct 4 and Sox2 demonstrated initial down-regulation of both Oct4 and Sox2 at early passages but maintained expression throughout all passages (Figure 5.7).

5.4.4 Immunocytochemical characterisation

Expression of different markers was investigated in both human islets and ISCs. Initial validation of key markers was carried out in human pancreas.

5.4.4.1 Validation of markers in the pancreas

Phenotypic marker validation was carried out in human normal pancreas and sections from a pancreas with pancreatitis. Staining was carried out for several markers including islet endocrine markers, ductal markers, exocrine markers, proliferation markers and stem / progenitor cell markers.

Expression of islet endocrine markers was confirmed with positive staining for insulin (Figures 5.8-10), C-peptide (Figure 5.8), somatostatin (Figure 5.9), and pancreatic polypeptide (PP) (Figure 5.10). The ductal tree was demonstrated by CK19 staining (Figure 5.11 A) while the exocrine compartment was characterised by amylase staining (Figure 5.11 B).

In sections from a pancreas of a patient with chronic pancreatitis who had undergone pancreatectomy, areas of cytoplasmic Nanog (Figure 5.12 A) and Sox2 (Figure 5.12 B) staining were observed. Sporadic single or small clusters of extra-islet insulin staining cells were also revealed in these sections (Figure 5.13 A). Proliferative

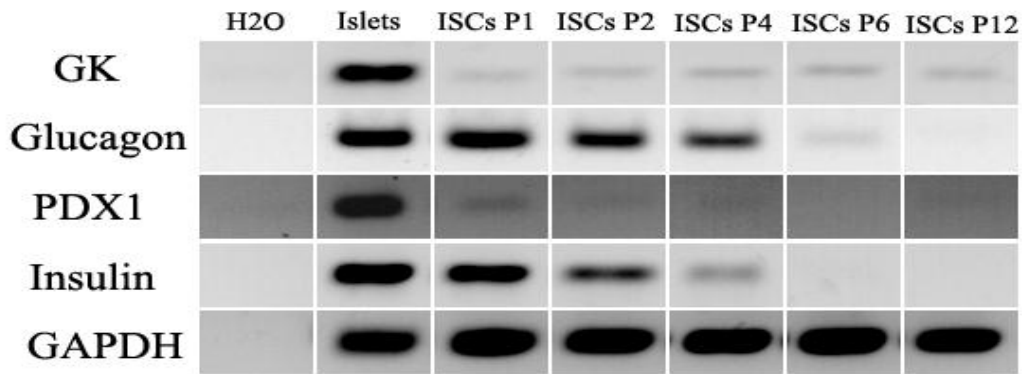


Figure 5.6 Time-course analysis for islet-specific gene expression profile in ISCs. Gradual reduction followed by loss of expression of insulin, PDX1 and glucagon were observed. (courtesy of H.R.H. Al-Turaifi).

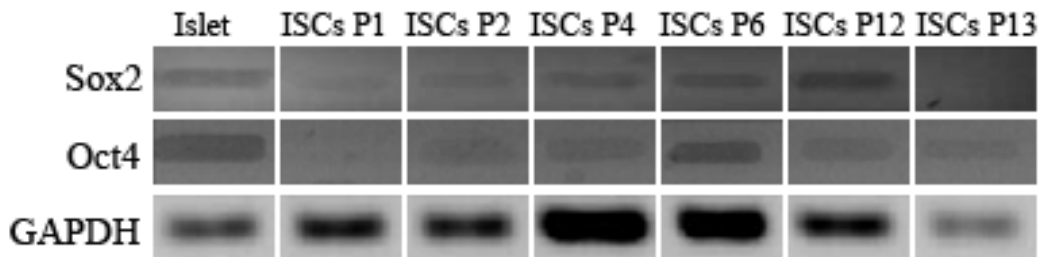


Figure 5.7 Time-course analysis for stem cell marker gene expression profile in ISCs. Despite initial down-regulation of Oct4 and Sox2, maintained expression was confirmed at all passages. (courtesy of H.R.H. Al-Turaifi).

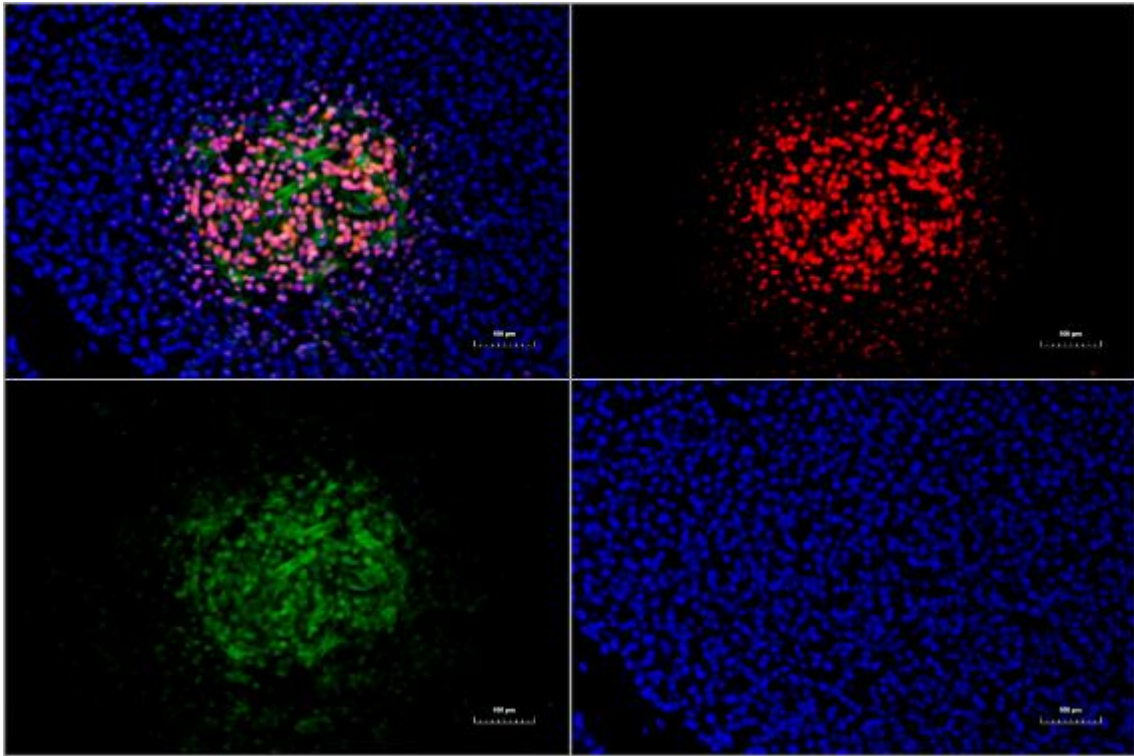


Figure 5.8 Insulin and C-peptide staining of human pancreas. The image demonstrates co-localisation of insulin (red) and C-peptide (green) in β -cells in an islet within a pancreas section. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

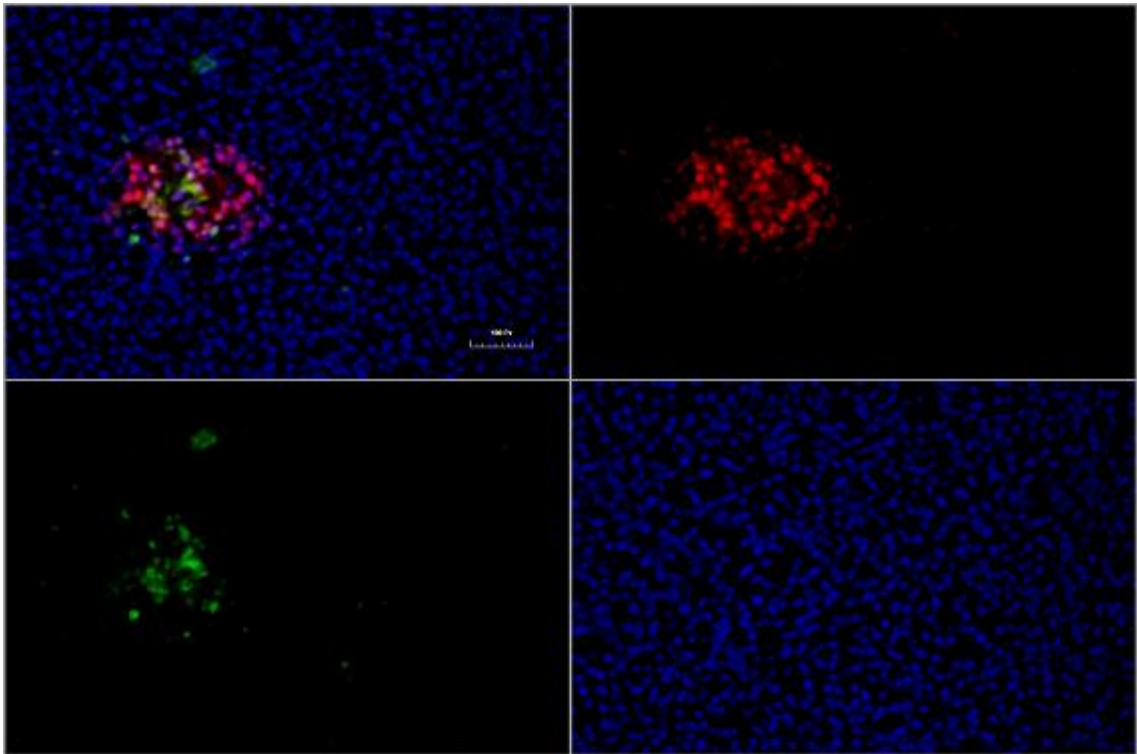


Figure 5.9 Insulin and somatostatin staining of human pancreas. The image demonstrates insulin (red) and somatostatin (green) in an islet within a pancreas section. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

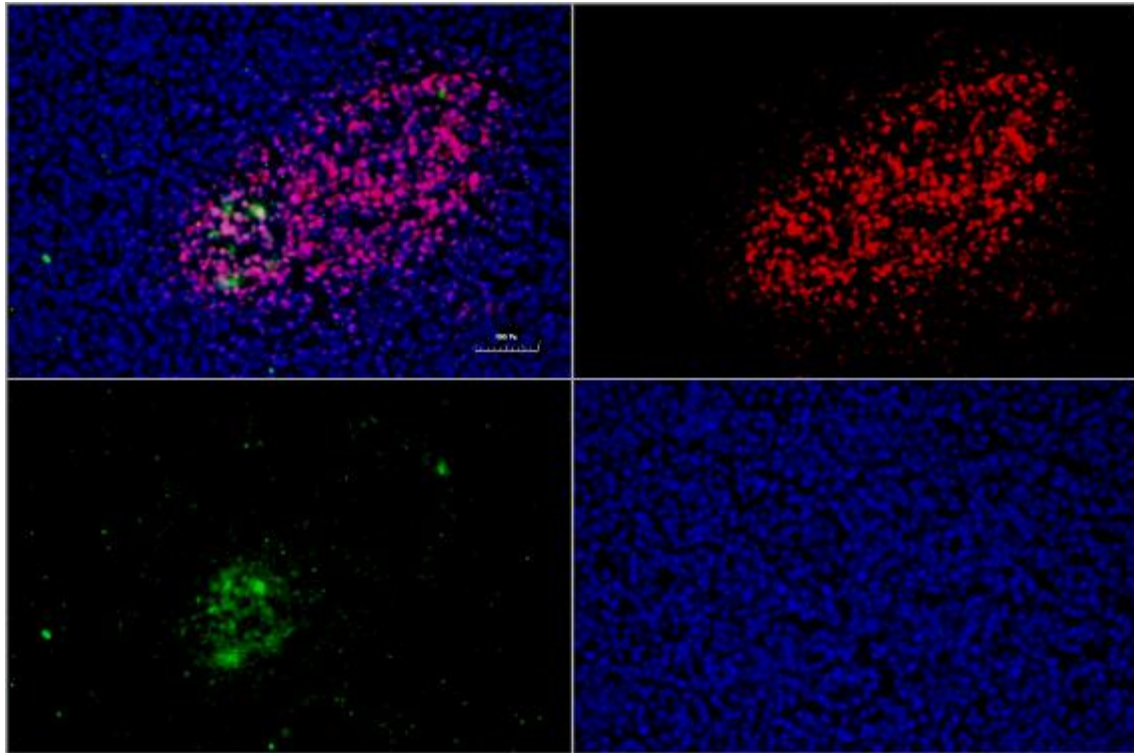


Figure 5.10 Insulin and pancreatic polypeptide staining of human pancreas. The image demonstrates insulin (red) and PP (green) in an islet within a pancreas section. Red: Cy3 green: FITC; nuclei were counter-stained with DAPI.

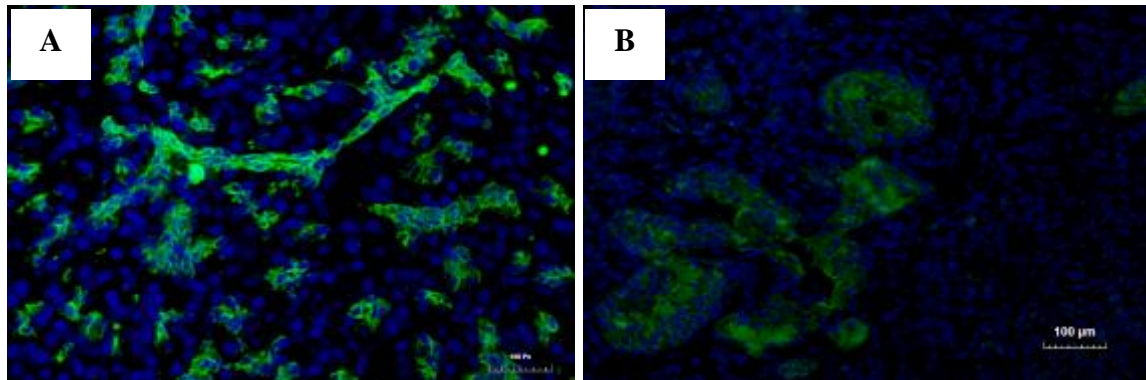


Figure 5.11 CK19 (A) and amylase (B) staining in human pancreas. CK19 (A) is stained in green (FITC) delineating the ductal tree in the pancreas section. Amylase (b) stained in green (FITC) outlining the acinar compartment within this pancreas section. Green: FITC; nuclei were counter-stained with DAPI.

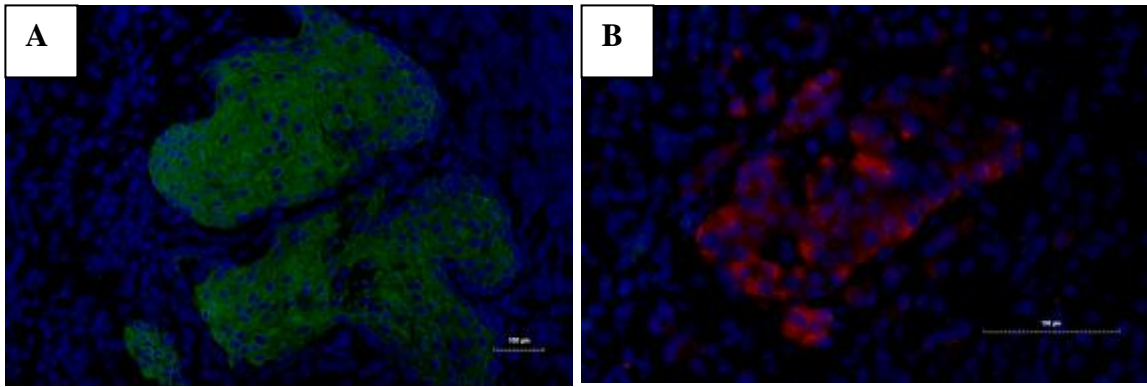


Figure 5.12 Nanog and Sox2 staining in human pancreas with chronic pancreatitis. IHC staining on these sections from a pancreatitis pancreas revealed cytoplasmic expression of stem cell marker Nanog (A, green) and Sox2 (B, red). Red: Cy3; green: FITC; nuclei were counter-stained with DAPI

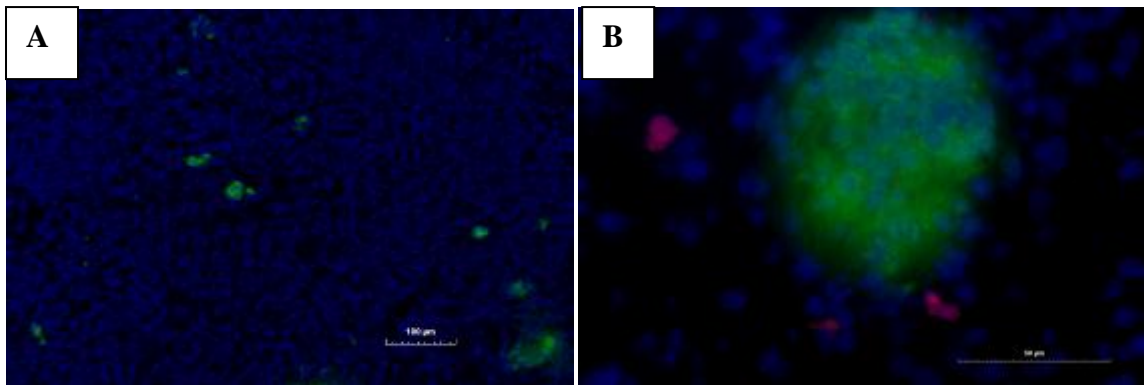


Figure 5.13 Proliferation in human pancreas with pancreatitis. Sporadic extra-islet insulin staining (A, green: FITC) was observed in addition to insulin staining within intact islets B, green: FITC) and peri-islet Ki67 (B, red: Cy3) activity. Nuclei were counter-stained with DAPI.

activity in the peri-islet regions was demonstrated by detection of Ki67 positive cells (Figure 5.13 B). Further assessment of normal human pancreas demonstrated proliferative activity within the ductal compartment although no co-localisation of insulin and Ki-67 was seen (Figure 5.14).

5.4.4.2 Immunocytochemical characterisation of fresh human islets

Study of cellular constitution of human islet preparations was carried out employing ICC staining on accutase-digested cytopun single cell preparations obtained from intact human islets (2.20.2). This confirmed staining of insulin (Figure 5.15 A, B, C), somatostatin (Figure 5.15 A), PP (Figure 5.15 B), glucagon (Figure 5.15 C) and amylase (Figure 5.15 D). Expression of stem cell markers Nanog co-localising with vimentin (Figure 5.15 E); and Oct4 (Figure 5.15 F) was confirmed. Proliferative activity was observed by detection of Ki67 (Figure 5.15 F). No Oct4, Nanog, insulin, PP, somatostatin or glucagon positive cell was observed to co-express Ki67.

5.4.4.3 Immunocytochemical characterisation of ISCs

Following passaging, ISCs grew actively in monolayer adherent culture. A tendency towards spontaneous 3D cluster / clump formation was noted, however. Clump formation and overall culture growth slowed down at late passages (beyond passage 9/10). Morphologies of both monolayer and 3D clusters were characterised by ICC.

5.4.4.3.1 Adherent ISCs

Active proliferation was confirmed in early ISCs by high expression of Ki67 (Figure 5.16). Expression of all differentiated islet markers was confirmed at passages 3 and 4 including insulin, glucagon, somatostatin and PP (Figure 5.17). True endogenous insulin staining was further validated by co-staining with C-peptide (Figure 5.17 A).

Expression of vimentin (Figure 5.18 A) and CK19 (Figure 5.18 B) was confirmed at early passages with co-expression seen frequently at later passages (P7/8)

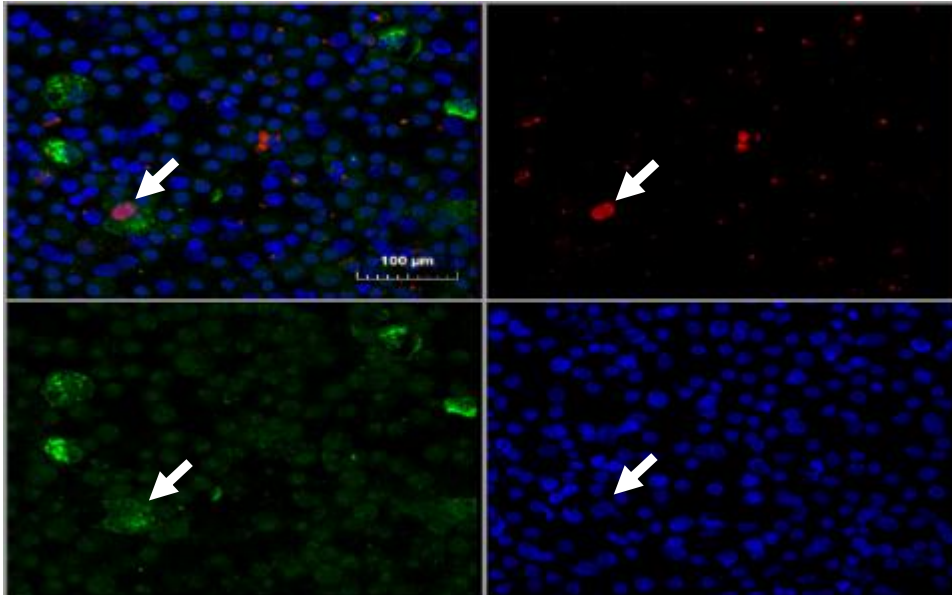


Figure 5.14 Proliferative activity in normal pancreas. Ki67 (red: Cy3) co-localised with Ck19 (green: FITC) indicating active proliferation within the ductal compartment. Nuclei were counter-stained with DAPI.

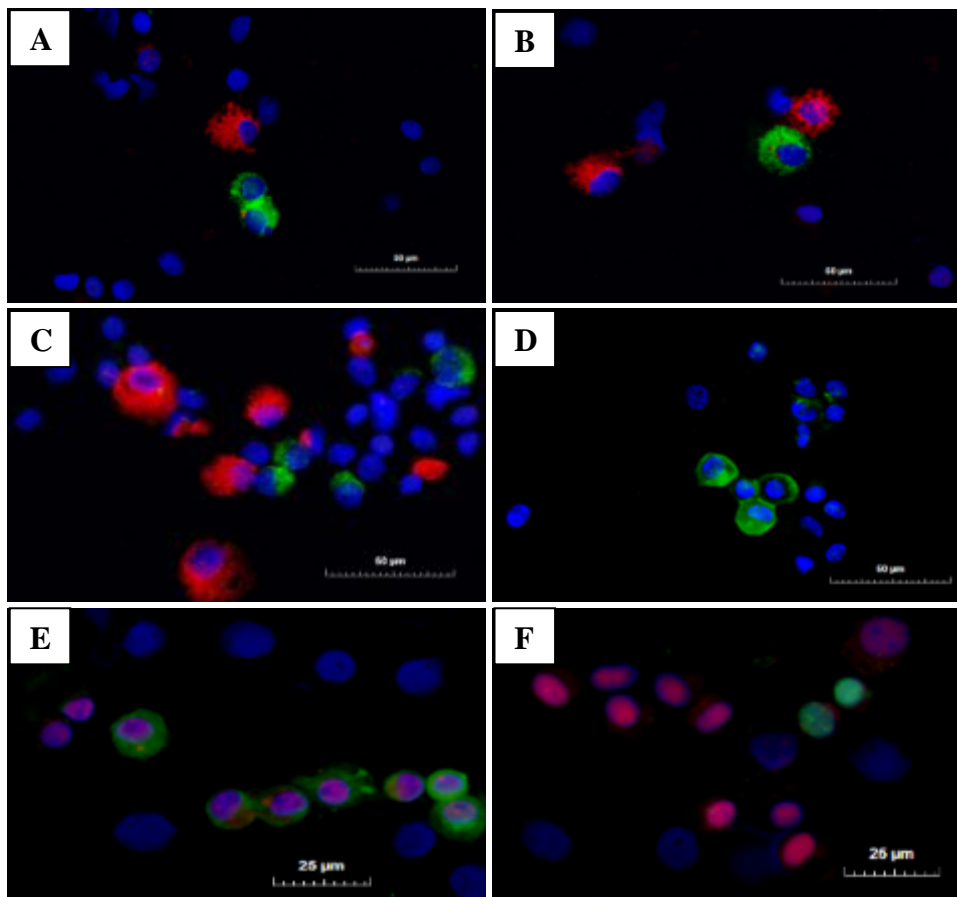


Figure 5.15 ICC characterisation of fresh islets. ICC carried out on fresh islets showed insulin (red: Cy3 in A, B, C), somatostatin (green: A), PP (green: B), glucagon (green: C) and amylase (green D). Nanog staining (E, red) co-localised with vimentin (E, green). Proliferative activity was observed with Ki67 staining (F, green); however there was no co-localisation with Oct4 (F, red). Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

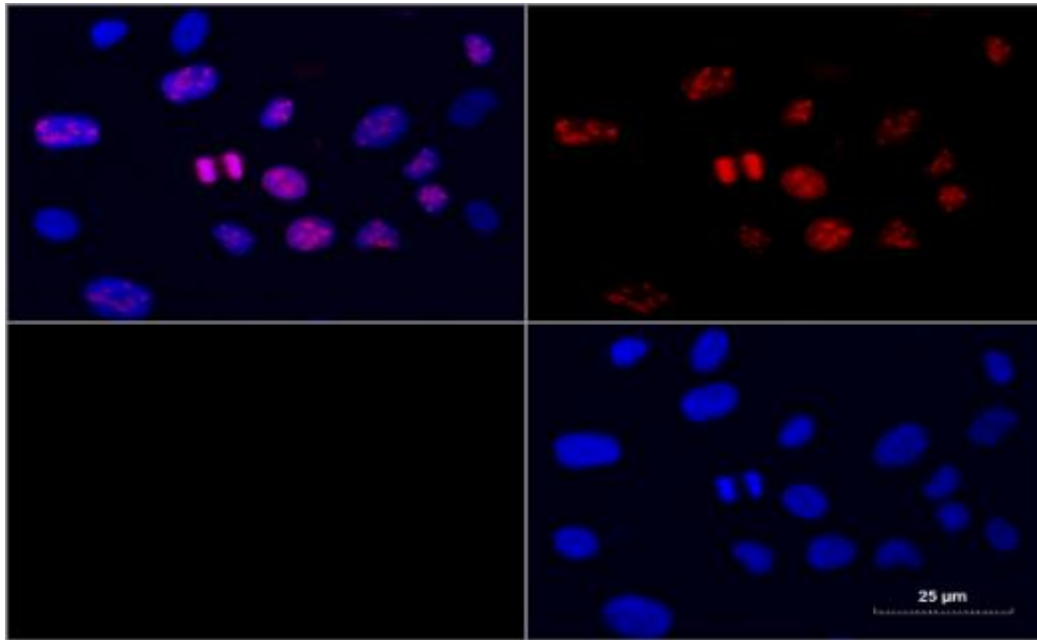


Figure 5.16 Proliferative activity in ISCs. Ki67 expression (red) in a high percentage of cells confirmed very active proliferative state of early ISCs. Red: Cy3; nuclei were counter-stained with DAPI.

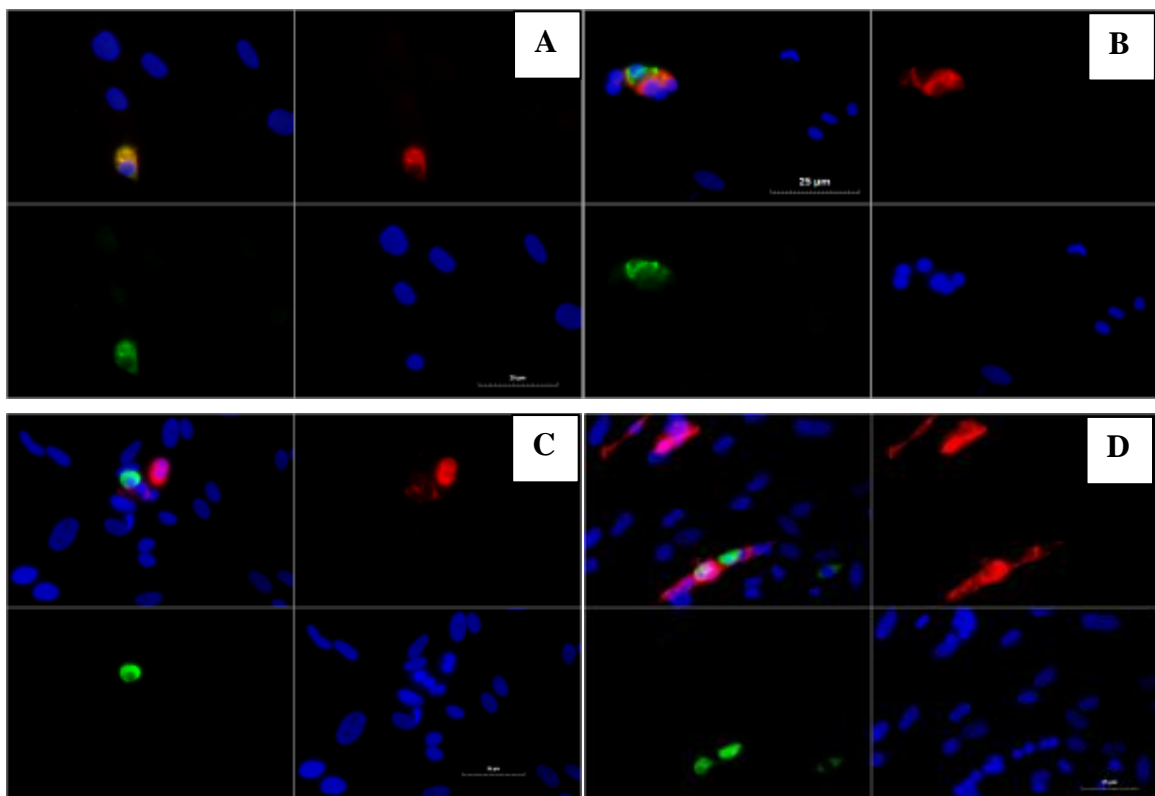


Figure 5.17 Hormone expression profile in ISCs at passage 4. Insulin expression was confirmed (A, B, C, D, red). Validity of insulin expression was further confirmed by detection of co-localised C-peptide (A, green). Other differentiated islet phenotype markers including glucagon (B, green), somatostatin (C, green) and PP (D, green) were detected but did not co-localise with insulin or C-peptide. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

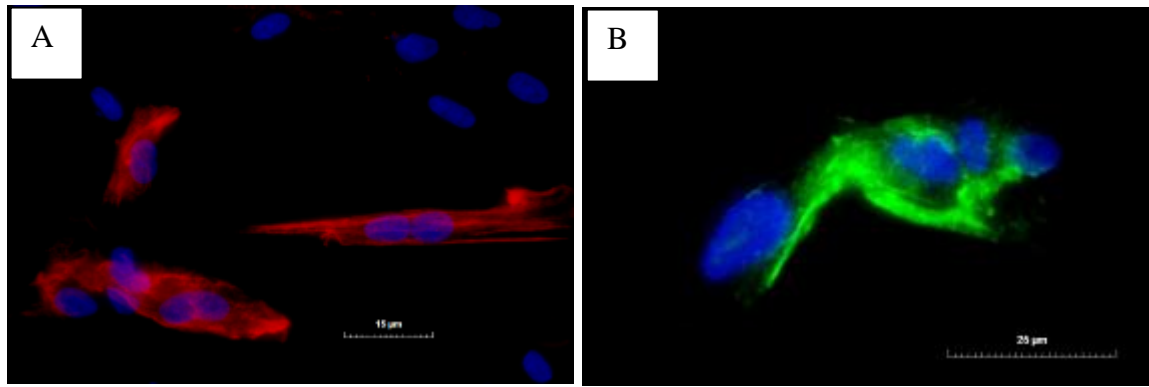


Figure 5.18 Expression of vimentin (A) and CK19 (B) in ISCs at passage 4. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

(Figure 5.19), a phenotype not seen in intact human pancreas. Nestin expression was also confirmed with infrequent co-expression with vimentin in early passages (P3/4) (Figure 5.20). Expression of other pancreatic cell phenotypes including exocrine (amylase, Figure 5.21) and endothelial (endoglin, Figure 5.22) was confirmed.

Proliferative activity was seen largely within the ductal phenotype with co-expression of CK19 and Ki67 (Figure 5.23).

Moreover, nuclear localisation of the embryonic stem cell marker Oct4 was detected (Figure 5.24) indicating potential progenitor/stem-like properties in ISCs. NGN3, a key transcription factor for endocrine development, was detected in early ISCs (Figure 5.25 A). Both islet markers Nkx6.2 (Figure 5.25 B) and PAX6 (Figure 5.25 C) were also detected. Interestingly albumin, a hepatocyte marker, was detected in a very small number of cells (Figure 5.25 D). No insulin or C-peptide positive cell was ever detected co-expressing other markers (examples glucagon, somatostatin or PP Figure 5.17, endoglin Figure 5.22 or Oct4 Figure 5.24).

Senescence of ISCs beyond passage 10 accompanied by slow growth was confirmed with marked reduction in Ki67 positive cells (Figure 5.26 A) and loss of differentiated marker expression including insulin, glucagon, PP, and somatostatin. Maintained expression of both CK19 (Figure 5.26 A) and vimentin (Figure 5.26 B) was confirmed.

5.4.4.3.2 *Spontaneous clustering in ISC culture*

During the early / middle passaging phase (up to passage 6-8) and soon after establishing the cells post passaging there was a spontaneous tendency in the culture for clump/aggregate formation (Figure 5.28). Staining of these clumps revealed pockets of C-peptide positive cells (Figure 5.28). Similar results were observed with insulin staining. However, no co-staining with Ki67 was seen. The origin of these

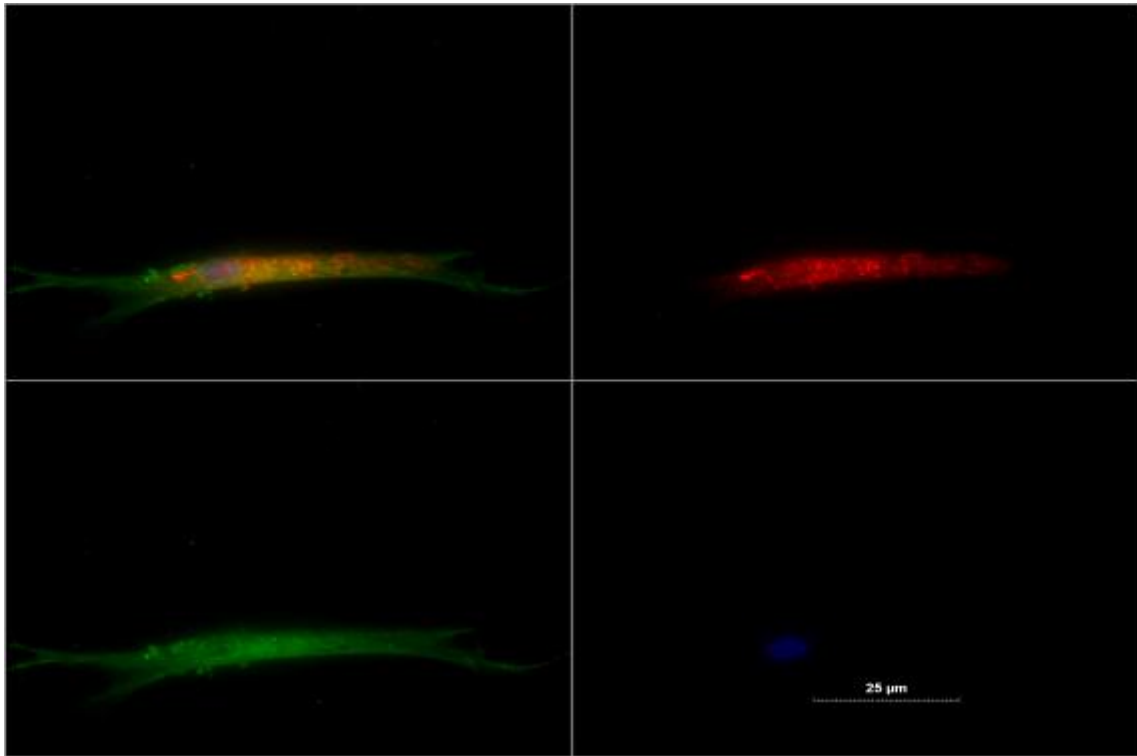


Figure 5.19 Co-expression of CK19 and vimentin in ISC at passage 8. CK19 (green) was co-localised with vimentin (red) in ISCs. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

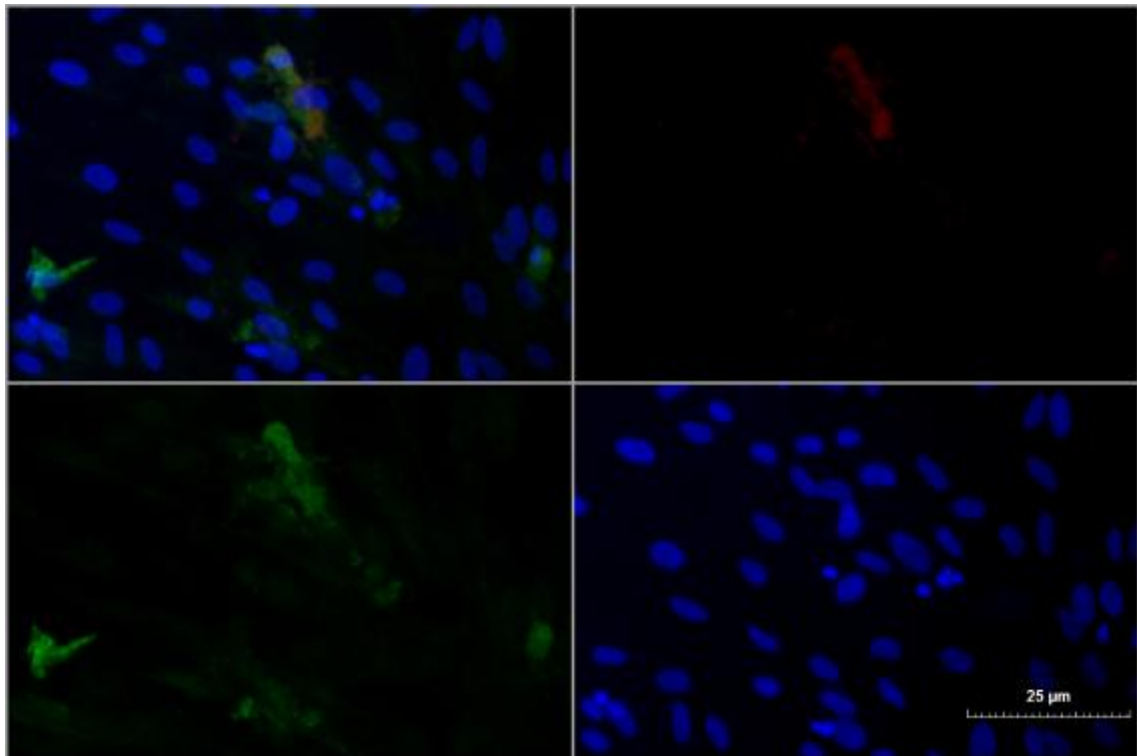


Figure 5.20 Expression of vimentin and nestin in ISC culture. Expression of nestin (green) was detected in early (passage 4) ISC culture. Infrequent nestin and vimentin (red) co-expression was observed. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

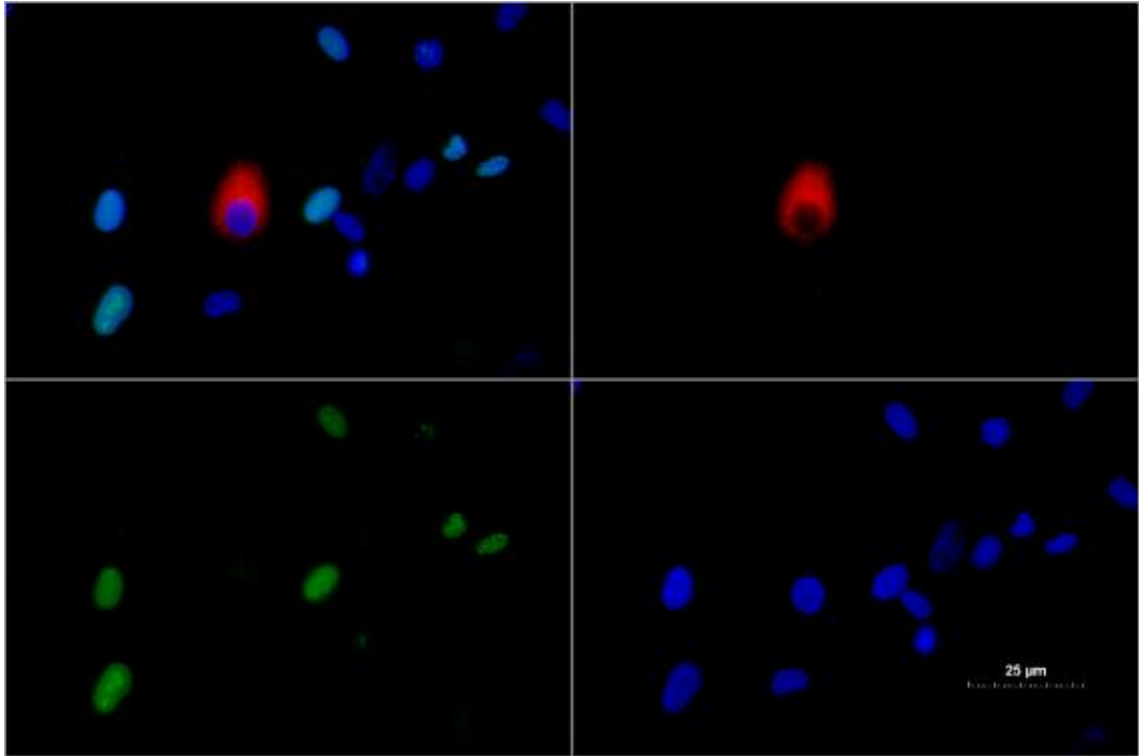


Figure 5.21 Amylase expression in ISCs. Expression of amylase (red) was maintained (passage 5). No co-localisation with Ki67 (green) was detected indicating either carryover of exocrine cells or differentiation / transdifferentiation in culture. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

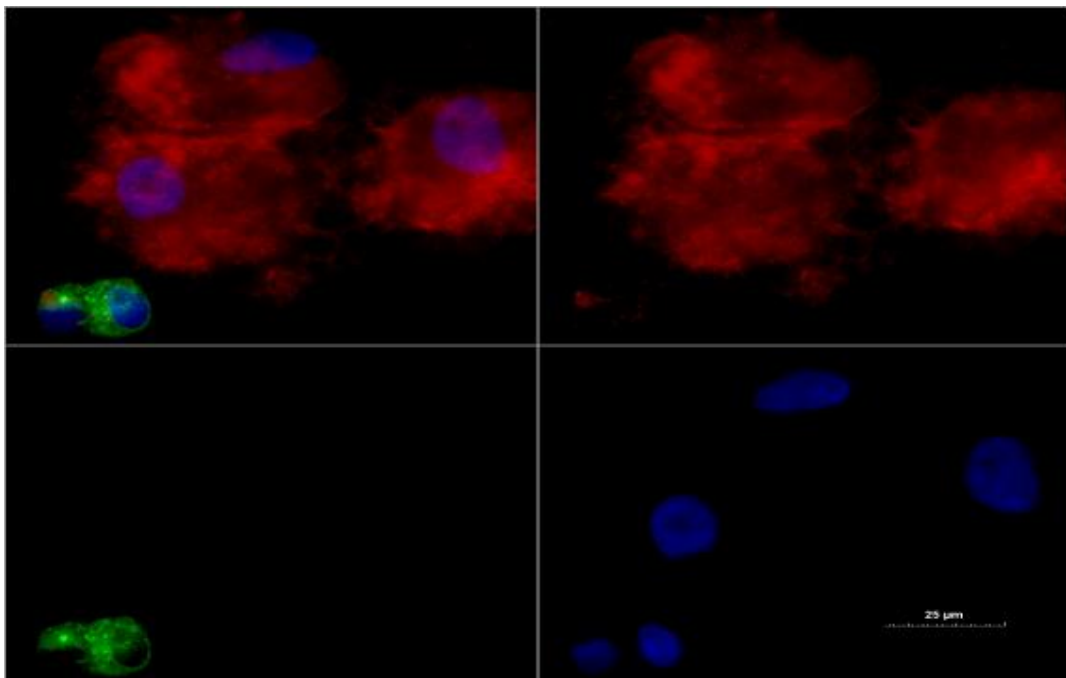


Figure 5.22 Endothelial marker expression in ISCs. Endoglin, an endothelial cell marker, was detected in culture (red) with no co-localisation with C-peptide (green). Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

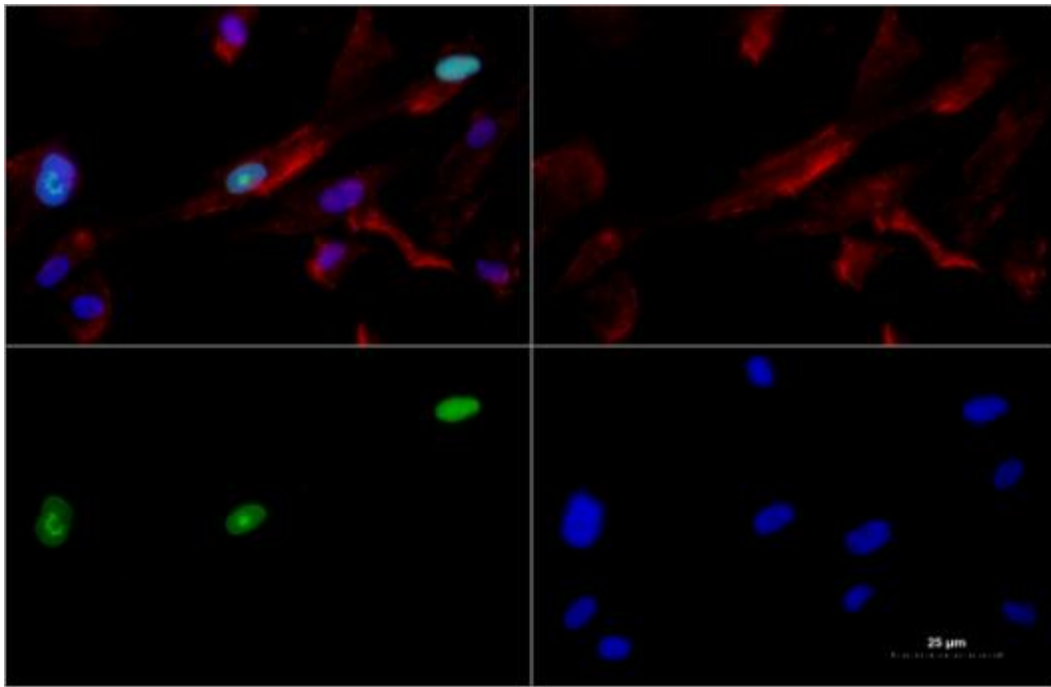


Figure 5.23 CK19 and Ki67 expression in ISCs. Co-localisation of Ki67 (green) and CK19 (red) indicates active proliferation within the ductal phenotype in ISC culture (passage 5). Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

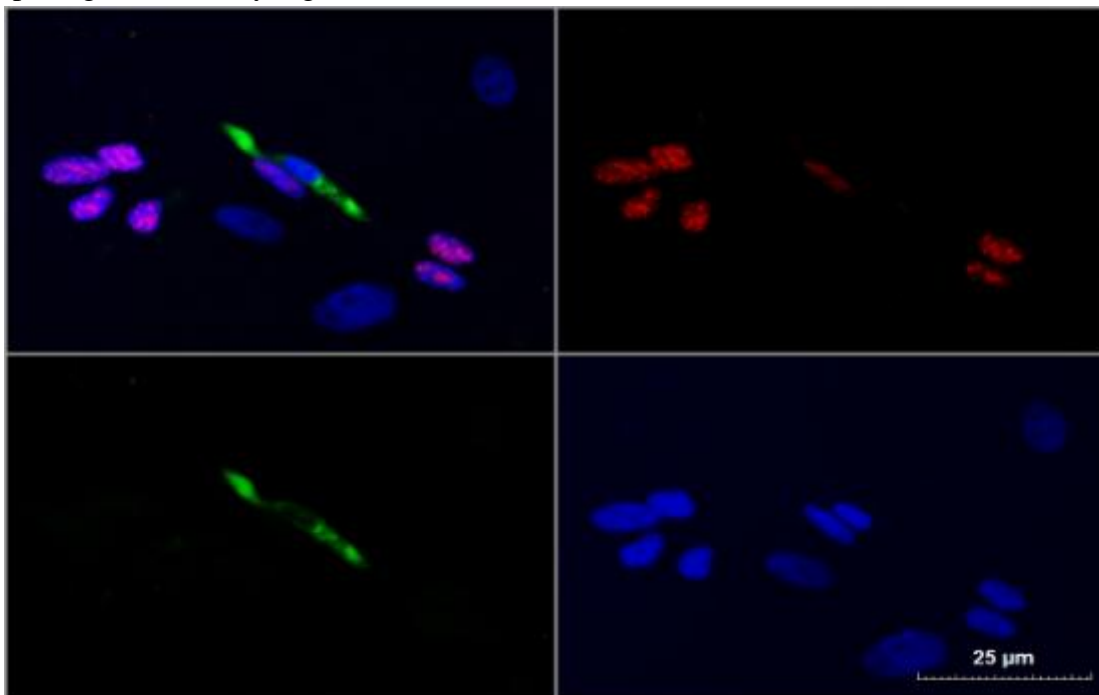


Figure 5.24 Oct4 expression in ISCs culture. Nuclear localisation of stem cell marker Oct4 was detected (red). No co-localisation of Oct4 and differentiated cell hormonal markers including C-peptide (green) was observed. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

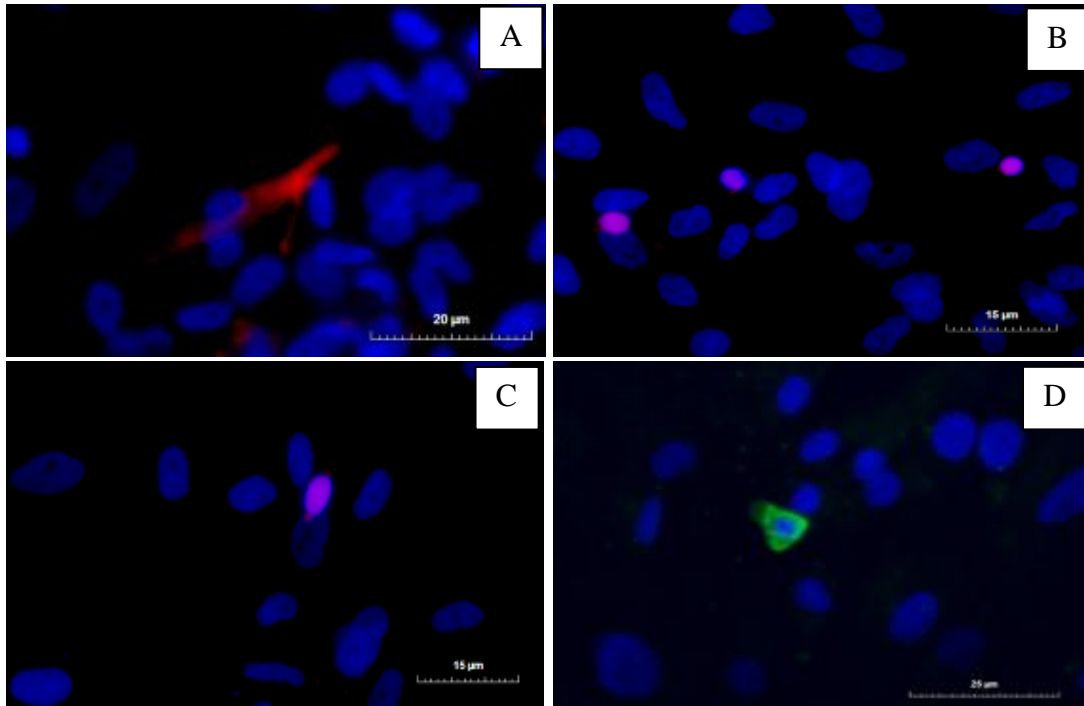


Figure 5.25 Developmental marker expression in ISCs culture. Expression of the endocrine-pancreas developmental marker NGN3 was detected in a small number of cells (A, red). Nkx6.1 (B, red) and PAX6 (C, red) were also detected. Interestingly albumin, a hepatocyte marker was also detected in low number of cells (D, green). Red: Cy3, green: FITC, nuclei were counter stained with DAPI.

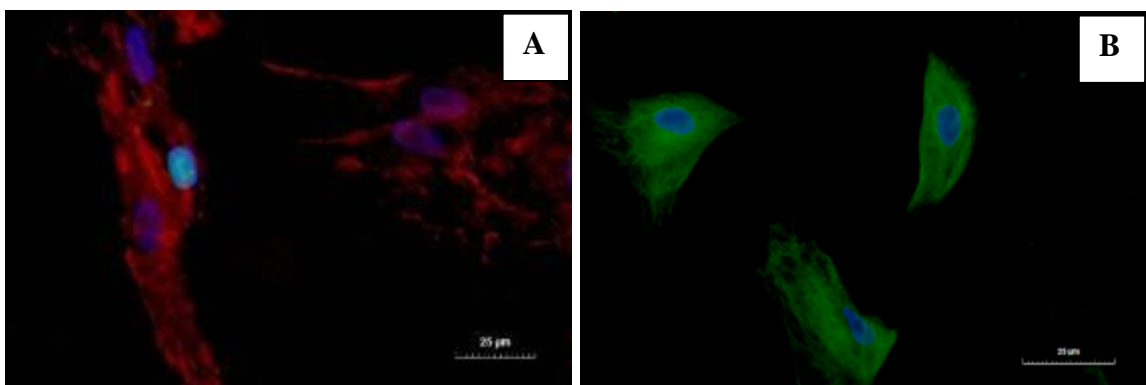


Figure 5.26 Characterisation of late ISCs. These show marked reduction in proliferation with less Ki67 positive cells (A, green). Expression of both Ck19 (A, red) and vimentin (B, green) was maintained. Red: Cy3; green: FITC; nuclei were counter-stained with DAPI.

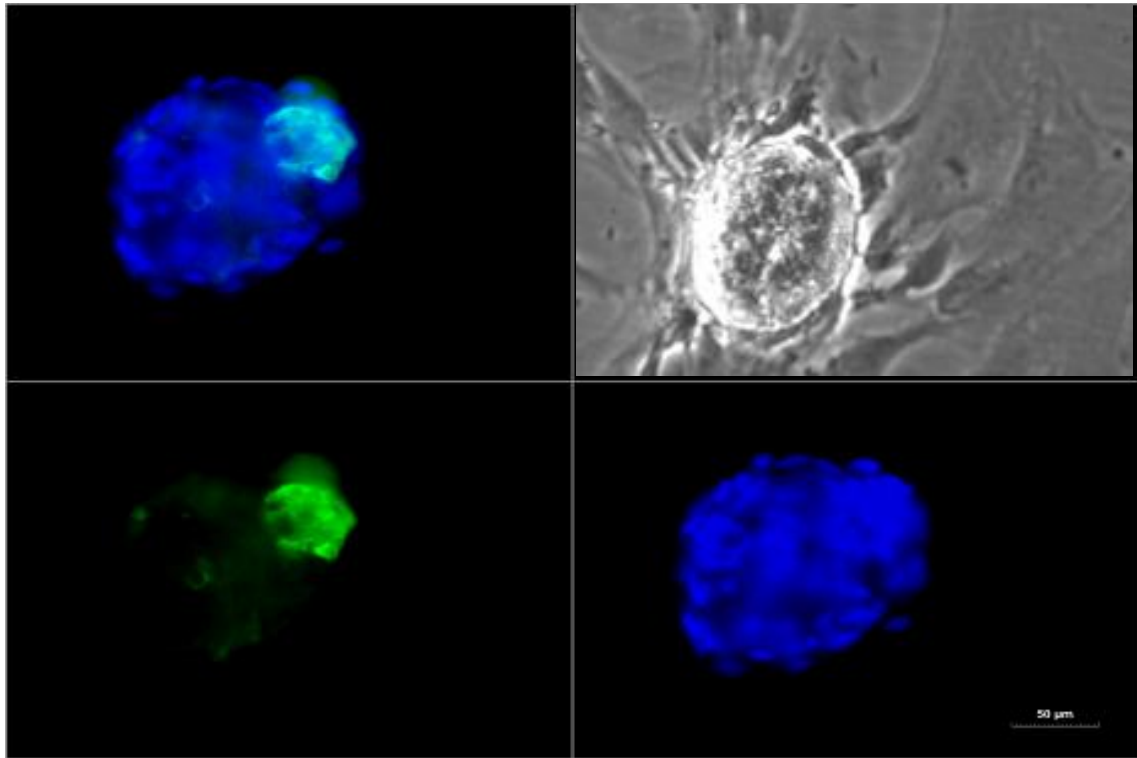


Figure 5.27 C-peptide expression in ISCs at passage 5. When ISCs approached confluence they formed cellular clumps. Pockets of C-peptide (green) expressing cells were detected indicating possible β -cell neogenesis or redifferentiation. Green: FITC; nuclei were counter-stained with DAPI.

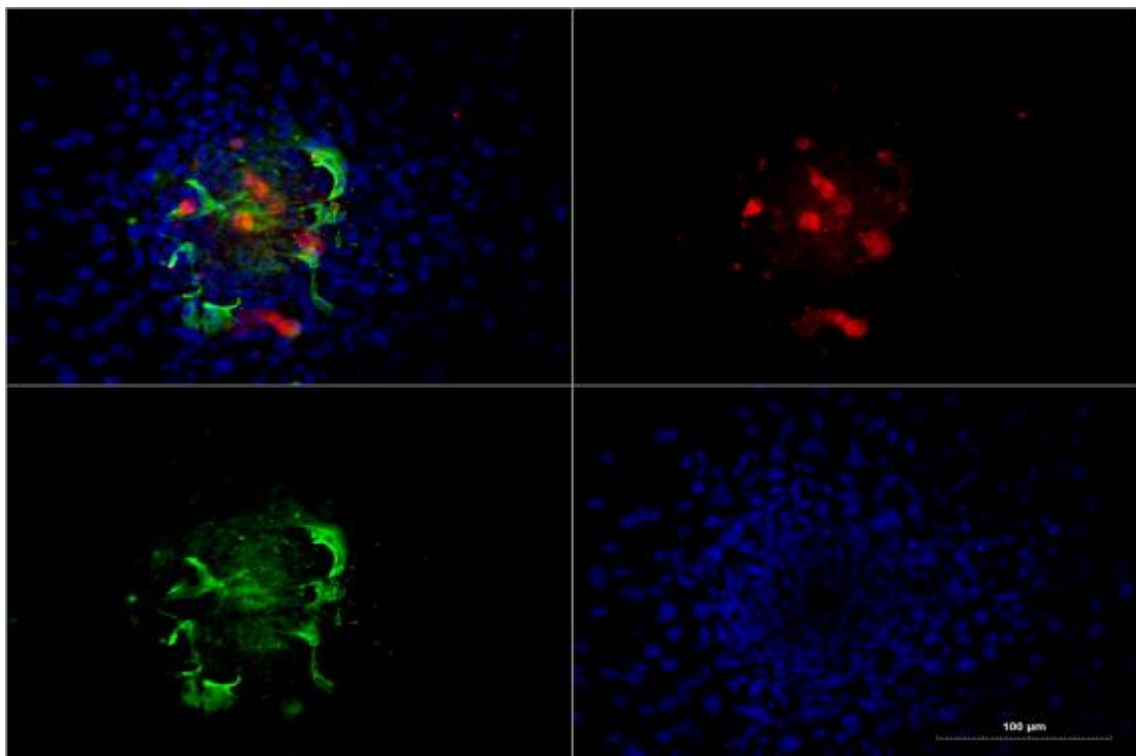


Figure 5.28 Vimentin and nestin in ISC clumps. Expression of vimentin (green) and nestin (red) was observed within the newly formed clumps.

pockets is not clear with several possibilities. This may be due to re-aggregation of primary differentiated β -cell which maintained their phenotype in culture over the preceding passages. A second possibility is these were new cells arising from β -cell replication. A third possibility is for these cells to arise from stem / progenitor cells proliferation and replication. Fourthly these cells may arise as a result of dedifferentiation (endothelial mesenchymal transition, EMT) / proliferation / redifferentiation. None of these possibilities can be confirmed or refuted with 100% certainty. However at least in this model absence of insulin and Ki67 co-expression suggests that self-replication of mature β -cells in culture is unlikely. Vimentin, nestin (Figure 5.28) and CK19 (Figure 5.29) expression was confirmed within newly formed aggregates.

5.4.4.4 Assessment of PDX1 expression

Expression of PDX1, a key β -cell transcription factor, was confirmed in RT-PCR studies (5.4.3) but it was not possible to meaningfully assess it in ICC studies due to lack of sufficient specificity in available antibodies, despite multiple attempts with several brands of commercial PDX1 antibodies. To further confirm presence of the signalling pathway necessary for endogenous PDX1 gene expression, transfection studies were carried out utilising pPDX1-eGFP, a PDX1 reporter plasmid in which a β -cell-specific fragment of the PDX1 promoter is sub-cloned upstream of eGFP (Campbell and Macfarlane, 2002) (Figure 2.9 A). Transfection efficiency in ISCs was investigated employing reporter plasmids driven by constitutively active promoters: pIRES-eGFP (Figure 2.9 B) and pCAG-DsRed (Figure 2.9 C).

Initial trials of transfecting ISCs using Lipofectamine2000™ (Invitrogen) were not satisfactory with very low transfection efficiency and high toxicity. Successful non-toxic transfection was achieved using the non-lipid based transfection agent JetPEI™

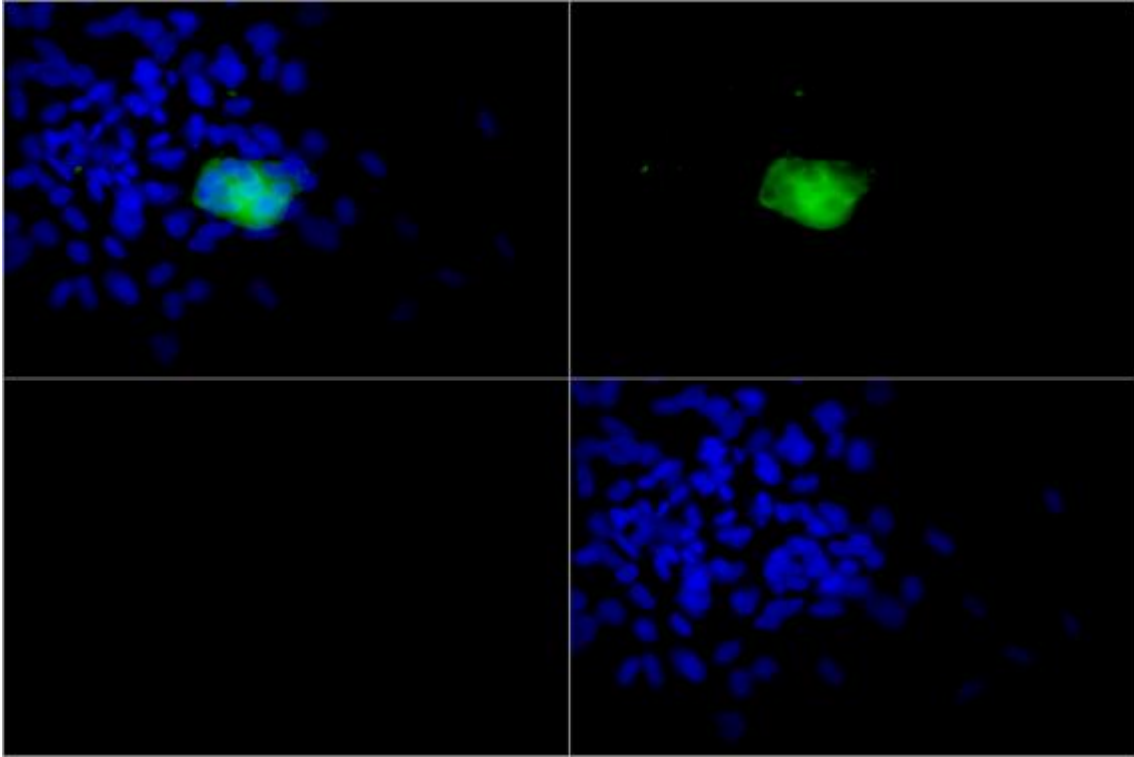


Figure 5.29 CK19 expression in ISCs. Pockets of CK19 (green) expressing cells were detected in the newly formed clumps.

with both pCAG-DsRed (Figure 5.30 A) and pIRES-eGFP (Figure 5.30 B) at an average efficiency of $7\pm 2\%$ (mean \pm SD) of cells. The superiority of JetPEI over Lipofectamine2000 in this setting could be a result of prolonged exposure to plasmid DNA in the case of JetPEI as opposed to relatively shorter exposure times with Lipofectamine2000 necessary to reduce toxicity. JetPEI was thus used for the PDX1 reporter gene analysis.

Expression of the beta-cell specific PDX1 reporter gene fragment in ISCs at passage 4 was confirmed at 48 hours post-transfection by detection of green fluorescence (Figure 5.31).

5.4.4.5 Development of functional live-cell markers for β -cells

Primary β -cells express and biosynthesize insulin and then store it inside the secretory granules as hexamers bound by a zinc molecule. Sulphonylurea receptor 1 (SUR1) is expressed in β -cells and plays a key role in insulin secretion.

Two methods for live detection of β -cells dependent on these functional markers were investigated to facilitate further elucidation of the fate of β -cells in culture.

5.4.4.5.1 Fluorescence-tagged sulphonylurea treatment

BODIPY®-tagged glibenclamide, a fluorescence-tagged sulphonylurea, was utilised for potential live labelling and visualisation of β -cells (2.14.1) as a novel approach for live β -cell imaging. Initial proof of staining was optimised in intact human islets (2.14.1.1). No meaningful staining was seen at concentrations equal or below 50 nM. Best labelling of islets was achieved with 100 nM but extensive non-specific background staining remained problematic. Concentrations equal to or above 500 nM resulted in universal labelling of all cells in the sample. Incubations for 30 minutes resulted in more non-specific staining compared to 15 minutes. Therefore a concentration of 100 nM with 15 minutes incubation was used in ISCs.

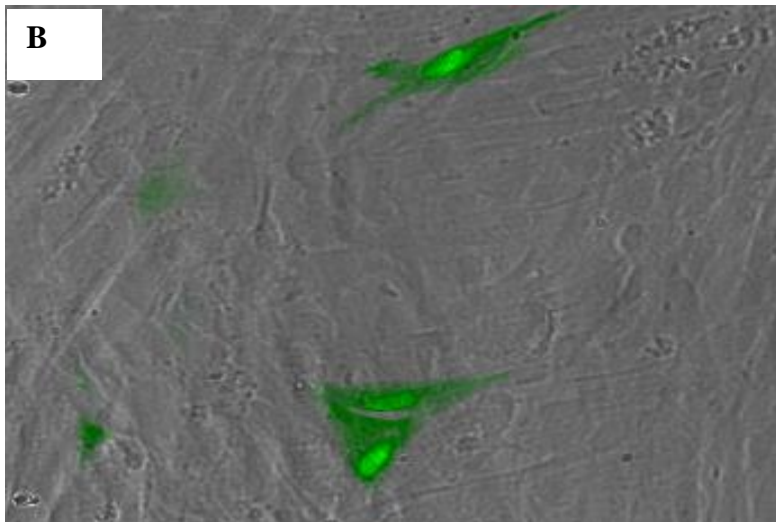
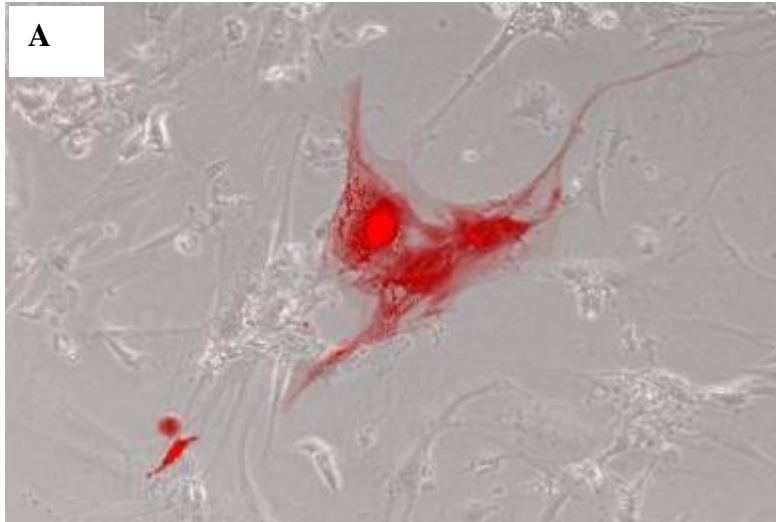


Figure 5.30 Reporter gene expression in ISCs at passage 4. Transfection with both DsRed reporter (A) and eGFP reporter (B) was successful using a transfection agent confirming transfectability of these primary cells.

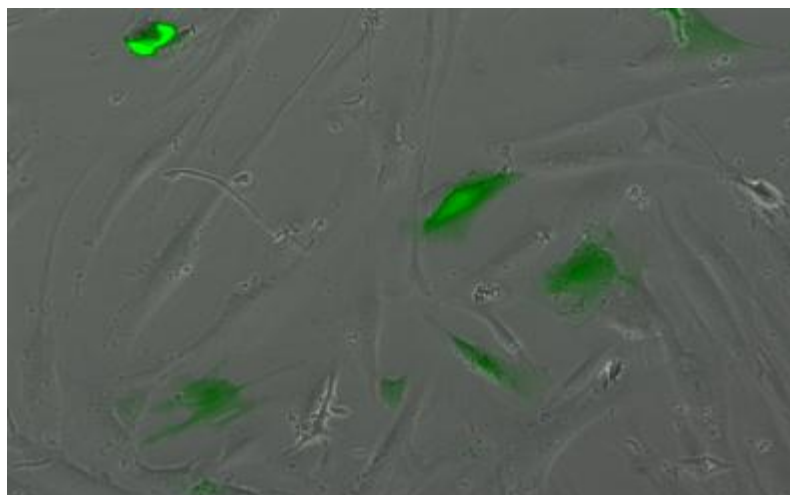


Figure 5.31 PDX1 reporter gene expression in ISCs. A selected representative field confirming expression of PDX1 in number of ISCs indicated by eGFP expression following transfection with PDX1 driven eGFP reporter plasmid.

Staining adherent ISCs with 100 nM BODIPY®-tagged glibenclamide resulted in optimal labelling with single defined cells in adherent culture and minimal non-specific background (Figure 5.32).

Trials of validating specificity of BODIPY®-tagged glibenclamide-labelled cells using the standard ICC protocol for insulin were not successful. It was not clear whether this was due to the glibenclamide secretagogue effect on β -cells resulting in relative depletion of insulin in insulin-expressing ISCs, in which storage capacity is already limited as shown before (5.4.2), or whether BODIPY-glibenclamide was washed off during the ICC staining protocol.

5.4.4.5.2 Newport green (NPG) labelling

NPG is a green fluorescent zinc indicator that has been used by several groups to enable assessment of live β -cells (Ichii et al., 2005; Lukowiak et al., 2001; Parnaud et al., 2008). Labelling optimisation of intact islets (2.14.2.1) showed 10 nM NPG to be the most acceptable labelling concentration (Figure 5.33). However, non-specific background staining was also present but to a lesser extent compared to SU labelling.

Zinc-rich cells in the ISCs culture were successfully labelled with 10 nM NPG (Figure 5.34) achieving cell-specific staining with minimal non-specific background staining. Further validation of specificity of NPG labelling for β -cells was not possible using standard ICC methods due to possible wash-out during the staining procedure.

5.5 Discussion

Establishment and optimisation of proliferative cultures from intact human islet preparations was achieved. Active proliferation was maintained up to passage 9/10, with an estimate of 20,000 doublings in cell number by this stage in the presence of foetal calf serum (FCS) and ITS-A supplement but no other extra-cellular matrices.

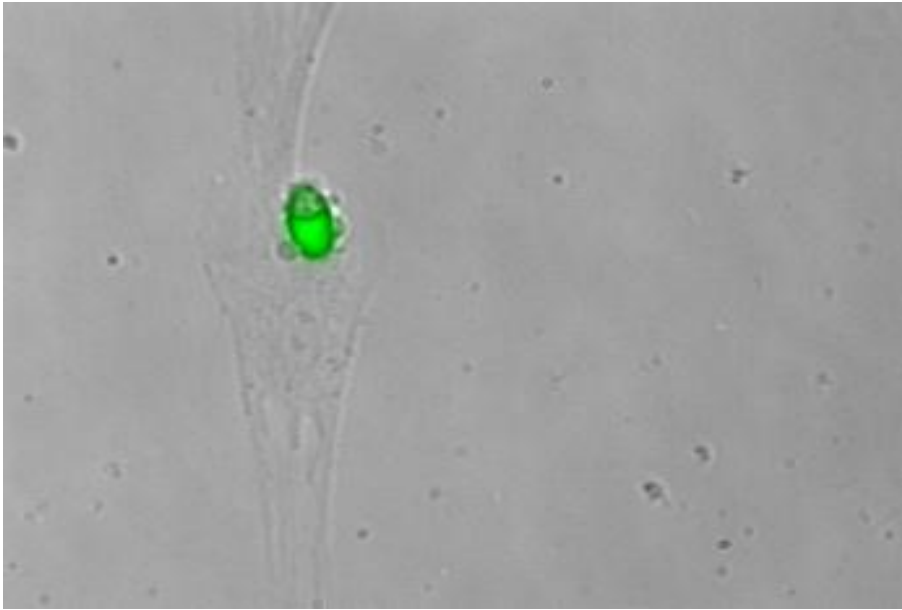


Figure 5.32 BODIPY®-tagged glibenclamide ISCs labelling. Live imaging of ISCs at passage 4 demonstrating one possible β -cell with uptake of green fluorescent-tagged SU.

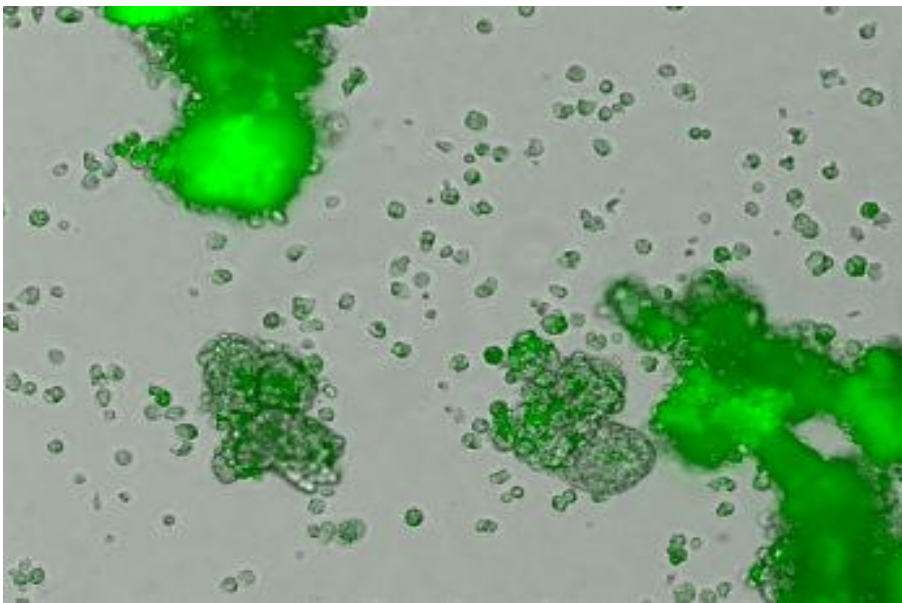


Figure 5.33 NPG staining of fresh human islets. This shows more islet specific staining but with significant background staining

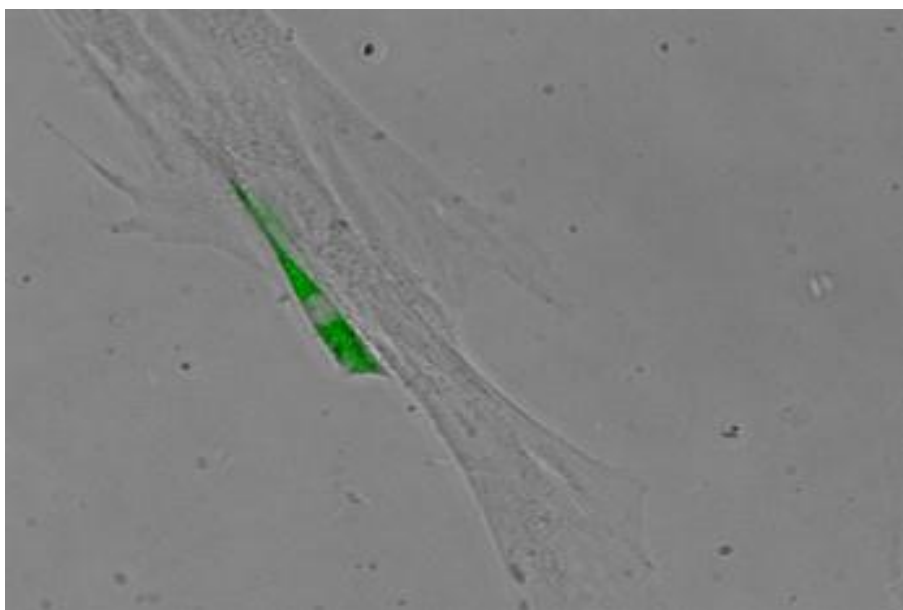


Figure 5.34 NPG staining of ISCs at passage 4. Cell specific staining was observed with incubations with NPG at 10 nM for 15 minutes.

Significant reduction in growth rate together with senescence was noted in later passages. Characterisation of early and middle passages, demonstrated ISCs to be a heterogeneous population of cells that contain all islet endocrine phenotypes including α -cells, β -cells, δ -cells and PP-cells, in addition to ductal, exocrine and endothelial cells. Further studies on (pro)insulin expression, production, storage and secretion demonstrated continued, but decreased, insulin expression and production during early and middle passages which was completely lost in later passages (passages beyond 6/7). At early passage, insulin synthesis was only equivalent to 5% of that of intact islets and there was evidence of impaired storage capacity in β -cells.

Further immunohistochemical characterisation of ISCs demonstrated expression of different phenotypic markers. These included the pluripotent stem cell markers Oct4, Nanog and Sox2 in addition to expression of nestin, a marker of neuro-endocrine progenitor cells; vimentin, a mesenchymal marker; NGN3, a developmental marker of the pancreatic endocrine compartment; and albumin, a hepatocyte marker. This demonstrates generation of an actively proliferative culture from human islet-rich pancreatic preparations producing heterogeneous population with potential progenitor / stemness capacity.

Establishment of proliferative cultures from human pancreas preparations has been reported by several groups with varying protocols and outcomes (Beattie et al., 1997; Gershengorn et al., 2004; Kayali et al., 2007; Lechner et al., 2005; Ouziel-Yahalom et al., 2006). There is overall agreement on use of FCS in culture media and allowing growth and expansion in 2D monolayer instead of maintaining the 3D structure of the islet, while there were significant differences in cell purity in the starting culture and use of different matrices and other growth factor supplements. Beattie stressed the importance of purity of human islets used in the proliferative culture and elected to

hand pick Dithizone-stained islets which were then established in HTB-9 extracellular matrix-coated plates using RPMI 1640 medium supplemented with FCS and HGF to (Beattie et al., 1997). Gershengorn accepted varying purities but attempted further enrichment of starting culture with islets by filtration through 45 μ m filter to reduce single cells and small clusters, then cultured islets in standard treated culture-ware using CMRL 1066 medium supplemented with FCS and L-glutamine (Gershengorn et al., 2004). On the other hand, Lechner accepted islet preparations as provided but dissociated these into single cells before establishing the culture in standard treated culture-ware using CMRL 1066 medium supplemented with FCS, ITS, EGF and β -NGF (Lechner et al., 2005). In the current project islet preparations regardless of purity were established in culture in non-adherent tissue culture flasks using CMRL 1066 medium supplemented with FCS, HEPES and ITS. All the above mentioned protocols resulted in active proliferation and gradual loss of insulin expression as culture progressed but with differences in proliferation capacity and culture phenotype. Beattie reported an estimate of 30,000 fold increase in cell number (~30 passages) accompanied by rapid loss of insulin expression but with persistence of PDX1 message (Beattie et al., 1999). Gershengorn observed active proliferation for more than 20 passages with similar rapid loss of the endocrine phenotype in addition to other epithelial markers while gaining expression of more mesenchymal markers including vimentin (Gershengorn et al., 2004). Lechner did not attempt to assess proliferative capacity over several passages but reported loss of insulin expression even when starting culture reaches confluence at first passage with an increase in nestin or CK19 expression (Lechner et al., 2005).

The above findings raise an important question about the origin of what appear to be differentiated cells in middle passages, including insulin expressing cells? The current

studies in this chapter and other mentioned reports (Beattie, Gershengorn, Lechner and others) confirm the eventual loss of differentiated cell phenotype including β -cells. Although Russ confirmed in elegant lineage tracing studies epithelial to mesenchymal transition (EMT) of β -cells in islet enriched proliferative cultures (Russ et al., 2009), redifferentiation of these into functional β -cells has not yet been confirmed. Dor showed in lineage tracing studies preserved self-replication capacity in mouse β -cells which was shown to be the primary mechanism for β -cell regeneration *in vivo* in adult mouse, in near-total pancreatectomy mouse model (Dor et al., 2004). In the human *in vitro* model established in this chapter, no evidence for β -cell replication was found with insulin-positive cells consistently being Ki67 negative.

Convincing nuclear expression of the classical pluripotency-associated marker Oct4 in pancreas-derived proliferative cell cultures has been demonstrated for the first time in the current studies (White et al., 2011). This work provided preliminary data for a successful Diabetes UK PhD studentship application to explore the potential stemness and differentiation capacity of these novel cells in lentiviral reporter gene lineage tracing studies using the *in vitro* human primary pancreatic cell model established in this chapter.

Preliminary data also for the utility of reporter gene transfections to identify specific phenotypes has also been provided with expression of a β -cell specific PDX1 promoter fragment plasmid in selected ISC cells at early to mid-passage. This provides a potential means of selecting and sorting live β -cells from these mixed cultures. Alternative approaches using fluorescence-tagged sulphonylurea and the fluorescent zinc indicator Newport green were also evaluated, again identifying a sub-population of cells in low to middle passage ISCs in live culture.

Spontaneous formation of cell clusters with ‘pockets’ of cells expressing differentiated ductal (CK19) and β -cell (insulin/C-peptide) markers was noted. This suggests the potential for redifferentiation or neogenesis following proliferation *in vitro* although confirmation of origin would necessitate *in vitro* lineage tracing studies. In the absence of specific differentiation protocols, insulin and C-peptide storage and secretion has remained very low and insufficient for clinical value without further steps to induce insulin biosynthesis and secretion in a much larger proportion of the proliferated population.

**Chapter 6. Islet survivor cell
differentiation and pseudo-islet studies**

6.1 Introduction

Heterogeneity and potential plasticity of ISCs has been demonstrated in Chapter 5 with confirmed evidence of progenitor/stem cell marker expression. Despite continued expression of insulin and other islet markers during early culture, level of expression was very limited and not sufficient for any therapeutic application. Several groups have attempted to differentiate pancreatic cell cultures towards more efficient and physiologically functional insulin expression. This typically involved treatment with specific factors and/or modification of culture conditions (Beattie et al., 1997; Bonner-Weir et al., 1993; Gershengorn et al., 2004; Ouziel-Yahalom et al., 2006). Bonner-Weir reported generation of islet buds from ductal-rich human pancreas cell culture employing Matrigel, an extracellular matrix, and keratinocyte growth factor to create 3 dimensional structures. Islet-bud structures showed improved insulin secretory response to glucose over 24 hours stimulation (Bonner-Weir et al., 2000). Beattie employed a matrix derived from the human bladder carcinoma cell line HTB 9 in addition to supplementation with HGF (Beattie et al., 1997). Gershengorn reported successful re-differentiation of expanded islet cells in culture when cells were treated with serum-free medium and EGF (Ouziel-Yahalom et al., 2006) however this could not be replicated by other groups (Kayali et al., 2007). Use of serum-free medium and supplementation with betacellulin resulted in successful re-differentiation of expanded cells towards β -cells by Ouziel-Yahalom (Ouziel-Yahalom et al., 2006). Despite relative success reported by different groups following different protocols there is still no optimal differentiation model for reproducibly achieving physiological or near physiological functional cells.

The three dimensional structure of intact islets has been shown to be very important for maintenance of physiological islet function (Halban et al., 1982; Hopcroft et al., 1985;

Pipeleers et al., 1982). Many mechanisms have been suggested to play a role in this including direct communication through adhesion molecules at the cell-cell contact points (Cirulli et al., 1994; Dahl et al., 1996); exchange of small molecules through gap junctions (Orci et al., 1973; Schofield and Orci, 1975); and paracrine interactions between different islet cells (Franklin et al., 2005; Ishihara et al., 2003). Artificial aggregation of the MIN6 β -cell line with formation of 3 dimensional islet-like structures has been shown to significantly improve β -cell function and insulin secretion in response to a range of stimuli (Brereton et al., 2007).

6.2 Aims

The overall aim of this chapter was to attempt differentiation of ISCs expanded in culture towards mature islet-like cells. Specific objectives were to:

1. Generate three dimensional structures (pseudo-islets) from ISCs
2. Characterise pseudo-islets
3. Evaluate function of pseudo-islet *in vivo*

6.3 Formation of pseudo-islets

Pseudo-islets were established and characterised through the hanging drop (HD) method. High throughput production was attempted employing micro-wells and a bioreactor approach.

6.3.1 Hanging drop method

Hanging drop is a widely used methodology for formation and study of 3 dimensional structures including mitogenic spheroids (Timmins et al., 2004; Valcarcel et al., 2008), embryoid bodies (Dang et al., 2002) and cell differentiation (Gutierrez et al., 2005;

Yoon et al., 2006). This concept has also been used to re-aggregate dissociated rat islets to generate consistent and standardised pseudo-islets (Cavallari et al., 2007).

The hanging drop technique employed in these studies was developed and tailored for the needs of this project from scratch and was not based on other published techniques. During the development of the method different drop volumes were tested and 35 μ l was found to be the largest optimal volume enabling efficient nutrient support for ISCs without risk of the drop falling during HD culture establishment. Different cellular densities in the HD were tested. Low densities (<1000 cells/drop) resulted in poor aggregation and fragility of the aggregates. Large densities (>4000 cells/drop) resulted in nutrient depletion and necrosis within 24 hours. Densities between 2000 and 3000 cells/drop resulted in optimal pseudo-islet formation with a size range from 50-300 μ m diameter (Figure 6.1). HD volume was standardised in all experiments to 35 μ l containing 2000-3000 cells (2.12.4.1). Characterisation was carried out on pseudo-islets to evaluate potential differentiation towards mature endocrine phenotype compared to adherent ISCs.

6.3.1.1 Stem cell gene expression evaluation

This was carried out to enable evaluation of the effect of 3D aggregation on expression of stem cell markers detected in adherent ISCs. Expression of Oct4 and Nanog was studied in PIs at passage 4 in comparison to adherent culture at passage 4 and cDNA obtained from human embryonic cell line H9 by semi-quantitative conventional RT-PCR. B2M was used as a reference gene. The results confirmed expression of Oct4 and Nanog in H9 and ISCs. However expression was lost in the PIs (Figure 6.2).

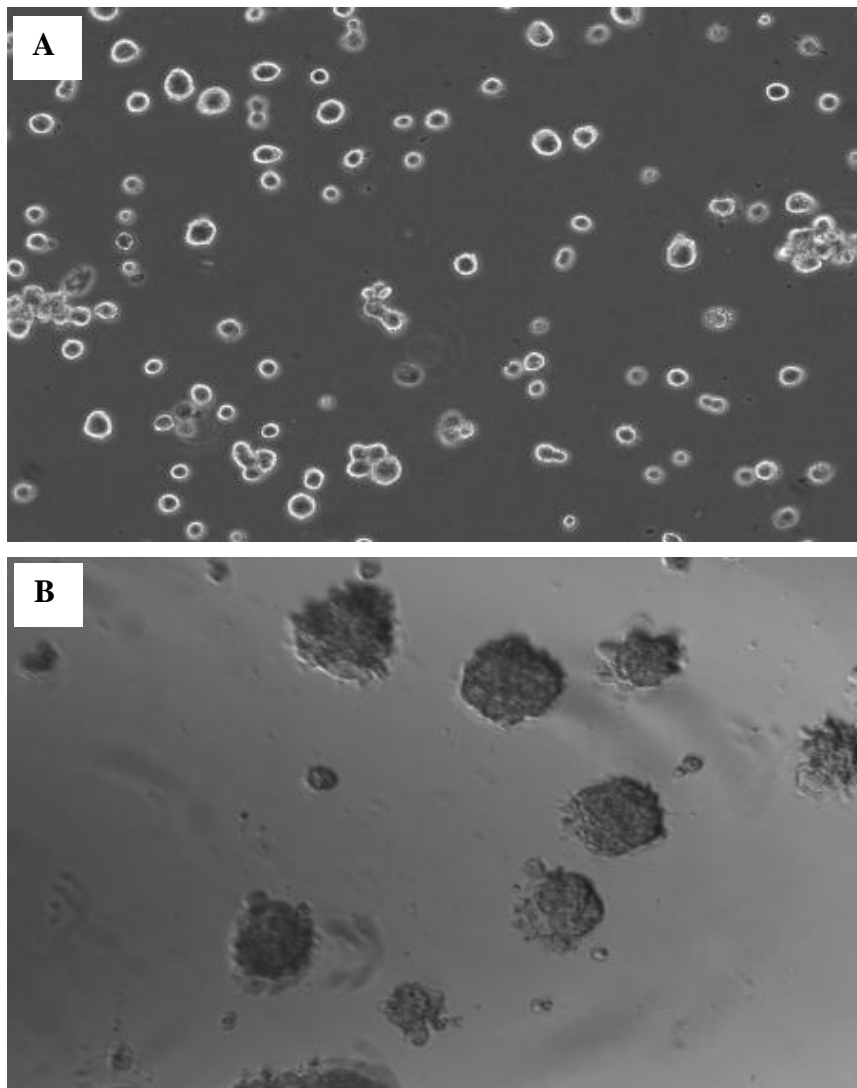


Figure 6.1 Pseudo-islet formation. ISCs are dispersed into single cells (A). Following 24 hour culture in hanging drops, the majority of cells aggregate into pseudo-islets (B).

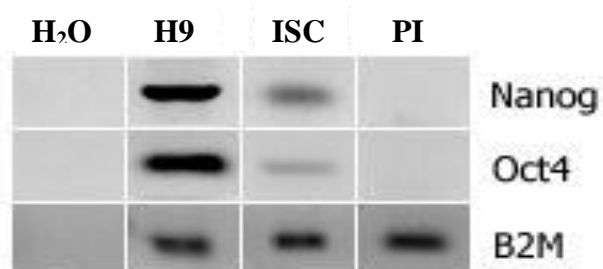


Figure 6.2 Stem cell marker expression. Expression of both Oct4 and Nanog was lost following formation of PIs. H9: human stem cells, ISC: islet survivor cell, PI: pseudo-islet.

This indicates a possible shift away from stem/progenitor state towards a more differentiated state.

6.3.1.2 Newport Green staining

NPG labelling was utilised to evaluate β -cell phenotype in PIs in comparison to ISCs in adherent culture and intact human islets (2.14.2). Adherent ISCs at passage 4 showed positive labelling with NPG but in a low density sporadic pattern (Figure 6.3 A). Following formation of PIs at passage 4, NPG labelling was observed across the aggregate with an overall enhanced uptake when compared to adherent ISCs (Figure 6.3 B). Intensity of NPG labelling was lower than that of intact primary islets (Figure 6.3 C). Interestingly, when the process was reversed and PIs were re-cultured in standard non-hanging drop culture in same serum-free medium they adhered within 24 hours and started to lose their 3D structure in a similar fashion to primary human islets upon establishment in adherent culture. Moreover, PIs which started to lose the 3 dimensional configuration showed lower NPG uptake (Figure 6.4).

These results indicate enhanced zinc content in PIs compared to adherent cells but this remained lower than that seen in intact islets.

6.3.1.3 Assessment of hormone profile

To evaluate the effect of PI formation on functionality of ISCs, insulin, proinsulin and C-peptide profiles were assessed in both PIs and adherent cells. Due to different culture conditions for adherent ISCs and PIs, experiments were carried out as follows. ISCs at passage 4 were washed and passaged in serum free medium for PI formation as before (2.12.4.1). Three groups of 15 drops were cultured using the hanging drop method (PIs) in serum-free medium (Table 2.2) and three groups of 15 drops were cultured in three wells in a 24 well plate (adherent cells) in same serum-free medium.

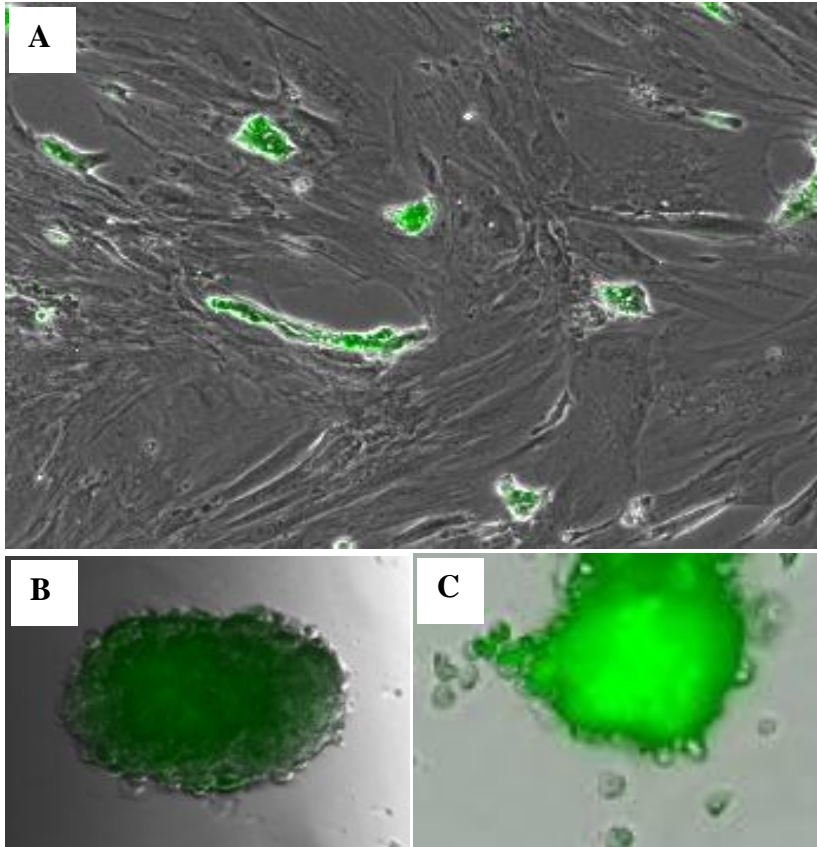


Figure 6.3 NPG studies in PIs. NPG labelling in adherent cells was sporadic (A). Following formation of PIs, NPG labelling was seen across the whole PI (B) and was comparable to, but less intense than, staining in intact human islets (C).

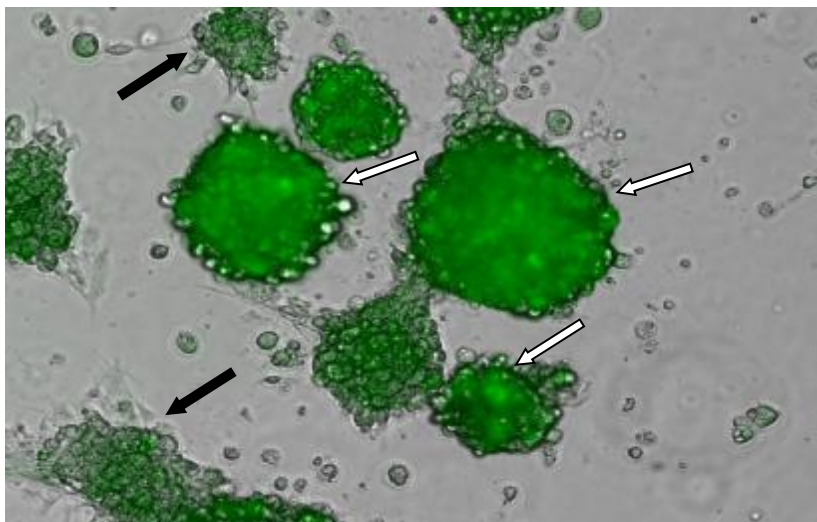


Figure 6.4 NPG staining of re-adhered ISC following formation of PIs. As PIs started to lose their 3D structure less NPG staining was observed (black arrows) compared with PIs maintaining the spherical structure (white arrows).

This ensured equal cell and medium loading in both groups. Cell culture was carried out at 37 °C in a 5% CO₂ humidified incubator for 24 hours following which each group of hanging drops was pooled into one tube and medium / PI pellet separated. Similarly, medium was recovered from adherent cells and pellet was retrieved by scraping. Insulin, proinsulin and C-peptide assays were carried out on medium and cell lysates. The results showed a significant increase in (pro)insulin biosynthesis in PIs confirmed for both insulin and C-peptide (Table 6.1). Moreover, there was a significant increase in insulin and C-peptide storage and near-significant proinsulin storage in PIs compared to adherent culture (Figure 6.5). The markedly higher insulin levels compared to proinsulin and C-peptide are likely to be, at least in part, false secondary to background carryover of exogenous insulin in the maintenance culture of adherent ISCs. Nevertheless, the increase in insulin levels in PIs is most likely reflecting true increase in insulin biosynthesis, especially that a similar trend is seen with proinsulin and C-peptide.

6.3.2 Micro-well studies

Micro wells (MWs) were developed by Dr Mark Ungrin from the University of Toronto in Canada to facilitate production of uniform and consistent embryoid bodies (Ungrin et al., 2008). Following a collaboration agreement, Dr Ungrin kindly provided trial samples to evaluate possible utilisation in high throughput production of PIs. MWs are made from Polydimethylsiloxane (PDMS) (2.12.4.2). Coating with Pluronic F-127 (Pluronic for short) was an essential to prevent adherence to the MW insert. Effect of Pluronic itself was not previously studied on ISCs therefore a control of standard wells coated with Pluronic was included in all studies in addition to standard adherent control.

	Insulin pmol/l	Proinsulin pmol/l	C-peptide pmol/l
PIs	1820±49	23.7±2.6	304.6±12.9
Adherent	1514±88	15.5±2.2	172.2±14.9
P value	0.039	0.07	0.0025

Table 6.1 Insulin, proinsulin and C-peptide levels in PIs and adherent ISCs. Results represent total insulin, proinsulin and C-peptide in both PIs and adherent ISCs cultured in serum-free medium. Statistical difference is evaluated with paired student test. n=3, mean±SEM.

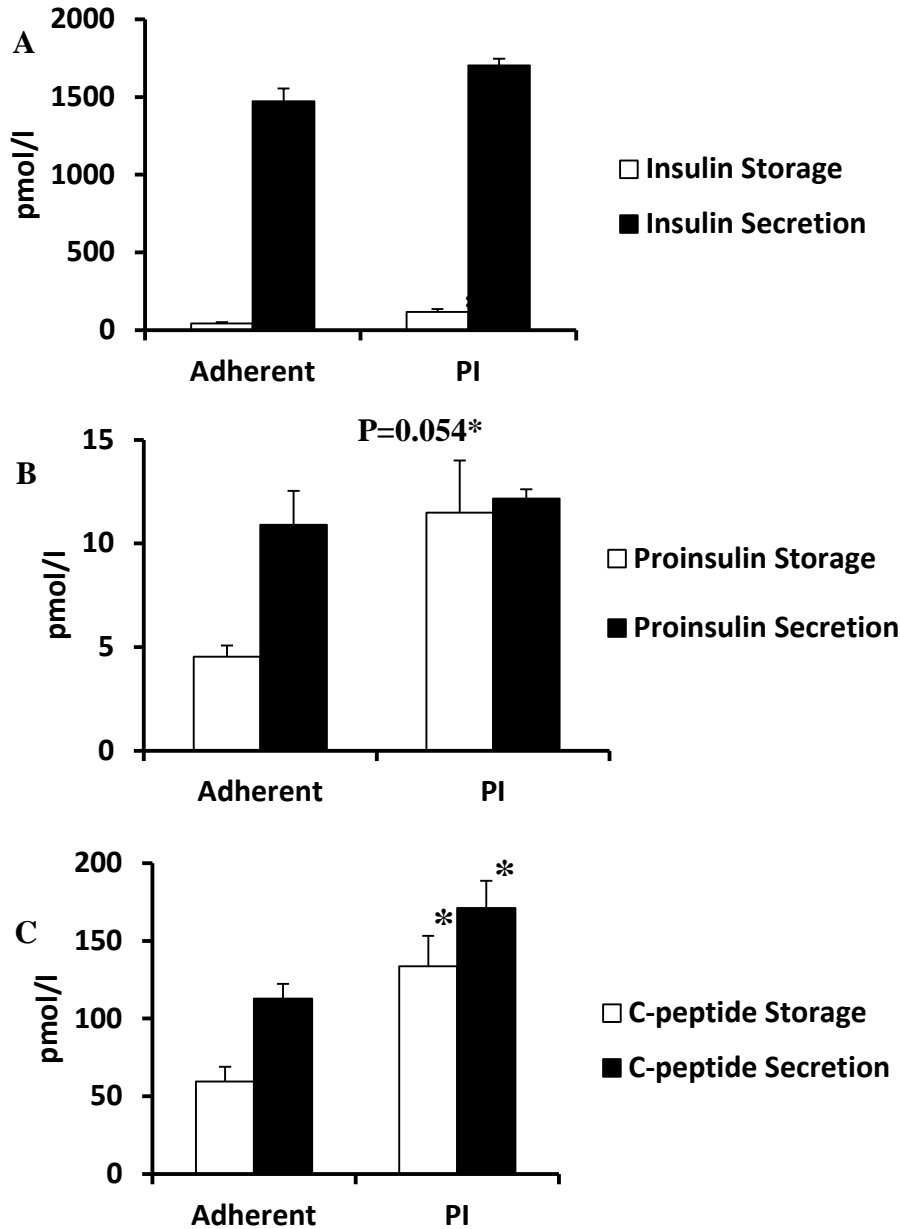


Figure 6.5 Changes in hormone storage and secretion following PI formation. There was >2 fold increase in storage of insulin (A), proinsulin (B) and C-peptide (C) in PIs compared to adherent ISCs. This was significant for all except for proinsulin which was not significant (p=0.054). Although secretion was increased in all assayed hormones/polypeptides but this was only significant in case of C-peptide. n=3, mean±SEM, *p <0.05 for secretion or storage in PIs vs adherent ISCs.

6.3.2.1 Micro-well validation for PI formation

At passage 4, three groups with equal loading of 5×10^5 cells per well were cultured in 24 well plates in wells with a MW insert (800 μm well size) coated with Pluronic (MW group), in standard wells coated with Pluronic (Pluronic control group) or standard non-coated wells (adherent control group). Each condition included 4 repeats. Visual observation under inverted microscope 24 hours post establishment in culture demonstrated formation of regularly-shaped PIs in the MW group with 1-2 PIs per MW (Figure 6.6 A). ISCs cultured in the Pluronic control group formed irregularly-shaped aggregates with varying sizes with many cells failing to form aggregates (Figure 6.6 B). ISCs cultured in the adherent control group adhered to the floor of the well and continued to grow with spontaneous clump formation (Figure 6.6 C). These morphological observations demonstrate the utility of MWs to generate uniform PIs in much larger quantities and less manipulations compared to hanging drop.

Medium samples from all wells were assessed for both insulin and C-peptide. In comparison to adherent control group, there was a reduction in insulin levels in the Pluronic group while insulin levels in the MW were slightly higher (Figure 6.7 A). C-peptide levels were comparable between all groups (Figure 6.7 B).

6.3.2.2 Effect of passage number on cellular proliferation and insulin synthesis in PIs

To determine effect of passage on cellular mass and insulin biosynthesis in PIs generated in MWs, ISCs from passages 4 (P4), 7 (P7), and 12 (P12) were employed in micro-well studies including Pluronic control (3 repeats) and MWs (800 μm per micro-well with 6 repeats). Medium and cell pellet were harvested at 24 hours.

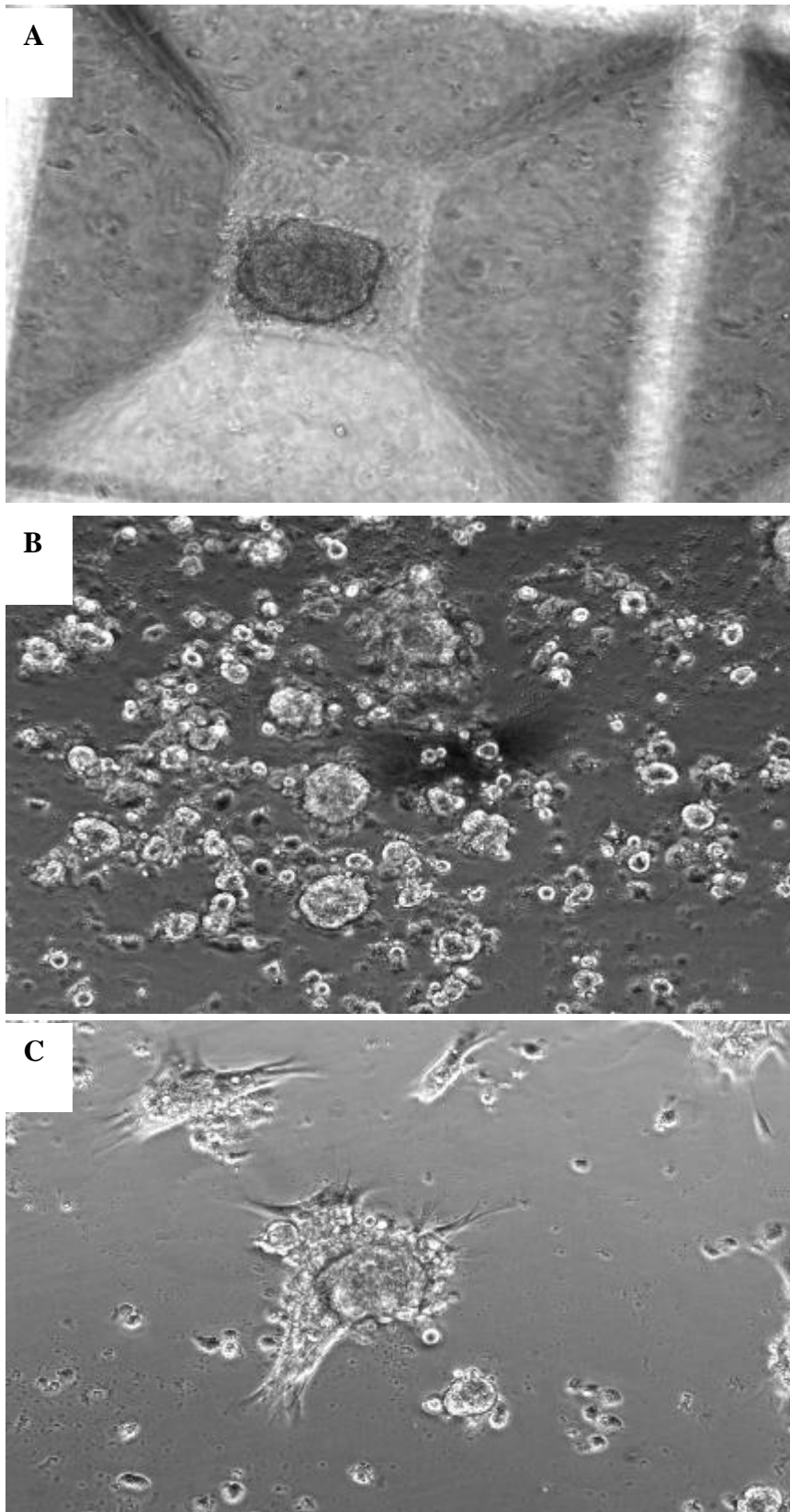


Figure 6.6 PI formation in micro wells. At 24 hours post culture establishment regular PIs were observed in Pluronic-coated MWs (A) while Pluronic-coated standard wells contained irregular PIs with varying shapes and sizes (B). ISCs in standard non-coated wells adhered to the well (C).

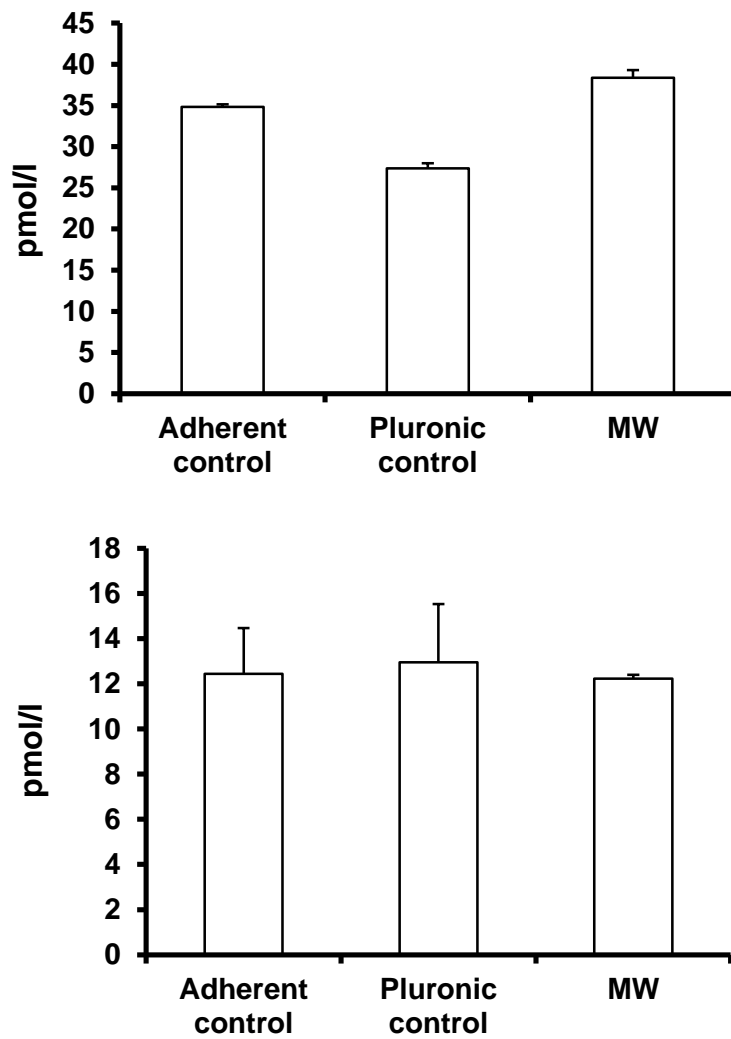


Figure 6.7 Insulin and C-peptide secretion from PIs formed in MWs in comparison to controls. Mean insulin secretion from the Pluronic control was lower than that of adherent control while insulin secreted from PIs in the MW group was higher than that both adherent and Pluronic controls (A). Low level C-peptide was detected in all three groups with differences between the groups (B). n=4, mean±SEM.

6.3.2.2.1 Proliferation

Difference in proliferative capacity was assessed by measuring protein mass in each group at different passages. There was a consistently lower protein content in the PIs grown in MWs compared to Pluronic control. This was statistically significant in passages 4 and 7 with a similar trend in passage 12 suggesting reduction in the proliferative capacity in PIs compared to Pluronic control (Figure 6.8).

6.3.2.2.2 Insulin secretion at different passages

Despite meaningful insulin secretion from passage 4 PIs grown in MWs there was a significant loss of insulin biosynthesis and secretion in PIs of passages 7 and 12 ISCs indicating loss of β -cell phenotype (Figure 6.9). Culture in MWs did not seem to have any positive effect, at least in this setting, on β -cell phenotype recovery. Further analysis was carried out on passage 4 only.

6.3.2.3 Insulin, proinsulin and C-peptide profiles in early PIs grown in MWs

Detailed insulin, proinsulin and C-peptide analysis was carried out on passage 4 samples of the above experiment. The results demonstrated an overall significant increase in insulin biosynthesis in PIs grown in MWs compared to control confirming the previous finding with PIs grown in hanging drops (6.3.1.3), with significantly increased insulin secretion and near significant increase in insulin storage (Figure 6.10 A). On the other hand, there was no meaningful change in proinsulin storage or secretion (Figure 6.10 B) but processing of proinsulin to insulin was significantly higher in PIs compared to control although processing in both groups was >90% (Figure 6.10 D). Secreted C-peptide levels were significantly higher in the PI group compared to control, in keeping with insulin findings, with no meaningful difference in stored C-peptide levels being observed (Figure 6.10 C).

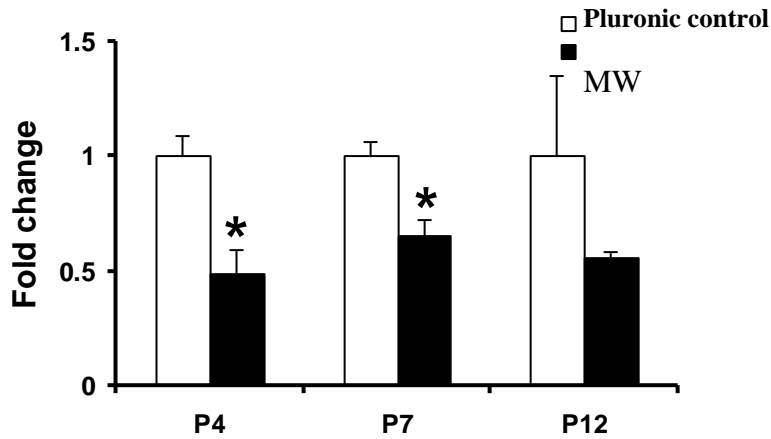


Figure 6.8 Cellular proliferation in PIs compared to Pluronic control. Results represent fold difference in protein content in PIs grown in MWs compared to Pluronic control. n=3 ±SEM, t test *= p < 0.05

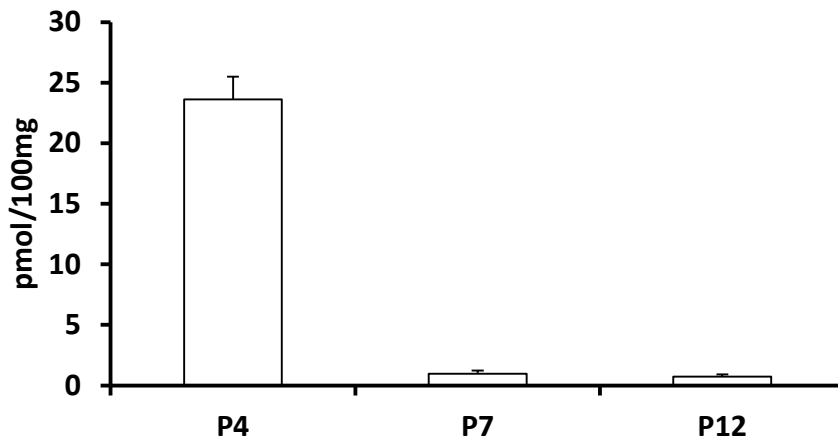


Figure 6.9 Insulin release from PIs at different passages. Results represent mean of total insulin released into media from PIs grown in MWs at passages 4 (P4), 7 (P7) and 12 (P12) corrected for protein content. n = 6, ± SEM, * = p < 0.05.

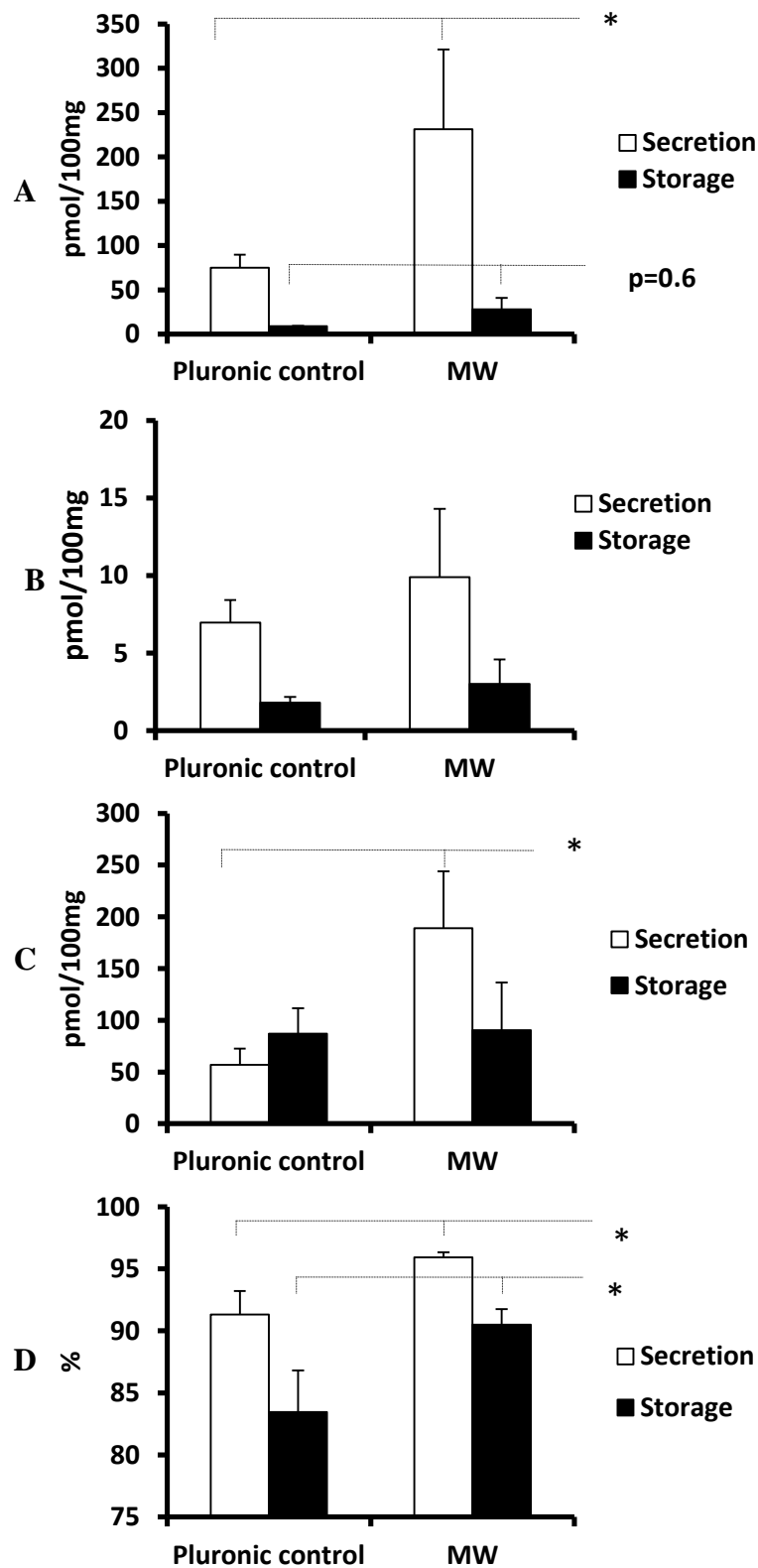


Figure 6.10 Pro(insulin) studies in PIs grown in MWs compared to Pluronic control. Storage and secretion of insulin (A), proinsulin (B) and C-peptide (C) in PIs grown in MWs at passage 4 compared to Pluronic control. Processing of proinsulin to insulin (D) was calculated as ratio of (insulin) / (insulin + proinsulin). Results represent mean of biological repeats at 24 hour post culture, corrected to protein content. n=3, mean±SEM, * = p < 0.05.

6.3.3 Extended culture in bioreactor setup

Bioreactor culture setup for maintenance culture of PIs generated in MWs was explored (2.12.4.3). Due to limited nutrient content in the MW setup it was not possible to extend culture further without risk of nutrient depletion and cellular loss. It is hypothesised that further extension culture in microgravity environment might maintain PI mass and allow for further differentiation towards mature β -cell phenotype.

Extension culture for a further 48 hours (total 72 hours) was attempted in high aspect ratio vessels (HARV) in the bioreactor running at 20 rpm inside the 37 °C tissue and 5% CO₂ culture incubator.

Assessment of cellular mass demonstrated significant loss of cells in the bioreactor culture similar to that seen in static culture in Pluronic control (Figure 6.11). Further assessment of insulin and C-peptide showed maintenance of hormone biosynthesis in remaining islets (Figure 6.12).

Within these experimental settings there were no further improvement / differentiation of ISCs following bioreactor extension culture. Moreover, this resulted in significant loss of cell mass. In order to further test the above hypothesis further optimisations including rotational speed, medium constitution and volume, frequency of medium change, cellular density, and oxygen pressure will be required.

6.4 *In vivo* functional assessment of PIs

To assess biological activity of ISCs; an *in vivo* transplantation assay was carried out in the SCID mouse C.B-17/Icr model. Two groups of 5 healthy SCID mice were employed in this experiment. One group was transplanted with PIs generated from ISCs at passage 6 following MW method of PI generation and one group was transplanted with count matched human skin fibroblast cells to provide a negative

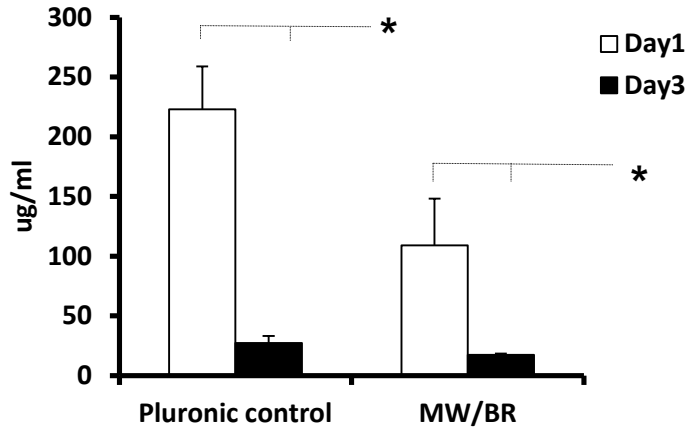


Figure 6.11 Cellular mass in PIs following 72 hour culture. There was a significant loss of mass in PIs established in Pluronic control at day 3 compared to day 1. Similarly, there was a significant loss in cellular mass in PIs established in MWs for 24 hours and further extended culture for total of 72 hours in bioreactor (BR). n=3, mean±SEM, * = p < 0.05.

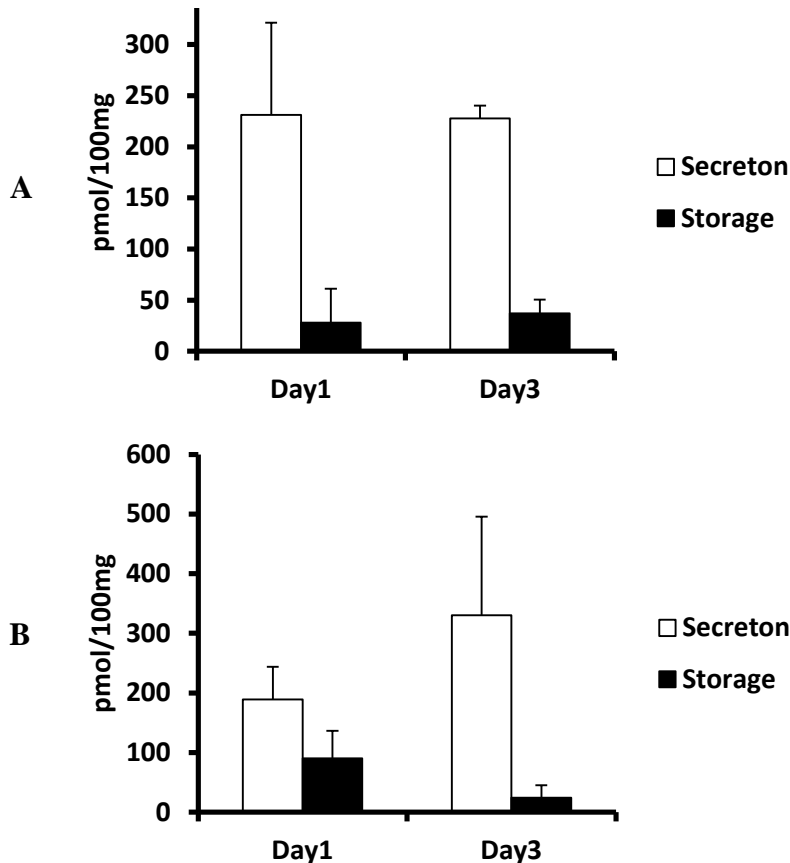


Figure 6.12 Effect of prolonged culture of PIs on hormone biosynthesis. There was no change in either insulin (A) or C-peptide (B) storage or secretion following at day 3 of extended culture of the PIs in bioreactors. n=3, mean±SEM, * = p < 0.05.

control with comparable metabolic demand as PIs (2.23.2.2). Due to logistical limitations with delayed animal delivery, ISCs were maintained in culture leading to higher passage number that was originally planned.

Sub-renal capsule transplantation was carried out as outlined before (2.23.2).

6.4.1 Effect of PIs on weight

Both control and test groups were comparable in starting weight (PI receiving group: 24.7 ± 0.2 grams, control group: 24.6 ± 0.5 grams, $p=0.8$, mean \pm SEM). Despite matching weight at the start of the experiment there was a notable weight increase in the control group but not in the PI group. On the contrary, the weight of PI group at the end of the experiment at day 17 was 2.6% lower than its weight at day 0 ($p=0.07$, $n=5$) while the control group had significantly higher weight at 3.6% of the starting weight at day 0 ($p=0.01$, $n=5$). The PI group was significantly lower in weight compared to the control group at the end of the experiment ($p=0.028$) (Figure 6.13).

6.4.2 Glucose homeostasis and glucose tolerance test

Random non-fasted morning glucose levels were comparable between both groups at base line (day 0: PI group 8.5 ± 1.1 mmol/l vs. 7.2 ± 0.6 mmol/l in control group) and at day 10 (PI group 8.4 ± 1.1 mmol/l vs. 9.6 ± 2.1 mmol/l in control group).

On day 17 all animals underwent an IPGTT with 1.5 g glucose /kg animal weight. Animal blood glucose was checked at 0, 30 and 60 minutes in each group to assess functionality of graft in response to glucose challenge. Basal blood glucose at 0 minutes was comparable in both groups (PI group 7.8 ± 1 , control 7.5 ± 1.4 mmol/l). In the control group there was an expected rise in blood glucose to 19.3 ± 3.9 mmol/l at 30 minutes. There was a significantly lower increase in blood glucose in the PI group to only 11.5 ± 2.2 mmol/l at 30 minutes ($p = 0.004$). At 60 minutes glucose levels in both

groups started to decrease but those in the PI group remained lower than that of control group (Figure 6.14).

6.4.3 Human insulin levels

Blood collection was limited to 15% of animal weight. This limited hormone analysis to a single target assay. Samples collected were assayed for human insulin using ultra-sensitive human insulin ELISA as outlined before (2.23.2.5). Blood samples were collected for baseline at day 0 before transplantation. Another blood sample was collected on day 10 for random glucose and insulin level. On Day 17 animals were fasted overnight to enable measurement of blood glucose and human insulin levels in the fasted state and after an intra-peritoneal glucose challenge. Following neck dislocation, the graft-bearing kidney was removed and homogenised for assessment of stored human insulin levels. Insulin level in medium of untransplanted PIs at 24 hour post culture was also included for comparison in the insulin analysis. All collected samples were analysed using ultra-sensitive human insulin ELISA kit with a limit of detection of 420 fmol/l (Table 2.9).

There was no detectable human insulin in all samples from the control animal group throughout the experiment. In the PI group there was no detectable human insulin at baseline (day 0), however at day 10 there was 727 ± 125 fmol/l human insulin (morning non-fasted, blood glucose was 8.4 ± 1.1 mmol/l). On Day 17 there were no detectable human insulin levels either in the fasted state or at 60 minutes post glucose challenge. Analysis of graft-bearing kidney lysates showed average of 402 ± 49 fmol/l human insulin. For comparison, 24 hours insulin secretion *in vitro* from a matching group of PIs that was not transplanted was 629 ± 111 fmol/l (Figure 6.15).

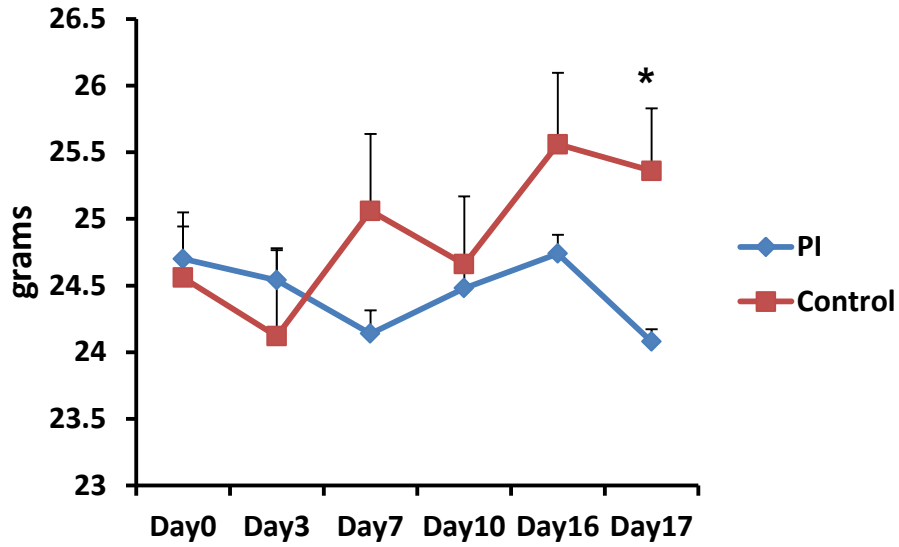


Figure 6.13 Weight assessment in SCID mice. Animal were transplanted on day 0 and experiment was terminated on day 17. Results represent mean weight in each group. N=5, \pm SEM, * = $p < 0.05$.

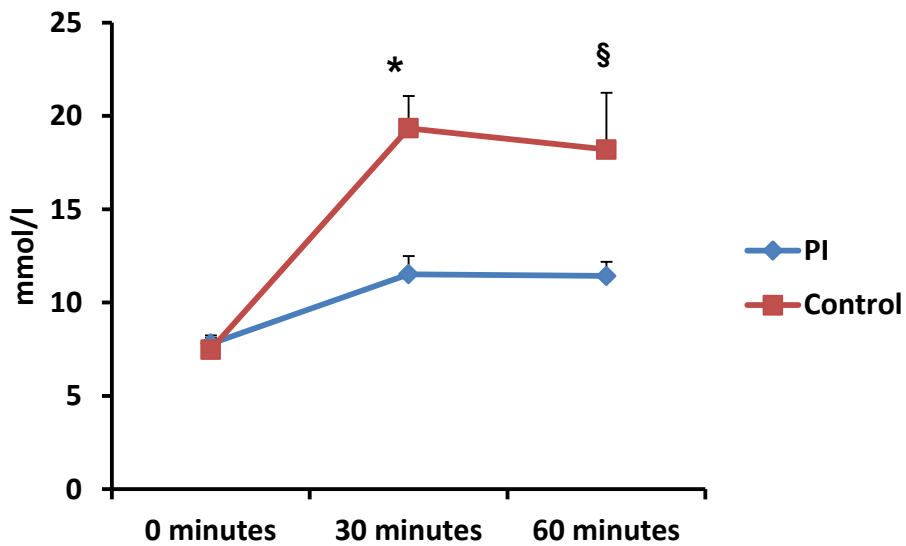


Figure 6.14 Intra-peritoneal glucose tolerance test. This was carried out on day 17. Results represent mean blood glucose at 0, 30 and 60 minutes in each group. n=5, mean \pm SEM, * = $p < 0.004$, § = 0.06

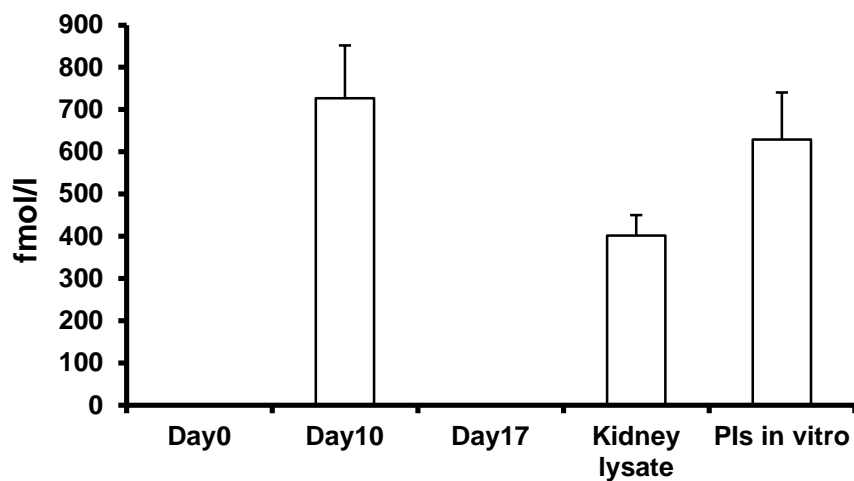


Figure 6.15 Human insulin production in SCID mice. Mouse plasma levels of human insulin at days 0, 10 and 17 post transplantation with PIs in addition to human insulin in graft-bearing kidney lysate and *in vitro* 24 hour insulin secretion from PIs. Results represent mean human insulin levels. n=5 except for PIs n=3, mean±SEM

6.5 Discussion

Islet survivor cells provide a readily available and expandable source of potential pancreas progenitor cells. Successful differentiation of these cells to mature endocrine phenotypes including β -cells has the potential to offer a plentiful source of cells for diabetes cellular therapy. Numerous publications have reported different methods and protocols for differentiation / re-differentiation / recovery of functional endocrine phenotype from pancreatic progenitor cells with varying success and lack of reproducibility (Bonner-Weir et al., 2000; Gallo et al., 2007; Gao et al., 2003; Kayali et al., 2007; Lechner et al., 2005; Ouziel-Yahalom et al., 2006). In this chapter enhanced insulin secretion has been investigated utilising formation of 3D pseudo-islets (PIs) from pancreatic islet survivor cells (ISCs) with deprivation from FCS and ITS supplement. Formation of PIs was successfully achieved using both the hanging drop method and MW. There was a trend towards functional enhancement of ISCs indicated by higher uptake of NPG and increased secretion and storage of insulin compared to adherent ISCs. Expression of stem cell markers Oct4 and Nanog was down-regulated with the formation of PIs in addition to reduced overall cellular proliferation compared to adherent ISCs. This indicates a shift from the active proliferative and progenitor state towards differentiation and maturity. However, generation of PIs through the hanging drop method was labour-intensive and not practical for large scale production. The collaboration that was established during these studies with University of Toronto enabled utilisation of a novel microwell (MW) system for generation of PIs in significantly higher quantities. The system was initially developed for generation of uniform embryoid bodies from human embryonic stem cells (Ungrin et al., 2008) and a number of prototype inserts were kindly provided by Dr Ungrin. Validation of the method and optimisation of an efficient PI generation protocol was attained. One

disadvantage of the system was the use of Pluronic for coating the PDMS material of MWs to prevent adherence. In a direct comparison between standard adherent culture of ISCs and culture in Pluronic coated wells, a negative effect of Pluronic was evident. Nevertheless ISCs grown in MWs showed higher insulin and C-peptide levels in comparison to cells grown in standard adherent culture and significantly higher levels in comparison to ISCs cultured in Pluronic coated wells though overall levels were still considerably lower than that of fresh islets. PIs formed in MWs showed significantly improved (pro)insulin biosynthesis, storage and secretion in comparison to adherent ISCs when tested at early passages. PIs generated from middle and late passages showed no meaningful detectable insulin. It is likely that the overall improvement in β -cell function seen in PIs of early passages is reflecting improved function of pre-existing β -cells in the adherent culture rather than re/differentiation of other cells. This phenomenon has indeed been seen in Min6 β -cell line with improved function and responsiveness following formation of pseudo-islets (Hauge-Evans et al., 1999). This, in fact, is an interesting view point should β -cell proliferation in adherent culture occur. It was not possible to confirm this in these studies and this very fact is a matter of controversy. While there is an agreement on ability of murine β -cell to proliferate both *in vitro* (Parnaud et al., 2008) and *in vivo* (Dor et al., 2004; Kim et al., 2001; Nir et al., 2007); this fact remains not agreed upon in human β -cells (Parnaud et al., 2008) although increasing evidence is accumulating in support of *in vitro* induced proliferation (Vasavada et al., 2006) and *in vivo* replication (Meier et al., 2008). Despite reported islet cell proliferation in culture by many groups (Beattie et al., 1997; Bonner-Weir et al., 1993; Gershengorn et al., 2004; Ouziel-Yahalom et al., 2006) there has been no reported success in recovering functional β -cell phenotype once the phenotype is lost in culture.

While initial culture of PIs for 24 hours demonstrated clear benefit over adherent culture there were a number of unresolved challenges. These included difficulty for scaling up PI production to enable any meaningful large animal or human studies, length of culture time is limited to 24 hours by the limit of medium content, need for pluronic to prevent adherence which might exert detrimental effect on cells, and potential risk of infection due to extensive manipulation. Thus, utilisation of microgravity bioreactor culture system for large scale expansion of ISCs and generation of PIs is an attractive approach with several potential benefits. These include ability for scaling up production without compromising sterility, very low risk of adherence due to constant movement precluding need for pluronic use, capacity for considerably larger medium volume potentially permitting longer culture periods, and relative ease of use. The current initial studies attempted to validate culture extension of PIs generated in MWs up to 72 hours in bioreactor HARV vessels. There was evident cellular loss, however remaining islets maintained comparable insulin biosynthesis to that after 24 hours post establishment in MWs. This was a proof of principle and further optimisation is required to determine optimal rotational speed, seeding density, type of vessel and type of medium and supplements to be used, frequency of medium change and oxygen pressure in the environment before microgravity bioreactor can be usefully implemented in this context.

An *in vivo* study was set up to assess biological functionality of PIs. Due to logistical restraints related to unexpected delays in animal delivery and availability of suitable early passage ISCs; PIs from middle passage (P6) were employed in this experiment. The experiment was carried out in healthy non-diabetic SCID mice to enable assessment of possible production of human insulin within an *in vivo* system. Control animals were transplanted with human skin fibroblasts. The experiment showed

maintenance of animal weight in the PI group while the control group gained significantly more weight over the study period. Moreover, by the end of the study an intraperitoneal glucose tolerance test showed evidence of human-like glucose regulation with maximum blood glucose detected at 30 minutes in an IPGTT reaching a small peak of 11.5 mmol/l compared to the expected higher peak of 19.3 mmol/l in the control group despite comparable fasting blood glucose between both groups. Animals in both groups were healthy and did not undergo streptozotocin treatment. Assessment of hormone profile was challenging due to limited blood volume allowed for sampling. Baseline samples before transplantation in both groups were negative for human insulin. Interestingly, samples collected on day 10 showed detectable, but low, human insulin levels. Homogenate of PI-bearing kidneys similarly showed detectable, but low, human insulin levels. Although not conclusive these results suggest potential biological activity of PIs *in vivo*. To study this biological activity in more detail further studies employing ISCs from an earlier passage with residual β -cells and/or progenitor cells should be attempted. Moreover, employing larger animals can enable more detailed analysis of hormonal and metabolic profiles. Transplantation in animal models of diabetes will also be of particular interest to evaluate whether hyperglycaemic state promotes further differentiation of progenitor cells or phenotype recovery of de-differentiated β -cells.

Varying success was reported in achieving (re)differentiation of islet/pancreas proliferative cells into β -cells by different groups. Almost all groups emphasised the need for serum-free media at the differentiation stage in addition to formation of cell clusters; although there was no agreement on either the expansion (discussed in details in (5.5)) or the differentiation protocols. Lechner cultured proliferating islet cells on Matrigel in presence of serum-free high glucose (17.5 mmol/l) DMEM medium and

ITS supplement to induce cluster formation. Insulin expression increased in these clusters in comparison to adherent cells but levels were relatively low with no glucose responsiveness and no *in vivo* validation was attempted (Lechner et al., 2005). Gershengorn cultured islet proliferative cells in serum-free CMRL 1066 medium (glucose content 5.6 mmol/l) supplemented with ITS to induce islet-like floating clusters. These clusters showed increased insulin messenger RNA levels and confirmed C-peptide staining. However insulin messenger RNA levels remained very low compared to fresh islets. No secretion studies or *in vivo* testing were reported (Gershengorn et al., 2004). Kayali attempted to replicate the same protocol as Gershengorn in comparison to the group's own protocol (Beattie et al., 1999). Insulin messenger RNA was profoundly down-regulated in both protocols at the end of the differentiation phase and *in vivo* transplantation failed to demonstrate any human C-peptide levels (Kayali et al., 2007). On the other hand Gallo observed a recovery of insulin and other endocrine markers expression in the islet proliferative culture upon induction of differentiation by deprivation from serum and addition of ITS supplement. Despite expression and detection of insulin, it was not glucose responsive (Gallo et al., 2007). Ouzeil-Yahalom elaborated on successful recovery of full β -cell phenotype from islet proliferative culture following treatment with betacellulin, either in presence or absence of serum, with normal (compared to fresh β -cells) insulin storage and secretion and glucose responsiveness (Ouziel-Yahalom et al., 2006). Despite the apparent success reported, no *in vivo* studies were undertaken and no other group was able to replicate this to date.

Chapter 7. Concluding discussion

7.1 General discussion

7.1.1 Islet transport programme

Widespread application of islet transplantation at remote transplant centres depends on feasible protocols to ensure safe and practical islet transport from central isolation centres. The third chapter of this thesis explored this in detail, for the UK setting.

7.1.1.1 Establishment of islet assessment and validation protocols

Islet assessment protocols were addressed and validated initially in rodent islets which were readily available in contrast to the human islet supply. These islets provided invaluable experimental material in enabling refinement of the assessment protocols. This led to addition of an extra washing step in the FDA/PI staining protocol to avoid false-positive PI staining occurring secondary to prolonged exposure to acetone, the FDA diluent. Also, another washing step was introduced in the Dithizone staining protocol to enhance the imaging quality. Moreover, static glucose stimulated insulin secretion experiments proved to be problematic and inconsistent necessitating an increase in number of repeats in further experiments in human islets to reduce variability in addition to establishing perfusion studies whereby many of the problems inherent to the static experiments were avoided. Rodent islets also enabled successful establishment of an *in vivo* transplant model in SCID mice for biological testing of islets and other cellular products.

7.1.1.2 Human islet transport

Ten human pancreases were processed following standard isolation protocols to produce clinical grade islets. Protocols for transport of islets from KCIIF to Newcastle were developed building on international experience (Benhamou et al., 2001; Ichii et al., 2007b; Kempf et al., 2005; Rabkin et al., 1999). Trials of different transport bags,

shipment temperature and medium volume were carried out. This work validated successful packaging and transport conditions for the setting in the UK comprising packaging islets in blood transfusion bags employing CMRL 1066 medium containing 0.5% HSA and 2 mM HEPES with a transport box that can maintain temperature lower than 24 °C. Islet quality was assessed at both KCIIF and Newcastle. This included viability / necrosis evaluation using the FDA/PI method, sterility confirmation using gram stain and bacterial culture, integrity scoring and islet count using Dithizone staining in addition to assessment of function in response to glucose stimulation in static and perfusion settings. Moreover, safety of direct transplantation following transport without washing or further processing was evaluated with assessment of transport medium sterility and content of insulin and a panel of proinflammatory markers including IL-6, IL-1 β , INF γ and TNF α . Sterility was confirmed with consistently negative Gram staining and absence of growth on bacterial cultures. Transported medium contained higher than physiological levels of the studied proinflammatory markers despite transport at low temperatures. However, the overall load of these cytokines and insulin in the bag was relatively trivial in view of the small volume of medium in the bag.

These optimised protocols were formulated as Standard Operating Procedures (SOPs) and have been adopted by the United Kingdom Islet Transplantation Consortium (UKITC) to unify the procedures across all centres enabling NHS funding of an integrated cost-effective and hub-and-spoke model ensuring geographical equity of access. A full list of these SOPs is included in Appendix 1.

7.1.1.3 Limitations

While successful islet transplantation was achieved following transplantation of islets immediately after isolation and following transportation there still remains

considerable immediate and on-going graft loss in recipients. Several factors contribute to this attrition in graft function including islet quality. The available tools for islet quality assessment might not be sufficiently robust and accurate in interrogating quality of islet preparations and still have poor correlation with transplantation outcome (Bertuzzi and Ricordi, 2007). Moreover, despite detection of relatively low proinflammatory cytokine levels in the transport/transplant medium it is expected that islets will secrete higher levels when transplanted as they become metabolically active with rewarming to 37 °C in the recipient. It has not been confirmed whether this contributes directly or indirectly to the inflammatory responses responsible for loss of a significant part of the graft soon after transplantation.

7.1.2 Maintenance / augmentation of islet mass and function

Despite numerous potential benefits of post-isolation islet culture; it is still thought that considerable transplantable mass may be lost (Kin et al., 2008). It is not known if islet number is reduced due to detrimental effects of culture conditions or whether lost islets were already damaged and were going to necrose / apoptose in any case after transplantation. Several groups have attempted to enhance islet mass and function through culture condition manipulation and treatment with different growth factors (Beattie et al., 2002; Brelje et al., 1993; Farilla et al., 2002; Hogg *et al.*, 1993; Liu et al., 2004; Vasavada et al., 2000). Experiments on treating human islets with a panel of pregnancy-related hormones and other growth factors were reported in Chapter 4. Islets were treated with factors either over 72 hours or acutely. Assessment of β -cell function was carried out for both hormone and gene expression. In the initial screening studies with 72 hour treatment there was evidence of enhanced insulin levels and β -cell function with the pregnancy related hormones. This was further confirmed in definitive studies with higher number of biological replicates treated with PRL resulted in a

significant increase in insulin but not PDX1 gene expression compared to vehicle. No consistent effect was observed on islet proliferation or islet stress markers. Acute treatment with PRL using higher hormone levels (200 nM) showed a potentiating effect on glucose stimulated insulin secretion demonstrated in perfusion studies.

The differences observed between these studies and published reports may, at least in part, be a result of the following: use of ultra-pure recombinant proteins in these studies as opposed to hormone extracts (Sorenson and Brelje, 1997); many studies were carried out on rodent animal models with potential species-specific response (Garcia-Ocana et al., 2000; Petrik et al., 1999); some reports achieved results through marker expression induction instead of protein treatment (Vasavada et al., 2000). Moreover, there is no good evidence of enhanced mass with any treatment in intact human islets to date.

Since the above treatments demonstrated no positive effect on enhancing mass and function, the next logical step would be to test whether treatment with these markers has any protective effect on islet mass and/or function. This may be tested in islets cultured in stressful / toxic conditions that may simulate the transplant environment including islets cultured in the presence of immunosuppressants (Ishizuka et al., 1993) used for both induction and maintenance immune-suppression post transplantation, islets cultured in presence of proinflammatory cytokines (John et al., 2000) or islets exposed to hypoxic conditions and other toxic metabolites (El-Assaad et al., 2003; Pipeleers and Van De Winkel, 1986). Such studies are currently being conducted in our lab in definitive studies involving treatment with pregnancy related hormones to evaluate any potential protective role of these hormones on cultured islets.

7.1.3 Islet survivor cell studies

Existence of true stem cells in the pancreas has been debated previously. Many groups have reported the potential for establishment of pancreatic cells in proliferative culture with varying outcomes with respect to differentiation towards mature β -cells. In the current studies consistent proliferative cultures were successfully established from human islet preparations and were fully characterised in experiments reported in Chapter 5. The proliferative culture was heterogeneous with expression of differentiated markers from a range of pancreatic phenotypes including islet hormones (insulin, glucagon, somatostatin, pancreatic polypeptide), ductal marker (CK19), acinar marker (amylase) and endothelial marker (endoglin) in addition to expression of developmental and mesenchymal markers (NGN3, nestin, vimentin). These studies showed for the first time expression of the stem cell marker Oct4 at both mRNA and protein levels (White et al., 2011) indicating the potential plasticity of these cultures. This heterogeneous population was shown to gradually lose expression of islet differentiated markers as the culture progresses *in vitro*. Insulin storage capacity of ISCs was very limited compared to cultured human islets. This actively proliferating heterogeneous culture provided a model for studying pancreatic progenitor cells in a human *in vitro* system. The potential for pseudo-islet generation to induce / enhance differentiation of ISCs was explored in Chapter 6. Three dimensional cellular aggregates were induced when ISCs were cultured in hanging-drops or in novel micro-wells (MWs) in serum-free medium. Pseudo-islets (PIs) showed a trend towards enhanced insulin biosynthesis, enhanced processing and enhanced storage in comparison to adherent ISCs. Nevertheless, insulin / C-peptide levels remained significantly lower than those of fresh islets and storage capacity remained impaired. A pilot *in vivo* study carried out on PIs generated from relatively late passage ISCs (P6)

transplanted in healthy SCID mice with no diabetes induction demonstrated possible *in vivo* activity with low but detectable human insulin in mice sera at 10 days post transplantation and in graft-bearing kidneys of PI transplanted animals. Moreover, PI transplanted animals showed significantly less weight gain compared to control and glucose levels following IPGTT were significantly lower. These encouraging, but not conclusive results merit further *in vivo* studies employing PIs generated from earlier passage ISCs with confirmed insulin expression and to include diabetic SCID mouse model.

7.2 Strengths and weaknesses of approaches adopted

Transportation of clinical grade human islets for transplantation at remote centres is an exciting concept. Despite the strong academic portfolio of β -cell and islet research in Newcastle; all research prior to this project was conducted in rodent models. In this project a human specific approach was followed. This necessitated establishment of protocols, techniques, knowledge and experience; benefiting from established experience within the group as well as initiating new and exciting collaborations with key groups in the field including islet groups in King's College, Diabetes Research institute in Miami, Alberta Diabetes Research Institute, and University of Toronto. The work followed an incremental adoption of a validated approach for clinical implementation including a number of novel features and culminated in validated protocols that led to successful clinical transplantation of transported islets at Newcastle as part of government-funded islet transplantation program. Moreover, these protocols provide a platform for further future refinement and development.

Trials for augmentation of function of intact islets employing a number of growth factors including pregnancy related hormones were carried out in a screening approach

experimental design. This showed no improvement of islet mass or function at standard optimal culture conditions. This approach was challenging, however assays established will enable *in vitro* evaluation of the potential effects of different agents on attenuating toxicity and proinflammatory signalling in models of transplantation as well as Type 1 and Type 2 diabetes. Indeed these skills and techniques enabled me to robustly demonstrate the effect of various factors on islet function during my PhD studies (Arden et al., 2008; Campbell et al., 2008).

The human specific approach, unlike other experimental animal models, proved to be challenging with limitation on tissue availability and unavoidable variability between different preparations. However, this approach was of particular importance as results obtained from non-human tissue might not necessarily be true on human tissue. Moreover, the current experiments have yielded methods underpinning informed future human studies.

Robust, reproducible and scalable protocols for generation of ISCs have been established. Characterisation of ISCs demonstrated actively proliferating culture with accelerated loss of β -cell phenotype but with confirmed expression of pluripotency markers, a novel and exciting finding (White et al., 2011). Differentiation studies adopted a minimally interventional approach through generation of 3 dimensional pseudo-islets employing hanging drop and novel micro-wells. Interestingly this led to enhanced islet-like phenotype *in vitro* and evidence of biological activity in a pilot *in vivo* study. Nevertheless the results were not conclusive and future studies are required to further elucidate *in vivo* activity.

A SCID mouse model for *in vivo* assessment of islet function was successfully set up and validated. Sub-renal capsule delivery was significantly facilitated by a micro-manipulator device engineered and kindly provided by Professor GC Korbitt from the

Alberta Diabetes Research Institute in Edmonton. SCID mouse transplantation is an important bioassay that has been setup and can be employed in testing other potential future cellular products.

7.3 Suggested future work

Although transport protocols established in this project led to successful clinical transplantations and enabled life changing treatments in several patients; they still represent a platform for further refinement and development. There is a clear need for better islet quality assessment tools to enable more accurate prediction of post-transplant function, thus avoiding transplantation of unfavourable preparations that may otherwise look to be of transplantable grade. Indeed, detailed studies are currently underway in our laboratory as part of a multi-centre collaborative project underpinned by protocols established in this project to devise a novel islet transport and assessment system. This involves a battery of robust and quality-assured assessment tools to estimate islet quality and predict outcome post transplantation.

Moreover, a new collaboration with Professor KK Papas in Arizona was established to explore possible implementation of several innovative technologies including oxygen persufflation of the pancreas during transport between procurement site and isolation facilities and an oxygen-permeable silicon rubber membrane device for islet transport / culture.

Plasticity / progenitor state and heterogeneity of ISCs was of interest and further lineage-tracing studies to investigate the fate of β -cells and origin of new ones as well as progenitor cells will be of particular importance in both standard adherent culture and in PI generation studies.

Further *in vivo* studies will be required for functional assessment of human islets following manipulation in culture as well as for studying biological effects of differentiated PIs. Moreover, moving to portal vein transplantation in rat models would provide a powerful model that simulates current clinical practice to enable study of engraftment and the inflammatory / thrombotic processes currently limiting clinical transplantation success.

Finally, established islet transplant protocols represent a conduit for further refinements and deeper understanding of the causes and possible prevention of beta-cell loss in Type 1 diabetes.

Appendices

Appendix 1.1

ITPO001: Sequence of action

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University of Newcastle upon Tyne: Diabetes Research Group/Haematology
Stem Cell Laboratory RVI

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DIABETES RESEARCH GROUP LABORATORY

STEM CELL LABORATORY

STANDARD OPERATING PROCEDURE

ITPO 001

SEQUENCE OF ACTION FOR ISLET ASSESSMENT AND RELEASE

Laboratory Procedure

Final Approve

Operative Date:

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1.0 PURPOSE OF PROCEDURE

To ensure smooth and systematic work starting with islet reception at Newcastle RVI hospital until islet issuing for clinical transplantation

2.0 PRINCIPLE OF PROCEDURE

Step by step action algorithm of work flow

3.0 PERSONNEL

All trained personnel working in the cGMP Facility

4.0 EQUIPMENT/MATERIALS

N/A

5.0 ATTACHMENTS

LSISOP001 Attachment I: Data collection sheet

6.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

1. Communication between Newcastle islet team, on call Islet Isolation facility and Newcastle transplantant coordinator is important. All relevant information needs to be communicated in time
2. Islet assessment at Newcastle is required before issuing islets for transplantation

7.0 PROCEDURE

A. Sequence of events and the corresponding SOP:

1. Islet packaged at the isolation facility and sent to Newcastle: ITSOP001
2. Islets are received at Newcastle and sample collection for sterility, viability and islet equivalent count estimation: ITSOP002
3. Assessment of viability at Newcastle: ITSOP003
4. Assessment of islet equivalent and integrity at Newcastle: ITSOP004 (can be delayed until post transplant)
5. Assessment of transport temperature: ITSOP005
6. Islet transfer to the Freeman Hospital, Interventional Radiology Theatre: ITSOP006

Laboratory Procedure

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Approved by: _____ Date: _____
Ali Aldibbiat, MD, MPhil
Research Associate
Diabetes Research Group

Approved by: _____ Date: _____
Janice Dunn,
Stem Cell Laboratory

Approved by: _____ Date: _____
James Shaw, PhD.
Consultant and Honorary Physician
Diabetes Research Group

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ITSOP001: Islet packaging

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DIABETES RESEARCH GROUP LABORATORY

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STANDARD OPERATING PROCEDURE

ITSOP001

ISLET PACKAGING AT ISOLATION CENTRE

Laboratory Procedure

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1.0 PURPOSE OF PROCEDURE

Package islets for transportation to Newcastle for clinical transplantation

2.0 PRINCIPLE OF PROCEDURE

Ensure optimal packing condition for islets can reduce islet stress during transport journey.

3.0 PERSONNEL

All trained personnel

4.0 EQUIPMENTS/MATERIALS

This procedure is carried out in a class II cabinet in the clean room under same strict cleaning procedures.

1. Transport bags (Baxter, cat. no. R8003)
2. Transport medium (CMRL1066 NCL1, PAA laboratories)
3. 20% Human serum albumin (HSA) (Bio Products laboratories, ZENLAB20)
4. 50ml syringe
5. Heat sealer
6. 8-12 cool-packs kept at 4 °C
7. Transport cool box with shelving unit inside
8. Hobo temperature logger
9. Sterile scissors

5.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

1. Bag may leak if not sealed securely
2. Temperature need to be maintained below 22 °C to reduce biological activity of islets, thus depletion of nutrients and above 5 °C to prevent cold damage

Laboratory Procedure

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3. PROCEDURE

A. Medium preparation

1. Open a new 500ml bottle of CMRL NCL1 medium in the Class II cabinet
2. Add 50ml of 20% HSA to make final CMRL supplemented with 2% HSA

B. Protocol

1. If islet yield is below 200,000 IEQs then resuspend in 150 ml of HSA supplemented CMRL medium
2. If islet yield is more than 200,000 IEQs then resuspend in 200 ml of HSA supplemented CMRL medium
3. If islet yield is more than 400,000 IEQs then divide the islets evenly into two transport bags using medium volume as advised in B 1 and B 2
4. Cut the tubing port of the transport bag using the sterile scissors
5. Remove the syringe plunger altogether
6. Connect the syringe to the bag tube
7. Dispense islet suspension into the syringe from the open end while holding it vertically and await suspension infusion into the bag by gravity
8. Repeat until all islet suspension is in the bag
9. Remove the Syringe and clamp the tube by the clamp provide with the bag to prevent leakage
10. Seal the bag with the heat sealer twice
11. Label the bag with preparation number, date and time, IEQ count, and site specific information
12. Repeat B4-10 using HSA supplemented CMRL medium and label as wash bag
13. Prepare one wash bag per islet bag
14. Place horizontally inside the middle shelf in the shelving unit
15. Place the Hobo logger inside the same shelf
16. Place the cool packs around the shelving unit
17. Place all paper work including islet release certificate inside a plastic envelope and place in the upper shelf in the shelving unit
18. Close the cool box and secure it with a plastic securing band
19. Hand the box to the courier
20. Inform the receiving centre of the details

Laboratory Procedure

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Approved by: _____ Date: _____
Ali Aldibbiat, MD, MPhil
Research Associate
Diabetes Research Group

Approved by: _____ Date: _____
Janice Dunn,
Stem Cell Laboratory

Approved by: _____ Date: _____
James Shaw, PhD.
Consultant and Honorary Physician
Diabetes Research Group

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ITSOP002: Receiving transported islets in Newcastle

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DIABETES RESEARCH GROUP LABORATORY
STEM CELL LABORATORY
STANDARD OPERATING PROCEDURE
ITSOP002
RECEIVING TRANSPORTED ISLETS IN NEWCASTLE

Laboratory Procedure	Final Approve	Operative Date:
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1.0 PURPOSE OF PROCEDURE

Receiving islets isolated at the on-call central islet isolation facility following transport to Newcastle

2.0 PRINCIPLE OF PROCEDURE

To outline the receiving procedure and assessment of the transportation bags for any possible damage incurred during the transportation

3.0 PERSONNEL

All trained personnel working in the GMP Facility

4.0 EQUIPMENT/MATERIALS

All these items are located in the preparation room unless otherwise specified:

1. Appropriate personal protective equipment (storage and liquid nitrogen room) and 70% ethanol spray
2. Plastic tray
3. Tube rack
4. Plastic envelope transparent for documents
5. Clean transparent bag with self sealing strip
6. Bacterial culture bottle (number required is equivalent to number of islet bags and wash medium bags) and specimen carrier bag
7. 30ml Universal tube (number required is equivalent to number of islet bags and wash medium bags +2)
8. 10ml Syringe and 20G needle
9. 2 way luer with stoppers
10. Permanent-ink marking pen

5.0 ATTACHMENTS

N/A

6.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

1. This procedure involves sampling the islets for gram stain, bacterial culture and islet quality assessment (viability, islet equivalent count and possibly islet function)
2. If islets fulfil transplantation criteria then they would be sent to the surgical theatre for clinical transplantation in diabetic patients
3. Expected time for this procedure is 30-50 minutes
4. Observations to be documented on Data Collection Sheet of ITSOP001 Attachment I
5. Protective clothing and gloves should be worn before entering the clean room according to the appropriate procedures (SCL/O/056)

Laboratory Procedure

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18. Remove the syringe and close the luer inlet with a stopper
19. Close syringe outlet with a stopper and place inside an open 30ml universal tube with inlet down to allow islets to settle down for 5 minutes
20. Dispense 1ml into a Universal tube. All islets removed from the islet bag should now be in the universal tube. Label the tube as **Islet tube** with UKT number and time and date
21. Dispense 1ml into a Universal tube and label as **For Gram stain** with UKT number and time and date
22. Dispense 2ml into the bacterial culture tube and label: UKT number and time and date
23. Inspect islet bag for any leaking while confirming the luer tab is closed and the luer outer opening is blocked with a stopper, spray with 70% sterile ethanol and place inside a clean transparent bag with self sealing strip and seal the plastic bag opening
24. Remove everything from the cabinet and place on the grey tray
25. Document time and date in the data collection sheet
26. Clean the cabinet and dispose of waste following the appropriate procedures (SCL/O/052 and SCL/O/054)
27. Place the tray in the hatch and close the handle properly
28. Clean benches that have been used following appropriate procedures (SCL/O/054)
29. Exit the clean room
30. Place islet bag and medium back in the tray unit in the cool box
31. Place bacterial culture and gram stain bottles in an appropriate microbiology bag and label the carrier bag following ITSOP002 Attachment I Filling microbiology specimen carrier sheet
32. Return all items used (tray, rack,etc) to their original locations
33. Follow on to islet viability assessment ITSOP003-4
34. Log Hobo data logger to the designated PC to confirm temperature control during the transport (should not be above 22^oC) using ITSOP005, if the logger show the temperature at any stage of transportation to be outside the above range follow instruction is D

C. Measures in the case of bag damage/suspected abnormality

1. Check and document the site of damage and/or other abnormalities
2. Try to identify possible cause of damage and/or other abnormalities
3. Take sample for bacterial gram stain assessment as in above
4. In case of spillage follow spillage SOP P/SOP/10/01 to ensure safety.
5. Inform the Diabetes Research Team leader and discuss possible cancellation. If cancellation is not decided then follow steps in section B otherwise go to C 6
6. Inform transplant coordinator and islet isolation team. Islets can be used for research if appropriate consent for research is in place

D. Measures in the case of loss of temperature control during the transportation

1. Document the time and period during which the temperature was above the acceptable range
2. Check for possible cause of the rise in temperature

Laboratory Procedure

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3. If the temperature was less than 4⁰C outside the acceptable range (4-22⁰C) for less than 30 minutes then continue with the islet assessment ITSOP003
4. If temperature was more than 4⁰C outside acceptable range (4-22⁰C) for more than 30 minutes then discuss with the Diabetes Research Team leader for possible cancellation. If cancellation is not decided then follow steps in section B otherwise go to D 5
5. Inform transplant coordinator and islet isolation team. Islets can be used for research if appropriate consent for research is in place

8.0 HEALTH AND SAFETY REQUIREMENTS

- 8.1 All used disposable equipment should be discarded according to current laboratory practice P/SOP/06/01 Department of Haematology.
- 8.2 All biological waste discarded according to P/SOP/07/01 Department of Haematology.
- 8.3 All spillages of non-biological material should be dealt with according to P/SOP/11/01 Department of Haematology.
- 8.4 All spillages of biological material should be dealt with according to P/SOP/10/01 Department of Haematology.
- 8.5 All accidents should be dealt with according to P/SOP/13/01 Department of Haematology.
- 8.6 See risk assessment data for use of Cutan and Tuffie wipes.

9.0 REFERENCES

- 9.1 "Rules and Guidance for Pharmaceutical Manufacturers and Distributors 2002"
The Stationary Office ISBN 011322559 8
- 9.2 "Cleanroom Cleaning Guidelines" 2003 PRIMARIUS Ltd

Laboratory Procedure

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ITSOP002, Attachment I: Microbiology specimen carrier filling

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DIABETES RESEARCH GROUP LABORATORY
STEM CELL LABORATORY
STANDARD OPERATING PROCEDURE
ITSOP002
Attachment I: Filling microbiology specimen carrier sheet

Laboratory Procedure	Final Approve	Operative Date:
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1.0 PURPOSE OF PROCEDURE

Filling the details of the microbiology specimen carrier bag

2.0 Procedure:

The field on the carrier bag (Figure 1) should be filled as follows:

Surname: UKT number

Forename: ICP x. ICP: stands for Islet Clinical Prep. X: stands for the serial number of number of the clinical prep at Newcastle.

Sex: sex of donor

Sate of Birth: islet isolation day

Specimen: Bag 1 islet medium. Bag 2 wash medium

Date: the date of sampling for microbiology

Tests required: Gram stain and bacterial culture

Hospital: Freeman Hospital

Ward: 6A

Consultant/GP: Dr Jim Shaw

Requesting Doctor (Block Capitals): name of person on call

Clinical Details: Sterility testing of clinical islet preparation for transplantation. Please report any positive results to Dr Jim Shaw via switchboard

The form is titled 'ROYAL VICTORIA INFIRMARY NEWCASTLE UPON TYNE MICROBIOLOGY SEROLOGY'. It includes fields for SURNAME, FORENAME(S), RECORD No., SEX, DATE OF BIRTH, LABORATORY NUMBER, HOSPITAL, WARD, CONSULTANT/GP, REQUESTING DOCTOR (BLOCK CAPITALS), CLINICAL DETAILS, and ANTIBIOTIC THERAPY. There are also biohazard symbols and instructions on how to use the carrier bag.

Figure 1. Microbiology specimen carrier bag.

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ITSOP002, attachment II: Data collection sheet

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STEM CELL LABORATORY

STANDARD OPERATING PROCEDURE

ITSOP002

Attachment II: Data Collection Sheet

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ITSOP003: Islet viability assessment

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STANDARD OPERATING PROCEDURE

ITSOP003

ISLET VIABILITY ASSESSMENT

Laboratory Procedure

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1.0 PURPOSE OF PROCEDURE

Assess transported islets viability and suitability for transplantation

2.0 PRINCIPLE OF PROCEDURE

Fluorescein diacetate (FDA) permeates living cells. Living cells hydrolyze fluorescein diacetate (not fluorescent) into fluorescein which has green fluorescence following excitation with UV light

Propidium iodide (PI) only enters cells with compromised cellular membrane and binds to the nucleic acid content resulting in red fluorescence following excitation with a UV light

3.0 PERSONNEL

All trained personnel

4.0 EQUIPMENTS/MATERIALS

Equipments and materials are located within Diabetes Research Laboratory M2019 or Diabetes tissue culture suite M2016 on the second floor Cookson Building, Newcastle University.

1. PBS (100mL) (Room M2019)
2. Fluorescein diacetate: stock solution 24 μM (9.9 mg/ml in acetone), Sigma or equivalent. Made up solution is located in the door compartment of tissue culture (room M2016) fridge
3. Propidium iodide: stock solution 750 μM (0.5 mg/ml in PBS, pH approximately 7.4), Sigma or equivalent. Made up solution is located in the door compartment of tissue culture (room M2016) fridge
4. P10, P200, P1000 pipettes and tips (Room M2019)
5. 1.5ml micro centrifuge tubes (3-5) (Room M2019)
6. 12 well tissue culture plate (1) (Tissue culture room M2016)
7. Sterile plastic Pasteur pipettes (2-4) (Tissue culture room M2016)
8. Inverted fluorescent microscope (Diabetes microscope room at M2016)
9. Microbiology Safety Cabinet (class 2) (Tissue culture room M2016)
10. Tabletop microcentrifuge (Sprout, Diabetes Microscope room, M2016)

5.0 ATTACHMENTS

Islet viability scoring sheet ITSOP003 Attachment I

6.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

1. Passing criteria is a viability score of 70% or more
2. If islets fail this test then they will not be sent for transplantation
3. Expected time for this procedure is 20 minutes
4. Procedure to be carried out in the Microscope room M2016

Laboratory Procedure

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PROCEDURE

A. Protocol

1. Islets are collected from the transfusion bag in a class II safety cabinet within the clean room of the GMP facility in the stem cell labs (ITSOP 002)
2. Sampled islets are taken to the microscope room (M2016) where viability staining is performed
3. Mix islets in the Universal tube labelled **Islets** manually and collect 100µl using a pipette, dispense in a 1.5 tube
4. Add 880µl PBS to make 980µl
5. Add 10µl working strength PI and 10µl working strength FDA
6. Incubate at room temperature for 3 minutes keeping the tube vertical
7. If tissue has not settled in the bottom of the tube then centrifuge the tube using a tabletop microcentrifuge for 30 seconds at maximum speed 6000rpm
8. Carefully remove 950µl of the supernatant making sure cells are not removed
9. Add 950µl PBS, mix and then transfer to a well in the 12 well plate and place plate on the stage of the inverted microscope
10. Count 50 consecutive cell clusters noting percent viability on the viability scoring sheet (ITSOP003 attachment I) using the fluorescence mode with the red and green filters
11. Criteria for transplantation is a score of 70% or more

Laboratory Procedure

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ITSOP003, Attachment I: Viability scoring chart

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DIABETES RESEARCH GROUP LABORATORY

STEM CELL LABORATORY

STANDARD OPERATING PROCEDURE

ITSOP003

ISLET VIABILITY ASSESSMENT Attachment I

Laboratory Procedure

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Operative Date:

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Review Date:

Viability scoring sheet:

UKT number:, Date: DD/MM/YY, Time: Hour Minute

Cell cluster	Viability score %	Cell cluster	Viability score %
1		26	
2		27	
3		28	
4		29	
5		30	
6		31	
7		32	
8		33	
9		34	
10		35	
11		36	
12		37	
13		38	
14		39	
15		40	
16		41	
17		42	
18		43	
19		44	
20		45	
21		46	
22		47	
23		48	
24		49	
25		50	
Mean			
Comments			

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ITSOP004 Islet equivalent count and integrity estimation

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DIABETES RESEARCH GROUP LABORATORY
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STANDARD OPERATING PROCEDURE
ITSOP004
ISLET EQUIVALENTS COUNT AND INTEGRITY ESTIMATION

Laboratory Procedure

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A. Procedure

1. Islets are in 900µl medium in the Universal tube
2. Mix gently but thoroughly, remove 500µl and dispense them in a 2mL tube
3. Add 1ml working strength Dithizone to the 2mL tube and leave to stain for 3-5 minutes, islets stain in red
4. Spin briefly with tabletop microcentrifuge for 30 seconds at maximum speed 6000rpm
5. Discard 1.2 ml and add 0.5ml PBS to the stained islets (total volume in tube is now 0.8ml) and transfer to a Petri dish and dispense in approximately 8 separate drops
6. Add extra 0.2ml of PBS to the tube to recover any remaining islets in the tube and transfer to the Petri dish like in 5 and dispense in 2 separate drops
7. Leave for 1 minute to enable islets to settle down to the bottom of the dish
8. Count all red-stained islets in each drop under the bright-field on the inverted microscope estimating islet size using the graticule on the eyepiece
9. Document readings on ITSOP004 Attachment I
10. Calculate the total number of IEQs in the preparation (to calculate the IEQs, multiply by the equivalence factor at the base of the column. Add the totals in each column to give the Total IEQs in the sample).

B. Calculations

1. Total IEQs in the 500µl sample reflects total IEQs per 2 ml of transported islets (500µl is 1/2 of 1ml and the 1ml sample has the islets of 4ml transport mix)
2. Divide total IEQs calculated in **A** by 2 to get IEQs in 1 ml and then multiply by total transported islet volume to get the total IEQs in the bag

C. Integrity scoring

1. Carry out integrity scoring while estimating IEQs
2. Score 50 different and unselected islets according to islet border regularity/continuity. An islet with regular and continuous boarder is scored as 100 % and an islet with significantly compromised boarder is scored 0 %, an islet with minimal to moderate disruption to its boarder is scored 50 %.
3. Calculate mean score for the 50 islets, this reflects the integrity score of the preparation

8.0 References

1. KING'S CELL ISOLATION UNIT, SOP: P005, Count islets and calculate IEQs

Laboratory Procedure

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ITSOP004, Attachment I: Working strength Dithizone preparation

The Newcastle upon Tyne Hospitals NHS Trust
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DIABETES RESEARCH GROUP LABORATORY
STEM CELL LABORATORY
STANDARD OPERATING PROCEDURE
ITSOP004
Attachment I: Working strength Dithizone preparation

Laboratory Procedure	Final Approve	Operative Date:
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PURPOSE OF PROCEDURE

Preparation of working strength of Dithizone to stain islets and determine islet equivalents

2.0 PRINCIPLE OF PROCEDURE

Dithizone is a zinc indicator. It can permeate living cells and stain zinc rich cells red. β -cells are rich in zinc which is required for the formulation of osmotically stable insulin hexamers in the secretory granules. Following incubation with Dithizone, islets stain red and non-islet tissue remains unstained. This enables simple and efficient calculation of islet size and integrity

3.0 PERSONNEL

All trained personnel working in the GMP Facility

4.0 EQUIPMENT/MATERIALS

1. Diphenyl Thiocarbazine (Dithizone, Sigma, Cat No.: D5130).
2. HBSS 200 ml
3. Dimethyl sulfoxide (DMSO, Sigma Cat. No.: D8779).
4. P1000 pipette and tips
5. 15ml conical tube (number required 2)
6. 250ml bottle (number required 2)
7. Magnetic stirrer
8. Fine scale
9. 0.45 μ m syringe filtration unit

5.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

1. Dithizone is toxic to humans. Avoid direct contact with the chemical
2. Dithizone stains surfaces and cloths with red. Careful handling is required
3. Dithizone is not water soluble and requires DMSO to dissolve it
4. Filtration enables removal of crystallised Dithizone in the final solution
5. Working strength solution is not stable for more than 24 hours
6. Expected time for this procedure is 20 minutes

6.0 PROCEDURE

1. Weigh 10mg of dithizone powder into 15ml falcon tube.
2. Add 4ml of DMSO to the dithizone tube and pipette the solution up and down to dissolve it.
3. Add the solution drop-wise into 200ml HBSS without HEPES while setting the stirring at maximum speed.
4. Continue stirring for around 10 min
5. Filter the solution using the 0.45 μ m filter
6. Dispense filtered Dithizone into a new tube and label appropriately with time and date

Laboratory Procedure

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7.0 References:

1. KING'S CELL ISOLATION UNIT, SOP: P010, Dithizone solution

Laboratory Procedure	Final Approve	Operative Date:
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ITSOP004, attachment II: Islet equivalent calculation chart

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DIABETES RESEARCH GROUP LABORATORY

STEM CELL LABORATORY

STANDARD OPERATING PROCEDURE

ITSOP004

Attachment II: Islet equivalents calculation chart

Laboratory Procedure	Final Approve	Operative Date:
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2.0 REFERENCES

- 9.1 KING'S CELL ISOLATION UNIT, SOP: P005, Count islets and calculate IEQ

Laboratory Procedure	Final Approve	Operative Date:
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ITSOP004, Attachment III: Islet integrity scoring chart

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DIABETES RESEARCH GROUP LABORATORY

STEM CELL LABORATORY

STANDARD OPERATING PROCEDURE

ITSOP004

Attachment III: Islet Integrity chart

Laboratory Procedure

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Operative Date:

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Review Date:

Integrity sheet:

UKT number:, Date: DD/MM/YY, Time: Hour Minute

Islet	Integrity score %	Islet	Integrity score %
1		26	
2		27	
3		28	
4		29	
5		30	
6		31	
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18		43	
19		44	
20		45	
21		46	
22		47	
23		48	
24		49	
25		50	
Mean			
Comments			

Laboratory Procedure

Final Approve

Operative Date:

Location of copies

Review Date:

ITSOP005 Downloading temperature from Hannah datalogger

The Newcastle upon Tyne Hospitals NHS Trust
University of Newcastle upon Tyne: Diabetes Research Group/Haematology
Stem Cell Laboratory RVI

Edition: 1.0
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DIABETES RESEARCH GROUP LABORATORY
STEM CELL LABORATORY
STANDARD OPERATING PROCEDURE
ITSOP005
DOWNLOADING TEMPERATURE FROM HANNA DATALOGGER

Laboratory Procedure

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1.0 PURPOSE OF PROCEDURE

Downloading temperature from Hanna data logger which accompanies the transported islets inside the coolbox

2.0 PRINCIPLE OF PROCEDURE

To outline steps for connecting the logger to the transmitting station and then use HI 141000 ver 0.9.4 software for downloading the temperature profile during transport

3.0 PERSONNEL

All trained personnel

4.0 EQUIPMENT/MATERIALS

1. Windows PC
2. HI 141000 ver 0.9.4 software installed on the PC
3. Transmitting station (HI 141001)
4. Hanna Data logger which did accompany the islets (HI 141AH)

5.0 ATTACHMENTS

N/A

6.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

1. Islets should be transported with temperature range of 4-22°C
2. If islets did go through a high temperature phase for prolonged period of time then their viability and function might become compromised and this will result in a negative impact on transplantation outcome

Laboratory Procedure

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7.0 PROCEDURE

1. Start the designated PC with the HI 141000 software on
2. Start the program HI 141000 (Figure 1)

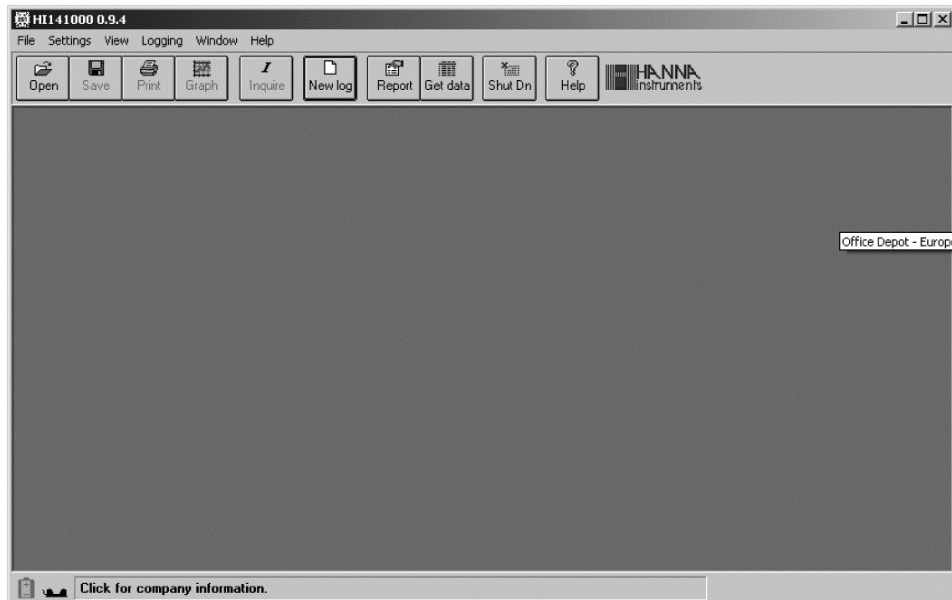


Figure 1.

3. Place a logger onto the transmitting station in the correct orientation (the arrow on the unit should point to the transmitter cable)
4. Press *Get data* and wait for the unit and transmitter to establish connection
5. The software might give the message “ the logger is currently logging” (Figure 2), do you want to continue” and for this click on yes

Laboratory Procedure

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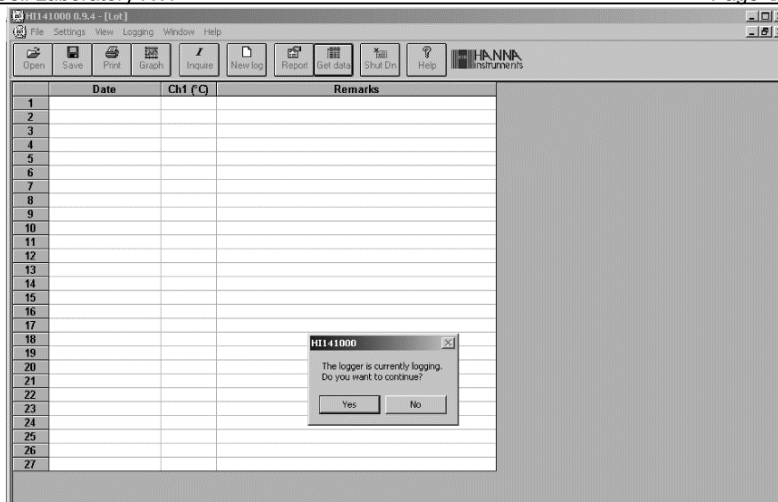


Figure 2.

6. Confirm that UKT, Isolation centre and destination centre match with the logger Lot ID
7. Click on *file* then *save lot*, use UKT number as file name
8. Click on *Graph*, the software will generate a graph of all temperatures of the transport journey
9. If temperature appears to have been outside the red lines (Acceptable temperature range of 4-22°C) for more than 30 minutes then discuss with the clinical team leader for possible cancellation
10. Click on *file* then *print graph* and attach this to the data collection sheet (ITSOP001 Attachment I)
11. Close the software
12. If temperature did fulfil temperature range requirement in addition to viability then processed with islet transfer to the operating theatre (ITSOP006)

Laboratory Procedure

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ITSOP006 Islet transfer to the Freeman Hospital

The Newcastle upon Tyne Hospitals NHS Trust
University of Newcastle upon Tyne: Diabetes Research Group/Haematology
Stem Cell Laboratory RVI

Edition: 1.0
Page 1 of 4

DIABETES RESEARCH GROUP LABORATORY
STEM CELL LABORATORY
STANDARD OPERATING PROCEDURE
ITSOP006
ISLET TRANSFER TO THE FREEMAN HOSPITAL

Laboratory Procedure

Final Approve

Operative Date:

Location of copies

Review Date:

1.0 PURPOSE OF PROCEDURE

Outline procedure for islet transfer to the operating theatre in the Freeman Hospital

2.0 PRINCIPLE OF PROCEDURE

Performing necessary communication and arranging for islet bags to be transferred to the Interventional Radiology Operating Theatre at the Freeman Hospital

3.0 PERSONNEL

All trained personnel

4.0 EQUIPMENT/MATERIALS

N/A

5.0 ATTACHMENTS

N/A

6.0 LIMITATIONS OR SPECIAL CONSIDERATIONS

N/A

7.0 PROCEDURE

1. If Islets fulfil safety requirements for transplantation then person on call should proceed with islet transfer to the Interventional Radiology Operating Theatre at the Freeman Hospital
2. Person on call should call transplant co-ordinator to confirm suitability of islets for transplantation and give estimated time of islet arrival at the operating Theatre
3. Transfer of islet box can be carried out using the private car of one of the islet team or by the hospital private taxi service
4. Hospital private taxi service can be arranged by person on call by contacting reception of Leazes wing at the RVI at extension number 25800 (From university lines dial 2125800) and ask for a taxi from the hospital taxi service for islet collection from Leazes wing. Collection should be within maximum of 30 minutes
5. Person on call should transfer cool box to the Leazes wing entrance in the RVI and wait for the collection vehicle
6. Islet box should be stored and secured in the car away from any heat sources during transfer to the Interventional Radiology Operating Theatre at the Freeman Hospital
7. Person on call should accompany the cool box to the Freeman Hospital
8. Upon arrival person on call should take islet box to the Interventional Radiology Operating Theatre
9. Islet box will be handed to the transplantation team while documenting date and time on the data collection sheet (ITSOP 001 attachment I)

Laboratory Procedure

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Appendix 2.1

Islet transport paper in Cell Medicine Part B Cell Transplantation

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Validation of Islet Transport From a Geographically Distant Isolation Center Enabling Equitable Access and National Health Service Funding of a Clinical Islet Transplant Program for England

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Islet transplantation has become established as a successful treatment for type 1 diabetes complicated by recurrent severe hypoglycemia. In the UK access has been limited to a few centrally located units. Our goal was to validate a quality-assured system for safe/effective transport of human islets in the UK and to successfully undertake the first transplants with transported islets. Pancreases were retrieved from deceased donors in the north of England and transported to King's College London using two-layer method (TLM) or University of Wisconsin solution alone. Islets were isolated and transported back to Newcastle in standard blood transfusion or gas-permeable bags with detailed evaluation pre- and posttransport. In the preclinical phase, islets were isolated from 10 pancreases with mean yield of 258,000 islet equivalents. No significant differences were seen between TLM and University of Wisconsin solution organ preservation. A significant loss of integrity was demonstrated in islets shipped in gas-permeable bags, whereas sterility, number, purity, and viability were maintained in blood transfusion bags. Maintenance of secretory granules and glucose-stimulated insulin secretion was confirmed following transport. A Standard Operating Procedure enabling final pretransplant quality control from a simple side-arm sample was validated. Moreover, levels of insulin and cytokines in transport medium were low, enabling transplant without centrifugation/resuspension at the recipient site. Six clinical transplants of transported islets were undertaken in five recipients with 100% primary graft function and resolution of severe hypoglycemia. Safe and clinically effective islet transport has been established facilitating sustainable NHS funding of a clinical islet transplant program for the UK.

Key words: Islet transplant; Islet isolation; Islet transport; Type 1 diabetes; Glucose-stimulated insulin secretion

INTRODUCTION

Successful islet transplantation for the treatment of type 1 diabetes complicated by recurrent severe hypoglycemia has been established for several years (15) with significant advances being achieved in the past decade in both islet isolation and transplant management (8). However, wide-spread adoption of and equitable access to islet transplantation has remained restricted due to a range of factors including shortage of suitable donor tissue (14) and the limited number of accredited clinical isolation units.

Islet isolation facilities are complex laboratories that

require extensively trained and highly skilled technical staff with 24-h on-call availability in addition to strict compliance with legislative standards for production of clinical grade tissue according to current Good Manufacturing Practice (cGMP) (12). Establishing programs for transporting islets isolated at a central isolation facility to a geographically distant transplant site would enable significantly more people to benefit from this treatment with considerable overall cost savings. The potential for successful islet isolation at a distant site, in the University of Minnesota facility 1,500 miles away from the transplant center, was initially demonstrated in the autotransplant setting following pancreatectomy for

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chronic pancreatitis (11). Since then, various models of islet transport for allotransplantation have been validated in centers in the US (5) and Europe (3) but not in the UK. Our goals were to develop and validate a quality-assured system for safe and effective transport of human islets in the UK; to implement and obtain legislative approval for Standard Operating Procedures underpinning this process; and to undertake the first UK transplants with transported clinical grade islets.

MATERIALS AND METHODS

Ethical Approval

National ethical and Trust Research and Development approval was obtained for all preclinical studies using deceased pancreases donated for this research with the specific consent of donor relatives.

Pancreas Retrieval and Transport

Pancreases were retrieved from deceased donors after brain death (DBD) in the north of England by the specialist multiorgan retrieval team according to standard procurement protocols for both vascularized pancreas and islet transplantation. This included cooling of the lesser sac with ice at the time of cold vascular perfusion with University of Wisconsin solution (UWS) without vascular perfusion of the portal venous system (13). The retrieved pancreas was perfused briefly with cold UWS on the back table and then placed in a 1-L polyethylene transport chamber (VWR, Leicestershire, UK) with either 600 ml of UWS (ViaSpan, DuPont Pharma Ltd, Herts, UK) alone or 300 ml UWS mixed with 300 ml freshly oxygenated perfluorodecalin (F2 Chemicals, Lancashire, UK). In the case of the latter two-layer method (TLM), a retention frame was employed to maintain the pancreas within the perfluorodecalin layer. The transport chamber was buried in ice inside an insulated transport cool-box (MGD Coolers, Staffordshire, UK) supplied with data-logger set to record temperature every 5 min (Hobo pendant data-logger, Tempcon Instrumentation Ltd, West Sussex, UK) and transported by road to the islet isolation facility at King's College Hospital NHS Foundation Trust, in London 300 miles away, by a dedicated organ courier (Lifeline Medical Transport Service, Northumberland, UK). In the clinical phase of the program, locally retrieved organs were transported in UWS to the Oxford Diabetes Endocrinology and Metabolism clinical islet isolation facility in addition to the King's College Hospital facility.

Islet Isolation

Islet isolations were carried out as previously described employing purified collagenase [Liberase HI (Roche Diagnostics Ltd, West Sussex, UK) for the preclinical program, and Collagenase NB 6 GMP Grade

(SERVA Electrophoresis GmbH, Heidelberg, Germany) for the clinical program] infusion for enzymatic digestion and mechanical dissociation in a Ricordi chamber, followed by purification on a continuous density gradient using a COBE 2991 processor (4).

Islet Packaging and Transport to Newcastle

Purified islets were resuspended in CMRL medium (PAA Laboratories Ltd, Somerset, UK) supplemented with 2% human serum albumin (ZENLAB20: Bio Products Laboratories, Herts, UK) and 2 mM HEPES (PAA Laboratories Ltd, Somerset, UK) and packaged in either 100 ml (for 300-ml capacity bags) or 400 ml (for 1000-ml capacity bags) transport medium in gas-permeable bags (Permalife, Origen Biomedical Europe, Helsingborg, Sweden) or blood transfusion bags (Baxter Healthcare Ltd, Berkshire, UK). For clinical implementation islets were packaged in 500-ml capacity bags in 200 ml medium. Twelve hours after packing was set as the latest time that dispatched islets could be transplanted clinically. Islet bags were placed horizontally in a shelving unit inside the transport cool-box containing ice packs on the base, avoiding direct contact with the cooled packs. Islets were transported by road to Newcastle using the Lifeline courier. Continuous temperature monitoring throughout the journey was carried out using the Hobo pendant data-logger. Reassessment of islets was undertaken within the Newcastle Biomanufacturing Cellular Therapies GMP suite.

Islet Quality Assessment

Gram staining and bacterial culture was carried out in the clinical microbiology laboratories at the isolation centre and in the recipient center at the Freeman Hospital, Newcastle.

Islet equivalent count (IEQ) corrected for a standard islet diameter of 150 μm and percentage purity was determined following dithizone staining (Sigma-Aldrich, Pool, UK) at a final concentration of 25 $\mu\text{g}/\text{ml}$ for 2–4 min (4). Mean percentage integrity was determined in 50 randomly selected dithizone-positive islets according to the intactness of the pseudocapsular border. Mean percentage viability was assessed in 50 randomly selected islets by fluorescein diacetate (FDA) (0.5 μM final concentration)/propidium iodide (PI) (15 μM final concentration) (Sigma-Aldrich, Poole, UK) staining and inverted fluorescence microscope imaging using a standard protocol (2), with the addition of an extra PBS washing step following 2-min incubation with the stains to minimize false-positive PI staining induced by acetone (the FDA solvent).

Static assays of glucose-stimulated insulin secretion (GSIS) were undertaken in a water bath at 37°C. Following preincubation for 1 h in DMEM containing 2

mM glucose and washing in PBS, 6 aliquots of 15 islets were incubated in DMEM containing 2 mM glucose and 6 aliquots were incubated in DMEM containing 20 mM glucose each for 2 h. Supernatant insulin concentration was determined by Dako human insulin ELISA (BioStat, Inverness, UK) with proinsulin concentration determined by human proinsulin ELISA (Mercodia, through Diagenics, Milton Keynes, UK). Intracellular insulin and proinsulin concentrations were assayed in islets lysed by sonication in PBS.

To determine glucose-stimulated insulin secretion in perfused islets, an equal number of islets (70–100) was dispensed into each of four perfusion chambers. Islets were perfused for 60 min using G&G buffer (0.18 M KCl, 1 mM MgCl₂, and 10 mM NaHCO₃) supplemented with 2 mM D-glucose. Perfusion with 2 mM D-glucose was continued for a further 20 min with sample collection from each chamber every 2 min. Islets were then perfused with G&G buffer supplemented with 18 mM D-glucose and samples were collected every 2 min for 20 min (9). Collected samples were assayed by homogeneous time resolved fluorescence (hTRF) insulin assay (Cisbio Bioassays, Bagnols-sur-Cèze, France) following the manufacturer's instructions. Fold change in insulin release in each chamber was calculated from mean insulin concentration over the 10 min before and after the change from 2 to 20 mM glucose.

Insulin and Proinflammatory Cytokine Quantification in Transport Medium

Medium samples from bags were collected upon arrival in Newcastle and were frozen at -20°C . Insulin concentration was assayed by ELISA (Dako) and proinflammatory cytokines [interferon- γ (INF- γ), interleukin-1 (IL-1), IL-6, tumor necrosis factor- α (TNF- α)] were quantified by multiplex assay (Meso-Scale Discovery, Gaithersburg, MD, USA).

Clinical Islet Transplant Criteria and Protocols

Inclusion criteria were C-peptide-negative type 1 diabetes complicated by recurrent severe hypoglycemia despite optimized conventional management, including a trial of continuous subcutaneous insulin infusion pump therapy or suboptimal glycemic control in the presence of a functioning renal graft. Contraindications included insulin resistance [body mass index (BMI) >28 or total daily insulin requirement >0.7 U/kg] and isotopic glomerular filtration rate <60 ml/min/1.73 m²/macroalbuminuria (albumin excretion rate >300 mg/24 h)—unless previous renal graft. Induction was with either daclizumab or alemtuzumab (single 15 mg subcutaneous dose administered immediately after completion of islet infusion preceded by 100 mg hydrocortisone and 10 mg chlorpheniramine intravenously and followed by 1 g

paracetamol orally every 6 h for 48 h) with maintenance immunosuppression comprising mycophenolate mofetil and tacrolimus (0.1 mg/kg) (target trough range 8–10 $\mu\text{g/L}$). Islets were infused under local anesthesia into the hepatic portal vein following ultrasound-guided percutaneous transhepatic cannulation.

Statistical Analysis

Data are reported as mean \pm SD with static and perfusion GSIS reported as a stimulation index (insulin secretion rate at high glucose/insulin secretion rate at low glucose). Differences were compared by paired/unpaired Student's *t*-test and values of $p < 0.05$ considered statistically significant.

RESULTS

Pancreas Preservation and Islet Isolation Outcome

Ten pancreases were evaluated in the preclinical phase. All fulfilled the inclusion criteria, in place at the time, for acceptance of an organ into the clinical islet transplant program but with no suitable recipient on the active transplant waiting list. These included donors aged 20–65 years with no history of diabetes, recurrent pancreatitis, chronic renal disease, malignancy, Intensive Therapy Unit stay >10 days, alcohol or drug abuse. Serology was negative for hepatitis B, hepatitis C, *Treponema*, and HIV with no evidence of nodularity, fibrosis, or severe fatty infiltration on macroscopic examination of the pancreas.

Six donors (60%) were male. Cause of death was intracranial hemorrhage (including subarachnoid hemorrhage) in nine (90%) and middle cerebral artery infarct in one (10%). Macroscopic appearance was normal in six (60%) with moderate fatty infiltration in four (40%).

Six organs (60%) were transported to the King's College Hospital isolation facility using TLM and four (40%) in UWS alone. Mean donor age was 48 years (range 30–54) and mean BMI 27 kg/m² (Table 1). Mean cold ischemic time (defined as cross-clamp until pancreas perfusion) with collagenase was 8.3 ± 4.1 h. Mean duration for islet isolation was 6.2 ± 0.9 h. Sterility was confirmed in all preparations. Islet equivalent (IEQ) yield varied widely from 60,000 to 580,000. Mean purity was 69% and viability 72%. There were no significant differences between donor organs preserved with the TLM or with UWS (Table 1).

Islet Transport and Reassessment at Satellite Site

In the preclinical phase, islets were packaged immediately following postisolation assessment without interim culture. Mean time from packaging in King's College Hospital to reassessment in Newcastle was 7.5 ± 3.3 h. Maintenance of stable temperature in the sealed

Table 1. Comparison of Pancreas Preservation Methods on Assessment at Isolation Center

	N (%)	Age	BMI	CIT	IEQ × 1000 (Range)	Purity	Viability
TLM	6 (60)	46 ± 9	27 ± 5	7.3 ± 1.6	300 (150–580)	65 ± 16	63 ± 24
UWS	4 (40)	52 ± 7	27 ± 4	9.8 ± 6.5	196 (60–350)	75 ± 13	85 ± 9
Total	10	48 ± 9	27 ± 5	8.3 ± 4.1	258 (60–580)	69 ± 15	72 ± 22
<i>p</i> -value		0.3	0.9	0.5		0.3	0.1

Values are mean ± SD or mean (range). BMI, body mass index; CIT, cold ischemic time; IEQ, islet equivalent; TLM, two-layer method; UWS, University of Wisconsin solution.

transport box was confirmed with mean temperature of $7.8 \pm 1.0^\circ\text{C}$.

Reassessment of islet sterility, number, purity, integrity, and viability was undertaken in the Newcastle Biomanufacturing Cellular Therapies GMP suite. Sterility was maintained in all preparations confirmed by negative gram staining and absence of growth in culture. In early studies, each isolation batch was divided into semipermeable and standard blood transfusion bags for transport. Viability was comparable in both types of bag, but a significant loss of integrity was demonstrated in islets shipped in semipermeable bags (Table 2). This was believed to be due to shear stresses induced by islet movement during the journey in these more brittle bags. Moreover, on one occasion a semipermeable bag ruptured during the journey, precluding further consideration for transport of clinical preparations. No clear cause for the rupture was identified. However, possible friction with the side wall of the plastic shelving unit during the journey may have contributed. Standard blood transfusion bags remained intact throughout all transport journeys and were thus adopted for the clinical program.

For definitive comparisons of the 10 islet preparations transported in standard blood transfusion bags, six preparations were evaluated following centrifugation and resuspension confirming no significant decrease in islet equivalent number (Table 3). In parallel, a Standard Operating Procedure was validated enabling a 4-ml

Table 2. Comparison of Islet Preparations in Semipermeable and Standard Blood Bags

Isolation Batch	Viability (%)		Integrity (%)	
	Blood Bag	Permalife Bag	Blood Bag	Permalife Bag
1	90	90	80	60
2	90	90	80	60
3	50	55	50	40
Mean ± SD	77 ± 23	78 ± 20	72 ± 19	53 ± 12
<i>t</i> -Test (<i>p</i>)	0.2	0.02		

'side-arm' sample to be taken from the bag port under sterile conditions without the requirement of a GMP clean room or manipulation of the product in vitro. The robustness and utility of this method for maintaining islet sterility, purity, integrity, and viability was demonstrated, with no evidence of deterioration during the transport journey (Table 3). Preservation of normal β -cell morphology without degranulation was confirmed before and after transport (Table 3, Fig. 1).

Preserved β -cell function in transported islets was confirmed by significant increases in insulin secretion in the presence of high glucose in both static incubation and dynamic perfusion studies (Table 3). Greater than 95% processing of proinsulin to insulin was demonstrated in secreted (pro)insulin, with a minor but significant increase in proinsulin processing on glucose stimulation.

To evaluate the potential of transplanting islets from the transport bag without a washing and resuspension step, insulin and proinflammatory cytokine levels in the media were assayed following the journey. Total insulin content was less than 2 units in all cases with cytokine concentrations low (Table 3).

Clinical Transplantation

Successful completion of the preclinical phase and approval of the validated Standard Operating Procedures provided sufficient evidence for National Health Service commissioning of a fully funded clinical islet transplant program in the UK for those with recurrent hypoglycemia despite optimal conventional therapy or suboptimal glycemic control with a functioning renal transplant. This was implemented on April 1, 2008, configured around central clinically certified islet isolation facilities in London and Oxford with the potential for transport of purified islets to additional satellite transplant centers in Newcastle, Manchester, and Bristol to ensure equity of access throughout England.

To date, five patients have received a total of six transplants in Newcastle with islets isolated in King's College Hospital and Oxford, transported and evaluated according to the protocols developed in the preclinical phase. Islet yields of 224,000–600,000 IEQ were attained with maintained sterility, purity, integrity, and

Table 3. Evaluation of Islet Quality Following Transport

	King's	Newcastle	<i>p</i> -Value
IEQ ($\times 1000$)	156 \pm 60	151 \pm 90	0.8
Purity (%)	69 \pm 12	69 \pm 13	1.0
Integrity (%)	58 \pm 16	58 \pm 21	1.0
Viability (%)	72 \pm 22	76 \pm 18	0.1
Secretory granules/ β -cell	177 \pm 63	207 \pm 66	0.2
GSIS stimulation index: static		1.3 \pm 0.2	0.006
GSIS stimulation index: perfusion		3.4 \pm 1.2	0.0002
Proinsulin/insulin processing (%) (intracellular)			0.03
2 mM glucose		70 \pm 3	
20 mM glucose		74 \pm 3	
Proinsulin/insulin processing (%) (secreted)			0.007
2 mM glucose		96 \pm 0.4	
20 mM glucose		97 \pm 0.2	
Insulin content in transport bag (U/100,000 IEQ)		0.6 \pm 0.2	
IFN- γ (pg/ml)		7.3 \pm 1.8	
IL-1 β (pg/ml)		1.1 \pm 0.1	
IL-6 (pg/ml)		1.1 \pm 0.05	
TNF- α (pg/ml)		5.3 \pm 0.7	

Values are mean \pm SD. GSIS, glucose-stimulated insulin secretion; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

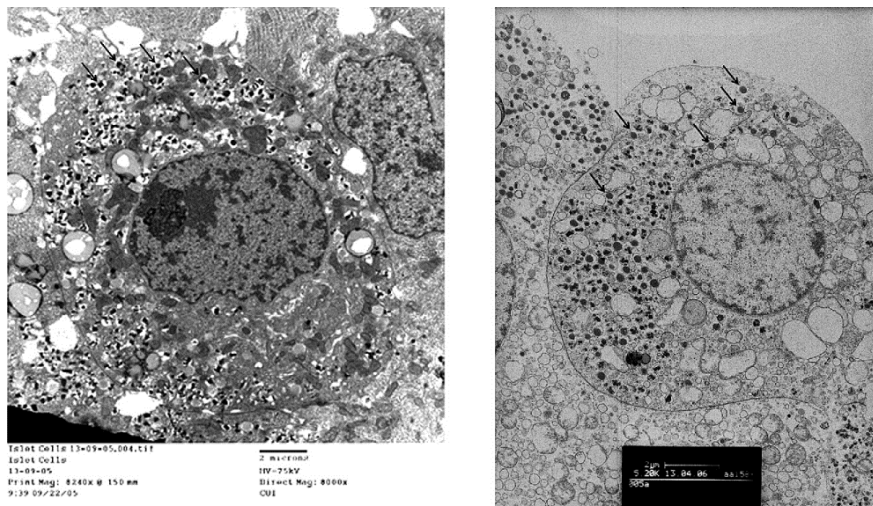


Figure 1. Electron microscopic images of islets at King's College islet isolation facility (left) and in Newcastle (right) demonstrating the presence of numerous secretory granules in both.

viability confirmed on arrival by 'side-arm' analysis (Table 4). Four female recipients have received single islet transplants alone (ITA), with a single female islet after kidney (IAK) recipient receiving two transplants (the second 3 months after the first). Induction for the first recipient was intravenous basiliximab, with all other recipients receiving subcutaneous alemtuzumab immediately after each islet infusion.

Alemtuzumab was well tolerated without adverse effects other than for transient asymptomatic pyrexia (single episode 37.5–38°C for less than 40 min) 24 h following administration. Target trough tacrolimus levels were maintained in all with no infusion- or immunosuppression-related serious adverse events.

Primary graft function (defined as positive C-peptide (>50 pmol/L) at 28 days after first transplant) in addition to prevention of recurrent severe hypoglycemia was achieved in all. In the first recipient, there was evidence of loss of islet integrity on a second in vitro assessment of the side-arm sample following transplant. This recipient rapidly developed donor-specific antibodies with loss of detectable C-peptide at 6 months posttransplant. However, the patient has maintained improvement in hypoglycemia awareness and reduction in severe hypoglycemia. In all subsequent transplants, an initial period of overnight culture at 37°C in CMRL medium supplemented with 5% human serum albumin in a 5% CO₂ incubator was added prior to islet transport, to enable any loss of viability or integrity to become manifest.

For the second transplant in the second recipient (performed at 3 months post-first transplant), although overall viability was maintained at 90% posttransport, occasional PI-positive cells were seen in the core of the largest islets (>300 µm diameter). This recipient incrementally lost graft function over the subsequent months, becoming C-peptide negative at 12 months after first graft. This was despite an HbA1c of 4.7% at 6 months.

Any evidence of central necrosis has since been added as an exclusion criterion for clinical islet transplantation in the UK program.

Good graft function after a single transplant has been maintained in all other recipients with absolute prevention of further severe hypoglycemia and HbA1c <7%, fulfilling National Institute for Health and Clinical Excellence (NICE) criteria for transplant success (10). Insulin requirements have been reduced, with insulin independence maintained for >24 months in recipient 3, who received >10,000 IEQ/kg (Table 4).

DISCUSSION

This study confirms the safety and utility of islet allotransplantation in the UK employing accredited clinically proven central isolation facilities and satellite transplant centers, ensuring equity of access regardless of geographical location. While a small number of transplant programs internationally use transported islets, protocols for these programs remain specific to the local setting. Transport of both whole organ pancreas and isolated islets was carried out using air chartered flights in the Miami–Houston program with cold ischemic times of less than 7 h (5), while the Groupe Rhin-Rhône-Alpes-Genève pour la Transplantation d'Ilots de Langerhans (GRAGIL) Swiss/French collaborative network employed road services with cold ischemic time of up to 8 h (3,7). In the UK the most suitable transport method was road ambulance, enabling target cold ischemic time of less than 9 h. Pancreases in the Miami–Houston program were packaged either in TLM or UWS (5), while in the GRAGIL program pancreas packaging was carried out using UWS (3,7). In the preclinical phase of the UK program, both TLM and UWS alone were evaluated with no significant differences in islet quality. Therefore, UWS, which is well established and currently used in the retrieval Standard Operating Proce-

Table 4. Islet Preparations Employed in the Clinical Transplant Program

Isolation Site	CIT	Packaging Until Transplant	Transplant Mass (IEQ/kg)	Viability (Pretransport)	Viability (Posttransport)	Recipient No.	Age at Transplant (Years)	Primary Graft Function	Loss of Graft Function	Prevention of Severe Hypoglycemia
King's	4.1	7.5	5,200	90	80	1	54	yes	6 months	yes
King's	4.3	9.5	5,368	90	90	2	44	yes	—	yes
King's	8.5	9.0	6,700	90	90	2	44	—	12 months	yes
Oxford	3.0	8.0	12,000	90	85	3	60	yes	no	yes
King's	3.1	9.8	6,349	90	90	4	64	yes	no	yes
Oxford	9.0	7.1	4,058	90	92	5	49	yes	no	yes
Mean ± SD	5.3 ± 2.7	8.5 ± 1.1	6,613 ± 2,799	90 ± 0	88 ± 5		54 ± 8			

ture for whole vascularized pancreas transplantation, was used to simplify the procedure for a national adoption.

Islets for the Miami–Houston program were packaged in gas-permeable bags in variable but relatively large volumes of MM1 medium at room temperature. Islet transport journey to transplantation center was by air and lasted <5 h (5). Upon arrival islets were washed and reassessed again prior to transplantation. In the GRAGIL program islets were initially packaged in 50-ml syringes in X-vivo medium but later changed to purpose made bags (7) in CMRL-1066 medium. Islets were transported by road at room temperature and the transport journey lasted <5 h. Islets were transplanted upon arrival directly without repeated quality assessments.

In the preclinical phase of our program both gas-permeable and blood bags were evaluated and transport was carried out at a relatively cool temperatures to reduce biological activity and thus enable transplantation without a further wash step at the receiving center. Within this setting, no meaningful advantage of gas-permeable bags on islet quality could be detected. Indeed, there was evidence of increased islet fragmentation and therefore only standard blood bags were used in the clinical program. Repeated assessment at Newcastle confirmed maintained sterility, number, purity, integrity, and function, together with the utility of using a sample obtained directly from the bag via a side arm to confirming quality immediately prior to transplantation. Moreover, safety of transplanting islets directly without a washing step at the receiving centre was supported by low levels of insulin and inflammatory cytokines in the transport/transplant medium.

As journey times of <10 h can always be achieved by road between all of the UK centers with no evidence of product deterioration over this period, there was no need to consider shipping by air. Equally there was no evidence that oxygen concentration or nutrient consumption were limiting at the relatively cool temperature employed with sufficient media volume and gas headspace. Semipermeable bags have been successfully used to maintain islets in longer transport journeys (6) but decreased number and viability in conventional versus semipermeable bags has only been confirmed in incubations of 18 h or more at 20°C (5). The possibility for puncturing of semipermeable bags and inadequate protection against hypoxia at the islet core, certainly at temperatures above 20°C, has been reported (1). Islet integrity following transport by road in semipermeable bags has not previously been reported to our knowledge. In the present studies, all journeys were associated with decreased integrity, and careful reevaluation of islet integrity and fragmentation following transport using semipermeable bags in other programs is recommended.

Preclinical evaluation underpinned approval, funding,

and implementation of a national program for islet transplantation in England providing equity of access. This enabled the first transplants of islets in the UK transported from a geographically distant isolation facility. Success was confirmed by attainment of primary graft function and resolution of severe hypoglycemia in all recipients.

Relatively early loss of C-peptide positivity in the first two recipients, despite maintenance of adequate immunosuppression throughout, underlines the importance of pre-transport culture to enable any loss of integrity or decreased viability to become manifest pretransplant (5), in addition to the necessity of careful reevaluation of quality posttransport with criteria precluding transplantation, including any evidence of islet central necrosis.

Administration of single dose alemtuzumab subcutaneously immediately following islet infusion avoided induction of T-cell depletion prior to confirmation of successful completion of the islet transplant procedure. Preceded by steroid/antihistamine “cover,” this was both well tolerated and effective with no evidence of cytokine release beyond transient asymptomatic pyrexia. Peritransplant serum cytokine profiles are being analyzed in ongoing studies to further characterize any potential for alemtuzumab-induced proinflammatory response during engraftment. This remains the favored induction agent in the UK clinical islet transplant program, with longer term outcome data in a larger number of recipients awaited.

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