Improving the function of islet and β-cell grafts

Michelle Beatrice Paget

Doctor of Philosophy

ASTON UNIVERSITY

December 2008

Thesis summary
Aston University

Thesis title: Improving the function of islet and β-cell grafts
Name of student: Michelle Beatrice Paget
Degree: Doctor of Philosophy
Year of submission: 2008

Background: Human islet transplantation would offer a less invasive and more physiological alternative than whole pancreas transplantation and insulin injections respectively for the treatment of diabetes mellitus if islet graft survival can be improved. Initial recipient post-transplant insulin independence declines to <10% after 5 years. Factors contributing to graft failure include enzymatic disruption of the islet microenvironment during isolation, diabetogenic effects of immunosuppressants and metabolic stress resulting from slow revascularisation.
Aims: To investigate the effect of co-culture in both static (SC) and rotational culture (RC) of BRIN-BD11 beta-cells (D11) and human umbilical vein endothelial cells (HUVEC) on D11 insulin secretion; and the effect of a thiazolidinedione (TZD) on D11 function and HUVEC proliferation. To assess the effect of culture media, SC, RC and a TZD on human islet morphology, insulin secretion and VEGF production. To initiate *in vivo* protocol development for assessment of revascularisation of human islet grafts.
Methods: D11 cells were cultured +/-TZD and co-cultured with HUVEC +/-TZD in SC and RC. D11 insulin secretion was induced by static incubation with low glucose (1.67mM), high glucose (16.7mM) and high glucose with 10mM theophylline (G+T) and determined by ELISA. HUVEC were cultured +/-TZD in SC and RC and proliferation was assessed by ATP luminescence assay and VEGF ELISA. D11 and HUVEC morphology was determined by immunocytochemistry.
Human islets were cultured in SC and RC in various media +/-TZD. Insulin secretion was determined as above and VEGF production by fluorescence immunocytochemistry (FI) and ELISA. Revascularisation of islet grafts was assessed by vascular corrosion cast and FI.
Results: D11 cultures showed significantly increased insulin secretion in response to 16.7mM and G+T over basal; this was enhanced by RC and further improved by adding 10mM TZD.
Untreated D11/HUVEC co-cultures displayed significantly increased insulin secretion in response to 16.7mM and G+T over basal, again enhanced by RC and improved with 10mM TZD. 10mM TZD significantly increased HUVEC proliferation over control.
Human islets maintained in medium 199 (m199) in SC and RC exhibited comparable maintenance of morphology and insulin secretory profiles compared to islets maintained in RPMI, endothelial growth media and dedicated islet medium Miami#1. All cultures showed significantly increased insulin secretion in response to 16.7mM and G+T over basal; this was enhanced by RC and in certain instances further improved by adding 25mM TZD. TZD increased VEGF production and release as determined by ELISA. Post-implant vascular corrosion casts of mouse kidneys analysed by x-ray micro tomography indicates a possible TZD enhancement of microvessel growth via VEGF upregulation.
Conclusions: D11/HUVEC co-culture in SC or RC does not alter the morphology of either cell type and supports D11 function. TZD improves D11 and D11/HUVEC SC and RC co-culture insulin secretion while increasing HUVEC proliferation.
Human islet RC supports islet functional viability and structural integrity compared to SC while the addition of TZD occasionally further improves secretagogue induced insulin secretion. Expensive, 'dedicated' islet media showed no advantage over m199 in terms of maintaining islet morphology or function. TZD upregulates VEGF in islets as shown by ELISA and suggested by x-ray micro tomography analysis of vascular corrosion casts.
Maintenance of islets in RC and treatment with TZD prior to transplant may improve the functional viability and revascularisation rate of islet grafts.

Key words/phrases:
Human islet isolation/culture, human islet transplantation/revascularisation, insulin secretion, thiazolidinedione/VEGF/insulin, BRIN-BD11/HUVEC co-culture

Acknowledgements

I would like to thank Dr Taghi Miri from the Chemical Engineering Department of the University of Birmingham for collaborating with regards to performing x-ray microtomography on the vascular corrosion casts for this thesis.

Many thanks to the staff at the pathology laboratory of Worcester Royal Hospital for performing tissue processing, paraffin wax embedding and tissue section cutting on the spleen samples for this thesis.

I would also like to express my thanks to Professor Cliff Bailey and Mr. Richard Downing for their patience and support as supervisors.

My grateful thanks to Mr. John Inglis for the time spent photographing the vascular corrosion casts for this thesis and for his help with printing.

List of Contents

List of Figures and Tables

Figures

Tables

List of abbreviations

ANOVA	analysis of variance
ATP	adenylate triphosphate
BSA	bovine serum albumin
CAT	computed axial tomography
cAMP	cyclic adenylate monophosphate
CSC	conventional static culture
DM	diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxynucleic acid
DTZ	diphenylthiocarbazone
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
ECM	extracellular matrix
FBS	foetal bovine serum
FCS	foetal calf serum
GLP-1	glucagon-like peptide 1
G+T	high glucose + 10mM theophylline
HARV	high aspect rotational vessel
HBSS	Hanks' balanced salt solution
HLA	human leucocyte antigen
HUVEC	human umbilical vein endothelial cells
IL-1	interleukin-1
IL-2	interleukin-2
IL-6	interleukin-6
IL-10	interleukin-10
IAPP	islet amyloid polypeptide
IDDM	insulin-dependent diabetes mellitus
IRL	Islet Research Laboratory
mRNA	messenger ribonucleic acid
NIDDM	non insulin-dependent diabetes mellitus

PBS	phosphate buffered saline
PPARγ	peroxisome proliferator-activated receptor gamma
RLU	relative light units
RT	room temperature
RCCS	rotational cell culture system
RC	rotational culture
SEM	scanning electron microscopy
SC	static culture
SNP	single nucleotide polymorphism
TZD	thiazolidinedione
TCM	tissue culture medium
TNF-α	tumour necrosis factor alpha
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
UKHTB	UK Human Tissue Bank
UW	University of Wisconsin solution
VEGF	vascular endothelial growth factor
XMT	x-ray microtomography

Chapter 1: Introduction

The purpose of this thesis

This thesis sets out to test the general hypothesis that by introducing *in vitro* pre-transplant intervention to islet/β-cell grafts, post-transplant vascularisation and consequently graft survival and function could be improved. It aims to do this by:

- developing an *in vitro* β-cell support and endothelial cell proliferation protocol using cell lines prior to modifying the technique for use with human islets
- maintaining the structural integrity and functional viability of cultured islets by employing a rotational cell culture system to provide a microgravity environment that enhances cell-cell contact and thorough circulation, thus improving the availability of trophic factors and nutrients
- increasing the expression of vascular endothelial growth factor (VEGF) in endogenous islet endothelial cells by employing a thiazolidinedione (rosiglitazone)
- initiating development of an *in vivo* protocol to assess revascularisation of human islets transplanted under the kidney capsule and into the spleens of mice.

The importance of this work stems from the urgent need to improve the quality and quantity of islets/β-cells for implant into diabetic patients, and to improve the revascularisation of islets grafts post transplant to support viability and function of the cells.

Background

Diabetes mellitus

Diabetes mellitus [from the Greek dia (through) betes (to go) and mellitus (sweet tasting)] describes a group of disorders characterised by hyperglycaemia (ADA 2008). The disorders are associated with various endocrine and metabolic abnormalities (Dupre et al. 1982; Begg 1984; Gerich 1986; Wautier and Guillausseau 1998), the risk of microvascular complications such as nephropathy, neuropathy and retinopathy (Engerman et al. 1977; Giugliano et al. 1996; Coccheri 2007; Gunduz and Bakri 2007; LeRoith and Rayfield 2007; Lockhart et al. 2008) as well as macrovascular complications like cardiac ischaemia (Giugliano et al. 1996; LeRoith and Rayfield 2007; Steiner 2007), stroke and peripheral vascular disease (Coccheri 2007; ADA 2008; Lockhart et al. 2008).

It is estimated that the prevalence of diabetes mellitus (DM) is currently 2.35 million in the UK (with a suspected 1 million as yet undiagnosed), and by 2010 this number will rise to more than 2.5 million (Roberts 2007). Worldwide prevalence increased from approximately 150 million in 2001 (Alberti 2001; Zimmet 2003) to 194 million in 2006 (Erdmann 2006). This pandemic is set to continue with an alarming 350 – 400 million people estimated to be suffering from diabetes by 2025 (Zimmet 2003; Erdmann 2006; Coccheri 2007).

The cost of treating diabetes and its complications consumes approximately 10% of the UK NHS yearly budget, amounting to £9.6 million per day (Roberts 2007). Also, the cost of inpatient care for people with diabetes is disproportionately higher than for those without the condition, and they occupy more bed days than non-diabetic patients with the same conditions that are unrelated to diabetes (Currie et al. 1997; Sampson et al. 2006).

The endocrinopathies responsible for the manifestation of hyperglycaemia typically involves absolute or relative impairment of insulin secretion and/or insulin action (Nerup and Lernmark 1981; Gerich 1986; Harris MI 1997). Due to the complexity of DM, various classification systems exist, but only the two major categories of DM, namely type 1 and type 2, will feature in this project.

Type 1 DM: aetiology and pathogenesis

Type 1 DM (T1DM), or insulin-dependent DM (IDDM), is a disease of β-cell destruction resulting in a loss of insulin secretion and effecting a chronic metabolic disorder that is characterised by hyperglycaemia (Gerich 1986). Most cases arise following T-cell dependent, autoimmune destruction of the β-cells in the pancreatic islets of Langerhans (Nerup and Lernmark 1981; Todd 1990; Haverkos et al. 2003; Kulmala 2003) and most of these cases result from a genetic susceptibility linked to the β-cell alloantigen HLA DRw4 (Solow et al. 1979; Deschamps et al. 1980; Shires et al. 1983; Wallden et al. 2008). Human leucocyte antigens (HLA) are cell surface molecules that present antigenic peptide to T cells and certain HLAs have been shown to be associated with type 1 diabetes (Singal and Blajchman 1973; Krach et al. 2003; Wallden et al. 2008). Some 80% of patients with type 1 DM have specific HLA phenotypes associated with detectable serum islet cell cytoplasmic antibodies and islet cell surface antibodies (Lipton and LaPorte 1989; Scheuner et al. 1997; Kulmala 2003). The genetics of type 1 DM cannot be classified according to a specific model of dominant, recessive or intermediate inheritance of a specific set of genes (Todd 1999) and a full discussion of the genetics of diabetes mellitus lies beyond the scope of this thesis.

A small proportion of diabetes is caused by exposure to herbicides or pesticides (Saldana et al. 2007; Michalek and Pavuk 2008) and arsenic (Liu et al. 2006; Paul et al. 2007; De Vizcaya-Ruiz et al. 2008). Three decades ago a group suggested that cyanide was

diabetogenic (McMillan and Geevarghese 1979), but others later refuted this statement (Cooles 1988; Swai et al. 1992; Soto-Blanco et al. 2001) and more recently yet another group showed that cyanide aggravated diabetes rather than causing it (Yessoufou et al. 2006).

Type 1 DM was also called juvenile onset DM as most cases are diagnosed before the age of 30 (but may be diagnosed in older adults), or insulin dependent DM, because, unless insulin treatment is provided, the patient will succumb to diabetic ketoacidosis (Harris MI 1997).

Type 1 DM accounts for 10-15% of all cases of diabetes mellitus and affects about 1 in 300 people in Europe (Gerich 1986; Winter and Schatz 2003) and was once considered to be a disease of acute onset, but evidence shows that it is mostly a genetically determined chronic autoimmune disorder with a long subclinical prodromal phase that can last for years (Eisenbarth 1986; Kulmala 2003). In genetically susceptible subjects a precipitating event, possibly an environmental impact such as coxsackie B4 virus, presumably triggers the autoimmune attack against the pancreatic β-cells (Bach 1997; Haverkos et al. 2003; Kulmala 2003; Dotta et al. 2007). As β-cell destruction progresses individuals show an increased loss of first-phase insulin release when subjected to intravenous glucose tolerance testing, but many patients remain euglycaemic during the early stages of loss of insulin secretion (Bleich et al. 1990; Keymeulen 2008).

During the preclinical phase of type 1 diabetes impaired insulin secretion is one of the first metabolic aberrations to occur and during the 18 months prior to clinical onset, a subclinical rise in blood glucose levels can be detected (Bleich et al. 1990; Kulmala 2003). Complete loss of first-phase insulin release is followed by hyperglycaemia and the clinical onset of the disease. The cleaving of proinsulin in the secretory vesicles of pancreatic β-cells produces insulin and C-peptide, which are secreted concurrently, and C-peptide secretion may still be

present for a period following the onset of diabetes, but once autoimmune destruction of the β-cells is complete all C-peptide production ceases.

When autoimmune destruction of the β-cells commences, so does infiltration of the islets with T-lymphocytes, B-lymphocytes and macrophages. Only β-cells are targeted, leaving the A-cells, D-cells, PP-cells and other cells (such as clear cells) intact (Guven and Kuenzi 1998).

T1DM remains incurable and treatment with parenteral insulin offers only partial reinstatement of glucose homeostasis. Despite considerable advances in the understanding of the aetiology, there is currently no real prospect of either prevention or cure by pharmacological treatment.

Type 2 DM: aetiology and pathogenesis

Type 2 DM (T2DM), also known as non insulin-dependent diabetes mellitus (NIDDM), is a heterogeneous group of disorders in which hyperglycaemia results from both an impaired insulin secretory response to glucose and decreased insulin effectiveness in stimulating glucose uptake by skeletal muscle and in restraining hepatic glucose production; a phenomenon known as insulin resistance (Bailey and Nattrass 1988; Guven and Kuenzi 1998; Perseghin et al. 2003; Hoppener and Lips 2006; Chang-Chen et al. 2008).

Historically most cases of type 2 DM were diagnosed after the age of 30 years, but due to factors such as a more sedentary lifestyle and obesity it is becoming more frequent in children and young adults (Aucouturier et al. 2008). An entirely separate group of disorders due to mutation of hepatocyte nuclear factors and glucokinase (Holmkvist et al. 2008; Karlsson et al. 2008) are termed 'maturity onset diabetes of the young [MODY] (Barness 2007; Kaufman and Schantz 2007; Lipton 2007).

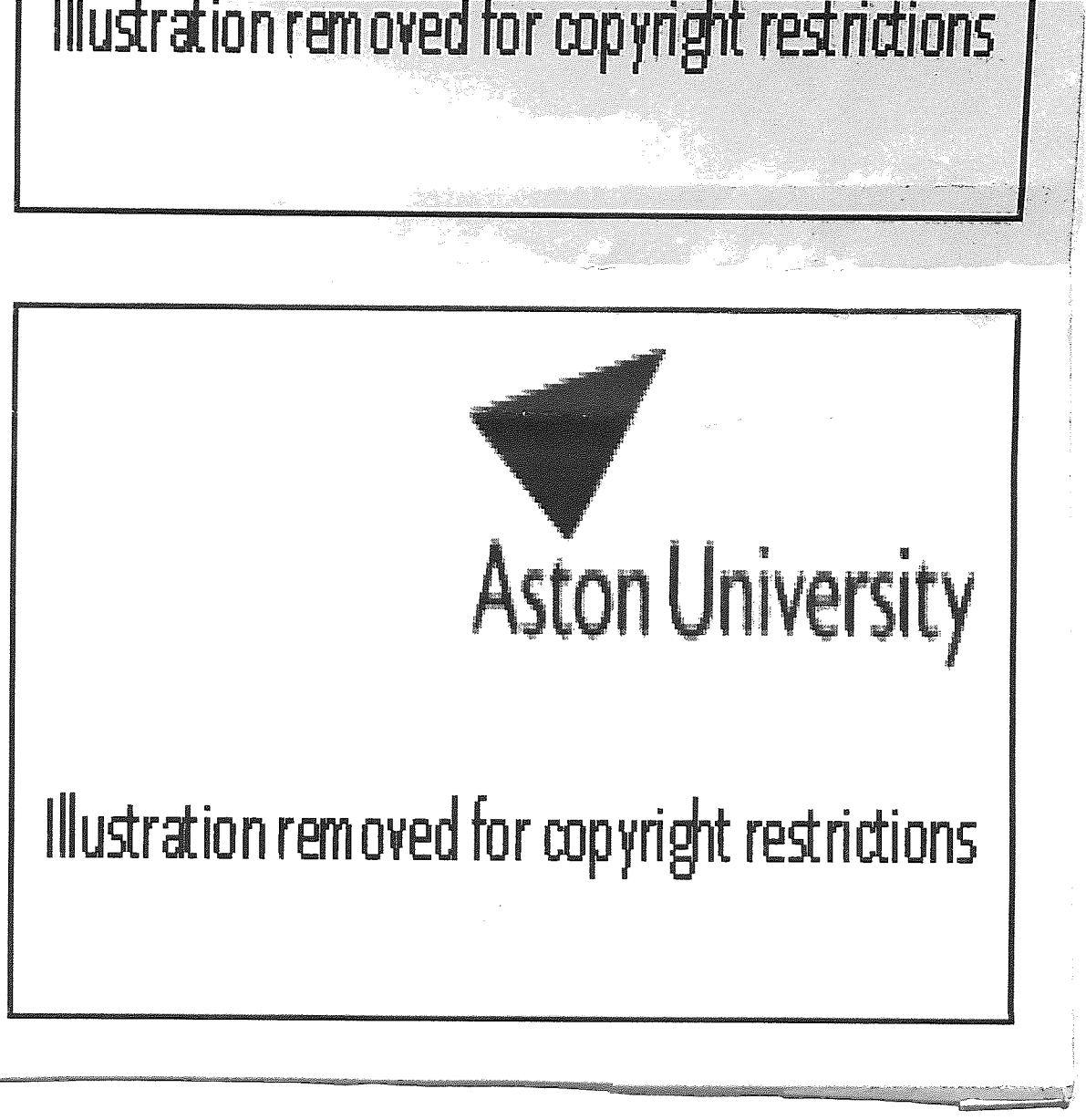


Figure 1. A schematic representation of glucose stimulated insulin secretion, compiled from various publications (Karl et al. 1975; Hedeskov 1980; Hellman et al. 1980; MacDonald and Fahien 1990; Garvey 1992; Petit and Loubatieres-Mariani 1992; Wang et al. 1993; Bailey 2000; Thorens 2001; Ren et al. 2007).

The number of β-cells in the pancreas influences how much insulin can be produced. Many studies have shown a modest (20-40%) reduction in β-cell mass in patients with longstanding T2DM (Saito et al. 1979; Clark et al. 1988; Porte and Kahn 2001). The islets have a normal morphology and no insulitis is observed and a greater than 70% loss of β-cell mass is required to cause overt diabetes (Kloppel et al. 1985; Sreenan et al. 1999). Moreover, concurrent obesity (another insulin resistant state) is often accompanied by a significant increase in β-cell mass (Kloppel et al. 1985; Dickson and Rhodes 2004; Paris et al. 2004; Prentki and Nolan

2006; Chang-Chen et al. 2008). Obesity results in a decrease in adiponectin secretion by up to 50%, leading to insulin resistance and beta cell dysfunction (Bacha et al. 2004). It thus seems likely that factors other than β-cell mass are responsible for impairment in insulin secretion in T2DM.

Studies in various populations have shown a positive correlation between low birth weight and the development of impaired glucose tolerance (IGT) and T2DM later in life (Barker et al. 1993; McCance et al. 1994; Wolf 2003; Simmons 2007). Hales and colleagues reported that men and women with IGT or newly diagnosed T2DM had a mean birth weight that was 0.3kg less than non-diabetic subjects, a higher placental to birth weight ratio, a smaller head circumference and a lower ponderal (weight/length) index (Hales et al. 1991; Phipps et al. 1993). Evidence exists that poor nutrition and impaired growth are associated with reduced insulin secretion and/or reduced β-cell mass (Hales and Barker 1992; Jaquet et al. 2003) and some studies have shown that foetal malnutrition may lead to insulin resistance later in life (Phillips 1996; Wolf 2003).

Islet amyloid polypeptide (IAPP), or amylin, is produced by the β-cell, packaged into secretory granules with insulin and co-secreted into the sinusoidal space (Clark 1989; Johnson et al. 1989). Amylin is the precursor for the amyloid deposits which are frequently observed in patients with T2DM (Nishi et al. 1990; Hoppener et al. 2002; Hoppener and Lips 2006) and while some researchers claim that amylin may be involved in causing β-cell dysfunction in T2DM (Howard 1986; Clark 1989; Johnson et al. 1989; Ohsawa et al. 1989; Nishi et al. 1990), others failed to find an inhibitory effect of amylin on insulin secretion when it was infused in pharmacological doses in rats, rabbits and humans (Bretherton-Watt et al. 1990; Ghatei et al. 1990).

Glucagon-like peptide 1 (GLP-1) is synthesized and secreted by the small intestine in response to ingested nutrients (Goke et al. 1991) and is a potent insulin secretagogue (Thorens and Waeber 1993). GLP-1 has a specific β-cell receptor that has been localized to chromosome 6 (Stoffel et al. 1993) and after binding of GLP-1 to its receptor; the adenylate cyclase system is stimulated. Cyclic AMP levels rise and cAMP-dependent protein kinase A augments glucose-induced insulin secretion by phosphorylation of key elements of the glucose signalling pathway. This includes the ATP-dependent K^+ channel, the voltage-dependent calcium channel and/or elements of the insulin-secretory machinery. The gluco-incretin effect of GLP-1 is reduced in T2DM (Nauck et al. 1986; Thorens and Waeber 1993), but GLP-1 levels are normal or increased (Nauck et al. 1986), suggesting β-cell resistance to GLP-1. Postprandial insulin secretory response and restoration of near normal glycaemia in T2DM patients can be achieved by pharmacological doses of GLP-1 (Nauck et al. 1993).

The acquired defect in insulin secretion may be explained by the concept of glucose toxicity (Unger and Grundy 1985; Leahy 1990). Tight metabolic control leads to improved insulin secretion, which shows that hyperglycaemia exerts a deleterious effect on insulin secretion. Evidence exists that impaired insulin gene transcription results from prolonged exposure of β-cells to high glucose concentrations (Robertson et al. 1994). This leads to decreased insulin synthesis and secretion and the term 'desensitization' was developed to explain a temporary, inducible and reversible state of impaired insulin secretion following prolonged exposure of β-cells to hyperglycaemia while the term glucose toxicity is reserved for the irreversible loss of insulin secretory capacity by β-cells (Robertson et al. 1994).

Environmental factors are important in the development of the type 2 diabetic phenotype (Lebovitz 1999), but epidemiological and familial studies show that there are also genetic influences on the development of type 2 diabetes (Mercado et al. 2002; Sanghera et al. 2008).

Multiple studies investigating single nucleotide polymorphisms (SNPs, where a single DNA nucleotide is replaced with another) in European (UK, Danish and French) and Japanese populations have identified that SNPs occurring in or within regulatory areas near the following genes represent novel risk loci for T2DM:

CAPN10, HHEX/KIF11/IDE, CDKN2A/B, IGF2BP2, TC7L2, SLC30A8, CDKAL1, PKN2, FLJ39370, EXT2/ALX4 JAZF1, CDC123-CAMK1D, THADA, ADAMTS9, NOTCH2 and TSPAN8-LGR5 (Horikawa et al. 2000; Ehrmann et al. 2002; Iwasaki et al. 2005; Groves et al. 2006; Grarup et al. 2007; Sladek et al. 2007; Zeggini et al. 2007; Zeggini et al. 2008).

Further studies have shown that genes contributing to T2DM may vary between individuals and populations (Groves et al. 2006; Kwan et al. 2007; Cauchi et al. 2008; Kwan et al. 2008; Lewis et al. 2008). Extreme forms of insulin resistance resulting from mutations in the genes for the insulin receptor and peroxisome proliferator-activated receptor gamma have been reported, but are rare (Mercado et al. 2002; Chistiakov 2008). New risk loci for T2DM are being discovered and a full discussion on the genetics involved in T2DM lies outside the remit of this thesis.

Current treatments for diabetes and their limitations

Insulin and oral anti-diabetic drugs

Insulin was one of the first proteins to be crystallised in pure form in 1926 and in 1952 Frederick Sanger made it the first protein to be fully sequenced, thus making its exact amino acid sequence known (Sanger 1959). This enabled chemically synthesised insulin to be produced by 1963 and the development of recombinant gene technology in 1978 allowed production of human insulin by inserting the human insulin gene into bacteria, which resulted in the large scale production of this hormone and, ultimately, revolutionised the use of peptides and proteins as therapeutic agents (Pillai and Panchagnula 2001). The development

of recombinant-DNA-derived insulin analogues gave rise to rapid-acting insulins (such as lispro and aspart) that are absorbed rapidly from the injection site, intermediate-acting (such as neutral protamine lispro) and long-acting insulins (such as glargine and determir) which are absorbed very slowly at physiological pH (Brange et al. 1992; Bristow 1993; Hirsch 1999). More recently, a rapid-acting insulin in powder form (Exubera) was developed for inhalation therapy (Mandal 2005; Odegard and Capoccia 2005). Figure 2 shows the amino acid sequence of human insulin.

Figure 2. The amino acid sequence of human insulin, compiled from various publications (Sanger 1959; Adams et al. 1969; Sanger 1988; Brange and Langkjoer 1993). Note the three disulfide bonds (red) between cystine amino acids. Two of these disulfide bonds serve as stabilising links between the alpha and beta chains of the insulin molecule.

Many insulin-replacement regimes utilise combinations of rapid-, intermediate- and/or long-acting forms of insulin in an attempt to replicate near-physiological insulin levels and improve patient compliance (Turner and Matthews 2000; Halbron et al. 2007; Hassan et al. 2008). The rapid-acting insulin is administered before or with a meal to provide postprandial (30-60 minutes after a meal) glycaemic control, intermediate-acting insulin action peaks 4 - 5

hours after injection and lasts for 10 – 15 hours, while the long-acting insulin is used in an attempt to mimic the basal insulin secretion of the islets in the hope of preventing hypoglycaemic incidents over a 24-hour period (Hoffman and Ziv 1997; Campbell et al. 2001; Heise and Heinemann 2001; Bolli 2002; Couper and Prins 2003).

The treatment of DM does not rely solely on insulin. Many forms of type 2 DM can be effectively treated with diet, exercise and drugs (Barnard et al. 1994; Edelman 1998; Tang and Reed 2001; Scheen 2003).

The biguanides, metformin and phenformin were introduced in 1957 and act by decreasing hepatic glucose output and increasing insulin sensitivity of the target tissues (Bailey and Nattrass 1988; Bailey 1992; Braunstein 2003; Bailey and Day 2004; Krentz and Bailey 2005). Phenformin was withdrawn due to an association with lactic acidosis, but metformin is used successfully as a monotherapy or in combination with a sulphonylurea (Bailey 1992; Bailey et al. 2005; Molavi et al. 2007) or a thiazolidinedione (Bailey and Day 2004; Lin et al. 2008).

The sulphonylureas, such as tolbutamide (now rarely used) and glimepiride were introduced in the 1950's and act by stimulating the pancreatic β-cells to increase insulin production by binding to the sulfonylurea receptor (Duhault and Lavielle 1991; Bailey 2000; Braunstein 2003; Krentz and Bailey 2005).

Insulin secretagogues like the meglitinide analogues (such as repaglinide), function by restoring early-phase insulin release by the islets (Bailey 2000; Dornhorst 2001; Braunstein 2003; Krentz and Bailey 2005).

The thiazolidinedione (TZD) class of oral antidiabetic agents, troglitazone (withdrawn in 1999), rosiglitazone and pioglitazone, targets insulin resistance by improving insulin sensitivity in the target tissues and TZDs have been shown to preserve ß-cell function (Bailey

1999; Bailey 2000; Cheng-Lai and Levine 2000; Braunstein 2003; Bailey and Day 2004; Krentz and Bailey 2005; Campbell and Mariz 2007).

The α-Glucosidase inhibitors (acarbose and miglitol) delay carbohydrate digestion which slows glucose absorption from the intestine and thus lower postprandial glucose levels (Braunstein 2003; Krentz and Bailey 2005).

The glucagon-like-peptide-1 (GLP-1) agonists (i.e. exendin-4) act by augmenting glucose stimulated insulin secretion (thus reducing plasma glucose concentrations) and increasing glucose uptake and metabolism by muscle and liver cells (Alcantara et al. 1997; Edwards et al. 2001; Bailey 2005; Doggrell 2007; Krentz et al. 2008; Martin 2008). Exendin-4 has also been shown to increase β-cell replication and neogenesis (Xu et al. 1999), modulate β-cell apoptosis (Li et al. 2003) and encourage differentiation of a human pancreatic duct cell line into endocrine cells containing insulin and glucagon (Zhou et al. 2002).

Dipeptidyl peptidase IV (DPP-4) is an enzyme that rapidly degrades GLP-1, thus ameliorating its glucose-lowering effect (McIntosh et al. 2006; Cheng et al. 2008; Flatt et al. 2008; Krentz et al. 2008). Inhibitors of DPP-4 (i.e. sitagliptin and vildagliptin) slow the rate of GLP-1 degradation by blocking the action of DPP-4, resulting in improved glucose homeostasis (McIntosh et al. 2006; Flatt et al. 2008; Krentz et al. 2008; Lamont and Drucker 2008).

Type 2 DM can be treated with these orally administered drugs as some functional islets remain, allowing the residual β-cells to be targeted with appropriate therapies. However, these oral anti-diabetic agents have limitations in that, individually, they contend with only part of the pathogenic process and work only to a partial extent (Bailey 2000). They also do not re-establish normal ß-cell function or normal insulin sensitivity and, while their usefulness depends on a critical mass of functional ß-cells, none have been shown to prevent (although some may slow) gradual ß-cell loss (Bailey 2000; Krentz and Bailey 2005). T2DM is a

progressive disease with many patients proceeding to exogenous insulin, which is also not a cure and does not reinstate normal glucose homeostasis, highlighting the requirement for research into cellular therapy for inadequately controlled T2DM.

Unfortunately, due to the near total destruction of β-cells, type 1 DM is not treatable with oral agents. Insulin itself cannot be given orally due to the fact that, as a protein, it will undergo rapid enzymatic digestion (Zinman and Tsui 1997). The most common form of treatment for type 1 DM remains insulin replacement via injection, mostly multiple daily subcutaneous injections (Chetty and Chien 1998; Bailey 2000).

Although insulin injections have served people with diabetes well, it cannot replace the 'closed loop' system that exists within the body. This closed loop system fine-tunes the release of insulin in response to factors such as intake of food and exercise (Zinman 1989; Bailey 2000). The release of this precious hormone from the beta cells in the pancreas is regulated by gut hormones (Vahl and D'Alessio 2004; Kruger et al. 2006; Holst et al. 2008), the composition of the absorbed nutrients (Gannon and Nuttall 2006) and the autonomic nervous system (Zinman and Tsui 1997; Ahren 2008). Glucose homeostasis is thus much more complex than a sudden bolus of insulin around mealtime.

Another disadvantage of the insulin injection regime lies in the many factors that affect the absorption rate of subcutaneously injected insulin, such as:

- The site of injection and the depth of the injection - how much of the injected insulin ends up in a fat depot as opposed to in muscle (from which absorption is more rapid).
- The temperature of the site as well as the ambient temperature (higher site temperature = faster absorption rate).
- Exercise just before or after injection will result in faster rates of absorption.

- The type of insulin used and the dose delivered – long-acting insulins are absorbed more slowly than their short-acting analogues.

Thus neither oral anti-diabetic agents nor intensive insulin therapy provide the ultimate answer to diabetes as physiological insulin release in response to energy intake cannot be fully replicated by these treatments (Bailey 2000). For this reason attention is directed to the use of transplant procedures for the treatment of diabetes.

Pancreas transplantation

In 1889, 33 years before insulin was successfully extracted for use, Minkowski and von Mering showed that an 'anti-diabetic' substance was produced in the pancreas by inducing diabetes in dogs following pancreatectomy (Botting 1996). In an attempt to restore the lost insulin secretory ability of a 15-year-old boy suffering from diabetes, Dr. P. Watson Williams implanted three pieces of fresh sheep's pancreas subcutaneously into the boy in the Bristol Royal Infirmary on 20 December 1893, unfortunately without success (Williams 1984).

In 1924 Pybus described a temporary reduction in urinary glucose levels in one of two patients prior to rejection of the subcutaneous implants of sliced human pancreas (Pybus 1924). Fichera transplanted foetal pancreatic tissue under the tunica vaginalis in 1928, but his patient died of ketotic coma, possibly due to acute rejection of the graft (Pozza et al. 1992).

These early failures did not, however, diminish the determination of the medical fraternity to make pancreatic transplants succeed, and in 1966 Lillehei performed the first vascularised pancreas transplant (Kelly et al. 1967). The following ten years saw only a few more pancreatic transplants, until Dubernard published the description of a new surgical technique, the segmental Neoprene injection technique, in 1978 (Dubernard et al. 1978). This technique

was much simpler and safer and resulted in pancreatic transplantation being more widely accepted. One of the difficulties encountered during the development of a successful technique for pancreas transplantation was exocrine drainage.

An investigation into duct ligation versus various drainage routes in dogs resulted in the following findings:

- Both duct ligation and drainage into the peritoneal cavity resulted in high morbidity rates, severe pancreatitis as well as exocrine and endocrine atrophy
- Pancreatic duct drainage into the stomach, small bowel (enteric) or bladder prevented pancreatitis and preserved exocrine and endocrine function (MacDonald et al. 1982; Sutherland 1988)
- Exocrine drainage into the bladder resulted in urologic complications; hence enteric drainage became the technique of choice (Adamec et al. 2004; Monroy-Cuadros et al. 2006).
- More recently, surgeons have developed a technique whereby the whole pancreas, together with a portion of duodenum, is anastomosed into the recipient's duodenum (Hummel et al. 2008). The safety of this new technique is being evaluated for future use (Squifflet et al. 2008).

The results of pancreatic transplants, in terms of patient survival and graft function, were documented by the ACS/NIH Organ Transplant Registry between 17th December 1966 and 30th June 1977. After this period the International Registry for Pancreas and Islet Transplantation in Minneapolis, under the guidance of Dr. David Sutherland, has taken on the task of monitoring patient survival and graft function post transplant (Sutherland 1980). The valuable data accumulated by these two registries have shown that both patient survival and pancreatic graft function have improved immensely since the first experimental transplants (Sutherland 1988).

This improvement in clinical results has been further consolidated since the introduction of cyclosporine (a potent immunosuppressant) in 1978, with patient survival rising to around 90% and 1-year pancreatic function to between 60 and 90% (Sutherland 1980). When the statistics concerning patient survival and graft function are considered, whole pancreas transplantation appears to be better than insulin injection regimes in terms of addressing physiological insulin replacement. However, there are limitations surrounding this procedure that result in it not being the perfect solution:

- The number of multi-organ donors per year does not nearly match the number of people who would benefit from a pancreatic transplant, thus there remains a huge tissue shortfall (Calafiore 1997).
- Whole pancreas transplantation requires major surgery and the risk of patient morbidity and even mortality has not been completely overcome (Rosen et al. 1991). Other methods of replacing insulin-secreting tissue, such as isolated islet transplantation, are much less invasive and dangerous.
- Although pancreatic transplantation, when successful, is able to restore glucose homeostasis it is a procedure that requires lifelong immunosuppression, thus rendering it unsuitable for the otherwise healthy insulin dependent patient (Calafiore 1997).
- Pancreatic transplantation is usually reserved for patients undergoing simultaneous kidney transplant for end-stage renal failure or for patients who have previously had a kidney transplant (Sutherland 1988).

Date	Pancreas transplantation milestone
1889	Minkowski and von Mering show that the 'anti-diabetic' substance is produced by the pancreas – by pancreatectomy in dogs
1893	Dr. P. Watson Williams implants pieces of fresh sheep's pancreas subcutaneously in a 15-year old boy at Bristol Royal Infirmary – without success
1924	Pybus implants sliced human pancreas subcutaneously in 2 patients – one shows a temporary reduction in urinary glucose levels
1928	Fichera transplants foetal pancreas under the tunica vaginalis - the patient dies of ketotic coma
1966	Lillehei performs the first vascularised pancreas transplant
1978	Dubernard employs the segmental Neoprene injection technique and whole pancreas transplants become more widely accepted
1978	The potent immunosuppressant cyclosporine is introduced and clinical results are significantly improved

Figure 3. A timeline of some of the milestones in the development of whole pancreas transplantation as a clinical procedure.

Islet transplantation

Whole pancreas transplantation has many logistical limitations for routine application in the treatment of diabetes (see summary above); hence researchers turned their attention to the possibility of transplanting only the insulin secreting tissue contained within the pancreas (Morris et al. 1989).

The early years of islet isolation and transplantation

The initial attempts to isolate islets from the pancreas began with Bensley in 1911 when he stained islets in the guinea pig pancreas with a number of vital dyes and subsequently removed them by microdissection (Bensley 1911). Norberg performed the first quantitative biochemical analyses of isolated islets in 1942 (Hellerstrom 1964) when he microdissected islets from pancreata of rats and guinea pigs as well as enlarged islets from obese, hyperglycaemic ob/ob mice. In 1965 Keen, Sells and Jarrett microdissected islets from duct-ligated pancreata of rats (Keen et al. 1965). While microdissection produced islets for biochemical and physiological study, traumatic isolation, small numbers of islets and the pathological state of the pancreas from which they were isolated limited use of these islets to *in vitro* research.

The need for a greater number of viable islets for *in vitro* study prompted the development of enzymatic dispersion of the pancreas to free the islets from the acinar tissue prior to isolation. Moskalewski first used the enzyme complex collagenase in 1965 to digest chopped guinea pig pancreas and although some islets were destroyed, the acinar tissue was preferentially digested, allowing for the isolation of larger numbers of viable islets that responded to glucose stimulation (Moskalewski 1965). In 1967 Lacy and Kostianovsky improved the technique by intraductal distension of the pancreas prior to digestion and by employing

sucrose gradients for centrifugal separation of the islets from the pancreatic digest (Lacy and Kostianovsky 1967). Sorenson achieved more efficient separation in 1968 with the use of Ficoll density gradients (Sorenson 1968).

Ballinger and Lacy reported the first major attempt at islet transplantation in 1972 when they performed intraperitoneal or intramuscular transplants of 400 – 600 islets into streptozotocin-diabetic rats and achieved long-term amelioration of hyperglycaemia, polyuria and glycosuria (Ballinger and Lacy 1972). Diabetes recurred after excision of the intramuscular transplants, and histological examination of the explanted tissue revealed intact alpha cells and degranulated β-cells. Various groups achieved more consistent successes between 1973 and 1977 when islets were transplanted into the portal vein or splenic pulp than at other sites; a suggestion arose that fewer islets could be used to ameliorate diabetes when they were transplanted into sites with portal venous drainage (Kemp et al. 1973; Federlin et al. 1976; Gray and Watkins 1976; Feldman et al. 1977; Finch et al. 1977).

Attempts were made to apply the technique developed by Lacy and Kostianovsky to large animal pancreata such as pig (Sutherland et al. 1974), monkey (Scharp et al. 1975) and dog (Lorenz et al. 1979). Despite claims of success, little evidence was presented to show post-transplant graft function (Sutherland 1981). Clinical trials of both auto- and allotransplantation were attempted and success was claimed (Najarian et al. 1977; Cameron et al. 1980; Valente et al. 1980; Traverso et al. 1981), but once again little evidence existed regarding graft function and long-term insulin independence. In 1979 Downing compared venous and ductal distension of the canine pancreas to improve islet isolation (Downing et al. 1979) and in 1981 Horaguchi and Merrell reported a technique for isolating canine islets in which the pancreas was retrogradely perfused through the pancreatic duct with collagenase at 37°C (Horaguchi and Merrell 1981). Noel, Mintz and colleagues later employed Ficoll density

gradients to purify islets obtained by a similar method (Noel et al. 1982). In 1984 Gray and colleagues successfully used a slight variation of this method on human pancreata (Gray et al. 1984) and in 1988 Ricordi and colleagues developed a semi-automated method in which the gland was placed into a sealed chamber during the digestion phase and constant agitation (enhanced by metal spheres) encouraged dispersion of the pancreas (Ricordi et al. 1988). In many centers around the world this method, or variations thereof, is still used to isolate islets for clinical transplantation (Boker et al. 2001; Ricordi 2003; Paget et al. 2007).

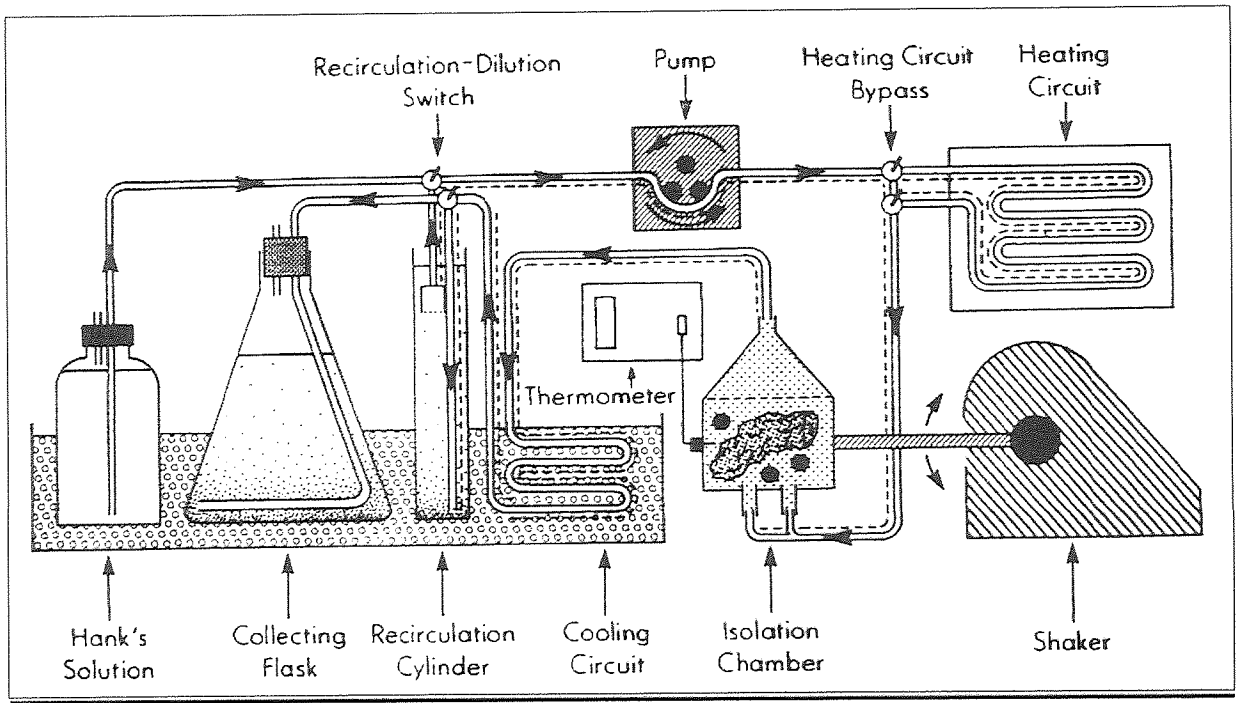


Figure 4. A diagram showing the semi-automated human islet isolation procedure. This image is reproduced with kind permission of Professor Camillo Ricordi of the Diabetes Research Institute at the University of Miami.

Date	Islet transplantation milestone
1869	Paul Langerhans first describes the islets in the pancreas
1911	Bensley stains islets in guinea pig pancreas and removes them by microdissection
1942	Norberg performs the first quantitative analysis of isolated islets following microdissection
1965	Keen, Sells and Jarrett microdissect islets from duct-ligated rat pancreata
1965	Moskalewski uses collagenase to digest chopped guinea pig pancreas and isolates viable islets
1967	Lacy and Kostianovsky develop intraductal distension of the pancreas prior to digestion and employ sucrose gradients for centrifugal separation of the islets form the pancreatic digest
1968	Sorenson achieves more efficient islet separation using Ficoll density gradients
1972	Ballinger and Lacy report the first major attempt at islet transplantation in rats
1973 - 1977	Various groups investigate different transplant sites – such as the portal vein and splenic pulp – with improved results
1979	Downing compares venous and ductal distension of the canine pancreas
1981	Horaguchi and Merrell isolate canine islets by retrograde perfusion of the pancreatic duct with collagenase at 37°C
1984	Gray and colleagues use a variation of this method to obtain human islets
1988	Ricordi and colleagues develop a sealed chamber with a 500μm mesh to improve the digestion phase of human islet isolation

Figure 5. A timeline of some of the milestones in the development of the technique for islet isolation.

Clinical islet transplantation

Clinical islet transplantation began in the early 1990s, but the success rate remained poor with only 8.2 percent of the 267 allografts transplanted between 1990 and 2000 achieving insulin independence for more than one year (Sutherland 1996; Shapiro et al. 2000). Early in 2000 Shapiro and colleagues in Edmonton (Canada) made several changes to previously used protocols to acquire highly purified endotoxin-free islets with minimal cold ischaemic time, they used a steroid-free immunosuppression regimen of sirolimus, tacrolimus and the anti-interleukin-2 receptor antibody daclizumab (Shapiro et al. 2000) and transplanted a large islet mass of more than 4,000 islet equivalents per kilogram of body mass per transplant, with a packed cell volume of less than 10ml. With this new protocol the Edmonton group reported 100% early success with islet transplants in seven type 1 patients (Shapiro et al. 2000; Reach 2001). The patients were selected only if they had type 1 diabetes for more than five years with uncontrolled serum glucose levels despite exogenous insulin therapy (Shapiro et al. 2000). Patients also had to have severe recurrent hypoglycaemia with metabolic instability or coma to ensure that the risk of transplantation and immunosuppression was less than that of continued uncontrolled diabetes (Shapiro et al. 2000). Six of the first seven patients received islets from two donors while the seventh required three islet implants to become insulin independent (Shapiro et al. 2000).

Of 35 patients transplanted by the Edmonton group by 2002, 87% remained insulin independent at one year (Burridge et al. 2002). Disappointingly, five years post-transplant only 10% of the islet graft recipients remained insulin independent even though 80% were producing C-peptide (Ryan et al. 2005).

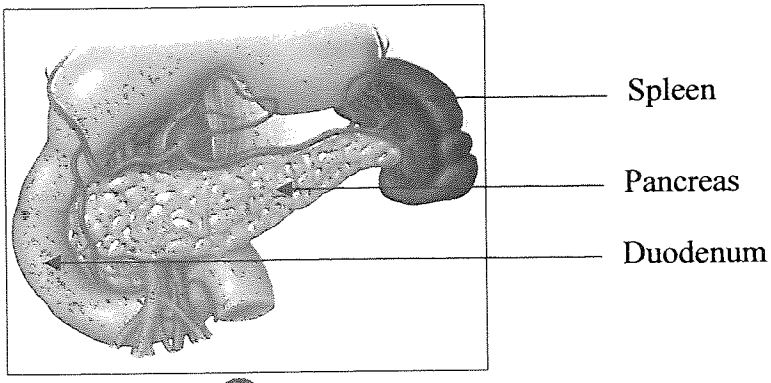
About 10 - 15% of people with diabetes have type 1 (Winter and Schatz 2003), and due to the strict selection criteria only 5 - 10% of type 1 diabetes sufferers are currently potentially

suitable for transplant (Breay 2003). The global lack of donors means that there are only enough pancreata to treat 0.5% of even this small group of type 1 patients (Shapiro et al. 2001).

A human pancreas contains about 1 million islets, of which less than half are usually recovered during isolation (Lakey et al. 2002). About 10 000 islets/kg of bodyweight are required to achieve insulin independence post transplant, thus more than one islet transplant per recipient is currently still required (Lakey et al. 2002).

In 2005 Shapiro and colleagues performed a distal pancreatectomy on the 56-year old mother of a 27-year-old woman with insulin-dependent diabetes since the age of 15 years, isolated the islets and successfully transplanted the graft into the portal vein of the recipient; who subsequently achieved insulin independence (Matsumoto et al. 2005). While this may be a significant advancement in the field, the long-term results are anxiously awaited with the disappointing 5-year data from recipients who have received islets from up to 3 donors lowering expectation in the minds of some scientists.

The donor pancreas is retrieved



Islets are isolated by enzyme digestion and density gradient centrifugation

Islets are infused into the portal vein of the transplant recipient. From here the insulin secreting structures enter the liver and lodge in the capillaries of the portal vein

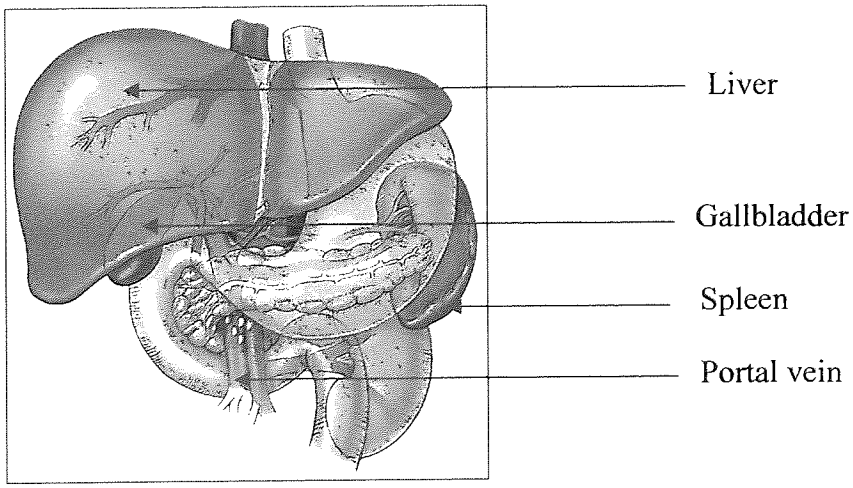


Figure 6. A diagram showing the process of islet transplantation.

Some possibilities for practical intervention

A major limitation to the success of islet transplants as a treatment for type 1 diabetes is the poor level of revascularisation and the time required to achieve it. Vascularisation of transplants is invariably less than endogenous native islets, which enjoy a disproportionally large and specifically routed blood supply (Samols et al. 1988). Damage sustained to the complex islet microvasculature during the isolation process, leaves the islets unable to induce re-vascularisation until about 10 - 14 days post transplant (Menger et al. 1992; Olsson and Carlsson 2006). This period of ischaemia clearly disadvantages the already traumatised islets, and constitutes a critical phase that will determine establishment of the transplant (Olsson and Carlsson 2006). A cocktail of toxic immunosuppressive drugs, which have been shown to impede revascularisation (Cross et al. 2007) and inhibit insulin secretion (Paty et al. 2002; Polastri et al. 2002; Cross et al. 2007), usually compounds the effect of ischaemia at this time. Enhancing the survival and function of transplanted islets could be achieved by interventions to:

- preserve functional viability and structural integrity of the islets and
- induce more rapid and greater revascularisation (Menger et al. 1992)

The development of improved transplantation and immunosuppression protocols suggest that transplantation of primary islets of Langerhans may offer a 'cure' for type 1 diabetes (Shapiro et al. 2000; Shapiro et al. 2001). However, the clinical impact of this treatment is limited by the availability of islet transplant material from human donors (Shapiro et al. 2001). Furthermore, most islet transplant recipients require islets from two to three donors in order to achieve insulin independence (Shapiro et al. 2000), placing an additional burden on the already inadequate donor pool. The limited donor material could be used more effectively by the development of procedures that increase the islet yield from donor pancreata (Tsujimura et al. 2002) and also by developing strategies to increase the biosynthesis and intracellular

storage of insulin in ß-cells while glucose responsiveness is maintained. If this can be achieved, a smaller number of islets would be required to restore glucose homeostasis in a graft recipient.

Preserving structural integrity and functional viability of islets/pseudoislets

Islet isolation employs a potent collagenase blend to enzymatically disrupt exocrine tissue, resulting in a pancreatic digest from which the islets can be extracted using density gradient centrifugation (Heald et al. 2000; Jay et al. 2004). The many variables between donors (i.e. age, medical history, method of pancreas retrieval, cold and warm ischaemia time, presence of fibrosis, damage to the organ/capsule etc.) affect the impact of enzymatic action on the tissue, hence incubation times vary significantly (from 20 minutes to 45 minutes) between pancreata to achieve an acceptable level of islet cleavage from exocrine tissue prior to purification (Heald et al. 2000; Balamurugan et al. 2003; Paget et al. 2007).

In the experience of the Islet Research Laboratory (IRL), even when extreme care is taken to quench enzyme activity promptly upon visualisation (using dithizone) of cleaved islets in the pancreatic digest, islet structure may become impaired during the purification procedure resulting in a frayed appearance and islet fragments of <50μm in the final preparation (Balamurugan et al. 2003; Murray et al. 2005). Furthermore, islets with a sound structural appearance upon isolation often disintegrate in conventional static culture (CSC, in 90mm Petri dishes) within 3 – 7 days and exhibit a corresponding loss of glucose stimulated insulin secretion (Beattie et al. 2002; Murray et al. 2005). This loss of insulin secretory capacity has been directly associated with the CSC method and a tendency for tissue aggregation that leads to central necrosis (Falorni et al. 1996). This phenomenon greatly reduces the post-isolation window of opportunity to utilise human islets for research, but a study by the IRL has shown

that employing a rotational cell culture system (RCCS) that maintains the islets in a microgravity environment supports insulin secretory function and preserves islet structure (Murray et al. 2005). The RCCS provides an opportunity to pre-treat islets prior to implantation to potentially assist both islet function and structure.

Employing the thiazolidinedione (TZD) class of anti-diabetic drugs together with the RCCS may improve the survival of islet grafts by:

- exploiting the improvement in insulin secretory function and structure of islets maintained in a RCCS (Murray et al. 2005)
- further enhancing insulin secretory capacity due to the beta-cell granulation and protective effect of TZDs (Buckingham et al. 1998; Campbell and Mariz 2007)
- possibly improving the rate of revascularisation of islet grafts due to upregulation of vascular endothelial growth factor (VEGF) by TZDs (Campbell and Mariz 2007), thus reducing central necrosis and supporting islet survival and function (see next section).

Improving the post-transplant rate of revascularisation

Damage to the islet microvasculature is an important factor leading to significant impairment of function in new transplants, and "the discovery of methods to accelerate vascularisation of the [islet] graft" has been identified as a crucial goal for the improvement of transplantation therapy of type 1 diabetes (Robertson 2002). The presence of necrotic exocrine tissue in islet preparations, enzymatic disruption of the endothelium during islet isolation, hypoxic and osmotic insults and lack of larger blood vessels are all believed to contribute to poor vascularisation of islet grafts and hinder the rate of revascularisation (Heuser et al. 2000).

Islets receive 5-10 times more blood per volume of tissue than the exocrine cells, and β-cells are often bordered by two or more capillaries (Lifson et al. 1980). Bonner-Weir and Orci (Bonner-Weir and Orci 1982) provided evidence for a preferred route of normal islet blood flow: arterioles penetrate the β-cell rich centre of the islet and form a capillary network that coalesces into collecting venules as they leave the islet. This intricate islet microvasculature is damaged during islet isolation, which may be one of the leading causes of central necrosis in cultured islets and of the loss and/or reduced function of transplanted islets.

Studies of transplanted mouse and human islets demonstrated marked decreases in vascular density and pO_2 of the transplanted islets when compared to endogenous islets (Carlsson et al. 2002), and this reduced vascularisation was observed whether the islets were grafted under the kidney capsule, intra-splenically, or intra-hepatically via the portal vein (Carlsson et al. 2002; Mattsson et al. 2002). Islet β-cells require a high oxygen consumption to allow glycolytic metabolism to regulate insulin secretion, so the reduced oxygenation as a consequence of reduced vascularisation is likely to be detrimental to physiological function. It is considered that high vascularisation of implanted islets would restore adequate oxygenation and largely re-establish lost physiological function as far as possible without re-innervation. Moreover, the route of blood flow through the tissue is probably not likely to be a limiting factor provided that the extent of vascularisation is sufficient to supply normal levels of oxygenation.

In most experimental studies to date the transplant material has been introduced without any targeted strategy to ensure revascularisation. Nonetheless, revascularisation of transplanted primary islets does occur with the development of a glomerulus-like network of capillaries similar to that seen in intact islets (Beger et al. 1998). However, in several studies Bailey and colleagues noted and reported poor vascularisation to the core of implanted islets and

aggregations of β-cells (Stewart et al. 1993; Stewart et al. 1994). Subsequent studies showed that growth of β-cell aggregates occurs mainly at the periphery and thus attracts new vascularisation at the periphery at the expense of the core (Bailey et al. 1999). When vascularisation is improved at the core, then peripheral growth of β-cell aggregates is reduced, potentially enabling greater overall insulin production from fewer cells.

The vascular endothelial growth factor (VEGF) family of genes plays an important role in the growth and differentiation of vascular endothelial cells (Ferrara 2000). $VEGF_{165}$ is the major isoform of the VEGF family, and has been used to enhance revascularisation in a variety of experimental models after administration of recombinant $VEGF_{165}$ (Takeshita et al. 1994), or by transfection with cDNAs encoding $VEGF_{165}$ (Mack et al. 1998). Gene transfer experiments with retroviral vectors containing VEGF showed enhanced microvessel sprouting from aortic rings embedded into a collagen matrix (Alian et al. 2002).

VEGF has also been shown to promote neovascularisation *in vivo* in a range of tissues. Intravitreal administration of VEGF by injection (Tolentino et al. 1996) or slow-release implants (Ozaki et al. 1997) induced retinal neovascularisation in rabbits and primates. In a pig model of chronic myocardial ischaemia VEGF has been shown to stimulate restorative myocardial angiogenesis (Sato et al. 2001). Furthermore, intra-arterial injection of adeno-associated viral vector mediated delivery of VEGF resulted in arteriogenesis in ischaemic rat hindlimbs (Chang et al. 2003).

VEGF appears to be important in regulating islet angiogenesis, since human islets contain the mRNAs for at least two isoforms, $VEGF_{121}$ and $VEGF_{165}$ (Gorden et al. 1997). Furthermore, islet VEGF mRNA levels were increased by hypoxia or by prolonged maintenance in culture *in vitro*, suggesting that conditions associated with a reduction or abolition of vascular flow

result in enhanced signals for islet angiogenesis (Gorden et al. 1997). Accordingly, it has been suggested that the decreased revascularisation of islets from older donors reflects an age-dependent reduction in VEGF production by the islets (Carlsson et al. 2002). Thus, VEGF mRNA is expressed in islets, and the VEGF protein produced is bioactive (Gorden et al. 1997).

Isolated islets contain their own vascular endothelium, but it seems likely that the revascularisation of transplanted islets occurs predominantly through the invasion of the islets by host endothelium. Interestingly, in non-human primates, the new capillary supply to transplanted islets persists after the immune destruction of the transplanted islets, suggesting that the vascular supply was of host origin (Hirshberg et al. 2002). Similarly, a capillary network formed after transplantation of islet-like structures that had been re-formed *in vitro* from only islet endocrine cells, excluding endothelial cells (Beger et al. 1998). This demonstrates that the capillary bed can form entirely from the host tissue. However, the capillary bed formed more rapidly when intact islets were transplanted (Beger et al. 1998), perhaps suggesting that the presence of donor endothelial cells facilitates the process of revascularisation (Olsson and Carlsson 2005).

Studies have noted that agonists of the peroxisome proliferator-activated receptor gamma (PPARγ), such as the thiazolidinediones rosiglitazone and pioglitazone, increase expression and action of VEGF by endothelial cells (Yamakawa et al. 2000; Biscetti et al. 2008). PPARγ is expressed by β-cells (Dubois et al. 2000) as well as by endothelium (Bishop-Bailey 2000; Biscetti et al. 2008). Thus, stimulation of PPARγ by a thiazolidinedione (TZD) provides a potential route to increase VEGF-mediated vascularisation of islets and β-cells. This approach has the additional advantage that TZDs have been reported to increase islet β-cell granulation (Buckingham et al. 1998; Campbell and Mariz 2007) and may support β-cell function after

the trauma of being isolated and transplanted. Also TZDs are already an established clinical class of agents to improve insulin action and glycaemic control in diabetic states where insulin is present (Bailey and Day 2001).

Pilot studies suggest that the concentration of TZDs required to hyperstimulate VEGF production and action in the islet may be greater than normal therapeutic concentrations. Also, prolonged high concentrations of TZDs with insulin are prone to cause capillary leakage and oedema (Idris et al. 2003). For this thesis, a study employing exposure to a TZD to promote VEGF production will be undertaken *in vitro* prior to implantation.

β-cell models

Due to the scarcity of human tissue for research, a number of cell lines have been developed for use as models for certain human cell types. Beta cell models available include:

- UMR407/3 rat beta cell line (Ng et al. 1987)
- βTC1, βTC2, βTC3, βTC-tet, βTC6-f7 mouse beta cell lines (Efrat et al. 1988; Brant et al. 1992; Efrat et al. 1993; Knaack et al. 1994)
- HIT-T15 hamster and RIN rat beta cell lines (Eddlestone et al. 1989; Brant et al. 1992)
- IgSV195 mouse beta cell line (Gilligan et al. 1989)
- MIN6 mouse beta cell line (Miyazaki et al. 1990)
- BRIN-BD11 rat beta cell line (McClenaghan et al. 1996)
- INS-1 rat beta cell line (Poitout et al. 1996)
- βlox5 human beta cell line (de la Tour et al. 2001)
- CM human beta cell line (Cavallo et al. 1996; Monetini et al. 1999)

This is not intended to be an exhaustive list of the existing beta cell lines, but an indication of the efforts that have been made to produce useful cells for research when human tissue is not available.

For the purposes of this study the MIN6 and BRIN-BD11 beta cell lines were assessed (see chapter 4 for more detail), resulting in the BRIN-BD11 cell line being selected as a beta cell model, kindly supplied by Professor Peter Flatt from the University of Ulster, Northern Ireland.

Hypothesis

The main aim of this research is to test the hypothesis that supporting β-cell structure and function as well as increasing the rate of islet revascularisation post-transplant will improve graft function and survival. The objectives are as follows:

- to develop a method to co-culture the rat-derived beta cell line BRIN-BD11 (D11) with human umbilical vein endothelial cells (HUVEC), both in static monolayer culture and rotational cell culture, and to investigate the effect of co-culture, culture medium and method of culture on both D11 and HUVEC morphology and viability; and on D11 function
- to apply this knowledge to the culture of human islets both in static and rotational cell culture, and to investigate the effect of culture medium and method of culture on human islets
- to assess *in vitro* stimulation of insulin production/secretion in human islets and D11 pseudoislets and also endothelial VEGF production – from HUVEC incorporated into D11 pseudoislets and from endogenous human islet endothelial cells - with a thiazolidinedione (TZD)
- to initiate development of an *in vivo* protocol for testing of the effect of culture method and TZD pre-treatment by transplantation

Chapter 2: Materials and methods

Cell preparation and tissue culture

MIN6 cells thawing and seeding

MIN6 cells were obtained from Dr. Steve Ashcroft at Oxford University by kind permission of Professor Jun-ichi Miyazaki from the Osaka University Medical School, Japan. A vial of MIN6 cells was removed from the liquid nitrogen store, transported to the Islet Research Laboratory (IRL) in the vapour phase of liquid nitrogen, sprayed with ethanol (Pharmacy Department, Worcestershire Royal Hospital, UK), briefly transferred to a tissue culture hood (Hereaus LaminAir HB2448, from Thermo Life Sciences Ltd, Essex, UK – now Thermo Fisher Scientific) and the cap loosened by a quarter turn to relieve pressure, then very quickly thawed in a 37°C water bath (Grant Instruments Cambridge Ltd, Cambridgeshire, UK) with care taken not to submerge the vial.

The cell suspension was transferred to a sterile 15ml centrifuge tube (Appleton Woods, Birmingham, UK) and 5ml Dulbecco's Modified Eagles Medium (DMEM with 25mM glucose, Sigma Chemical Company, Dorset, UK), supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin (all from Sigma) was added very slowly in a drop-wise manner, as rapid dilution of dimethyl sulphoxide (DMSO) would cause severe osmotic damage to the cells. The cell suspension was centrifuged (Mistral 3000i with swing-out rotor, Meadowrose Scientific, Oxfordshire, UK) at 300 x g and the supernatant was discarded. The cell pellet was resuspended in 5ml tissue culture medium (TCM) and transferred to a sterile T25 flask (Appleton Woods). Incubation was at 37°C in a humidified incubator with 5% CO_2 (Nuaire 5500E, Triple Red Laboratory Technology, Oxfordshire, UK). Medium was changed the following day by transferring the culture flask to a sterile tissue culture hood and aspirating the medium with a sterile 10ml plastic pipette (Triple Red

Laboratory Technology). The medium was discarded and fresh medium slowly added with a new sterile pipette.

MIN6 cells monolayer and rotational culture and passage

MIN6 cells were cultured in DMEM supplemented as described above. They were maintained in T25 (5ml medium) tissue culture flasks and incubated at 37°C in a humidified incubator with 5% CO_2. Medium was changed 24h post-thawing and then every second day by transferring the culture flask to a sterile tissue culture hood and aspirating the medium with a sterile 10ml plastic pipette. The medium was discarded and fresh medium slowly added with a new sterile pipette. Once a flask reached 80% confluence (figure 7), the cells were passaged as follows: the flask was placed in a sterile tissue culture hood and the medium was removed, 5 ml sterile phosphate buffered saline (PBS, from Sigma) was added, removed and discarded and this rinsing procedure was repeated. 0.5ml Trypsin-EDTA solution (Sigma) was added and the flask was left at room temperature for 3 - 5 minutes. The flask was then firmly tapped against the palm of the hand and when the cells showed evidence of slipping away from the surface, 5ml medium with 10% FBS was added to quench the action of trypsin.

The contents of the flask were transferred to a sterile 15ml centrifuge tube and centrifuged at 300 x g for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in 3ml medium. 1ml of the cell suspension was added to each of three T25 flasks containing 4ml medium (final volume 5ml/flask). The flasks were placed into a humidified incubator at 37°C with 5% CO_2.

For rotational culture (figure 7) of MIN6 cells, the rotator base of a Synthecon rotational cell culture system (RCCS- Cellon, Luxembourg) was thoroughly cleaned and placed in a humidified incubator at 37°C with 5% CO_2. A 10ml capacity, sterile high aspect rotational

vessel (HARV - Cellon) was transferred to a tissue culture hood, the end caps were removed and placed into a sterile petri dish and the syringe ports were opened. Using a sterile pastette (Appleton Woods), 5ml DMEM tissue culture medium was slowly inserted into the vessel via the 0.5-inch port (see figure 8). Next, the cells from two 80% confluent T25 flasks were placed into a sterile 15ml tube and centrifuged 300 x g for 3 minutes. The cells were resuspended in 4ml medium and added to the HARV via the 0.5-inch port and finally, 1ml FBS was added to provide the required 10% serum content of the medium. The port was wiped with an alcohol pad (NHS Supplies, Worcestershire Royal Hospital, UK), the cap replaced and tightened, and the syringe ports closed.

A 10ml syringe (NHS Supplies) was filled with DMEM tissue culture medium, the left syringe port was wiped with an alcohol pad and the syringe was attached to the port. The right syringe port was wiped with an alcohol pad and an empty 5ml syringe (NHS Supplies) was attached. Both ports were opened and the vessel was slowly tilted and gently tapped to manoeuvre air bubbles under the right port with the empty 5ml syringe. By gently depressing the plunger of the 10ml syringe that contained medium, the air bubbles were expelled through the right port and into the empty 5ml syringe.

Once all bubbles had been removed, the right port was closed and the 5ml syringe was discarded. The right port was once again cleaned with an alcohol pad and the end cap was replaced. The 10ml syringe was left in place with the port open (as the volume of medium in the vessel may vary with a temperature change) and the vessel was transferred to the incubator and attached to the rotator base. The rotation speed was set to 8 rotations per minute as per manufacturer's instructions for suspension cell cultures.

The medium was changed every second day by detaching the vessel from the rotator base and transferring it to a sterile tissue culture hood. The cells were allowed to settle to the bottom of the vessel with the ports facing up. The 10ml syringe that was attached to the left port was discarded, the end cap of the right port removed, and both ports wiped with alcohol pads and opened. An empty 20ml syringe (NHS Supplies) was attached to the right port and the plunger was very slowly withdrawn to remove approximately half of the conditioned medium, while care was taken not to remove any cells. A 10ml syringe filled with medium was attached to the left port and an empty 5ml syringe to the right port. The vessel was once again filled with medium and the procedure for removing bubbles was repeated.

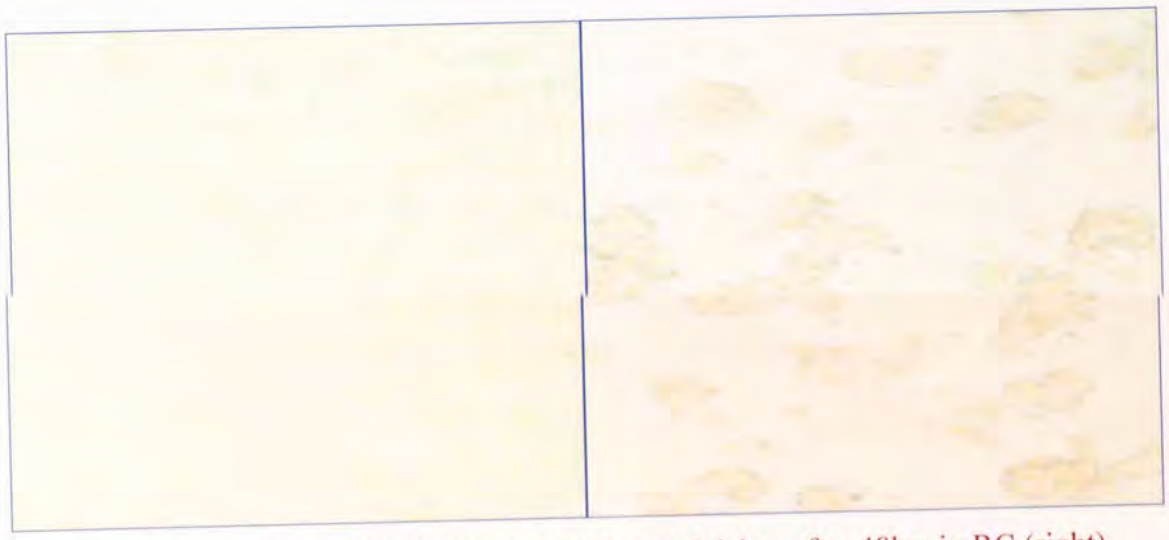


Figure 7. MIN6 monolayer cells (left) and MIN6 pseudoislets after 48hrs in RC (right).

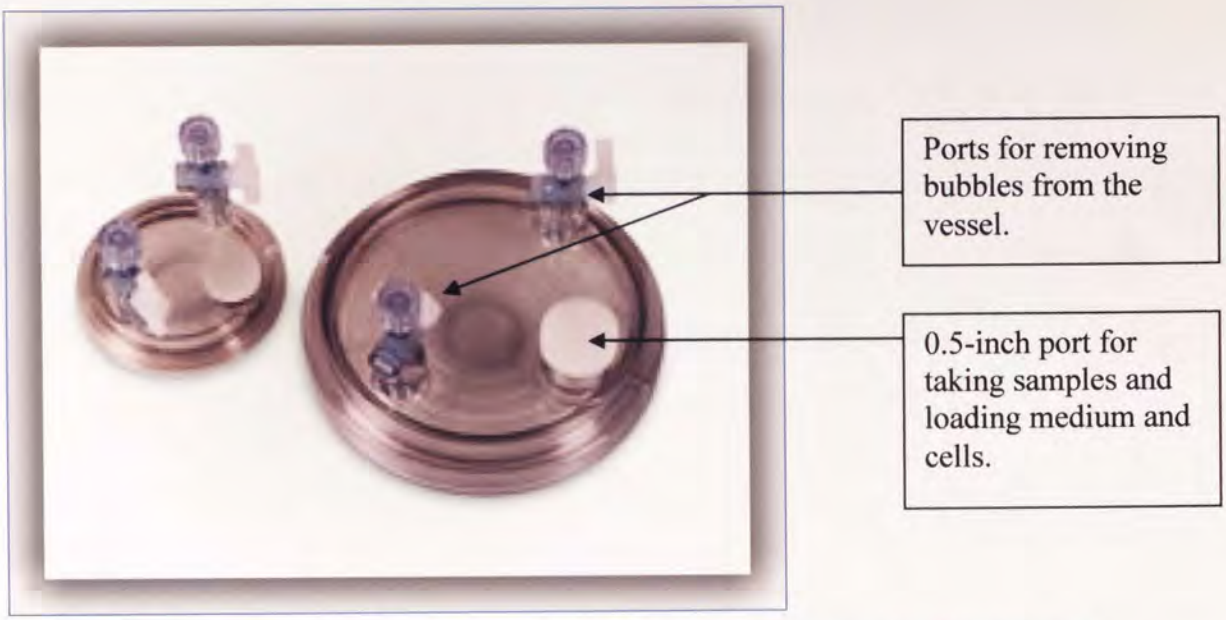


Figure 8. A high aspect rotational vessel (HARV) used for rotational cell culture in conjunction with a Synthecon rotational cell culture system (RCCS).

MIN6 cells cryopreservation

The medium from an 80% confluent T25 flask was removed and discarded, 5ml sterile PBS was added as a rinse, removed and the procedure was repeated. 0.5ml Trypsin-EDTA was added and the flask was left at room temperature for 3 - 5 minutes. After 3 minutes the flask was firmly tapped against the palm of the hand and as soon as cells showed evidence of slipping away from the plastic surface, 5ml medium with FBS was added to quench the action of trypsin. The cell suspension was transferred to a sterile 15ml centrifuge tube and centrifuged at 300 x g for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in 1ml FBS. 1ml of a 20% DMSO (sterile, Sigma) and 80% FBS was prepared and added very slowly in a drop-wise manner to the cell suspension (on ice). 1ml of the cell suspension was transferred to each of 2 labelled (cell-line, date, passage number) cryovials, which were then placed into a cryo freezing container (Nalge Europe Ltd, Hereford, UK) that employs 100% isopropyl alcohol (Sigma) to allow a temperature decrease of 1°C/minute. The container was placed into a -80°C freezer (Sanyo VIP Series, MJ Patterson Scientific Ltd, Luton, UK) overnight. The following day the vials were transferred to a liquid nitrogen store.

BRIN-BD11 cells thawing and seeding

BRIN-BD11 cells were provided by Professor P. R. Flatt, University of Ulster, Northern Ireland. A cryovial of D11 cells, containing the cells from a confluent T75 tissue culture flask, was removed from the liquid nitrogen store and thawed using the same method described above.

The cell suspension was transferred to a sterile 15ml centrifuge tube with 5ml RPMI 1640 TCM (supplemented with 10% foetal bovine serum, 100U/ml penicillin, 100μg/ml streptomycin - all from Sigma), centrifuged at 300 x g for 3 minutes and the supernatant was discarded to remove DMSO. The cell pellet was resuspended in 5ml medium and transferred to three sterile T75 flasks (Appleton Woods). Incubation was at 37°C in a humidified incubator with 5% CO_2. Medium was changed the following day by transferring the culture flask to a sterile tissue culture hood and aspirating the medium with a sterile 10ml plastic pipette. The medium was discarded and fresh medium added slowly with a new sterile pipette.

BRIN-BD11 cells monolayer and rotational culture and passage

D11 were maintained in RPMI 1640 TCM as a monolayer (figure 9) in T75 flasks or in a rotational cell culture system. BRIN-BD11 cells were passaged when ~80% confluence was reached. Medium was removed; the cells were rinsed with sterile PBS and detached from the culture plastic by adding 1ml trypsin-EDTA solution, gently swirling it around the flask, removing the excess, checking under a microscope for signs of the cells rounding up then firmly tapping the flask against a hard surface to detach the cells. TCM was added to form a cell suspension after which cells from one T75 tissue culture flask were plated into three T75 tissue culture flasks. Medium was changed at 24hrs post passage, then every 48hrs.

For rotational culture of D11 cells (figure 9), the same method as for MIN6 (as described above) was used with the following differences: D11 cells were cultured in RPMI 1640 culture medium and one RC vessel contained D11 cells from one 80% confluent T75 flask.

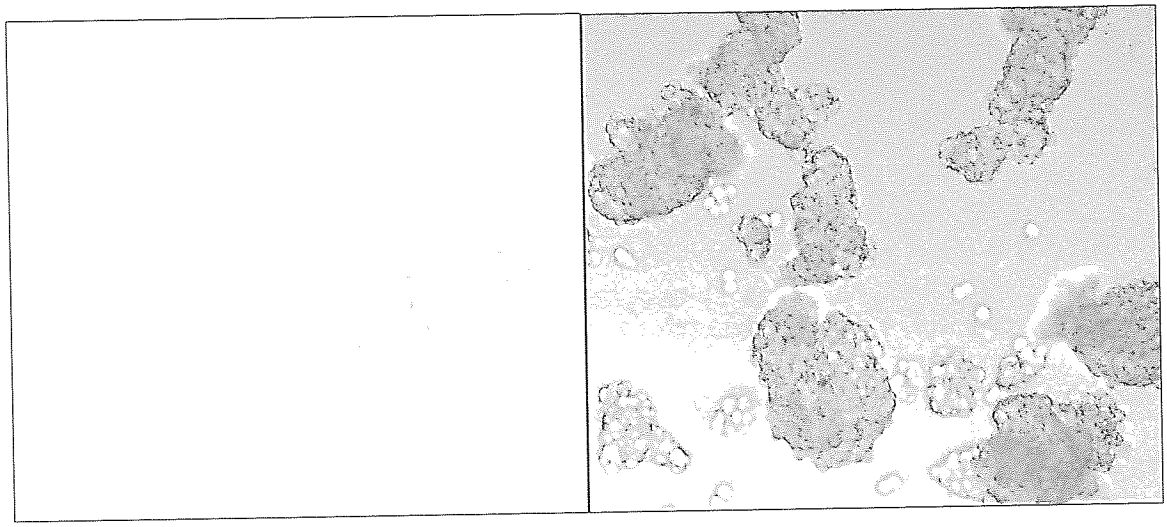


Figure 9. BRIN-BD11 cells grown as a monolayer (left) and in RC to form pseudoislets (right).

BRIN-BD11 cells cryopreservation

D11 cells from an 80% confluent T75 flask were harvested as for passage, centrifuged at 300 x g for 3 minutes. The cell pellet was suspended in 1ml freezing media containing 10%DMSO, 80% FBS and 10% TCM (RPMI 1640 + additives), transferred to a labelled cryovial (cell line, date, passage number) and placed into a cryo freezing container overnight in a -80°C freezer as for MIN6 cell cryopreservation. D11 were stored for up to 6 months in -80°C without loss of function/viability. A substantial stock of D11 was stored in liquid nitrogen.

HUVEC thawing and seeding

Human umbilical vein endothelial cells (HUVEC) and endothelial growth medium (EGM) were purchased from Lonza Wokingham Ltd., Berkshire, UK (formerly Cambrex Bio Science, Wokingham, UK). EGM was prepared by adding all the supplements to the basal

medium under sterile conditions. HUVEC were seeded at a density of ~3333 cells/cm^2 (the suggested range = 2500 - 5000 cells/cm^2), hence for each cryovial containing ~500 000 cells, two T75 tissue culture flasks were prepared. Each T75 flask received 15ml EGM and were placed in a 37°C, 5% CO_2 humidified incubator for at least 30 minutes to allow the vessels to equilibrate.

The cryovial was sprayed with alcohol before the cap was briefly twisted a quarter turn in a sterile field to relieve the pressure. The cap was retightened and the cryovial was quickly thawed in a 37°C water bath, with care taken not to submerge the entire vial. The cells in the cryovial were resuspended using a cell saver pipette tip and dispensed into the culture flasks set up earlier. The flasks were gently rocked to evenly distribute the cells and then returned to the incubator. Centrifugation was not performed to remove cells from the cryoprotectant cocktail as Lonza suggested that this action is more damaging to the cells than the effect of DMSO residue in the culture medium. Growth medium was changed the day after seeding and every second day thereafter.

HUVEC monolayer and rotational culture and passage

HUVEC were grown as a monolayer (figure 10) in T75 flasks in EGM and were passaged when ~80% confluence was reached. Medium was removed; the cells were rinsed with sterile PBS and detached from the culture plastic with 1ml/flask trypsin-EDTA solution, checking under a microscope for signs of the cells rounding up then firmly tapping the flask against a hard surface to detach the cells. EGM was added to form a cell suspension and HUVEC from one tissue culture flask were then replated into 3 tissue culture flasks of the same size or loaded into rotational culture vessels (as described above). Medium was changed at 24hrs post passage, then every 48hrs.

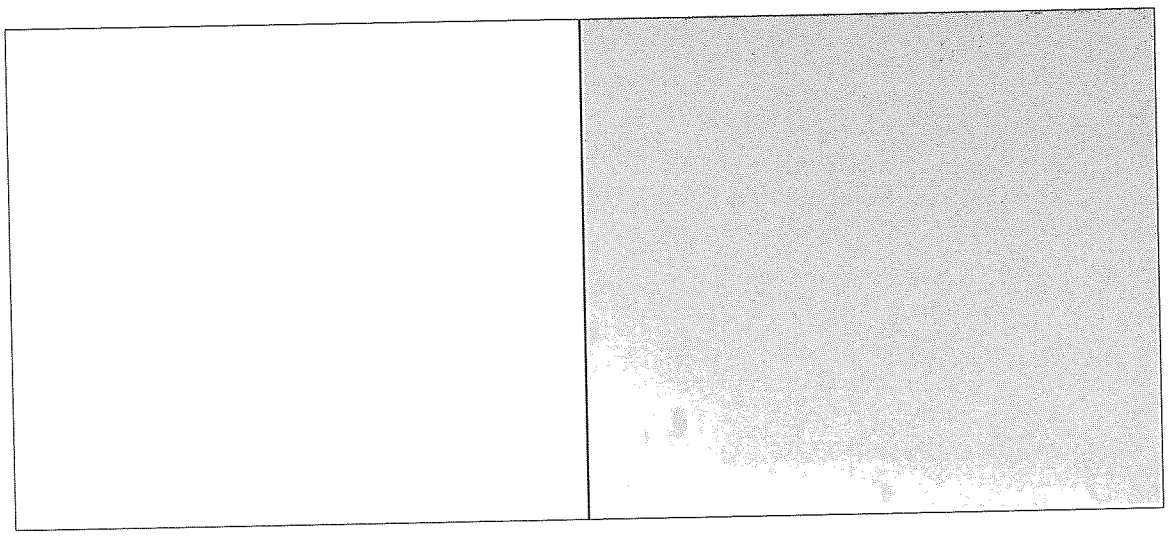


Figure 10. HUVEC grown as a monolayer (left) and in RC (right). Note that HUVEC in RC did not form aggregates and remained rounded, while in tissue culture flasks as a monolayer the cells adhered and flattened.

HUVEC cryopreservation

HUVEC were harvested from an 80% confluent T75 flask as for passage, centrifuged at 300 x g for 3 minutes and the cell pellet resuspended in freezing media consisting of 10% DMSO, 80% EGM and 10% FBS. The cell suspension was transferred to a labelled cryovial (cell type, date, passage number) and frozen in the same way as D11 overnight then transferred to liquid nitrogen as HUVEC did not respond well to storage at -80°C.

BRIN-BD11 and HUVEC co-culture monolayer and rotational culture

BRIN-BD11 and HUVEC were also co-cultured as a monolayer or in rotational culture (figure 11). Once co-culture commenced all experimental groups, including the controls, were grown in a 50:50 mixture of RPMI: EGM as this combination supported growth and morphology of both cell types.

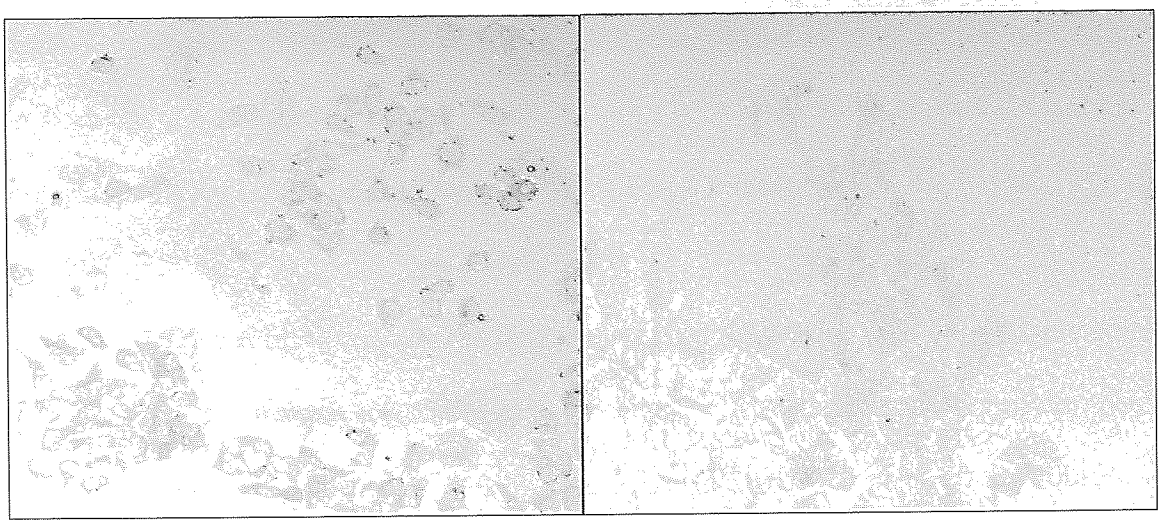


Figure 11. BRIN-BD11 and HUVEC co-culture as a monolayer (left) and in RC (right).

Human islet isolation

Human pancreata were obtained through the UK Human Tissue Bank (UKHTB), Leicester, England. The IRL at Worcestershire Clinical Research Unit has ethical approval from the Worcestershire Local Research Ethics Committee to isolate human islets for research purposes – 'A study of human islets for the treatment of diabetes mellitus'. LREC number: 47/98.

2L of Hanks' balanced salt solution (HBSS, Sigma) was prepared by adding 17ml/L of a sterile 30% solution of bovine serum albumin (BSA, Sigma) and cooling to 4°C. On delivery of the pancreas, 0.5g of Liberase (Roche Diagnostics Ltd., Sussex, UK) was reconstituted in 15ml sterile distilled, deionised water and placed in a refrigerator (Thermo Life Sciences) at 4°C for 30 minutes to disperse and equilibrate. During this time the pancreas was inspected visually for condition, colour, size and shape in a tissue culture hood. Adipose tissue, remnants of the spleen and duodenum, and blood vessels were trimmed away with sterile surgical instruments while care was taken to keep the delicate membranous capsule surrounding the pancreas intact. The weight of the 'cleaned' pancreas was noted and one sample (approximately 5g from the head of the gland) was fixed in formalin (Pharmacy

Department, Worcestershire Royal Hospital, UK). Two further samples, of the same size and also from the head, were taken for microbiological and virology assessment and stored in a refrigerator at 4°C until its delivery to the microbiology laboratories.

The 15ml Liberase solution was mixed with 135ml HBSS (without BSA), the pancreatic duct was cannulated, the enzyme solution was slowly injected into the duct to distend the gland and any leaks were clamped to aid distension. Upon completion the pancreas was placed into a sterile 1L glass beaker and a further 500ml HBSS (without BSA) was added. The beaker was sealed with parafilm (Appleton Woods) and placed into the 37°C water bath to warm the tissue and enzyme. Gentle manual agitation was employed and the temperature was checked periodically with a sterile probe (Jenway series 2000, Bibby Scientific, Essex, UK) and once the tissue reached 37°C, the beaker was transferred to the stirrer-incubator (Stuart Scientific, Surrey, UK) with a magnetic stirrer to ensure continuous agitation.

Digestion was allowed to take place for ~20 minutes before the pancreas was cut into approximately 6 pieces with sterile scissors to allow further access of the enzyme to undigested tissue. Digestion was not allowed to continue for longer than 40 minutes as islet fragmentation would occur. Once visual inspection under an Olympus CK2 inverted microscope (Olympus, Herts, UK) at x 10 magnification of a sample of the pre-separation digest stained with dithizone (DTZ, Sigma) revealed islets being freed from the exocrine tissue, the process was stopped by adding 2L cold HBSS with BSA.

The digest was transferred to sterile 250ml centrifuge tubes (Triple Red Laboratory Technology) and centrifuged at 150 x g for 3 minutes. The supernatant was removed and discarded and the pellet was resuspended in HBSS with BSA to further dilute and inactivate any remaining enzyme. Dispersion of the pellet was achieved by gently passing the cell

suspension up and down a sterile 10ml plastic pipette (Triple Red Laboratory Technology). In order to remove larger, undigested pieces of tissue the cell suspension was then passed through a sterile 500μm stainless steel mesh (constructed in-house), followed by centrifugation in sterile 50ml tubes (Triple Red Laboratory Technology) at 150 x g for 3 minutes.

The cell pellet was then resuspended in cold (4°C) University of Wisconsin (UW) solution (DuPont UK, Hertfordshire, UK) and kept on ice for 30 - 45 minutes to facilitate increased cell density of the acinar tissue in order to achieve better purification of the islet preparation with density gradient centrifugation (Robertson et al. 1992). A sample of this pre-separation digest was taken for microbiological assessment and a further sample was taken to perform islet counts. Figure 12 shows DTZ-stained islets from a pre-separation pancreatic digest.

The cell suspension in UW was centrifuged at 4°C for 2 minutes at 400 x g. For each 0.5ml of pellet obtained, enough Histopaque (Sigma) was prepared for one isolation column as follows: 10.4ml Histopaque + 2.6ml HBSS (with BSA) to give a total of 13ml. Thus if the UW pellet was 5ml, 130 ml Histopaque/HBSS was prepared.

The pellet was resuspended in the Histopaque/HBSS and aliquots of 13.5ml of the new suspension were transferred to sterile 50ml tubes. 5ml of a HBSS with BSA was slowly layered over the Histopaque/HBSS/cell suspension and subsequently centrifuged at 800 x g for 5 minutes. The top layer of HBSS, the interface between the two layers and ~2ml below the interface were removed with a sterile pastette and this fraction (containing the islets) was transferred into sterile 50ml tubes (3 fractions of about 8ml per 50ml tube). 25ml HBSS with BSA was added to each 50ml tube and they were centrifuged at 300 x g for 3 minutes. The supernatants were discarded and the pellets were resuspended in m199 tissue culture medium

(Sigma) supplemented with 10% foetal calf serum (FCS, First Link UK, Birmingham, UK), 100U/ml penicillin, 100µg/ml streptomycin, 10µg/ml amphotericin B (all from Sigma). A sample of the isolated islets was taken for microbiological assessment. Figure 13 shows post-separation islets stained with dithizone.

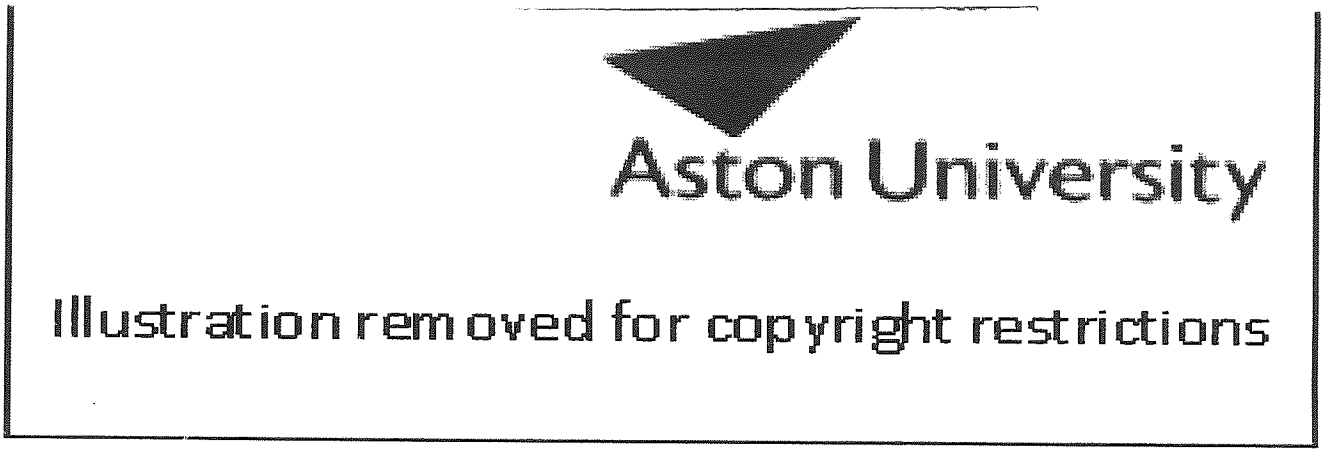


Figure 12. A sample of pre-separation human pancreatic digest stained with DTZ to distinguish islets from the exocrine tissue (Islet Research Laboratory, Worcester, UK).

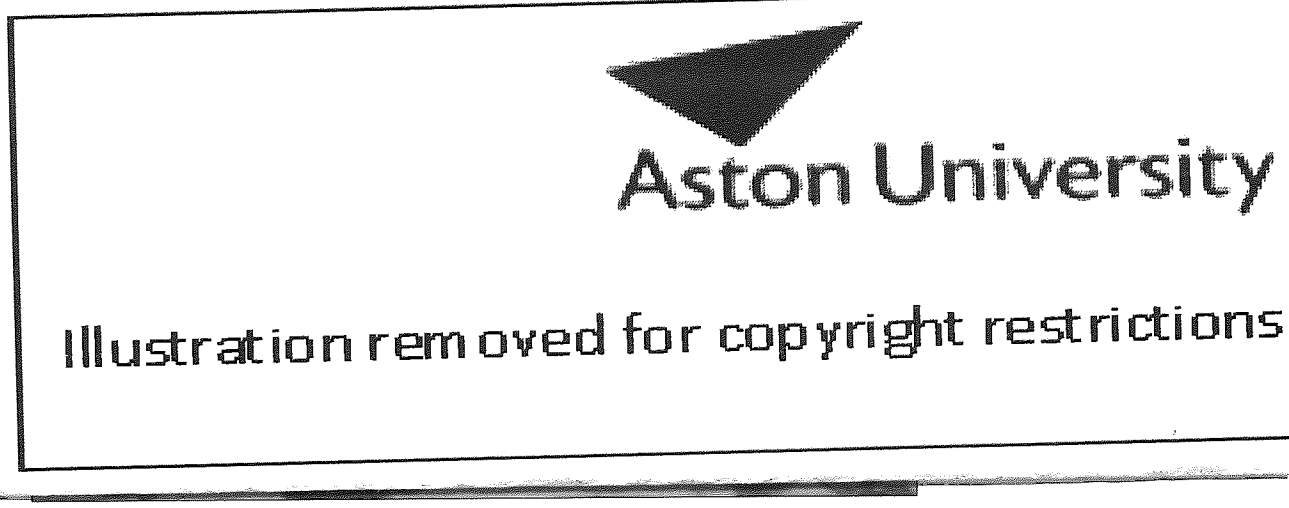


Figure 13. A sample of isolated human islets stained with DTZ to show the purity of the islet preparation (Islet Research Laboratory, Worcester, UK).

Diphenylthiocarbazone (Dithizone, DTZ) preparation

DTZ is a specific stain for islet tissue as it stains the zinc in the insulin granules, resulting in a characteristic orange-red stain. The acinar tissue is left unstained; hence both islet numbers and purity of the islet preparation can be assessed using this stain.

1% w/v stock solution:

15mg of DTZ powder was dissolved in 1.5ml of DMSO in a 15ml tube. After thorough mixing the solution was left for 5 minutes.

Working solution:

500µl of the stock solution (above) was added to 100ml HBSS, mixed well and left for 5 minutes. The solution was then filtered and kept for up to 1 week at 4°C and in the dark. A few drops of this solution were used to stain each of three 100µl aliquots of the islet preparation for counting.

Trypan blue preparation and islet viability scoring

Trypan Blue is a vital stain recommended for use in estimating the proportion of viable cells in a population. The principle is based on the fact that the chromophore is negatively charged and does not react with the cell unless the membrane is damaged. Staining facilitates the visualisation of cell morphology. Live cells do not take up the dye, while non-viable cells do.

1ml of a 0.4% Trypan Blue solution (Sigma) was added to 9ml saline solution to produce the working solution. A few drops of the working solution were added to the wells of a 24-well plate that contained the islet samples that had previously been stained with DTZ for counting. Ten random islets were scored as follows (Hesse *et al*, 1992):

- If none of the cells within the islet had taken up the dye, the islet received a score of 3
- If less than half of the cells within the islet had taken up the dye, the islet was given a score of 2
- If more than half of the cells had taken up the dye, the islet was allocated a score of 1
- When all 10 scores were added together, a total score of greater than 18 indicated a viable cell preparation.

Human islets static culture

3 x 100µl samples of the islet suspension were stained with DTZ, the islets were counted and the number of islet equivalents/ml calculated (see section 'Insulin secretion' below). Figure 12 shows islets stained with DTZ in a sample of the pre-separation digest, while figure 13 shows DTZ stained isolated islets. The same three samples were then subjected to a Trypan Blue test to give an indication of their membrane integrity and hence viability. The islet suspension was plated into 90mm Petri dishes (Appleton Woods) at a cell density of about 750 islet equivalents/ml of m199 with additives and placed into a humidified incubator (Heraeus B5060 from Thermo Life Sciences Ltd, Essex, UK) at 30°C with 5% CO_2. Medium was changed every 48 hours to maintain adequate levels of nutrients and to remove waste products and cell debris. Details of islet yield; viability and other characteristics are presented in the results section of this thesis.

Human islets rotational culture

The islets from one Petri dish (10ml, containing approximately 7500 islets) were transferred to a 15ml centrifuge tube, centrifuged at 300 x g for 3 minutes, the supernatant removed and the cell pellet was resuspended in 4ml m199. The islets were then loaded into a HARV vessel as described above with the following differences: the culture medium used for islets was m199 and the incubation temperature was 30°C.

Human islet cryopreservation

781µl DMSO was added to 4 219µl m199 tissue culture medium (supplemented as described above) to provide a 2M DMSO solution while a 3M solution was prepared by adding 1 170µl DMSO to 3 830µl supplemented m199. 30 000 islet equivalents (IEQ) were suspended in 1ml supplemented m199 in a sterile 15ml tube, 0.5ml 2M DMSO was added very slowly and the islets left at room temperature (RT) for 5 minutes before a further 0.5ml 2M DMSO was added and left for 25 minutes. 2ml 3M DMSO was added and left for 15 minutes prior to the tube being placed into ice slush for 5 minutes.

The islets were gently brought into suspension by careful use of a sterile pastette before being transferred (2 x 2ml) aliquots into sterile, labelled cryovials and placed into a cryo freezing container that ensures slow freezing of the cells by a temperature reduction of 1°C/minute. The container was placed into a -80°C freezer where the cells were stored until required.

Human islet thawing

A vial of frozen islets were selected and rapidly thawed in a 37°C water bath after which 20ml supplemented m199 TCM was added very slowly to prevent osmotic damage to the cells as the DMSO cryoprotectant diffused out of the islets. Centrifugation was not performed at this stage to remove DMSO as it was highly diluted by the volume of medium added. The islet preparation was allowed to stand at RT for 1hr prior to being placed into either static or rotational culture.

Insulin secretion

MIN6 cells monolayer

MIN6 cells were plated out into a sterile 24-well plate at a density of about 300 small cell clumps (<50μm) per well. 2ml DMEM, supplemented as described above, was added to each well. The plate was then placed into a humidified incubator overnight to allow the cells to adhere and recover from trypsinisation.

Insulin secretagogues (2mM glucose, 15mM glucose and 15mM glucose + 10mM theophylline – glucose and theophylline both from Sigma) were prepared in Hepes buffered Hanks' solution with 0.5% BSA, pH 7.4 at 37°C.

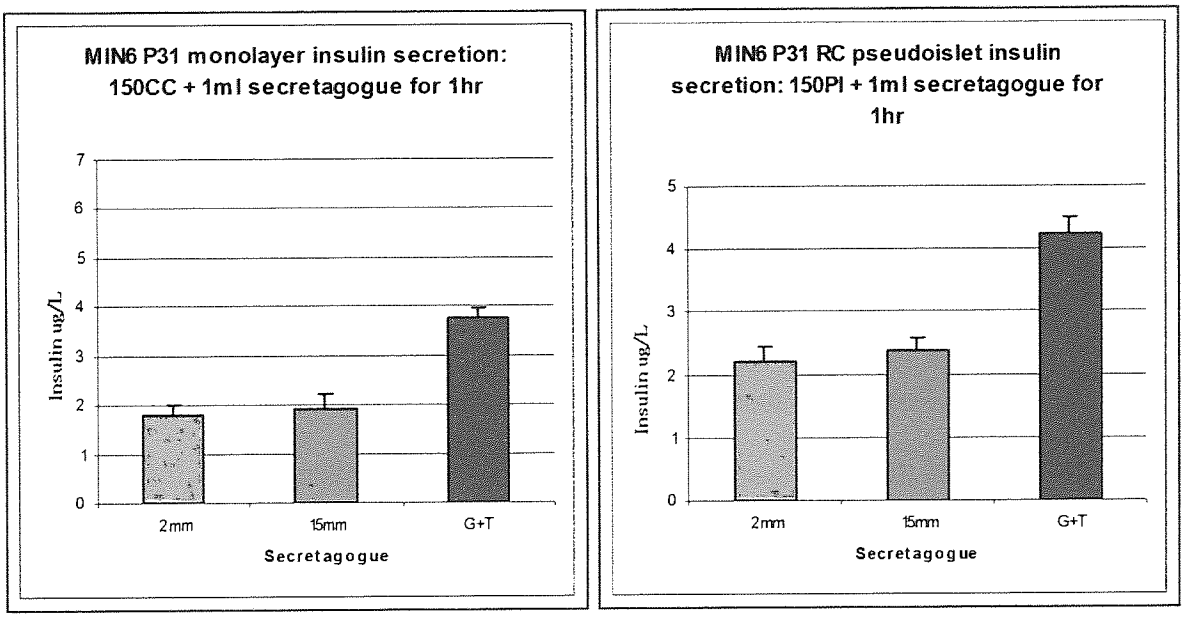
Prior to commencement of the static challenge, the culture medium was removed with a pipette and 1.5ml of a 2mM glucose solution added to each well. The solution was then removed and this wash step repeated. All wells then received 1.5ml of a 2mM glucose solution for 1 hour at 37°C to return the cells to basal insulin secretion.

Following the 1-hour pre-incubation period the 2mM glucose solution was removed and 1ml/well of the appropriate challenge solution was added for 1 hour at 37°C, after which the solutions were transferred to eppendorf tubes and frozen for future analysis (figure 14) by enzyme-linked immunosorbant assay (ELISA, Mercodia kit from Diagenics Ltd., Milton Keynes, UK).

MIN6 cells rotational culture

MIN6 pseudoislets were harvested from a rotational cell culture vessel and approximately 150 pseudoislets/well were placed into sterile 24-well plates (Appleton Woods) with 1ml/well of the culture medium and allowed to adhere by outgrowth for 24hrs. Insulin secretion

experiments were performed as for MIN6 monolayer described above and insulin determined by ELISA (figure 14).



Key:
2mM = 2mM glucose in Hepes buffered Hanks' solution with 0.5% BSA (HBSS + BSA)
15mM = 15mM glucose in HBSS
G+T = 15mM glucose + 10mM theophylline in HBSS
CC = cell clumps
PI = pseudoislets
Figure 14. An example of insulin secretion by static incubation from MIN6 monolayer cells (left) and RC pseudoislets (right).

BRIN-BD11 cells monolayer

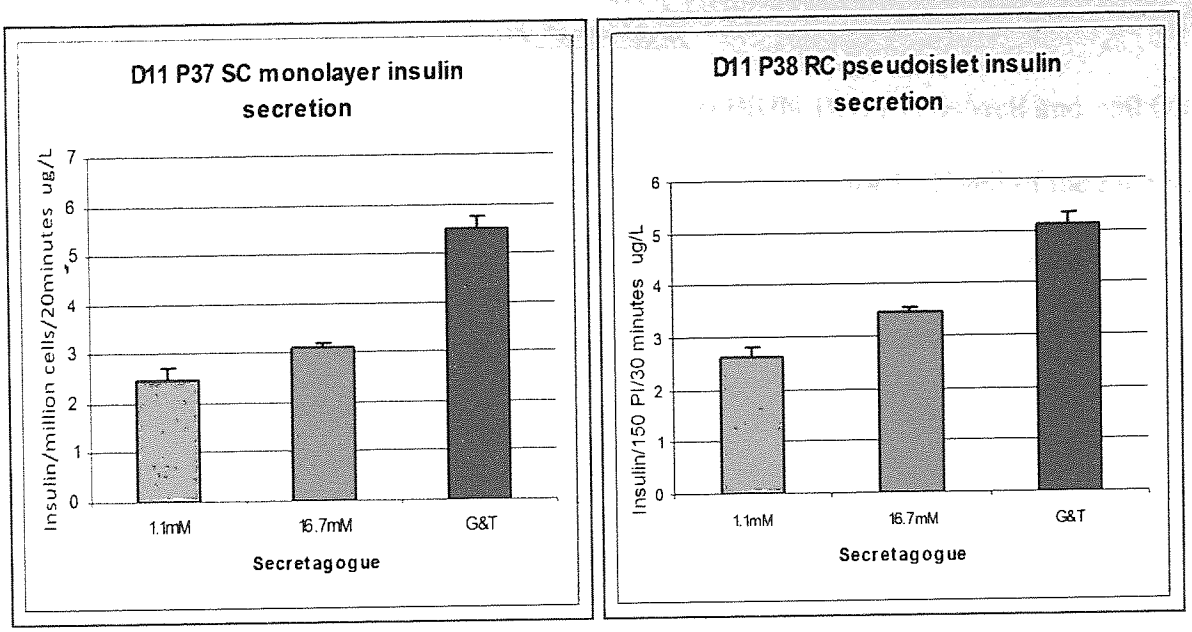
Approximately 175 000 BRIN-BD11 cells/well (SC) were placed into sterile 24-well plates with 1ml/well of the relevant medium (RPMI 1640 +/- 10mM of the TZD rosiglitazone) and allowed to adhere and grow for 24hrs.

Insulin secretagogues were prepared in Hepes buffered Krebs Ringer solution: 115mM NaCl, 4.7mM KCL, 1.28mM $CaCl.6H_2O$, 1.2mM KH_2PO_4, 1.2mM $MgSO_4.7H_2O$, 10mM $NaHCO_3$, 9.9mM Hepes, 0.1% BSA (all from Sigma Chemical Company). The secretagogues used were 1.1mM glucose, 16.7mM glucose and 16.7mM glucose + 10mM theophylline.

On the day of the insulin secretion experiment the culture medium was removed from the 24-well plate and the wells were washed once with 1.1mM glucose prior to receiving 1ml/well 1.1mM glucose. The plate was placed into an incubator at 37°C for an hour after which the 1.1mM glucose was removed and 0.5ml/well of the relevant secretagogue was added to the wells for 20 minutes with gentle agitation every five minutes. The supernatants were removed and insulin content (figure 15) was determined by ELISA.

BRIN-BD11 cells rotational culture

BRIN-BD11 pseudoislets were harvested from a rotational cell culture vessel and approximately 150 pseudoislets/well were placed into sterile 24-well plates with 1ml/well of the relevant medium (RPMI 1640 +/- 10mM rosiglitazone) and allowed to adhere by outgrowth for 24hrs. Static incubation insulin secretion (figure 15) was performed as for D11 monolayer with the exception of the incubation time with insulin secretagogues being 30 minutes as opposed to 20 minutes. This was to allow insulin from within the construct to diffuse into the supernatant.



Key:
SC = static culture
RC = rotational culture
PI = pseudoislets
1.1mM = 1.1mM glucose in Hepes buffered Krebs Ringer solution with 0.5% BSA
16.7mM = 16.7mM glucose in Krebs
G+T = 16.7mM glucose + 10mM theophylline in Krebs
Figure 15. An example of insulin secretion by static incubation from BRIN-BD11 SC monolayer cells (left) and RC pseudoislets (right).

HUVEC monolayer

Approximately 50 000 cells/well of HUVEC were placed into sterile 24-well plates with 1ml/well of the relevant medium (EGM +/- 10mM rosiglitazone) and allowed to adhere to the plastic overnight. Although it was not expected that these cells would secrete insulin, for control purposes the insulin secretion experiments and insulin detection were performed as described above.

HUVEC rotational culture

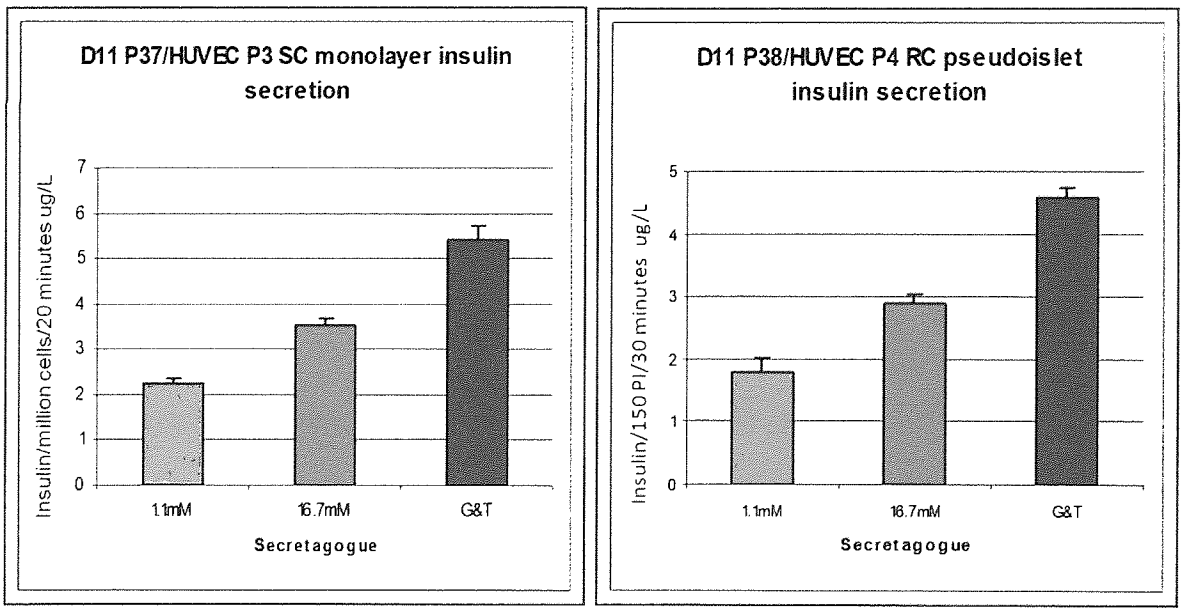
HUVEC were harvested from a rotational culture vessel and ~50 000 cells/well were placed into sterile 24-well plates, cultured and subjected to insulin secretion experiments and insulin detection as for monolayer above.

BRIN-BD11 and HUVEC co-culture monolayer

For monolayer co-culture insulin secretion ~175 000 BRIN-BD11 cells/well and ~50 000 cells/well of HUVEC were placed into sterile 24-well plates with 1ml/well of the relevant medium (RPMI: EGM +/- 10mM rosiglitazone) and allowed to adhere overnight prior to insulin secretion experiments and insulin detection being performed as described above (figure 16).

BRIN-BD11 and HUVEC co-culture rotational culture

For the rotational co-culture insulin secretion, ~ 150 pseudoislets/well from BRIN-BD11/HUVEC RC were placed into sterile 24-well plates with 1ml/well of the appropriate medium (RPMI: EGM +/- 10mM rosiglitazone) and allowed to adhere overnight prior to insulin secretion and detection being performed as described above (figure 16).



Key:
SC = static culture
RC = rotational culture
PI = pseudoislet
1.1mM = 1.1mM glucose in Hepes buffered Krebs Ringer solution with 0.5% BSA
16.7mM = 16.7mM glucose in Krebs
G+T = 16.7mM glucose + 10mM theophylline in Krebs

Figure 16. An example of insulin secretion by static incubation from BRIN-BD11 and HUVEC SC monolayer co-culture (left) and RC co-culture pseudoislets (right).

Human islets static and rotational culture

Islet preparations are very rarely 100% pure as there is inevitably a degree of exocrine tissue 'contamination'. In order to establish the purity of the isolated islet preparation, as well as the number of islets isolated, samples of the cell suspension were stained with DTZ. This stain binds specifically to the zinc found in insulin storage vesicles (red colour) and hence distinguishes islets/β-cells from exocrine tissue. Prior to performing insulin secretion experiments, an accurate count was required for all treatment groups in static and rotational culture to ensure equal numbers of islets used per ml of secretagogue.

Hepes buffered Hanks' solution was prepared with 0.5% BSA and adjusted to pH 7.4 at 37°C using a 2M solution of NaOH (Sigma). The following insulin secretagogues were prepared in the Hank's solution: 1.67mM glucose, 16.7mM glucose, 16.7mM glucose + 10mM theophylline. Islets were removed from the incubator (in static and/or rotational culture, +/- 25mM rosiglitazone) and, in a sterile environment; aliquots were transferred to 15ml tubes and centrifuged at 300 x g for 3 minutes. The supernatants were removed; the pellets were resuspended in 1.67mM glucose and placed into a rack in a 37°C water bath for one hour to ensure that the islets return to basal insulin secretion.

During this time a count was performed for each of the islet groups:
3 x 100µl samples were taken from each islet group and placed into wells of a 24-well plate with a grid on its base (grid drawn in-house). 0.5ml of the DTZ working solution were added to each well and left for 2-3 minutes to allow the characteristic red staining of the islets to develop. The islet sample counts were performed using the 10x eyepiece and the x4 objective to give a total magnification of x40. A calibrated grid was placed in one eyepiece of the inverted microscope to allow the islets to be assessed for size while the grid on the bottom of the 24-well plate allowed systematic counting of all the islets in each 100µl sample. The islets

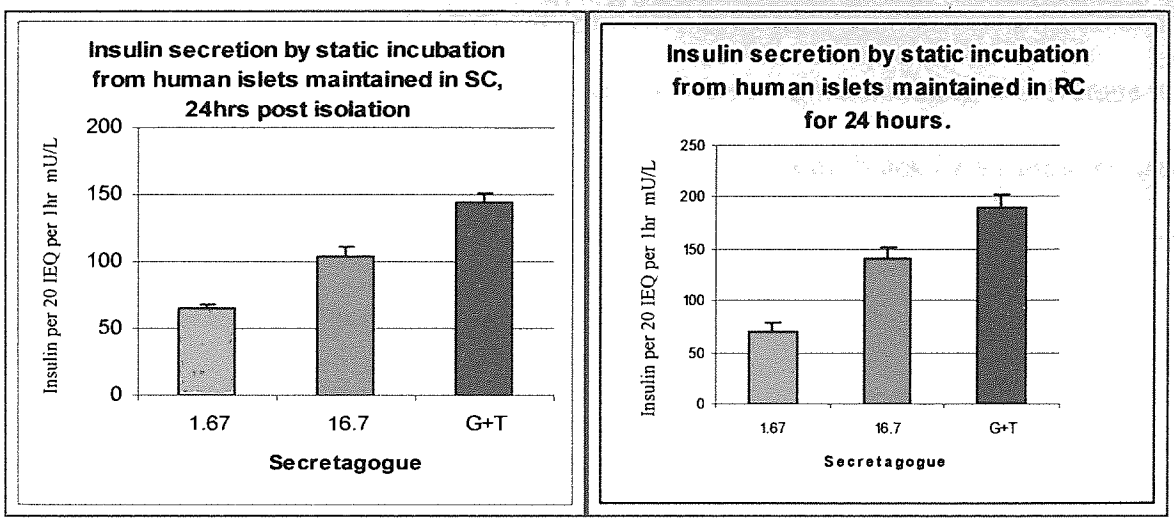
were grouped into size ranges and the mean of 3 samples for each islet size range was calculated. From these islet numbers, the number of islet equivalents (IEQ) was calculated by using the conversion factors shown in table 1 below. The purpose of converting islet numbers into IEQs is to indicate the number of islets of 150μm diameter represented by the isolated islet preparation as a means of standardisation (Ricordi et al. 1990). The total obtained from the last column provided the total number of IEQs in 100μl of the islet preparation, hence if the isolated islets were suspended in 40ml of tissue culture medium and 100μl contained 200 IEQs, then 40ml contained: 400 x 200 = 80 000 IEQs.

The volume of each islet preparation in the pre-incubation 1.67mM glucose solution was adjusted to allow for ~20 IEQ/50μl of cell suspension. For each islet group 6 LP2 tubes (NHS Supplies) were labelled for each of the 3 secretagogues and 50μl of islet suspension was transferred to the tubes. 2.5ml of the relevant secretagogues was added to the tubes, parafilm was used to cover the tubes and a one hour static incubation followed in the 37°C water bath to allow insulin secretion in response to the secretagogues.

After the 1-hour challenge, the tubes were removed from the rack; the contents agitated and then centrifuged at 300 x g for 3 minutes. The supernatants were transferred to eppendorf tubes and frozen for insulin analysis by ELISA (figure 17).

Islet size group (µm)	**Mean number of islets from 3 X 100µl samples (n)**	**IEQ conversion factor (to convert all islets to 150µm):**	**Number of IEQs per size group/100µl: n X factor**
50 – 100		0.167	
101 - 150		0.667	
151 - 200		1.685	
201 - 250		3.500	
251 - 300		6.315	
301 - 350		10.352	
351 - 400		15.883	
>400		22.750	
Sum of IEQs per 100µl of isolated islet preparation: Σ of last column			

Table 1. Conversion factors used to calculate the number of islet equivalents per size group and total number of IEQs for the islet preparation.



Key:
1.67mM = 1.67mM glucose in Hepes buffered Hanks' solution with 0.5% BSA (HBSS + BSA)
16.7mM = 16.7mM glucose in HBSS
G+T = 16.7mM glucose + 10mM theophylline in HBSS
Figure 17. An example of insulin secretion by static incubation from human islets in SC (left) and RC (right).

Immunostaining

BRIN-BD-11 and HUVEC monolayer, rotational culture and co-culture

Cells were grown as for the insulin secretion studies, both in the presence and absence of 10mM of the TZD rosiglitazone. Once the cells had been treated as per experimental protocol and were ready to be stained for the marker/s of interest, they were fixed as follows:

- culture medium was removed from the wells and the cells were washed 1 x with 0.5ml PBS/well for 3 minutes on a plate shaker (Scientific Laboratory Supplies, Yorkshire, UK) on a slow setting
- the PBS wash was removed and 0.5ml/well of cold 70% ethanol (Hayman Ltd, Witham, Essex, UK) was added to allow fixation of the cells at room RT for 10 minutes on a plate shaker
- 3 x 0.5ml/well PBS washes were performed for 3 minutes each after the ethanol had been removed.

If staining was to be done at a later date, 1ml/well PBS or deionised water was added and the plate was stored in a refrigerator (maximum 1 week). When immunostaining was commenced the PBS or water was removed from the wells and an avidin/biotin block (Vectastain kit from Vector Laboratories, Peterborough, UK) was performed to help block non-specific sites that could result in background/non-specific staining. 4 drops of avidin was added to each well and the plate was placed on a shaker at RT for 15 minutes. The avidin was removed, a wash step followed with 1 x 0.5ml/well PBS, 4 drops of biotin was placed into each well and the plate was again placed onto the shaker at RT for 15 minutes. The biotin was removed and 2 x 0.5ml/well PBS washes were performed for 2 minutes each.

PBS was removed and 0.5ml/well of PBS with 5% of the normal serum from the relevant Vectastain kit was added (if a mouse monoclonal antibody was used, the mouse kit contained normal horse serum as the secondary antibody in this kit has been raised in a horse). The plate was allowed to incubate at RT for 20 minutes on a shaker. During this time, the primary antibody was prepared in PBS containing 5% normal serum from the applicable kit. Table 2 shows the antibody dilutions used.

After the 20 minute incubation the excess PBS/serum was removed, but no wash was performed as the primary antibody was also prepared with PBS containing 5% serum. 250µl/well PBS was added to all wells (including negative control wells to check for non-specific or background staining) not receiving the antibody of interest and 250µl/well of the prepared antibody to the relevant wells. The plate was allowed to incubate on a shaker at RT for 1 hour after which the antibody solution was removed and the plate was washed 3 times with 0.5ml/well PBS, allowing 3 minutes for each wash on the shaker at RT. The secondary, biotinylated antibody was prepared from the relevant kits (mouse or rabbit). The final PBS wash was removed, 250µl/well of the secondary, biotinylated antibody was added and the

plate was allowed to incubate at RT on the shaker for 30 minutes. During this incubation the avidin/biotinylated enzyme (alkaline phosphatase) complex was prepared by adding 2 drops of solution A and 2 drops of solution B to 5 ml PBS and allowing it to equilibrate for 30 minutes prior to use.

After the 30 minute incubation of the secondary, biotinylated antibody the solution was removed from the wells and 2 x 0.5ml/well PBS washes were performed, allowing 3 minutes per wash. 250µl/well of the prepared A+B solution was added and allowed to incubate on the shaker for 30 minutes at RT. The A+B solution was removed and 3 x 0.5ml/well PBS washes followed, allowing 3 minutes per wash with the last wash being left in the wells while the stain was prepared.

For insulin (to stain D11 cells and human β-cells in islets), the Vector Red substrate for alkaline phosphatase was prepared by mixing 2 drops from each of the reagents in the kit in 5ml of a 100mM Tris-HCL buffer (pH 8.2) and the solution was mixed well.

For CD31 (to stain human endothelial cells), the Vector Blue substrate for alkaline phosphatase was prepared by mixing 2 drops from each of the reagents in the kit in 5ml of a 100mM Tris-HCL buffer (pH 8.2) and the solution was mixed well.

The last PBS wash was removed from the plate and 250µl/well of the relevant substrate was added to the wells and the plate was wrapped in foil and placed on a plate shaker for 20 – 30 minutes to enhance colour development. Colour intensity was checked every 5 minutes and development was stopped when the colour was satisfactory. The substrate was removed, followed by a wash with 0.5ml/well Tris-HCl for 2 minutes after which the Tris-HCl was replaced with 0.5ml/well PBS and photographs were taken using a Zeiss Axioskop 40

microscope fitted with an Axiocam camera linked to a computer with Axiovision software (microscope, camera and software from Carl Zeiss Ltd, Herts, UK).

When dual staining was required to show both insulin (for the presence of β-cells) and CD31 (an endothelial cell marker) the monoclonal antibody was used first (in this case mouse anti-human CD31 for HUVEC) followed by the polyclonal antibody (rabbit anti-pig insulin for BRIN-BD11 β-cells). Dual staining was performed by repeating the process described above with a different colour endpoint for the second antibody used. In this project HUVEC were stained first for CD31 using Vector Blue to visualise, followed by insulin staining with visualisation using Vector Red.

Antibody	**Supplier**	**Antibody dilution**	**Visualisation Colour**
Mouse anti-human CD31	Dako Cytomation, Glostrup, Denmark	1:100	Vector Blue
Rabbit anti-pig insulin (cross reacts with rat insulin)	Abcam, Cambridge, UK	1:200	Vector Red

Table 2. Antibody dilutions used to stain for insulin in BRIN-BD11 cells and CD31 in HUVEC.

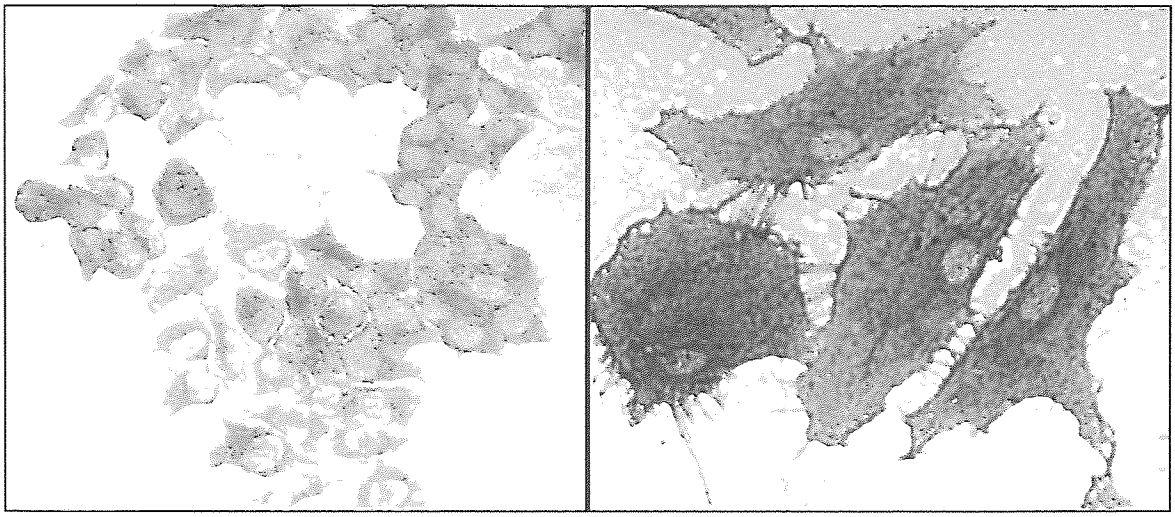


Figure 18. Immunostaining of BRIN-BD11 for insulin, visualised with Vector Red (left) and HUVEC for CD31, visualised with Vector Blue (right).

Fluorescence immunostaining

Human islets dual staining

Sterile 24-well plates were prepared by placing one autoclaved glass coverslip (VWR International, Leicestershire, UK) per well, followed by 1ml m199 tissue culture medium (supplemented as described above) and placing the plates into a humidified incubator with 5% CO_2 at 30°C for one hour to equilibrate. Islets were subsequently plated at a density of 20 islets/well and allowed to adhere for 24 hours prior to experimental conditions (i.e. addition of TZD) being initiated. A further 24 hours of culture was allowed prior to the medium being removed and one 0.5ml PBS wash being performed for 3 minutes on a plate shaker on a slow setting. The islets were fixed as follows:

The PBS wash was removed and 0.5ml/well of cold 4% w/v paraformaldehyde (Sigma) was added to allow fixation of the islets at room temperature (RT) for 10 minutes on a plate shaker 3 x 0.5ml/well PBS washes were performed for 3 minutes each after the paraformaldehyde had been removed.

If staining was to be done at a later date, 1ml/well PBS or deionised water was added and the plate was stored in a refrigerator.

When immunostaining was commenced the PBS or water was removed from the wells and antigen retrieval/membrane permeabilisation was performed by adding 0.5ml/well of 0.05% Triton X-100 (TX-100 from Sigma) in PBS for 30 minutes at room temperature on a plate shaker. The TX-100 was removed and 2 x 0.5ml/well PBS washes were performed for 3 minutes each prior to 250µl/well of a blocking solution consisting of 2% Roche blocking buffer (Roche Diagnostics Ltd.) and 5% normal goat serum (Sigma) in PBS being added and allowed to incubate at room temperature on a plate shaker for 1 hour.

A primary antibody mixture was prepared in the blocking buffer as follows:

- Rabbit anti-pig polyclonal insulin (Abcam, Cambridge, UK) + mouse anti-human monoclonal VEGF (R&D Systems, Minneapolis, USA), both at 1:100 dilution
- Rabbit anti-pig polyclonal insulin + mouse anti-human monoclonal CD31 (Dako Cambridgeshire, UK), both at 1:100 dilution

300µl/well of the appropriate antibody mixture was added and allowed to incubate at room temperature on a plate shaker for 1 hour prior to being placed at 4°C overnight. Suitable controls were employed to rule out non-specific/background staining.

The following day the antibody mixture was removed and 3 x 0.5ml/well PBS washes were performed for 3 minutes each on a plate shaker prior to addition of a fluorescent-labelled secondary antibody mixture as follows:

Goat anti-rabbit TRITC and goat anti-mouse FITC (both from Southern Biotech, Alabama, USA) were diluted 1:100 in PBS with 0.05% tween-20 (Sigma). TRITC was selected to result in a red stain for insulin and FITC to produce green staining for VEGF and CD31.

300µl/well of the appropriate secondary antibody mixture was added and allowed to incubate at room temperature on a plate shaker for three hours prior to 3 x 0.5ml/well PBS washes being performed for 3 minutes each. A further 0.5ml/well PBS was added, the coverslips were gently lifted with an angled 25G needle and lifted with forceps, dipped into deionised water and mounted onto cleaned glass slides (VWR International). Images were captured using the equipment described in D11/HUVEC immunostaining above.

Development of an immunostaining technique to detect human islets in paraffin wax sections of formalin-fixed mouse spleens harvested at three time points post-implant is discussed in chapter 6.

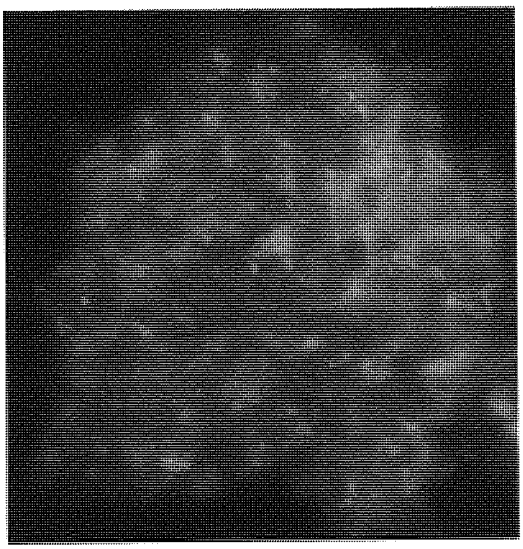


Figure 19. An example of a human islet dual stained for insulin (red, TRITC) and VEGF (green, FITC).

Adenylate triphosphate luminescence

This technique is based upon the bioluminescent measurement of adenylate triphosphate (ATP) that is present in all metabolically active cells (Lemasters and Hackenbrock 1977; Ido et al. 2002). The bioluminescent method utilises the enzyme luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:

$$ATP + Luciferin + O_2 \xrightarrow[Mg^{++}]{Luciferase} Oxyluciferin + AMP + PP_i + CO_2 + Light$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer (Stratec Biomedical Systems, Birkenfeld, Germany).

MIN6 cells monolayer and rotational culture

As this mouse beta cell line did not prove to be reliably glucose responsive, it was replaced with the BRIN-BD11 beta cell line and no further experiments were performed using MIN6; hence no ATP luminescence data have been collected for MIN6.

BRIN-BD11 cells monolayer and rotational culture

BRIN-BD11 cells were grown as for the insulin secretion studies detailed above. On the day of harvesting ATP samples, culture medium was removed from the wells and 1ml PBS was added per well and then removed as a wash. This process was repeated once more prior to 250µl nucleotide releasing reagent (from a ViaLight kit supplied by Lonza) being added to each well for 7 minutes to allow lysis of the cells. The content of each well was transferred to a microcentrifuge tube (VWR International) and centrifuged at 500 x g for 5 minutes to pellet any remaining cell debris. The supernatant was transferred to clean, labelled tubes and either frozen for future analysis or assayed immediately.

ATP detection by bioluminescence was performed as follows:

180µl of each ATP sample was loaded into a clean, white, flat-bottomed 96-well plate (Porvair Sciences Ltd, Leatherhead, UK), leaving the first 3 columns free to serve as a brief injector test. The plate was placed into the luminometer and the lid was closed and locked to seal out light. The VialLight protocol was selected, a file name was allocated and the assay

was initiated. The relative light unit (RLU) results were printed and the data collated and subjected to statistical analysis in Excel.

HUVEC monolayer and rotational culture

HUVEC were grown as for the insulin secretion studies detailed above and ATP bioluminescence was determined as for BRIN-BD11 cells.

BRIN-BD11 and HUVEC co-culture monolayer and rotational culture

BRIN-BD11 and HUVEC were grown as co-cultures in both CSC and RC as for the insulin secretion studies detailed above and ATP bioluminescence was determined as for BRIN-BD11 cells.

Human islets

Due to the heterogeneous cell population of human islets and the varying percentage of exocrine tissue present ATP bioluminescence assays were not performed on islet preparations as it would be impossible to determine from which cell population any increase/decrease in ATP content originated.

VEGF production

HUVEC monolayer and rotational culture

HUVEC were grown as monolayers and in a RCCS and plated into 24-well plates as for insulin secretion studies. After 24 hours of culture +/- 10mM TZD, the tissue culture medium was harvested for VEGF detection by ELISA [RayBiotech Inc., purchased from Insight Biotechnology, Wembley, UK] (Cross et al. 2007; Laugharne et al. 2007).

BRIN-BD11 monolayer and rotational culture

B-cells do not manufacture VEGF; hence this group was not included in the VEGF production experiments.

BRIN-BD11/ HUVEC monolayer and rotational culture

BRIN-BD11/HUVEC monolayer and rotational culture co-cultures were plated into 24-well plates as for insulin secretion studies. After 24 hours of culture +/- 10mM TZD, the tissue culture medium was harvested for VEGF detection by ELISA.

Human islets static and rotational culture

Human islets maintained in SC and RC +/- TZD for 24 hours (48 hours post-isolation) were plated at a density of 20 IEQ/well in 24-well plates (receiving m199 +/- TZD) and allowed to adhere by outgrowth for 24 hours. Tissue culture medium was harvested at 24 hours (72 hours post isolation) and 48 hours (4 days post isolation) and VEGF detection was performed by ELISA.

Chapter 3: Vascular corrosion cast method development

Introduction

Despite considerable progress being made in the understanding of angiogenesis and vasculogenesis, quantification of vascular changes remains a challenge (Sharma et al. 2005). One characteristic of the vasculature that impedes precise measurement is the irregular shape of its vessels and distinct differences between individuals (Grizzi et al. 2001; Sharma et al. 2005).

Several methods have been developed to study alterations in vascular density and vessel growth, which include:

- Microvessel counting under a dissecting microscope in a murine model of intradermal tumour angiogenesis (Runkel et al. 1991; Choi et al. 2005)
- Infusion of a dye into the vasculature of interest followed by colour flow Doppler ultrasonography to visualise vessels (Mari et al. 1995; Aardema et al. 2000; Huang et al. 2008)
- Immunohistochemical staining of appropriately preserved samples for antigens relating to vascular growth, such as CD31, CD34, VEGF, VEGF receptors, which can be quantified by manual cell/vessel counting within a given field or by computer using image analysis software (Schoell et al. 1997; Hopkins et al. 1998; Aronsson and Muhr 2002; McGough et al. 2002; Choi et al. 2005; Campos et al. 2006)
- Use of contrast enhanced computer tomography (Miles 1999; Rodallec et al. 2006; Jinzaki and Kuribayashi 2007) and magnetic resonance imaging (Hoang et al. 2004; Gambarota et al. 2008; Oberholzer et al. 2008) to study tumour angiogenesis can quantify physiological changes such as increased perfusion, blood volume and capillary permeability

- Electron microscopy has been used to provide information about capillary numbers, vessel wall thickness, vessel lumen diameter, capillary surface area (Parlange and Sims 1993; Warley et al. 1995; Kong et al. 1998; Polykandriotis et al. 2007)
- Plastic vascular corrosion casts have been used extensively to study and depict vasculature by infusion of a monomer-catalyst-promoter mixture into the vessels of interest, allowing polymerisation of the plastic followed by corrosion of the tissue to leave a cast of the vasculature (Nopanitaya et al. 1979; Burger et al. 1984; Fahrenbach et al. 1988; Lametschwandtner et al. 1990; Pollitt and Molyneux 1990; Schraufnagel et al. 1996; Macchiarelli et al. 2006; Whiteley et al. 2006)
- X-ray microtomography has been used successfully to study the microcirculation in rat kidneys perfused with a lead chromate containing, silicon based solution (Ortiz et al. 2000), barium sulphate filled microangioarchitecture of tumours in rabbits (Maehara 2003), and silicon polymer filled rat renal vasculature (Nordsletten et al. 2006).

Although other techniques exist for studying vasculature, an exhaustive list lies outside the remit of this thesis. Certain techniques such as magnetic resonance imaging, contrast enhanced computer tomography and Doppler ultrasonography were unsuitable as access to the required equipment was unavailable. A novel technique was sought to study the vasculature of human islets transplanted under the kidney capsule of mice, hence the possibility of combining vascular corrosion casting and x-ray microtomography was investigated. The procedure employed to implant human islets under the kidney capsules and into the spleens of mice is discussed in chapter 6.

Method development of vascular corrosion casting

Batson's No.17 corrosion kit (Sudwan et al. 1991) was purchased from Polysciences Europe, Eppelheim, Germany and initial casts were performed in 12 – 16 week old male rats to develop the technique before application to the intricate microvasculature in mice.

The monomer-catalyst-promoter-pigment solution was prepared as per manufacturer's instructions and the animal was euthanized by CO_2 followed by cervical dislocation. A ventral midline incision exposed the abdominal cavity and a 20G needle was inserted into the left renal vein. A 5ml syringe containing the cast solution was attached to the needle and gentle pressure was applied to allow infusion. However, the mixture polymerised rapidly and no cast was achieved.

Prior to the second attempt, a saline flush was administered via the inferior vena cava after termination by cervical dislocation. The flush was partially successful as the kidneys and liver took on a mottled appearance. The casting solution was prepared using half the suggested amount of catalyst and promoter as this allowed a more generous working time before polymerisation set in. A 20G needle was inserted into the left renal vein and the monomer solution was infused resulting in partial kidney and liver casts (figure 20).

A saline flush, containing 10units/ml heparin, via the left renal vein preceded the third vascular corrosion cast attempt. This resulted in a well flushed kidney and the needle was left in situ for attachment of the syringe containing the monomer solution. An air lock formed, resulting in a partial kidney cast.

The same procedure was followed for the fourth attempt, but care was taken to prevent an air lock during exchange of the syringes. This resulted in a complete rat kidney vascular cast.

The kidney was excised and placed into cold saline on ice for 2 – 3 hours to dissipate heat formed during polymerisation and prevent expansion and distortion of the specimen. The kidney was then placed into a maceration solution consisting of 20% w/v NaOH to remove the tissue from the vascular corrosion cast (figure 21).

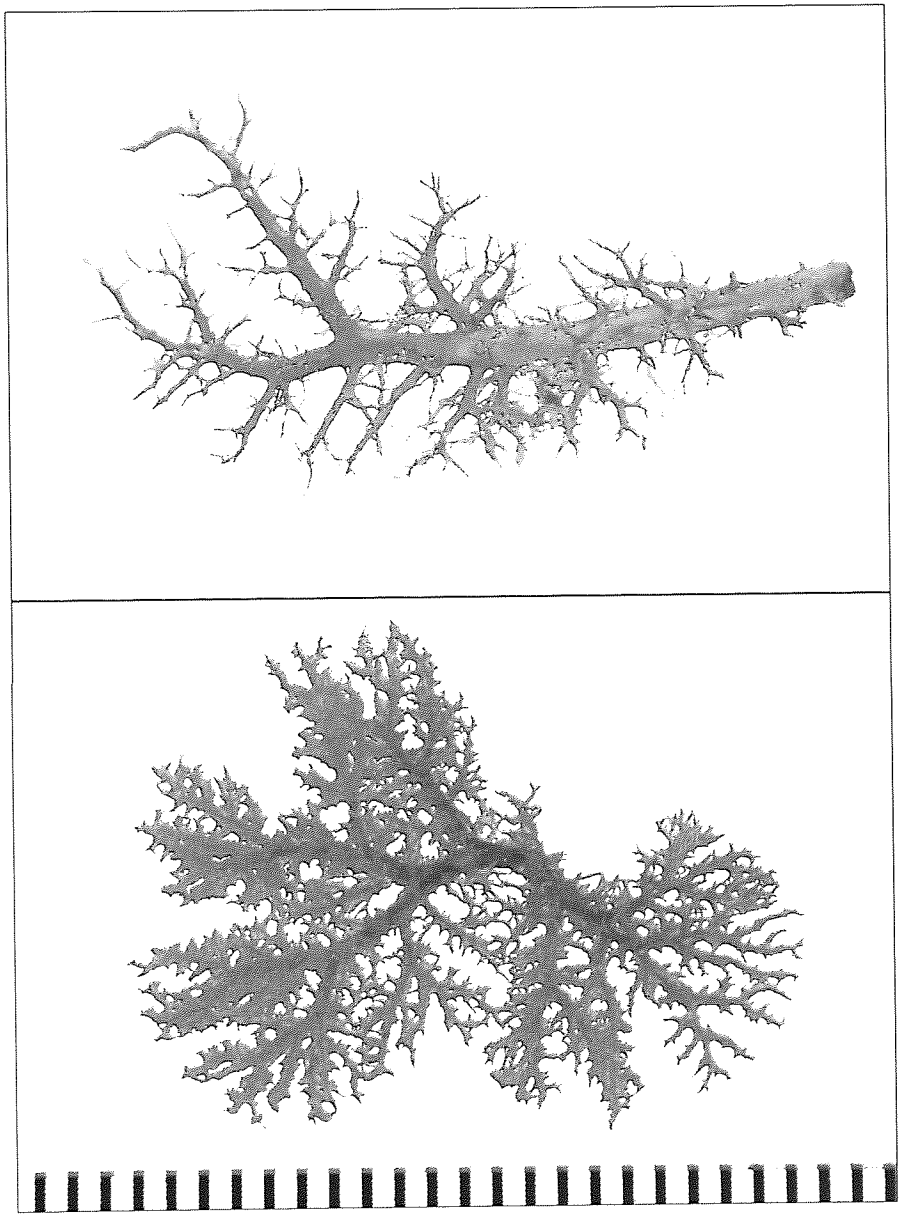


Figure 20. Photographs of partial rat liver lobe vascular corrosion casts.

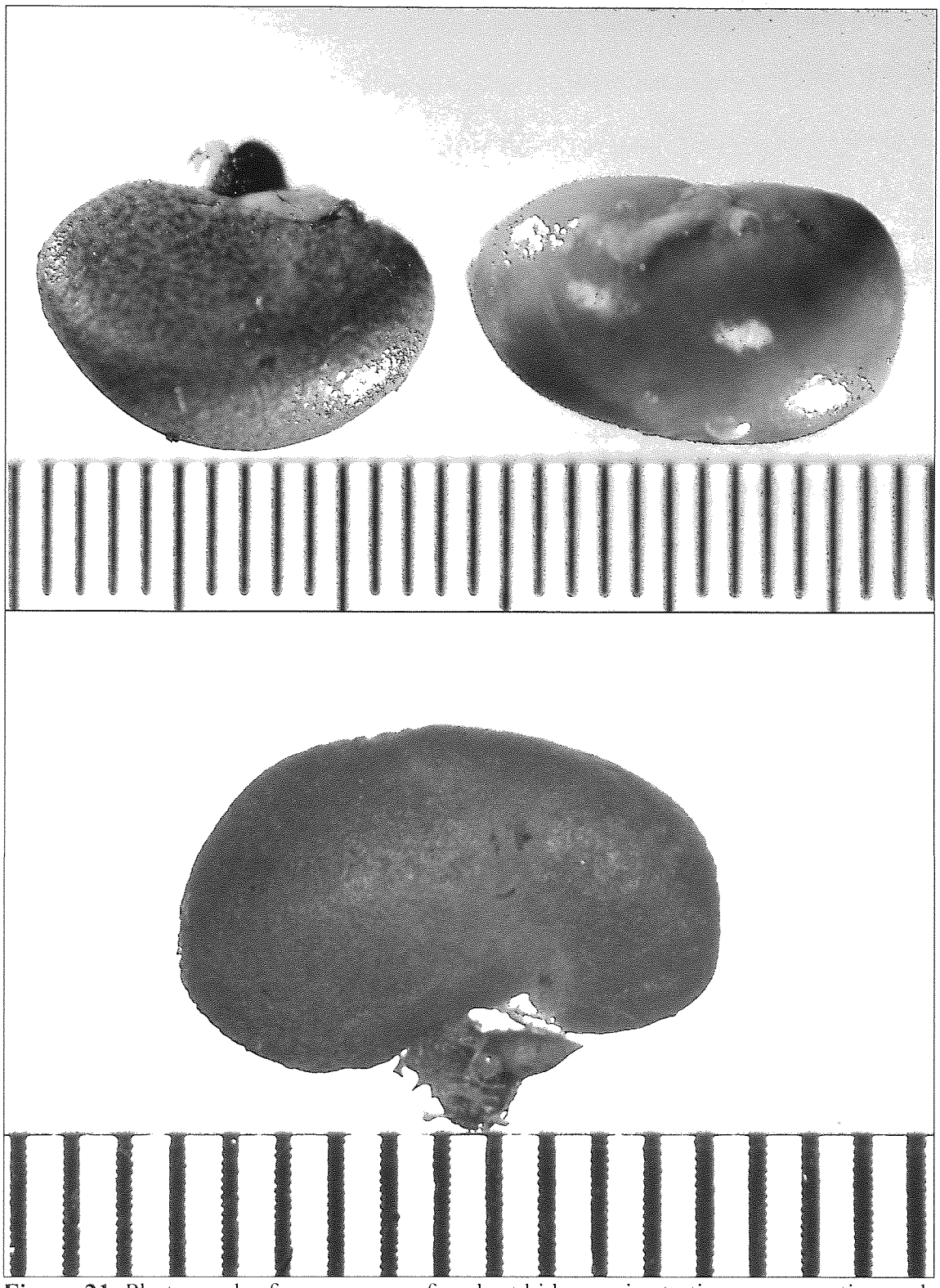


Figure 21. Photograph of monomer perfused rat kidney prior to tissue maceration and non-perfused rat kidney (top) and vascular corrosion cast post maceration (bottom).

The technique was transferred to 12 – 16 week old male mice, using a 25G needle to administer both the saline/heparin flush as well as the monomer solution. Initial attempts did not result in successful casts, mostly due to rupture of the renal vein. The entry positions of the needles for the saline/heparin flush and monomer infusion were changed to alleviate some of the pressure on the renal vein and small bulldog clamps were used to keep the needles in place. Table 3 summarises the site selection and cast outcome information obtained.

Saline flush site	Flush outcome	Monomer infusion site	Cast outcome
Inferior vena cava	Very good liver flush, partial kidney flush	Same needle as for saline flush	Partial kidney and liver casts
Inferior vena cava	Very good liver flush, partial kidney flush	Renal vein	Partial kidney cast
Aorta, below aortic arch	Very good liver and kidney flush	Renal vein	Very good kidney and liver lobe casts

Table 3. Site selection for saline flush and monomer infusion and the resulting flush and cast outcomes.

The most successful flush was achieved via the aorta, while monomer infusion via the renal vein resulted in very good kidney and liver lobe casts (figures 22 - 23). This method was used to successfully obtain vascular corrosion casts of mouse kidneys at days 6, 9 and 12 after implantation of 50 human islets (see chapter 6).

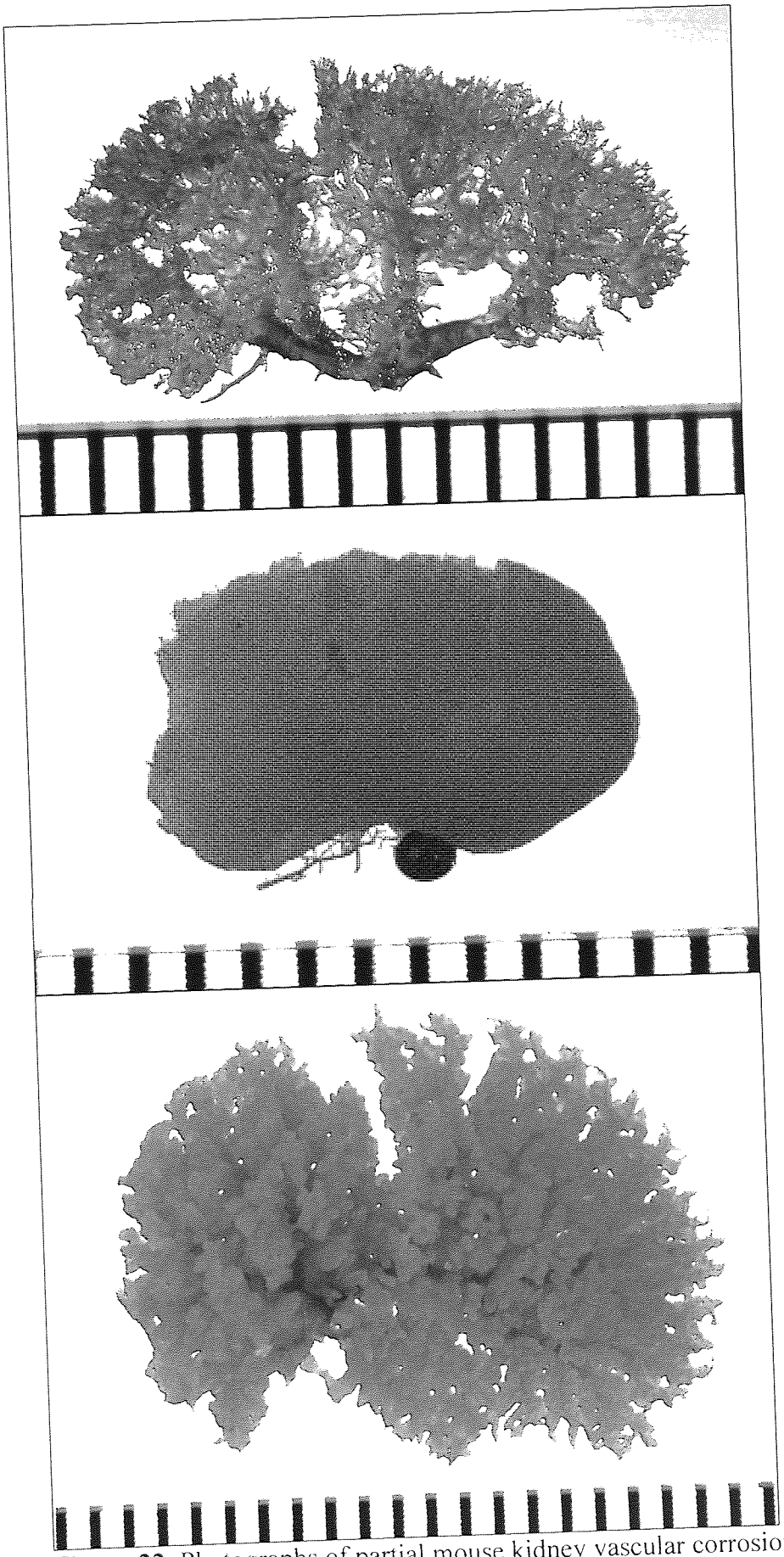


Figure 22. Photographs of partial mouse kidney vascular corrosion casts (top and middle) and a partial mouse liver lobe cast (bottom).

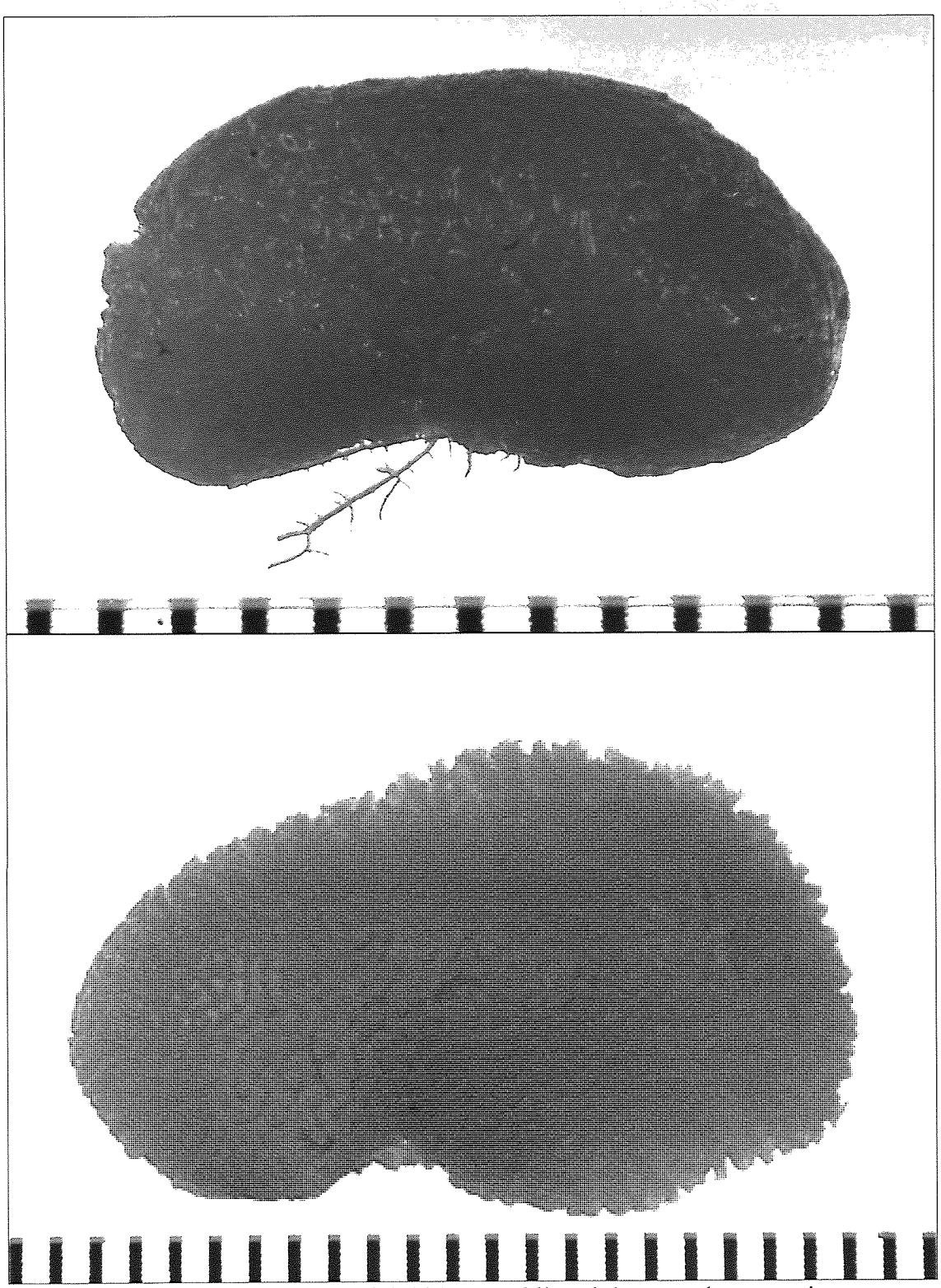


Figure 23. Photograph of mouse full kidney and liver lobe vascular corrosion cast.

CHAPTER 4: BRIN-BD11 and HUVEC monolayer and rotational culture +/- 10mM rosiglitazone

Introduction

The success of islet transplants as a treatment for type 1 diabetes is restricted by a number of complex problems such as a limited donor pool (Kapur et al. 1999; Buckley 2000; Brasile et al. 2001; Mathew 2004; Balamurugan et al. 2006; Monaco 2006), beta cell toxicity of immunosuppressants (Shapiro et al. 2002; Nanji and Shapiro 2004; Du and Xu 2006), relatively low retrieval percentage of islets from donor pancreata (Shapiro et al. 2000; Lakey et al. 2002) and the poor level and slow rate of revascularisation (Menger et al. 1992). Damage to the complex islet microvasculature during the isolation process results in transplanted islets requiring approximately 2 weeks to revascularise (Menger et al. 1992). This detrimental period of ischaemia constitutes a key phase that will influence establishment of the transplant and presents an opportunity to improve the survival and function of transplanted islets via interventions that would result in an increased revascularisation rate (Menger et al. 1992).

Most experimental islet transplant studies to date have not included strategies to enhance the revascularisation rate of the graft. However, transplanted islets do revascularise with the development of a capillary network similar to that of intact islets (Beger et al. 1998).

Evidence shows that the growth and differentiation of vascular endothelial cells are, in part, regulated by the VEGF group of genes (Ferrara 2000). VEGF-A regulates vasculogenesis (new blood vessel development from endothelial cells in the absence of pre-existing vasculature) and angiogenesis (new blood vessel development from pre-existing vasculature) as well as microvessel permeability (Burant and Simeone 2002; Konstantinova and Lammert

2004; Del Moral et al. 2006), VEGF-B directs embryonic angiogenesis and VEGF-C and D specifically regulate lymphangiogenesis (Rubbia-Brandt et al. 2004; Byrne et al. 2005).

Human VEGF-A consists of 5 major isoforms having 121, 145, 165, 189, or 206 amino acid residues in the mature monomer, with $VEGF_{165}$, a heavily glycosylated protein of about 45kDa, being the most abundant form and $VEGF_{206}$ being very rare (Houck et al. 1992; Ferrara 1999). VEGF-A_{121} is a soluble and freely diffusible protein and, being weakly acidic, does not bind to heparin (Houck et al. 1992). VEGF-A_{165} is also secreted, but a significant fraction remains bound to the cell surface and the extracellular matrix (ECM), while VEGF-A_{189} and VEGF-A_{206} are more basic and bind to heparin, resulting in these isoforms being sequestered in the ECM (Houck et al. 1992).

Beta cells have been shown to stain positively for VEGF-A (Kuroda et al. 1995), signifying a role for VEGF-A in islet angiogenesis and a possibility that beta cells sequester VEGF-A to aid in the regulation of microvessel permeability to facilitate rapid uptake of released insulin into the blood (Kuroda et al. 1995; Konstantinova and Lammert 2004). In addition, islet VEGF-A mRNA levels were increased by hypoxia or by prolonged maintenance in culture *in vitro*, suggesting that conditions associated with a reduction or abolition of vascular flow result in enhanced signals for islet angiogenesis (Gorden et al. 1997).

Endothelial expression and action of VEGF-A has been experimentally increased by agonists of the peroxisome proliferator-activated receptor gamma (PPARγ), such as the TZDs rosiglitazone and pioglitazone (Yamakawa et al. 2000). PPARγ is expressed by β-cells (Dubois et al. 2000) as well as by endothelium (Bishop-Bailey 2000), consequently stimulation of PPARγ by a TZD provides a potential route to increase VEGF-mediated vascularisation of islets and β-cells. Additional advantages to this approach are that TZDs

have been reported to increase islet β-cell granulation (Buckingham et al. 1998) and they are currently utilised clinically to improve insulin action and glycaemic control in diabetic states where insulin is present (Bailey and Day 2001).

Due to the paucity of human tissue for research, a number of cell lines have been developed for use as models for certain human cell types. Initially, the MIN6 cell line was investigated for use as a beta cell model for this project. This cell line was established from insulinomas obtained by targeted expression of the simian virus 40 T-antigen genes in transgenic mice and these cells have the morphological characteristics of pancreatic beta cells (Miyazaki et al. 1990).

Professor Jun-ichi Miyazaki from Osaka University Medical School in Japan kindly gave his consent for the IRL to use MIN6 cells for research. Two vials of MIN6 cells at passage 29 were obtained from Dr. Steve Ashcroft at Oxford University, UK. The literature suggests that MIN6 cells have been used successfully as a model for pancreatic beta cells and have consistently shown glucose-stimulated insulin secretion in a concentration-dependent response similar to that of normal islets (Hayashi et al. 1996; Kawakami et al. 1997; Minami et al. 2000). However, after extensive experimentation on this cell line in the IRL, insulin secretion in response to various secretagogues did not prove to be robust and reproducible enough to ensure sound scientific data (see results below). It is postulated that the various clones within a cell line perform differently when confronted with a physiological challenge and that the clone received by the IRL was unfortunately not significantly glucose responsive.

MIN6 cells have proven to be very useful for studying other aspects of β-cell physiology such as the expression of mitogen-activated protein kinases (MAPKs) and MAPK kinases (MEKs) in rat islets of Langerhans and the involvement of MAPKs in regulated insulin secretion

(Persaud et al. 1996); calcium signalling in MIN6 pseudoislets (Squires et al. 2000); calcium-dependent translocation of cytosolic phospholipase A2 in pancreatic beta-cells (Persaud et al. 2003); the influence of cell-to-cell contact on proliferative marker expression and apoptosis in MIN6 pseudoislets (Luther et al. 2005) and beta-cell proliferation in MIN6 pseudoislets (Carvell et al. 2007).

For the purposes of this study the BRIN-BD11 cell line was selected as a beta cell model to develop experimental protocols when human pancreata were not available for the isolation of human islets for research. BRIN-BD11 cells were generously supplied by Professor Peter Flatt from the University of Ulster, Northern Ireland.

The novel insulin-secreting BRIN-BD11 cell line was established following electrofusion of New England Deaconess Hospital rat pancreatic islet cells and tumoural rat insulinoma RINm5F cells (McClenaghan et al. 1996; Rasschaert et al. 1996). Wells of cell fusion mixture with insulin output 5 – 10 times greater than parent RINm5F cells were subcultured with eventual establishment of clones, including BRIN-BD11 (McClenaghan et al. 1996; Rasschaert et al. 1996). Morphological studies established that these cells grow as monolayers with epitheloid characteristics, maintaining stability in tissue culture for >50 passages (McClenaghan et al. 1996; Rasschaert et al. 1996).

Human Umbilical Vein Endothelial Cells (HUVEC) were selected to represent the vascular component of this study as they have been used successfully and extensively in other areas of research requiring vascular endothelial cells (Garcia et al. 1992; Tranqui et al. 1992; Villasante et al. 2007; Cheng et al. 2008; Chikaraishi et al. 2008; Czabanka et al. 2008). HUVEC express the CD31 endothelial cell marker and retain their morphology and stability in tissue culture for at least 5 passages (guaranteed by Lonza).

Methods

Chapter 2 of this thesis contains detailed materials and methods information, hence experimental protocols will be described only briefly in this chapter.

MIN6 cells were grown in T25 culture flasks with DMEM TCM (with 25mM glucose), supplemented with 10% FBS, 100U/ml penicillin, and 100μg/ml streptomycin until ~80% confluent. The cells were then harvested by trypsinisation and plated into 24-well plates for monolayer insulin secretion experiments or placed into rotational culture (RC) to form pseudoislets for use in RC insulin secretion studies.

D11 were grown in T75 flasks in RPMI 1640 TCM with 10% FBS + 100U/ml penicillin + 100μg/ml streptomycin until 80% confluent, after which the cells were harvested by trypsinisation. For SC, 175 000 D11/well were plated into 24-well plates (+/- 10mM TZD) and allowed to adhere for 24 hours prior to insulin secretion, ATP release, immunostaining or VEGF production and release experiments being performed. For RC, 150 D11 pseudoislets/well were plated into 24-well plates (+/- TZD) and allowed to adhere for 24 hours prior to experimentation as above.

HUVEC were grown in EGM in T75 flasks until 80% confluent prior to being harvested by trypsinisation. For both SC and RC 50 000 HUVEC/well were plated into 24-well plates (+/- 10mM TZD) and allowed to adhere for 24 hours prior to insulin secretion, ATP release, immunostaining or VEGF production and release experiments being performed.

For D11/HUVEC co-culture experiments D11 and HUVEC were grown as above. After trypsinisation, 175 000 D11 and 50 000 HUVEC were placed into the wells of a 24 well plate (in 50:50 RPMI: EGM, +/- 10mM TZD) for SC experiments and allowed to adhere for 24

hours prior to experimentation. For RC, D11, HUVEC and a mixture of D11 and HUVEC were placed into RC vessels in 50:50 RPMI: EGM for 24 hours. D11 and D11/HUVEC RC co-culture formed pseudoislets, while HUVEC RC did not. 150 pseudoislets/well of D11 RC and D11/HUVEC RC were transferred into 24-well plates in 50:50 RPMI: EGM +/- 10mM TZD and allowed to adhere for 24 hours prior to experimentation. HUVEC RC were plated at a density of 50 000 cells/well into 24-well plates in 50:50 RPMI: EGM +/- 10mM TZD and allowed to adhere for 24 hours prior to experimentation.

MIN6 results and discussion

Several methods were investigated to characterise the insulin secretory profile of MIN6 cells in response to insulin secretagogues. SC monolayer cells and RC pseudoislets were exposed to low glucose (2mM), high glucose (15mM) and high glucose + 10mM theophylline. Table 4 summarises the initial combinations of pre-incubation and incubation times and volumes tested and figures 24 and 25 show the insulin secretion data.

Culture method	Number of cell clumps (CC) or pseudoislets (PI)	Insulin secretion in LP2 tubes or 24-well plates	Pre-incubation time	Pre-incubation volume	Incubation time	Incubation volume
SC	150 CC	LP2	60 min	1ml	60 min	1ml
SC	300 CC	LP2	60 min	2ml	60 min	0.5ml
SC	150 CC	plates	60 min	1ml	60 min	1ml
SC	300 CC	plates	60 min	2ml	60 min	0.5ml
RC	150 PI	LP2	60 min	1ml	60 min	1ml
RC	300 PI	LP2	60 min	2ml	60 min	0.5ml
RC	150 PI	plates	60 min	1ml	60 min	1ml
RC	300 PI	plates	60 min	2ml	60 min	0.5ml

Key:

SC = Static culture in T25 tissue culture flasks, RC = Rotational culture

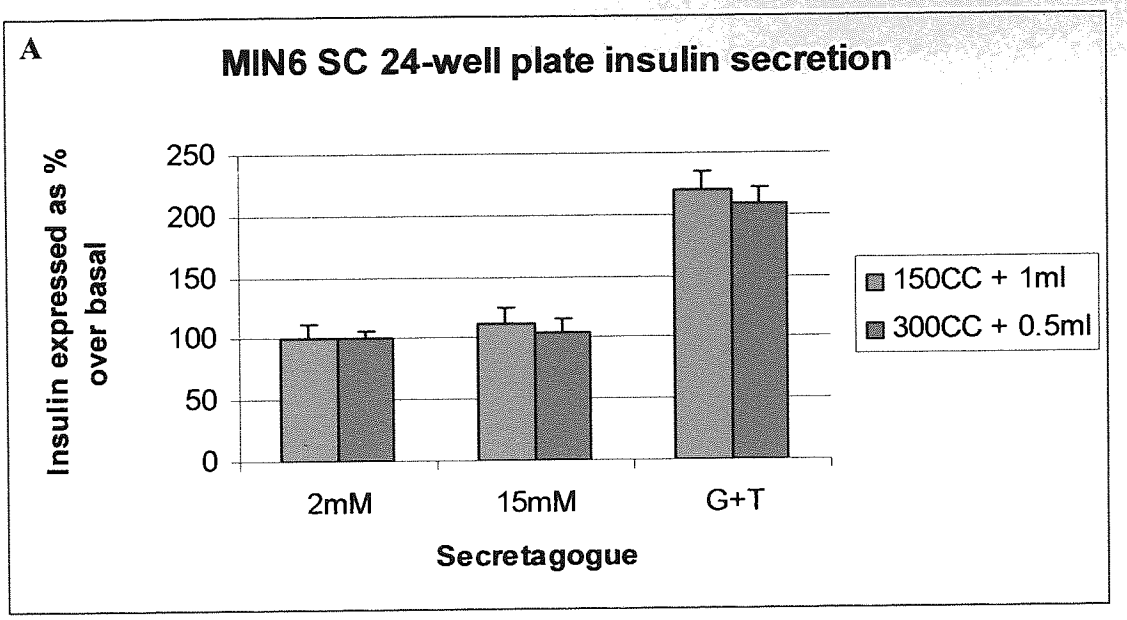
CC = small cell clumps formed after SC trypsinisation

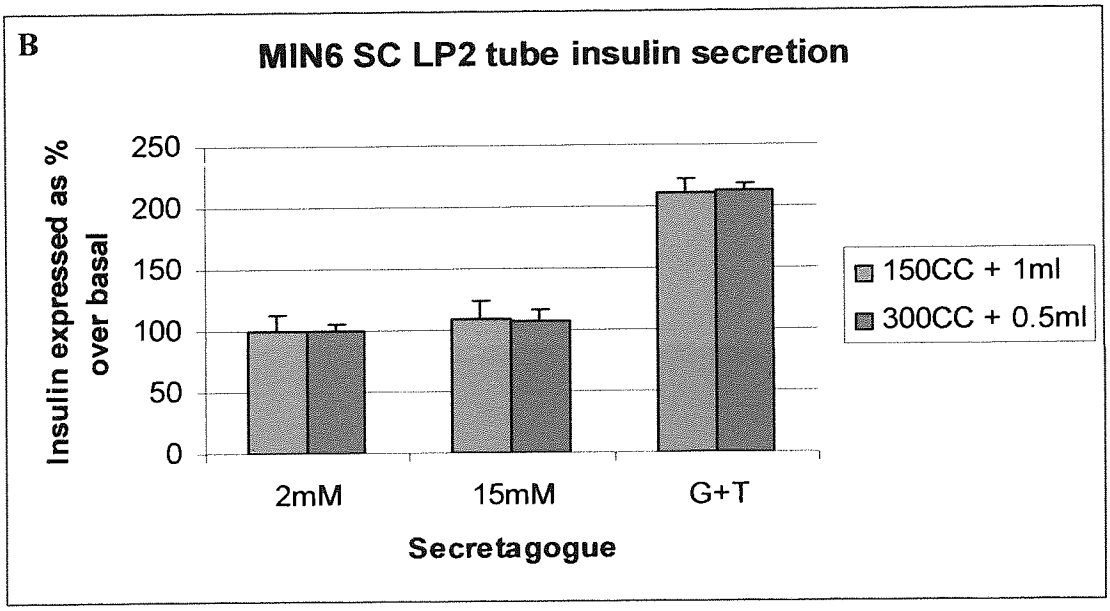
PI = pseudoislets formed in RC

Pre-incubation = a period during which all cells are exposed to low glucose to ensure basal insulin secretion prior to stimulation

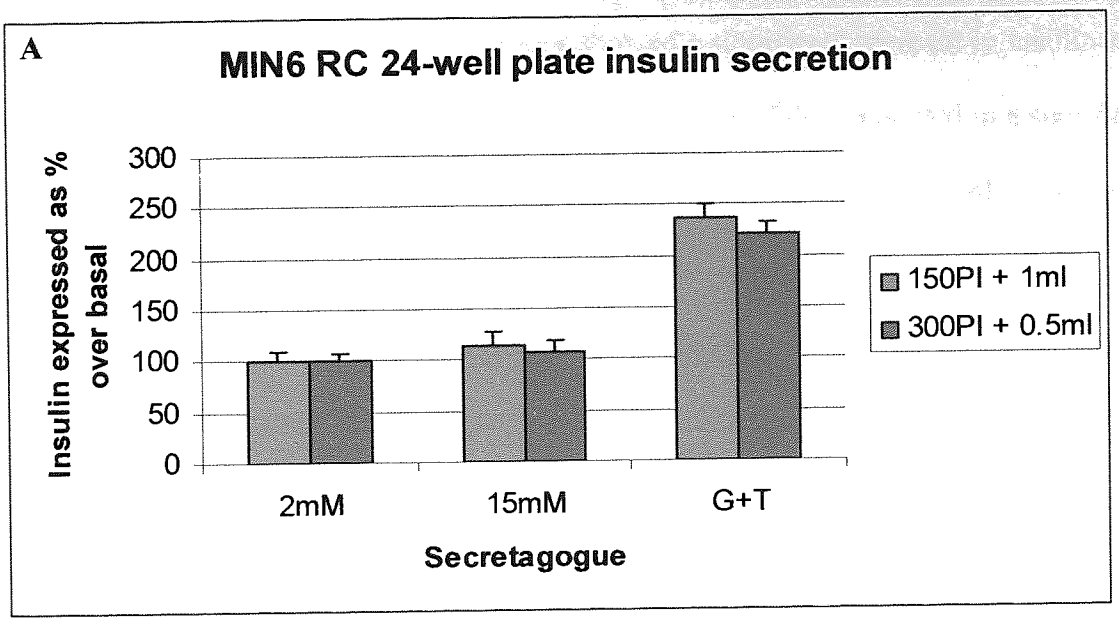
Incubation = a period during which cells are exposed to insulin secretagogues

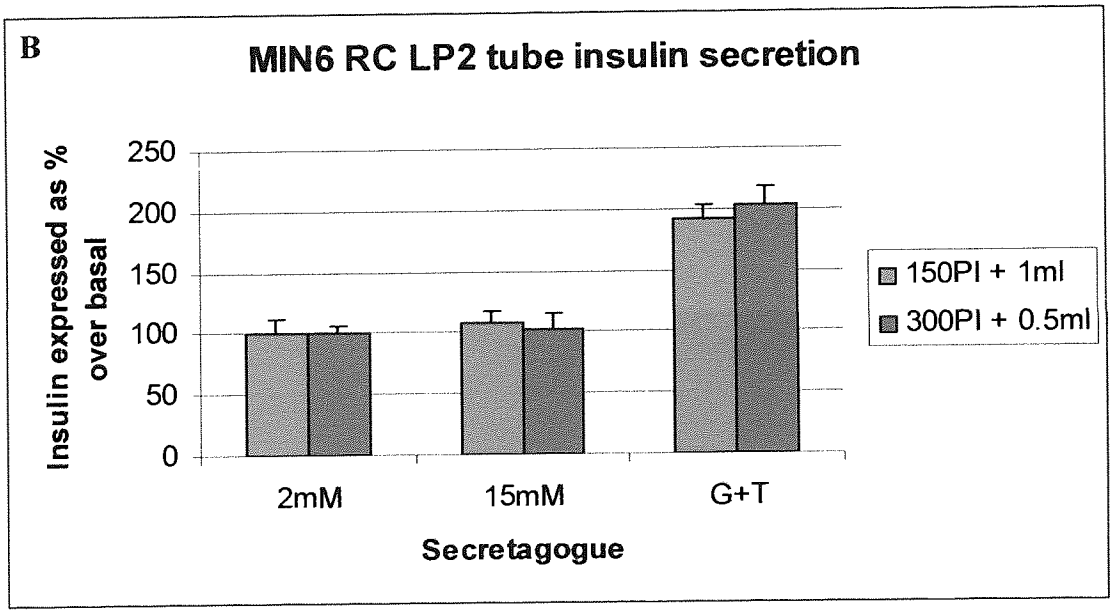
Table 4. Initial experimental protocols employed to assess the insulin secretory capacity of MIN6 monolayers and pseudoislets.





Key:
SC = static culture
150CC + 1ml = 150 small cell clumps formed after SC trypsinisation challenged with 1ml of insulin secretagogue
300CC + 0.5ml = 300 small cell clumps formed after SC trypsinisation challenged with 0.5ml of insulin secretagogue
Figure 24. Insulin secretion by static incubation from SC MIN6 in 24-well plates as an adherent monolayer (A) or as a suspension in LP2 tubes (B).





Key:
RC = rotational culture
150PI + 1ml = 150 pseudoislets challenged with 1ml of insulin secretagogue
300PI + 0.5ml = 300 pseudoislets challenged with 0.5ml of insulin secretagogue
Figure 25. Insulin secretion by static incubation from RC MIN6 pseudoislets in 24-well plates as an adherent culture (A) or as a suspension in LP2 tubes (B).

While all eight MIN6 experimental groups showed a significant increase in insulin secretion in response to high glucose + 10mM theophylline, none of them resulted in a significant response to high glucose alone. In an attempt to elicit a more physiological response from this beta-cell line the following changes were made to the existing protocol:

- 4 groups of culture medium (DMEM) containing 0, 1, 8 and 25mM glucose were tested to assess if the high glucose concentration (25mM) of the suggested growth medium for these cells inhibited glucose sensitivity
- Pre-incubation times were lengthened to 2 hours and glucose was omitted from pre-incubation medium to assess if this change would result in lower levels of basal insulin secretion or increase insulin responses to secretagogues
- Incubation times of 30, 60, 90 and 120 minutes in the presence of insulin secretagogues were implemented to establish optimal exposure times
- MIN6 pseudoislets were produced by a method provided by Professor Peter Jones, King's College, London
- MIN6 cells from various passage numbers (29 – 35) were assessed.

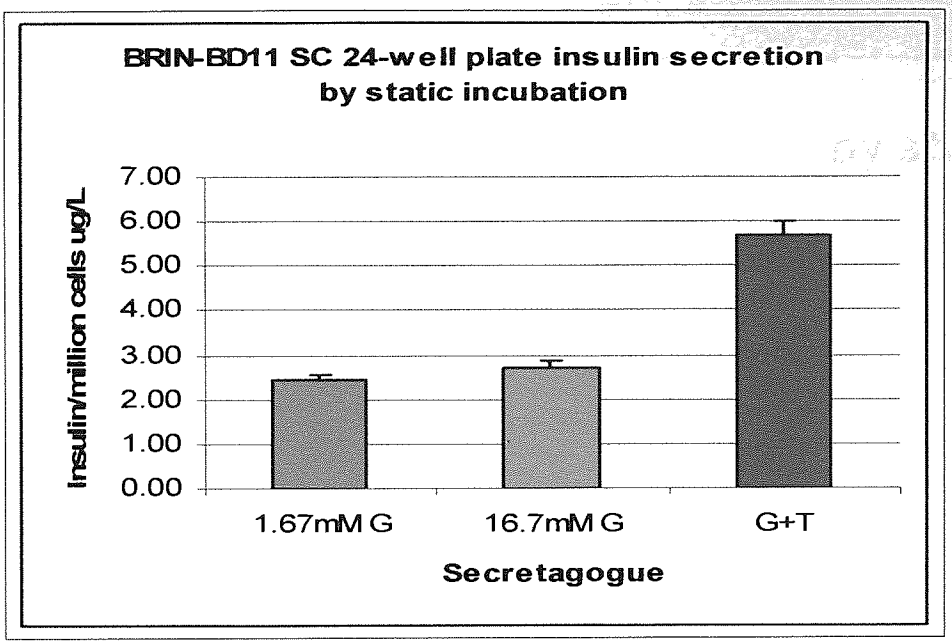
None of the interventions resulted in a significant increase in insulin secretion in response to high glucose while high glucose + 10mM theophylline continued to result in significantly increased insulin secretion. After careful consideration, use of this cell line as a beta cell model was discontinued in the IRL and BRIN-BD11 cells were acquired.

Results BRIN-BD11 and HUVEC

Beta cells play an important role in glucose homeostasis by fine-tuning their insulin secretion in response to glucose and other insulin secretagogues, consequently D11 cells were characterised by experiments to determine their secretagogue-induced insulin secretion profile and by immunostaining for the presence of stored, intracellular insulin. Initial experiments returned disappointing insulin secretory results for D11 (see figure 26), hence the following changes were made to the standard protocol:

- Pre-incubation time in low glucose was increased from 40 minutes to 1hr
- The incubation volume of insulin secretagogues was reduced from 1ml to 0.5ml/well in 24-well plates and incubation time for RC was increased from 20 to 30 minutes
- The plate was agitated gently every five minutes during the secretagogue incubation period to ensure dispersion of any secreted insulin to prevent activation of a negative feedback mechanism that could result in blunted responses
- Upon harvesting the supernatant a plastic pastette was used to aspirate the sample and gently rinse it over the adhered cells to capture secreted insulin that may have settled into 'unstirred layers' between the cells.

These protocol changes resulted in a marked improvement in the insulin secretory results from D11 cells; figure 27 shows the typical insulin secretion profile of D11 cells in response to 3 secretagogues and an image of D11 immunostained for insulin.



Key:
1.67mM G = 1.67mM glucose in Krebs Ringer with 0.2% BSA
16.7mM G = 16.7mM glucose in Krebs Ringer with 0.2% BSA
G+T = 1.67mM glucose + 10mM theophylline in Krebs Ringer with 0.2% BSA
Figure 26. Insulin secretion from static culture D11 cells, passage 35, using the standard protocol.

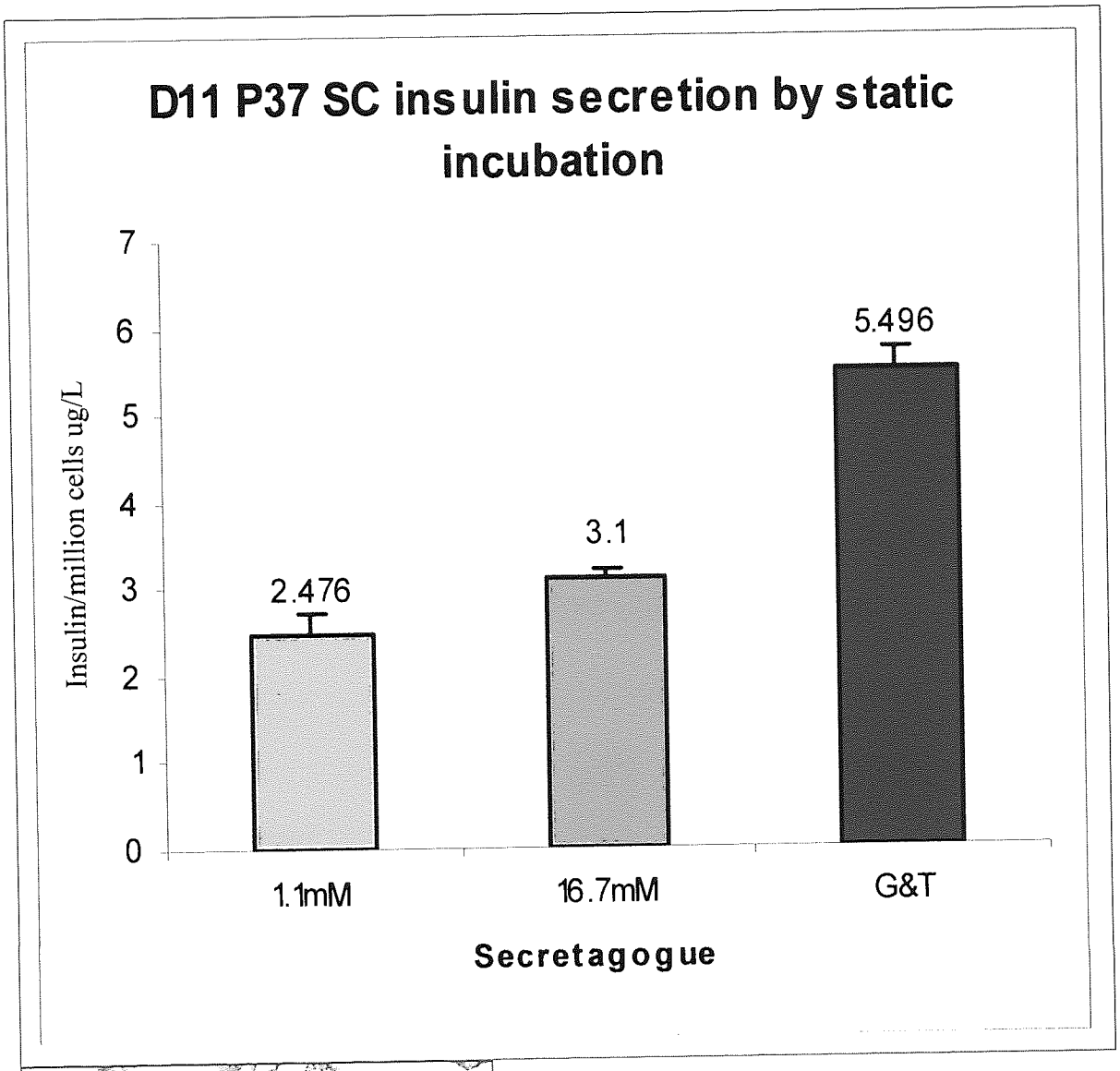


Figure 27. A typical insulin secretion profile for static culture D11 after protocol modifications mentioned above (top) and D11 monolayer stained positively for insulin (bottom).

Vascular endothelial cells express CD31 and their proliferation rate can be increased by the addition/upregulation of VEGF. ATP is present in all metabolically active cells and an increase in proliferation results in a significant increase in ATP levels. This increase in ATP can be measured by chemiluminescence and provides reliable information about the rate of proliferation of the cells. Figure 28 shows variations in HUVEC proliferation rates in response to a VEGF inducer (rosiglitazone, concentration selection discussed under VEGF section below) and an image of HUVEC immunostained for CD31.

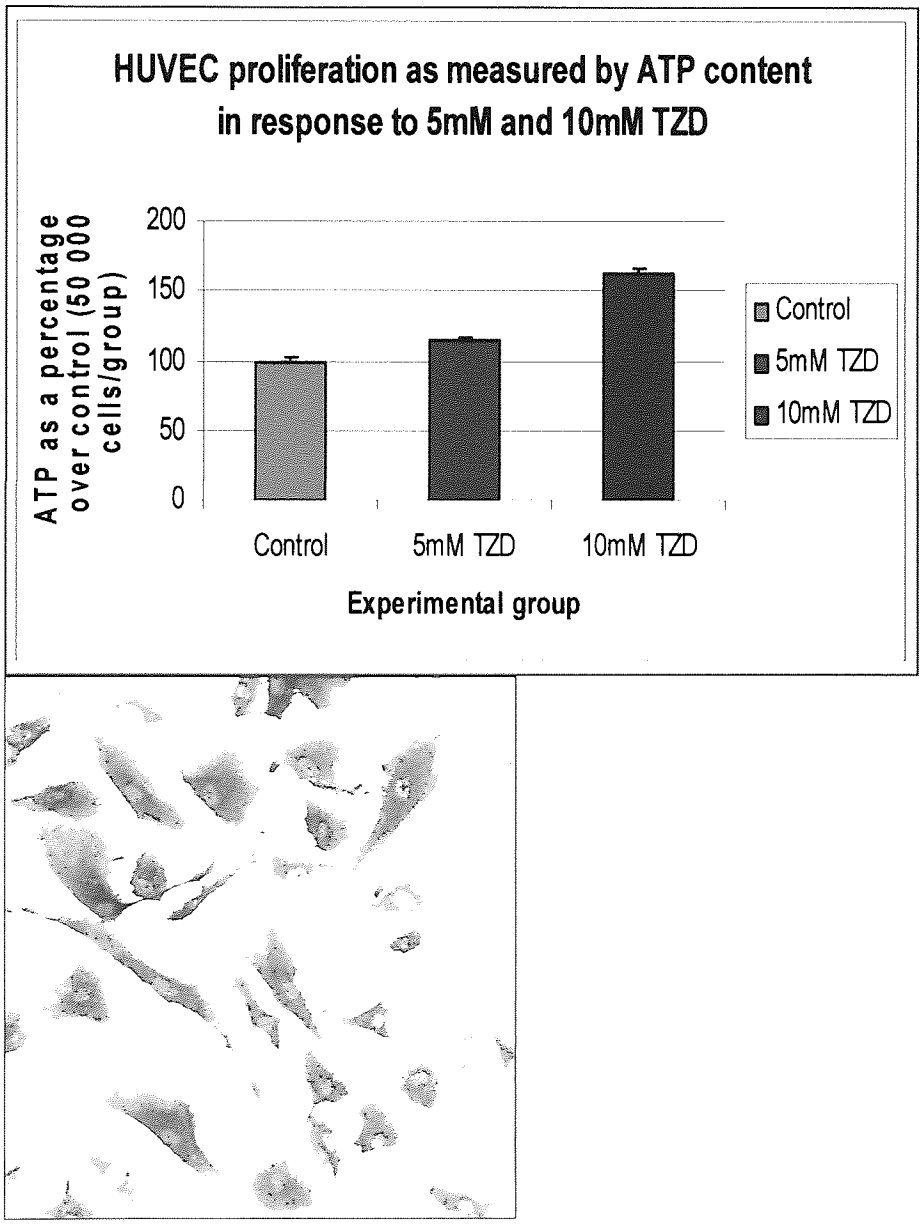


Figure 28. Proliferation rate of HUVEC monolayer, as determined by intracellular ATP concentration, in response to 5mM and 10mM rosiglitazone (top) and HUVEC monolayer positively stained for the vascular endothelial marker CD31 (bottom).

Cell culture

BRIN-BD11 and HUVEC proliferated well in static culture (SC) both in the presence and absence of 10mM TZD with no visual alteration to morphology. Also, a co-culture of the two cell types in SC +/- 10mM TZD did not appear to affect morphology or viability as both cell types survived and proliferated.

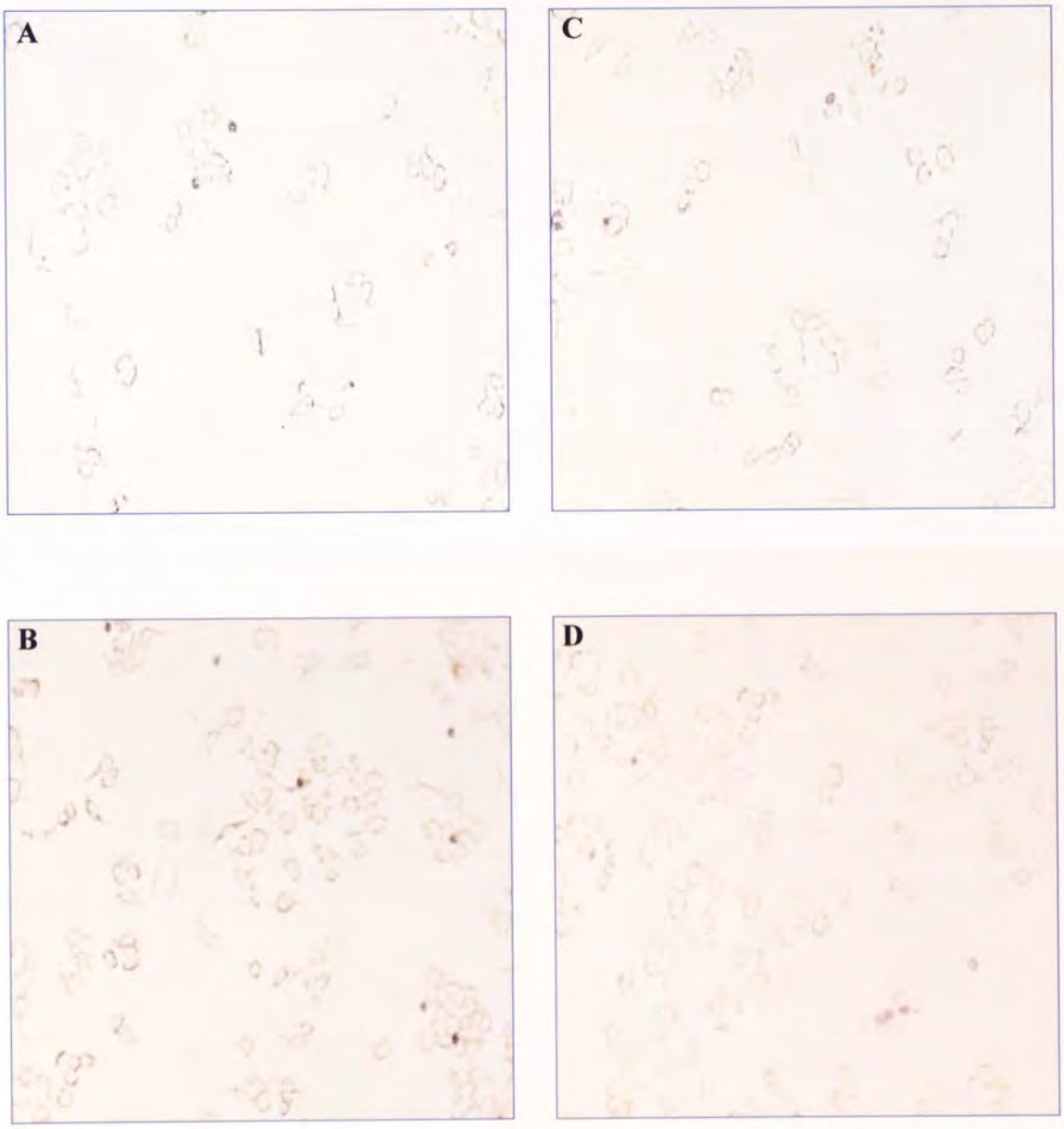


Figure 29. Images showing SC D11 cells at 24hrs culture (A) and 48hrs culture (B), 24hrs culture with 10mM TZD (C) and 48hrs culture with 10mM TZD (D). All images x10 magnification.

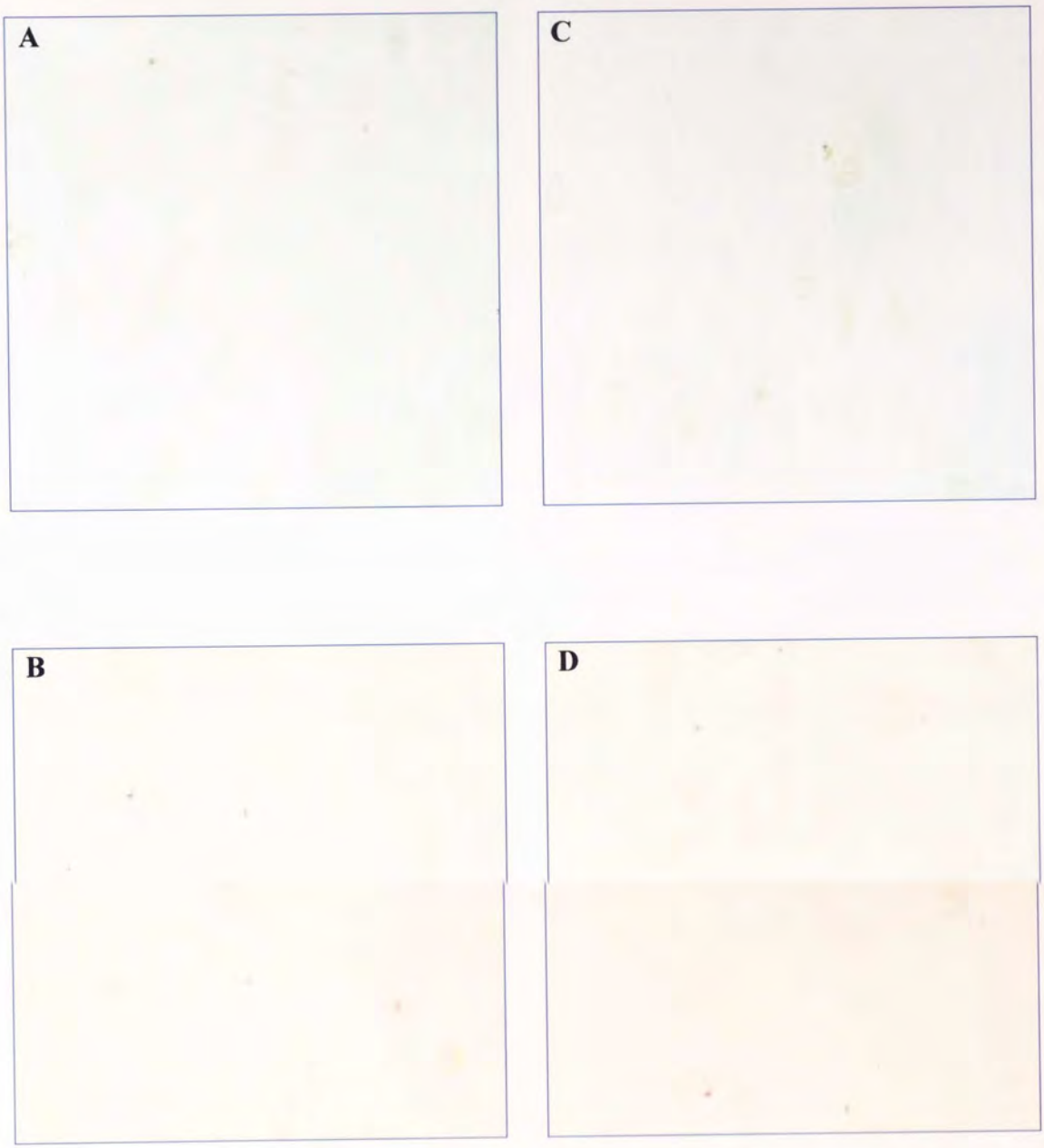


Figure 30. Images showing SC HUVEC at 24hrs culture (A) and 48hrs culture (B) HUVEC cultured with 10mM TZD at 24hrs (C) and 48hrs (D). All images x 10 magnification.

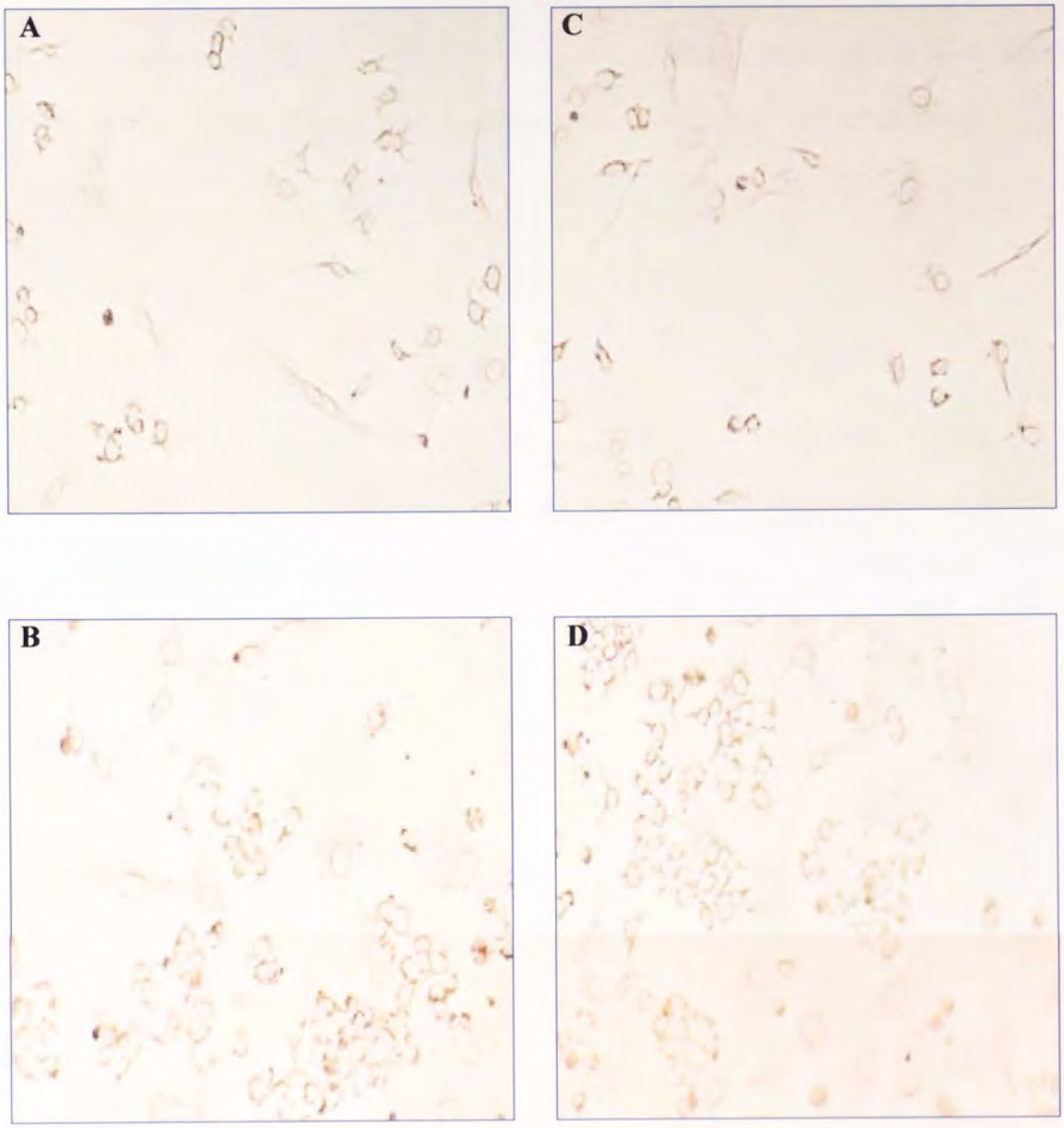


Figure 31. Images showing SC D11/ HUVEC co-culture at 24hrs culture (A) and 48hrs culture (B) and D11/HUVEC co-cultured with 10mM TZD at 24hrs (C) and 48hrs (D). All images x10 magnification.

BRIN-BD11 cells formed islet-like structures within 24 hours when cultured in a rotational cell culture system +/- HUVEC and +/- TZD. These structures varied in size from 50μm - 300μm. HUVEC alone did not form three dimensional structures in RC.

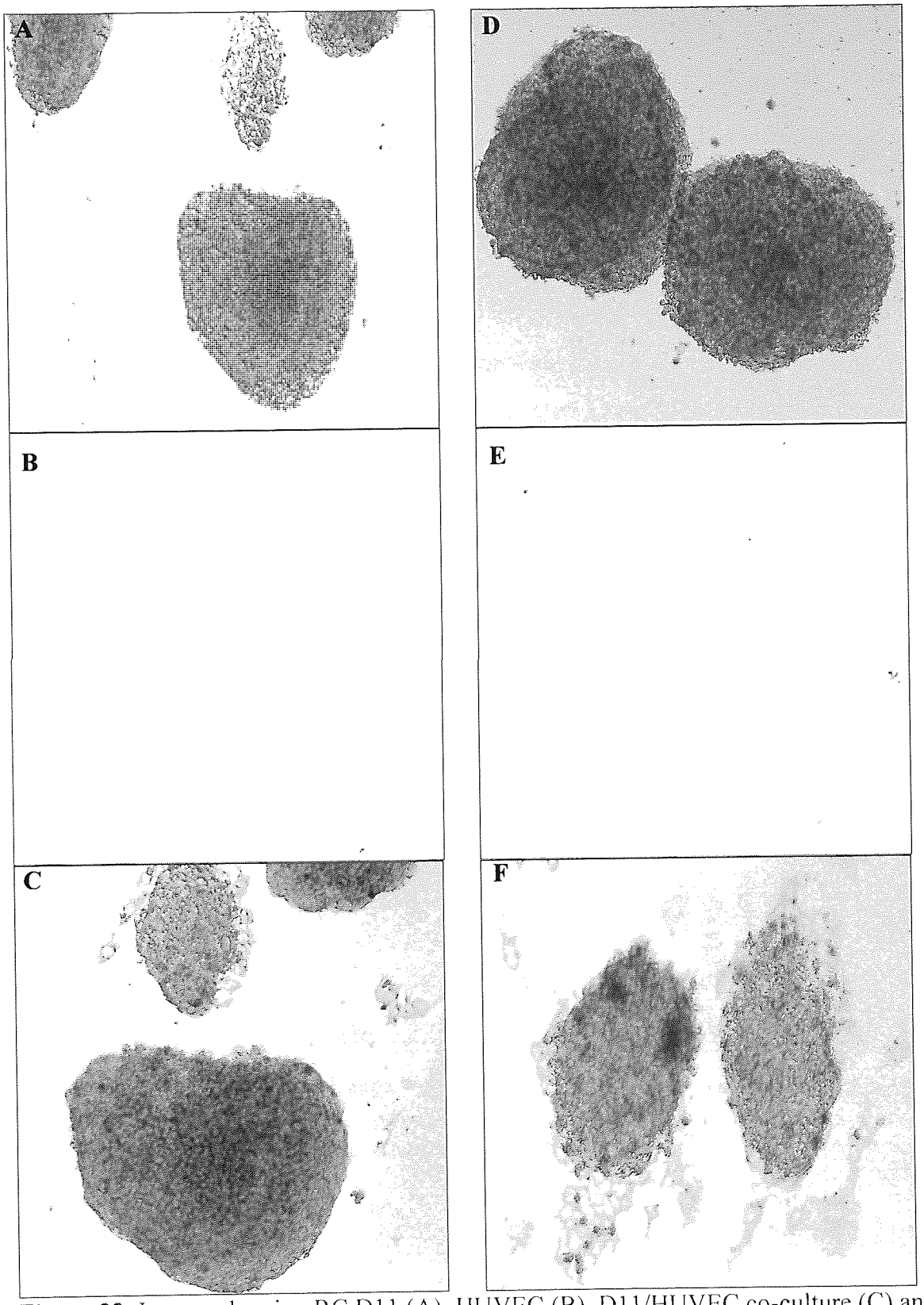


Figure 32. Images showing RC D11 (A), HUVEC (B), D11/HUVEC co-culture (C) and in the presence of 10mM TZD D11 (D), HUVEC (E), D11/HUVEC co-culture (F). All images x10 magnification.

Insulin secretion

As HUVEC did not secrete insulin in response to insulin secretagogues, data are not shown. Figure 33 graphically represents insulin secretion from D11 SC and D11/HUVEC co-culture SC +/- 10mM TZD, while table 5 summarises the statistically significant differences found in and between experimental groups. For each group the experiment was repeated three times with n = 6/secretagogue/treatment group each time. A Student's t-test was employed to determine statistically significant differences in and between treatment groups with $p<0.05$ being considered significant.

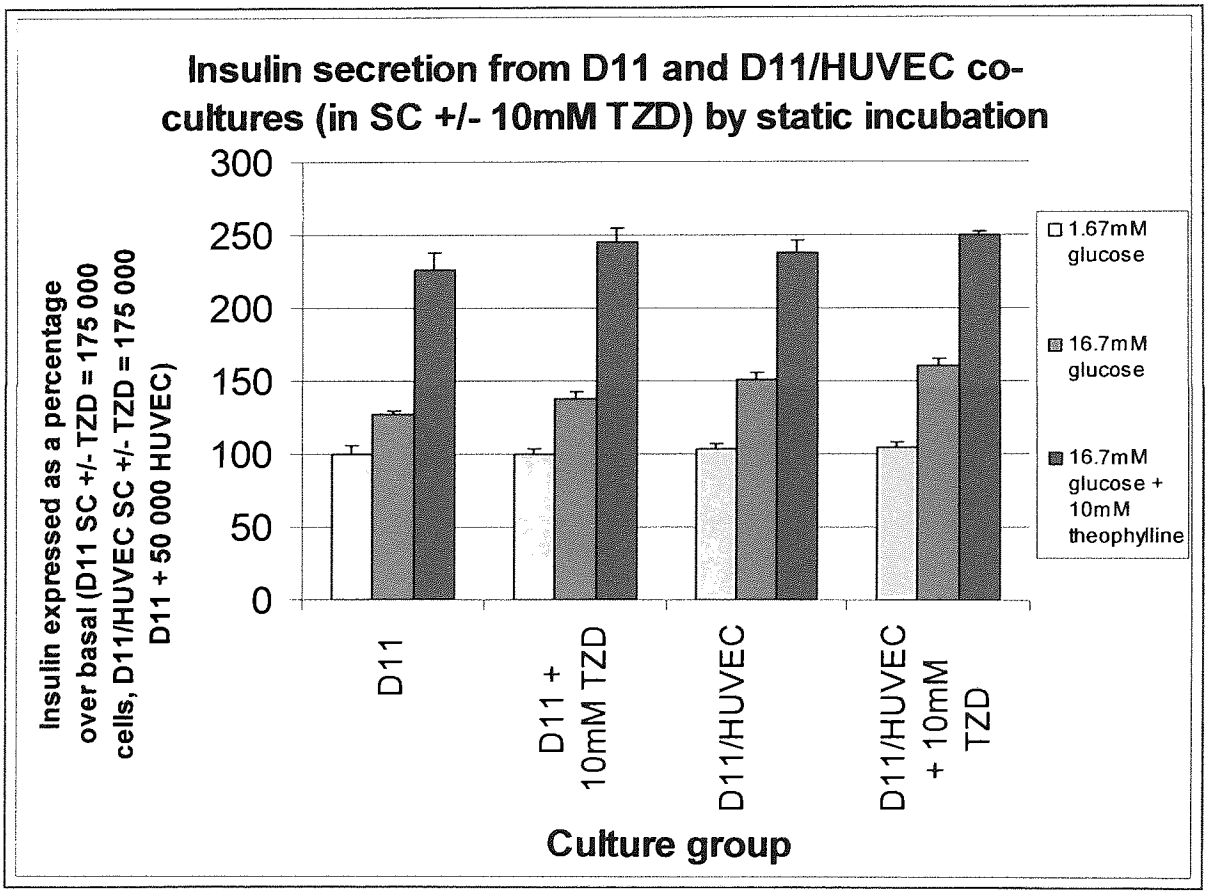


Figure 33. Insulin secretion from SC BRIN-BD11 cells and a co-culture of BRIN-BD11 cells with HUVEC +/- 10mM TZD. The results of Student's t-test for various comparisons are recorded in table 1 ($p<0.05$ = statistically significant), non-significant comparisons are not listed.

Comparison by Student's t-test	p-value
D11 16.7 v 1.67	<0.001
D11 G+T v 1.67	<0.001
D11 G+T v 16.7	<0.001
D11 TZD 16.7 v 1.67	<0.001
D11 TZD G+T v 1.67	<0.001
D11 TZD G+T v 16.7	<0.001
D11/HUVEC 16.7 v 1.67	<0.001
D11/HUVEC G+T v 1.67	<0.001
D11/HUVEC G+T v 16.7	<0.001
D11/HUVEC TZD 16.7 v 1.67	<0.001
D11/HUVEC TZD G+T v 1.67	<0.001
D11/HUVEC TZD G+T v 16.7	<0.001
D11/HUVEC 16.7 v D11 16.7	<0.001
D11/HUVEC TZD 16.7 v D11 TZD 16.7	<0.05

Table 5. Results of Student's t-test for figure 33.

Under RC conditions HUVEC also did not secrete insulin, hence data are not shown. Figure 34 graphically represents insulin secretion from D11 RC and D11/HUVEC co-culture RC +/- 10mM TZD, while table 6 summarises the statistically significant differences found in and between experimental groups.

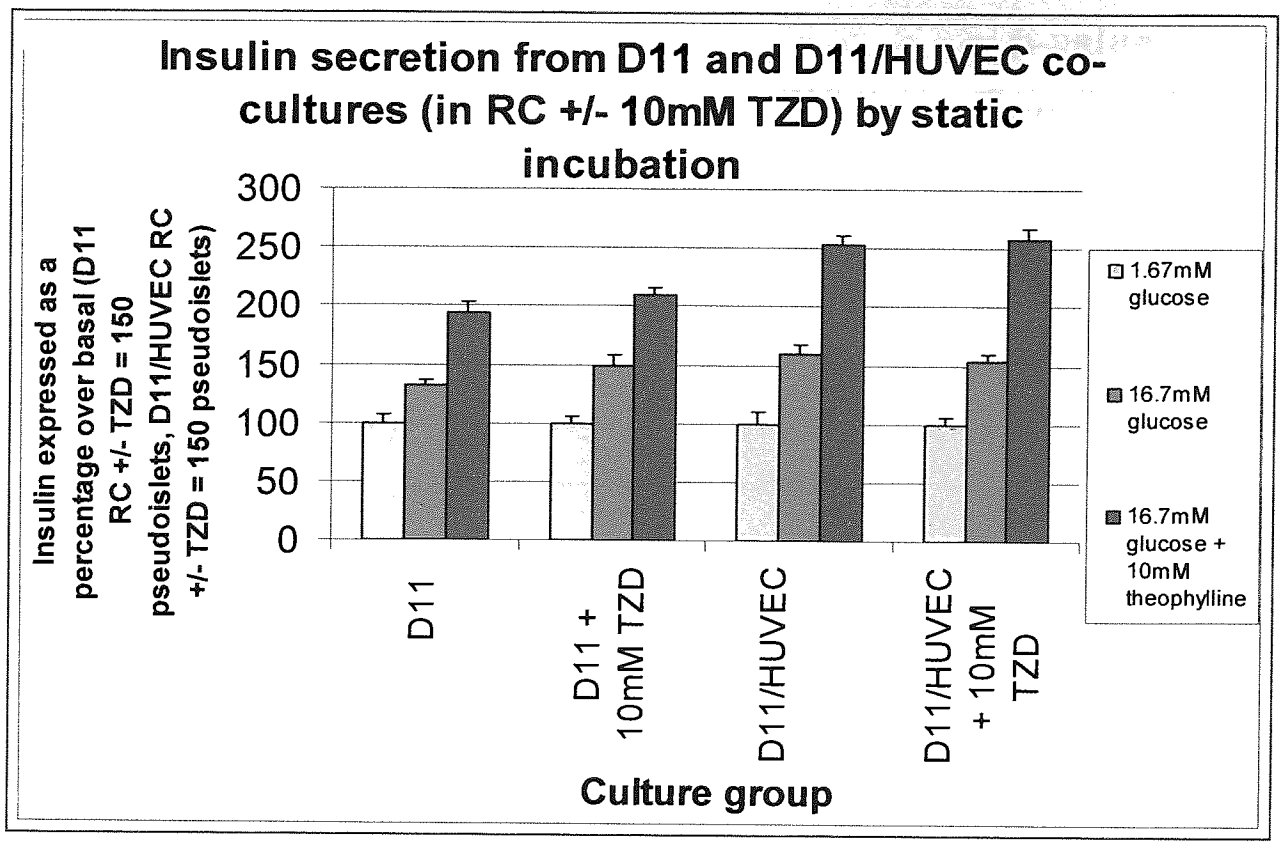


Figure 34. Insulin secretion from RC BRIN-BD11 cells and a co-culture of BRIN-BD11 cells with HUVEC +/- 10mM TZD. The results of Student's t-test for various comparisons are recorded in table 6 ($p<0.05$ = statistically significant), non-significant comparisons are not listed.

Comparison by Student's t-test	p-value
D11 16.7 v 1.67	<0.05
D11 G+T v 1.67	<0.001
D11 G+T v 16.7	<0.001
D11 TZD 16.7 v 1.67	<0.05
D11 TZD G+T v 1.67	<0.001
D11 TZD G+T v 16.7	<0.05
D11/HUVEC 16.7 v 1.67	<0.001
D11/HUVEC G+T v 1.67	<0.05
D11/HUVEC G+T v 16.7	<0.001
D11/HUVEC TZD 16.7 v 1.67	<0.001
D11/HUVEC TZD G+T v 1.67	<0.001
D11/HUVEC TZD G+T v 16.7	<0.001
D11/HUVEC 16.7 v D11 16.7	<0.05
D11/HUVEC G+T v D11 G+T	<0.05
D11/HUVEC TZD 16.7 v D11 TZD 16.7	<0.05
D11/HUVEC TZD G+T v D11 TZD G+T	<0.05

Table 6. Results of Student's t-test for figure 34.

ATP bioluminescence

Preliminary experiments to assess HUVEC ATP content in response to 5mM and 10mM TZD (figure 28) showed that 10mM TZD resulted in a more robust increase in ATP content as compared to control HUVEC, hence 10mM TZD was used to stimulate HUVEC proliferation in both SC and RC. For each group the experiment was repeated three times with n =

6/treatment group each time. A Student's t-test was used to determine statistically significant differences between groups with $p<0.05$ being considered significant.

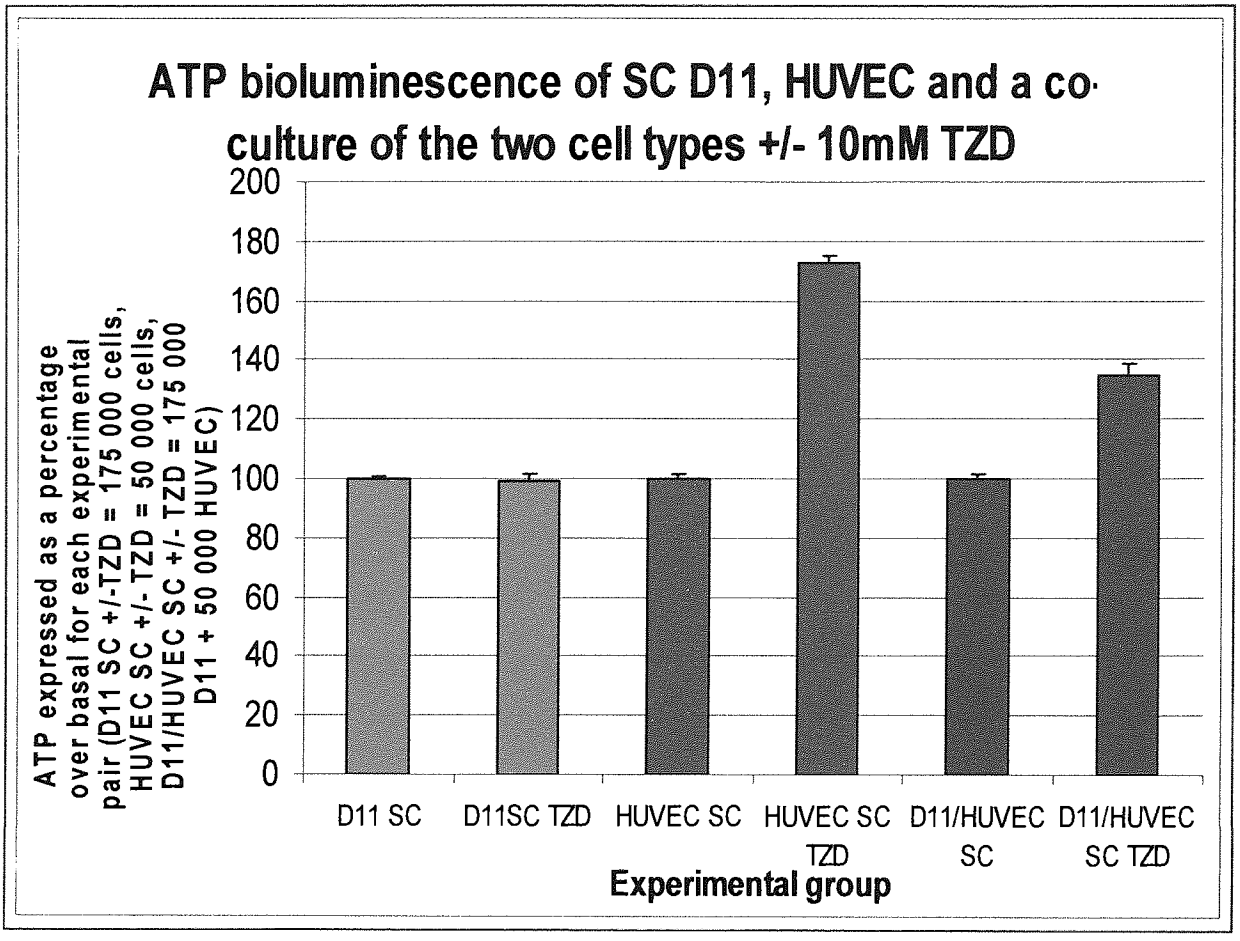


Figure 35. ATP bioluminescence results for SC D11, HUVEC and a co-culture of the two cell types +/- 10mM TZD. A Student's t-test returned a p-value of 0.91 when comparing D11 SC TZD to D11 SC and $p<0.001$ for both HUVEC SC TZD v HUVEC SC and D11/HUVEC SC TZD v D11/HUVEC SC.

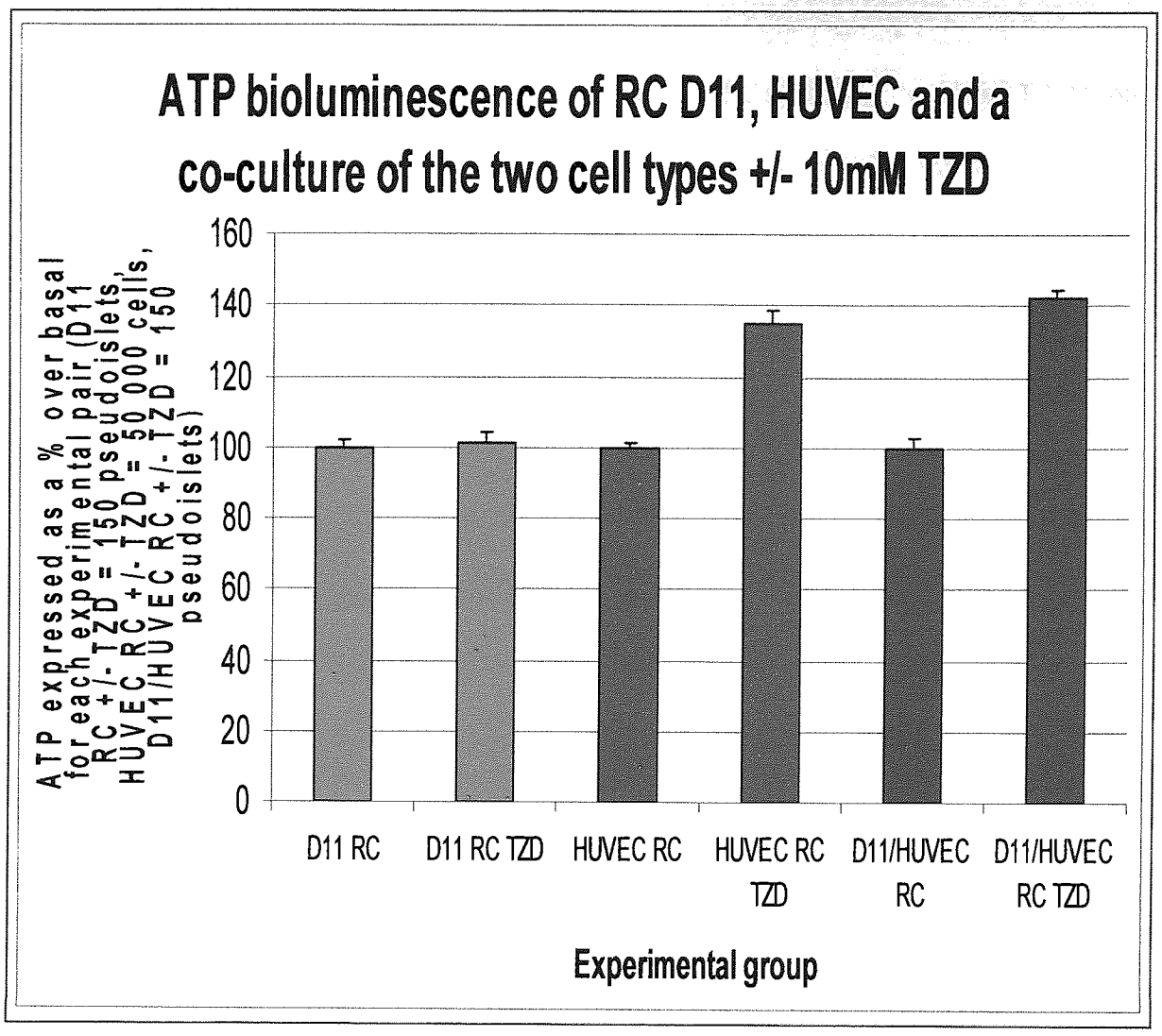


Figure 36. ATP bioluminescence results for RC D11, HUVEC and a co-culture of the two cell types +/- 10mM TZD. A Student's t-test returned a p-value of 0.62 when comparing D11 RC TZD to D11 RC and $p<0.001$ for both HUVEC RC TZD v HUVEC RC and D11/HUVEC RC TZD v D11/HUVEC RC.

Immunostaining

D11 cells, HUVEC and a co-culture of the two cell types (SC and RC, +/- 10mM TZD) were plated and cultured as for insulin secretion. After 24 hours medium was removed and cells were rinsed with PBS prior to being fixed with cold 70% ethanol.

D11 were immunostained using a polyclonal rabbit anti-rat insulin antibody and visualised using the Vector Labs ABC-AP rabbit kit and Vector Red. HUVEC were immunostained using a monoclonal mouse-anti-human CD31 antibody. Visualisation was performed using the Vector Labs ABC-AP mouse kit and Vector Blue. Appropriate controls were used to rule out non-specific staining.

Figure 37 shows immunostaining of D11 SC +/- 10mM TZD while figure 38 shows the results for D11 RC +/- 10mM TZD. Figures 39 - 42 show results for HUVEC SC +/- 10mM TZD, HUVEC RC +/- 10mM TZD, D11/HUVEC SC +/- 10mM TZD and D11/HUVEC RC +/- 10mM TZD.

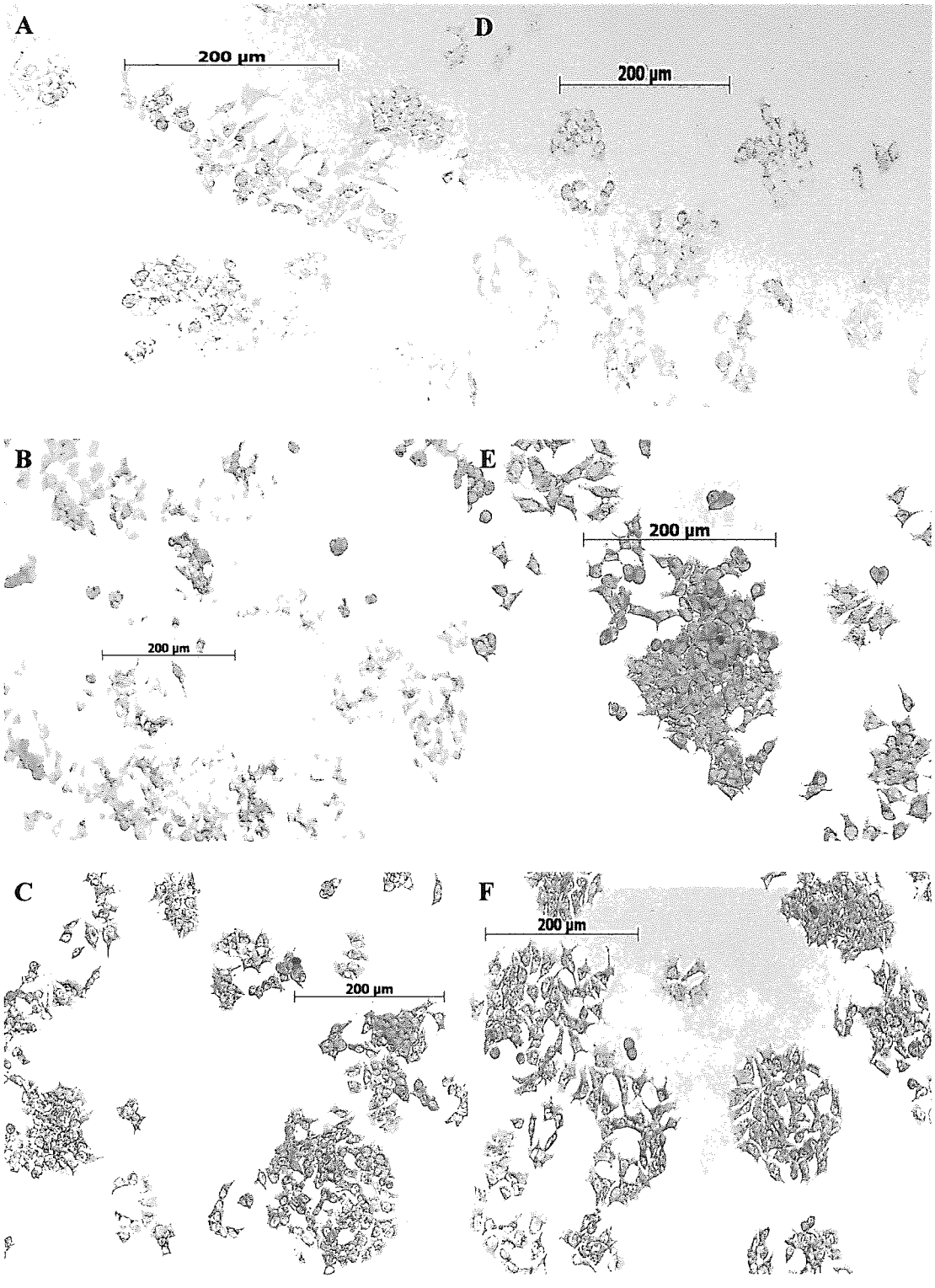


Figure 37. D11 SC cultured without 10mM TZD. Immunostaining for CD31 (A), for insulin (B) and dual stained for insulin and CD31 (C). D11 SC cultured with 10mM TZD. Immunostaining for CD31 (D), for insulin (E) and dual stained for insulin and CD31 (F). All images x20 magnification.

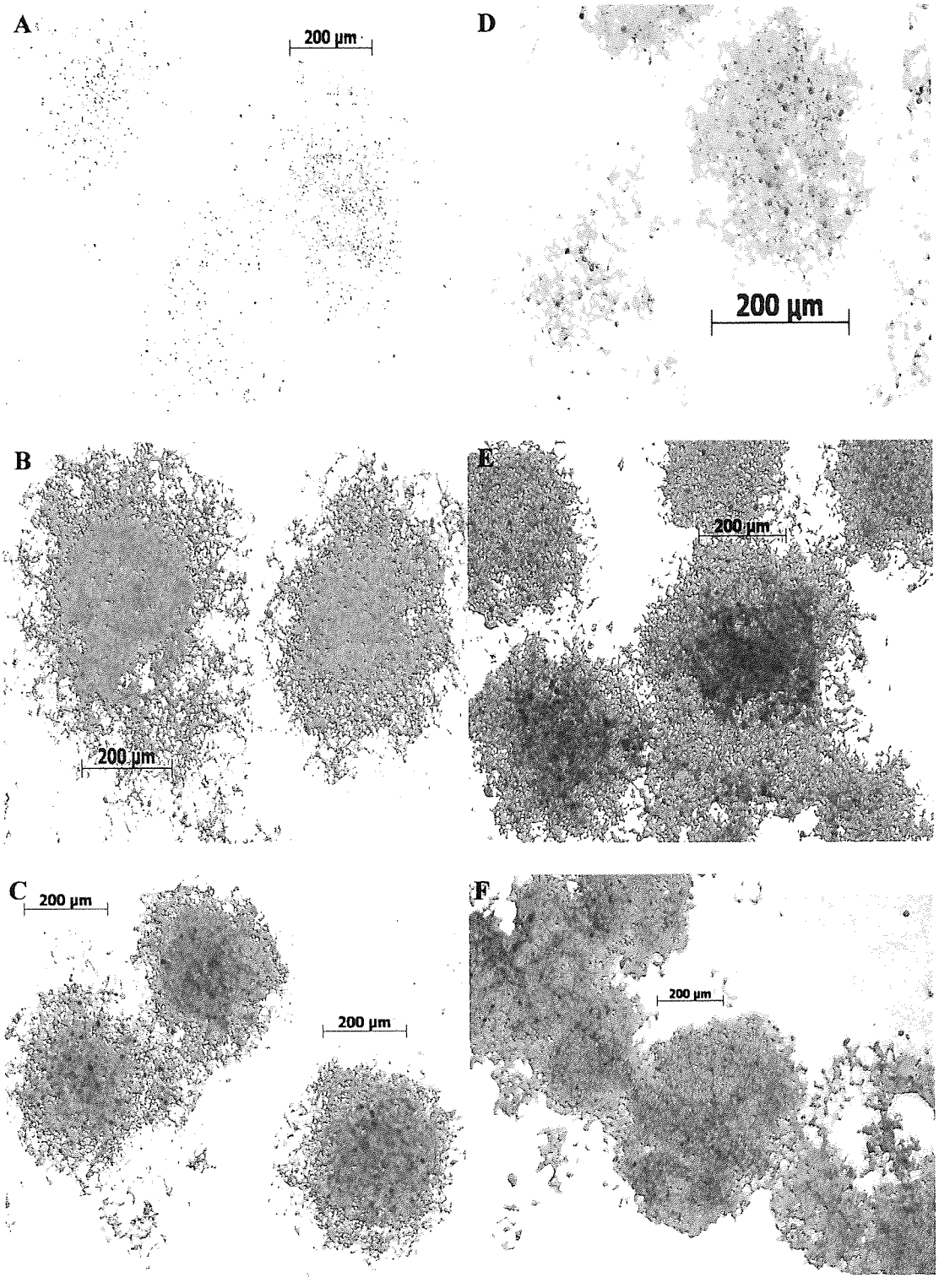


Figure 38. D11 RC cultured without 10mM TZD. Immunostaining for CD31 (A), for insulin (B) and dual stained for insulin and CD31 (C). D11 RC cultured with 10mM TZD. Immunostaining for CD31 (D), for insulin (E) and dual stained for insulin and CD31 (F). All images x10 magnification.

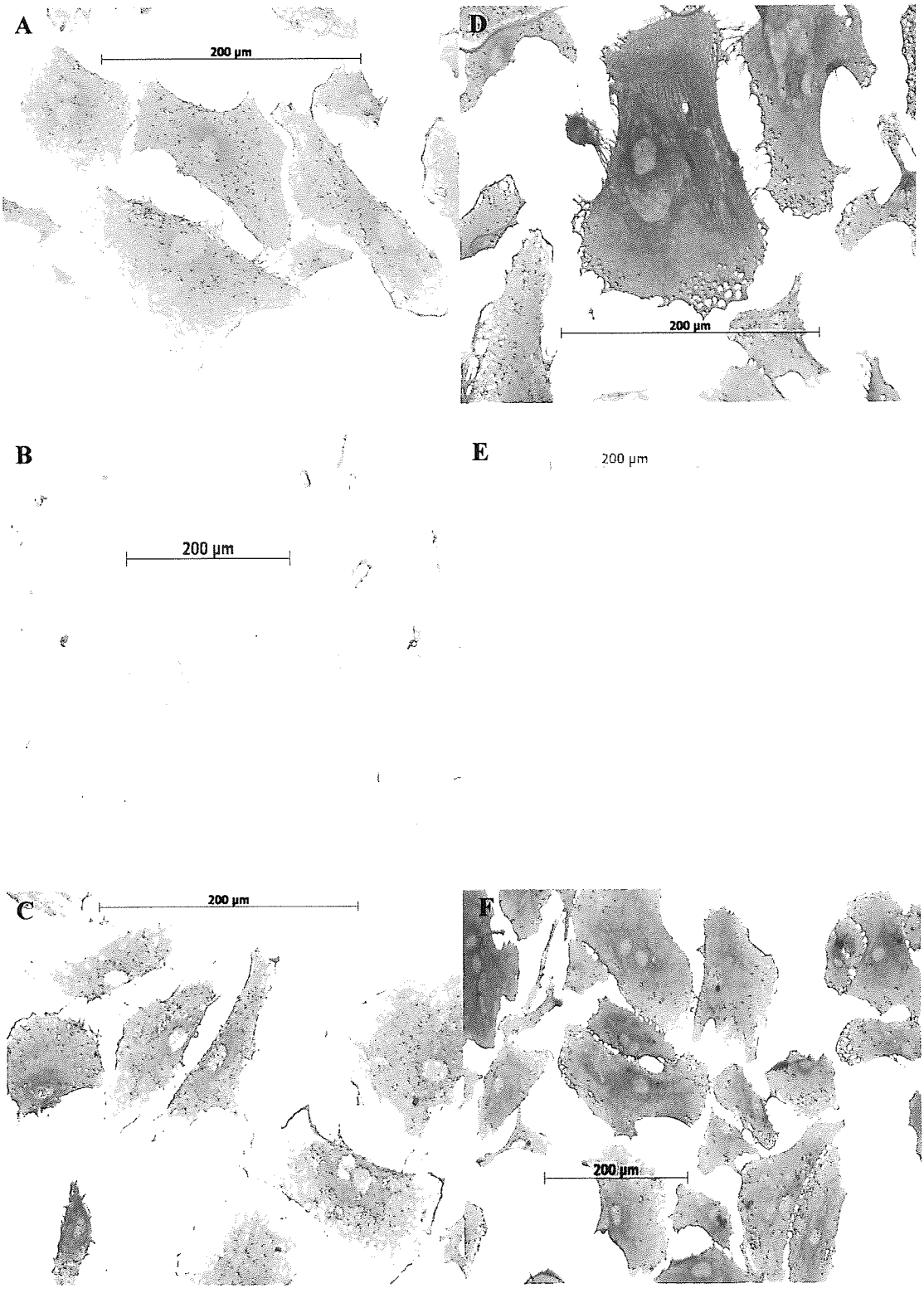


Figure 39. HUVEC SC cultured without 10mM TZD. Immunostaining for CD31 (A), for insulin (B) and dual stained for insulin and CD31 (C). HUVEC SC cultured with 10mM TZD. Immunostaining for CD31 (D), for insulin (E) and dual stained for insulin and CD31 (F). Images A, D, C & F x40 magnification and images B & E x20 magnification.

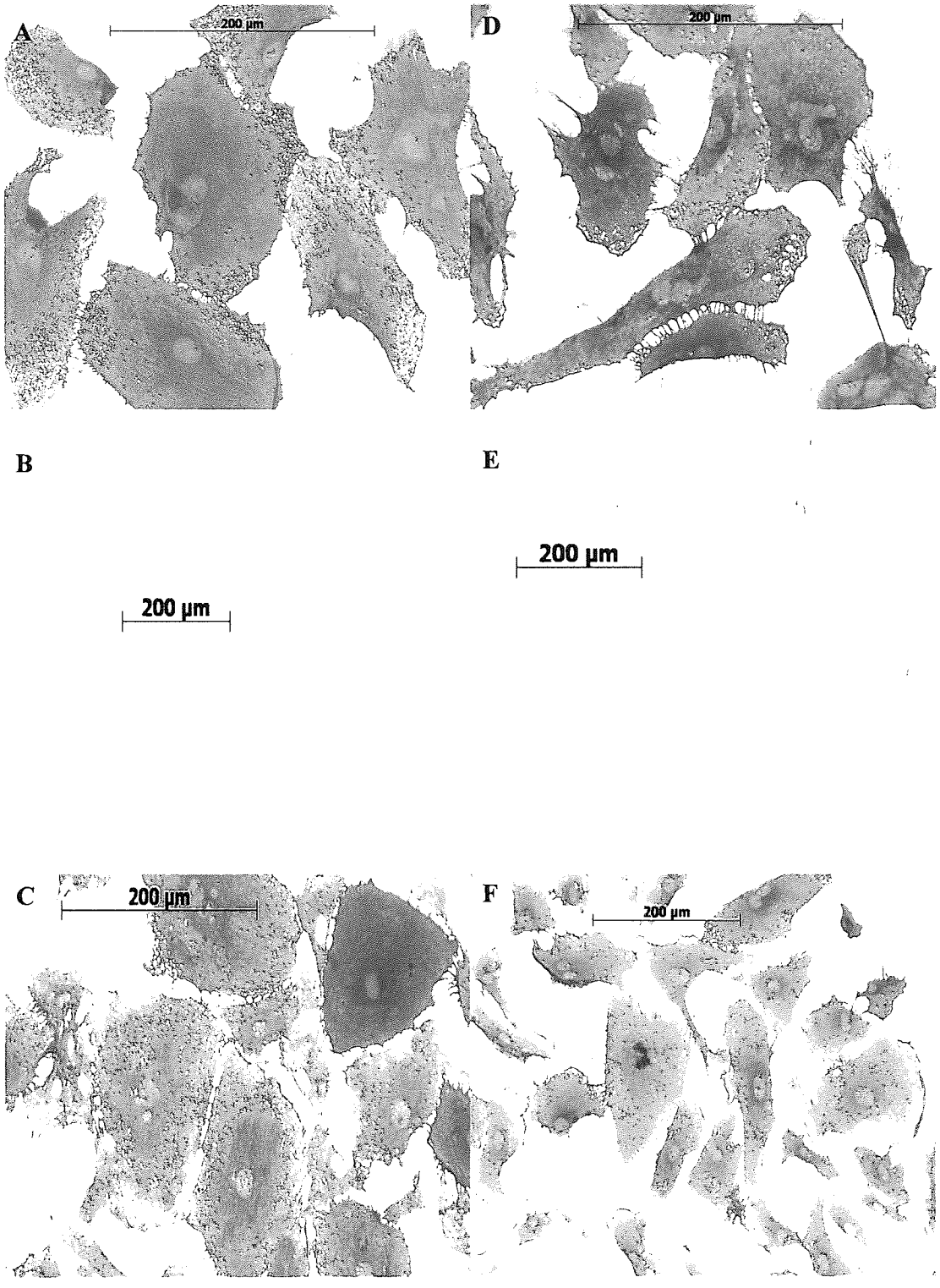


Figure 40. HUVEC RC cultured without 10mM TZD. Immunostaining for CD31 (A), for insulin (B) and dual stained for insulin and CD31 (C). HUVEC RC cultured with 10mM TZD. Immunostaining for CD31 (D), for insulin (E) and dual stained for insulin and CD31 (F). All images x20 magnification.

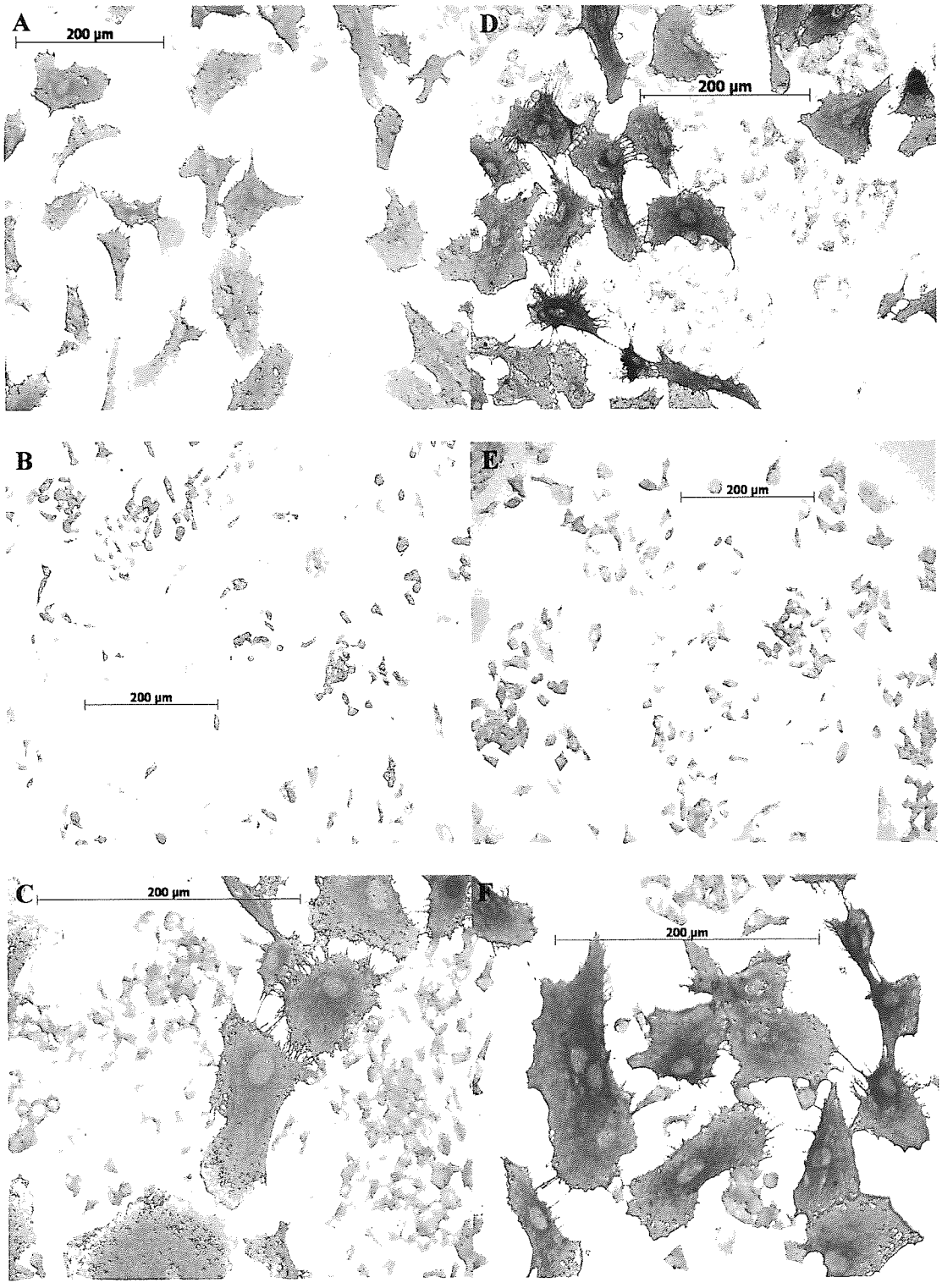


Figure 41. D11/HUVEC SC co-culture without 10mM TZD. Immunostaining for CD31 (A), for insulin (B) and dual stained for insulin and CD31 (C). D11/HUVEC SC co-culture with 10mM TZD. Immunostaining for CD31 (D), for insulin (E) and dual stained for insulin and CD31 (F). Images A, B, D & E x20 magnification and images C & F x40 magnification.

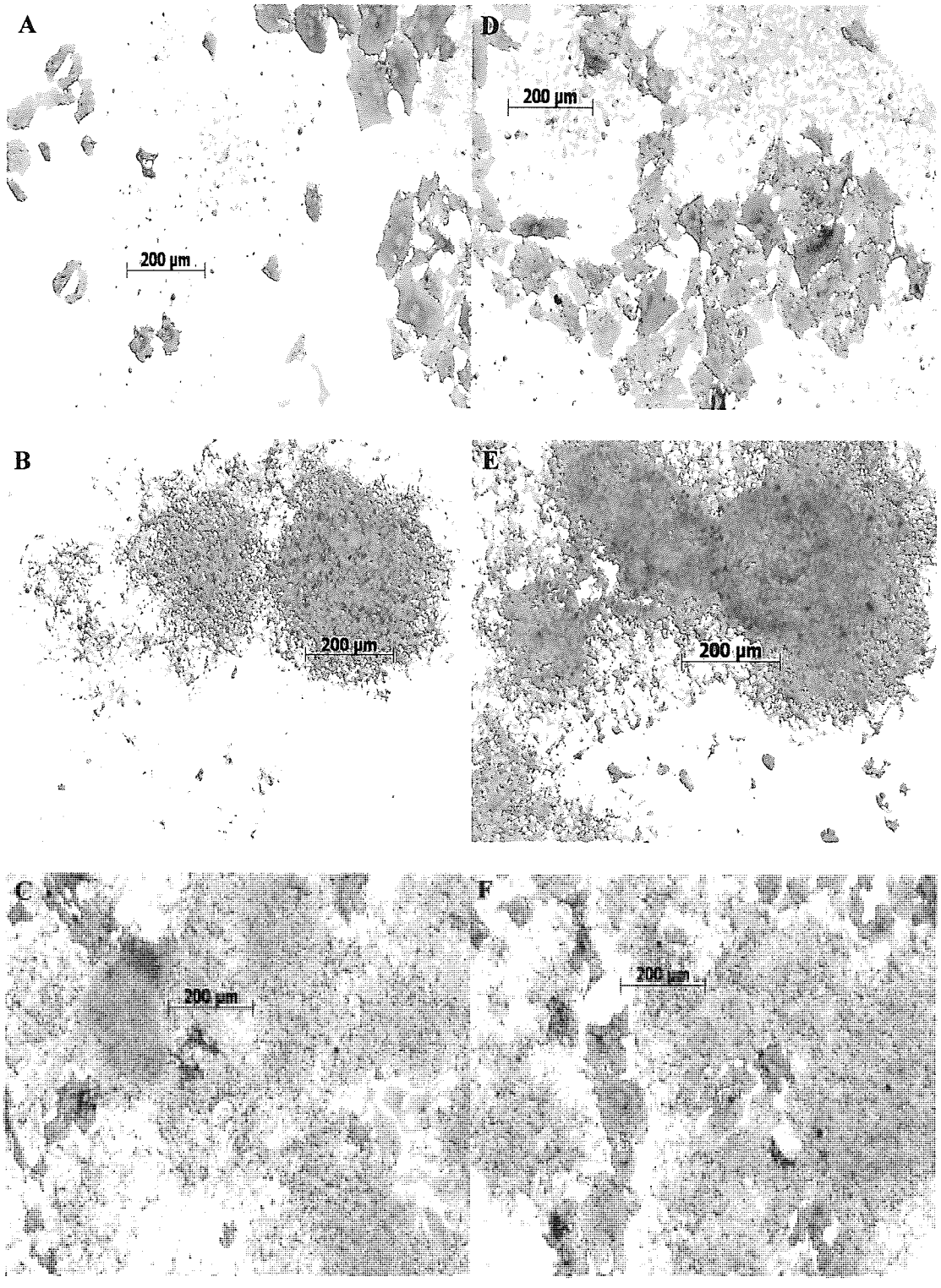


Figure 42. D11/HUVEC RC cultured without 10mM TZD. Immunostaining for CD31 (A), for insulin (B) and dual stained for insulin and CD31 (C). D11/HUVEC RC cultured with 10mM TZD. Immunostaining for CD31 (D), for insulin (E) and dual stained for insulin and CD31 (F). All images x10 magnification.

VEGF production and release

Upregulation of VEGF expression by a TZD via the PPARγ pathway requires supraphysiological doses of the agonist, with researchers using concentrations up to 25mM TZD *in vitro* to elicit the desired response (Yamakawa et al. 2000). *In vivo* studies have shown that concentrations of TZDs up to 1500 times the human therapeutic dose were well tolerated (Lewis et al. 2001; Mody et al. 2007; Lewis et al. 2008). A dose response experiment indicated that HUVEC responded best to a 10mM concentration of TZD in terms of intracellular ATP content, while this dose also supported secretagogue-induced D11 insulin secretion.

HUVEC were grown in both static and rotational culture +/- BRIN-BD11 cells, after which they were transferred to 24-well plates +/- 10mM TZD. After 24 hours of treatment the tissue culture medium was harvested and VEGF was detected by ELISA (Cross et al. 2007; Laugharne et al. 2007). Figure 43 graphically represents VEGF release from SC HUVEC and D11/HUVEC +/- 10mM TZD, while figure 44 represents VEGF release from RC HUVEC and D11/HUVEC +/- 10mM TZD. For each group the experiment was repeated three times with n = 4/treatment group each time. A Student's t-test was used to determine statistically significant differences in VEGF release in and between experimental groups, with $p<0.05$ being considered significant.

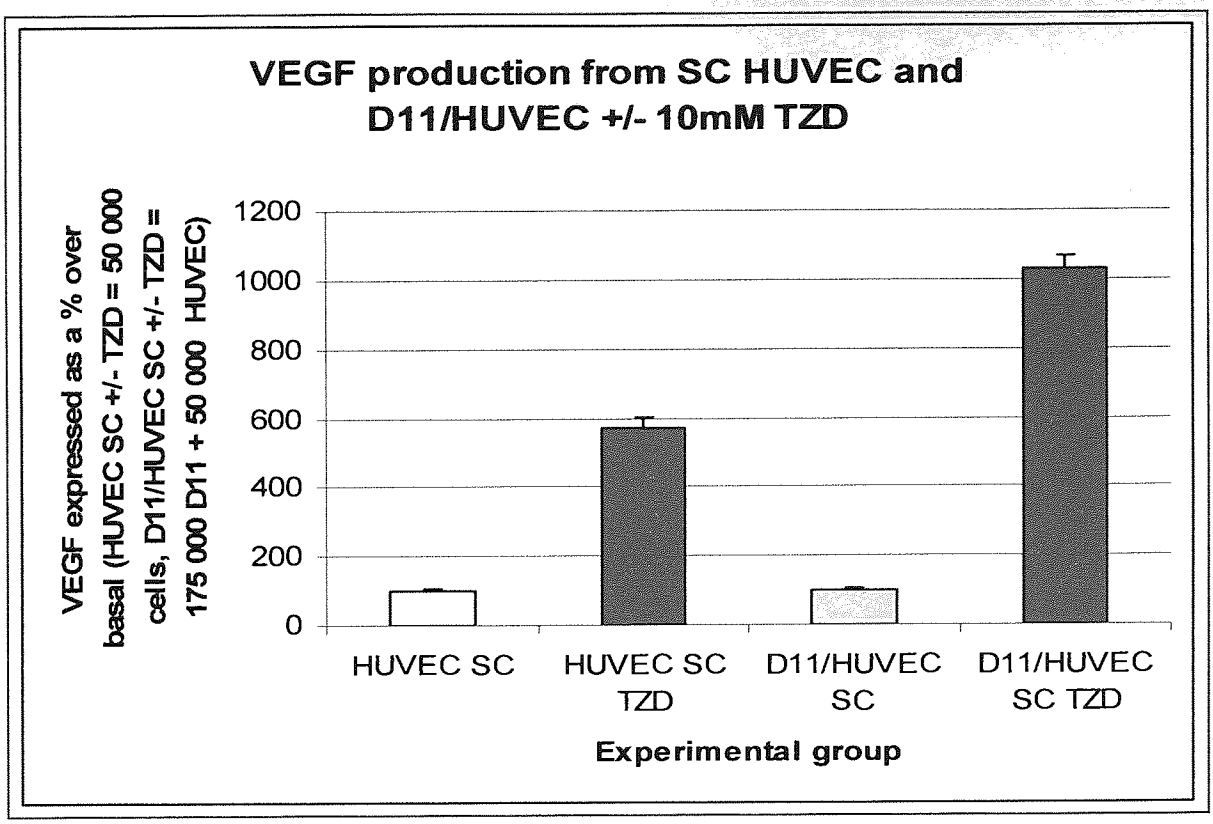


Figure 43. VEGF release from SC HUVEC and a co-culture of BRIN-BD11 cells with HUVEC +/- 10mM TZD. A Student's t-test ($p<0.05$ = statistically significant), returned $p<0.01$ for both HUVEC SC TZD compared to HUVEC SC and D11/HUVEC SC TZD compared to D11/HUVEC SC.

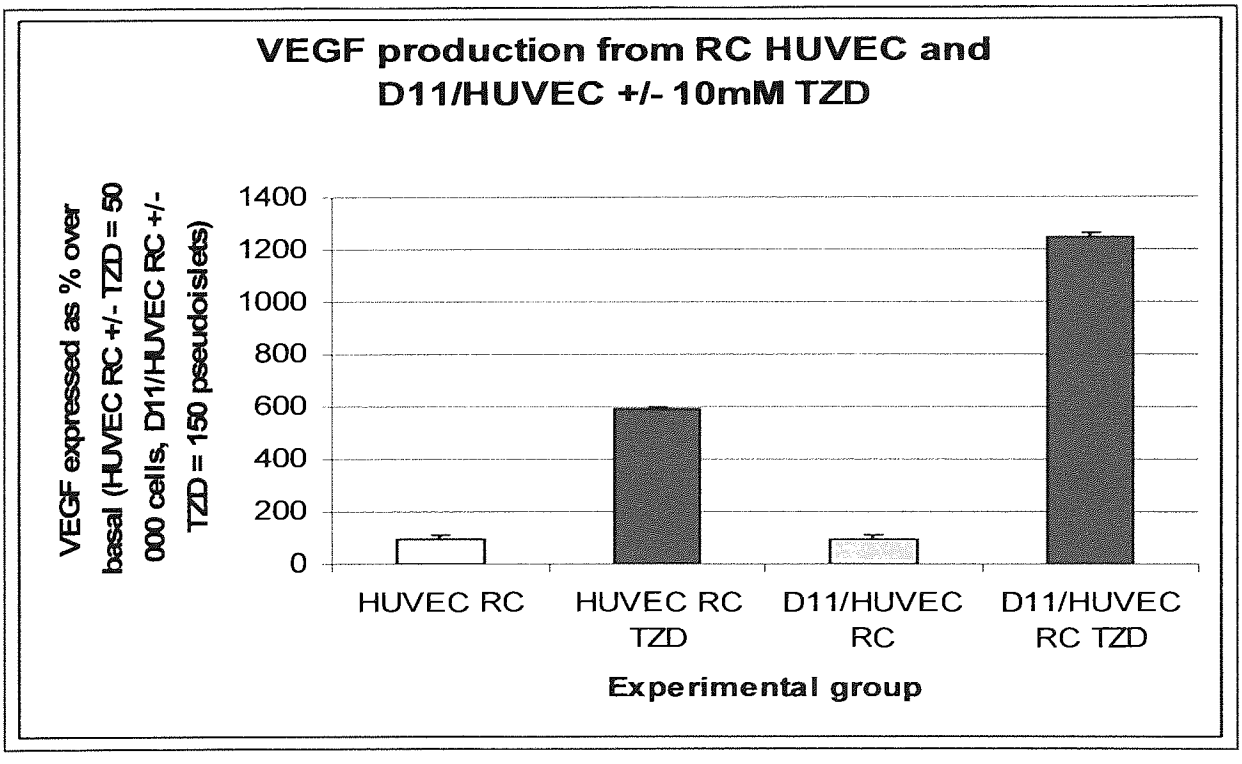


Figure 44. VEGF release from RC HUVEC and a co-culture of BRIN-BD11 cells with HUVEC +/- 10mM TZD. A Student's t-test ($p<0.05$ = statistically significant), returned $p<0.01$ for both HUVEC RC TZD compared to HUVEC RC and D11/HUVEC RC TZD compared to D11/HUVEC RC.

Discussion

Cell culture

The culture medium of choice for HUVEC endothelial growth medium (EGM) supplemented with 1% foetal bovine serum (FBS), while D11 are cultured in RPMI + 10% FBS. In order to determine the optimal culture medium for the co-culture of these two cell types, the following combinations of the two media were tested:

- 100% EGM
- 75% EGM : 25% RPMI
- 50% EGM : 50% RPMI
- 25% EGM : 75% RPMI
- 100% RPMI

As expected, both cell types grew well in the medium suggested for their growth and maintenance. HUVEC did not perform well in 100% or 75% RPMI as many cells failed to adhere to the tissue culture flask and proliferation of the adhered cells was poor, requiring more than twice as long (two weeks as opposed to 6 days) to reach confluence as cells in 100% EGM. 50% and 25% RPMI resulted in improved proliferation of HUVEC with only a slight delay (8 days required) in reaching confluence when compared to 100% EGM.

D11 did not perform well in 100% or 75% EGM, as indicated by retarded growth rate with 10 days being required instead of the customary 5 to reach confluence and a significant proportion of cells failing to adhere to the culture flask. 50% and 25% EGM resulted in improved D11 proliferation with confluence being achieved in 7 days.

One of the major differences between the two culture media is the percentage of FBS used, with HUVEC requiring only 1% and D11 10%. The combination of media that supported

acceptable growth rates of both cell types was 50% EGM: 50% RPMI containing 5.5% FBS. Endothelial cells are generally grown under low serum (1 – 2%) conditions (Bishop et al. 1999; Lang et al. 2001), however, blood serum is defined as plasma without fibrinogen and other clotting factors and typically contains 6 – 8% protein. Normal blood thus contains more than 1 – 2% serum and endothelial cells proliferate well under these conditions physiologically to repair injury and form new blood vessels when required. This could explain why HUVEC proliferation *in vitro* was supported by medium with 5.5% FBS. Endothelial cells have been shown to proliferate well in the presence of 10% serum when angiogenic factors were present (Davison et al. 1980; Gospodarowicz and Ill 1980). EGM contains VEGF, fibroblast growth factor, insulin-like growth factor and epidermal growth factor. The presence of these stimulators of endothelial proliferation in the culture medium may also explain the sustained HUVEC proliferation in the presence of 5.5% FBS. Also, the addition of 10mM TZD upregulated the expression of VEGF by HUVEC via the PPARγ pathway (discussed in more detail in chapter 5), further enhancing proliferative ability in the presence of 5.5% serum.

When D11 were grown in RC (+/- HUVEC, +/- TZD) cell constructs (pseudoislets) resembling human islets were formed (figure 32). In the pancreas, most of the beta cells form part of the three dimensional structure of islets, with only a relatively low percentage of lone beta cells scattered throughout the exocrine component of the pancreas (Bouwens 2004). Also, both islets and beta cells from cell lines have been shown to possess a more sensitive and potent insulin secretory response to secretagogues when contained within islets or pseudoislets (Hauge-Evans et al. 1999; Squires et al. 2000; Hauge-Evans et al. 2002; Squires et al. 2002; Brereton et al. 2007), indicating an important communicative connection requirement for optimal function.

Insulin secretion

As shown in table 5 and figure 33, all the D11 static cultures (including co-cultures with HUVEC and +/- 10mM TZD) showed a significant increase in insulin secretion over basal (1.67mM glucose) when challenged with high glucose (16.7mM). For all of these groups a challenge with high glucose and 10mM theophylline (G+T) resulted in significantly more insulin being released when compared to both the basal and high glucose results. These results indicate that the D11 cells used in these experiments were healthy and functional.

Comparisons between the SC groups revealed that D11/HUVEC co-culture insulin secretion in response to 16.7mM glucose was significantly higher than that of D11 alone with the same being true for D11/HUVEC TZD 16.7 versus D11 16.7 and D11/HUVEC TZD 16.7 versus D11 TZD 16.7. These findings imply that addition of HUVEC to a D11 culture supports the function of the beta cells as the addition of TZD without HUVEC did not result in a significant increase in insulin secretion with either 16.7 or G+T. No significant differences were found between groups when the G+T insulin secretion results were compared. This propensity of D11 cells to become more responsive to secretagogues when co-cultured with another cell type has been noted by the IRL with at least two cell types other than HUVEC (data not yet published).

In the rotational culture (RC) groups significant increases in insulin secretion was observed when both 16.7 and G+T were compared to basal (1.67) glucose and also when G+T was compared to 16.7, again confirming viability of the D11 cells used and indicating that RC supported D11 insulin secretory function.

As in the SC groups a significant increase in insulin secretion was seen when the following comparisons between groups were performed: D11/HUVEC 16.7 versus D11 16.7, D11 and

D11/HUVEC TZD 16.7 versus D11 TZD 16.7 (table 6 and figure 34). These results suggest that the addition of HUVEC to a D11 rotational culture improves the function of the beta cells while the addition of TZD alone does not. This observation again supports the findings that beta cells perform more effectively when they form part of a three dimensional structure. The exact influences of the close proximity of other cell types are yet to be elucidated.

While the SC groups showed no significant difference in the G+T results between groups, the RC groups resulted in the following significant G+T increases: D11/HUVEC G+T versus D11 G+T, D11/HUVEC TZD G+T versus D11 G+T and D11/HUVEC TZD G+T versus D11 TZD G+T. As these differences were not seen in the SC groups, these results indicate a possible beta cell supportive effect of the rotational cell culture method. The positive effects of RC on human islet insulin secretion (Murray et al. 2005) is shown and discussed in chapter 5.

ATP bioluminescence

Static culture experiments employing 10mM TZD in an attempt to enhance HUVEC proliferation via an upregulation in VEGF biosynthesis resulted in a significant ($p<0.001$) increase in intercellular ATP of HUVEC and D11/HUVEC co-culture ($p<0.001$), but no significant difference was found in D11 alone cultures ($p = 0.91$).

A significant increase in ATP levels between treated and control groups indicated an enhanced proliferation rate. This experiment shows that addition of 10mM TZD to D11 culture did not increase the rate of cellular proliferation, while both the HUVEC and D11/HUVEC co-cultures experienced increased proliferation rates when exposed to 10mM TZD. Although the increase in ATP levels over control was highly significant for both HUVEC and D11/HUVEC co-cultures, the HUVEC TZD alone culture ATP was significantly

higher ($p<0.001$) than the D11/HUVEC TZD co-culture. This could be due to the fact that the D11 in the co-culture were not proliferating at an enhanced rate while the HUVEC proliferation was upregulated, thus the D11 'diluted' the effect of TZD on HUVEC growth as indicated by ATP content.

In RC experiments, D11 alone did not show increased ATP levels in response to 10mM TZD ($p = 0.62$), while HUVEC and D11/HUVEC co-culture showed significantly increased ATP levels ($P<0.001$ for both). However, HUVEC TZD alone cultures showed a significantly lower percentage increase in ATP content over control than D11/HUVEC TZD co-culture ($p<0.05$). This implies a supportive effect of D11 to HUVEC in RC. However, this comparison should be viewed with caution as RC results in D11 pseudoislet formation with unquantifiable incorporation of HUVEC, while the HUVEC TZD group contained known numbers (50 000) of HUVEC.

Also, when %ATP increase over control is compared HUVEC SC TZD shows a significantly enhanced increase in ATP levels over HUVEC RC TZD ($p<0.001$). This indicates that HUVEC perform better under SC conditions than in RC, but that the addition of D11 to HUVEC RC partially reverses this phenomenon.

Immunostaining

D11expressed insulin and HUVEC stained positively for CD31; with no non-specific background staining occurring in either culture when grown under static culture conditions both individually and as co-cultures (figures 37, 39, 41). D11 did not express the endothelial cell marker CD31, but HUVEC showed a faint positive staining for insulin (figures 39, 41). It is possible that HUVEC sequestered small quantities of insulin from the culture medium (Paek et al. 2005), but did not release any in response to insulin secretagogues. Also,

endothelial cells possess insulin receptors (Zeng and Quon 1996; Nitert et al. 2005), explaining the weak insulin stain seen in HUVEC. Similar results were obtained when the cells were exposed to 10mM TZD for 24 - 48 hours, thus neither the addition of TZD or co-culture of the two cell types in SC had any effect on the morphology of either cell type.

However, when HUVEC (both SC and RC) were exposed to 10mM TZD for 4 days or longer the cells appeared to develop fissures (figure 45 below). This observation is consistent with published information indicating that prolonged, high concentrations of TZD with insulin are prone to cause capillary leakage and oedema (Idris et al. 2003).

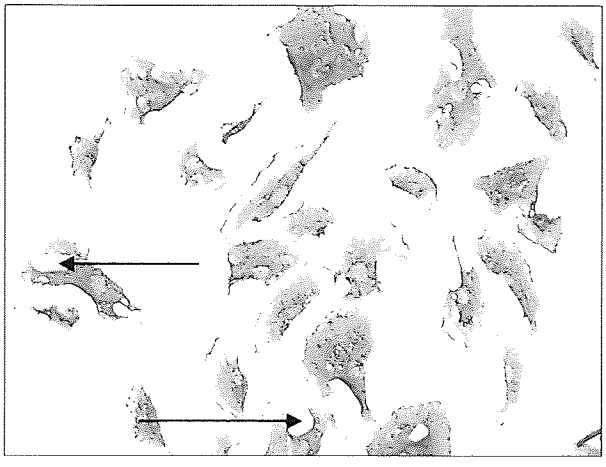


Figure 45. HUVEC exposed to 10mM TZD for 5 days. Note the fissures (black arrows) indicating a compromise in the structural integrity of the cells.

Immunostaining of D11 and HUVEC, both individually and as co-cultures, in RC produced similar results to those found for SC. D11 expressed insulin when cultured +/- HUVEC and +/- 10mM TZD but did not stain positively for CD31 (figures 38, 42). HUVEC expressed CD31 when cultured +/- D11 and +/- 10mM TZD, and also stained faintly for insulin (figures 40, 42). Thus growing the cells in RC, adding 10mM TZD to the culture medium and growing D11 and HUVEC as co-cultures did not affect the morphology of the cells.

VEGF production and release

HUVEC maintained as a monolayer in static culture responded to 10mM TZD by significantly increasing VEGF production and release into the tissue culture medium ($p<0.01$, Student's t-test) and this was also true for the monolayer co-culture of D11 cells with HUVEC in a static culture environment ($p<0.01$, Student's t-test). A significant difference was also found when D11/HUVEC TZD VEGF release was compared to HUVEC TZD VEGF release in SC ($P<0.05$), indicating that the presence of D11 cells enhanced VEGF production from SC HUVEC exposed to 10mM TZD. This comparison was made as both groups contained the same number of HUVEC (50 000 cells). However, beta cells do sequester VEGF (Kuroda et al. 1995) and it is possible that the 175 000 D11 cells used in the co-culture with 50 000 HUVEC contributed to the total amount of VEGF detected in the tissue culture medium.

HUVEC maintained in a rotational cell culture system significantly increased VEGF production and release when exposed to 10mM TZD ($p<0.01$, Student's t-test) and a co-culture of D11 cells and HUVEC responded in a similar manner ($p<0.01$, Student's t-test). A comparison between RC D11/HUVEC TZD and RC HUVEC TZD returned $p<0.01$ (Student's t-test). This result relates positively to the same comparison made for ATP content, indicating a possible supportive effect of adding D11 to HUVEC proliferation (ATP) and function (VEGF production and release) in rotational culture. Once more it should be emphasised that D11 cells formed pseudoislets in RC that incorporated HUVEC in unquantifiable numbers (see immunostaining), hence it was not possible to determine if the 150 pseudoislets used contained 50 000 HUVEC, as in the RC HUVEC TZD group.

No significant difference was found in VEGF release between HUVEC RC TZD and HUVEC SC TZD, indicating that the culture method had no effect on the function of HUVEC in terms of producing and releasing VEGF in response to 10mM TZD.

Conclusions

These results show that RC improves the insulin secretory capacity of beta cells and that TZD inclusion in the culture medium has an additional supportive effect. Also, exposure of HUVEC to a TZD enhances endothelial proliferation and VEGF production. These findings underpin the decision to adapt the techniques developed thus far to study the effects of RC and a TZD on insulin secretion and VEGF release from human islets.

CHAPTER 5: The effect of rosiglitazone on human islet insulin secretion and VEGF production

Introduction

The IRL has ethical approval to isolate human islets for research from donor pancreata sourced by the UK Human Tissue Bank (UKHTB) at De Montford University, Leicester. Often, the pancreas was retrieved for transplant and had been transported to the transplant centre, where the organ was examined and deemed unsuitable for transplant. The transplant co-coordinator then referred the organ to UKHTB which, in turn, contacted the IRL to arrange delivery of the gland.

This sequence of events frequently resulted in the cold ischaemia time of the pancreas greatly exceeding the 6 – 8 hour limit suggested by the literature for isolating good quality, functional islets (Benhamou et al. 1994; Lakey et al. 1996; Lakey et al. 2002). In an attempt to make use of this rare resource, the IRL had undertaken a project to isolate islets from pancreata with cold ischaemia times ranging between 10 and 16 hours and have isolated viable islets from such pancreata as well as from a pancreas after 19 hours cold ischaemia (data not yet published).

Much research has been conducted to optimise culture conditions for human islets. A number of studies showed that by reducing the culture temperature from 37°C to 24°C for at least 24 hours prior to transplant, antigen presenting leukocytes died while the islet insulin secretion profile remained good and post-transplant islet graft survival improved (Faustman et al. 1982; Ricordi et al. 1987; Scharp et al. 1987). At the IRL human islets are routinely cultured at 30°C, resulting in very good insulin secretion profiles (Jay et al. 2004; Murray et al. 2005; Paget et al. 2007).

The conventional medium for human islet culture at the IRL is medium199 supplemented with 10% FCS, 100U/ml penicillin, 100μg/ml streptomycin and 10μg/ml amphotericin B (Jay et al. 2004; Murray et al. 2005). Several groups developed defined media for islet/beta cell culture with some including additives to support islet function (Buitrago et al. 1975; Gylfe 1977; Clark and Chick 1990; Ling and Pipeleers 1994; Schmied et al. 2000; Tillotson et al. 2001), while others omitted serum due to the possibility of pathogen contamination (Clayton et al. 1998; Ogawa et al. 2004). Phenol red was excluded from some media as it is an estrogen mimic (Huff et al. 1988) although other studies have shown that estrogens protect islets from amyloid and preserves glucose-induced insulin secretion in the presence of high glucose (Lenzen and Bailey 1984; Kooptiwut et al. 2007). The study for this thesis evaluated four culture media for use with human islets (m199, Miami#1, RPMI and EGM). In addition, a rotational cell culture (RC) system was employed, as a study by the IRL has shown that RC significantly improves islet function and maintains the structure and viability of human islets for a greater period than does SC (Murray et al. 2005).

Studies have shown that adding VEGF to islet culture increased the sprouting of capillaries from mouse and human islets (Linn et al. 2003; Konstantinova and Lammert 2004; Kidszun et al. 2006) prior to transplantation (Hsu et al. 2005; Cross et al. 2007). Also, administering rosiglitazone systemically prior to and after transplant (Hsu et al. 2005) improved islet graft survival, moreover, ex vivo transfection for human vascular endothelial growth factor gene expression prior to islet transplantation improves graft revascularisation (Narang et al. 2006). One of the aims of this thesis is to investigate if pre-treatment of human islets with a TZD can induce enhanced proliferation of endogenous endothelial cells by upregulating VEGF synthesis prior to transplant rather than by adding exogenous VEGF or TZDs systemically. Also, TZDs have been shown to support ß-cell function in terms of insulin secretion (Buckingham et al. 1998; Campbell and Mariz 2007). The results from chapters 4 and 5 of

this thesis corroborate these findings. A recent publication indicated that rosiglitazone preserved ß-cell function in patients with adult-onset latent autoimmune diabetes (Yang et al. 2008), again strengthening the argument for incorporating this drug into an islet pre-treatment in order to facilitate preserved islet function post-transplant. Rosiglitazone was added to islets at a concentration of 25mM in SC and RC to assess the effect on VEGF production and insulin secretion. Results obtained from studies using D11 beta cells and HUVEC (chapter 4) suggested that an acute exposure (24 hours) to a supraphysiological dose of TZD produced the most effective increase in endothelial proliferation (ATP data, figures 35 & 36), while prolonged exposure resulted in compromised structural integrity of HUVEC (figure 45).

Methods

Human islets were maintained in both SC and RC as described in chapter two; however, three additional culture media were assessed for their efficacy in supporting islet function and structure (see results section below).

The immunostaining technique used to obtain visualisation of insulin, VEGF and CD31 was based on the principal of using a suitable fixative (4% formaldehyde), a primary antibody to detect the ligand of interest (insulin, VEGF and CD31), followed by a fluorescence labelled secondary antibody resulting in visualisation of the signal obtained. The technique used is discussed fully in chapter two.

A static incubation method for was employed to assess insulin secretion in response to three secretagogues. Again, a full description of the method is recorded in chapter 2, but it briefly comprised:

- A period of pre-incubation, allowing the islets to settle to a state of basal insulin secretion prior to being stimulated.

- A period of exposure to one of three secretagogues to induce insulin secretion and detection of the amount of insulin released by ELISA.

Chapter 2 provides a full description of the method used to induce and detect VEGF production and release. In brief, islets were allowed to equilibrate in supplemented medium 199 for 24 hours after isolation after which experimental groups comprising of m199 SC +/- 25mM TZD and m199 RC +/- 25mM TZD were set up. After 24 hours the tissue culture medium was harvested and assessed for VEGF content by ELISA.

Results

Human islet culture

Human islets were isolated and allowed to equilibrate in SC (90mm petri dishes) in supplemented medium 199 in a humidified incubator with 5% CO_2 at 30°C for 24 hours prior to being assigned to one of four tissue culture media:

- Medium 199
- Endothelial growth medium
- RPMI1640 medium
- Miami#1 medium

Each medium group was duplicated to allow islet culture in the presence and absence of 25mM TZD, resulting in eight static culture experimental groups.

Due to the paucity of human islets for research three rotational culture groups were evaluated:

- Medium 199
- Medium 199 + TZD
- Endothelial growth medium

Medium 199 was selected as the main medium for use in RC as no advantage was seen with more expensive media, see insulin secretion data below. EGM was selected as this medium has been developed to support endothelial growth, see HUVEC data, chapter 4, and one of the objectives of this study was to promote enhanced endothelial proliferation.

At 48 hours treatment a sample from each culture group was stained with dithizone and photographs were taken to record the morphology of islets (figures 46 and 47). Photographs were also taken at 5 days treatment of islets from the m199 SC and m199 RC groups to show the loss of structural integrity of islets maintained in SC (figure 48).

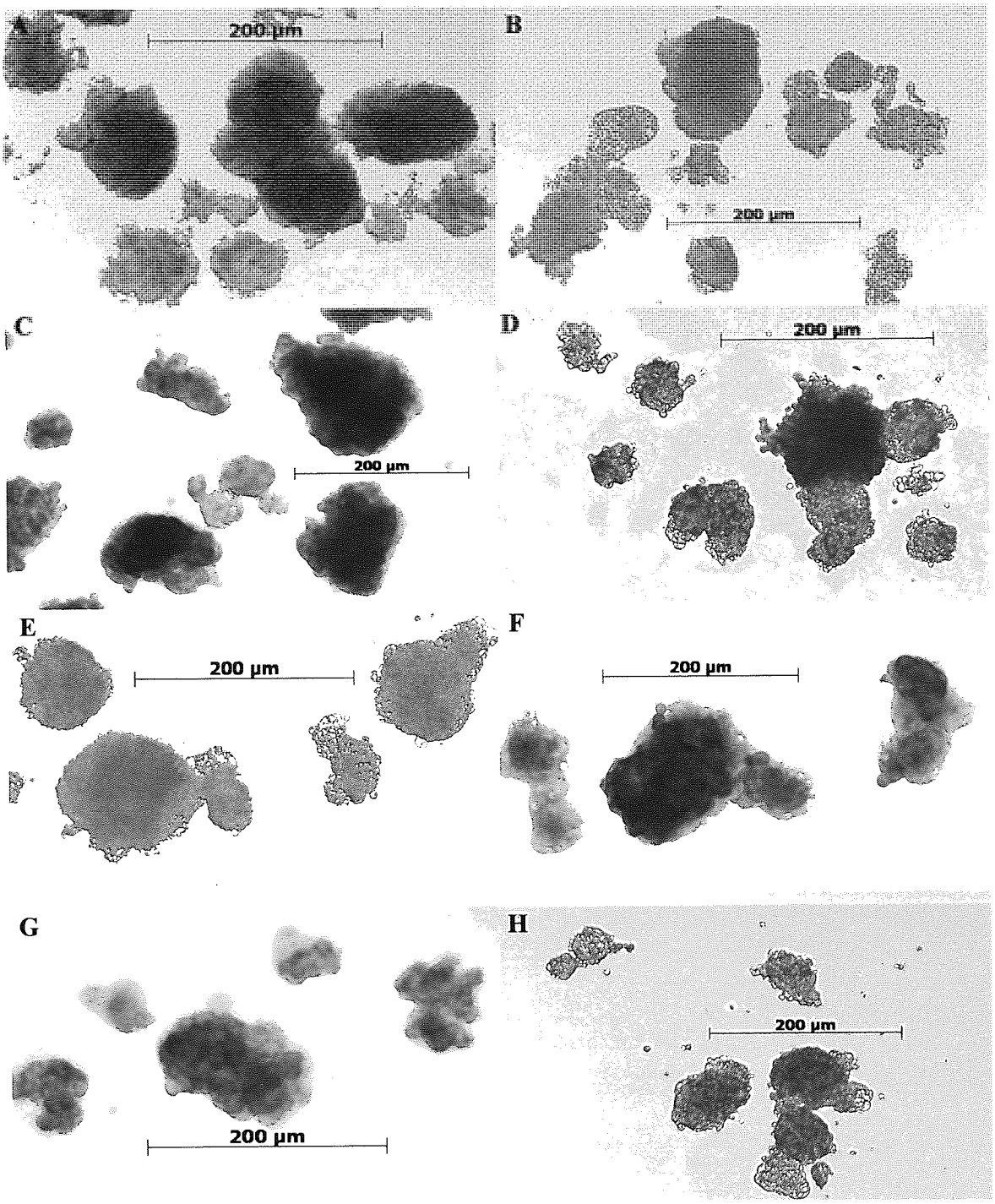


Figure 46. Human islets from eight experimental groups at 48 hours post isolation, 24 hours treatment. A = m199 SC, B = m199 SC TZD, C = EGM SC, D = EGM SC TZD, E = RPMI SC, F = RPMI SC TZD, G = Miami#1 SC and H = Miami#1 SC TZD. All photographs were taken at x 20 magnification.

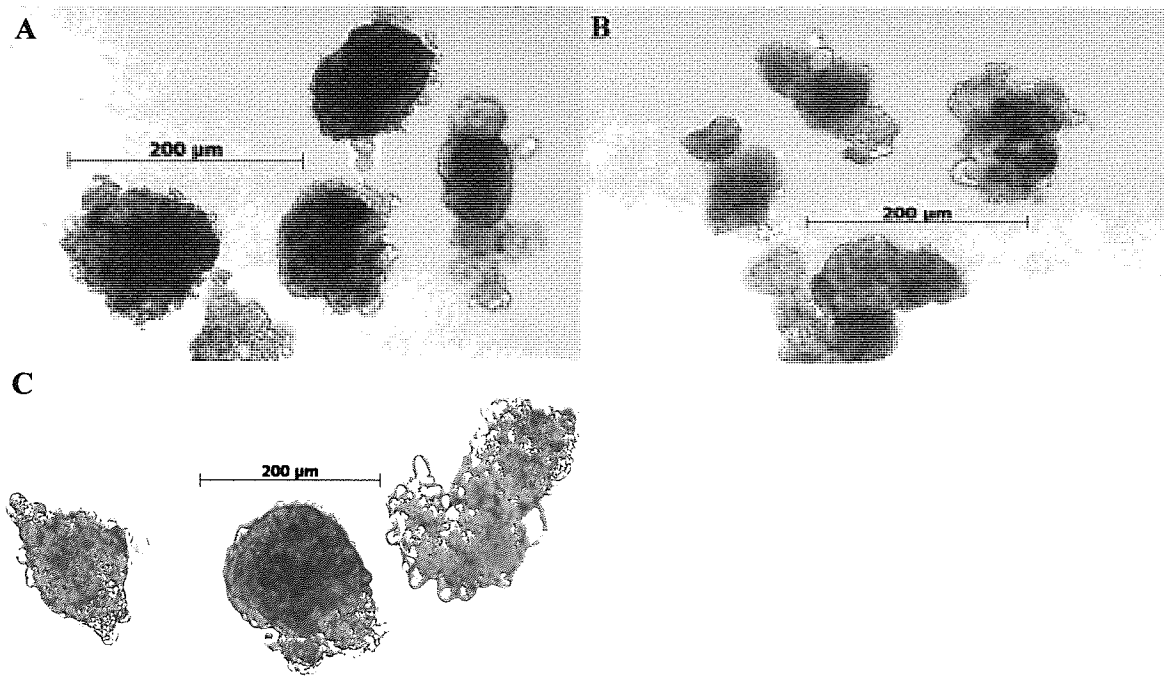


Figure 47. Human islets maintained in RC. 48 hours post isolation, 24 hours treatment. A = m199 RC, B = m199 RC TZD and C = EGM RC. All three photographs were taken at x 20 magnification.

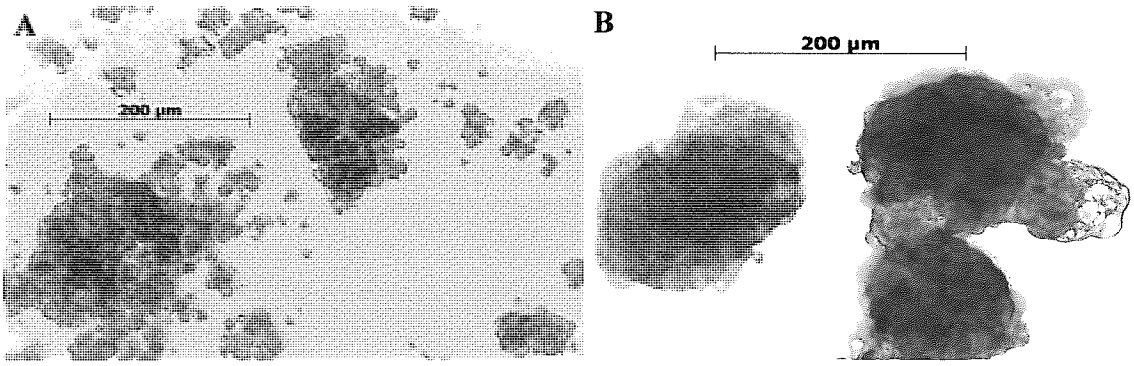


Figure 48. Human islets maintained in m199 for 5 days. A = SC and B = RC. Both photographs were taken at x 20 magnification.

Human islet insulin secretion

Islets from the eight SC and three RC groups described above were subjected to insulin secretion experiments at 48 hours (24 hours treatment), 72 hours (48 hours treatment) and 4 days (72 hours treatment) post isolation as detailed in chapter 2. Figure 49 shows the combined 24 hour post isolation insulin secretion data for the 4 donors used and figures 50 - 52 show the insulin secretion data for the eleven islet groups over three days. Tables 7 - 9 show statistically significant differences found in and between groups.

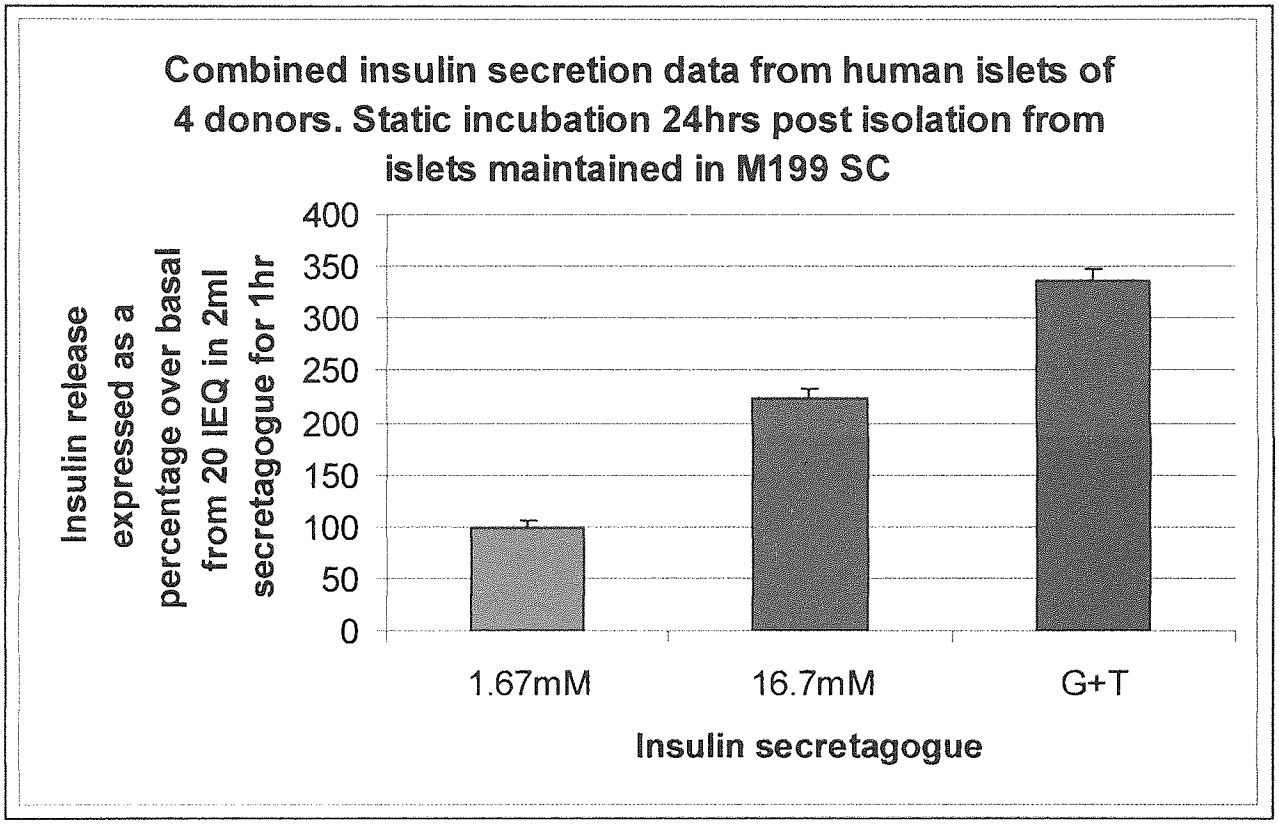


Figure 49. Insulin secretion from human islets of 4 donors 24 hours post isolation, with islets maintained in m199 SC. A Student's t-test returned $p<0.01$ for comparisons between 16.7mM and 1.67mM, G+T and 1.67mM; as well as G+T and 16.7mM. These statistically significant differences indicate functionally viable islets.

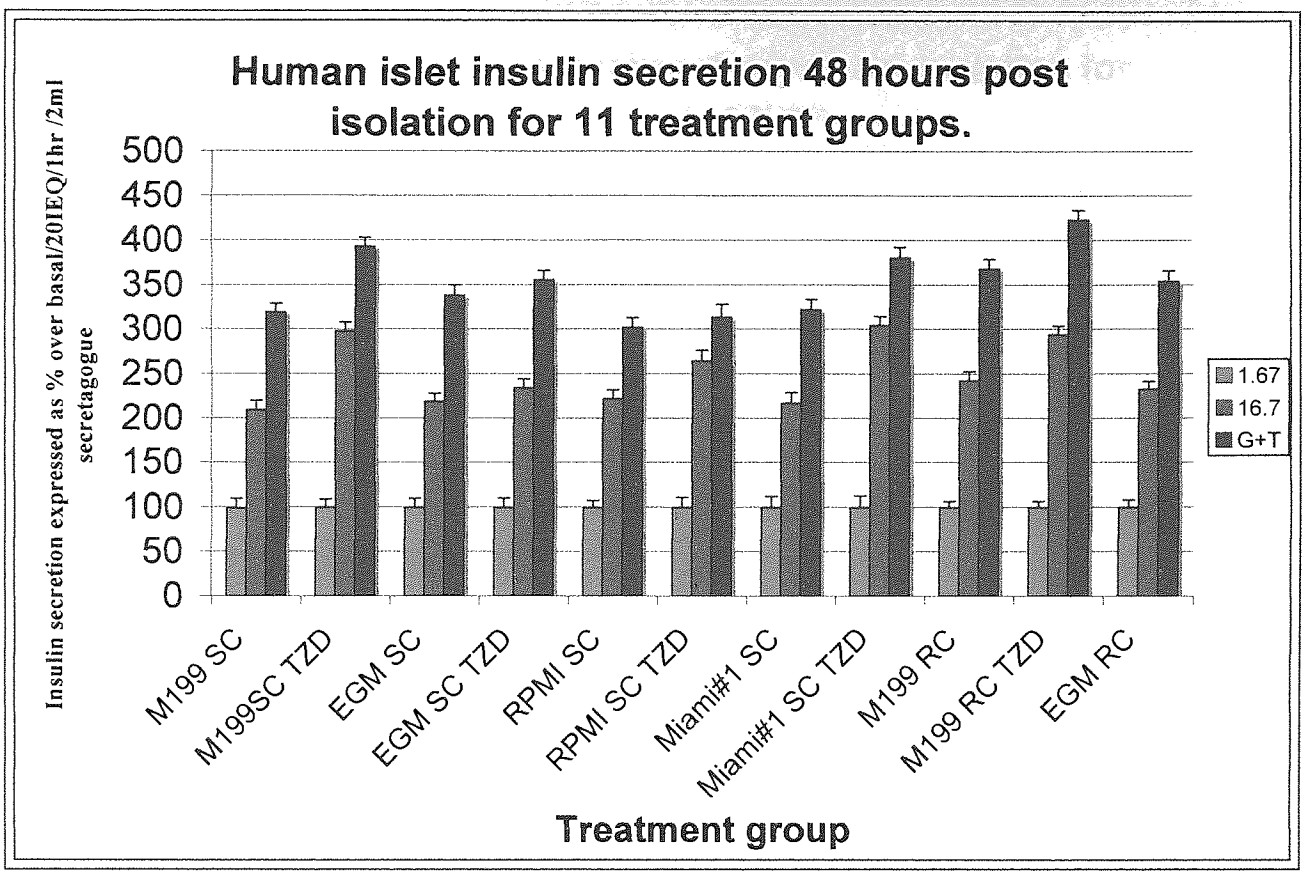


Figure 50. Insulin secretion results for human islets 48 hours post isolation (24 hours treatment) from eleven experimental groups. Table 7 shows the relevant statistics.

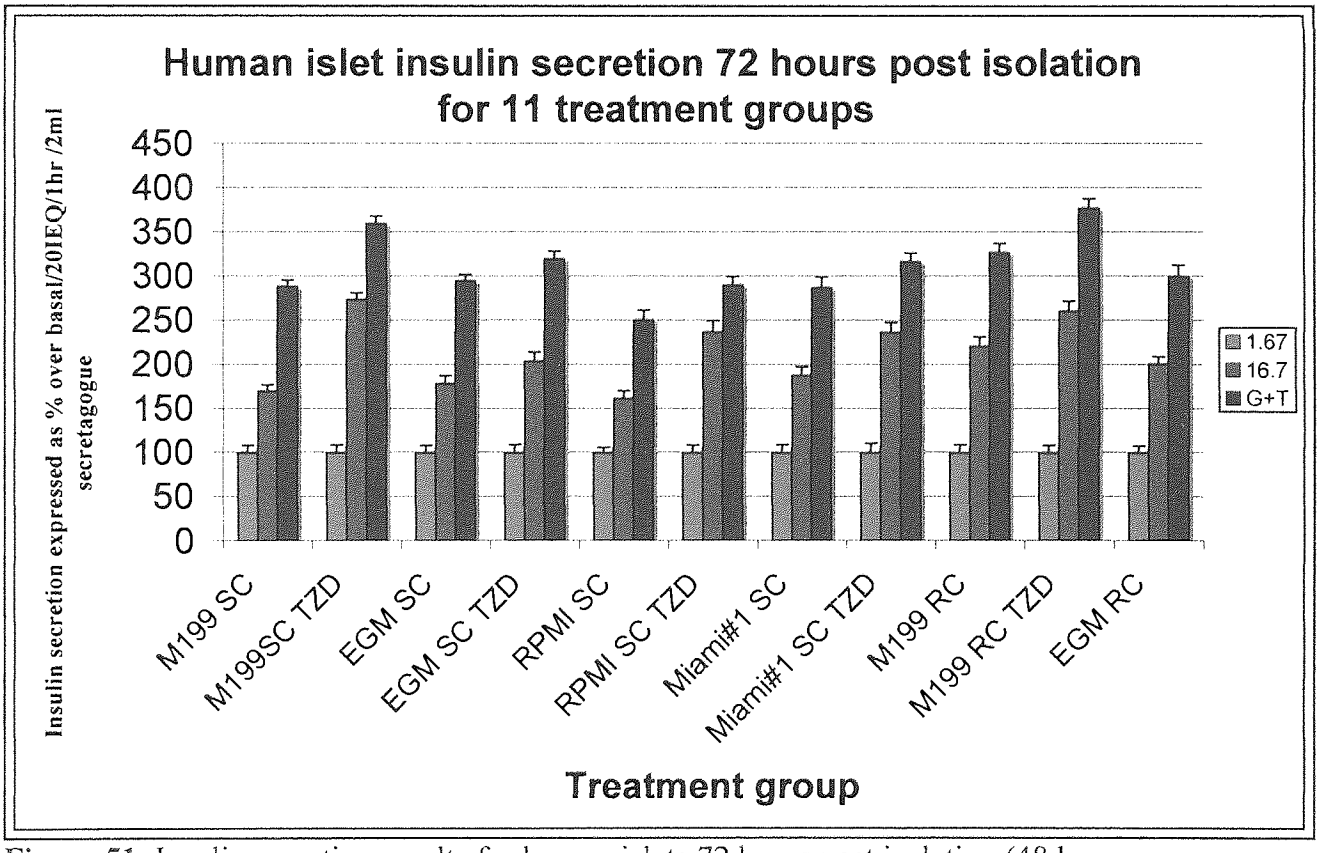


Figure 51. Insulin secretion results for human islets 72 hours post isolation (48 hours treatment) from eleven experimental groups. Table 8 shows the relevant statistics.

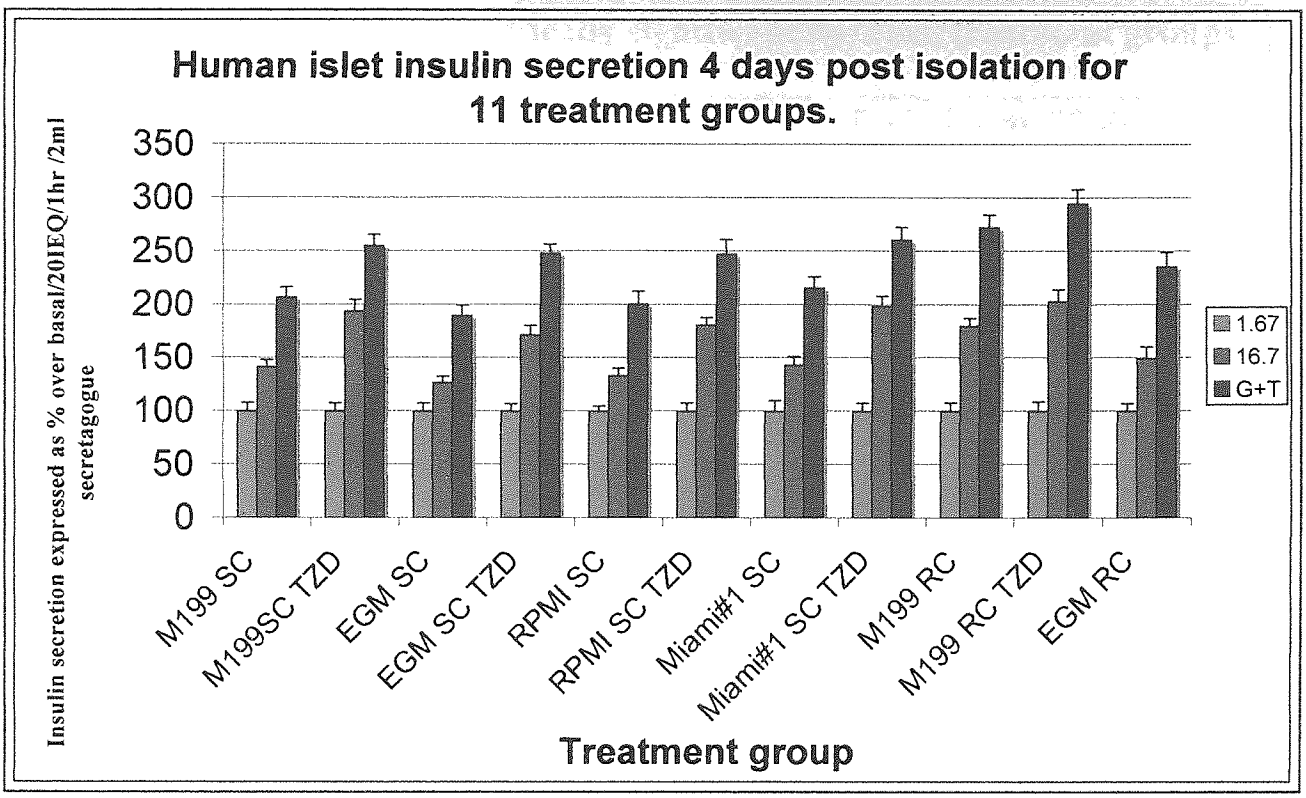


Figure 52. Insulin secretion results for human islets 4 days post isolation (72 hours treatment) from eleven experimental groups. Table 9 shows the relevant statistics.

A one-way analysis of variance (ANOVA, SigmaStat) was used to determine if there were significant differences between the 16.7mM and G+T insulin secretion results of eleven treatment groups. A significant difference was found on each of the three days; hence a post hoc test was performed. A Holm-Sidak test was used to perform multiple comparisons as the eleven groups consisted of varying numbers of replicates:

- n = 20 per secretagogue (4 pancreata, 5 replicates/secretagogue) for m199 SC, m199 SC TZD, EGM SC, EGM SC TZD, m199 RC, m199 RC TZD and EGM RC
- n = 15 per secretagogue (3 pancreata, 5 replicates/secretagogue) for RPMI SC and RPMI SC TZD
- n = 12 per secretagogue (2 pancreata, 6 replicates/secretagogue) for Miami#1 SC and Miami#1 SC TZD

Comparisons found to be statistically significant between treatment groups at 48 hours culture	
m199 SC TZD 16.7 v m199 SC 16.7	m199 RC TZD G+T v m199 RC G+T
m199 SC TZD 16.7 v EGM SC TZD 16.7	m199 SC TZD G+T v m199 SC G+T
Miami SC TZD 16.7 v Miami SC 16.7	m199 SC TZD G+T v RPMI SC TZD G+T
Miami SC TZD 16.7 v EGM SC TZD 16.7	m199 RC G+T v m199 SC G+T
Miami SC TZD G+T v RPMI SC TZD G+T	

Table 7. Statistically significant comparisons between the eleven treatment groups for 16.7mM and G+T induced insulin secretion at 48 hours culture, using a Holm-Sidak test for multiple comparisons (SigmaStat).

Comparisons found to be statistically significant between treatment groups at 72 hours culture	
m199 SC TZD 16.7 v m199 SC 16.7	m199 RC TZD G+T v m199 RC G+T
m199 SC TZD 16.7 v EGM SC TZD 16.7	m199 SC TZD G+T v m199 SC G+T
m199 RC 16.7 v m199 SC 16.7	m199 SC TZD G+T v RPMI SC TZD G+T
RPMI SC TZD 16.7 v RPMI SC 16.7	

Table 8. Statistically significant comparisons between the eleven treatment groups for 16.7mM and G+T induced insulin secretion at 72 hours culture, using a Holm-Sidak test for multiple comparisons (SigmaStat).

Comparisons found to be statistically significant between treatment groups at 4 days culture	
Miami SC TZD 16.7 v Miami SC 16.7	EGM SC TZD 16.7 v EGM SC 16.7
m199 SC TZD 16.7 v m199 SC 16.7	EGM SC TZD G+T v EGM SC G+T
m199 RC G+T v m199 SC G+T	

Table 9. Statistically significant comparisons between the eleven treatment groups for 16.7mM and G+T induced insulin secretion at 4 days culture, using a Holm-Sidak test for multiple comparisons (SigmaStat).

The addition of 25mM TZD to the culture media increased insulin secretion in response to 16.7mM glucose and G+T in all the groups with significant increases seen as follows at 48 hours post isolation (24 hours treatment):

- m199 SC TZD 16.7 compared with m199 SC 16.7
- m199 RC TZD 16.7 compared with m199 SC 16.7
- m199 SC TZD G+T compared with m199 SC G+T
- m199 RC TZD G+T compared with m199 RC G+T
- Miami#1 SC TZD 16.7 compared with Miami#1 SC 16.7

Significant increases in insulin secretion resulting from the addition of 25mM TZD was seen as follows at 72 hours post isolation (48 hours treatment):

- m199 SC TZD 16.7 compared with m199 SC 16.7
- m199 SC TZD G+T compared with m199 SC G+T
- m199 RC TZD G+T compared with m199 RC G+T
- RPMI SC TZD 16.7 compared with RPMI SC 16.7

Significant increases in insulin secretion resulting from the addition of 25mM TZD was seen as follows at 4 days post isolation (72 hours treatment):

- m199 SC TZD 16.7 compared with m199 SC 16.7
- Miami#1 SC TZD 16.7 compared with Miami#1 SC 16.7
- EGM SC TZD 16.7 compared with EGM SC 16.7
- EGM SC TZD G+T compared with EGM SC G+T

Insulin secretion from islets maintained in m199 was never significantly lower than islets maintained in any of the other three culture media on a like-for-like basis (i.e. m199 SC 16.7

compared with EGM SC 16.7 or RPMI SC 16.7). On four occasions m199 outperformed EGM and RPMI:

- At 48 hours and 72 hours post isolation m199 SC TZD 16.7 resulted in significantly higher insulin secretion than EGM SC TZD 16.7
- At 48 hours and 72 hours post isolation m199 SC TZD G+T resulted in significantly higher insulin secretion than RPMI SC TZD G+T

Islets maintained in m199 RC resulted in significantly increased insulin secretion when compared to islets from the m199 SC groups on many occasions with the following comparisons resulting in statistically significant differences:

- m199 RC G+T compared to m199 SC G+T at 48 hours culture
- m199 RC 16.7 compared to m199 SC 16.7 at 72 hours culture
- m199 RC G+T compared to m199 SC G+T at 4 days culture

Human islet immunostaining

As no advantage was found using RPMI1640, EGM or Miami#1 tissue culture media over medium 199, it was deemed unnecessary to subject islets from all eleven culture groups to the expensive technique of fluorescence immunostaining. Islets from m199 SC +/-TZD and m199 RC+/-TZD were plated onto glass coverslips in 24-well plates. Islets were allowed to adhere to the glass for 24 hours prior to being fixed in cold 4% paraformaldehyde.

Islets were stained for the presence of insulin (rabbit anti-pig polyclonal) and VEGF (mouse anti-human monoclonal). Insulin was visualised with a goat anti-rabbit TRITC (red), while VEGF was visualised using FITC (green). Figures 53 - 56 show the images obtained, while figure 57 shows an islet from the m199 RC TZD group stained for insulin and CD31 (mouse anti-human monoclonal, visualized with a goat anti-mouse FITC).

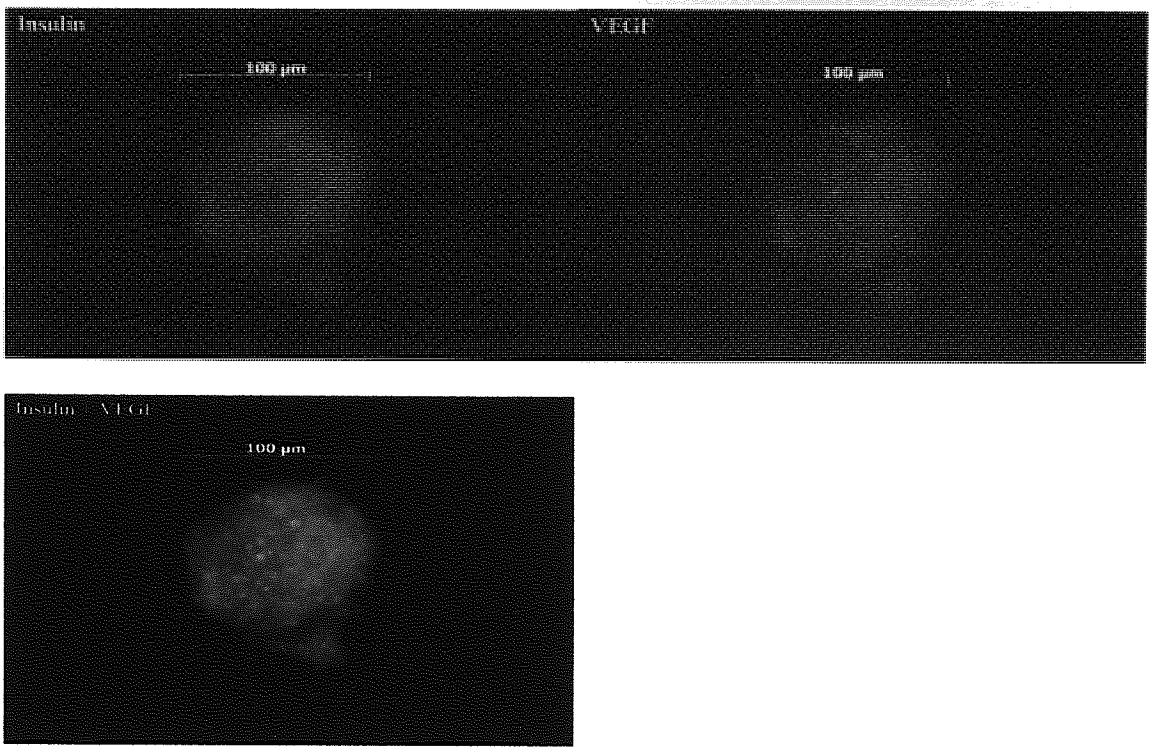


Figure 53. Fluorescence immunostaining for insulin and VEGF in a human islet maintained in m199 SC. The photograph was taken at x 20 magnification.

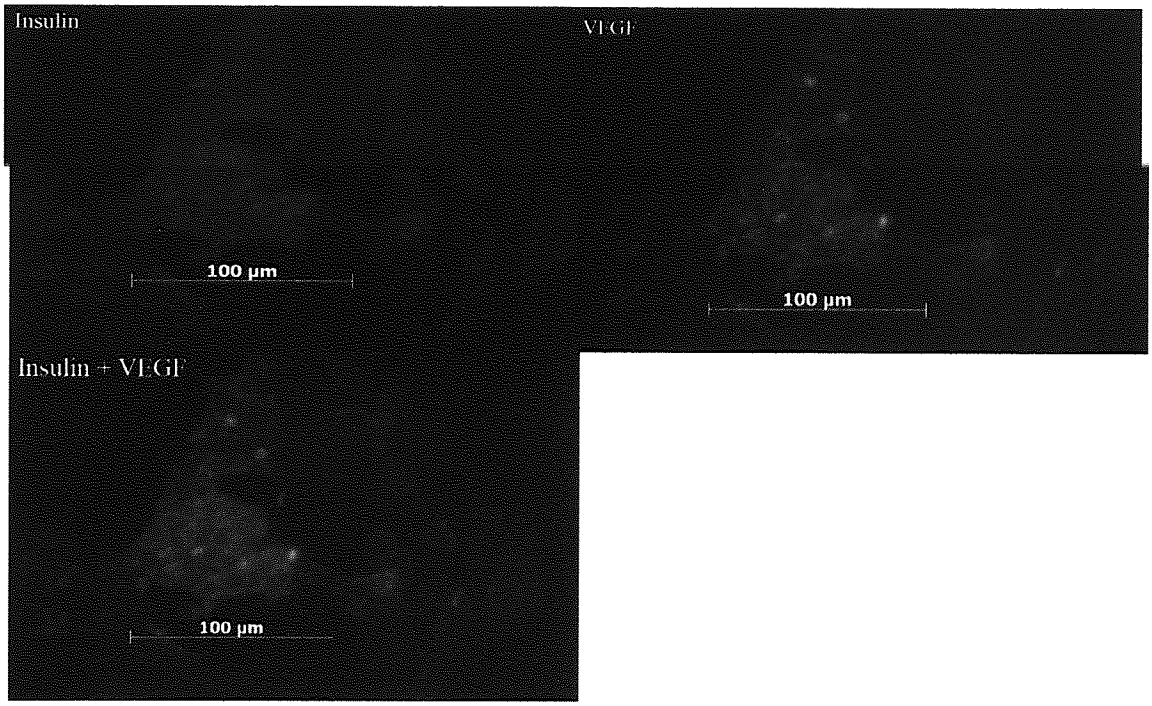


Figure 54. Fluorescence immunostaining for insulin and VEGF in a human islet maintained in m199 SC TZD. The photograph was taken at x 20 magnification.

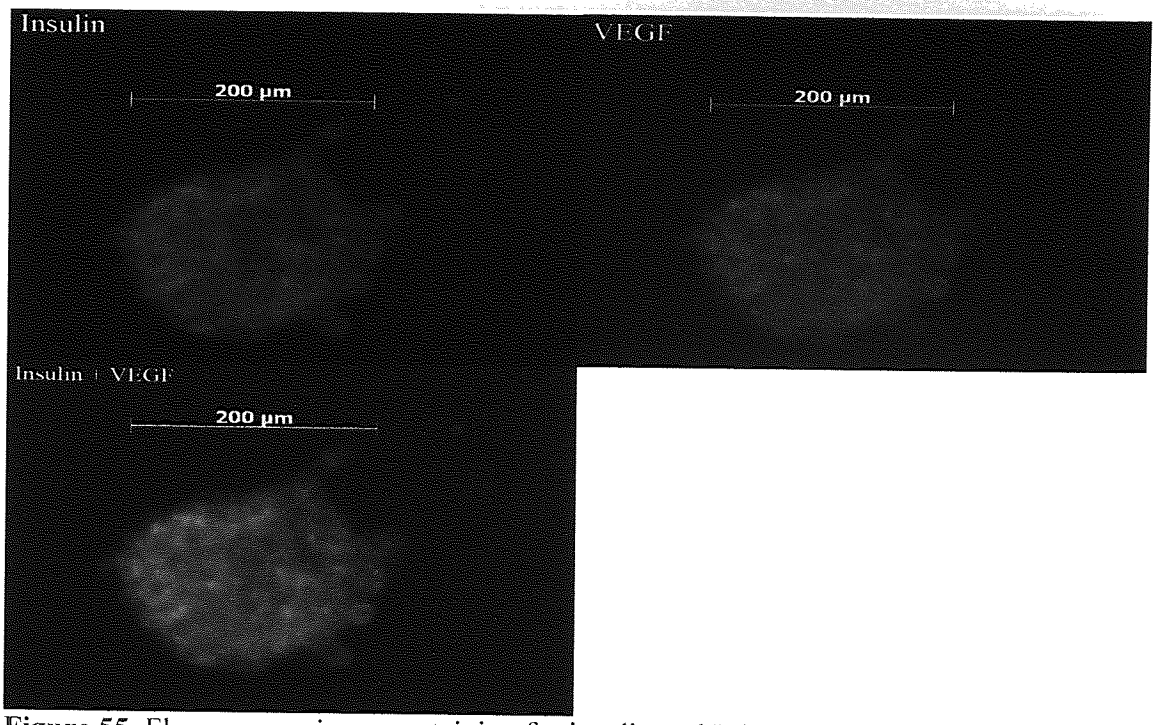


Figure 55. Fluorescence immunostaining for insulin and VEGF in a human islet maintained in m199 RC. The photograph was taken at x 20 magnification.

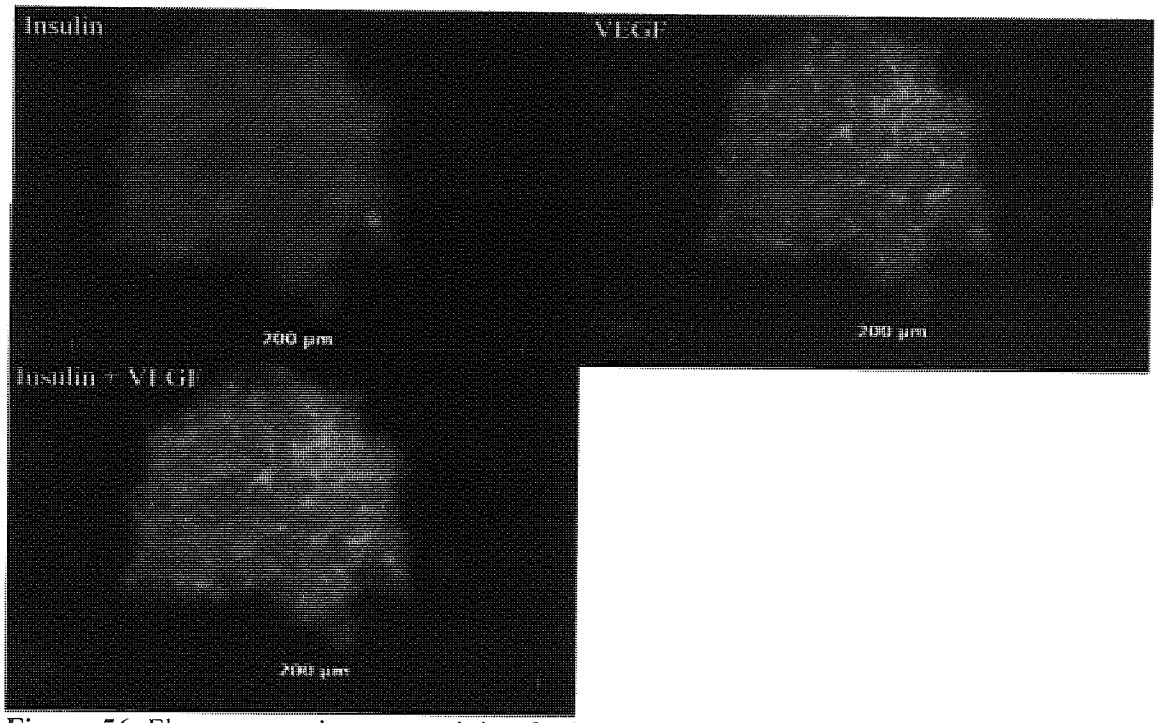


Figure 56. Fluorescence immunostaining for insulin and VEGF in a human islet maintained in m199 RC TZD. The photograph was taken at x 20 magnification.

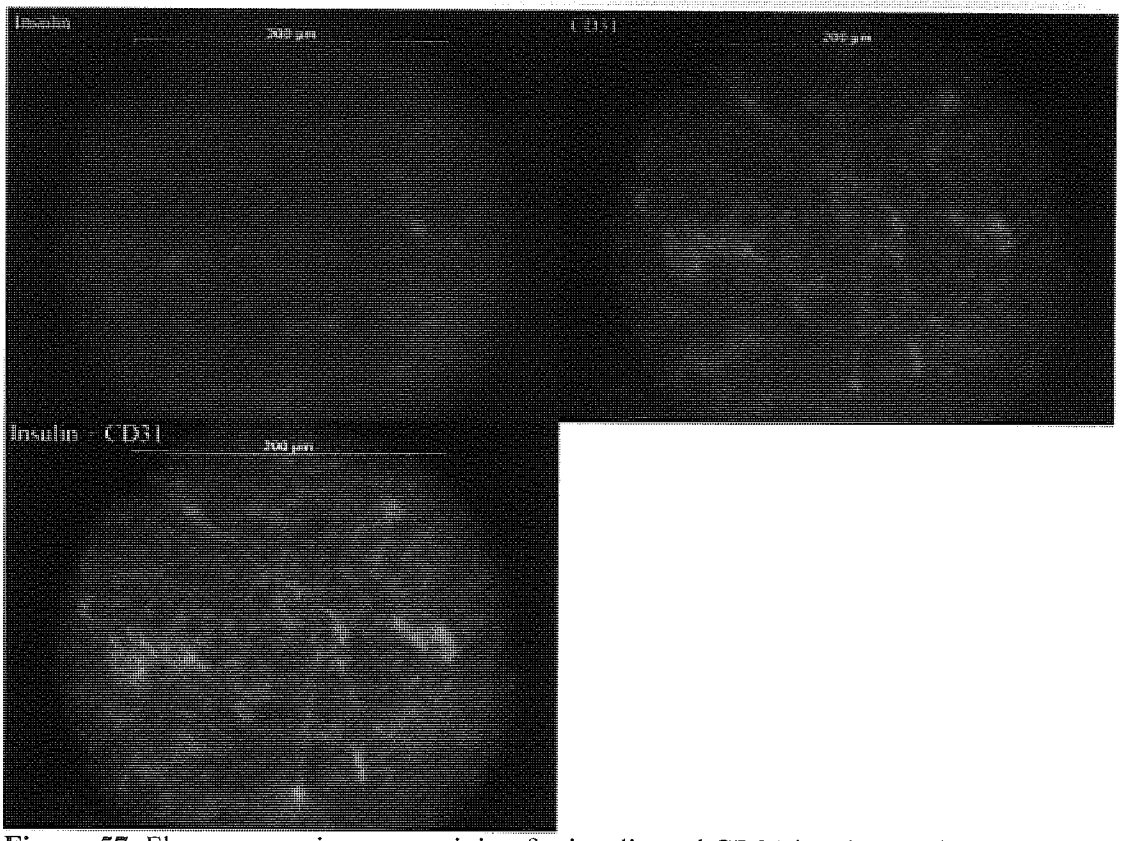


Figure 57. Fluorescence immunostaining for insulin and CD31 in a human islet maintained in m199 RC TZD. The photograph was taken at x 20 magnification.

Human islet VEGF production and release

Human islets maintained in m199 in SC and RC +/- TZD for 24 hours (48 hours post-isolation) were plated into 24-well plates at a density of 20 IEQ/well to ensure tight control of islet mass. The islets received m199 +/-TZD as per their experimental group and tissue culture medium was harvested at 24 hours (72 hours post isolation) and 48 hours (4 days post isolation). VEGF concentration was determined by ELISA (Cross et al. 2007; Laugharne et al. 2007), figures 58 and 59 show graphic representations of the results while statistically significant differences in and between groups are shown in table 10.

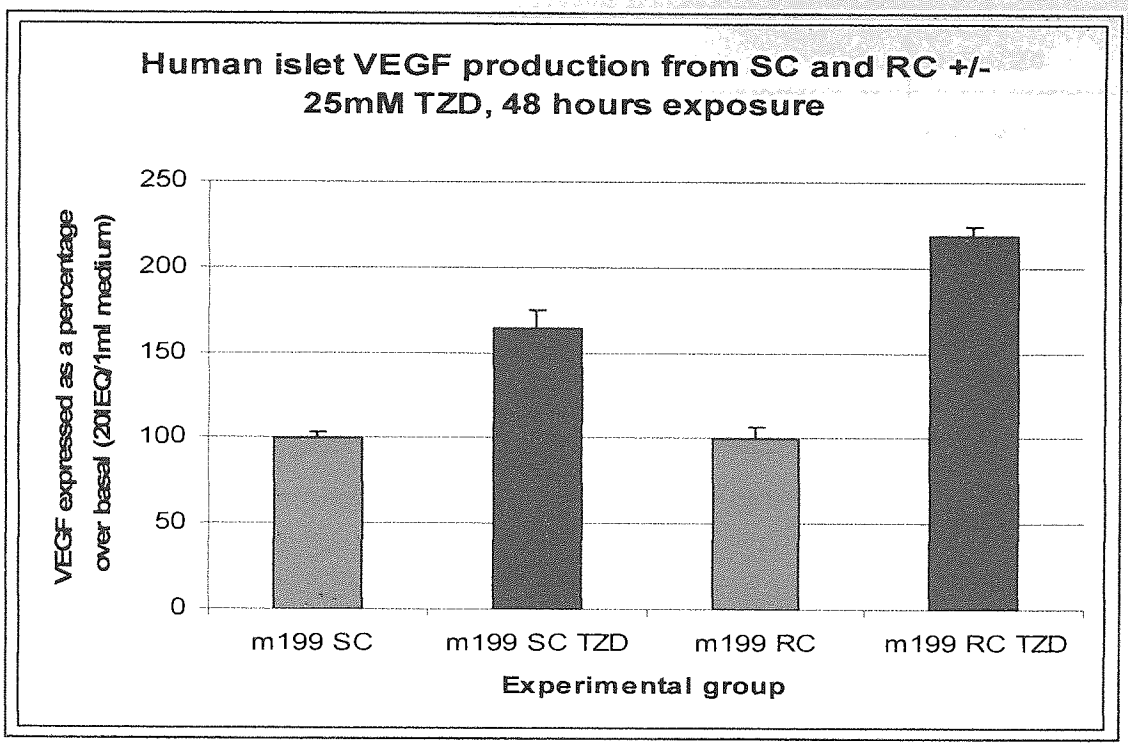


Figure 58. VEGF production and release from human islets in SC and RC +/- 25mM TZD with 48 hours exposure to TZD (n=8 per experimental group). A Student's t-test returned $p<0.01$ for m199 SC TZD v m199 SC and m199 RC TZD v m199 RC. A comparison between m199 RC TZD and m199 SC TZD returned $p<0.05$.

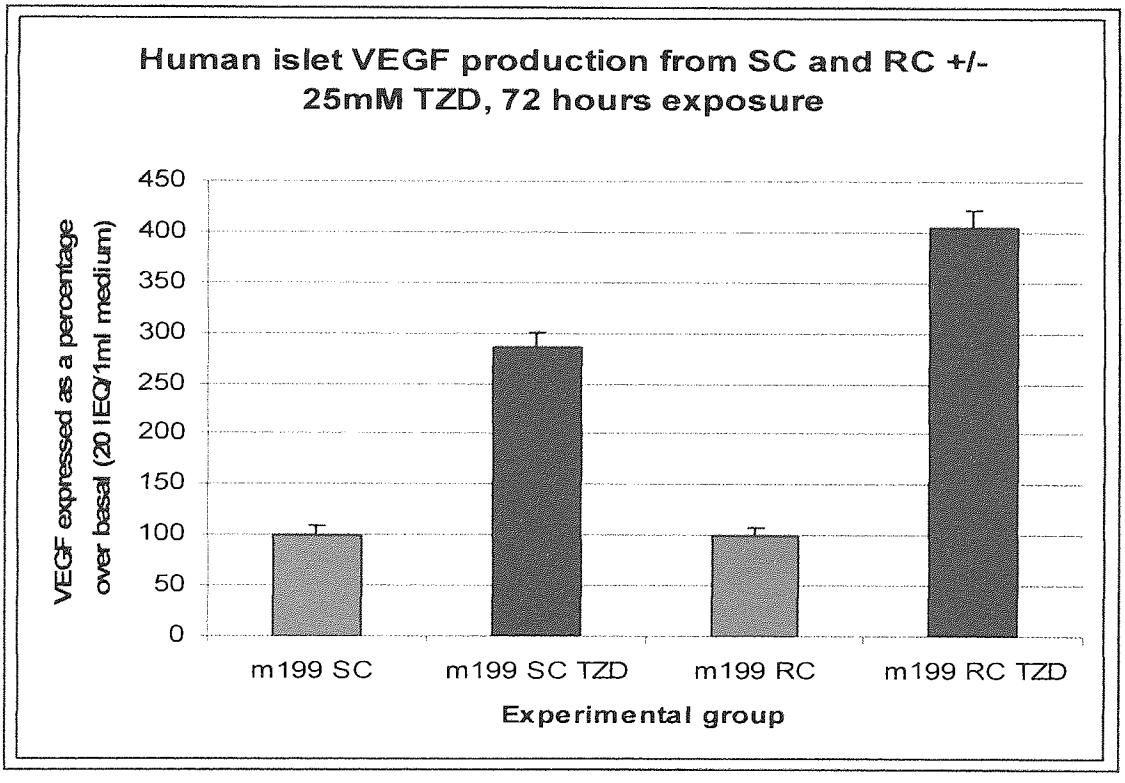


Figure 59. VEGF production and release from human islets in SC and RC +/- 25mM TZD with 72 hours exposure to TZD (n=8 per experimental group). A Student's t-test returned $p<0.01$ for m199 SC TZD v m199 SC and m199 RC TZD v m199 RC. A comparison between m199 RC TZD and m199 SC TZD returned $p<0.05$.

Comparison by Student's t-test	p-value
m199 SC TZD v m199 SC (both 48hrs treatment)	0.0087
m199 RC TZD v m199 RC (both 48hrs treatment)	0.0026
m199 RC TZD v m199 SC TZD (both 48hrs treatment)	0.032
m199 SC TZD v m199 SC (both 72hrs treatment)	0.0011
m199 RC TZD v m199 RC (both 72hrs treatment)	0.00099
m199 RC TZD v m199 SC TZD (both 72hrs treatment)	0.028
m199 SC TZD 72hrs treatment v m199 SC TZD 48hrs treatment	0.01
m199 RC TZD 72hrs treatment v m199 RC TZD 48hrs treatment	0.0029

Table 10. Comparisons in and between experimental groups of islets +/- 25mM TZD at 48 hours and 72 hours treatment in SC and RC. The results in this table relate to figures 58 and 59 above.

Discussion

Human islet culture

Islets from all of the eleven treatment groups stained well with dithizone at 48 hours post isolation (24 hours treatment), indicating the presence of zinc that is packaged with insulin in secretory vesicles (Wolters et al. 1983; Shiroi et al. 2002). Thus islets from all of the experimental groups showed evidence of insulin content, suggesting that the culture media and conditions were supporting insulin storage. Indeed islet morphology appeared 'normal' for all the groups (figures 46, 47) with no apparent differences in the amount of fragmentation, although islets in the three RC groups did appear slightly more 'rounded' (figure 47). This is an important observation as islet fragmentation has been shown to impair insulin secretory capacity and lead to beta cell apoptosis (Rosenberg et al. 1999; Balamurugan et al. 2003).

At 5 days culture the islets maintained in m199 SC continued to stain with dithizone, but had suffered extensive fragmentation. The m199 RC islets retained a more 'normal' three dimensional structure, with no significant fragmentation occurring (figure 48). Islet fragmentation destroys the important physical connections between beta cells, leading to impaired cell-cell communication. Studies have revealed that aggregated beta cells function more effectively than lone beta cells (Hauge-Evans et al. 1999; Squires et al. 2000; Hauge-Evans et al. 2002; Squires et al. 2002). Calcium flux studies within beta cell clusters have shown that some beta cells within a cluster respond before others to a secretory signal, subsequently 'recruiting' the dormant beta cells to secrete insulin (Squires et al. 2002), thus indicating the importance of intercellular communication within islets in terms of regulating appropriate levels of insulin secretion in response to secretagogues. These observations, together with the findings of this study, support evidence previously published by the IRL regarding the positive effect of RC on maintaining human islet structural integrity (Murray et al. 2005).

Human islet insulin secretion

Islets were maintained in m199 SC for 24 hours post isolation, after which an insulin secretion experiment was performed prior to allocation into experimental groups. The islets from all four of the donors used for the study in this thesis were functionally viable, significantly increasing insulin secretion in response to 16.7mM glucose and G+T when compared to secretion resulting from exposure to 1.67mM glucose ($p<0.01$ for both comparisons, Student's t-test with $p<0.05$ being significant). Also, insulin secretion in response to G+T was significantly higher than that evoked by exposure to 16.7mM glucose ($p<0.01$, Student's t-test). These results indicate that the islets were fundamentally functional as they were able to detect both nutrient (glucose) and non-nutrient (theophylline) signals, and

that their insulin secretory mechanisms were able to respond by ensuring appropriate levels of insulin secretion in response to the secretagogue detected.

At 48 hours post isolation (24 hours treatment), islets from all of the eleven experimental groups significantly increased insulin secretion in response to both 16.7mM glucose and G+T when compared to secretion resulting from 1.67mM glucose ($p<0.01$ for all groups for both treatments, Student's t-test). All groups also showed significantly raised insulin secretion when G+T was compared to 16.7mM ($p<0.05$, Student's t-test). This trend was maintained for all the groups at 72 hours post isolation (48 hours treatment) and 4 days post isolation (72 hours treatment), with significant differences being found when both 16.7mM and G+T insulin secretion results were compared to 1.67mM glucose insulin release ($p<0.05$, Student's t-test). Again, insulin secretion resulting from exposure to G+T was significantly higher than that evoked when 16.7mM glucose was employed as a secretagogue ($p<0.05$, Student's t-test). These results indicate that the islets remained functionally viable in all four tissue culture media in SC and in m199 and EGM in RC for at least 4 days post isolation (72 hours treatment), both in the presence and absence of 25mM TZD. Thus there was no negative effect on islet function from either culture method (SC or RC) or any of the four culture media assessed in this study.

Previous studies have elucidated the basic tissue culture component requirements to sustain islet function, indicating that addition of 10% foetal calf serum was beneficial while high glucose content (>5.5mM) was shown to be detrimental (Andersson 1976; Collier et al. 1982). Clayton *et al* later showed that a serum-free medium could sustain islets and that albumin, ethanolamine, phosphoethanolamine, and prostaglandins were beneficial to islets, while deoxynucleotides, biotin, fetuin, sodium selenite, and zinc were detrimental (Clayton et al. 1998). The addition of insulin to islet medium did not show a significant benefit in terms of

islet function (Clayton et al. 2001). Thus many of the basic tissue culture media appear to be adequate for islet survival and function.

A one-way ANOVA, using SigmaStat software, was employed to determine if any significant differences occurred when insulin secretion in response to either 16.7mM glucose or G+T was compared across the eleven treatment groups. Significant differences were found between the experimental groups for both 16.7mM glucose and G+T at all three time points. A Holm-Sidak post hoc test was used to make multiple comparisons and the statistically significant differences are listed in tables 7 – 9, with $p<0.05$ being considered significant. Although significant differences in insulin secretion were found (and are recorded in tables 7 – 9) in response to 16.7mM glucose and G+T between the EGM, RPMI and Miami#1 culture groups, these will not be discussed in this thesis as the focus was directed at the performance of m199 versus these three media. Also, comparisons between results from SC or RC TZD of one media and SC or RC of another media will not be discussed (i.e. EGM SC TZD v RPMI SC or m199 RC TZD v EGM RC etc.) as the effect of a TZD should be assessed within the treatment group to gain accurate information about its action on insulin secretion (i.e. m199 SC TZD 16.7mM v m199 SC 16.7mM).

The results show that the addition of 25mM TZD increased insulin secretion in all four culture media and for both culture methods (SC and RC), with significant differences seen in insulin secretion in response to 16.7mM glucose in the m199 SC, m199 RC and Miami#1 SC groups. TZD addition significantly increased insulin secretion in response to G+T in the m199 SC and m199 RC groups.

Medium 199 compared well with the other three media tested, never delivering inferior results in terms of islet function and outperforming EGM and RPMI on four occasions, indicating

that m199 was a suitable choice of tissue culture medium for the maintenance of human islets under both static and rotational culture conditions.

Islets maintained in m199 RC secreted significantly more insulin in response to 16.7mM glucose when compared to the m199 SC group at 72 hours post isolation (48 hours treatment). Also, insulin secretion in response to G+T from m199 RC islets was significantly higher when compared to m199 SC islets at 48 hours and 4 days post isolation. These results support data previously published by the IRL, indicating a role for RC in maintaining functional viability of human islets during prolonged periods of culture, thus extending the insulin secretory lifespan of this precious resource to facilitate experimentation beyond 3 – 4 days post isolation (Murray et al. 2005).

The main differences between m199 and the other three media tested in this study are:

- RPMI contained 11mM glucose as opposed to the 5.5mM of the other three media
- EGM was designed to support endothelial growth, hence included only 1% serum, but also contained 0.04% hydrocortisone, 0.4% fibroblast growth factor, 0.1% VEGF, 0.1% insulin-like growth factor, 0.1% epidermal growth factor and 0.1% heparin
- Miami#1 medium included 5mM sodium pyruvate, 16.7μm zinc sulphate, 6.25mg/L insulin and transferrin, 6.25μg/L selenium and 25ml/L human serum albumin

Interestingly, this study supports the findings of others that many basic media containing inorganic salts, amino acids and vitamins will support islet function satisfactorily when compared to more expensive media containing additives purported to be required for normal islet function (Lucas-Clerc et al. 1993; Clayton et al. 1998; Clayton et al. 2001; Jay et al. 2004), although others maintain that a defined human islet culture medium is required (Bottino et al. 1997; Gaber and Fraga 2004; Murdoch et al. 2004).

Endothelial cell growth can be induced by conditions involving hypoxia (Gorden et al. 1997; Mizukami et al. 2006; Ruas et al. 2007), by direct application of endothelial growth factors (Hopkins et al. 1998; Del Moral et al. 2006) or by induction of growth factors like VEGF (Biscetti et al. 2008; Dandona et al. 2008). Most human islet transplant studies to date do not employ interventions directed at promoting endothelial cell health and proliferation to aid post transplant islet revascularisation. This study attempts to ensure the health of endogenous islet endothelial cells prior to implantation by upregulation of VEGF expression in response to TZD exposure. The upregulation of VEGF release seen after exposure to rosiglitazone supports the findings of other groups (Jozkowicz et al. 2000; Huang et al. 2008).

Human islet immunostaining

Islets from the m199 SC +/- TZD and m199 RC +/- TZD groups all stained positively for insulin and VEGF, both individually and when dual staining was performed (figures 53 – 56). The dual staining images suggest a high degree of co-localisation of insulin and VEGF. This could be explained by the fact that vascular endothelial cells contain insulin receptors (Kondo and Kahn 2004; Nitert et al. 2005; Chen and Michel 2006; Kearney et al. 2008) and ß-cells have been shown to sequester VEGF (Kuroda et al. 1995).

Interestingly, both the insulin and VEGF signals appeared to be stronger in the SC and RC groups with 25mM TZD and the dual staining showed more distinct localisation of the two signals in certain areas of the islet. While this was an observation, and thus not quantitative, the finding is supported by both the insulin secretion and VEGF 'production and release' data.

The increased intensity of VEGF staining in the TZD treated islets may be explained by the fact that TZDs have been shown to upregulate VEGF expression in endothelial cells via the peroxisome proliferator-activated receptor gamma (PPARγ) pathway (Yamakawa et al. 2000).

Also, the enhanced insulin staining seen may be due to the ability of TZDs to rejuvenate beta cells (Bell 2003) and increase beta cell granulation (Buckingham et al. 1998).

The nuclear receptor PPARγ is a ligand-dependent transcription factor that controls the expression of specific target genes involved in adipogenesis, inflammatory responses, and lipid metabolism (Jiang et al. 1998; Shao et al. 1998; Rocchi and Auwerx 1999; Stumvoll 2003). Several studies have shown that PPARγ also inhibits tissue injury associated with immune activation, exhibiting potent anti-inflammatory action (Ji et al. 2001; Landreth and Heneka 2001; Wada et al. 2001; Cuzzocrea et al. 2004). Cytokines such as interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α) and interferon gamma have been shown to reduce insulin secretory capacity of islets and combinations of these cytokines induced islet necrosis (Eizirik 1988; Rabinovitch et al. 1992; Yamada et al. 1993). Interleukin-2 (IL-2), a cytokine released by T-lymphocytes, has also been implicated in islet inflammation during diabetes development (Allison et al. 1994; Hayashi et al. 1999). In a mouse study of diabetes, employment of a monoclonal antibody against IL-2 prevented insulitis and development of glucose intolerance (Hayashi et al. 1999). Many other cytokines such as Fas, perforin, interleukin-10, Th1 and FAM3B have been implicated in islet inflammation, dysfunction and death thus promoting the development of diabetes (Yamada et al. 1996; Moriwaki et al. 1999; Balasa et al. 2000; Marselli et al. 2001; Cao et al. 2003). Interestingly, the cytokine iterleukin-6 (IL-6) has been shown to protect islets from the effects of the damaging cytokines (Park et al. 2003; Choi et al. 2004). Therefore it is important to note that PPARγ activation by a TZD has been shown to modulate proliferation of macrophages, B-cells and T-lymphocytes, thus mediating cytokine-induced inflammation driven by these cells (Marx et al. 2002; Kostadinova et al. 2005). These findings strongly support the use of a TZD prior to and after islet implantation as it may aid islet viability and survival by inhibition of inflammation due to either implant injury or indeed autoimmune driven inflammation in type 1 diabetes.

The RC TZD islet stained for insulin and CD31 showed substantial signals for both markers with more distinct differences in their localisation within the islet (figure 57). Unlike VEGF, CD31 is a dedicated endothelial cell marker and is not sequestered by beta cells, possibly explaining the more distinct CD31 staining.

Human islet VEGF production and release

Islets maintained in m199 in SC responded to 25mM TZD by significantly increasing VEGF production and release into the tissue culture medium. This response was seen at 48 hours and 72 hours treatment with $p<0.01$ (employing a Student's t-test) for both time points. Interestingly, VEGF production and secretion from SC TZD at 72 hours treatment was significantly increased when compared with SC TZD at 48 hours treatment ($p<0.05$, Student's t-test). This indicates that the effect of a TZD on VEGF production from SC human islets increased over the 72 hours exposure period. However, exposure to supraphysiological doses of TZD lead to fissures in HUVEC after 4 days exposure (see chapter 4, figure 45). It is possible that the compromised endothelial structural integrity could result in leakage from endothelial cells by 72 hours exposure, thus adding to the increase in VEGF release seen over time.

Islets maintained in RC with 25mM TZD also responded with a significant increase in VEGF production and release at both 48 hours and 72 hours treatment when RC TZD groups were compared to control ($p<0.01$ at both time points, Student's t-test). A comparison between RC TZD 72 hours and RC TZD 48 hours showed a significant increase in VEGF production and release between the two time points ($p<0.05$, Student's t-test). This result again implies that the effect of a TZD on VEGF production and release from RC human islets is increased over a 72 hour exposure period.

When RC TZD was compared to SC TZD, VEGF production and release was found to be significantly higher in RC TZD at both time points with a Student's t-test returning $p<0.05$ for 48 hours treatment and $p<0.01$ for 72 hours treatment. This result implies an enhancing effect of RC on the ability of human islet endothelial cells to produce and release VEGF in response to TZD.

Rotational cell culture has proven to be useful in maintaining the health and function of islet beta cells (Murray et al. 2005) and it is therefore possible that the endogenous islet endothelial cells also benefit from the unique microgravity environment provided by RC. This theory is supported by the VEGF data shown above, as human islets released significantly more VEGF in response to TZD stimulation in rotational culture than when they were maintained in static culture. RC is a dynamic culture system that allows dispersion of released growth factors such as VEGF, thus possibly enhancing the effect by increasing the opportunity for cells to make contact with it. This upregulation of VEGF by islet endothelial cells may impact positively on engraftment of the transplant as many studies have shown that VEGF drives angiogenesis (Takeshita et al. 1994; Ferrara 2000; Kanazawa 2007).

Conclusions

This study supports evidence in the literature that human islets can be successfully maintained in various tissue culture media (Lucas-Clerc et al. 1993; Brendel et al. 1994; Clayton et al. 1998; Jay et al. 2004). However, no advantage was seen over medium 199 when more expensive, commercially available media were assessed in terms of maintaining islet structure or function.

Islet structure and insulin secretory profiles deteriorate significantly after approximately 3 - 4 days in conventional static culture (Brendel et al. 1994; Matta et al. 1994; Bottino et al. 1997;

Murray et al. 2005). Several phenomena, including activation of free radicals and nitric oxide production, may be responsible for these effects (Bottino et al. 1997; Murray et al. 2005). Also, the fragmentation of the three dimensional structure of the islets initiated by enzymatic disruption of the pancreas during islet isolation was exacerbated by conventional static culture methods (Matta et al. 1994; Rosenberg et al. 1999; Balamurugan et al. 2003). Employing a bioreactor culture system or a rotational cell culture system that provided a microgravity environment, had a significant effect on maintaining islet structure and supported islet function for longer periods than conventional static culture (Matta et al. 1994; Murray et al. 2005).

TZDs have been shown to have a beneficial effect on both insulin secretion (Lupi et al. 2004; Zhou et al. 2005; Campbell and Mariz 2007) and the production and release of VEGF (Emoto et al. 2001). In this study, the addition of 25mM TZD (rosiglitazone) to the culture medium increased human islet insulin secretion and VEGF production and release.

These results indicate the potential application of a combination of rotational cell culture and a TZD as an effective pre-treatment for human islets prior to implantation to support the insulin secretory function of the beta cells and prime the endothelial component of the islet to proliferate more rapidly, potentially facilitating enhanced islet revascularisation.

Chapter 6: Protocol development for assessing human islet vascularisation

Introduction

A small pilot study was undertaken to commence protocol development for *in vivo* assessment of the vascularisation of human islets cultured under conventional SC conditions +/- TZD. Although the results from chapters 4 and 5 show a beneficial effect of RC on maintaining islet structure and viability, use of SC would be required as a control against which to measure differences encountered *in vivo* by employing RC and was thus used as a starting point for protocol development. Due to unavailability of human pancreata, frozen islets were used for this study, table 11 summarises the isolation procedure and outcome.

The addition of VEGF to human islet culture medium has been shown to preserve the beta cell insulin secretory response in the presence of toxic immunosuppressants such as tacrolimus, sirolimus and rapamycin (Cross et al. 2007; Laugharne et al. 2007), while the delivery of human VEGF cDNA to murine islets resulted in improved islet function and revascularisation after implantation under the kidney capsule of diabetic mice (Zhang et al. 2004). A recent study showed that VEGF-A deficient mice exhibited reduced insulin gene expression levels and impaired glucose tolerance, indicating a role for VEGF in promoting healthy islet function (Jabs et al. 2008). Thus, the evidence in the literature suggesting the benefits of VEGF in terms of supporting both islet function and revascularisation, together with the results from chapters 4 and 5, strengthened the argument for upregulation of VEGF expression via TZD exposure prior to islet transplantation.

Vascular corrosion casts have been used extensively to investigate vasculature (Nopanitaya et al. 1979; Whiteley et al. 2006), including that of the kidney (Wei et al. 2006) and the casts

have traditionally been analysed by scanning electron microscopy (Burger et al. 1984; Fahrenbach et al. 1988; Macchiarelli et al. 2006; Whiteley et al. 2006).

For the purposes of this study, x-ray microtomography was investigated as a possible means of analysing the vascular corrosion casts. X-ray microtomography (XMT) is a miniaturised version of medical CT or CAT (computed axial tomography) scanning. The specimen is mounted on a turntable between an x-ray source and an x-ray sensitive camera and a series of X-ray projections are recorded at a number of angles around the specimen, usually over a range of either 180 or 360 degrees. In XMT, unlike medical CT, the specimen is usually rotated, rather than the X-ray source and detector. As the projections are taken through a single plane in the specimen, it is possible to reconstruct a cross sectional image of that plane. In most XMT scanners today, two dimensional images are recorded, making it possible to reconstruct a complete three dimensional (3D) map of X-ray attenuation (personal communication with Dr. Taghi Miri, Department of Chemical Engineering, University of Birmingham, September 2008).

X-ray microtomography has been used to produce 3D images of structures as diverse as ice crystals formed during food freezing (Mousavi et al. 2007), bone ingrowth into porous biomaterials (Jones et al. 2007), barium sulphate filled microangioarchitecture of tumours in rabbits (Maehara 2003), microcirculation in rat kidneys perfused with a lead chromate containing, silicon based solution (Ortiz et al. 2000), and formalin fixed, excised inner ears of mice (Van Spaendonck et al. 2000).

Methods

Post-thawing, the islets were divided into two groups and maintained in static culture (90mm petri dishes) in medium 199 (supplemented with 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin and 10µg/ml amphotericin B) +/- 25mM TZD. A trypan blue membrane integrity test returned a post-thawing score of 22 indicating viable islets. An insulin secretion experiment was performed on both groups at 24hrs culture to establish islet function, figure 61 shows the insulin secretion data obtained.

Islets were transplanted under the kidney capsules (Lacy et al. 1982; Davalli et al. 1995; Jaeger et al. 1995; Mattsson et al. 2006; Caiazzo et al. 2008) and into the spleens (Andersson 1982; Sandler and Andersson 1982) of six 13 week old, non-diabetic, lean male mice using keyhole surgery, see figure 60. As the purpose of this pilot study was an initial investigation into revascularisation it was deemed unnecessary to use diabetic mice at this stage, however, once a suitable protocol has been established it is envisaged that diabetic mice will be used to assess graft function in conjunction with engraftment.

Cold ischaemia time	The pancreas arrived at the IRL at 17.20 with a cold ischaemia time of 10.5 hours
Organ description	The organ was well packaged: double bagged with sufficient ice. A section of spleen and a large amount of fat were attached; the pancreas was a healthy pink-brown colour. There was no visible damage to the organ or capsule after removal of the fat and spleen tissue.
Digestion phase	84g of tissue was available for islet isolation and a very good degree of ductal distension was achieved with infusion of Liberase (lot no: 93597920). The pancreas was left intact for the initial 30 minute digestion phase after which it was cut into 6 pieces and allowed to disperse for a further 10 minutes
Pre-separation digest	The pancreatic tissue was well dispersed with many exocrine clumps visible and free islets ranging from 50 - 600μm in diameter. A trypan blue score of 22 indicated viable islets.
Isolated islet preparation	The post-separation islet preparation purity was ~85% with islets varying in size from 50 - 600μm. A trypan blue score of 22 indicated viable islets. 314 000 IEQ were isolated of which 6 vials containing 20 000 IEQ/vial were cryopreserved for future use.

Table 11. A summary of the islet isolation procedure and the outcome obtained from the donor pancreas used to produce the islets for this transplant study.

Anaesthesia was induced using an isofluorane/oxygen combination (Chung et al. 2007; Taylor 2007; Valentim et al. 2008) and the abdominal area was swabbed with an iodine solution prior to a small area of fur being removed in the upper left quadrant of the abdominal area. A 1cm incision was made with sterilised surgical scissors and the left kidney was exposed using sterilised forceps to gently part the abdominal wall.

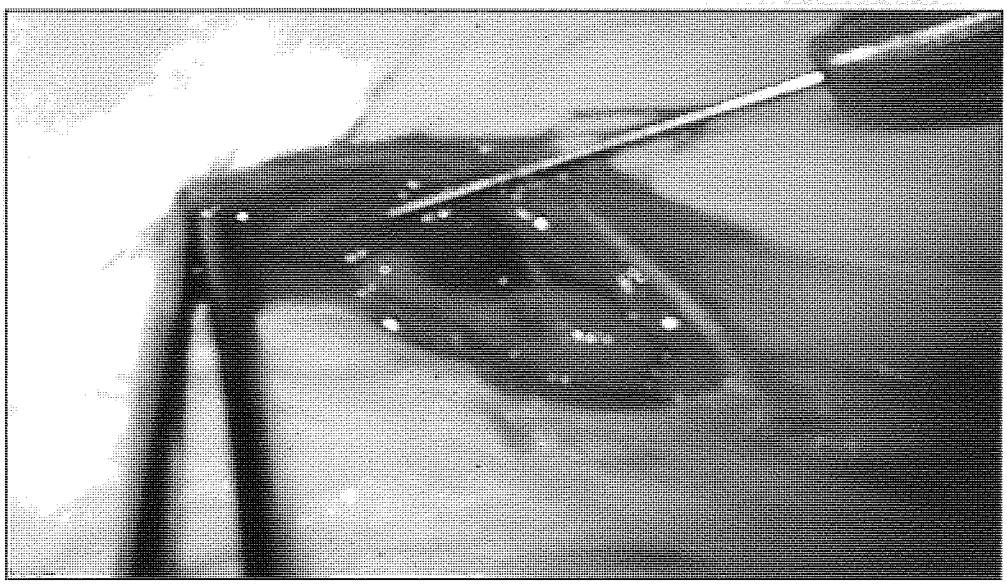


Figure 60. Keyhole surgery technique used for depositing human islets into a channel under the kidney capsule. All surgical procedures were performed by Professor C.J.Bailey.

The islets were contained in a sterile 1ml syringe with a 26G needle that was used (as opposed to the 25G needle shown in figure 60) to pierce the kidney capsule and create a small channel into which the 50 islets were injected. The same procedure was followed to place 50 islets into the spleen before the abdominal wall was closed with 3 sutures and the skin with one suture and two stainless steel clips.

Three of the mice received 50 islets from the m199 SC group under the left kidney capsule and a further 50 islets into their spleen. The procedure was repeated on the remaining 3 mice with islets from the m199 SC TZD group. Prior to recovery from anaesthesia each mouse received 5mg/kg azathioprine immunosuppressant and 10µg/kg Vetergesic analgesia subcutaneously as per manufacturer's instructions.

The animals were placed on warming plates and observed at regular intervals after surgery to monitor post-operative recovery. All six mice recovered rapidly, moving freely around their cages and eating.

Analgesia was administered as above at 24 hours post-transplant after which it was withdrawn as the animals showed no sign of discomfort, continued to consume food and water and the incision sites were healing well. Azathioprine was administered daily at 5mg/kg subcutaneously and body weights and general observations were recorded daily. All 6 mice lost weight within 48 hours after the procedure, but recovered their body weight by day 5 post transplant.

One animal from each group was terminated by cervical dislocation on days 6, 9 and 12 post islet implant. A systemic saline flush was administered, the spleen was harvested and fixed in formalin for histological examination, and a polymer was infused into the left kidney to yield a vascular corrosion cast.

Results

Pre-transplant islet assessment

The islets were assessed for glucose stimulated insulin secretion at 24 hours culture, a few hours prior to implant. In brief, a sample of islets from each group was placed into a low glucose solution (1.67mM) for 1 hour at 37°C to allow the islets to settle to a state of basal insulin secretion. For each group 20 islets/tube were placed into 18 LP2 tubes after the 1 hour pre-incubation and 2ml/tube of the relevant secretagogue – low glucose (1.67mM), high glucose (16.7mM) and G&T (16.7mM glucose + 10mM theophylline) – was added (n = 6 per secretagogue). The islets were incubated for 1 hour at 37°C after which the supernatants were removed and analysed for insulin content by ELISA assay (see chapter 2). A Student's t-test returned $p<0.05$ (statistically significant) for both groups when insulin secretion in response to 16.7mM glucose and G+T were compared to insulin secretion from islets challenged with 1.67mM glucose. Figure 61 shows the insulin secretion results for both the m199 SC and m199 SC+TZD groups.

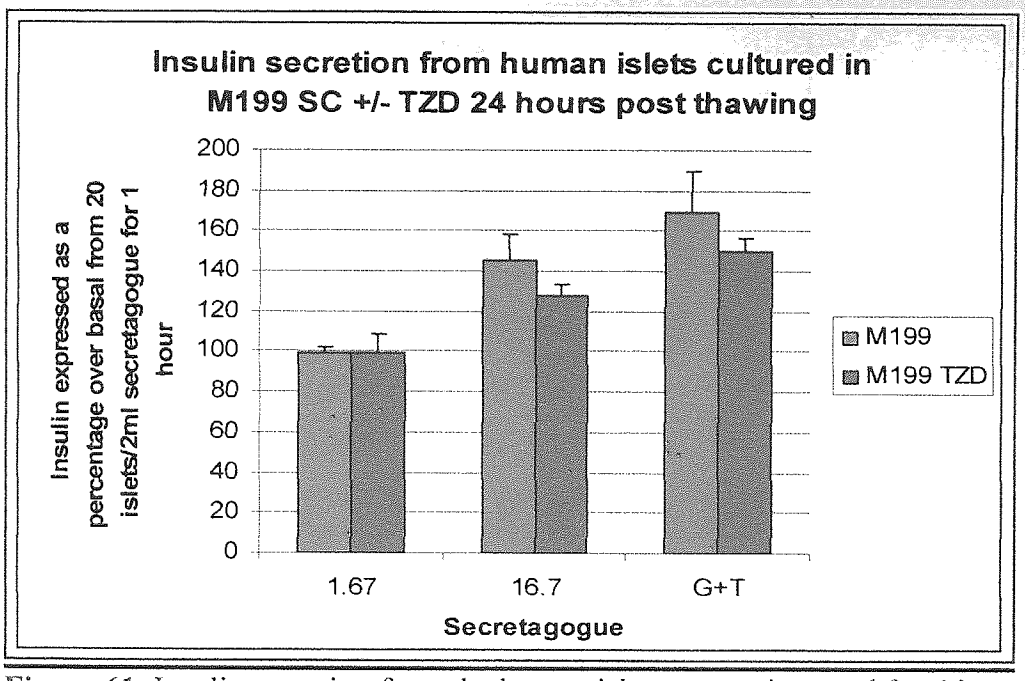


Figure 61. Insulin secretion from the human islet preparation used for this transplant study. Data represent insulin secretion 24 hours post thawing from islets maintained in medium 199 under static culture conditions +/- 25mM TZD.

Kidney vascular corrosion casts

On days 6, 9 and 12 post transplant one mouse from each group was terminated by cervical dislocation. A vascular corrosion cast was prepared from the left kidney of each mouse as described in chapter 3, the kidney tissue was macerated in a 20% w/v NaOH solution the casts were cleaned under warm, running water and then stored in filter sterilised deionised water prior to being photographed (figures 62, 63).

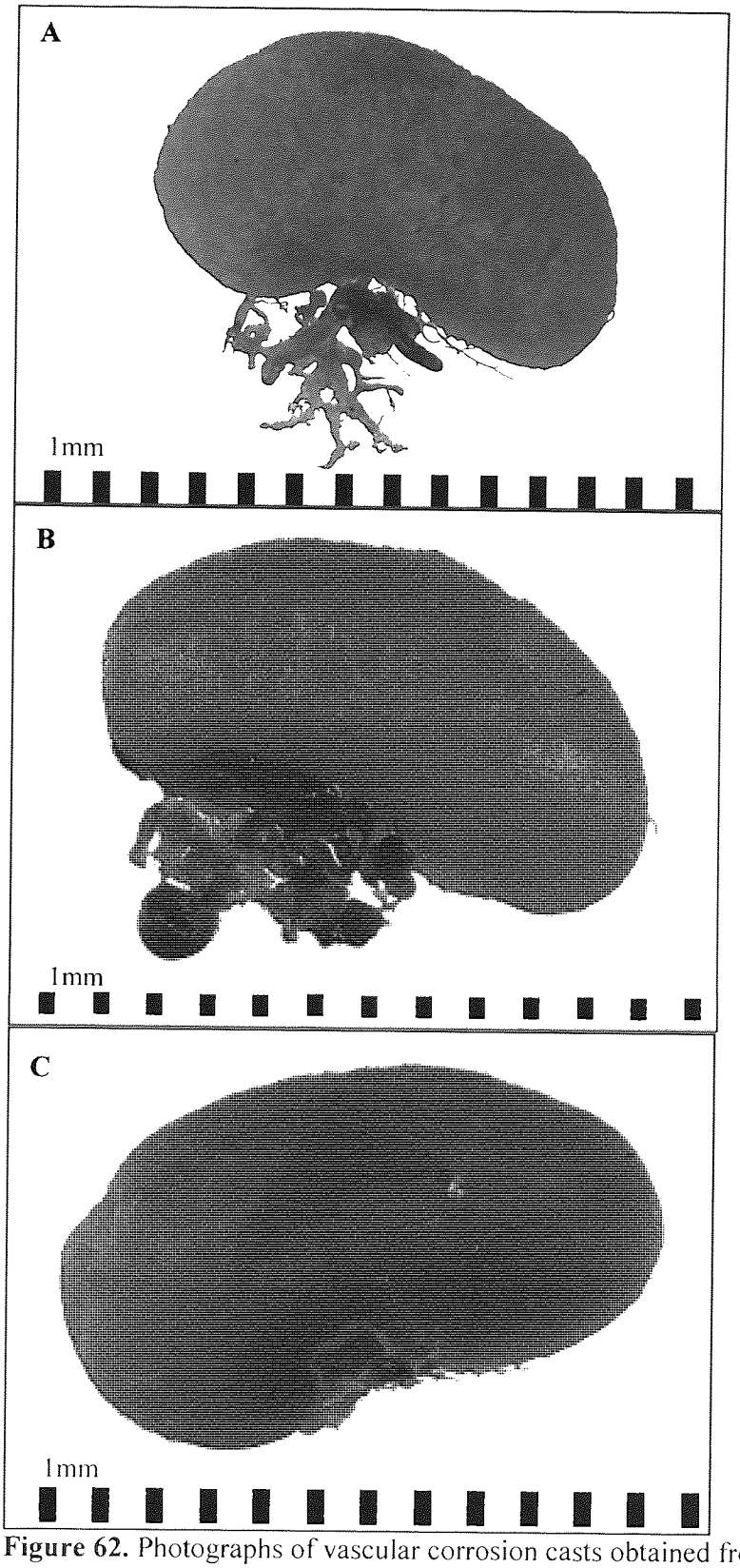


Figure 62. Photographs of vascular corrosion casts obtained from polymer infusion into the left kidneys of mice at days 6, 9 and 12 post islet implant from the control (m199 SC) group. Image A depicts the vascular corrosion cast from control 1, while B and C show the casts obtained from control 2 and 3 respectively.

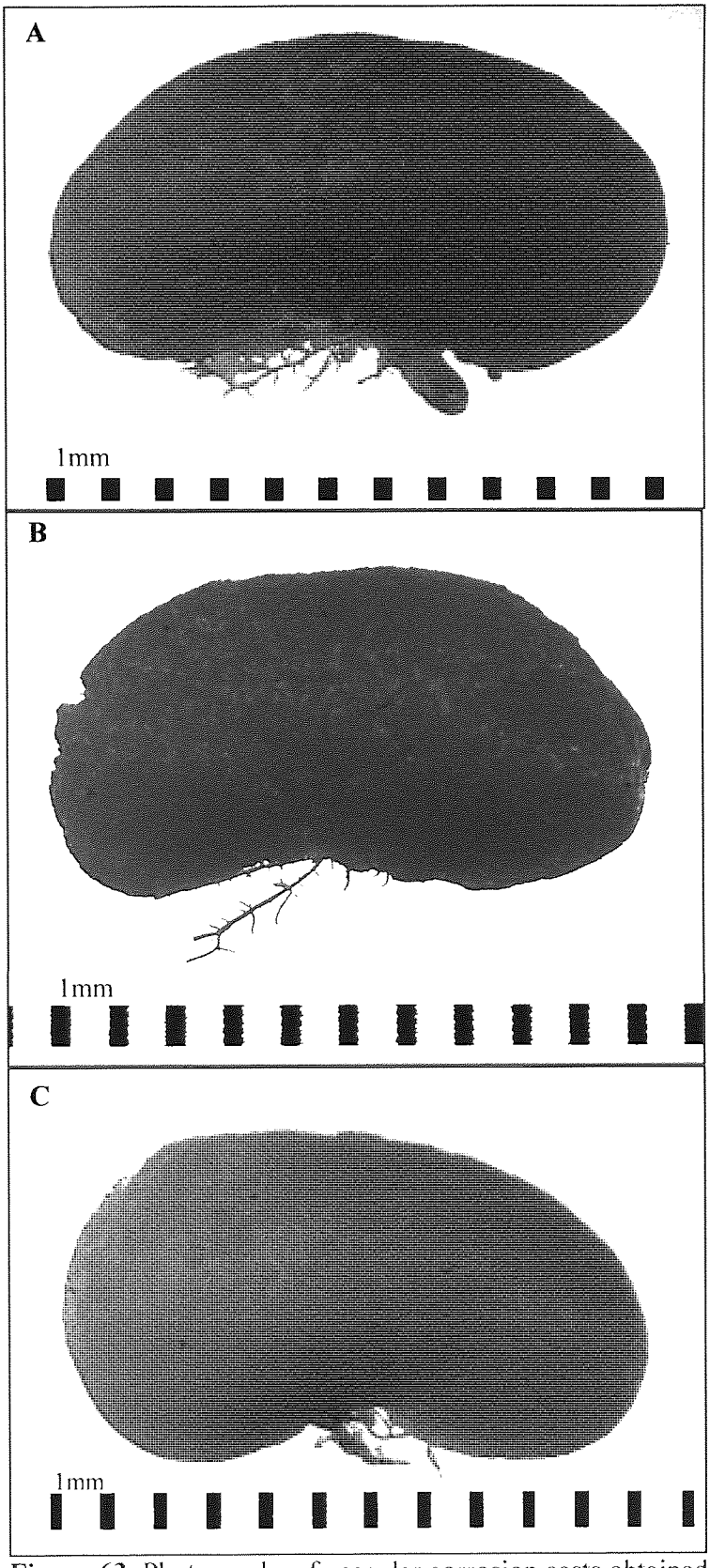


Figure 63. Photographs of vascular corrosion casts obtained from polymer infusion into the left kidneys of mice at days 6, 9 and 12 post islet implant from the treatment (m199 SC TZD) group. Image A depicts the vascular corrosion cast from TZD 1, while B and C show the casts obtained from TZD 2 and 3 respectively.

Spleen histology

Upon termination of the relevant mice on days 6, 9 and 12 post islet-implant, the spleens were excised and fixed in formalin after administration of the systemic saline flush, but prior to initiation of polymer infusion to produce vascular corrosion casts. A 5mm section through the area of islet implantation of each spleen was sent to the Worcester Royal Hospital Histopathology Laboratory for tissue processing, paraffin wax embedding and sectioning onto glass slides.

Microwave antigen retrieval was performed on all sections in a 10mM sodium citrate buffer for 40 minutes prior to commencement of insulin staining. Both fluorescence and enzyme-substrate based staining methods were tested to assess suitability for use on formalin fixed, paraffin wax embedded mouse spleen samples. Image 64 shows staining achieved in the control 3 spleen with TMB used as a substrate for horseradish peroxidase, and DAB visualisation of human insulin in the TZD 1 spleen. Image 65 shows fluorescence staining for insulin achieved in the control 1 and 2 as well as in the TZD 2, and 3 spleens with TRITC visualisation of insulin in the control 1 spleen and FITC employed for the remaining experimental groups.

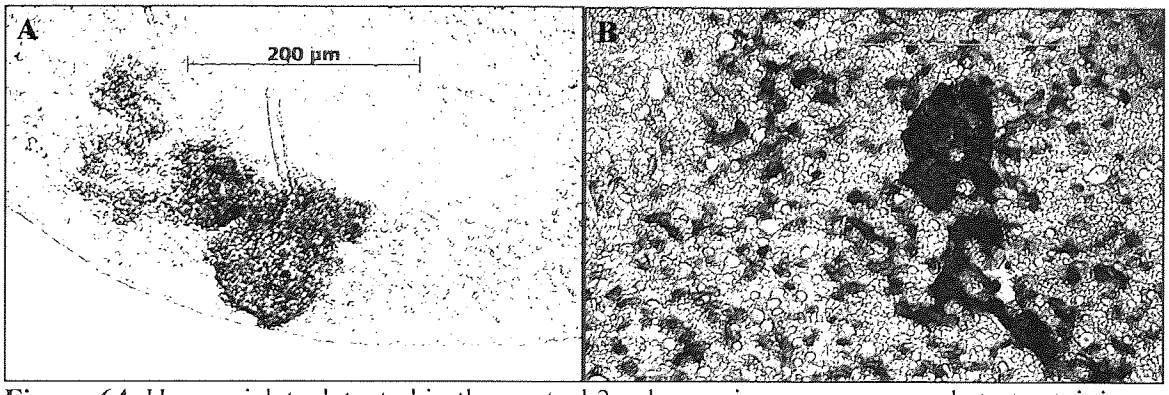


Figure 64. Human islets detected in the control 3 spleen using an enzyme-substrate staining technique with TMB resulting in blue visualisation of human insulin (A). DAB was used to visualise insulin in the TZD 1 spleen (B). Photographs were taken at x20 magnification.

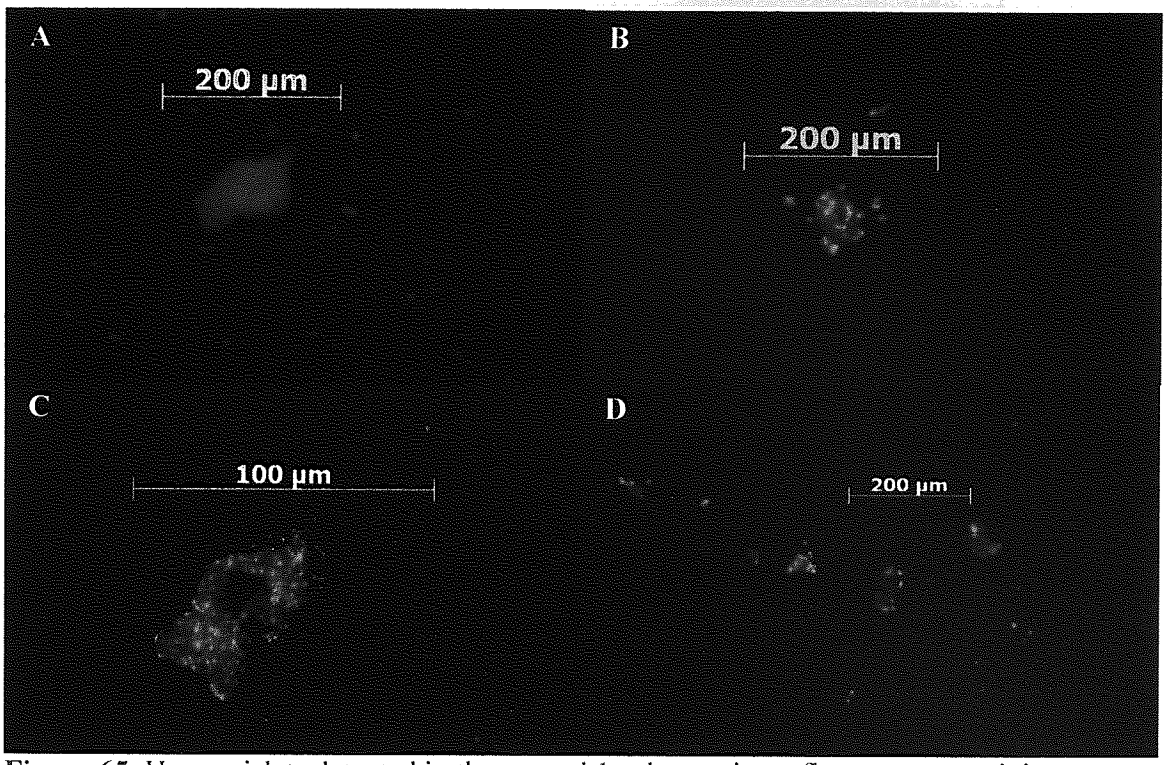


Figure 65. Human islets detected in the control 1 spleen using a fluorescence staining technique with TRITC resulting in red visualisation of human insulin (A). FITC was used to obtain green visualisation of insulin in the control 2 (B), TZD 2 (C) and TZD 3 (D) spleens. Photographs were taken at x20 magnification.

X-ray microtomography of vascular corrosion casts

X-ray microtomography (SkyScan-1072 microtomograph from SkyScan, Antwerpen, Belgium) of each cast yielded ~600 frames of information relating to the vasculature of the kidney. The tomography data were processed by the Department of Chemical Engineering at the University of Birmingham to produce images representing 'slices' through each kidney cast on the z-axis (see figures 68 – 73). As the University of Birmingham do not currently possess software to quantify the data relating to vasculature, 10 frames per cast were selected from the central section of the kidneys (where most of the islets were expected to be located, see figure 66). Five vessel measurements were performed per frame in the area shown in figure 67, employing the CTAn SkyScan volumetric reconstruction software supplied by the Department of Chemical Engineering, University of Birmingham.

While the newly formed vascular supply to the implanted islets was expected to have a diameter as small as 5.27μm (Henderson and Moss 1985), the means to measure and quantify these vessels were not available. Thus it was decided that unusual 'hot spots' or larger than expected vessels, possibly indicating increased blood flow to sites of injury or ischaemia, would be measured in the areas where islet location was anticipated.

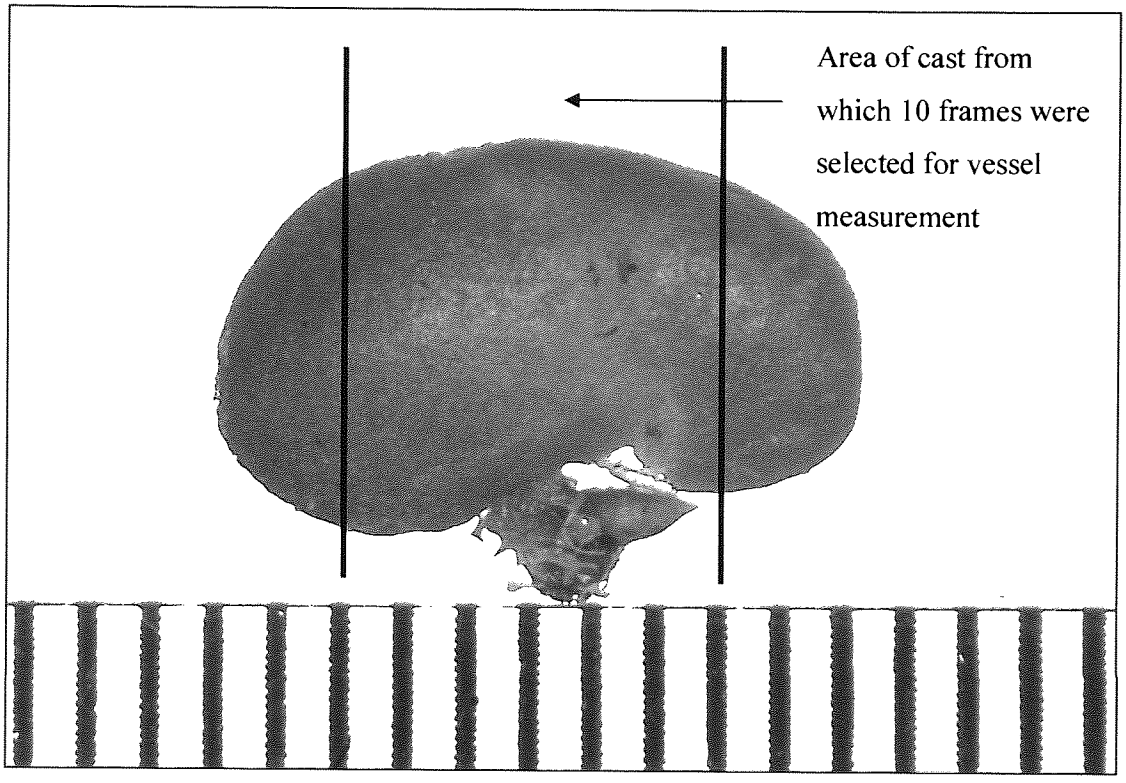


Figure 66. Ten frames from the central section of each cast were selected for vessel measurement.

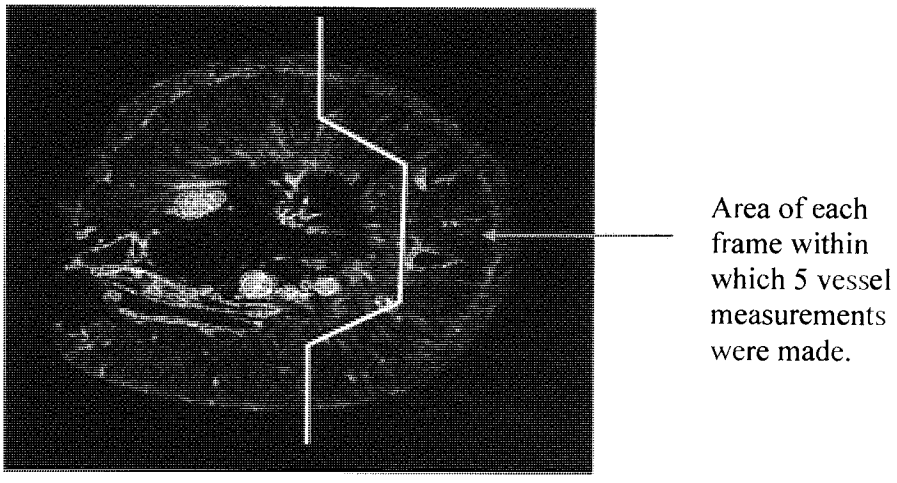


Figure 67. Five vessels from each of the ten frames selected per cast were sized within the area shown using the CTAn SkyScan volumetric reconstruction software.

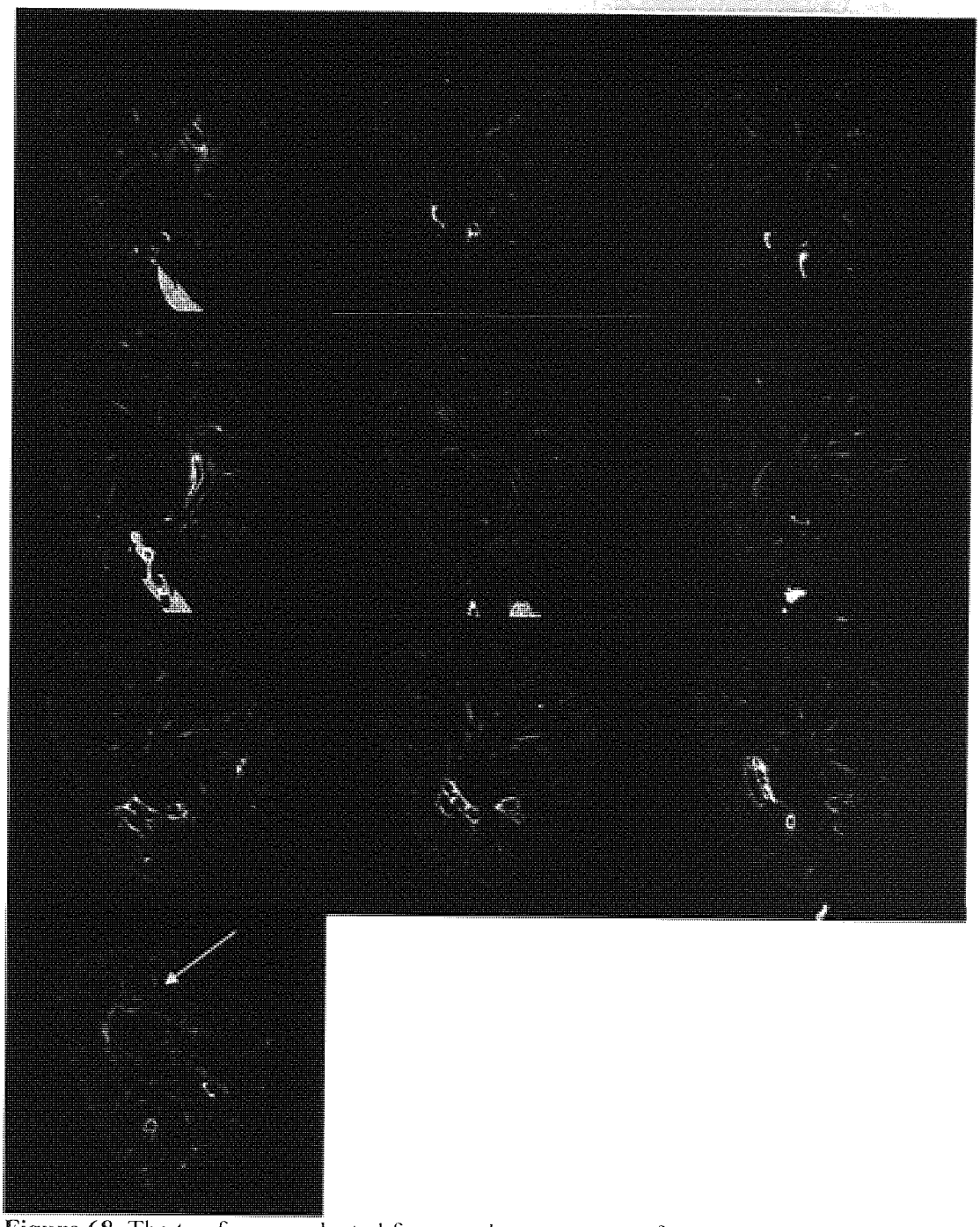


Figure 68. The ten frames selected for vessel measurement from control 1. The position of the yellow arrow indicates the area of the cortex where islets were implanted.

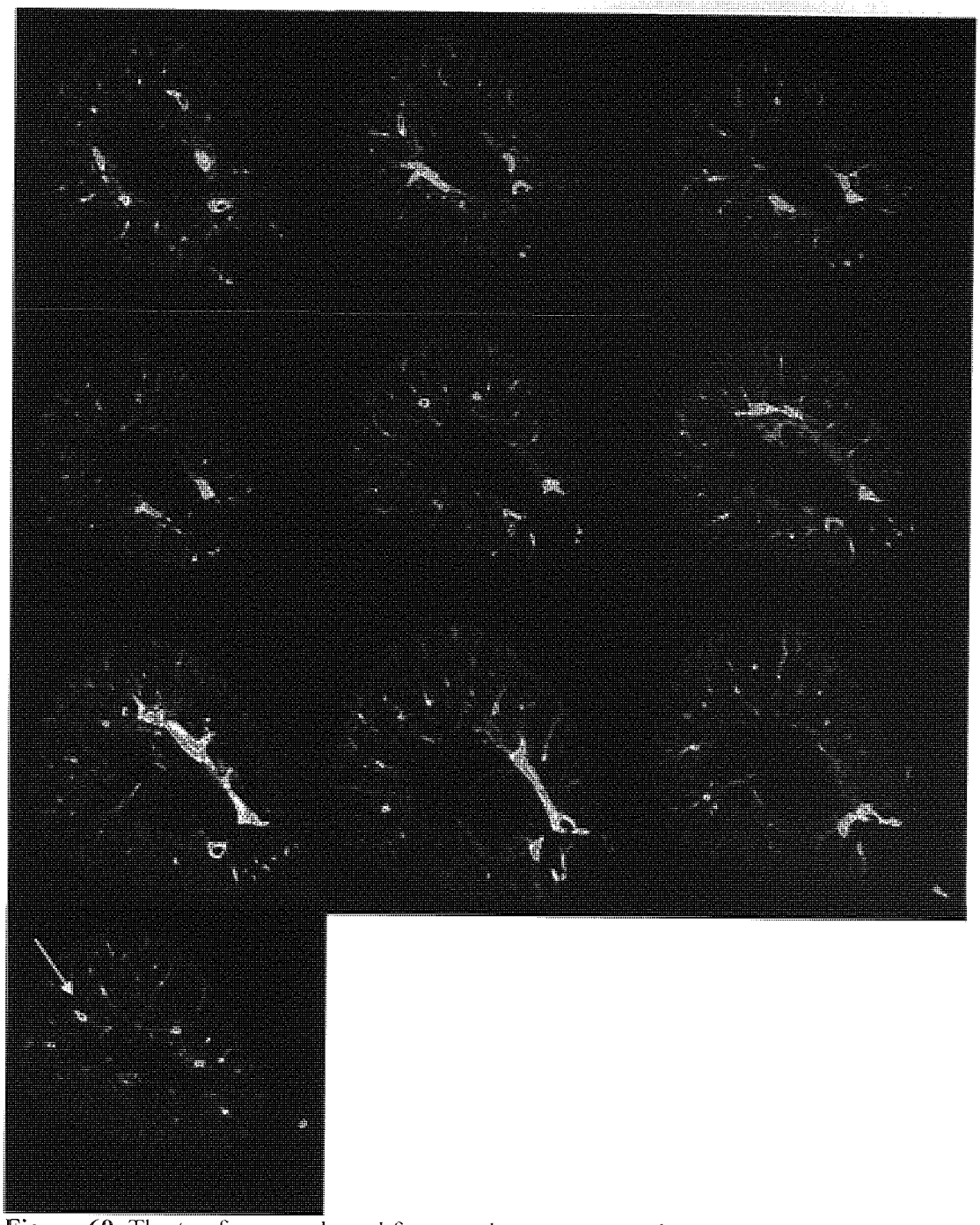


Figure 69. The ten frames selected for vessel measurement from control 2. The position of the yellow arrow indicates the area of the cortex where islets were implanted.

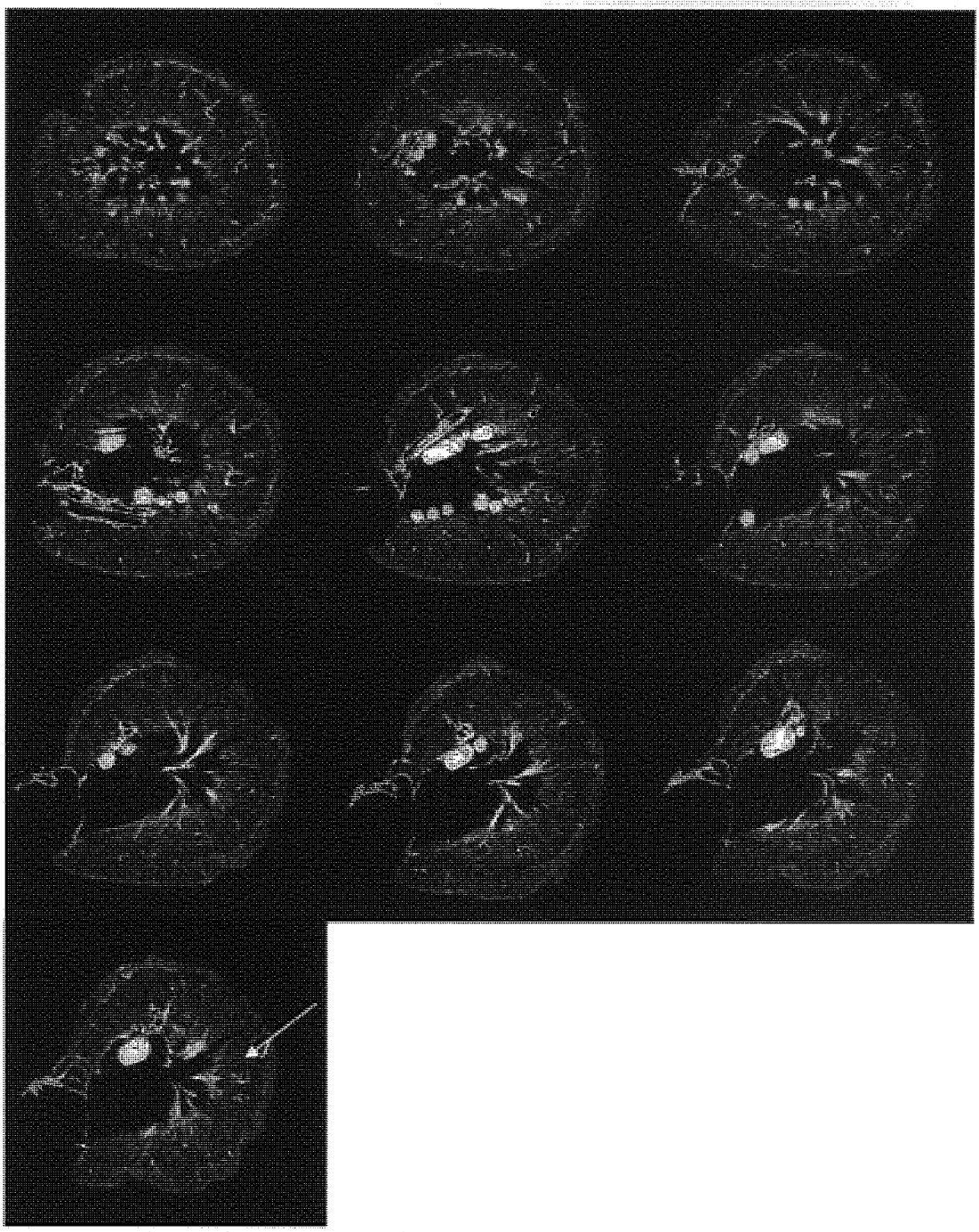


Figure 70. The ten frames selected for vessel measurement from control 3. The position of the yellow arrow indicates the area of the cortex where islets were implanted.

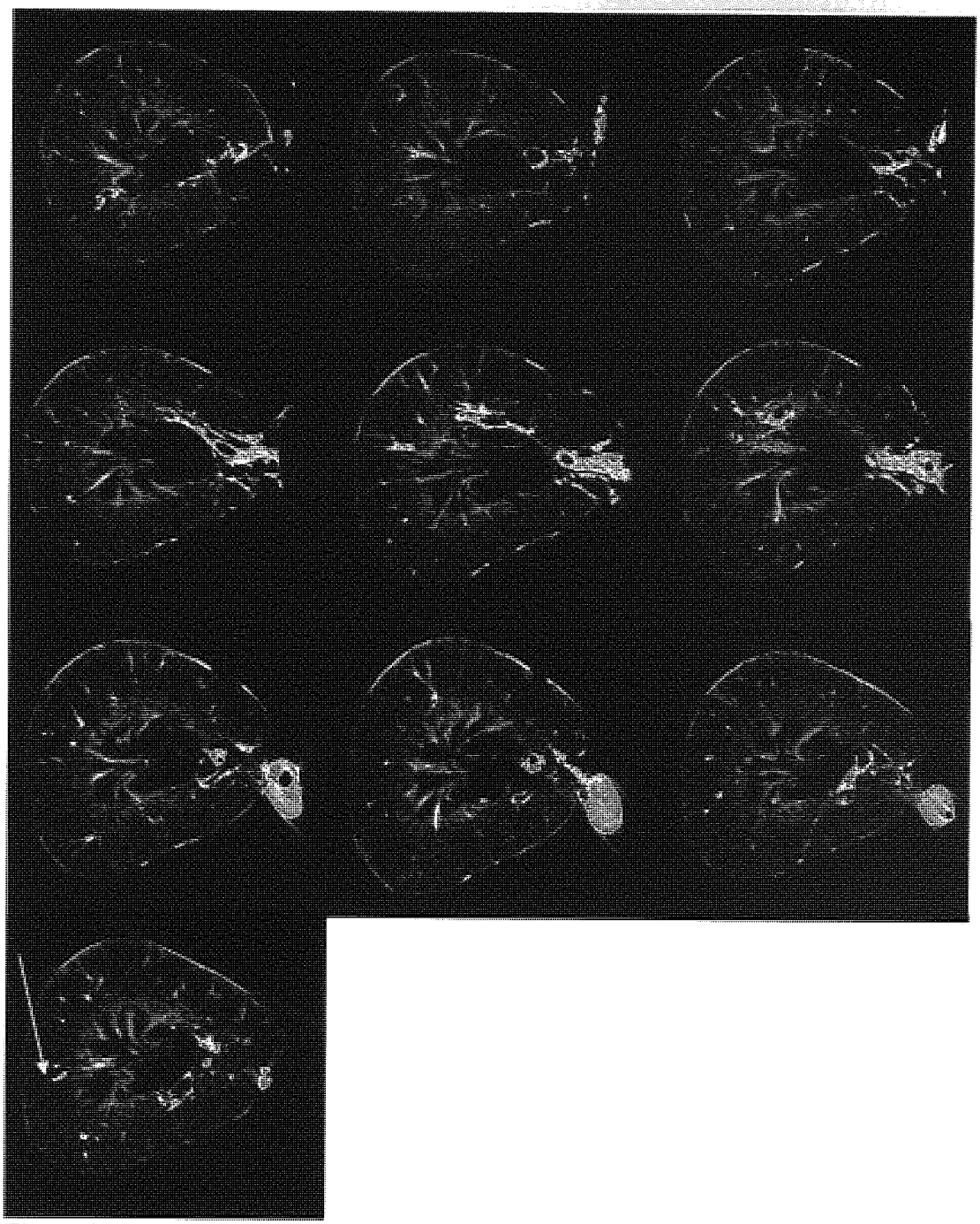


Figure 71. The ten frames selected for vessel measurement from TZD 1. The position of the yellow arrow indicates the area of the cortex where islets were implanted.

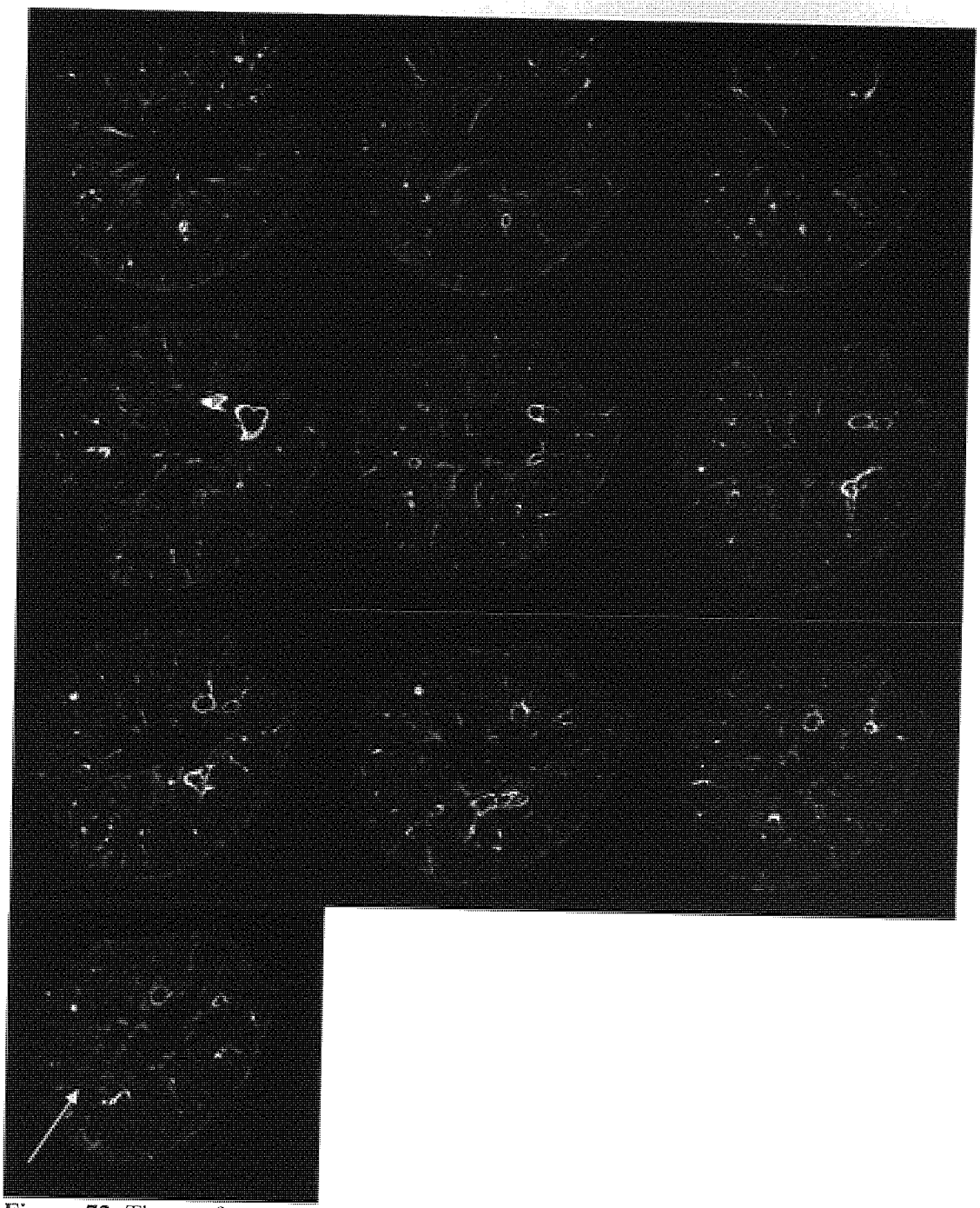


Figure 72. The ten frames selected for vessel measurement from TZD 2. The position of the yellow arrow indicates the area of the cortex where islets were implanted.

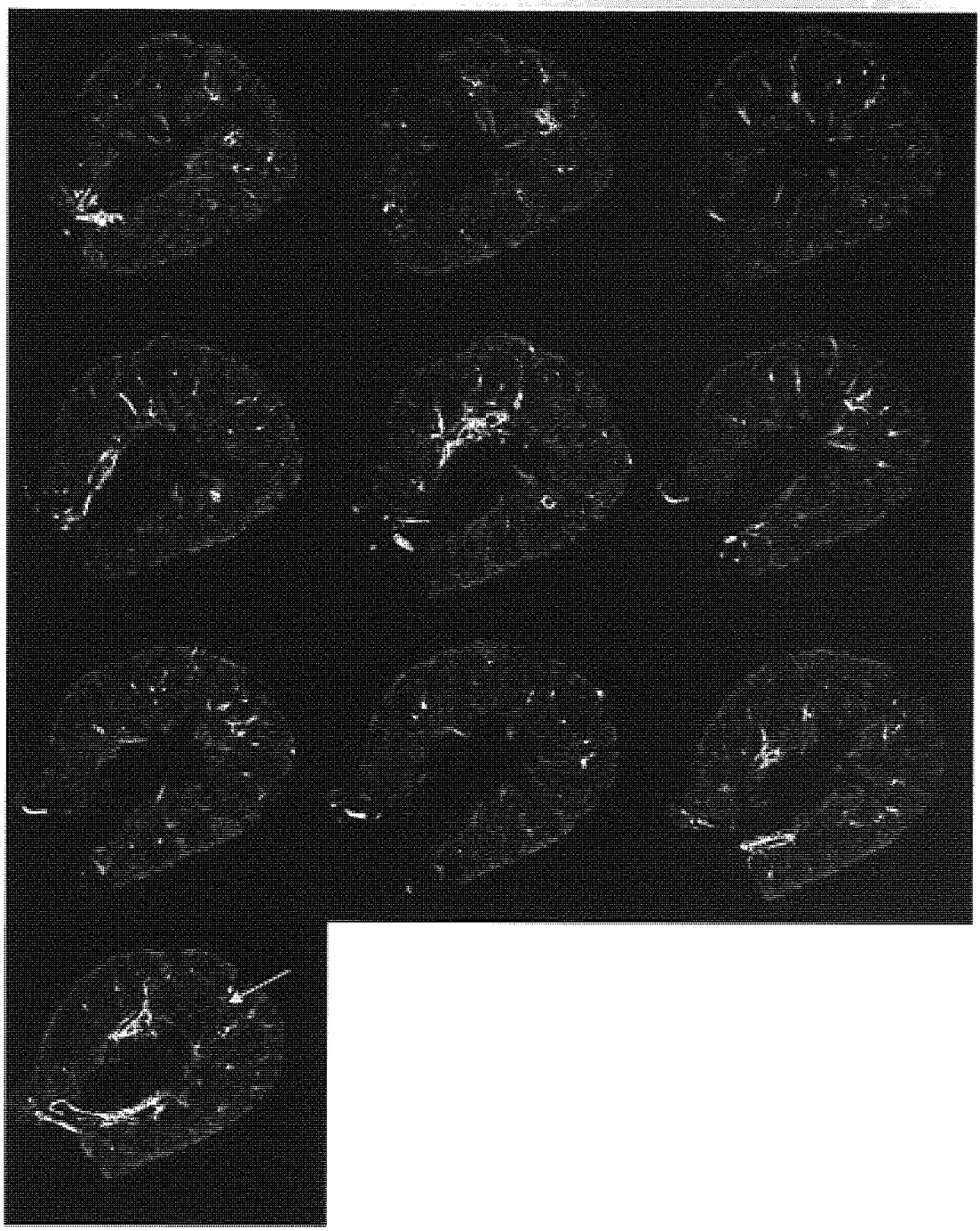


Figure 73. The ten frames selected for vessel measurement from TZD 3. The position of the yellow arrow indicates the area of the cortex where islets were implanted.

	Vessel size groups				
Corrosion cast	**No of vessels <50 μm**	**No of vessels 50 - 75 μm**	**No of vessels 75 - 100 μm**	**No of vessels 100 - 125 μm**	**No of vessels >125 μm**
Control 1	6	30	8	4	2
Control 2	5	29	9	5	2
Control 3	6	23	10	8	3
Control totals	**17**	**82**	**27**	**17**	**7**
TZD 1	6	23	12	6	3
TZD 2	4	21	14	7	4
TZD 3	4	19	13	8	6
TZD totals	**14**	**43**	**39**	**21**	**13**

Table 12. Vessel sizing results for 5 vessels per frame from 10 frames per cast.

Discussion

Pre-transplant islet function

Both groups showed a significant increase in insulin secretion in response to high glucose (16.7mM) and G&T when compared to low glucose. A Student's t-test returned $p<0.05$ for 16.7mM v 1.67mM and G&T v 1.67mM for both groups. Unusually for human islets, there was no significant difference in insulin secretion when G&T was compared to 16.7mM employing a Student's t-test ($p>0.05$ for both groups). In addition, there was no significant difference in insulin secretion for the same secretagogue between the two groups.

Cryopreservation has been shown to compromise islet function, blunting the insulin secretory ability in response to high glucose (Piemonti et al. 1999; Gatto et al. 2003) and other secretagogues such as theophylline (Hou et al. 2006), hence fresh islets will be used wherever

possible for subsequent transplant studies. Also, islets were placed into experimental groups (SC and SC TZD) immediately after thawing and subjected to an insulin secretion experiment 24 hours later. It is possible that exposure to a TZD directly upon thawing may have added to islet stress post-thawing, resulting in a detrimental effect on islet function. McKay and Karow showed that islets were functional post-thawing but that the insulin secretory response was improved by increasing the post-thaw culture period, thus providing proof that islets require a recovery period after cryopreservation in order to return to a normal functional state (McKay and Karow 1983). This evidence, together with the IRL results showing that rotational culture preserves islet structure and supports islet function (Murray et al. 2005), suggests that a 24 – 48 hour period of rotational culture post thawing, prior to commencement of experimentation, may aid the restoration of islet function after cryopreservation.

Kidney vascular corrosion casts

The renal cortex of mice has been shown to be a favourable islet implantation site, supporting the insulin secretory profile of the islets, thus resulting in reversal of diabetes (Mellgren et al. 1986; Hayek and Beattie 1997). At least part of the explanation for the success of implants into animal kidneys is believed to be the nature and extent of the vascular supply, especially to the renal cortex: an extensive arteriolar supply that is not subject to strong local regulation. A study by Carlsson *et al* showed that capillary pressure in revascularised islets implanted under the kidney capsule of rats was similar to that of the kidney, indicating good islet revascularisation and perfusion (Carlsson et al. 1998). Also, islets implanted into the renal cortex showed a higher level of revascularisation than intra-splenic islet implants (Mattsson et al. 2002).

The kidneys are highly vascularised organs receiving a substantial proportion of cardiac output via the renal artery, which branches and supplies the glomeruli in the renal cortex via

the afferent arterioles (Brenner and Beeuwkes 1978; Rosivall et al. 1979). Post-glomerulus, the efferent arterioles supply the medulla with blood (Brenner and Beeuwkes 1978; Rosivall et al. 1979). A study by Geraghty *et al* showed that mean cortical blood flow is three times higher than medullary blood flow (Geraghty et al. 1992). This renal microvascular arrangement indicates that the cortex receives a higher proportion of oxygen-rich arterial blood than the medulla, and the cortical arterial blood has a higher oxygen tension than medullary arterial blood (Nelimarkka et al. 1982), thus supporting the implantation of islets into the cortex. The interstitial osmotic gradients into the depth of the medulla further reduce this as a suitable site compared with the cortex (Geraghty et al. 1992; Pallone et al. 1998).

Vascular corrosion casts have been used extensively to depict and study the vasculature of kidneys in many species; including angiogenesis resulting from polycystic kidney disease in humans (Moore et al. 1992), the vasculature of the kidney in pigs (Moore et al. 1992), renal vasculature of rabbits (Wagner et al. 2006), renal microvasculature in rats (Manelli et al. 2007; Sricharoenvej et al. 2007) and guinea pigs (Sangiorgi et al. 2004), as well as in mice (Ninomiya et al. 1998; Drevs et al. 2002).

Scanning electron microscopy of corrosion casts from a study investigating the anatomy of capillaries in the rat heart showed that corrosion casts were able to depict vessels with a diameter as low as 5.6μm (Hossler et al. 1986). Vascular corrosion casts from a study of the three-dimensional micromorphology of vessels in the growth zone of rat femurs were able depict vessels with a diameter of around 5μm (Aharinejad et al. 1995). These studies support the use of vascular corrosion casting as a means of depicting mouse renal vasculature and possibly detecting islet microvasculature post islet implant.

Successful vascular corrosion casts were obtained from all three mice in each of the two experimental groups. The renal veins of control 1 and 2 and TZD 1 mice ruptured upon completion of polymer infusion, leading to the additional cast section in the position of the renal vein. The polymer infused into the TZD 2 failed to fill the small vessels in one minor area directly under the kidney capsule. Fortunately, these imperfections occurred outside the area of the casts where the islets were expected to be located. All the casts were thus deemed suitable for further analysis by x-ray micro tomography at the Chemical Engineering Department of Birmingham University.

A study by Morini showed that the pressure used for polymer infusion was crucial for obtaining a complete vascular cast, with a pressure of 100 +/- 5 mm Hg providing the best results (Morini et al. 2000). In an attempt to avoid renal vein rupture and to ensure complete filling of the kidney microvasculature with the polymer, future studies will employ a syringe pump to regulate the filling pressure (Fahrenbach et al. 1988). Although the polymer used in this study is a low-viscosity methyl methacrylate plastic (Sudwan et al. 1991), for future studies its viscosity will be monitored and, if necessary, adjusted to more closely resemble that of blood (Lametschwandtner et al. 1990).

Spleen histology

The spleen has been used successfully as a transplant site for islets and beta cell aggregates, with islet morphology remaining unaffected by the intra-splenic environment (Janney et al. 1982; Sandler and Andersson 1982; Warnock et al. 1983; Mellgren et al. 1986; Hayek and Beattie 1997; Horton et al. 2000; Aoki et al. 2005). Also, intra-splenic islet implants have been detected by immunostaining for insulin (Banks et al. 1982; Warnock et al. 1983; Horton et al. 2000; Aoki et al. 2005), supporting the utilisation of the spleen in this study as an islet depot for histological examination at given time points post implant.

Both of the techniques used in this study to detect insulin via immunostaining have been documented in the literature (Banks et al. 1982; Cross et al. 2007; Dickens et al. 2008; Passam et al. 2009). The enzyme-substrate staining method yielded satisfactory results when employed to visualise human insulin to detect islets implanted into the spleens of mice. Both TMB and DAB were used as substrates for horseradish peroxidase, with TMB resulting in blue insulin staining in the control 3 spleen and DAB producing a brown stain in the TZD 1 spleen (figure 64).

Fluorescence staining produced good quality visualisation of human insulin in the remaining spleens, with TRITC resulting in a red stain for insulin in the control 1 spleen and FITC staining insulin green in the control 2 as well as the TZD 2 and 3 spleens (figure 65).

Both methods proved to be useful for detecting human islets implanted into mouse spleens, with clear staining and minimal background or non-specific staining. However, fluorescence staining may lend itself more readily to quantification with the intensity of the fluorescent signal relating to the quantity of antibody-bound ligand (Phillips et al. 1991; Antonini et al. 2000; Lopez et al. 2005). Image analysis software available from Zeiss is compatible with the fluorescence microscopy system currently in use at the IRL.

X-ray microtomography of vascular corrosion casts

The process of x-ray microtomography (XRM) is potentially ideal as it is non-invasive (Cano et al. 2007; Kuhn et al. 2007; Heethoff et al. 2008) and does not require the sample to be destroyed by sectioning as does scanning electron microscopy (SEM) or any form of histological processing.

XRM was able to accurately depict vessels with a diameter as low as 14 μm during a study of the microcirculation in a mouse placenta (Langheinrich et al. 2004), while XRM of a polymer sponge returned structure diameters as low as 12.5 μm (Muller et al. 2002). The smallest vessels detected in the corrosion casts from this study had a diameter of 7 μm, with the possibility of smaller vessels being visualised by XRM in future studies (personal communication with Dr Taghi Miri, Chemical Engimneering Department, University of Birmingham, November 2008), suggesting that XRM would be a suitable means of non-invasive corrosion cast examination.

The purpose of employing x-ray microtomography was to assess its potential as an alternative to SEM; however, until an automated method is developed to quantify the data generated, it would be prudent to use these two methods in conjunction with quantitative fluorescence microscopy to accurately elucidate the effects of TZD islet pre-treatment on post-implant engraftment.

The control 3 and TZD 3 casts were damaged during mounting for x-ray microtomography, unfortunately in the area relating to islet location. However, it was still possible to measure 5 of the largest vessels in the designated area (figure 67) for each of the ten frames selected per cast. Such small areas of damage will have less impact on the outcome of engraftment studies if an automated method can be developed to assess vessels in all of the relevant frames (~200).

To assess if there was an increase in blood supply in response to implant injury or islet ischaemia, the five largest vessels were measured using the CTAn component of the SkyScan software developed for use with x-ray microtomography (Foo et al. 2007). Care was taken to measure diameter as accurately as possible by not measuring vessels obviously in the

horizontal plane. This method of assessment was not quantitative and had limitations as it was not possible to manually score all of the relevant frames (~200) for each cast.

Table 12 shows the size distribution of the vessels measured for the control and TZD casts. The results indicate that the TZD casts generated 73 vessels in the size groups with a vessel diameter in excess of 75μm compared to 51 vessels >75μm in the control casts. Approximately 50% of the vessels in the TZD casts had diameters in excess of 75μm, while the control casts yielded around 33.33% vessels >75μm. This result could point to increased blood flow, via angiogenesis, to the area of injury caused by needle insertion for implantation of islets and the stress signals from ischaemic islets (Linn et al. 2006). While it is again stressed that these results were not quantitative, they do seem to substantiate the quantitative results from VEGF analysis that indicates a considerable increase in the growth factor that drives vascular growth.

Recent technological advances have resulted in a method to quantify X-ray microtomography images in 3D (Jones et al. 2008), supporting the decision to investigate this non-invasive imaging technique.

Conclusions

While the cryopreserved islets used for this study were viable, the robust insulin secretory response to secretagogues was somewhat blunted as neither the SC or the SC TZD group showed significantly increased insulin secretion in response to G+T when compared with insulin secreted in response to 16.7mM glucose. Wherever possible, fresh islets should be used for future studies to ensure the best possible condition prior to treatment and implantation.

The presence of insulin staining in the spleens from both groups indicates that the transplant method was successful; and that the transplant site selected to harvest islets for histological examination was appropriate.

The success of the kidney vascular corrosion casts for both groups indicates that the method developed for corrosion casting and the polymer selection were both suitable. However, due to renal vein rupture, the polymer viscosity will be altered for future studies to resemble that of blood more closely and a syringe pump will be employed to maintain a suitable filling pressure.

Recent advances in x-ray microtomography suggest that this method may be used to quantify data gained from vascular corrosion casts. It is envisaged that quantitative fluorescence microscopy will be used in conjunction with x-ray microtomography to study the revascularisation of human islets maintained in RC and treated with a TZD prior to implantation.

Chapter 7: Overview and conclusions

Introduction

Islet transplantation shows promise as a potential cure for insulin dependent diabetes and may have applications in certain patients with severe or labile non-insulin dependent forms of diabetes. The research performed for this thesis was aimed at certain aspects of the islet transplant process which require improvement. These include efforts to increase the islet yield from donor pancreata (Lakey et al. 2002; Brandhorst et al. 2005; Miki et al. 2008), reducing the beta cell toxicity of immunosuppressants (Laugharne et al. 2007), immunomodulation of islets prior to implantation (Varotto et al. 2006; Giannoukakis et al. 2008), enhancing islet viability pre and post implantation (Hanley et al. 2006; Yamamoto et al. 2008) and improving the rate of islet engraftment post transplant (Del Bo et al. 2006; Johansson et al. 2008). A particular problem is the failure of islets to vascularise promptly after implantation, which is necessary to avoid the detrimental effects of post-implant hypoxia and to re-establish waste removal and nutrient supply. Thus the main purpose of the work for this thesis was to investigate the possibility of developing an *in vitro* islet pre-treatment to ensure the health and viability of both the endocrine islet and its endogenous vasculature to support post-transplant insulin secretory capacity and enhance revascularisation.

Methodology development

Due to a paucity of human tissue for research, particularly after the introduction of new legislation relating to the Human Tissue Act, initial experiments were performed using the BRIN-BD11 beta cell line to represent the insulin secretory fraction of the islet (McClenaghan et al. 1996). Additionally, human umbilical vein endothelial cells (HUVEC) were selected to simulate the vascular component. A rotational cell culture system was employed to provide a microgravity environment for more efficient nutrient and oxygen

distribution, to allow cell-cell contact during culture that encouraged D11 pseudoislet formation and maintenance of human islet structure, to facilitate cell contact with released growth factors such as VEGF, and to enhance functional viability of D11 pseudoislets and human islets (Murray et al. 2005). Furthermore, the addition of the TZD rosiglitazone to culture media was assessed as a potential means to upregulate endothelial VEGF expression, thereby enhancing endothelial proliferation (Biscetti et al. 2008). An additional benefit of TZD employment was the β-cell protective effect of the drug and its ability to increase β-cell granulation (Yang et al. 2008).

Insulin secretion from D11 cells and human islets in both conventional static culture and rotational culture was induced by secretagogues during static incubation experiments. Secreted insulin was detected by ELISA, while insulin expression was determined by immunostaining.

VEGF production and release from HUVEC and islet endothelium was induced by addition of a TZD to the culture medium, which was subsequently assessed for VEGF content by ELISA. VEGF expression in HUVEC and human islets was determined by immunostaining.

Initiation of protocol development to assess human islet revascularisation post-transplant was achieved by intra-splenic and renal subcapsular implantation of human islets into male mice. Detection of human islets in the excised, formalin-fixed mouse spleens was performed by immunostaining, while the kidney vasculature was visualised by vascular corrosion casting and subsequent cast analysis by x-ray microtomography.

The main problems encountered by application of the above methodologies to my investigations included:

- optimising culture conditions to support the viability of rat-derived beta cells and human endothelial cells in both static and rotational culture
- determining appropriate TZD concentration to support beta cell function and enhance endothelial proliferation
- developing a dual staining technique for both enzyme-substrate and fluorescence based immunostaining in order to visualise insulin and CD31 or insulin and VEGF simultaneously in D11/HUVEC co-cultures and human islets
- selection of two appropriate islet implantation sites to allow examination of vasculature and histological detection of human islets
- choice of a novel yet suitable technique to visualise renal/islet vascular morphology post islet implant

Culture conditions were optimised for the co-culture of D11 and HUVEC by assessing HUVEC morphology and D11 morphology and insulin secretory capacity during culture in various combinations of RPMI and EGM tissue culture media as well as varying serum concentrations. The media combination that yielded the best results in both static and rotational culture was a 50:50 ratio of RPMI and EGM with 5.5% foetal bovine serum (see chapter 4 discussion). Endothelial cells are traditionally cultured with 1 – 2% serum (Bishop et al. 1999; Lang et al. 2001), but have been reported to proliferate very well in media containing 10% serum when angiogenic factors such as VEGF are present (Davison et al. 1980; Gospodarowicz and Ill 1980). EGM contains VEGF, fibroblast growth factor, insulin-like growth factor and epidermal growth factor, thus encouraging HUVEC proliferation in the presence of 5.5% FBS. This combination of RPMI and EGM with 5.5% FBS also effectively maintained D11 insulin secretion in response to secretagogues, thus proving it to be a suitable medium for D11 and HUVEC co-culture.

The appropriate TZD concentration to support D11 function and HUVEC proliferation was determined by assessing D11 insulin secretion in response to three secretagogues, and HUVEC ATP content after exposure to a TZD. 10mM TZD significantly enhanced HUVEC proliferation and improved D11 insulin secretion, however, human islet insulin secretion was best supported by 25mM TZD. Studies have shown that supraphysiological doses of TZDs are required to upregulate VEGF expression via the PPARγ pathway, with researchers using concentrations up to 25mM TZD *in vitro* to elicit the desired response (Yamakawa et al. 2000). *In vivo* studies have shown that concentrations of TZDs up to 1500 times the human therapeutic dose were well tolerated (Lewis et al. 2001; Mody et al. 2007; Lewis et al. 2008). A dose response experiment indicated that HUVEC responded best to a 10mM concentration of TZD in terms of intracellular ATP content, while this dose also supported secretagogue-induced D11 insulin secretion. Interestingly, human islet insulin secretion and VEGF production and release were best supported by 25mM TZD. Human β-cells and islet endothelium thus required a higher concentration of TZD than D11 and HUVEC. This was possibly due to fundamental differences in signal handling between human and rat-derived β-cells and between HUVEC and endogenous islet endothelium. For the clinical setting, it would be suggested that islets undergo a 24 hour period of RC pre-treatment with a supraphysiological dose of TZD to initiate accelerated endothelial growth and support islet viability. However, islets would be removed from the high TZD exposure prior to implantation to avoid a possible carry-over effect of high TZD concentrations post-implant, such as detrimental angiogenesis potentially exacerbating conditions such as retinopathy – although both rosiglitazone and troglitazone have been shown to prevent retinopathy in mice at doses of 100 micromol/L (Murata et al. 2001) by inhibition of retinal endothelial cells. A clinically relevant dose of TZD may be administered to the recipient for a period of two to four weeks post implant to support islet revascularisation and function.

An enzyme-substrate based immunostaining protocol was developed by testing a range of antigen retrieval techniques, as well as antibody concentrations and incubation times. The protocol described in chapter 2 reproducibly results in high quality staining for insulin in D11 cells and CD31 in HUVEC, allowing for dual staining of these two antigens with no evidence of non-specific or background staining. This process was repeated to produce a robust fluorescence immunostaining protocol to allow for dual staining of insulin and either VEGF or CD31 in human islets.

The liver, kidney, spleen, pancreas, omental pouch and peritoneum have been explored as potential sites for islet implantation (Merani et al. 2008). As this study required access to the islets at various time points post implant, the liver was ruled out due to the dispersal of islets after release into the portal vein. The pancreas was excluded due to its tendency to develop pancreatitis following injury. While adipose tissue has been shown to release angiogenic factors, the omentum and peritoneum were rejected as implant sites due to poor vascular supply as post-transplant TZD therapy will be a consideration in future studies and vascular corrosion casting would be impractical. The kidney offers a highly vascularised site that has been used with much success and subcapsular implantation would ensure a degree of islet confinement for post-implant study (Mellgren et al. 1986; Hayek and Beattie 1997). However, nephropathy in human diabetes sufferers reduces the likelihood of clinical renal islet implantation, thus a second site was explored during this study. The spleen has been utilised as an islet implantation site, successfully trapping islets for histological examination (Scharp et al. 1992; van der Burg et al. 1996; Hayek and Beattie 1997; Horton et al. 2000; Aoki et al. 2005). However, the relative risk of haemorrhage and the improved access of lymphocytes to transplanted tissue in the spleen would limit its use as a clinical choice for islet transplantation (Merani et al. 2008).

From an immunologic perspective, the ideal site would provide immunoprivilege to protect newly implanted islets from early inflammatory attack. From the surgical viewpoint, the ideal site would allow easy access to minimise potential implantation complications and a transplant site requiring the least number of islets would be advantageous to reduce the need for high transplant volume or for infusions from multiple donors (Merani et al. 2008). Good access of transplanted tissue to oral immunosuppressive drugs and ease of monitoring would also be useful (Merani et al. 2008). The gastric submucosa and subserosa have been investigated as potential islet implantation sites, with both locations generating encouraging results (Sageshima et al. 2001; Tchervenivanov et al. 2002; Caiazzo et al. 2007). The advantages of these sites include ease of access for implant, good vascularisation and ready access to oral therapeutic agents (Merani et al. 2008). However, a full evaluation of metabolic function and morphology, as well as comparison with the portal and other sites, is yet to be completed (Merani et al. 2008). Due to convenient accessibility and monitoring through biopsy, skeletal muscle in the forearm has been investigated in three humans as a potential clinical implantation site (Stegall 1997). However, this site resulted in more leucocytic infiltration than others and biopsies showed that two of the three patients transplanted showed infiltrate consistent with autoimmune disease (Stegall 1997). Thus, for the purposes of continuing the research performed for this thesis, the kidney and spleen would remain a suitable choice of islet implantation site.

Various techniques have been developed to visualise and study vasculature. Certain techniques such as magnetic resonance imaging, contrast enhanced computer tomography and Doppler ultrasonography were unsuitable as access to the required equipment was unavailable. Vascular corrosion casts provide a three dimensional representation of the polymer infused vasculature and have traditionally been examined by scanning electron microscopy. A novel method of corrosion cast analysis was sought for this study to

complement histological examination of implanted islets. Selection criteria for the new technique included accurate, three dimensional and non-invasive imaging of the cast to allow for additional, invasive scanning electron microscopy if required. Thus x-ray microtomography, an emerging technique allowing non-invasive three dimensional reconstruction of x-ray data, was assessed.

Key findings and implications

Cross-species co-culture of rat-derived D11 cells and HUVEC was successful with the morphology of both cells being preserved and the insulin secretory profile of D11 being supported under both static and rotational cell culture conditions. Interestingly, the presence of HUVEC improved D11 insulin secretion in response to high glucose in both static and rotational culture. This phenomenon has been observed in the IRL when cell types other than HUVEC have been co-cultured with D11. The underlying mechanisms are not yet fully understood, but the release of trophic factors (such as hormones, cytokines and growth factors) and the physical contact of the additional non-beta-cells will be investigated as beta cells have been shown to perform more efficiently when arranged into three dimensional clusters (Hauge-Evans et al. 1999; Squires et al. 2000; Hauge-Evans et al. 2002; Squires et al. 2002). The effective distribution of oxygen, nutrients and released trophic factors as well as physical cell-cell contact during RC could all play a part in the support of the islet three dimensional arrangement and formation of D11 pseudoislets by encouraging 'binding and communication' structures such as gap junctions between beta cells and other cell types in co-culture (i.e. HUVEC).

Employment of a rotational cell culture system resulted in D11 pseudoislet formation and improved secretagogue-induced insulin secretion in D11 and human islets. Addition of 10mM TZD increased D11 insulin secretion, although not enough to be statistically significant. The

same trend was seen when 25mM TZD was added to human islet culture, although the increases were significant in several instances. These findings suggest that a period of islet pre-treatment utilising both TZD and rotational culture may benefit the overall health of islets prior to implantation. Also, TZD benefits both β-cell function and islet endothelium by improving β-cell granulation (Buckingham et al. 1998; Campbell and Mariz 2007; Yang et al. 2008) and upregulating VEGF expression by endothelial cells (Yamakawa et al. 2000; Biscetti et al. 2008). The results of the study for this thesis support these published findings. HUVEC responded to 10mM TZD by significantly increasing VEGF production and release into the culture media with a concomitant increase in intracellular ATP, indicating increased proliferation. Human islets also significantly increased VEGF production and release when stimulated with 25mM TZD in static culture, with rotational culture further enhancing VEGF release. Thus, rotational culture together with TZD impacted positively on endothelial health as well as supporting beta cell function, further strengthening the argument for their combined utilisation as a means of *in vitro* priming of islet beta and endothelial cells prior to transplantation.

Cryopreservation impeded islet function, resulting in a blunted insulin secretory response to 16.7mM glucose + 10mM theophylline. Freshly isolated islets respond to this secretagogue by significantly increasing insulin secretion when compared to 16.7mM glucose without 10mM theophylline, suggesting that cryopreservation impacts negatively on the islet insulin secretory machinery (Piemonti et al. 1999; Gatto et al. 2003) by blunting the usual potentiating effect of theophylline on insulin secretion induced by high glucose (Hou et al. 2006). Theophylline acts by increasing intracellular cyclic AMP (Giugliano et al. 1979) which, in turn results in more effective closure of K^+ ATP channels in the cell membrane, allowing prolonged membrane depolarisation and subsequent Ca^{2+} influx with a concomitant

insulin secretion (Yajima et al. 1999). The positive effects of rotational culture and TZD might be usefully employed to restore normal function to these compromised islets.

Human islets were successfully implanted under the kidney capsule and into the spleens of lean male mice, with all six mice recovering well from surgery and thriving for the duration of the study. Histological examination of the formalin-fixed excised spleens resulted in positive staining for human insulin, indicating the presence of human islets and supporting the selection of the spleen as an implant site for the purposes of this study.

Vascular corrosion casts were successfully obtained from the left kidneys of all six mice, validating the development of the corrosion casting technique as a means of depicting mouse renal vasculature. Additionally, x-ray microtomography analysis of the corrosion casts yielded interesting results, allowing the use of dedicated software to determine vessel diameters in the islet implant areas. Although the data obtained were not quantifiable with the current software, recent advances in the field have resulted in quantitative x-ray microtomography development (Jones et al. 2008), suggesting an improved application for corrosion cast analysis. The casts obtained during this study suggested, even by superficial and non-quantitative examination, that renal vasculature may have been enhanced at the implantation site of islets treated with 25mM TZD for 24 hours prior to implantation compared to control islets.

Conclusion

This programme of work has provided evidence for the value of utilising a combination of rotational culture and a thiazolidinedione as an *in vitro* pre-treatment to benefit islet viability, function and revascularisation post transplant. The results of this study form a foundation for

undertaking pre-implant scientific manoeuvres with islets to improve graft performance and clinical transplantation outcomes, and provide a basis for further studies.

Future studies

Future studies could focus on improving islet cryopreservation to minimise functional loss and assess the potential benefit of rotational culture and TZD therapy as a post-thawing recovery strategy.

Employing a syringe pump to regulate polymer infusion pressure and adjusting polymer viscosity to more closely resemble that of blood may offer improvements to vascular corrosion casting of the mouse kidney. Also, by taking advantage of recent technological advances in the field of x-ray microtomography, a sensitive and quantitative method of cast analysis could be developed to provide extensive, non-invasive information about islet engraftment.

Further *in vivo* studies are required to validate a protocol allowing accurate investigation of post transplant islet function and revascularisation. This could be achieved by a program of islet transplants into diabetic mice, with rigorous post-implant monitoring of glucose homeostasis and islet engraftment. Also, the effect of RC and TZD on islet revascularisation may be explored as this preliminary transplant study investigated only SC islets +/- TZD with promising results.

References

Aardema, M. W., B. T. De Wolf, M. C. Saro, H. Oosterhof, V. Fidler and J. G. Aarnoudse (2000). "Quantification of the diastolic notch in Doppler ultrasound screening of uterine arteries." Ultrasound Obstet Gynecol **16**(7): 630-4.

ADA (2008). "Diagnosis and Classification of Diabetes Mellitus." Diabetes Care **31**(Supplement 1): S55 - 60.

Adamec, M., L. Janousek, K. Lipar, F. Hampl, F. Saudek, R. Koznarova, P. Boucek and T. Havrdova (2004). "A prospective comparison of bladder versus enteric drainage in vascularized pancreas transplantation." Transplant Proc **36**(4): 1093-4.

Adams, M. J., T. Blundell, E. Dodson, G. Dodson, M. Vijayan, E. Baker, M. Harding, B. Rimmer and S. Sheat (1969). "Structure of rhombohedral 2-zinc insulin crystals." Nature **224**: 491.

Aharinejad, S., S. C. Marks, Jr., P. Bock, C. A. MacKay, E. K. Larson, A. Tahamtani, A. Mason-Savas and W. Firbas (1995). "Microvascular pattern in the metaphysis during bone growth." Anat Rec **242**(1): 111-22.

Ahren, B. (2008). "Evidence that autonomic mechanisms contribute to the adaptive increase in insulin secretion during dexamethasone-induced insulin resistance in humans." Diabetologia **51**(6): 1018-24.

Alberti, G. (2001). "Noncommunicable diseases: tomorrow's pandemics." Bulletin of the World Health Organization **79**(10): 907.

Alcantara, A. I., M. Morales, E. Delgado, M. I. Lopez-Delgado, F. Clemente, M. A. Luque, W. J. Malaisse, I. Valverde and M. L. Villanueva-Penacarrillo (1997). "Exendin-4 agonist and exendin(9-39)amide antagonist of the GLP-1(7-36)amide effects in liver and muscle." Arch Biochem Biophys **341**(1): 1-7.

Alian, A., A. Eldor, H. Falk and A. Panet (2002). "Viral mediated gene transfer to sprouting blood vessels during angiogenesis." J Virol Methods **105**(1): 1-11.

Allison, J., L. Oxbrow and J. F. Miller (1994). "Consequences of in situ production of IL-2 for islet cell death." Int Immunol **6**(4): 541-9.

Andersson, A. (1976). "Tissue culture of isolated pancreatic islets." Acta Endocrinol Suppl (Copenh) **205**: 283-94.

Andersson, A. (1982). "Reversal of hyperglycemia by intrasplenic transplantation of 4-week-cultured allogeneic mouse islets." Diabetes **31 Suppl 4**: 55-9.

Antonini, J. M., D. R. Hemenway and G. S. Davis (2000). "Quantitative image analysis of lung connective tissue in murine silicosis." Exp Lung Res **26**(2): 71-88.

Aoki, T., H. Hui, Y. Umehara, S. LiCalzi, A. A. Demetriou, J. Rozga and R. Perfettit (2005). "Intrasplenic transplantation of encapsulated genetically engineered mouse insulinoma cells reverses streptozotocin-induced diabetes in rats." Cell Transplant **14**(6): 411-21.

Aronsson, D. E. and C. Muhr (2002). "Quantification of sensitivity of endothelial cell markers for the astrocytoma and oligodendroglioma tumours." Anticancer Res **22**(1A): 343-6.

Aucouturier, J., J. S. Baker and P. Duche (2008). "Fat and carbohydrate metabolism during submaximal exercise in children." Sports Med **38**(3): 213-38.

Bach, J. (1997). "Autoimmunity and Type 1 Diabetes." Trends in Endocrinology and Metabolism **8**: 71 - 74.

Bacha, F., R. Saad, N. Gungor and S. A. Arslanian (2004). "Adiponectin in youth: relationship to visceral adiposity, insulin sensitivity, and beta-cell function." Diabetes Care **27**(2): 547-52.

Bailey, C. J. (1992). "Biguanides and NIDDM." Diabetes Care **15**(6): 755-72.

Bailey, C. J. (1999). "Insulin resistance and antidiabetic drugs." Biochem Pharmacol **58**(10): 1511-20.

Bailey, C. J. (2000). "Potential new treatments for type 2 diabetes." Trends Pharmacol Sci **21**(7): 259-65.

Bailey, C. J. (2005). "Drugs on the horizon for diabesity." Curr Diab Rep **5**(5): 353-9.

Bailey, C. J., A. Bagdonas, J. Rubes, S. O. McMorn, J. Donaldson, N. Biswas and M. W. Stewart (2005). "Rosiglitazone/metformin fixed-dose combination compared with uptitrated metformin alone in type 2 diabetes mellitus: a 24-week, multicenter, randomized, double-blind, parallel-group study." Clin Ther **27**(10): 1548-61.

Bailey, C. J., E. L. Davies and K. Docherty (1999). "Prospects for insulin delivery by ex-vivo somatic cell gene therapy." J Mol Med **77**(1): 244-9.

Bailey, C. J. and C. Day (2001). "Thiazolidinediones today." British Journal Of Diabetes and Vascular Disease **1**: 7 - 13.

Bailey, C. J. and C. Day (2004). "Avandamet: combined metformin-rosiglitazone treatment for insulin resistance in type 2 diabetes." Int J Clin Pract **58**(9): 867-76.

Bailey, C. J. and M. Nattrass (1988). "Treatment--metformin." Baillieres Clin Endocrinol Metab **2**(2): 455-76.

Balamurugan, A. N., Y. Chang, S. Bertera, A. Sands, V. Shankar, M. Trucco and R. Bottino (2006). "Suitability of human juvenile pancreatic islets for clinical use." Diabetologia **49**(8): 1845-54.

Balamurugan, A. N., Y. Chang, J. J. Fung, M. Trucco and R. Bottino (2003). "Flexible management of enzymatic digestion improves human islet isolation outcome from sub-optimal donor pancreata." Am J Transplant **3**(9): 1135-42.

Balasa, B., K. Van Gunst, N. Jung, D. Balakrishna, P. Santamaria, T. Hanafusa, N. Itoh and N. Sarvetnick (2000). "Islet-specific expression of IL-10 promotes diabetes in nonobese diabetic mice independent of Fas, perforin, TNF receptor-1, and TNF receptor-2 molecules." J Immunol **165**(5): 2841-9.

Ballinger, W. F. and P. E. Lacy (1972). "Transplantation of intact pancreatic islets in rats." Surgery **72**(2): 175-86.

Banks, I. G., J. M. Sloan and K. D. Buchanan (1982). "A histological study of intrasplenic transplanted neonatal rat pancreas and of adjacent adipose tissue proliferation." Diabetologia **22**(2): 128-33.

Barker, D. J., C. N. Hales, C. H. Fall, C. Osmond, K. Phipps and P. M. Clark (1993). "Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth." Diabetologia **36**(1): 62-7.

Barnard, R. J., T. Jung and S. B. Inkeles (1994). "Diet and exercise in the treatment of NIDDM. The need for early emphasis." Diabetes Care **17**(12): 1469-72.

Barness, L. A. (2007). "Obesity in children." Fetal Pediatr Pathol **26**(2): 75-85.

Beattie, G. M., A. M. Montgomery, A. D. Lopez, E. Hao, B. Perez, M. L. Just, J. R. Lakey, M. E. Hart and A. Hayek (2002). "A novel approach to increase human islet cell mass while preserving beta-cell function." Diabetes **51**(12): 3435-9.

Beger, C., V. Cirulli, P. Vajkoczy, P. A. Halban and M. D. Menger (1998). "Vascularization of purified pancreatic islet-like cell aggregates (pseudoislets) after syngeneic transplantation." Diabetes **47**(4): 559-65.

Begg, I. S. (1984). "Diabetic retinopathy: a review of general medical factors in patient care." Can J Ophthalmol **19**(4): 159-68.

Bell, D. S. (2003). "Beta-cell rejuvenation with thiazolidinediones." Am J Med **115 Suppl 8A**: 20S-23S.

Benhamou, P. Y., P. C. Watt, Y. Mullen, S. Ingles, Y. Watanabe, Y. Nomura, C. Hober, M. Miyamoto, T. Kenmochi, E. P. Passaro and et al. (1994). "Human islet isolation in 104 consecutive cases. Factors affecting isolation success." Transplantation **57**(12): 1804-10.

Bensley, R. R. (1911). "Studies on the pancreas of the guinea pig." American Journal of Anatomy **12**: 297 - 388.

Biscetti, F., E. Gaetani, A. Flex, T. Aprahamian, T. Hopkins, G. Straface, G. Pecorini, E. Stigliano, R. C. Smith, F. Angelini, J. J. Castellot, Jr. and R. Pola (2008). "Selective activation of peroxisome proliferator-activated receptor (PPAR)alpha and PPAR gamma induces neoangiogenesis through a vascular endothelial growth factor-dependent mechanism." Diabetes **57**(5): 1394-404.

Bishop, E. T., G. T. Bell, S. Bloor, I. J. Broom, N. F. Hendry and D. N. Wheatley (1999). "An in vitro model of angiogenesis: basic features." Angiogenesis **3**(4): 335-44.

Bishop-Bailey, D. (2000). "Peroxisome proliferator-activated receptors in the cardiovascular system." Br J Pharmacol **129**(5): 823-34.

Bleich, D., R. A. Jackson, J. S. Soeldner and G. S. Eisenbarth (1990). "Analysis of metabolic progression to type I diabetes in ICA+ relatives of patients with type I diabetes." Diabetes Care **13**(2): 111-8.

Boker, A., L. Rothenberg, C. Hernandez, N. S. Kenyon, C. Ricordi and R. Alejandro (2001). "Human islet transplantation: update." World J Surg **25**(4): 481-6.

Bolli, G. B. (2002). "Clinical strategies for controlling peaks and valleys: type 1 diabetes." Int J Clin Pract Suppl(129): 65-74.

Bonner-Weir, S. and L. Orci (1982). "New perspectives on the microvasculature of the islets of Langerhans in the rat." Diabetes **31**(10): 883-9.

Botting, J. (1996). "Animal experiments and the production of insulin." Research Defense Society News: 9 - 14.

Bottino, R., L. Inverardi, U. Valente and C. Ricordi (1997). "Serum-free medium and pyruvate improve survival and glucose responsiveness of islet beta cells in culture." Transplant Proc **29**(4): 1978.

Bouwens, L. (2004). "Islet morphogenesis and stem cell markers." Cell Biochem Biophys **40**(3 Suppl): 81-8.

Brandhorst, H., M. D. Brendel, M. Eckhard, R. G. Bretzel and D. Brandhorst (2005). "Influence of neutral protease activity on human islet isolation outcome." Transplant Proc **37**(1): 241-2.

Brange, J., J. F. Hansen, L. Langkjaer, J. Markussen, U. Ribel and A. R. Sorensen (1992). "Insulin analogues with improved absorption characteristics." Horm Metab Res Suppl **26**: 125-30.

Brange, J. and L. Langkjoer (1993). "Insulin structure and stability." Pharm Biotechnol **5**: 315-50.

Brant, A. M., S. McCoid, H. M. Thomas, S. A. Baldwin, A. Davies, J. C. Parker, E. M. Gibbs and G. W. Gould (1992). "Analysis of the glucose transporter content of islet cell lines: implications for glucose-stimulated insulin release." Cell Signal **4**(6): 641-50.

Brasile, L., B. Stubenitsky, M. Booster and G. Kootstra (2001). "The cadaveric kidney and the organ shortage--a perspective review." Clin Transplant **15**(6): 369-74.

Braunstein, S. (2003). "New developments in type 2 diabetes mellitus: combination therapy with a thiazolidinedione." Clin Ther **25**(7): 1895-917.

Breay, P. (2003). Due to the strict selection criteria only 5 - 10% of type 1 diabetes sufferers are currently considered for transplant. P. Breay - Clinical Endocrinologist for the islet transplant program at the University of Alberta, Edmonton, Canada.

Brendel, M. D., S. S. Kong, R. Alejandro and D. H. Mintz (1994). "Improved functional survival of human islets of Langerhans in three-dimensional matrix culture." Cell Transplant **3**(5): 427-35.

Brenner, B. M. and R. Beeuwkes, 3rd (1978). "The renal circulations." Hosp Pract **13**(7): 35-46.

Brereton, H., M. J. Carvell, S. J. Persaud and P. M. Jones (2007). "Islet alpha-cells do not influence insulin secretion from beta-cells through cell-cell contact." Endocrine **31**(1): 61-5.

Bretherton-Watt, D., S. G. Gilbey, M. A. Ghatei, J. Beacham and S. R. Bloom (1990). "Failure to establish islet amyloid polypeptide (amylin) as a circulating beta cell inhibiting hormone in man." Diabetologia **33**(2): 115-7.

Bristow, A. F. (1993). "Recombinant-DNA-derived insulin analogues as potentially useful therapeutic agents." Trends Biotechnol **11**(7): 301-5.

Buckingham, R. E., K. A. Al-Barazanji, C. D. Toseland, M. Slaughter, S. C. Connor, A. West, B. Bond, N. C. Turner and J. C. Clapham (1998). "Peroxisome proliferator-activated receptor-gamma agonist, rosiglitazone, protects against nephropathy and pancreatic islet abnormalities in Zucker fatty rats." Diabetes **47**(8): 1326-34.

Buckley, T. A. (2000). "The shortage of solid organs for transplantation in Hong Kong: part of a worldwide problem." Hong Kong Med J **6**(4): 399-408.

Buitrago, A., E. Gylfe, B. Hellman, L. A. Idahl and M. Johansson (1975). "Function of microdissected pancreatic islets cultured in a chemically defined medium. I. Insulin content and release." Diabetologia **11**(6): 535-40.

Burant, C. F. and D. M. Simeone (2002). "Connecting islet development and developing useful islets." Trends Endocrinol Metab **13**(2): 48-9.

Burger, P. C., D. B. Chandler, D. B. Drysdale, Y. Tano, J. D. Crapo, B. A. Freeman and G. K. Klintworth (1984). "Scanning electron microscopy of vascular casts in experimental ocular vasoproliferation." Scan Electron Microsc(Pt 4): 1893-8.

Burridge, P. W., A. M. Shapiro, E. A. Ryan and J. R. Lakey (2002). "Future trends in clinical islet transplantation." Transplant Proc **34**(8): 3347-8.

Byrne, A. M., D. J. Bouchier-Hayes and J. H. Harmey (2005). "Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF)." J Cell Mol Med **9**(4): 777-94.

Caiazzo, R., V. Gmyr, T. Hubert, N. Delalleau, R. Lamberts, E. Moerman, J. Kerr-Conte and F. Pattou (2007). "Evaluation of alternative sites for islet transplantation in the minipig: interest and limits of the gastric submucosa." Transplant Proc **39**(8): 2620-3.

Caiazzo, R., V. Gmyr, B. Kremer, T. Hubert, B. Soudan, B. Lukowiak, B. Vandewalle, M. C. Vantyghem, F. Pattou and J. Kerr-Conte (2008). "Quantitative in vivo islet potency assay in normoglycemic nude mice correlates with primary graft function after clinical transplantation." Transplantation **86**(2): 360-3.

Calafiore, R. (1997). "Perspectives in pancreatic and islet cell transplantation for the therapy of IDDM." Diabetes Care **20**(5): 889-96.

Cameron, J. L., D. G. Mehigan, D. P. Harrington and G. D. Zuidema (1980). "Metabolic studies following intrahepatic autotransplantation of pancreatic islet grafts." Surgery **87**(4): 397-400.

Campbell, I. W. and S. Mariz (2007). "Beta-cell preservation with thiazolidinediones." Diabetes Res Clin Pract **76**(2): 163-76.

Campbell, R. K., J. R. White, T. Levien and D. Baker (2001). "Insulin glargine." Clin Ther **23**(12): 1938-57; discussion 1923.

Campos, M., J. Amaral, S. P. Becerra and R. N. Fariss (2006). "A novel imaging technique for experimental choroidal neovascularization." Invest Ophthalmol Vis Sci **47**(12): 5163-70.

Cano, J., J. Campo, J. J. Vaquero, J. M. Martinez and A. Bascones (2007). "High resolution image in bone biology I. Review of the literature." Med Oral Patol Oral Cir Bucal **12**(6): E454-8.

Cao, X., Z. Gao, C. E. Robert, S. Greene, G. Xu, W. Xu, E. Bell, D. Campbell, Y. Zhu, R. Young, M. Trucco, J. F. Markmann, A. Naji and B. A. Wolf (2003). "Pancreatic-derived factor (FAM3B), a novel islet cytokine, induces apoptosis of insulin-secreting beta-cells." Diabetes **52**(9): 2296-303.

Carlsson, P. O., L. Jansson, A. Andersson and O. Kallskog (1998). "Capillary blood pressure in syngeneic rat islets transplanted under the renal capsule is similar to that of the implantation organ." Diabetes **47**(10): 1586-93.

Carlsson, P. O., F. Palm and G. Mattsson (2002). "Low revascularization of experimentally transplanted human pancreatic islets." J Clin Endocrinol Metab **87**(12): 5418-23.

Carvell, M., P. Marsh, S. Persaud and P. Jones (2007). "E-cadherin Interactions Regulate beta-Cell Proliferation in Islet-like Structures." Cell Physiol Biochem **20**(5): 617-26.

Cauchi, S., D. Meyre, E. Durand, C. Proenca, M. Marre, S. Hadjadj, H. Choquet, F. De Graeve, S. Gaget, F. Allegaert, J. Delplanque, M. A. Permutt, J. Wasson, I. Blech, G. Charpentier, B. Balkau, A. C. Vergnaud, S. Czernichow, W. Patsch, M. Chikri, B. Glaser, R. Sladek and P. Froguel (2008). "Post genome-wide association studies of novel genes associated with type 2 diabetes show gene-gene interaction and high predictive value." PLoS ONE **3**(5): e2031.

Cavallo, M. G., F. Dotta, L. Monetini, S. Dionisi, M. Previti, L. Valente, A. Toto, U. Di Mario and P. Pozzilli (1996). "Beta-cell markers and autoantigen expression by a human insulinoma cell line: similarities to native beta cells." J Endocrinol **150**(1): 113-20.

Chang, D. S., H. Su, G. L. Tang, L. S. Brevetti, R. Sarkar, R. Wang, Y. W. Kan and L. M. Messina (2003). "Adeno-associated viral vector-mediated gene transfer of VEGF normalizes skeletal muscle oxygen tension and induces arteriogenesis in ischemic rat hindlimb." Mol Ther **7**(1): 44-51.

Chang-Chen, K. J., R. Mullur and E. Bernal-Mizrachi (2008). "beta-cell failure as a complication of diabetes." Rev Endocr Metab Disord **9**(4): 329-43.

Chen, H. and T. Michel (2006). "Insulin signaling in vascular endothelial cells: a key role for heterotrimeric G proteins revealed by siRNA-mediated Gbeta1 knockdown." Biochemistry **45**(26): 8023-33.

Cheng, Q., P. K. Law, M. de Gasparo and P. S. Leung (2008). "Combination of the dipeptidyl peptidase IV inhibitor LAF237 [(S)-1-[(3-hydroxy-1-adamantyl)ammo]acetyl-2-cyanopyrrolidine] with the angiotensin II type 1 receptor antagonist valsartan [N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl]methyl]-L- valine] enhances pancreatic islet morphology and function in a mouse model of type 2 diabetes." J Pharmacol Exp Ther **327**(3): 683-91.

Cheng, Z. D., M. Y. Liu, G. Chen, H. M. Zhang, G. J. Qin, G. Liang and D. X. Liu (2008). "Anti-vascular permeability of the cleaved reactive center loop within the carboxyl-terminal domain of C1 inhibitor." Mol Immunol **45**(6): 1743-51.

Cheng-Lai, A. and A. Levine (2000). "Rosiglitazone: an agent from the thiazolidinedione class for the treatment of type 2 diabetes." Heart Dis **2**(4): 326-33.

Chetty, D. J. and Y. W. Chien (1998). "Novel methods of insulin delivery: an update." Crit Rev Ther Drug Carrier Syst **15**(6): 629-70.

Chikaraishi, Y., N. Matsunaga, M. Shimazawa and H. Hara (2008). "Rifampicin inhibits the retinal neovascularization in vitro and in vivo." Exp Eye Res **86**(1): 131-7.

Chistiakov, D. A. (2008). "The peroxisomal proliferator-activated receptor gamma isoform 2 (PPARgamma2) is an established susceptibility marker for type 2 diabetes (T2D)." Autoimmunity **41**(5): 338-9.

Choi, H. J., I. H. Choi, N. H. Cho and H. K. Choi (2005). "Color image analysis for quantifying renal tumor angiogenesis." Anal Quant Cytol Histol **27**(1): 43-51.

Choi, S. E., K. M. Choi, I. H. Yoon, J. Y. Shin, J. S. Kim, W. Y. Park, D. J. Han, S. C. Kim, C. Ahn, J. Y. Kim, E. S. Hwang, C. Y. Cha, G. L. Szot, K. H. Yoon and C. G. Park (2004). "IL-6 protects pancreatic islet beta cells from pro-inflammatory cytokines-induced cell death and functional impairment in vitro and in vivo." Transpl Immunol **13**(1): 43-53.

Chung, J. W., J. H. Ahn, J. Y. Kim, H. J. Lee, H. H. Kang, Y. K. Lee, J. U. Kim and S. W. Koo (2007). "The effect of isoflurane, halothane and pentobarbital on noise-induced hearing loss in mice." Anesth Analg **104**(6): 1404-8, table of contents.

Clark, A. (1989). "Islet amyloid and type 2 diabetes." Diabet Med **6**(7): 561-7.

Clark, A., C. A. Wells, I. D. Buley, J. K. Cruickshank, R. I. Vanhegan, D. R. Matthews, G. J. Cooper, R. R. Holman and R. C. Turner (1988). "Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes." Diabetes Res **9**(4): 151-9.

Clark, S. A. and W. L. Chick (1990). "Islet cell culture in defined serum-free medium." Endocrinology **126**(4): 1895-903.

Clayton, H., J. Turner, S. Swift, R. James and P. Bell (2001). "Supplementation of islet culture medium with insulin may have a beneficial effect on islet secretory function." Pancreas **22**(1): 72-4.

Clayton, H. A., Y. M. Goward, P. Prevost, S. M. Swift, D. Kimber, H. H. Contractor and N. J. London (1998). "In vitro islet culture: development of a serum-free medium." Transplant Proc **30**(2): 379.

Coccheri, S. (2007). "Approaches to prevention of cardiovascular complications and events in diabetes mellitus." Drugs **67**(7): 997-1026.

Collier, S. A., T. E. Mandel and W. M. Carter (1982). "Detrimental effect of high medium glucose concentration on subsequent endocrine function of transplanted organ-cultured foetal mouse pancreas." Aust J Exp Biol Med Sci **60 Pt 4**: 437-45.

Cooles, P. (1988). "Diabetes and cassava in Dominica." Trop Geogr Med **40**(3): 272-3.

Couper, J. J. and J. B. Prins (2003). "2: Recent advances in therapy of diabetes." Med J Aust **179**(8): 441-7.

Cross, S. E., S. K. Richards, A. Clark, A. V. Benest, D. O. Bates, P. W. Mathieson, P. R. Johnson, S. J. Harper and R. M. Smith (2007). "Vascular endothelial growth factor as a survival factor for human islets: effect of immunosuppressive drugs." Diabetologia **50**(7): 1423-32.

Currie, C. J., D. Kraus, C. L. Morgan, L. Gill, N. C. Stott and J. R. Peters (1997). "NHS acute sector expenditure for diabetes: the present, future, and excess in-patient cost of care." Diabet Med **14**(8): 686-92.

Cuzzocrea, S., B. Pisano, L. Dugo, A. Ianaro, P. Maffia, N. S. Patel, R. Di Paola, A. Ialenti, T. Genovese, P. K. Chatterjee, M. Di Rosa, A. P. Caputi and C. Thiemermann (2004). "Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-gamma, reduces acute inflammation." Eur J Pharmacol **483**(1): 79-93.

Czabanka, M., C. Korherr, U. Brinkmann and P. Vajkoczy (2008). "Influence of TBK-1 on tumor angiogenesis and microvascular inflammation." Front Biosci **13**: 7243-9.

Dandona, P., H. Ghanim, A. Chaudhuri and P. Mohanty (2008). "Thiazolidinediones-improving endothelial function and potential long-term benefits on cardiovascular disease in subjects with type 2 diabetes." J Diabetes Complications **22**(1): 62-75.

Davalli, A. M., Y. Ogawa, L. Scaglia, Y. J. Wu, J. Hollister, S. Bonner-Weir and G. C. Weir (1995). "Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice." Diabetes **44**(1): 104-11.

Davison, P. M., K. Bensch and M. A. Karasek (1980). "Isolation and growth of endothelial cells from the microvessels of the newborn human foreskin in cell culture." J Invest Dermatol **75**(4): 316-21.

de la Tour, D., T. Halvorsen, C. Demeterco, B. Tyrberg, P. Itkin-Ansari, M. Loy, S. J. Yoo, E. Hao, S. Bossie and F. Levine (2001). "Beta-cell differentiation from a human pancreatic cell line in vitro and in vivo." Mol Endocrinol **15**(3): 476-83.

De Vizcaya-Ruiz, A., O. Barbier, R. Ruiz-Ramos and M. E. Cebrian (2008). "Biomarkers of oxidative stress and damage in human populations exposed to arsenic." Mutat Res.

Del Bo, R., M. Scarlato, S. Ghezzi, A. Maestroni, L. Sjolind, C. Forsblom, M. Wessman, P. H. Groop, G. P. Comi, N. Bresolin, L. Luzi and G. Zerbini (2006). "VEGF gene variability and type 1 diabetes: evidence for a protective role." Immunogenetics **58**(2-3): 107-12.

Del Moral, P. M., F. G. Sala, D. Tefft, W. Shi, E. Keshet, S. Bellusci and D. Warburton (2006). "VEGF-A signaling through Flk-1 is a critical facilitator of early embryonic lung epithelial to endothelial crosstalk and branching morphogenesis." Dev Biol **290**(1): 177-88.

Deschamps, I., H. Lestradet, C. Bonaiti, M. Schmid, M. Busson, A. Benajam, A. Marcelli-Barge and J. Hors (1980). "HLA genotype studies in juvenile insulin-dependent diabetes." Diabetologia **19**(3): 189-93.

Dickens, S., P. Vermeulen, B. Hendrickx, S. Van den Berge and J. J. Vranckx (2008). "Regulable vascular endothelial growth factor165 overexpression by ex vivo expanded keratinocyte cultures promotes matrix formation, angiogenesis, and healing in porcine full-thickness wounds." Tissue Eng Part A **14**(1): 19-27.

Dickson, L. M. and C. J. Rhodes (2004). "Pancreatic beta-cell growth and survival in the onset of type 2 diabetes: a role for protein kinase B in the Akt?" Am J Physiol Endocrinol Metab **287**(2): E192-8.

Doggrell, S. A. (2007). "Is exenatide improving the treatment of type 2 diabetes? Analysis of the individual clinical trials with exenatide." Rev Recent Clin Trials **2**(1): 77-84.

Dornhorst, A. (2001). "Insulinotropic meglitinide analogues." Lancet **358**(9294): 1709-16.

Dotta, F., S. Censini, A. G. van Halteren, L. Marselli, M. Masini, S. Dionisi, F. Mosca, U. Boggi, A. O. Muda, S. D. Prato, J. F. Elliott, A. Covacci, R. Rappuoli, B. O. Roep and P. Marchetti (2007). "Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients." Proc Natl Acad Sci U S A **104**(12): 5115-20.

Downing, R., D. W. Scharp, R. C. Merrell, M. Greider and W. F. Ballinger (1979). "Venous vs ductal distention of the canine pancreas for isolation of islets." Surg Forum **30**: 422-4.

Drevs, J., R. Muller-Driver, C. Wittig, S. Fuxius, N. Esser, H. Hugenschmidt, M. A. Konerding, P. R. Allegrini, J. Wood, J. Hennig, C. Unger and D. Marme (2002). "PTK787/ZK 222584, a specific vascular endothelial growth factor-receptor tyrosine kinase inhibitor, affects the anatomy of the tumor vascular bed and the functional vascular properties as detected by dynamic enhanced magnetic resonance imaging." Cancer Res **62**(14): 4015-22.

Du, C. Y. and E. K. Xu (2006). "Protective effect of glucocorticoid-free immunosuppressive regimen in allogenic islet transplantation." Hepatobiliary Pancreat Dis Int **5**(1): 43-7.

Dubernard, J. M., J. Traeger, P. Neyra, J. L. Touraine, D. Tranchant and N. Blanc-Brunat (1978). "A new method of preparation of segmental pancreatic grafts for transplantation: trials in dogs and in man." Surgery **84**(5): 633-9.

Dubois, M., F. Pattou, J. Kerr-Conte, V. Gmyr, B. Vandewalle, P. Desreumaux, J. Auwerx, K. Schoonjans and J. Lefebvre (2000). "Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in normal human pancreatic islet cells." Diabetologia **43**(9): 1165-9.

Duhault, J. and R. Lavielle (1991). "History and evolution of the concept of oral therapy in diabetes." Diabetes Res Clin Pract **14 Suppl 2**: S9-13.

Dupre, J., M. C. Champion and N. W. Rodger (1982). "Replacement treatment with insulin in diabetes mellitus: problems and promise." Clin Invest Med **5**(2-3): 109-19.

Eddlestone, G. T., B. Ribalet and S. Ciani (1989). "Comparative study of K channel behavior in beta cell lines with different secretory responses to glucose." J Membr Biol **109**(2): 123-34.

Edelman, S. V. (1998). "Type II diabetes mellitus." Adv Intern Med **43**: 449-500.

Edwards, C. M., S. A. Stanley, R. Davis, A. E. Brynes, G. S. Frost, L. J. Seal, M. A. Ghatei and S. R. Bloom (2001). "Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers." Am J Physiol Endocrinol Metab **281**(1): E155-61.

Efrat, S., M. Leiser, M. Surana, M. Tal, D. Fusco-Demane and N. Fleischer (1993). "Murine insulinoma cell line with normal glucose-regulated insulin secretion." Diabetes **42**(6): 901-7.

Efrat, S., S. Linde, H. Kofod, D. Spector, M. Delannoy, S. Grant, D. Hanahan and S. Baekkeskov (1988). "Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene." Proc Natl Acad Sci U S A **85**(23): 9037-41.

Ehrmann, D. A., P. E. Schwarz, M. Hara, X. Tang, Y. Horikawa, J. Imperial, G. I. Bell and N. J. Cox (2002). "Relationship of calpain-10 genotype to phenotypic features of polycystic ovary syndrome." J Clin Endocrinol Metab **87**(4): 1669-73.

Eisenbarth, G. S. (1986). "Type I diabetes mellitus. A chronic autoimmune disease." N Engl J Med **314**(21): 1360-8.

Eizirik, D. L. (1988). "Interleukin-1 induced impairment in pancreatic islet oxidative metabolism of glucose is potentiated by tumor necrosis factor." Acta Endocrinol (Copenh) **119**(3): 321-5.

Emoto, M., T. Anno, Y. Sato, K. Tanabe, S. Okuya, Y. Tanizawa, A. Matsutani and Y. Oka (2001). "Troglitazone treatment increases plasma vascular endothelial growth factor in diabetic patients and its mRNA in 3T3-L1 adipocytes." Diabetes **50**(5): 1166-70.

Engerman, R., J. M. Bloodworth, Jr. and S. Nelson (1977). "Relationship of microvascular disease in diabetes to metabolic control." Diabetes **26**(8): 760-9.

Erdmann, E. (2006). "Worldwide spread of lifestyle diseases and its importance for cardiovascular events." Drugs Today (Barc) **42 Suppl C**: 5-8.

Fahrenbach, W. H., D. R. Bacon, J. C. Morrison and E. M. Van Buskirk (1988). "Controlled vascular corrosion casting of the rabbit eye." J Electron Microsc Tech **10**(1): 15-26.

Falorni, A., G. Basta, F. Santeusanio, P. Brunetti and R. Calafiore (1996). "Culture maintenance of isolated adult porcine pancreatic islets in three-dimensional gel matrices: morphologic and functional results." Pancreas **12**(3): 221-9.

Faustman, D., P. E. Lacy and J. M. Davie (1982). "Transplantation without immunosuppression." Diabetes **31 Suppl 4**: 11-6.

Federlin, K., M. Slijepcevic and K. Helmke (1976). "Islet transplantation in experimental diabetes of the rat. IV. The influence of transplantation site and of histocompatibility on islet function." Horm Metab Res **8**(2): 97-101.

Feldman, S. D., G. E. Hirshberg, G. Dodi, M. E. Raizman, D. W. Scharp, W. F. Ballinger and P. E. Lacy (1977). "Intrasplenic islet isografts." Surgery **82**(3): 386-94.

Ferrara, N. (1999). "Role of vascular endothelial growth factor in the regulation of angiogenesis." Kidney Int **56**(3): 794-814.

Ferrara, N. (2000). "VEGF: an update on biological and therapeutic aspects." Curr Opin Biotechnol **11**(6): 617-24.

Finch, D. R., P. H. Wise and P. J. Morris (1977). "Successful intra-splenic transplantation of syngeneic and allogeneic isolated pancreatic islets." Diabetologia **13**(3): 195-9.

Flatt, P. R., C. J. Bailey and B. D. Green (2008). "Dipeptidyl peptidase IV (DPP IV) and related molecules in type 2 diabetes." Front Biosci **13**: 3648-60.

Foo, M., A. Jones and M. A. Darendeliler (2007). "Physical properties of root cementum: Part 9. Effect of systemic fluoride intake on root resorption in rats." Am J Orthod Dentofacial Orthop **131**(1): 34-43.

Gaber, A. O. and D. Fraga (2004). "Advances in long-term islet culture: the memphis experience." Cell Biochem Biophys **40**(3 Suppl): 49-54.

Gambarota, G., W. Leenders, C. Maass, P. Wesseling, B. van der Kogel, O. van Tellingen and A. Heerschap (2008). "Characterisation of tumour vasculature in mouse brain by USPIO contrast-enhanced MRI." Br J Cancer **98**(11): 1784-9.

Gannon, M. C. and F. Q. Nuttall (2006). "Control of blood glucose in type 2 diabetes without weight loss by modification of diet composition." Nutr Metab (Lond) **3**: 16.

Garcia, J. G., J. W. Fenton, 2nd and V. Natarajan (1992). "Thrombin stimulation of human endothelial cell phospholipase D activity. Regulation by phospholipase C, protein kinase C, and cyclic adenosine 3'5'-monophosphate." Blood **79**(8): 2056-67.

Garvey, W. T. (1992). "Glucose transport and NIDDM." Diabetes Care **15**(3): 396-417.

Gatto, C., M. Callegari, M. Folin, M. Conconi, A. Paolin, G. Di Falco, S. Bredariol, R. Spinazzi, P. P. Parnigotto and G. G. Nussdorfer (2003). "Effects of cryopreservation and coculture with pancreatic ductal epithelial cells on insulin secretion from human pancreatic islets." Int J Mol Med **12**(6): 851-4.

Geraghty, J. G., M. Nsubuga, W. J. Angerson, N. N. Williams, A. A. Sarazen, P. A. Dervan and J. M. Fitzpatrick (1992). "A study of regional distribution of renal blood flow using quantitative autoradiography." Am J Physiol **263**(5 Pt 2): F958-62.

Gerich, J. E. (1986). "Insulin-dependent diabetes mellitus: pathophysiology." Mayo Clin Proc **61**(10): 787-91.

Ghatei, M. A., H. K. Datta, M. Zaidi, D. Bretherton-Watt, S. J. Wimalawansa, I. MacIntyre and S. R. Bloom (1990). "Amylin and amylin-amide lack an acute effect on blood glucose and insulin." J Endocrinol **124**(2): R9-11.

Giannoukakis, N., B. Phillips and M. Trucco (2008). "Toward a cure for type 1 diabetes mellitus: diabetes-suppressive dendritic cells and beyond." Pediatr Diabetes **9**(3 Pt 2): 4-13.

Gilligan, A., L. Jewett, D. Simon, I. Damjanov, F. M. Matschinsky, H. Weik, C. Pinkert and B. B. Knowles (1989). "Functional pancreatic beta-cell line from SV40 T-antigen transgenic mouse." Diabetes **38**(8): 1056-62.

Giugliano, D., A. Ceriello and G. Paolisso (1996). "Oxidative stress and diabetic vascular complications." Diabetes Care **19**(3): 257-67.

Giugliano, D., R. Torella, N. Passariello and S. Sgambato (1979). "Somatostatin and insulin secretion in man. II. The effect of theophylline." Acta Diabetol Lat **16**(4): 353-8.

Goke, R., H. C. Fehmann and B. Goke (1991). "Glucagon-like peptide-1(7-36) amide is a new incretin/enterogastrone candidate." Eur J Clin Invest **21**(2): 135-44.

Gorden, D. L., S. J. Mandriota, R. Montesano, L. Orci and M. S. Pepper (1997). "Vascular endothelial growth factor is increased in devascularized rat islets of Langerhans in vitro." Transplantation **63**(3): 436-43.

Gospodarowicz, D. and C. Ill (1980). "Extracellular matrix and control of proliferation of vascular endothelial cells." J Clin Invest **65**(6): 1351-64.

Grarup, N., C. S. Rose, E. A. Andersson, G. Andersen, A. L. Nielsen, A. Albrechtsen, J. O. Clausen, S. S. Rasmussen, T. Jorgensen, A. Sandbaek, T. Lauritzen, O. Schmitz, T. Hansen and O. Pedersen (2007). "Studies of association of variants near the HHEX, CDKN2A/B, and IGF2BP2 genes with type 2 diabetes and impaired insulin release in 10,705 Danish subjects: validation and extension of genome-wide association studies." Diabetes **56**(12): 3105-11.

Gray, B. N. and E. Watkins, Jr. (1976). "Prevention of vascular complications of diabetes by pancreatic islet transplantation." Arch Surg **111**(3): 254-7.

Gray, D. W., P. McShane, A. Grant and P. J. Morris (1984). "A method for isolation of islets of Langerhans from the human pancreas." Diabetes **33**(11): 1055-61.

Grizzi, F., P. Colombo, B. Barbieri, B. Franceschini, M. Roncalli, M. Chiriva-Internati, P. C. Muzzio and N. Dioguardi (2001). "Correspondence re: E. Sabo et al., Microscopic Analysis and Significance of Vascular Architectural Complexity in Renal Cell Carcinoma. Clin. Cancer Res., 7: 533-537, 2001." Clin Cancer Res **7**(10): 3305-7.

Groves, C. J., E. Zeggini, J. Minton, T. M. Frayling, M. N. Weedon, N. W. Rayner, G. A. Hitman, M. Walker, S. Wiltshire, A. T. Hattersley and M. I. McCarthy (2006). "Association analysis of 6,736 U.K. subjects provides replication and confirms TCF7L2 as a type 2 diabetes susceptibility gene with a substantial effect on individual risk." Diabetes **55**(9): 2640-4.

Gunduz, K. and S. J. Bakri (2007). "Management of proliferative diabetic retinopathy." Compr Ophthalmol Update **8**(5): 245-56.

Guven, S. and J. Kuenzi (1998). Diabetes Mellitus. Pathophysiology: Concepts of Altered Health States. New York, Lippencott-Raven: 805 - 830.

Gylfe, E. (1977). "Specific glucose protection of pancreatic beta-cell function during culture in chemically defined medium." Endocrinology **101**(4): 1281-5.

Halbron, M., S. Jacqueminet, C. Sachon, F. Bosquet, A. Hartemann-Heurtier and A. Grimaldi (2007). "Insulin therapy for type 2 diabetes: premixed or basal-prandial?" Diabetes Metab **33**(4): 316-20.

Hales, C. N. and D. J. Barker (1992). "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis." Diabetologia **35**(7): 595-601.

Hales, C. N., D. J. Barker, P. M. Clark, L. J. Cox, C. Fall, C. Osmond and P. D. Winter (1991). "Fetal and infant growth and impaired glucose tolerance at age 64." Bmj **303**(6809): 1019-22.

Hanley, S., S. Liu, M. Lipsett, M. Castellarin, L. Rosenberg, J. Tchervenkov and S. Paraskevas (2006). "Tumor necrosis factor-alpha production by human islets leads to postisolation cell death." Transplantation **82**(6): 813-8.

Harris MI, Z. P. (1997). Classification of Diabetes Mellitus and Other Categories of Glucose Intolerance, John Wiley and Sons Ltd.

Hassan, K., L. M. Rodriguez, S. E. Johnson, S. Tadlock and R. A. Heptulla (2008). "A randomized, controlled trial comparing twice-a-day insulin glargine mixed with rapid-acting insulin analogs versus standard neutral protamine Hagedorn (NPH) therapy in newly diagnosed type 1 diabetes." Pediatrics **121**(3): e466-72.

Hauge-Evans, A. C., P. E. Squires, V. D. Belin, H. Roderigo-Milne, R. D. Ramracheya, S. J. Persaud and P. M. Jones (2002). "Role of adenine nucleotides in insulin secretion from MIN6 pseudoislets." Mol Cell Endocrinol **191**(2): 167-76.

Hauge-Evans, A. C., P. E. Squires, S. J. Persaud and P. M. Jones (1999). "Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets." Diabetes **48**(7): 1402-8.

Haverkos, H. W., N. Battula, D. P. Drotman and O. M. Rennert (2003). "Enteroviruses and type 1 diabetes mellitus." Biomed Pharmacother **57**(9): 379-85.

Hayashi, H., K. Inoue, T. Aung, T. Tun, G. Yuanjun, W. Wenjing, S. Shinohara, H. Kaji, R. Doi, H. Setoyama, M. Kato, M. Imamura, S. Maetani, N. Morikawa, H. Iwata, Y. Ikada and J. Miyazaki (1996). "Application of a novel B cell line MIN6 to a mesh-reinforced polyvinyl alcohol hydrogel tube and three-layer agarose microcapsules: an in vitro study." Cell Transplant **5**(5 Suppl 1): S65-9.

Hayashi, T., H. Iwata and T. Onodera (1999). "Role of interleukin-2 in pancreatic islet-cell destruction in reovirus type 2-infected DBA/1 suckling mice." J Comp Pathol **120**(3): 313-20.

Hayek, A. and G. M. Beattie (1997). "Experimental transplantation of human fetal and adult pancreatic islets." J Clin Endocrinol Metab **82**(8): 2471-5.

Heald, K. A., T. R. Jay and R. Downing (2000). "A simple method of human islet isolation." Diabetic Medicine **17(suppl 1)**: 123-124.

Hedeskov, C. J. (1980). "Mechanism of glucose-induced insulin secretion." Physiol Rev **60**(2): 442-509.

Heethoff, M., L. Helfen and P. Cloetens (2008). "Non-invasive 3D-visualization with sub-micron resolution using synchrotron-X-ray-tomography." J Vis Exp(15).

Heise, T. and L. Heinemann (2001). "Rapid and long-acting analogues as an approach to improve insulin therapy: an evidence-based medicine assessment." Curr Pharm Des **7**(14): 1303-25.

Hellerstrom, C. (1964). "A method for the microdissection of intact pancreatic islets of mammals." Acta Endocrinologica **45**: 122 - 322.

Hellman, B., H. Abrahamsson, T. Andersson, P. O. Berggren, P. Flatt, E. Gylfe and H. J. Hahn (1980). "Calcium movements in relation to glucose-stimulated insulin secretion." Horm Metab Res Suppl **Suppl 10**: 122-30.

Henderson, J. R. and M. C. Moss (1985). "A morphometric study of the endocrine and exocrine capillaries of the pancreas." Q J Exp Physiol **70**(3): 347-56.

Heuser, M., B. Wolf, B. Vollmar and M. D. Menger (2000). "Exocrine contamination of isolated islets of Langerhans deteriorates the process of revascularization after free transplantation." Transplantation **69**(5): 756-61.

Hirsch, I. B. (1999). "Type 1 diabetes mellitus and the use of flexible insulin regimens." Am Fam Physician **60**(8): 2343-52, 2355-6.

Hirshberg, B., S. Mog, N. Patterson, J. Leconte and D. M. Harlan (2002). "Histopathological study of intrahepatic islets transplanted in the nonhuman primate model using edmonton protocol immunosuppression." J Clin Endocrinol Metab **87**(12): 5424-9.

Hoang, B. H., J. P. Dyke, J. A. Koutcher, A. G. Huvos, H. Mizobuchi, B. A. Mazza, R. Gorlick and J. H. Healey (2004). "VEGF expression in osteosarcoma correlates with vascular permeability by dynamic MRI." Clin Orthop Relat Res(426): 32-8.

Hoffman, A. and E. Ziv (1997). "Pharmacokinetic considerations of new insulin formulations and routes of administration." Clin Pharmacokinet **33**(4): 285-301.

Holmkvist, J., P. Almgren, V. Lyssenko, C. M. Lindgren, K. F. Eriksson, B. Isomaa, T. Tuomi, P. Nilsson and L. Groop (2008). "Common variants in maturity-onset diabetes of the young genes and future risk of type 2 diabetes." Diabetes **57**(6): 1738-44.

Holst, J. J., T. Vilsboll and C. F. Deacon (2008). "The incretin system and its role in type 2 diabetes mellitus." Mol Cell Endocrinol.

Hopkins, S. P., J. P. Bulgrin, R. L. Sims, B. Bowman, D. L. Donovan and S. P. Schmidt (1998). "Controlled delivery of vascular endothelial growth factor promotes neovascularization and maintains limb function in a rabbit model of ischemia." J Vasc Surg **27**(5): 886-94; discussion 895.

Hoppener, J. W. and C. J. Lips (2006). "Role of islet amyloid in type 2 diabetes mellitus." Int J Biochem Cell Biol **38**(5-6): 726-36.

Hoppener, J. W., M. G. Nieuwenhuis, T. M. Vroom, B. Ahren and C. J. Lips (2002). "Role of islet amyloid in type 2 diabetes mellitus: consequence or cause?" Mol Cell Endocrinol **197**(1-2): 205-12.

Horaguchi, A. and R. C. Merrell (1981). "Preparation of viable islet cells from dogs by a new method." Diabetes **30**(5): 455-8.

Horikawa, Y., N. Oda, N. J. Cox, X. Li, M. Orho-Melander, M. Hara, Y. Hinokio, T. H. Lindner, H. Mashima, P. E. Schwarz, L. del Bosque-Plata, Y. Oda, I. Yoshiuchi, S.

Colilla, K. S. Polonsky, S. Wei, P. Concannon, N. Iwasaki, J. Schulze, L. J. Baier, C. Bogardus, L. Groop, E. Boerwinkle, C. L. Hanis and G. I. Bell (2000). "Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus." Nat Genet **26**(2): 163-75.

Horton, P. J., W. J. Hawthorne, S. N. Walters, A. T. Patel, P. J. O'Connell, J. R. Chapman and R. D. Allen (2000). "Induction of allogeneic islet tolerance in a large-animal model." Cell Transplant **9**(6): 877-87.

Hossler, F. E., J. E. Douglas and L. E. Douglas (1986). "Anatomy and morphometry of myocardial capillaries studied with vascular corrosion casting and scanning electron microscopy: a method for rat heart." Scan Electron Microsc(Pt 4): 1469-75.

Hou, J., H. L. Xiang, X. S. Feng, X. H. Tian, X. L. Pang and Y. Teng (2006). "[Co-culture of cryopreserved rat islets with small intestinal submucosa]." Nan Fang Yi Ke Da Xue Xue Bao **26**(8): 1121-3.

Houck, K. A., D. W. Leung, A. M. Rowland, J. Winer and N. Ferrara (1992). "Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms." J Biol Chem **267**(36): 26031-7.

Howard, C. F., Jr. (1986). "Longitudinal studies on the development of diabetes in individual Macaca nigra." Diabetologia **29**(5): 301-6.

Hsu, B. R., S. H. Fu, K. W. Ku, T. Y. Yang, J. H. Juang and S. T. Chen (2005). "Enhancing islet engraftment with rosiglitazone." Transplant Proc **37**(1): 245-7.

Huang, P. H., M. Sata, H. Nishimatsu, M. Sumi, Y. Hirata and R. Nagai (2008). "Pioglitazone ameliorates endothelial dysfunction and restores ischemia-induced angiogenesis in diabetic mice." Biomed Pharmacother **62**(1): 46-52.

Huang, S. F., R. F. Chang, W. K. Moon, Y. H. Lee, D. R. Chen and J. S. Suri (2008). "Analysis of tumor vascularity using three-dimensional power Doppler ultrasound images." IEEE Trans Med Imaging **27**(3): 320-30.

Huff, K. K., C. Knabbe, R. Lindsey, D. Kaufman, D. Bronzert, M. E. Lippman and R. B. Dickson (1988). "Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells." Mol Endocrinol **2**(3): 200-8.

Hummel, R., M. Langer, H. H. Wolters, N. Senninger and J. G. Brockmann (2008). "Exocrine drainage into the duodenum: a novel technique for pancreas transplantation." Transpl Int **21**(2): 178-81.

Ido, Y., D. Carling and N. Ruderman (2002). "Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation." Diabetes **51**(1): 159-67.

Idris, I., S. Gray and R. Donnelly (2003). "Rosiglitazone and pulmonary oedema: an acute dose-dependent effect on human endothelial cell permeability." Diabetologia **46**(2): 288-90.

Iwasaki, N., Y. Horikawa, T. Tsuchiya, Y. Kitamura, T. Nakamura, Y. Tanizawa, Y. Oka, K. Hara, T. Kadowaki, T. Awata, M. Honda, K. Yamashita, N. Oda, L. Yu, N. Yamada, M. Ogata, N. Kamatani, Y. Iwamoto, L. Del Bosque-Plata, M. G. Hayes, N. J. Cox and G. I. Bell (2005). "Genetic variants in the calpain-10 gene and the development of type 2 diabetes in the Japanese population." J Hum Genet **50**(2): 92-8.

Jabs, N., I. Franklin, M. B. Brenner, J. Gromada, N. Ferrara, C. B. Wollheim and E. Lammert (2008). "Reduced insulin secretion and content in VEGF-a deficient mouse pancreatic islets." Exp Clin Endocrinol Diabetes **116 Suppl 1**: S46-9.

Jaeger, C., M. Wohrle, K. Federlin and R. G. Bretzel (1995). "Pancreatic islet xenografts at two different transplantation sites (renal subcapsular versus intraportal): comparison of graft survival and morphology." Exp Clin Endocrinol Diabetes **103 Suppl 2**: 123-8.

Janney, C. G., P. E. Lacy, E. H. Finke and J. M. Davie (1982). "Prolongation of intrasplenic islet xenograft survival." Am J Pathol **107**(1): 1-5.

Jaquet, D., J. Leger, C. Levy-Marchal and P. Czernichow (2003). "Low birth weight: effect on insulin sensitivity and lipid metabolism." Horm Res **59**(1): 1-6.

Jay, T. R., M. B. Paget, K. A. Heald and R. Downing (2004). "Are organ preservation solutions useful for the storage of isolated human islets?" Transplant Proc **36**(4): 1130-2.

Ji, J. D., H. Cheon, J. B. Jun, S. J. Choi, Y. R. Kim, Y. H. Lee, T. H. Kim, I. J. Chae, G. G. Song, D. H. Yoo, S. Y. Kim and J. Sohn (2001). "Effects of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) on the expression of inflammatory cytokines and apoptosis induction in rheumatoid synovial fibroblasts and monocytes." J Autoimmun **17**(3): 215-21.

Jiang, C., A. T. Ting and B. Seed (1998). "PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines." Nature **391**(6662): 82-6.

Jinzaki, M. and S. Kuribayashi (2007). "Dynamic contrast-enhanced CT of renal cell carcinoma for evaluation of tumor vascularity: analysis of single-phase or multiphase scanning." AJR Am J Roentgenol **188**(6): W569; author reply W570.

Johansson, M., J. Olerud, L. Jansson and P. O. Carlsson (2008). "Prolactin Treatment Improves Engraftment and Function of Transplanted Pancreatic Islets." Endocrinology.

Johnson, K. H., T. D. O'Brien, C. Betsholtz and P. Westermark (1989). "Islet amyloid, islet-amyloid polypeptide, and diabetes mellitus." N Engl J Med **321**(8): 513-8.

Jones, A. C., C. H. Arns, A. P. Sheppard, D. W. Hutmacher, B. K. Milthorpe and M. A. Knackstedt (2007). "Assessment of bone ingrowth into porous biomaterials using MICRO-CT." Biomaterials **28**(15): 2491-504.

Jones, J. R., R. C. Atwood, G. Poologasundarampillai, S. Yue and P. D. Lee (2008). "Quantifying the 3D macrostructure of tissue scaffolds." J Mater Sci Mater Med.

Jozkowicz, A., J. Dulak, E. Piatkowska, W. Placha and A. Dembinska-Kiec (2000). "Ligands of peroxisome proliferator-activated receptor-gamma increase the generation of vascular endothelial growth factor in vascular smooth muscle cells and in macrophages." Acta Biochim Pol **47**(4): 1147-57.

Kanazawa, H. (2007). "VEGF, angiopoietin-1 and -2 in bronchial asthma: new molecular targets in airway angiogenesis and microvascular remodeling." Recent Pat Inflamm Allergy Drug Discov **1**(1): 1-8.

Kapur, S., C. A. Bonham, S. F. Dodson, I. Dvorchik and R. J. Corry (1999). "Strategies to expand the donor pool for pancreas transplantation." Transplantation **67**(2): 284-90.

Karl, R. C., W. S. Zawalich, J. A. Ferrendelli and F. M. Matschinsky (1975). "The role of Ca-2+ and cyclic adenosine 3':5'-monophosphate in insulin release induced in vitro by the divalent cation ionophore A23187." J Biol Chem **250**(12): 4575-9.

Karlsson, E., N. Shaat and L. Groop (2008). "Can complement factors 5 and 8 and transthyretin be used as biomarkers for MODY 1 (HNF4A-MODY) and MODY 3 (HNF1A-MODY)?" Diabet Med **25**(7): 788-91.

Kaufman, F. R. and S. Schantz (2007). "Current clinical research on type 2 diabetes and its prevention in youth." School Nurse News **24**(3): 13-6.

Kawakami, Y., K. Inoue, H. Hayashi, W. J. Wang, H. Setoyama, Y. J. Gu, M. Imamura, H. Iwata, Y. Ikada, M. Nozawa and J. Miyazaki (1997). "Subcutaneous xenotransplantation of hybrid artificial pancreas encapsulating pancreatic B cell line (MIN6): functional and histological study." Cell Transplant **6**(5): 541-5.

Kearney, M. T., E. R. Duncan, M. Kahn and S. B. Wheatcroft (2008). "Insulin resistance and endothelial cell dysfunction: studies in mammalian models." Exp Physiol **93**(1): 158-63.

Keen, H., R. Sells and R. J. Jarrett (1965). "A method for the study of the metabolism of isolated mammalian islets of Langerhans and some preliminary results." Diabetalogia **1**: 28 - 32.

Kelly, W. D., R. C. Lillehei, F. K. Merkel, Y. Idezuki and F. C. Goetz (1967). "Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy." Surgery **61**(6): 827-37.

Kemp, C. B., M. J. Knight, D. W. Scharp, W. F. Ballinger and P. E. Lacy (1973). "Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats." Diabetologia **9**(6): 486-91.

Keymeulen, B. (2008). "Therapies aimed at preservation or restoration of beta cell function in type 1 diabetes." Verh K Acad Geneeskd Belg **70**(2): 85-103.

Kidszun, A., D. Schneider, D. Erb, G. Hertl, V. Schmidt, M. Eckhard, K. T. Preissner, G. Breier, R. G. Bretzel and T. Linn (2006). "Isolated pancreatic islets in three-dimensional matrices are responsive to stimulators and inhibitors of angiogenesis." Cell Transplant **15**(6): 489-97.

Kloppel, G., M. Lohr, K. Habich, M. Oberholzer and P. U. Heitz (1985). "Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited." Surv Synth Pathol Res **4**(2): 110-25.

Knaack, D., D. M. Fiore, M. Surana, M. Leiser, M. Laurance, D. Fusco-DeMane, O. D. Hegre, N. Fleischer and S. Efrat (1994). "Clonal insulinoma cell line that stably maintains correct glucose responsiveness." Diabetes **43**(12): 1413-7.

Kondo, T. and C. R. Kahn (2004). "Altered insulin signaling in retinal tissue in diabetic states." J Biol Chem **279**(36): 37997-8006.

Kong, H. L., D. Hecht, W. Song, I. Kovesdi, N. R. Hackett, A. Yayon and R. G. Crystal (1998). "Regional suppression of tumor growth by in vivo transfer of a cDNA

encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor." Hum Gene Ther **9**(6): 823-33.

Konstantinova, I. and E. Lammert (2004). "Microvascular development: learning from pancreatic islets." Bioessays **26**(10): 1069-75.

Kooptiwut, S., N. Semprasert and S. Chearskul (2007). "Estrogen increases glucose-induced insulin secretion from mouse pancreatic islets cultured in a prolonged high glucose condition." J Med Assoc Thai **90**(5): 956-61.

Kostadinova, R., W. Wahli and L. Michalik (2005). "PPARs in diseases: control mechanisms of inflammation." Curr Med Chem **12**(25): 2995-3009.

Krach, K., K. Badenhoop and R. R. Tonjes (2003). "The IDDM-associated solitary retroviral promoters DQ-LTR3 and DQ-LTR13 have a distinct impact on the expression of selected DQB1 genes in different cell lines in vitro." Immunogenetics **55**(8): 521-9.

Krentz, A. J. and C. J. Bailey (2005). "Oral antidiabetic agents: current role in type 2 diabetes mellitus." Drugs **65**(3): 385-411.

Krentz, A. J., M. B. Patel and C. J. Bailey (2008). "New Drugs for Type 2 Diabetes Mellitus : What is their Place in Therapy?" Drugs **68**(15): 2131-62.

Kruger, D. F., C. L. Martin and C. E. Sadler (2006). "New insights into glucose regulation." Diabetes Educ **32**(2): 221-8.

Kuhn, G., M. Schultz, R. Muller and F. J. Ruhli (2007). "Diagnostic value of micro-CT in comparison with histology in the qualitative assessment of historical human postcranial bone pathologies." Homo **58**(2): 97-115.

Kulmala, P. (2003). "Prediabetes in children: natural history, diagnosis, and preventive strategies." Paediatr Drugs **5**(4): 211-21.

Kuroda, M., T. Oka, Y. Oka, T. Yamochi, K. Ohtsubo, S. Mori, T. Watanabe, R. Machinami and S. Ohnishi (1995). "Colocalization of vascular endothelial growth factor (vascular

permeability factor) and insulin in pancreatic islet cells." J Clin Endocrinol Metab **80**(11): 3196-200.

Kwan, T., D. Benovoy, C. Dias, S. Gurd, C. Provencher, P. Beaulieu, T. J. Hudson, R. Sladek and J. Majewski (2008). "Genome-wide analysis of transcript isoform variation in humans." Nat Genet **40**(2): 225-31.

Kwan, T., D. Benovoy, C. Dias, S. Gurd, D. Serre, H. Zuzan, T. A. Clark, A. Schweitzer, M. K. Staples, H. Wang, J. E. Blume, T. J. Hudson, R. Sladek and J. Majewski (2007). "Heritability of alternative splicing in the human genome." Genome Res **17**(8): 1210-8.

Lacy, P. E., E. H. Finke, C. G. Janney and J. M. Davie (1982). "Prolongation of islet xenograft survival by in vitro culture of rat megaislets in 95% O2." Transplantation **33**(6): 588-92.

Lacy, P. E. and M. Kostianovsky (1967). "Method for the isolation of intact islets of Langerhans from the rat pancreas." Diabetes **16**(1): 35-9.

Lakey, J. R., G. L. Warnock, R. V. Rajotte, M. E. Suarez-Alamazor, Z. Ao, A. M. Shapiro and N. M. Kneteman (1996). "Variables in organ donors that affect the recovery of human islets of Langerhans." Transplantation **61**(7): 1047-53.

Lakey, J. R. T., T. Tsujimura, A. M. J. Shapiro and Y. Kuroda (2002). "Human Pancreas Preservation Prior to Islet Isolation." Cell Preservation Techniques **1**(1): 81 - 87.

Lametschwandtner, A., U. Lametschwandtner and T. Weiger (1990). "Scanning electron microscopy of vascular corrosion casts--technique and applications: updated review." Scanning Microsc **4**(4): 889-940; discussion 941.

Lamont, B. J. and D. J. Drucker (2008). "Differential antidiabetic efficacy of incretin agonists versus DPP-4 inhibition in high fat fed mice." Diabetes **57**(1): 190-8.

Landreth, G. E. and M. T. Heneka (2001). "Anti-inflammatory actions of peroxisome proliferator-activated receptor gamma agonists in Alzheimer's disease." Neurobiol Aging **22**(6): 937-44.

Lang, I., C. Hoffmann, H. Olip, M. A. Pabst, T. Hahn, G. Dohr and G. Desoye (2001). "Differential mitogenic responses of human macrovascular and microvascular endothelial cells to cytokines underline their phenotypic heterogeneity." Cell Prolif **34**(3): 143-55.

Langheinrich, A. C., R. M. Bohle, A. Breithecker, D. Lommel and W. S. Rau (2004). "[Micro-computed tomography of the vasculature in parenchymal organs and lung alveoli]." Rofo **176**(9): 1219-25.

Laugharne, M., S. Cross, S. Richards, C. Dawson, L. Ilchyshyn, M. Saleem, P. Mathieson and R. Smith (2007). "Sirolimus toxicity and vascular endothelial growth factor release from islet and renal cell lines." Transplantation **83**(12): 1635-8.

Leahy, J. L. (1990). "Natural history of beta-cell dysfunction in NIDDM." Diabetes Care **13**(9): 992-1010.

Lebovitz, H. E. (1999). "Type 2 diabetes: an overview." Clin Chem **45**(8 Pt 2): 1339-45.

Lemasters, J. J. and C. R. Hackenbrock (1977). "Kinetics of product inhibition during firefly luciferase luminescence." Biochemistry **16**(3): 445-7.

Lenzen, S. and C. J. Bailey (1984). "Thyroid hormones, gonadal and adrenocortical steroids and the function of the islets of Langerhans." Endocr Rev **5**(3): 411-34.

LeRoith, D. and E. J. Rayfield (2007). "The benefits of tight glycemic control in type 2 diabetes mellitus." Clin Cornerstone **8 Suppl 7**: S19-29.

Lewis, J. D., G. R. Lichtenstein, J. J. Deren, B. E. Sands, S. B. Hanauer, J. A. Katz, B. Lashner, D. H. Present, S. Chuai, J. H. Ellenberg, L. Nessel and G. D. Wu (2008). "Rosiglitazone for active ulcerative colitis: a randomized placebo-controlled trial." Gastroenterology **134**(3): 688-95.

Lewis, J. D., G. R. Lichtenstein, R. B. Stein, J. J. Deren, T. A. Judge, F. Fogt, E. E. Furth, E. J. Demissie, L. B. Hurd, C. G. Su, S. A. Keilbaugh, M. A. Lazar and G. D. Wu (2001). "An open-label trial of the PPAR-gamma ligand rosiglitazone for active ulcerative colitis." Am J Gastroenterol **96**(12): 3323-8.

Lewis, J. P., N. D. Palmer, P. J. Hicks, M. M. Sale, C. D. Langefeld, B. I. Freedman, J. Divers and D. W. Bowden (2008). "Association analysis in african americans of European-derived type 2 diabetes single nucleotide polymorphisms from whole-genome association studies." Diabetes **57**(8): 2220-5.

Li, Y., T. Hansotia, B. Yusta, F. Ris, P. A. Halban and D. J. Drucker (2003). "Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis." J Biol Chem **278**(1): 471-8.

Lifson, N., K. G. Kramlinger, R. R. Mayrand and E. J. Lender (1980). "Blood flow to the rabbit pancreas with special reference to the islets of Langerhans." Gastroenterology **79**(3): 466-73.

Lin, K. D., Y. H. Chang, C. L. Wang, Y. H. Yang, P. J. Hsiao, T. H. Li and S. J. Shin (2008). "Thiazolidinedione addition reduces the serum retinol-binding protein 4 in type 2 diabetic patients treated with metformin and sulfonylurea." Transl Res **151**(6): 309-14.

Ling, Z. and D. G. Pipeleers (1994). "Preservation of glucose-responsive islet beta-cells during serum-free culture." Endocrinology **134**(6): 2614-21.

Linn, T., J. Schmitz, I. Hauck-Schmalenberger, Y. Lai, R. G. Bretzel, H. Brandhorst and D. Brandhorst (2006). "Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets." Clin Exp Immunol **144**(2): 179-87.

Linn, T., K. Schneider, H. P. Hammes, K. T. Preissner, H. Brandhorst, E. Morgenstern, F. Kiefer and R. G. Bretzel (2003). "Angiogenic capacity of endothelial cells in islets of Langerhans." Faseb J **17**(8): 881-3.

Lipton, R. and R. E. LaPorte (1989). "Epidemiology of islet cell antibodies." Epidemiol Rev **11**: 182-203.

Lipton, R. B. (2007). "Incidence of diabetes in children and youth--tracking a moving target." Jama **297**(24): 2760-2.

Liu, Z., M. A. Sanchez, X. Jiang, E. Boles, S. M. Landfear and B. P. Rosen (2006). "Mammalian glucose permease GLUT1 facilitates transport of arsenic trioxide and methylarsonous acid." Biochem Biophys Res Commun **351**(2): 424-30.

Lockhart, C. J., P. K. Hamilton, K. A. McVeigh and G. E. McVeigh (2008). "A cardiologist view of vascular disease in diabetes." Diabetes Obes Metab **10**(4): 279-92.

Lopez, C., M. N. Pons and E. Morgenroth (2005). "Evaluation of microscopic techniques (epifluorescence microscopy, CLSM, TPE-LSM) as a basis for the quantitative image analysis of activated sludge." Water Res **39**(2-3): 456-68.

Lorenz, D., H. Lippert, W. Tietz, V. Worm, H. J. Hahn, A. Dorn, G. Koch, M. Ziegler and K. D. Rosenbaum (1979). "Transplantation of isolated islets of Langerhans in diabetic dogs. I. Results after allogeneic intraportal islet transplantation." J Surg Res **27**(3): 181-92.

Lucas-Clerc, C., C. Massart, J. P. Campion, B. Launois and M. Nicol (1993). "Long-term culture of human pancreatic islets in an extracellular matrix: morphological and metabolic effects." Mol Cell Endocrinol **94**(1): 9-20.

Lupi, R., S. Del Guerra, L. Marselli, M. Bugliani, U. Boggi, F. Mosca, P. Marchetti and S. Del Prato (2004). "Rosiglitazone prevents the impairment of human islet function induced by fatty acids: evidence for a role of PPARgamma2 in the modulation of insulin secretion." Am J Physiol Endocrinol Metab **286**(4): E560-7.

Luther, M. J., E. Davies, D. Muller, M. Harrison, A. J. Bone, S. J. Persaud and P. M. Jones (2005). "Cell-to-cell contact influences proliferative marker expression and apoptosis

in MIN6 cells grown in islet-like structures." Am J Physiol Endocrinol Metab **288**(3): E502-9.

Macchiarelli, G., J. Y. Jiang, S. A. Nottola and E. Sato (2006). "Morphological patterns of angiogenesis in ovarian follicle capillary networks. A scanning electron microscopy study of corrosion cast." Microsc Res Tech **69**(6): 459-68.

MacDonald, A. S., J. G. McKinnon and D. A. Malatajalian (1982). "Pancreatic autotransplantation: duct drainage to bladder, duodenum, stomach, compared to duct ligation or free drainage into the peritoneal cavity." Transplant Proc **14**(4): 705-8.

MacDonald, M. J. and L. A. Fahien (1990). "Insulin release in pancreatic islets by a glycolytic and a Krebs cycle intermediate: contrasting patterns of glyceraldehyde phosphate and succinate." Arch Biochem Biophys **279**(1): 104-8.

Mack, C. A., C. J. Magovern, K. T. Budenbender, S. R. Patel, E. A. Schwarz, P. Zanzonico, B. Ferris, T. Sanborn, P. Isom, O. W. Isom, R. G. Crystal and T. K. Rosengart (1998). "Salvage angiogenesis induced by adenovirus-mediated gene transfer of vascular endothelial growth factor protects against ischemic vascular occlusion." J Vasc Surg **27**(4): 699-709.

Maehara, N. (2003). "Experimental microcomputed tomography study of the 3D microangioarchitecture of tumors." Eur Radiol **13**(7): 1559-65.

Mandal, T. K. (2005). "Inhaled insulin for diabetes mellitus." Am J Health Syst Pharm **62**(13): 1359-64.

Manelli, A., S. Sangiorgi, E. Binaghi and M. Raspanti (2007). "3D analysis of SEM images of corrosion casting using adaptive stereo matching." Microsc Res Tech **70**(4): 350-4.

Mari, G., A. Z. Abuhamad, B. Uerpairojkit, E. Martinez and J. A. Copel (1995). "Blood flow velocity waveforms of the abdominal arteries in appropriate- and small-for-gestational-age fetuses." Ultrasound Obstet Gynecol **6**(1): 15-8.

Marselli, L., F. Dotta, S. Piro, C. Santangelo, M. Masini, R. Lupi, M. Realacci, S. del Guerra, F. Mosca, U. Boggi, F. Purrello, R. Navalesi and P. Marchetti (2001). "Th2 cytokines have a partial, direct protective effect on the function and survival of isolated human islets exposed to combined proinflammatory and Th1 cytokines." J Clin Endocrinol Metab **86**(10): 4974-8.

Martin, C. L. (2008). "Beyond glycemic control: the effects of incretin hormones in type 2 diabetes." Diabetes Educ **34 Suppl 3**: 66S-72S.

Marx, N., B. Kehrle, K. Kohlhammer, M. Grub, W. Koenig, V. Hombach, P. Libby and J. Plutzky (2002). "PPAR activators as antiinflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis." Circ Res **90**(6): 703-10.

Mathew, T. (2004). "The Australian experience in organ donation--2003." Ann Transplant **9**(1): 28-30.

Matsumoto, S., T. Okitsu, Y. Iwanaga, H. Noguchi, H. Nagata, Y. Yonekawa, Y. Yamada, K. Fukuda, K. Tsukiyama, H. Suzuki, Y. Kawasaki, M. Shimodaira, K. Matsuoka, T. Shibata, Y. Kasai, T. Maekawa, J. Shapiro and K. Tanaka (2005). "Insulin independence after living-donor distal pancreatectomy and islet allotransplantation." Lancet **365**(9471): 1642-4.

Matta, S. G., J. D. Wobken, F. G. Williams and G. E. Bauer (1994). "Pancreatic islet cell reaggregation systems: efficiency of cell reassociation and endocrine cell topography of rat islet-like aggregates." Pancreas **9**(4): 439-49.

Mattsson, G., A. Danielsson, V. Kriz, P. O. Carlsson and L. Jansson (2006). "Endothelial cells in endogenous and transplanted pancreatic islets: differences in the expression of angiogenic peptides and receptors." Pancreatology **6**(1-2): 86-95.

Mattsson, G., L. Jansson and P. O. Carlsson (2002). "Decreased vascular density in mouse pancreatic islets after transplantation." Diabetes **51**(5): 1362-6.

McCance, D. R., D. J. Pettitt, R. L. Hanson, L. T. Jacobsson, W. C. Knowler and P. H. Bennett (1994). "Birth weight and non-insulin dependent diabetes: thrifty genotype, thrifty phenotype, or surviving small baby genotype?" Bmj **308**(6934): 942-5.

McClenaghan, N. H., C. R. Barnett, E. Ah-Sing, Y. H. Abdel-Wahab, F. P. O'Harte, T. W. Yoon, S. K. Swanston-Flatt and P. R. Flatt (1996). "Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion." Diabetes **45**(8): 1132-40.

McGough, R. L., B. I. Aswad and R. M. Terek (2002). "Pathologic neovascularization in cartilage tumors." Clin Orthop Relat Res(397): 76-82.

McIntosh, C. H., H. U. Demuth, S. J. Kim, J. A. Pospisilik and R. A. Pederson (2006). "Applications of dipeptidyl peptidase IV inhibitors in diabetes mellitus." Int J Biochem Cell Biol **38**(5-6): 860-72.

McKay, D. B. and A. M. Karow (1983). "A functional analysis on isolated rat islets of Langerhans: effects of dimethylsulfoxide and low-temperature preservation." Cryobiology **20**(1): 41-50.

McMillan, D. E. and P. J. Geevarghese (1979). "Dietary cyanide and tropical malnutrition diabetes." Diabetes Care **2**(2): 202-8.

Mellgren, A., A. H. Schnell Landstrom, B. Petersson and A. Andersson (1986). "The renal subcapsular site offers better growth conditions for transplanted mouse pancreatic islet cells than the liver or spleen." Diabetologia **29**(9): 670-2.

Menger, M. D., P. Vajkoczy, R. Leiderer, S. Jager and K. Messmer (1992). "Influence of experimental hyperglycemia on microvascular blood perfusion of pancreatic islet isografts." J Clin Invest **90**(4): 1361-9.

Merani, S., C. Toso, J. Emamaullee and A. M. Shapiro (2008). "Optimal implantation site for pancreatic islet transplantation." Br J Surg **95**(12): 1449-61.

Mercado, M. M., J. C. McLenithan, K. D. Silver and A. R. Shuldiner (2002). "Genetics of insulin resistance." Curr Diab Rep **2**(1): 83-95.

Michalek, J. E. and M. Pavuk (2008). "Diabetes and cancer in veterans of Operation Ranch Hand after adjustment for calendar period, days of spraying, and time spent in Southeast Asia." J Occup Environ Med **50**(3): 330-40.

Miki, A., C. Ricordi, T. Yamamoto, A. Mita, S. Barker, A. Khan, R. Alejandro and H. Ichii (2008). "Effect of human islet rescue gradient purification on islet yield and fractional Beta cell viability." Transplant Proc **40**(2): 360-1.

Miles, K. A. (1999). "Tumour angiogenesis and its relation to contrast enhancement on computed tomography: a review." Eur J Radiol **30**(3): 198-205.

Minami, K., H. Yano, T. Miki, K. Nagashima, C. Z. Wang, H. Tanaka, J. I. Miyazaki and S. Seino (2000). "Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines." Am J Physiol Endocrinol Metab **279**(4): E773-81.

Miyazaki, J., K. Araki, E. Yamato, H. Ikegami, T. Asano, Y. Shibasaki, Y. Oka and K. Yamamura (1990). "Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms." Endocrinology **127**(1): 126-32.

Mizukami, Y., K. Fujiki, E. M. Duerr, M. Gala, W. S. Jo, X. Zhang and D. C. Chung (2006). "Hypoxic regulation of vascular endothelial growth factor through the induction of phosphatidylinositol 3-kinase/Rho/ROCK and c-Myc." J Biol Chem **281**(20): 13957-63.

Mody, M., N. Dharker, M. Bloomston, P. S. Wang, F. S. Chou, T. S. Glickman, T. McCaffrey, Z. Yang, A. Pumfery, D. Lee, M. D. Ringel and J. J. Pinzone (2007). "Rosiglitazone sensitizes MDA-MB-231 breast cancer cells to anti-tumour effects of tumour necrosis factor-alpha, CH11 and CYC202." Endocr Relat Cancer **14**(2): 305-15.

Molavi, B., N. Rassouli, S. Bagwe and N. Rasouli (2007). "A review of thiazolidinediones and metformin in the treatment of type 2 diabetes with focus on cardiovascular complications." Vasc Health Risk Manag **3**(6): 967-73.

Monaco, A. P. (2006). "Rewards for organ donation: the time has come." Kidney Int **69**(6): 955-7.

Monetini, L., M. G. Cavallo, F. Barone, L. Valente, M. Russo, B. Walker, R. Thorpe and P. Pozzilli (1999). "T cell reactivity to human insulinoma cell line (CM) antigens in patients with type 1 diabetes." Autoimmunity **29**(3): 171-7.

Monroy-Cuadros, M., A. Salazar, S. Yilmaz and K. McLaughlin (2006). "Bladder vs enteric drainage in simultaneous pancreas-kidney transplantation." Nephrol Dial Transplant **21**(2): 483-7.

Moore, B. J., K. R. Holmes and L. X. Xu (1992). "Vascular anatomy of the pig kidney glomerulus: a qualitative study of corrosion casts." Scanning Microsc **6**(3): 887-97; discussion 897-8.

Morini, S., W. Yacoub, C. Rastellini, E. Gaudio, S. C. Watkins and L. Cicalese (2000). "Intestinal microvascular patterns during hemorrhagic shock." Dig Dis Sci **45**(4): 710-22.

Moriwaki, M., N. Itoh, J. Miyagawa, K. Yamamoto, A. Imagawa, K. Yamagata, H. Iwahashi, H. Nakajima, M. Namba, S. Nagata, T. Hanafusa and Y. Matsuzawa (1999). "Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus." Diabetologia **42**(11): 1332-40.

Morris, P. J., D. W. Gray and R. Sutton (1989). "Pancreatic islet transplantation." Br Med Bull **45**(1): 224-41.

Moskalewski, S. (1965). "Isolation and Culture of the Islets of Langerhans of the Guinea Pig." Gen Comp Endocrinol **44**: 342-53.

Mousavi, R., T. Miri, P. W. Cox and P. J. Fryer (2007). "Imaging of food freezing using X-ray microtomography." International Journal of Food Science and Technology **42**(6): 714 - 727.

Muller, B., F. Beckmann, M. Huser, F. Maspero, G. Szekely, K. Ruffieux, P. Thurner and E. Wintermantel (2002). "Non-destructive three-dimensional evaluation of a polymer sponge by micro-tomography using synchrotron radiation." Biomol Eng **19**(2-6): 73-8.

Murata, T., Y. Hata, T. Ishibashi, S. Kim, W. A. Hsueh, R. E. Law and D. R. Hinton (2001). "Response of experimental retinal neovascularization to thiazolidinediones." Arch Ophthalmol **119**(5): 709-17.

Murdoch, T. B., D. McGhee-Wilson, A. M. Shapiro and J. R. Lakey (2004). "Methods of human islet culture for transplantation." Cell Transplant **13**(6): 605-17.

Murray, H. E., M. B. Paget and R. Downing (2005). "Preservation of glucose responsiveness in human islets maintained in a rotational cell culture system." Mol Cell Endocrinol **238**(1-2): 39-49.

Najarian, J. S., D. E. Sutherland, A. J. Matas, M. W. Steffes, R. L. Simmons and F. C. Goetz (1977). "Human islet transplantation: a preliminary report." Transplant Proc **9**(1): 233-6.

Nanji, S. A. and A. M. Shapiro (2004). "Islet transplantation in patients with diabetes mellitus: choice of immunosuppression." BioDrugs **18**(5): 315-28.

Narang, A. S., O. Sabek, A. O. Gaber and R. I. Mahato (2006). "Co-expression of vascular endothelial growth factor and interleukin-1 receptor antagonist improves human islet survival and function." Pharm Res **23**(9): 1970-82.

Nauck, M., F. Stockmann, R. Ebert and W. Creutzfeldt (1986). "Reduced incretin effect in type 2 (non-insulin-dependent) diabetes." Diabetologia **29**(1): 46-52.

Nauck, M. A., N. Kleine, C. Orskov, J. J. Holst, B. Willms and W. Creutzfeldt (1993). "Normalization of fasting hyperglycaemia by exogenous glucagon-like peptide 1 (7-36

amide) in type 2 (non-insulin-dependent) diabetic patients." Diabetologia **36**(8): 741-4.

Nelimarkka, O., L. Halkola and J. Niinikoski (1982). "Distribution of renal cortical and Medullary tissue oxygenation in hemorrhagic shock." Acta Chir Scand **148**(3): 213-9.

Nerup, J. and A. Lernmark (1981). "Autoimmunity in insulin-dependent diabetes mellitus." Am J Med **70**(1): 135-41.

Ng, K. W., P. R. Gummer, B. L. Grills, V. P. Michelangeli and M. E. Dunlop (1987). "Insulin release from a cloned precursor beta cell line." J Endocrinol **113**(1): 3-10.

Ninomiya, H., T. Inomata and K. Ogihara (1998). "Glomerular vascular changes in KK-Ay mice with early diabetes: scanning electron microscopy of vascular resin casts." J Vet Med Sci **60**(1): 103-6.

Nishi, M., T. Sanke, S. Nagamatsu, G. I. Bell and D. F. Steiner (1990). "Islet amyloid polypeptide. A new beta cell secretory product related to islet amyloid deposits." J Biol Chem **265**(8): 4173-6.

Nitert, M. D., S. I. Chisalita, K. Olsson, K. E. Bornfeldt and H. J. Arnqvist (2005). "IGF-I/insulin hybrid receptors in human endothelial cells." Mol Cell Endocrinol **229**(1-2): 31-7.

Noel, J., A. Rabinovitch, L. Olson, G. Kyriakides, J. Miller and D. H. Mintz (1982). "A method for large-scale, high-yield isolation of canine pancreatic islets of Langerhans." Metabolism **31**(2): 184-7.

Nopanitaya, W., J. G. Aghajanian and L. D. Gray (1979). "An improved plastic mixture for corrosion casting of the gastrointestinal microvascular system." Scan Electron Microsc(3): 751-5.

Nordsletten, D. A., S. Blackett, M. D. Bentley, E. L. Ritman and N. P. Smith (2006). "Structural morphology of renal vasculature." Am J Physiol Heart Circ Physiol **291**(1): H296-309.

Oberholzer, K., A. Pohlmann, W. Schreiber, P. Mildenberger, P. Kunz, H. Schmidberger, T. Junginger and C. Duber (2008). "Assessment of tumor microcirculation with dynamic contrast-enhanced MRI in patients with esophageal cancer: initial experience." J Magn Reson Imaging **27**(6): 1296-301.

Odegard, P. S. and K. L. Capoccia (2005). "Inhaled insulin: Exubera." Ann Pharmacother **39**(5): 843-53.

Ogawa, A., S. Terada, T. Kanayama, M. Miki, M. Morikawa, T. Kimura, A. Yamaguchi, M. Sasaki and H. Yamada (2004). "Improvement of islet culture with sericin." J Biosci Bioeng **98**(3): 217-9.

Ohsawa, H., A. Kanatsuka, T. Yamaguchi, H. Makino and S. Yoshida (1989). "Islet amyloid polypeptide inhibits glucose-stimulated insulin secretion from isolated rat pancreatic islets." Biochem Biophys Res Commun **160**(2): 961-7.

Olsson, R. and P. O. Carlsson (2005). "Better vascular engraftment and function in pancreatic islets transplanted without prior culture." Diabetologia **48**(3): 469-76.

Olsson, R. and P. O. Carlsson (2006). "The pancreatic islet endothelial cell: emerging roles in islet function and disease." Int J Biochem Cell Biol **38**(4): 492-7.

Ortiz, M. C., A. Garcia-Sanz, M. D. Bentley, L. A. Fortepiani, J. Garcia-Estan, E. L. Ritman, J. C. Romero and L. A. Juncos (2000). "Microcomputed tomography of kidneys following chronic bile duct ligation." Kidney Int **58**(4): 1632-40.

Ozaki, H., H. Hayashi, S. A. Vinores, Y. Moromizato, P. A. Campochiaro and K. Oshima (1997). "Intravitreal sustained release of VEGF causes retinal neovascularization in rabbits and breakdown of the blood-retinal barrier in rabbits and primates." Exp Eye Res **64**(4): 505-17.

Paek, H. J., L. J. Moise, J. R. Morgan and M. J. Lysaght (2005). "Origin of insulin secreted from islet-like cell clusters derived from murine embryonic stem cells." Cloning Stem Cells **7**(4): 226-31.

Paget, M., H. Murray, C. J. Bailey and R. Downing (2007). "Human islet isolation: semi-automated and manual methods." Diab Vasc Dis Res **4**(1): 7-12.

Pallone, T. L., E. P. Silldorff and M. R. Turner (1998). "Intrarenal blood flow: microvascular anatomy and the regulation of medullary perfusion." Clin Exp Pharmacol Physiol **25**(6): 383-92.

Paris, M., C. Tourrel-Cuzin, C. Plachot and A. Ktorza (2004). "Review: pancreatic beta-cell neogenesis revisited." Exp Diabesity Res **5**(2): 111-21.

Park, H., Y. Ahn, C. K. Park, H. Y. Chung and Y. Park (2003). "Interleukin-6 protects MIN6 beta cells from cytokine-induced apoptosis." Ann N Y Acad Sci **1005**: 242-9.

Parlange, L. M. and M. R. Sims (1993). "A T.E.M. stereological analysis of blood vessels and nerves in marmoset periodontal ligament following endodontics and magnetic incisor extrusion." Eur J Orthod **15**(1): 33-44.

Passam, F. H., M. G. Alexandrakis, M. Kafousi, M. Fotinou, K. Darivianaki, G. Tsirakis, P. A. Roussou, E. N. Stathopoulos and N. M. Siafakas (2009). "Histological expression of angiogenic factors: VEGF, PDGFRalpha, and HIF-1alpha in Hodgkin lymphoma." Pathol Res Pract **205**(1): 11-20.

Paty, B. W., J. S. Harmon, C. L. Marsh and R. P. Robertson (2002). "Inhibitory effects of immunosuppressive drugs on insulin secretion from HIT-T15 cells and Wistar rat islets." Transplantation **73**(3): 353-7.

Paul, D. S., A. W. Harmon, V. Devesa, D. J. Thomas and M. Styblo (2007). "Molecular mechanisms of the diabetogenic effects of arsenic: inhibition of insulin signaling by arsenite and methylarsonous acid." Environ Health Perspect **115**(5): 734-42.

Persaud, S. J., P. M. Jones, H. M. Roderigo-Milne, A. M. Buchan and P. E. Squires (2003). "Calcium-dependent translocation of cytosolic phospholipase A2 in pancreatic beta-cells." Biochem Biophys Res Commun **300**(4): 889-93.

Persaud, S. J., C. P. Wheeler-Jones and P. M. Jones (1996). "The mitogen-activated protein kinase pathway in rat islets of Langerhans: studies on the regulation of insulin secretion." Biochem J **313 (Pt 1)**: 119-24.

Perseghin, G., K. Petersen and G. I. Shulman (2003). "Cellular mechanism of insulin resistance: potential links with inflammation." Int J Obes Relat Metab Disord **27 Suppl 3**: S6-11.

Petit, P. and M. M. Loubatieres-Mariani (1992). "Potassium channels of the insulin-secreting B cell." Fundam Clin Pharmacol **6**(3): 123-34.

Phillips, D. I. W. (1996). "Insulin resistance as a programmed response to fetal undernutrition." Diabetalogia **39**: 1119 - 1122.

Phillips, R. P., P. G. Ross, M. Tyska, P. F. Sharp and J. V. Forrester (1991). "Detection and quantification of hyperfluorescent leakage by computer analysis of fundus fluorescein angiograms." Graefes Arch Clin Exp Ophthalmol **229**(4): 329-35.

Phipps, K., D. J. Barker, C. N. Hales, C. H. Fall, C. Osmond and P. M. Clark (1993). "Fetal growth and impaired glucose tolerance in men and women." Diabetologia **36**(3): 225-8.

Piemonti, L., F. Bertuzzi, R. Nano, B. E. Leone, C. Socci, G. Pozza and V. Di Carlo (1999). "Effects of cryopreservation on in vitro and in vivo long-term function of human islets." Transplantation **68**(5): 655-62.

Pillai, O. and R. Panchagnula (2001). "Insulin therapies - past, present and future." Drug Discov Today **6**(20): 1056-1061.

Poitout, V., L. K. Olson and R. P. Robertson (1996). "Insulin-secreting cell lines: classification, characteristics and potential applications." Diabetes Metab **22**(1): 7-14.

Polastri, L., F. Galbiati, F. Bertuzzi, P. Fiorina, R. Nano, S. Gregori, L. Aldrighetti, G. Pozza, A. Secchi, L. Adorini and A. M. Davalli (2002). "Secretory defects induced by

immunosuppressive agents on human pancreatic beta-cells." Acta Diabetol **39**(4): 229-33.

Pollitt, C. C. and G. S. Molyneux (1990). "A scanning electron microscopical study of the dermal microcirculation of the equine foot." Equine Vet J **22**(2): 79-87.

Polykandriotis, E., A. Arkudas, J. P. Beier, A. Hess, P. Greil, T. Papadopoulos, J. Kopp, A. D. Bach, R. E. Horch and U. Kneser (2007). "Intrinsic axial vascularization of an osteoconductive bone matrix by means of an arteriovenous vascular bundle." Plast Reconstr Surg **120**(4): 855-68.

Porte, D., Jr. and S. E. Kahn (2001). "beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms." Diabetes **50 Suppl 1**: S160-3.

Pozza, G., J. Traeger and A. Secchi (1992). Pancreas Transplantation: Whole Organ and Segmental. International Textbook of Diabetes Mellitus. Chichester, John Wiley and Sons Ltd: 9 - 24.

Prentki, M. and C. J. Nolan (2006). "Islet beta cell failure in type 2 diabetes." J Clin Invest **116**(7): 1802-12.

Pybus, F. C. (1924). "Notes on suprarenal and pancreatic grafting." Lancet **2**: 550 - 551.

Rabinovitch, A., W. L. Suarez, P. D. Thomas, K. Strynadka and I. Simpson (1992). "Cytotoxic effects of cytokines on rat islets: evidence for involvement of free radicals and lipid peroxidation." Diabetologia **35**(5): 409-13.

Rasschaert, J., P. R. Flatt, C. R. Barnett, N. H. McClenaghan and W. J. Malaisse (1996). "D-glucose metabolism in BRIN-BD11 islet cells." Biochem Mol Med **57**(2): 97-105.

Reach, G. (2001). "Islet transplantation: a field on the move." Nephrol Dial Transplant **16**(5): 893-6.

Ren, J., P. Jin, E. Wang, E. Liu, D. M. Harlan, X. Li and D. F. Stroncek (2007). "Pancreatic islet cell therapy for type I diabetes: understanding the effects of glucose stimulation on islets in order to produce better islets for transplantation." J Transl Med **5**: 1.

Ricordi, C. (2003). "Islet transplantation: a brave new world." Diabetes 52(7): 1595-603.

Ricordi, C., D. W. Gray, B. J. Hering, D. B. Kaufman, G. L. Warnock, N. M. Kneteman, S. P. Lake, N. J. London, C. Socci, R. Alejandro and et al. (1990). "Islet isolation assessment in man and large animals." Acta Diabetol Lat 27(3): 185-95.

Ricordi, C., P. E. Lacy, E. H. Finke, B. J. Olack and D. W. Scharp (1988). "Automated method for isolation of human pancreatic islets." Diabetes 37(4): 413-20.

Ricordi, C., P. E. Lacy, K. Sterbenz and J. M. Davie (1987). "Low-temperature culture of human islets or in vivo treatment with L3T4 antibody produces a marked prolongation of islet human-to-mouse xenograft survival." Proc Natl Acad Sci U S A 84(22): 8080-4.

Roberts, S. (2007). "Working together for better diabetes care." Department of Health Report: Diabetes National Service Framework Document.

Robertson, G. S., D. Chadwick, H. Contractor, S. Rose, R. Chamberlain, H. Clayton, P. R. Bell, R. F. James and N. J. London (1992). "Storage of human pancreatic digest in University of Wisconsin solution significantly improves subsequent islet purification." Br J Surg 79(9): 899-902.

Robertson, R. P. (2002). "Intrahepatically transplanted islets--strangers in a strange land." J Clin Endocrinol Metab 87(12): 5416-7.

Robertson, R. P., L. K. Olson and H. J. Zhang (1994). "Differentiating glucose toxicity from glucose desensitization: a new message from the insulin gene." Diabetes 43(9): 1085-9.

Rocchi, S. and J. Auwerx (1999). "Peroxisome proliferator-activated receptor-gamma: a versatile metabolic regulator." Ann Med 31(5): 342-51.

Rodallec, M., V. Vilgrain, A. Couvelard, P. Rufat, D. O'Toole, V. Barrau, A. Sauvanet, P. Ruszniewski and Y. Menu (2006). "Endocrine pancreatic tumours and helical CT:

contrast enhancement is correlated with microvascular density, histoprognostic factors and survival." Pancreatology **6**(1-2): 77-85.

Rosen, C. B., P. P. Frohnert, J. A. Velosa, D. E. Engen and S. Sterioff (1991). "Morbidity of pancreas transplantation during cadaveric renal transplantation." Transplantation **51**(1): 123-7.

Rosenberg, L., R. Wang, S. Paraskevas and D. Maysinger (1999). "Structural and functional changes resulting from islet isolation lead to islet cell death." Surgery **126**(2): 393-8.

Rosivall, L., E. Posch, G. Simon, E. Laszlo and L. Harsing (1979). "Intrarenal distribution of renal blood flow in the rat." Acta Physiol Acad Sci Hung **53**(4): 389-97.

Ruas, J. L., U. Lendahl and L. Poellinger (2007). "Modulation of vascular gene expression by hypoxia." Curr Opin Lipidol **18**(5): 508-14.

Rubbia-Brandt, L., B. Terris, E. Giostra, B. Dousset, P. Morel and M. S. Pepper (2004). "Lymphatic vessel density and vascular endothelial growth factor-C expression correlate with malignant behavior in human pancreatic endocrine tumors." Clin Cancer Res **10**(20): 6919-28.

Runkel, S., N. Hunter and L. Milas (1991). "An intradermal assay for quantification and kinetics studies of tumor angiogenesis in mice." Radiat Res **126**(2): 237-43.

Ryan, E. A., B. W. Paty, P. A. Senior, D. Bigam, E. Alfadhli, N. M. Kneteman, J. R. Lakey and A. M. Shapiro (2005). "Five-year follow-up after clinical islet transplantation." Diabetes **54**(7): 2060-9.

Sageshima, J., N. Kirchhof, S. Shibata, K. Hiraoka, D. E. Sutherland and B. J. Hering (2001). "Small bowel subserosal space as a site for islet transplantation and local drug delivery." Transplant Proc **33**(1-2): 1710.

Saito, K., N. Yaginuma and T. Takahashi (1979). "Differential volumetry of A, B and D cells in the pancreatic islets of diabetic and nondiabetic subjects." Tohoku J Exp Med **129**(3): 273-83.

Saldana, T. M., O. Basso, J. A. Hoppin, D. D. Baird, C. Knott, A. Blair, M. C. Alavanja and D. P. Sandler (2007). "Pesticide exposure and self-reported gestational diabetes mellitus in the Agricultural Health Study." Diabetes Care **30**(3): 529-34.

Samols, E., J. I. Stagner, R. B. Ewart and V. Marks (1988). "The order of islet microvascular cellular perfusion is B----A----D in the perfused rat pancreas." J Clin Invest **82**(1): 350-3.

Sampson, M. J., T. Crowle, K. Dhatariya, N. Dozio, R. H. Greenwood, P. J. Heyburn, C. Jones, R. C. Temple and E. Walden (2006). "Trends in bed occupancy for inpatients with diabetes before and after the introduction of a diabetes inpatient specialist nurse service." Diabet Med **23**(9): 1008-15.

Sandler, S. and A. Andersson (1982). "Survival of intrasplenically implanted islets in mice with experimental insulitis and hyperglycemia." Diabetes **31 Suppl 4**: 78-83.

Sanger, F. (1959). "Chemistry of insulin; determination of the structure of insulin opens the way to greater understanding of life processes." Science **129**(3359): 1340-4.

Sanger, F. (1988). "Sequences, sequences, and sequences." Annu Rev Biochem **57**: 1-28.

Sanghera, D. K., L. Ortega, S. Han, J. Singh, S. K. Ralhan, G. S. Wander, N. K. Mehra, J. J. Mulvihill, R. E. Ferrell, S. K. Nath and M. I. Kamboh (2008). "Impact of nine common type 2 diabetes risk polymorphisms in Asian Indian Sikhs: PPARG2 (Pro12Ala), IGF2BP2, TCF7L2 and FTO variants confer a significant risk." BMC Med Genet **9**: 59.

Sangiorgi, S., A. Manelli, M. Protasoni, M. Reguzzoni, T. Congiu and M. Raspanti (2004). "Structure and ultrastructure of microvessels in the kidney seen by the corrosion casting method." Ital J Anat Embryol **109**(1): 35-44.

Sato, K., T. Wu, R. J. Laham, R. B. Johnson, P. Douglas, J. Li, F. W. Sellke, S. Bunting, M. Simons and M. J. Post (2001). "Efficacy of intracoronary or intravenous VEGF165 in a pig model of chronic myocardial ischemia." J Am Coll Cardiol **37**(2): 616-23.

Scharp, D. W., P. E. Lacy, E. Finke and B. Olack (1987). "Low-temperature culture of human islets isolated by the distention method and purified with Ficoll or Percoll gradients." Surgery **102**(5): 869-79.

Scharp, D. W., P. Marchetti, C. Swanson, M. Newton, C. S. McCullough and B. Olack (1992). "The effect of transplantation site and islet mass on long-term survival and metabolic and hormonal function of canine purified islet autografts." Cell Transplant **1**(2-3): 245-54.

Scharp, D. W., J. J. Murphy, W. T. Newton, W. F. Ballinger and P. E. Lacy (1975). "Transplantation of islets of Langerhans in diabetic rhesus monkeys." Surgery **77**(1): 100-5.

Scheen, A. J. (2003). "Current management strategies for coexisting diabetes mellitus and obesity." Drugs **63**(12): 1165-84.

Scheuner, M. T., L. J. Raffel and J. I. Rotter (1997). Genetics of Diabetes. International Textbook of Diabetes Mellitus. Chichester, John Wiley and Sons Ltd: 37 - 88.

Schmied, B. M., A. Ulrich, H. Matsuzaki, X. Ding, C. Ricordi, M. P. Moyer, S. K. Batra, T. E. Adrian and P. M. Pour (2000). "Maintenance of human islets in long-term culture." Differentiation **66**(4-5): 173-80.

Schoell, W. M., D. Pieber, O. Reich, M. Lahousen, M. Janicek, F. Guecer and R. Winter (1997). "Tumor angiogenesis as a prognostic factor in ovarian carcinoma: quantification of endothelial immunoreactivity by image analysis." Cancer **80**(12): 2257-62.

Schraufnagel, D. E., M. Sekosan, T. McGee and M. B. Thakkar (1996). "Human alveolar capillaries undergo angiogenesis in pulmonary veno-occlusive disease." Eur Respir J **9**(2): 346-50.

Shao, D., S. M. Rangwala, S. T. Bailey, S. L. Krakow, M. J. Reginato and M. A. Lazar (1998). "Interdomain communication regulating ligand binding by PPAR-gamma." Nature **396**(6709): 377-80.

Shapiro, A. M., E. Geng Hao, J. R. Lakey, D. T. Finegood, R. V. Rajotte and N. M. Kneteman (2002). "Defining optimal immunosuppression for islet transplantation based on reduced diabetogenicity in canine islet autografts." Transplantation **74**(11): 1522-8.

Shapiro, A. M., J. R. Lakey, E. A. Ryan, G. S. Korbutt, E. Toth, G. L. Warnock, N. M. Kneteman and R. V. Rajotte (2000). "Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen." N Engl J Med **343**(4): 230-8.

Shapiro, A. M., E. A. Ryan and J. R. Lakey (2001). "Clinical islet transplant--state of the art." Transplant Proc **33**(7-8): 3502-3.

Shapiro, A. M., E. A. Ryan and J. R. Lakey (2001). "Diabetes. Islet cell transplantation." Lancet **358 Suppl**: S21.

Sharma, S., M. C. Sharma and C. Sarkar (2005). "Morphology of angiogenesis in human cancer: a conceptual overview, histoprognostic perspective and significance of neoangiogenesis." Histopathology **46**(5): 481-9.

Shires, R., G. Maier, A. Lustig, P. Barnett, B. I. Joffe and H. C. Seftel (1983). "HLA antigens in White and Black South African diabetics." S Afr Med J **64**(28): 1087-9.

Shiroi, A., M. Yoshikawa, H. Yokota, H. Fukui, S. Ishizaka, K. Tatsumi and Y. Takahashi (2002). "Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone." Stem Cells **20**(4): 284-92.

Simmons, R. A. (2007). "Role of metabolic programming in the pathogenesis of beta-cell failure in postnatal life." Rev Endocr Metab Disord **8**(2): 95-104.

Singal, D. P. and M. A. Blajchman (1973). "Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus." Diabetes **22**(6): 429-32.

Sladek, R., G. Rocheleau, J. Rung, C. Dina, L. Shen, D. Serre, P. Boutin, D. Vincent, A. Belisle, S. Hadjadj, B. Balkau, B. Heude, G. Charpentier, T. J. Hudson, A. Montpetit, A. V. Pshezhetsky, M. Prentki, B. I. Posner, D. J. Balding, D. Meyre, C. Polychronakos and P. Froguel (2007). "A genome-wide association study identifies novel risk loci for type 2 diabetes." Nature **445**(7130): 881-5.

Solow, H., R. Hidalgo and D. P. Singal (1979). "Juvenile-onset diabetes HLA-A, -B, -C, and -DR alloantigens." Diabetes **28**(1): 1-4.

Sorenson, R. L. (1968). "Isolation of an insulin secretion granule rich fraction from rat islets." Anatomical Record **160**: 498.

Soto-Blanco, B., A. B. Sousa, H. Manzano, J. L. Guerra and S. L. Gorniak (2001). "Does prolonged cyanide exposure have a diabetogenic effect?" Vet Hum Toxicol **43**(2): 106-8.

Squifflet, J. P., R. W. Gruessner and D. E. Sutherland (2008). "The history of pancreas transplantation: past, present and future." Acta Chir Belg **108**(3): 367-78.

Squires, P. E., A. C. Hauge-Evans, S. J. Persaud and P. M. Jones (2000). "Synchronization of Ca(2+)-signals within insulin-secreting pseudoislets: effects of gap-junctional uncouplers." Cell Calcium **27**(5): 287-96.

Squires, P. E., S. J. Persaud, A. C. Hauge-Evans, E. Gray, H. Ratcliff and P. M. Jones (2002). "Co-ordinated Ca(2+)-signalling within pancreatic islets: does beta-cell entrainment require a secreted messenger." Cell Calcium **31**(5): 209-19.

Sreenan, S., A. J. Pick, M. Levisetti, A. C. Baldwin, W. Pugh and K. S. Polonsky (1999). "Increased beta-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse." Diabetes **48**(5): 989-96.

Sricharoenvej, S., Y. Tongpob, P. Lanlua, S. Piyawinijwong, J. Roongruangchai and I. Phoungpetchara (2007). "Renal microvascular changes in streptozotocin-induced, long-termed diabetic rat." J Med Assoc Thai **90**(12): 2677-82.

Stegall, M. D. (1997). "Monitoring human islet allografts using a forearm biopsy site." Ann Transplant **2**(3): 8-11.

Steiner, G. (2007). "Atherosclerosis in type 2 diabetes: a role for fibrate therapy?" Diab Vasc Dis Res **4**(4): 368-74.

Stewart, C., N. A. Taylor, K. Docherty and C. J. Bailey (1993). "Insulin delivery by somatic cell gene therapy." J Mol Endocrinol **11**(3): 335-41.

Stewart, C., N. A. Taylor, I. C. Green, K. Docherty and C. J. Bailey (1994). "Insulin-releasing pituitary cells as a model for somatic cell gene therapy in diabetes mellitus." J Endocrinol **142**(2): 339-43.

Stoffel, M., R. Espinosa, 3rd, M. M. Le Beau and G. I. Bell (1993). "Human glucagon-like peptide-1 receptor gene. Localization to chromosome band 6p21 by fluorescence in situ hybridization and linkage of a highly polymorphic simple tandem repeat DNA polymorphism to other markers on chromosome 6." Diabetes **42**(8): 1215-8.

Stumvoll, M. (2003). "Thiazolidinediones -- some recent developments." Expert Opin Investig Drugs **12**(7): 1179-87.

Sudwan, P., P. Chunhabundit, S. Bamroongwong, P. Rattanachaikunsopon and R. Somana (1991). "Hypophyseal angioarchitecture of common tree shrew (Tupaia glis) revealed by scanning electron microscopy study of vascular corrosion casts." Am J Anat **192**(3): 263-73.

Sutherland, D. E. (1980). "International human pancreas and islet transplant registry." Transplant Proc **12**(4 Suppl 2): 229-36.

Sutherland, D. E. (1981). "Pancreas and islet transplantation. I. Experimental studies." Diabetologia **20**: 161 - 185.

Sutherland, D. E. (1988). "An update on pancreatic transplantation." Surg Annu **20**: 331-61.

Sutherland, D. E. (1996). "Pancreas and islet cell transplantation: now and then." Transplant Proc **28**(4): 2131 - 2133.

Sutherland, D. E., M. W. Steffes, G. E. Bauer, D. McManus, B. D. Noe and J. S. Najarian (1974). "Isolation of human and porcine islets of Langerhans and islet transplantation in pigs." Journal of Surgical Research **16**: 102 - 111.

Sutherland, D. E. R. (1980). "Pancreas and islet transplantation. Clinical results." Diabetologia **20**: 435 - 450.

Swai, A. B., D. G. McLarty, B. L. Mtinangi, S. Tatala, H. M. Kitange, N. Mlingi, H. Rosling, W. P. Howlett, G. R. Brubaker and K. G. Alberti (1992). "Diabetes is not caused by cassava toxicity. A study in a Tanzanian community." Diabetes Care **15**(10): 1378-85.

Takeshita, S., L. P. Zheng, E. Brogi, M. Kearney, L. Q. Pu, S. Bunting, N. Ferrara, J. F. Symes and J. M. Isner (1994). "Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model." J Clin Invest **93**(2): 662-70.

Tang, T. and M. J. Reed (2001). "Exercise adds to metformin and acarbose efficacy in db/db mice." Metabolism **50**(9): 1049-53.

Taylor, D. K. (2007). "Study of two devices used to maintain normothermia in rats and mice during general anesthesia." J Am Assoc Lab Anim Sci **46**(5): 37-41.

Tchervenivanov, N., S. Yuan, M. Lipsett, D. Agapitos and L. Rosenberg (2002). "Morphological and functional studies on submucosal islet transplants in normal and diabetic hamsters." Cell Transplant **11**(6): 529-37.

Thorens, B. (2001). "GLUT2 in pancreatic and extra-pancreatic gluco-detection (review)." Mol Membr Biol **18**(4): 265-73.

Thorens, B. and G. Waeber (1993). "Glucagon-like peptide-I and the control of insulin secretion in the normal state and in NIDDM." Diabetes **42**(9): 1219-25.

Tillotson, L. G., C. Lodestro, M. Hocker, B. Wiedenmann, C. E. Newcomer and L. M. Reid (2001). "Isolation, maintenance, and characterization of human pancreatic islet tumor cells expressing vasoactive intestinal peptide." Pancreas **22**(1): 91-8.

Todd, J. A. (1990). "Genetic control of autoimmunity in type 1 diabetes." Immunol Today **11**(4): 122-9.

Todd, J. A. (1999). "From genome to aetiology in a multifactorial disease, type 1 diabetes." Bioessays **21**(2): 164-74.

Tolentino, M. J., J. W. Miller, E. S. Gragoudas, F. A. Jakobiec, E. Flynn, K. Chatzistefanou, N. Ferrara and A. P. Adamis (1996). "Intravitreous injections of vascular endothelial growth factor produce retinal ischemia and microangiopathy in an adult primate." Ophthalmology **103**(11): 1820-8.

Tranqui, L., S. Soyez and M. R. Block (1992). "An in vitro model giving access to adhesion plaques." In Vitro Cell Dev Biol **28A**(1): 17-23.

Traverso, L. W., A. M. Abou-Zamzam and W. P. Longmire, Jr. (1981). "Human pancreatic cell autotransplantation following total pancreatectomy." Ann Surg **193**(2): 191-5.

Tsujimura, T., Y. Kuroda, T. Kin, J. G. Avila, R. V. Rajotte, G. S. Korbutt, E. A. Ryan, A. M. Shapiro and J. R. Lakey (2002). "Human islet transplantation from pancreases with prolonged cold ischemia using additional preservation by the two-layer (UW solution/perfluorochemical) cold-storage method." Transplantation **74**(12): 1687-91.

Turner, H. E. and D. R. Matthews (2000). "The use of fixed-mixture insulins in clinical practice." Eur J Clin Pharmacol **56**(1): 19-25.

Unger, R. H. and S. Grundy (1985). "Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes." Diabetologia **28**(3): 119-21.

Vahl, T. P. and D. A. D'Alessio (2004). "Gut peptides in the treatment of diabetes mellitus." Expert Opin Investig Drugs **13**(3): 177-88.

Valente, U., M. Ferro, S. Barocci, C. Campisi, F. Parodi, L. Cataldi, V. Arcuri and E. Tosatti (1980). "Report of clinical cases of human fetal pancreas transplantation." Transplant Proc **12**(4 Suppl 2): 213-7.

Valentim, A. M., H. C. Alves, I. A. Olsson and L. M. Antunes (2008). "The effects of depth of isoflurane anesthesia on the performance of mice in a simple spatial learning task." J Am Assoc Lab Anim Sci **47**(3): 16-9.

van der Burg, M. P., O. R. Guicherit, J. B. Jansen, M. Frolich, C. B. Lamers, H. H. Lemkes, J. A. Bruijn and H. G. Gooszen (1996). "Function and survival of intrasplenic islet autografts in dogs." Diabetologia **39**(1): 37-44.

Van Spaendonck, M. P., K. Cryns, P. H. Van De Heyning, D. W. Scheuermann, G. Van Camp and J. P. Timmermans (2000). "High resolution imaging of the mouse inner ear by microtomography: a new tool in inner ear research." Anat Rec **259**(2): 229-36.

Varotto, S. M., S. M. Basso, M. Ermani, D. Bordin, D. Pizzuti and F. Lumachi (2006). "Immunomodulation techniques in a pig-to-rat model of xenoislet transplantation to prolong graft survival." In Vivo **20**(6B): 857-60.

Villasante, A., A. Pacheco, A. Ruiz, A. Pellicer and J. A. Garcia-Velasco (2007). "Vascular endothelial cadherin regulates vascular permeability: Implications for ovarian hyperstimulation syndrome." J Clin Endocrinol Metab **92**(1): 314-21.

Wada, K., A. Nakajima and R. S. Blumberg (2001). "PPARgamma and inflammatory bowel disease: a new therapeutic target for ulcerative colitis and Crohn's disease." Trends Mol Med **7**(8): 329-31.

Wagner, R. C., K. Czymmek and F. E. Hossler (2006). "Confocal microscopy, computer modeling, and quantification of glomerular vascular corrosion casts." Microsc Microanal **12**(3): 262-8.

Wallden, J., J. Ilonen, M. Roivainen, J. Ludvigsson and O. Vaarala (2008). "Effect of HLA genotype or CTLA-4 polymorphism on cytokine response in healthy children." Scand J Immunol **68**(3): 345-50.

Wang, J. L., J. A. Corbett, C. A. Marshall and M. L. McDaniel (1993). "Glucose-induced insulin secretion from purified beta-cells. A role for modulation of Ca2+ influx by cAMP- and protein kinase C-dependent signal transduction pathways." J Biol Chem **268**(11): 7785-91.

Warley, A., J. M. Powell and J. N. Skepper (1995). "Capillary surface area is reduced and tissue thickness from capillaries to myocytes is increased in the left ventricle of streptozotocin-diabetic rats." Diabetologia **38**(4): 413-21.

Warnock, G. L., R. V. Rajotte and A. W. Procyshyn (1983). "Normoglycemia after reflux of islet-containing pancreatic fragments into the splenic vascular bed in dogs." Diabetes **32**(5): 452-9.

Wautier, J. L. and P. J. Guillausseau (1998). "Diabetes, advanced glycation endproducts and vascular disease." Vasc Med **3**(2): 131-7.

Wei, W., V. Popov, J. A. Walocha, J. Wen and E. Bello-Reuss (2006). "Evidence of angiogenesis and microvascular regression in autosomal-dominant polycystic kidney disease kidneys: a corrosion cast study." Kidney Int **70**(7): 1261-8.

Whiteley, K. J., C. D. Pfarrer and S. L. Adamson (2006). "Vascular corrosion casting of the uteroplacental and fetoplacental vasculature in mice." Methods Mol Med **121**: 371-92.

Williams, P. W. (1984). "Notes on diabetes treated with extract and by grafts of sheep's pancreas." British Medical Journal **2**: 1303 - 1304.

Winter, W. E. and D. Schatz (2003). "Prevention strategies for type 1 diabetes mellitus: current status and future directions." BioDrugs **17**(1): 39-64.

Wolf, G. (2003). "Adult type 2 diabetes induced by intrauterine growth retardation." Nutr Rev **61**(5 Pt 1): 176-9.

Wolters, G. H., A. Pasma, J. B. Wiegman and W. Konijnendijk (1983). "Changes in histochemically detectable calcium and zinc during tolbutamide-induced degranulation and subsequent regranulation of rat pancreatic islets." Histochemistry **78**(3): 325-38.

Xu, G., D. A. Stoffers, J. F. Habener and S. Bonner-Weir (1999). "Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats." Diabetes **48**(12): 2270-6.

Yajima, H., M. Komatsu, T. Schermerhorn, T. Aizawa, T. Kaneko, M. Nagai, G. W. Sharp and K. Hashizume (1999). "cAMP enhances insulin secretion by an action on the ATP-sensitive K+ channel-independent pathway of glucose signaling in rat pancreatic islets." Diabetes **48**(5): 1006-12.

Yamada, K., N. Takane, S. Otabe, C. Inada, M. Inoue and K. Nonaka (1993). "Pancreatic beta-cell-selective production of tumor necrosis factor-alpha induced by interleukin-1." Diabetes **42**(7): 1026-31.

Yamada, K., N. Takane-Gyotoku, X. Yuan, F. Ichikawa, C. Inada and K. Nonaka (1996). "Mouse islet cell lysis mediated by interleukin-1-induced Fas." Diabetologia **39**(11): 1306-12.

Yamakawa, K., M. Hosoi, H. Koyama, S. Tanaka, S. Fukumoto, H. Morii and Y. Nishizawa (2000). "Peroxisome proliferator-activated receptor-gamma agonists increase vascular endothelial growth factor expression in human vascular smooth muscle cells." Biochem Biophys Res Commun **271**(3): 571-4.

Yamamoto, T., C. Ricordi, A. Mita, A. Miki, Y. Sakuma, R. D. Molano, A. Fornoni, A. Pileggi, L. Inverardi and H. Ichii (2008). "beta-Cell specific cytoprotection by prolactin on human islets." Transplant Proc **40**(2): 382-3.

Yang, Z., Z. Zhou, X. Li, G. Huang and J. Lin (2008). "Rosiglitazone preserves islet beta-cell function of adult-onset latent autoimmune diabetes in 3 years follow-up study." Diabetes Res Clin Pract.

Yessoufou, A., J. M. Ategbo, A. Girard, J. Prost, K. L. Dramane, K. Moutairou, A. Hichami and N. A. Khan (2006). "Cassava-enriched diet is not diabetogenic rather it aggravates diabetes in rats." Fundam Clin Pharmacol **20**(6): 579-86.

Zeggini, E., L. J. Scott, R. Saxena, B. F. Voight, J. L. Marchini, T. Hu, P. I. de Bakker, G. R. Abecasis, P. Almgren, G. Andersen, K. Ardlie, K. B. Bostrom, R. N. Bergman, L. L. Bonnycastle, K. Borch-Johnsen, N. P. Burtt, H. Chen, P. S. Chines, M. J. Daly, P. Deodhar, C. J. Ding, A. S. Doney, W. L. Duren, K. S. Elliott, M. R. Erdos, T. M. Frayling, R. M. Freathy, L. Gianniny, H. Grallert, N. Grarup, C. J. Groves, C. Guiducci, T. Hansen, C. Herder, G. A. Hitman, T. E. Hughes, B. Isomaa, A. U. Jackson, T. Jorgensen, A. Kong, K. Kubalanza, F. G. Kuruvilla, J. Kuusisto, C. Langenberg, H. Lango, T. Lauritzen, Y. Li, C. M. Lindgren, V. Lyssenko, A. F. Marvelle, C. Meisinger, K. Midthjell, K. L. Mohlke, M. A. Morken, A. D. Morris, N. Narisu, P. Nilsson, K. R. Owen, C. N. Palmer, F. Payne, J. R. Perry, E. Pettersen, C. Platou, I. Prokopenko, L. Qi, L. Qin, N. W. Rayner, M. Rees, J. J. Roix, A. Sandbaek, B. Shields, M. Sjogren, V. Steinthorsdottir, H. M. Stringham, A. J. Swift, G. Thorleifsson, U. Thorsteinsdottir, N. J. Timpson, T. Tuomi, J. Tuomilehto, M. Walker, R. M. Watanabe, M. N. Weedon, C. J. Willer, T. Illig, K. Hveem, F. B. Hu, M. Laakso, K. Stefansson, O. Pedersen, N. J. Wareham, I. Barroso, A. T. Hattersley, F. S. Collins, L. Groop, M. I. McCarthy, M. Boehnke and D. Altshuler (2008). "Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes." Nat Genet **40**(5): 638-45.

Zeggini, E., M. N. Weedon, C. M. Lindgren, T. M. Frayling, K. S. Elliott, H. Lango, N. J. Timpson, J. R. Perry, N. W. Rayner, R. M. Freathy, J. C. Barrett, B. Shields, A. P.

Morris, S. Ellard, C. J. Groves, L. W. Harries, J. L. Marchini, K. R. Owen, B. Knight, L. R. Cardon, M. Walker, G. A. Hitman, A. D. Morris, A. S. Doney, M. I. McCarthy and A. T. Hattersley (2007). "Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes." Science **316**(5829): 1336-41.

Zeng, G. and M. J. Quon (1996). "Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells." J Clin Invest **98**(4): 894-8.

Zhang, N., A. Richter, J. Suriawinata, S. Harbaran, J. Altomonte, L. Cong, H. Zhang, K. Song, M. Meseck, J. Bromberg and H. Dong (2004). "Elevated vascular endothelial growth factor production in islets improves islet graft vascularization." Diabetes **53**(4): 963-70.

Zhou, J., M. A. Pineyro, X. Wang, M. E. Doyle and J. M. Egan (2002). "Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors." J Cell Physiol **192**(3): 304-14.

Zhou, Z., X. Li, G. Huang, J. Peng, L. Yang, X. Yan and J. Wang (2005). "Rosiglitazone combined with insulin preserves islet beta cell function in adult-onset latent autoimmune diabetes (LADA)." Diabetes Metab Res Rev **21**(2): 203-8.

Zimmet, P. (2003). "The burden of type 2 diabetes: are we doing enough?" Diabetes and Metabolism **29**: 6S9 - 6S18.

Zinman, B. (1989). "The physiologic replacement of insulin. An elusive goal." N Engl J Med **321**(6): 363-70.

Zinman, B. and E. Y. L. Tsui (1997). Alternative Routes for Insulin Delivery. International Textbook of Diabetes Mellitus. Chichester, John Wiley and Sons Ltd: 929 - 936.

Appendix 1: Publications to date

REVIEW

Advances in islet transplantation and the UK Islet Transplant Consortium

MB Paget, R Downing

ELSEVIER

ISOLATION/PRESERVATION/CULTURE

Are Organ Preservation Solutions Useful for the Storage of Isolated Human Islets?

T.R. Jay, M.B. Paget, K.A. Heald, and R. Downing

ELSEVIER

Molecular and Cellular Endocrinology 238 (2005) 39–49

MCE

www.elsevier.com/locate/mce

Preservation of glucose responsiveness in human islets maintained in a rotational cell culture system

Hilary E. Murray *, Michelle B. Paget, Richard Downing

Islet Research Laboratory, Worcestershire Clinical Research Unit, Worcestershire Acute Hospitals NHS Trust, Newtown Road, Worcester WR5 1HN, UK

Received 26 November 2004; received in revised form 8 February 2005; accepted 19 March 2005

Human islet isolation: semi-automated and manual methods

MICHELLE PAGET, HILARY MURRAY, CLIFFORD J BAILEY, RICHARD DOWNING

Diabetologia (2009) 52:477–485
DOI 10.1007/s00125-008-1247-x

ARTICLE

Sustained insulin secretory response in human islets co-cultured with pancreatic duct-derived epithelial cells within a rotational cell culture system

H. E. Murray & M. B. Paget & C. J. Bailey & R. Downing

Appendix 2: Human pancreas donor and islet isolation information.

	Donor 1	Donor 2	Donor 3	Donor 4
Donor age and sex	20 years, male	47 years, male	54 years, male	42 years, female
Organ cold ischaemia time	11 hours	9 hours	10.5 hours	13 hours
Organ packaging and ice supply	Good, double bagged with sufficient ice.	Good, double bagged with sufficient ice.	Good, double bagged with sufficient ice.	Good, double bagged with sufficient ice.
Organ appearance and condition	Healthy pink-brown with sections of spleen and duodenum attached. Substantial amount of hard, yellow fat attached and damage to capsule in the head to the centre of the organ.	Healthy pink-brown with section of duodenum attached. Substantial amount of hard and soft fat attached. Organ capsule intact.	Healthy pink-brown with section of spleen attached. Substantial amount of hard, yellow fat attached. Organ capsule intact.	Healthy pink-brown with sections of spleen and duodenum attached. Very little fat attached, slight damage to capsule in the centre of the organ.
Weight of organ used for isolation	89g	83g	84g	56g
Liberase lot number and degree of organ distension	93288220. Good distension after clamping of the damaged area.	93288220. Good distension.	93597920. Very good distension.	93597920. Very good distension.
Digestion time and pre-separation digest observation	35 minutes. Well digested, but most islets remained mantled with exocrine tissue. Islets ranged from 50 - 150μm.	35 minutes. Well digested with many free islets ranging from 100 - 300μm.	30 minutes. Well digested with many free islets ranging from 50 - >600 μm.	40 minutes. Well digested with many free islets ranging from 50 - 250 μm
Isolated islet size and degree of purity	Most isolated islets were mantled and ranged from 100 - 175μm. Purity was ~85%.	Most isolated islets were well cleaved and intact with sizes ranging from 100 - 300μm and purity ~85%.	Most isolated islets were well cleaved and intact with sizes ranging from 50 - >600μm and purity ~80%.	Most isolated islets were well cleaved and intact with sizes ranging from 50 - 200μm and purity ~80%.
Islet function and viability score	Trypan blue score of 22 indicated a viable islet preparation and 24hr insulin secretion profile was confirmed viability.	Trypan blue score of 23 indicated a viable islet preparation and 24hr insulin secretion profile was confirmed viability.	Trypan blue score of 21 indicated a viable islet preparation and 24hr insulin secretion profile was confirmed viability.	Trypan blue score of 22 indicated a viable islet preparation and 24hr insulin secretion profile was confirmed viability.