BETA CELL VIABILITY AND FUNCTION IN HYPOXIA: TOWARDS A CLINICALLY REFLECTIVE MODEL OF BETA CELL TRANSPLANTATION

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UNIVERSITY OF BRIGHTON
For my Father & Mother
# Table of Contents

Abstract .......................................................................................................................... vii  
Declaration ...................................................................................................................... viii  
Acknowledgements ........................................................................................................ ix  
List of Figures ................................................................................................................ x  
List of Main Abbreviations ............................................................................................ xiii  

Chapter One: Introduction .............................................................................................. 1  

1.1 Diabetes Epidemiology ............................................................................................ 1  
1.2 The History of Diabetes .......................................................................................... 1  
1.3 The Pancreas ............................................................................................................ 2  

1.3.1 Carbohydrate metabolism ................................................................................ 3  
1.3.2 Insulin Secretion ............................................................................................... 5  

1.4 Insulin Receptor ....................................................................................................... 6  

1.5 Diabetes Mellitus ..................................................................................................... 6  

1.5.1 Type 1 Diabetes ............................................................................................... 8  
1.5.2 Type 2 Diabetes ............................................................................................... 9  

1.6 Treatment of Diabetes ........................................................................................... 10  

1.6.1 Type 2 Diabetes .............................................................................................. 10  
1.6.2 Glucagon-like peptide-1 (GLP-1) ................................................................. 11  
1.6.3 Thiazolidinediones ......................................................................................... 13  
1.6.4 Type 1 Diabetes .............................................................................................. 15  

1.7 Hypoxia ................................................................................................................... 18  

1.7.1 Oxygen Sensing and Metabolism ................................................................. 19  
1.7.2 Hypoxia Inducible factor (HIF) ...................................................................... 20  
1.7.3 Vascular Endothelial Growth Factor (VEGF) and Angiogenesis ............... 21  
1.7.4 5’ Adenosine monophosphate-activated protein kinase (AMPK) .............. 22  
1.7.5 Mammalian target of rapamycin (mTOR) ................................................. 23
1.7.6 Phosphatidylinositol 3-kinase (PI3K) ............................................................ 25
1.7.7 Akt, Protein Kinase B .................................................................................... 26
1.7.8 Ribosomal S6 Kinase ..................................................................................... 27
1.7.9 Nuclear Factor kappa B.................................................................................. 27
1.8 Laboratory investigations of Diabetes ............................................................ 28
1.9 Aims ...................................................................................................................... 29

Chapter Two: Materials and Methods ............................................................... 31
2.1 Materials ............................................................................................................ 31
2.2 Methods .............................................................................................................. 31
  2.2.1 Culture Conditions ......................................................................................... 31
  2.2.3 Culture Conditions ......................................................................................... 32
  2.2.6 Hoechst Propidium Iodide (HPI) ................................................................... 34
Table 2.1: Reagents used in 10% SDS-PAGE resolving gel .......................................... 37
Table 2.2: Reagents used in 10% SDS-PAGE stacking gel ............................................ 37
  2.2.15 Reverse Transcription .................................................................................. 42
  2.2.16 Real-time Polymerase-chain Reaction ......................................................... 43
  2.2.17 Real-Time PCR Calculation.......................................................................... 43
  2.2.18 Data & statistical Analysis: .......................................................................... 46
Chapter Three: β-cell Viability in Hypoxia ............................................................ 47
  3.1 Introduction ....................................................................................................... 47
  3.2 Viability of β-cells in Hypoxia........................................................................... 49
  3.3 Growth of β-cells in Hypoxia............................................................................ 52
  3.4 Hoechst Propidium Iodide Staining of MIN6 β-cells in Normoxia and Hypoxia. 52
  3.5 Morphology of MIN6 β-cells in Normoxia and Hypoxia by light microscopy .... 54
  3.6 Cell Quiescence and Oxygen Re-stimulation ................................................... 57
  3.7 Nuclear Factor kappa B....................................................................................... 58
  3.8 5-bromodeoxyuridine, BrdU ............................................................................. 62
Chapter Four: Molecular Pathway in Hypoxia

4.1 Introduction

4.2 Ribosomal S6 Kinase, S6K

4.3 Phosphatidylinositol 3-kinase (PI3K)

4.4 Protein Kinase B (Akt)

4.5 GLUT2

4.6 Adenosine monophosphate-activated protein kinase (AMPK)

4.7 Discussion

Chapter Five: Revascularisation and β-cell Function

5.1 Introduction

5.2 Revascularisation of the β-cell

5.2.1 hif

5.2.2 vegf

5.3 GLP-1 and Hypoxia

5.3.1 GLP-1 & hif

5.3.2 GLP-1 and vegf

5.3.3 GLP-1 and Beta-cell viability

5.3.4 GLP-1 and S6 Kinase

5.4 Rosiglitazone and Hypoxia

5.4.1 Rosiglitazone and hif

5.4.2 Rosiglitazone and vegf

5.4.3 Rosiglitazone and Beta-cell viability

5.4.4 Rosiglitazone and S6 Kinase

5.5 Beta-cell function in hypoxia

5.5.1 PDX-1

5.5.2 Glucose-Stimulated Insulin-response
Chapter Six: Conclusions and Future Work

6.1 Aim of research

6.2 Summary of main findings

6.3 Growth, viability and revascularisation in hypoxia; 24 hours

6.4 Growth, viability and revascularisation in hypoxia; 48 hours

6.5 Growth, viability and revascularisation in hypoxia; 72 hours

6.6 Therapeutic compounds and hypoxia

6.7 Hypoxia and β-cell function

6.8 Future work

6.9 Implications of this research

References

Publications
Abstract

Beta-cell survival is low following islet transplantation and this may be linked to a delay in revascularisation of donor cells. This decrease in oxygen supply is termed hypoxia, the result of which is detrimental to beta-cell survival. The current research sought to investigate post-transplant beta-cell viability and function by investigating the effects of low oxygen levels on MIN6 pancreatic beta-cells. MIN6 beta-cells were exposed to 1% oxygen (hypoxia) or 21% oxygen (normoxia) over a period of 72 hours. Viability was assessed by MTT assay and cell number was determined by haemocytometer count at 0 hours (normoxia), 24 hours, 48 hours and 72 hours. A Hoechst propidium iodide (HPI) stain was used to identify beta-cell apoptosis or necrosis during hypoxia. Western blot analyses were performed to determine the PI3K, pAkt, pAMPK, PDX-1, GLUT2 and pS6K protein levels. Real time PCR was used to estimate glut2, vegf, hif and insulin gene expression by MIN6 cells following exposure to hypoxia over various time points.

Cell viability was reduced at 48 hours and 72 hours of hypoxia. Cell number in hypoxia was significantly lower than in normoxia at 48 hours and 72 hours. MIN6 cells showed a significant increase in viability when re-stimulated with oxygen for a period of 24 hours following previous 24 hours and 48 hours exposure to hypoxia. HPI staining demonstrated an absence of apoptosis or necrosis in MIN6 beta-cells exposed to hypoxia over 72 hours. BrdU analysis of S-phase cell cycle progression indicated that progression through the cell cycle may be protracted during hypoxia. Western blot analysis of phosphorylated S6 kinase protein confirmed a decrease in pS6K protein expression in response to low oxygen. PI3K and Akt protein was unchanged in hypoxia. AMPK phosphorylation was increased at 24 hours and 48 hours of hypoxia and an inhibition of this protein resulted in an increase in S6K at 24 hours of hypoxia but this did not result in an increase in MIN6 β-cell viability. hif gene expression was significantly decreased at 24 hours hypoxia while vegf mRNA was significantly increased at 24 hours and 48 hours of hypoxia. Treatment of these cells with Rosiglitazone while being exposed to hypoxia resulted in a significant increase in revascularisation markers over 72 hours. insulin gene expression and insulin secretion in response to high glucose was lower in MIN6 cells exposed to hypoxia and we demonstrated that these cells remain non-responsive to high glucose following 24 hours and 48 hours of hypoxia.

Results presented here have established and validated a novel in vitro clinically relevant model to study the effects of hypoxia on pancreatic beta-cell function. We have demonstrated that low oxygen does have a detrimental effect on the growth of pancreatic β-cells and this effect is reversed upon re-stimulation of cells with normoxic oxygen concentration. Furthermore, we have validated the use of therapeutic Rosiglitazone in the pre-incubation of these cells post isolation and pre-transplantation as a mechanism of increasing the regulation of revascularisation markers. These findings may prove essential to the future of the islet transplantation process. The use of this system provides a unique opportunity to begin to delineate the cell signalling and cell cycling events controlling beta cell function in the acute period of tissue hypoxia immediately following islet transplantation.
Declaration

I declare that the thesis entitled “Beta cell viability and function in hypoxia: towards a clinically reflective model of beta cell transplantation” is the original work of the author, unless otherwise stated. This thesis does not contain material that has been previously submitted in whole or in part for the award of any other academic degree or diploma.

Michelle Barry
October 2013
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List of Figures

Chapter One Introduction

Figure 1. 1 Glucose metabolism and insulin release in the beta cell. ______________ 5
Figure 1. 2 Diagram demonstrating the process if islet transplantation ___________ 17
Figure 1. 3 Schematic representation of the AMPK/mTOR/S6K pathway __________ 22
Figure 1. 4 Schematic representation of the PI3K/Akt/mTOR/S6K pathway ________ 25

Chapter Two Material & Methods

Figure 2.1 Picture of Hypoxic Glove Box from COY laboratories ________________ 32

Chapter Three β-Cell Viability in Hypoxia

Figure 3. 1 MTT assay of MIN6 cells exposed to 1% oxygen over 8 hours ________ 50
Figure 3. 2 MTT assay of MIN6 cells exposed to 1% oxygen over 72 hours ________ 51
Figure 3.3 MIN6 β-cell number following exposure to 1% oxygen over 72 hours. __ 53
Figure 3. 4 HPI Staining of MIN6 cells exposed to 21% and 1% oxygen for 72 hours. 55
Figure 3. 5 Morphology imagery of MIN6 cells exposed to 21% and 1% oxygen for 72 hours. __ 56
Figure 3. 6 MIN6 re-stimulation with 21% oxygen following 24hrs exposure to 1% oxygen. ________________________________ 59
Figure 3. 7 MIN6 re-stimulation with 21% oxygen following 48hrs exposure to 1% oxygen. ________________________________ 60
Figure 3. 8 NFκB expression in MIN6 cells exposed to hypoxia over 72 hours. _____ 61
Figure 3. 9 BrdU staining of MIN6 cell exposed to normoxic or hypoxic conditions over 48 hours. ____________ 63
Figure 3.10 Cell count of BrdU stained MIN6 cells exposed to normoxic or hypoxic conditions over 48 hours. ___________ 64

Chapter Four Molecular Pathway in Hypoxia

Figure 4.1 Phosphorylation of S6K in MIN6 β-cells exposed to hypoxia over 72 hours. __ 72
Figure 4.2 Schematic representation of the PI3K/Akt/mTOR/S6K pathway __________ 74
Figure 4.3 PI3K expression in MIN6 cells exposed to hypoxia over 72 hours. ________ 75
Figure 4.4 pAktthr308 expression in MIN6 cells exposed to hypoxia over 72 hours. ____ 77
Figure 4.5 pAktser473 expression in MIN6 cells exposed to hypoxia over 72 hours. ___ 78
Figure 4.6 Expression of glut2 gene in MIN6 cells exposed to hypoxia over 72 hours. 80
Figure 4.7 GLUT2 protein expression in MIN6 cells exposed to hypoxia over 72 hours 82
Figure 4.8 Schematic representation of the AMPK/mTOR/S6K pathway. 85
Figure 4.9 pAMPKα expression in MIN6 cells exposed to hypoxia over 72 hours. 86
Figure 4.10 pS6K expression in MIN6 cells exposed to hypoxia over 72 hours with and without incubation with 2.5 µM AMPK inhibitor Compound C. 88
Figure 4.11 MTT assay of MIN6 β-cells exposed to 1% oxygen 72 hours and incubated with 2.5 µM AMPK inhibitor compound C. 90

Chapter Five Revascularisation and β-Cell Function

Figure 5.1 The effect of 1% oxygen on hif-1α gene expression over 72 hours exposure to hypoxia. 98
Figure 5.2 The effect of 1% oxygen on vegf gene expression over 72 hours exposure to hypoxia. 100
Figure 5.3 The regulation of hif-1α gene expression in MIN6 β-cells exposed to 1% or 21% oxygen in the presence of 10 nM GLP-1. 102
Figure 5.4 The regulation of the vegf gene expression in MIN6 β-cells exposed to 1% or 21% oxygen in the presence of 10 nM GLP-1. 104
Figure 5.5 MTT assay of MIN6 β-cells exposed to 1% oxygen over 72 hours with or without 10 nM GLP-1/10 nM GLP-1 activity verification. 106
Figure 5.6 Phosphorylation of S6K in MIN6 β-cells exposed to hypoxia over 72 hours and treated with or without 10 nM GLP-1. 108
Figure 5.7 The regulation of hif-1α gene expression in MIN6 β-cells exposed to hypoxia in the presence of 10 µM Rosiglitazone. 112
Figure 5.8 The effect of 10 µM Rosiglitazone on vegf gene expression in MIN6 cells cultured in 21% and 1% oxygen over 72 hours. 114
Figure 5.9 MTT assay of MIN6 β-cells exposed to 1% oxygen over 72 hours with or without 10 µM Rosiglitazone. 115
Figure 5.10 Phosphorylation of S6K in MIN6 β-cells exposed to hypoxia over 72 hours and treated with or without 10 µM Rosiglitazone. 117
Figure 5.11 Regulation of PDX-1 protein in MIN6 cells exposed to hypoxia for 72 hours. 119
Figure 5.12 Insulin gene transcription in response to 25 mM glucose in hypoxia and normoxia at 24 hours. 123
Figure 5.13 Insulin gene transcription in response to 25 mM glucose in hypoxia and normoxia at 48 hours. 124
Figure 5.14 Insulin secretion in hypoxia and normoxia at 24 hours. 126
Figure 5.15 Insulin secretion in response to 25 mM glucose in hypoxia and normoxia at 24 hours. 127

Chapter Six Conclusions & Future Work

Figure 6.1 Schematic demonstrating MIN6 response to hypoxia at 24 hours. 142
Figure 6.2 Schematic demonstrating MIN6 response to hypoxia at 48 hours. 145
Figure 6.3 Schematic demonstrating MIN6 response to hypoxia at 72 hours. 147
Figure 6.4 Schematic demonstrating MIN6 response to hypoxia with the treatment of Rosiglitazone. 151
# List of Main Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate Activated Kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaMKK</td>
<td>Calcium/calmodulin-dependent protein kinase kinase</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DEPTOR</td>
<td>DEP domain-containing mTOR-interacting protein</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factors</td>
</tr>
<tr>
<td>HM</td>
<td>Hydrophobic motif</td>
</tr>
<tr>
<td>HNF-α</td>
<td>Hepatocyte nuclear factor alpha</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response elements</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>K-channel</td>
<td>Potassium Channel</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MHRA</td>
<td>Medicines and Healthcare products Regulatory Agency</td>
</tr>
<tr>
<td>MLST8</td>
<td>Mammalian lethal with SEC13 protein 8</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>NFkB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute of Clinical Excellence</td>
</tr>
<tr>
<td>PAK</td>
<td>Pancreas after Kidney</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate of 40 kilodaltons</td>
</tr>
<tr>
<td>PTA</td>
<td>Pancreas transplant alone</td>
</tr>
<tr>
<td>RAPTOR</td>
<td>Regulatory associated protein of mTOR</td>
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<tr>
<td>RICTOR</td>
<td>Rapamycin-insensitive companion of mTOR</td>
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<tr>
<td>S6K</td>
<td>S6 kinase</td>
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<tr>
<td>SGLT</td>
<td>Sodium-glucose linked transporter</td>
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<tr>
<td>T1D</td>
<td>Type 1 Diabetes</td>
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<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
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<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TORC</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>UKPDS</td>
<td>UK Prospective Diabetes Study</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
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Chapter One: Introduction

1.1 Diabetes Epidemiology

Diabetes is defined as a decrease in insulin secretion and action. It affects approximately 347 million people worldwide and an estimated 3.7 million people here in the UK. The growing rates of diabetes both worldwide and here in the UK are of major concern. The World Health Organisation (WHO) predicts that diabetes will be the seventh leading cause of death in the world by 2030. NHS figures published in 2010 indicate that 10% of the total NHS budget is spent on treating diabetes and its complications (Department of Health, 2006). For these reasons there is an immediate need to find a cure for this epidemic problem. But first, let us look at the background to this phenomenal disease.

1.2 The History of Diabetes

One of the first medical descriptions of diabetes dates back to 200AD from the Greek physician, Aretaeus of Cappadocia, who describes it as a disease of the kidneys and the bladder. In his own words, “patients never stop making water; the flow is incessant, as if from the flow of aqueducts”. Aretaeus was the first to name Diabetes, the name coming from the Greek word meaning siphon (Gemmill, 1972). Many years of diabetes research ensued in an attempt to identify the various cells contained within the pancreas. In 1869, the islets of Langerhans were discovered by the German medical student, Paul Langerhan (Palmer, Hsu et al., 1998). In 1889, one of the first experiments ever carried
out in the area of diabetes was performed by Oskar Minkowski and Josef von Mering. Here, Minkowski and von Mering were able to induce all the symptoms of diabetes in a dog by performing a total pancreatectomy. For the first time the essential role of the pancreas in diabetes was established (Brogard, 1992). Another discovery by Eugene Lindsay Opie in 1900 identified that in patients with diabetes, tissue degeneration in the islet of Langerhans was clear (Braissant et al., 2011). However, it is the work of Banting and Best in the MacLeod laboratories within the University of Toronto which has received the greatest accolade in the world of diabetes research. In 1921, Banting and Best observed a reduction in blood glucose in a depancreatized dog following the administration of a macerated pancreas (Palmer et al., 1998). This work is the first known in the discovery of insulin and is so greatly acknowledged it was awarded the Nobel Prize in Physiology and Medicine in 1923.

1.3 The Pancreas

The pancreas consists of two main structures, the exocrine and the endocrine pancreatic tissue (Slack, 1995). The exocrine gland secretes digestive enzymes, such as amylase, lipase and trypsin to the gut which aid digestion (Rosen et al., 1999). In conditions of pancreatic exocrine deficiency, such as chronic pancreatitis, insufficient absorption of key macronutrients is observed (Bastie et al., 2000). In diabetes research, it is the work and function of the endocrine tissue which is of most importance. The endocrine pancreas consists of approximately 1 million islets of Langerhans, each containing α-cells, β-cells, δ-cells and pancreatic polypeptide cells PPs (Pickup, 2003). Each cell type is known to have its own specific function, secreting glucagon, insulin, somatostatin and
pancreatic polypeptide respectively (Schwitzgebel, 2001). β-cells comprise approximately 60-70% of the total islet cell number and thus are the predominant cell found in the islet (Stefan, 1983). The other cells found in the islet of Langerhans are in much smaller proportions, α-cells encompass 15-20%, δ-cells comprise of 3-10% and pancreatic polypeptides (PP-cells) are present in the smallest proportion at approximately 1% (Gulick et al., 1994). The control of glucose levels in the body is dependent on the related response of both the α-cells and β-cells. During times of hypoglycaemia α-cells secrete glucagon and during times of hyperglycaemia β-cells secrete insulin (Gulick et al., 1994). Their role in the secretion of insulin and regulation of glucose metabolism is one that has been widely investigated.

1.3.1 CARBOHYDRATE METABOLISM

In a healthy individual, pancreatic β-cells secrete insulin in response to a carbohydrate-based meal. Insulin allows the uptake of glucose from carbohydrates by muscles and cells throughout the body. Here, glucose is either stored or utilised as a form of energy. Insulin is stored within intracellular vesicles in the β-cell. Following ingestion of a carbohydrate based meal, membrane depolarisation is observed upon glucose stimulation of the ATP-sensitive K⁺ channel (Dunne, 2000). This results in the influx of Ca²⁺ through voltage-dependent Ca²⁺ channels and subsequently insulin release (Matthews, 1975). While insulin is itself dependent on glucose to stimulate secretion, insulin is the key hormone regulating glucose metabolism. The rate of glucose metabolised by the body is dependent on the amount of insulin acting on the insulin receptor (White, 1994).
The metabolism of glucose is a complex process. Transporters known as GLUT (glucose transporter) and SGLT (sodium glucose transport proteins) transporters are responsible for the transfer of glucose to areas within the body requiring energy (Wood, 2003). Until recently, it was believed that just five glucose transporters (GLUT transporters) existed. However, it is now believed that the process of glucose metabolism is far more complex and may involve as many as fourteen facilitative glucose transporters (Wood, 2003; Augustin 2010). GLUT1 facilitated transporters are generally located in the brain and erythrocytes (Wood, 2003). GLUT2 transporters on pancreatic β-cells serve as a glucose signalling system as well as a facilitative glucose transporter and play a key role in the glucose-induced insulin-secretion properties of pancreatic β-cells (Thorens, 1996). GLUT4 transporters are the key glucose transporters in skeletal muscle, adipose tissue, heart and brain and are highly responsive to insulin. Following insulin stimulation, intracellular GLUT4 transporters are translocated to the extracellular membrane. Here, they are responsible for the rapid transport of glucose into the cell (Shepherd, 1999; Bryant 2002). Although it would seem that GLUT4 is essential for insulin-signalling, the metabolism of glucose is not greatly altered by GLUT4 down-regulation (Katz, 1995). Following this study, and many others like it, glucose transporters have been grouped into three classes based on protein similarities. Class one consists of GLUT1-4 and GLUT14, class two incorporates the odd transporters GLUT5, 7, 9 and 11 and class three includes GLUT6, 8, 10, 12 and 13 (Augustin, 2010). All transporters function to increase the uptake of glucose across cell membranes in a process of energy production and lipid and protein synthesis.
1.3.2 INSULIN SECRETION

Insulin is a hormone secreted in response to carbohydrate metabolism. In the pancreatic β-cell, glucose molecules bind to GLUT2 transporters and in response intracellular ratio of ATP/ADP rise (Schuit et al., 2002). This then leads to the closure of ATP-sensitive potassium-channels, K\textsuperscript{ATP} channels on the cell membrane (Ashcroft et al., 1984). Closure of these channels results in the repolarisation of the cell membrane and the opening of voltage-gated calcium-channels, increasing the concentration on Ca\textsuperscript{2+} intracellularly (Gembal et al., 1003). Intracellular insulin granules are excited by this increase in Ca\textsuperscript{2+} and thus results in insulin release from the β-cell (Henquin, 2000). This process is demonstrated schematically on Figure 1.1.

![Figure 1.1 Glucose metabolism and insulin release in the beta cell.](image)

Glucose enters the cell via the GLUT2 glucose transporter which causes an increase in the intracellular ATP:ADP ratio (1), closing K\textsubscript{ATP} channels (2) and triggering the opening of Ca\textsuperscript{2+} channels (3). This induces exocytosis of insulin containing granules and insulin release (4).
1.4 Insulin Receptor

The metabolic effect of insulin on carbohydrates is mediated via insulin receptor response on several sites in the body. The insulin receptor is composed of two external α-subunits which are linked to two transmembrane β-subunits by disulphide bonds. The α-subunits serve as the insulin binding site while the β-subunits are the domain for autophosphorylation upon insulin binding. Insulin binds to the α-subunit of the insulin receptor which in turn activates the β-subunit of this tyrosine kinase receptor (Sun, 1991). This receptor binding stimulates phosphorylation of proteins within the cell such as insulin receptor substrate IRS-1 (Kasugu, 1983; Sun, 1991). Phosphorylation of intracellular proteins results in a change in biological activity and an increase in insulin response. An example of this is the activation of the phosphatidylinositol 3-kinase (PI3K) protein which results in the translocation of glucose transporters to the cell membrane and allows for glucose uptake and storage (Kotani et al., 1995).

1.5 Diabetes Mellitus

Diabetes Mellitus defines a metabolic disorder resulting in hyperglycaemia. This may be as a result of the β-cells of the pancreas not producing enough insulin or the glucose requiring target tissues not responding to the insulin being produced. This results in high blood levels of glucose, termed hyperglycaemia, which can have detrimental effects on several organs in the body, including the heart (Ido, 2002), kidneys (Ritz, 1999) and nerves (Cameron, 2003). As a result, people with diabetes may concurrently suffer from cardiovascular disease (Ceriello, 2005), nephropathy with a potential risk of
renal failure (Ritz, 1999), visual impairment (Pollreisz, 2010) and neuropathy (Tesfaye, 1996).

Several different types of diabetes mellitus exist and are classified according to their respective causal agents. The two main classifications of diabetes mellitus are type 1 diabetes (T1D) and type 2 diabetes (T2D). The World Health Organisation (WHO) estimates that approximately 10% of cases of diabetes is attributed to T1D with approximately 90% of diabetes cases being attributed to T2D (World Health Organisation, 2009). The exact causes of each disease are yet to be fully identified but the resulting insulin loss eventually leads to a rise in postprandial glucose levels in both diseases. Other variations of the disease also exist. Gestational diabetes can develop during pregnancy and occurs when women cannot produce the surplus insulin required during this time (Girling, 2004). This form of the disease usually exists short-term and insulin secretion and function return to normal following the birth. Maturity-onset diabetes of the young (MODY) results from an autosomal dominant monogenic defect of the parent chromosome and is associated with a deficiency in insulin secretion (Velho, 1998). MODY is classified as a subclass of T2D but, unlike T2D, it is associated with an earlier onset with symptoms usually developed before the age of 25 years (Fajans, 2001). Six genes have been identified in the pathogenesis of the different types of MODY. A defect in the glucokinase gene results in the development of MODY 2 (Froguel, 1992). MODY 1 and MODY 3 can result from a mutation on the HNF-4α and HNF-1α genes respectively (Yamagata, 1996). A defect in the PDX-1 gene, a key gene involved in the development of the pancreas, can lead to the development of MODY4 (Stoffers, 1997). MODY5 and 6 are very rare forms of the disease. All these
gene defects result in a decrease in transcription factors responsible for the regular development and function of the β-cell and pancreas.

**1.5.1 TYPE 1 DIABETES**

T1D is classified as an autoimmune disease resulting in the total destruction of pancreatic β-cells and eventually results in complete insulin deficiency. This process is known as insulitis. The role of varying immune markers have been identified from several animal studies (Wong, 1999; Yang, 2006), however, the precise immunological substance of importance has yet to be identified in human models of diabetes. Scientists have several theories as to why insulitis is destroying pancreatic β-cells in type 1 diabetes. Several reports suggest viral infections (Filippi, 2005), environmental triggers (Mead, 2004) and genetics (Fugger, 2000) may signal immune markers which activate this autoimmune response. The resultant decrease in β-cell activity renders the patient with no insulin producing capability and requiring an external source of insulin. The presenting symptoms of T1D include polyuria, polydipsia, enuresis and weight loss (Roche, 2005). Hypoglycaemia is a term used to describe low plasma glucose which may result from an increase in insulin or a deficiency in glucagon production and is a common problem experienced by patients with T1D (Oskarsson, 1999). The resulting complications such as arrhythmias, cognitive function or even more serious brain damage and coma have been reported by Halami et al in severe hypoglycaemia (Halimi, 2010).
1.5.2 TYPE 2 DIABETES

Type 2 diabetes, like type 1 diabetes, is a disease ultimately characterised by a deficiency of insulin and the discussion of one without the other would seem inappropriate. Type 2 diabetes, is a disease of prolonged hyperglycaemia coupled with insulin resistance (Reaven, 1988). In a recent publication by the WHO, the number of people diagnosed with type 2 diabetes in the UK was reported as over 2.5 million. The numbers of people diagnosed with T2D has increased in recent years which is, in part, due to the rising epidemic of obesity (Wannamethee, 1999; Resnick, 2000). Insulin resistance is an interesting phenomenon linked with type 2 diabetes and is greatly associated with obesity (Weyer, 2001). Not only is obesity and high circulating levels of fat detrimental to the insulin receptors, it may also have negative effects on the pancreatic β-cell. The chronic exposure of β-cells to high circulating fatty acids, as seen in high fat diets, has been identified as a key player in the development of β-cell dysfunction (Zhou, 1994; Shimabukuro, 1998; Meadler 2001). Chronic exposure of the β-cell to high levels of fatty acids can lead to the induction of apoptosis (Lupi, 2002). The result is decreased insulin secretion coupled with increased insulin resistance and decreased glucose uptake. Secondary complications associated with type 2 diabetes are similar to those associated with type 1 diabetes and in some cases patients have been known to present with secondary complication symptoms before actually being diagnosed with type 2 diabetes.
1.6 Treatment of Diabetes

The treatment of diabetes varies between type 1 diabetes and type 2 diabetes. This is due to the fact that the β-cell in type 2 diabetes may have residual insulin secretory function and thus complete insulin replacement therapy is not a requirement, although progressive type 2 diabetes may require concomitant insulin therapy alongside insulin sensitizers.

1.6.1 TYPE 2 DIABETES

The United Kingdom Prospective Diabetes Study (UKPDS) study has shown a reduction in secondary complications in patients with type 2 diabetes with tightly controlled blood glucose (UKPDS, 1993). Current therapies to treat type 2 diabetes begin with a controlled diet where possible. The 2008 NICE guidelines on the use of medication to treat type 2 diabetes indicated the use of oral medication first and in cases where further intervention is required then intravenous medication should be used. Finally insulin replacement therapy should be used in progressive forms of the disease. As insulin loss is gradual in type 2 diabetes, medication which stimulates insulin secretion from the β-cell or mimics insulin signalling at the insulin receptor can improve glycaemic control. Type 2 diabetes therapy is divided into two main groups; the insulin secretagogues and the insulin sensitisers. The insulin secretagogues stimulate insulin secretion from the β-cells of the pancreas, examples include the Sulfonylureas and Meglitinides (Henquin, 2004). Insulin sensitizers vary in their mechanism of action, but as the name suggests, increase the sensitivity of tissues to insulin. Some insulin
sensitizers, such as the biguanides, inhibit hepatic gluconeogenesis whilst also increasing insulin sensitivity in muscle and fat (McIntyre, 1991; Hundal, 2000).

1.6.2 GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

The response of gut derived hormones, such as Glucagon-like peptide-1 (GLP-1), to ingested glucose has been termed the “incretin effect” and the secretion of GLP-1 is as a postprandial response by the gastro-intestinal tract (Elliott, 1993). In people with type 2 diabetes the incretin effect has been documented to be reduced (Nauck et al., 1986; Knop et al., 1986). GLP-1 is a hormone which is expressed in the intestinal L-cells of the gut (Drucker, 2006). Here cleavage of pro-glucagon by prohormone convertase 1 or PC1 results in the production of the GLP-1 hormone (Ugleholdt et al., 2004; Zhu, 2004). Endogenous GLP-1 has a short half-life of approximately 2 minutes which is mainly due to the rapid degradation by dipeptidyl peptidase IV or DPP-IV (Chia, 2005). GLP-1 response is glucose dependent and upon glucose ingestion, GLP-1 regulates insulin release from the β-cell in a dose-dependent manner (Kruger, 2006). In fact the secretion of GLP-1 is so dependent on glucose that upon achieving euglycaemia, GLP-1 secretion ceases. As an anti-diabetic agent, this greatly reduces the potential of patients experiencing hypoglycaemia. GLP-1 also demonstrates some beneficial regulatory effects on feeding and weight control and is seen to have a central effect on controlling satiety (Turton, 1996). For these reasons, interest has grown in this GLP-1 compound as a therapeutic agent to treat diabetes. Several publications indicate that GLP-1 has a role in β-cell protection and in increasing β-cell mass and proliferation (Wang, 1999; Perfetti, 2000; Drucker, 2003; Li Yanhou, 2003). Interestingly, the secretion of the GLP-1 hormone is decreased in type-2 diabetics compared with a healthy non-diabetic
population (Toft-Nielsen, 2001). This, and for many other reasons makes GLP-1 an ideal treatment for type 2 diabetes.

The response to GLP-1 is mediated by binding to the GLP-1 receptor, which is found on cells in the brain, stomach and adipocytes and on β-cells (Ranganath, 2008). The GLP-1 receptor is a seven transmembrane G-protein coupled receptor. Upon activation, adenylate cyclase is phosphorylated and this results in an activation of cyclic AMP (cAMP) (Holz, 2004; Ramos, 2008). This initial reaction is thought to be responsible for activating a number of pathways which are responsible for insulin secretion from the β-cell, glucose uptake from muscle and adipocytes, slowing gastric emptying and reducing appetite by both central and peripheral mechanisms. GLP-1 receptors on the pyloric sphincter of the stomach delay gastric emptying which may also increase satiety (Naslund, 1999). GLP-1 also controls satiety via a central mechanism. Diffusion allows GLP-1 receptors in the hypothalamus of the brain to be accessed by peripheral GLP-1 (Kastin, 2002). Here, GLP-1 is thought to exert an anorectic effect which may be responsible for weight loss (Alvarez, 2005).

In the β-cell, GLP-1 up-regulates the pancreatic duodenal homeobox-1 or PDX-1 transcriptional factor which is a key regulator of β-cell function (McKinnon, 2001; Yazhou, 2005). PDX-1 has been described as the master regulator of β-cell function. Glucose-stimulated insulin-secretion, via the GLUT2 transporter, is directly controlled by PDX-1 expression and the inactivation of this gene eventually results in the development of diabetes (Waeber, 1996). In other cell lines, GLP-1 has been shown to increase revascularisation markers which provides new therapeutic options for the use
of GLP-1 and outlines a potential role for GLP-1 in islet transplantation (Xiao-Yun et al., 2011; Wang, Qi et al. 2013).

DPP-IV degradation of GLP-1 renders this native compound useless as a therapeutic agent of which GLP-1 has a half-life of approximately 2 minutes (Hii, 2002). For this reason, DPP-IV inhibitors have been manufactured as a therapeutic option in the treatment regime for type 2 diabetes (Deacon et al., 1998; Green et al., 2006). DPP-IV inhibitors, also called “the gliptins”, must be administered orally in order to elicit their therapeutic effect and as such they offer an increase in patient compliance over intravenous GLP-1 agonists (Idris and Donnelly, 2007).

1.6.3 THIAZOLIDINEDIONES

The thiazolidinediones, such as Pioglitazone and Rosiglitazone, are nuclear peroxisome proliferator-activated receptor gamma (PPARγ) agonists which function to increase insulin sensitivity in muscles and adipose tissue (Smith et al., 2000). For this reason, the thiazolidinediones are known as the “insulin sensitizers”. The TZDs function also to lower circulating fatty acids which have a known contribution to the development of type 2 diabetes (Way et al., 2001). While TZDs are classed as insulin sensitizers, these drugs have demonstrated some protective effects directly on the pancreatic β-cell and for this reason have been incorporated into the treatment regimen to treat type 2 diabetes (Yang et al., 2003; Lupi et al., 2004).

Three known isoforms of the PPAR family of nuclear receptors are documented, PPARα, PPARβ/δ and PPARγ (Kliewer et al., 1994). PPARs bind to a specific DNA
sequence called the peroxisome proliferator response element (PPRE) as heterodimers with the retinoid X receptor RXR (DiRenzo et al., 1997; Juge-Aubry et al., 1997). The PPARα receptor is commonly expressed in cells responsible for the metabolism of fatty acids such as the liver, muscle and cardiac cells and is normally the target of lipid lowering medications (Gulick et al., 1994; Braissant et al., 1996; Palmer et al., 1998). The least well understood of these isoforms is the PPARβ/δ which has been associated with increased adipogenesis and decreased triglyceride formation (Bastie et al., 1999; Oliver et al., 2001). The PPARγ receptor is expressed on smooth muscle cells and adipose tissues and is involved in differentiation of adipocytes (Rosen et al., 1999).

Investigations into the mechanism of action of the antidiabetic TZDs identified that the mechanism of insulin sensitization was via direct binding with the PPARγ receptor (Lehmann et al., 1005; Berger et al., 1996). This process prevented the expression of tumour necrosis factor alpha (TNFα) in adipocytes and thus reduced the incidence of insulin resistance (Hotamisligil et al., 1995; Peraldi et al., 1997). TZDs have also been shown to increase key diabetes regulatory proteins GLUT4, GLUT2 and PDX-1 (Shimaya et al., 1997; Richardson et al., 2006, Kim et al., 2008). The use of the thiazolidinediones in treating diabetes however has diminished with the recent health warning from the MHRA regarding the cardiovascular risk associated with Rosiglitazone (MHRA, 2010). Alongside the anti-diabetic effects of TZDs, other laboratories have identified a role for Rosiglitazone in the regulation of angiogenic markers in various cell lines (Chu et al., 2006). This laboratory identified in 2011 that the addition of Rosiglitazone to the MIN6 pancreatic β-cell resulted in an increase in VEGF mRNA (Ferris, Marriott et al., 2011).
1.6.4 TYPE 1 DIABETES

1.6.4.1 Insulin

In 1993, the Diabetes Control and Complications Trial Research Group demonstrated the importance of tight glucose control on the prevention of secondary complications associated with T1D (Diabetes Control and Complications Trial Research Group, 1993). This study identified the absolute need for a replacement therapy that would not only provide insulin but also to act as a sensor which recognises low glucose levels, as a β-cell would do. The only therapy currently to treat Type 1 Diabetes is insulin. Insulin analogues are made in laboratories and are genetically or chemically modified to alter the speed of insulin release. This therapy, while adequate as an insulin replacement therapy, does not offer the patient the ability to tightly control glucose metabolism and has also been associated with increased incidences of hypoglycaemia (Diabetes Control and Complications Trial, 1991; Diabetes Control and Complications Trial Research Group, 1997). The necessity to provide a β-cell which can sensor circulating glucose and in response secrete appropriate correlating insulin levels is essential to the future of type 1 diabetes treatment and potentially a type 1 diabetes cure.

1.6.4.2 Transplantation

The process of transplantation dates back to the late 1960’s and early 1970’s. Surgeons performed whole pancreas transplants either simultaneously alongside the kidney, after kidney transplantation (PAK) or as a pancreas alone transplantation (PTA) (Sutherland, Goetz et al., 1984). An alternative method to treat and potentially cure type 1 diabetes is islet transplantation. Islet transplantation is a relatively new concept in the world of
diabetes and involves the isolation of islet cells from a deceased donor and the transplantation of these cells into the subsequent diabetic patient as demonstrated in Figure 1.2. It is the only treatment for type 1 diabetes which offers the patient the possibility of total insulin independence (Robertson, 2000). Nowadays transplantation for type 1 diabetes focuses on the transplantation of the specific insulin-producing islets of Langerhans. Numerous research groups worldwide are searching for the optimum protocol for islet transplantation, and leading the field is the Edmonton group who have reported seeing 100% insulin independence following transplantation (Shapiro, 2000). In this transplantation protocol, the pancreas is digested using the Liberase Purified Enzyme Blend which is a combination of collagenase isoforms I and II (Olack, Swanson et al., 1999) and approximately 5,000 islets per kilogram of recipient body weight are transplanted to the hepatic portal vein without culture and immediately following isolation (Shapiro, Ricordi et al., 2006; Pepper, Gala-Lopez et al., 2013). Under the guidance of radiography, islets are infused through a catheter into the portal vein and carried to the liver by the blood (Owen, Ryan et al., 2003). This protocol also requires a stringent corticosteroid-free immunosuppressive regimen of sirolimus once daily for three months post transplantation, tacrolimus is administered twice daily and five doses of daclizumab, a monoclonal antibody, is administered over a period of eight weeks to prevent organ rejection (Shapiro, 2000). Current data vary between clinical trials, however results show that insulin independence was achieved in certain patients and this complete independence was reduced by approximately 80% at 2 years (Gaglia, 2006; Shapiro, Ricordi et al., 2006). However, the incidence of hypoglycaemic attacks in patients with some residual function was reduced (Shapiro, Ricordi et al., 2006). The development of the process of islet transplantation is currently ongoing and ever-
changing (Froud, Baidal et al., 2008; Senior, Kin et al., 2012; Padmasekar, Lingwal et al., 2013; Rackman, Dhada et al., 2013; Benedini, Caumo et al. 2013).


Scientists and clinicians worldwide are currently in a desperate search to identify firstly the problems that are associated with the islet transplantation process and also what interventions are necessary to better the outcomes for type 1 diabetic patients opting to undergo islet transplantation. The need for more than one donor per recipient limits the amount of people who can receive this therapy. Scientists across the world are at present designing an optimum protocol for the differentiation of stem cells to pancreatic β-cells for transplantation which may ease the problem of insufficient donor pancreata (Shim, Kim et al., 2007; Bernardo, Cho et al., 2009).
Another problem associated with islet transplantation is the devascularisation of the pancreatic islet following isolation. Decreased vasculature leads to insufficient oxygen supply to the islet and may potentially result in decreased islet survival (Carlsson, Liss et al., 1998). Under the current protocol, revascularization of pancreatic islets may take several days following transplantation (Brissova, 2008). Thus it has been hypothesised that the β-cells may experience a period of hypoxia post isolation and that this hypoxic environment may be detrimental to β-cell function in the long term (Carlsson, Liss et al., 1998; Itoh, Iwahashi et al., 2011; Wang, Khan et al., 2013). The investigation of such on the pancreatic β-cell is imperative to the optimisation of the current islet transplantation protocol. Researchers worldwide are in a desperate search currently to develop oxygen delivery strategies which will increase reoxygenation of pancreatic β-cells and thus provide protection from hypoxic stress (Lazard, Vardi et al., 2012).

1.7 Hypoxia

A decrease in oxygen level is termed hypoxia, and the effect of such on pancreatic β-cells has yet to be investigated. Cellular adaptation to hypoxia plays a key role in the development of several diseases such as cancer (Salt, Johnson et al., 1998) and ischaemia (Buse, Henry et al., 2004). The effect of hypoxia on pancreatic islets has expanded in interest in recent times. This is mainly due to a reduction in β-cell survival post transplantation which has been associated with the low oxygenation of pancreatic islets (Carlsson, Liss et al., 1998). During the process of islet transplantation, cells are isolated from the pancreas and as a result are removed from their supporting vasculature (Mattsson, Jansson et al., 2002). A study using intravital fluorescence microscopy
identified that the length of time that islet cells remain without a distinctive vascular supply can be up to ten days with initial signs of revascularisation between days two and four (Vajkoczy, Menger et al., 1995). These results suggest that the islet cells are undergoing a period of hypoxia immediately post isolation. In hypoxia, cells can increase oxygen delivery by the induction of revascularisation genes such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α (HIF 1α) (Forsythe, Jiang et al., 1996; Semenza, 1998). The essential biological response of cells to hypoxia is to reduce oxygen consumption in the oxygen limited environment by switching to anaerobic metabolism (Skelin, Rupnik et al., 2010).

**1.7.1 OXYGEN SENSING AND METABOLISM**

Oxygen is essential to the maintenance of life and the control of cellular processes, such as metabolism. It is used by cells in the generation of ATP molecules from carbohydrates (Taylor, 2007). The ability of cells to sense oxygen concentration and respond is essential to the study of hypoxia. Although the exact mechanism each cell adopts in the oxygen response is unknown, it has been hypothesised that the mitochondria may play an important part in oxygen sensing, being the key oxygen consumers and ATP generators within the cell (Duchen, 1999). In a normoxic environment, in a process known as oxidative phosphorylation, glucose is metabolised to CO₂ and water in the presence of oxygen and will generate 38 ATP molecules (Brahini-Horn, Cliche et al., 2007). In a hypoxic low oxygen environment, a notable reduction in ATP generation has been confirmed in various cells (Kwast and Hand, 1996; Julian, Fraisl et al., 2009). Thus cells will need to turn to an alternative source by which they can generate ATP. In previous investigations, this source has been
documented to be glucose which, in contrast to oxidative phosphorylation, generates only 2 molecules of ATP (Brahimi-Horm and Pouyssegur, 2007). Thus, this hypoxic switch to a glycolytic method of ATP generation may explain the reduction in ATP generated in cells exposed to hypoxia.

1.7.2 HYPOXIA INDUCIBLE FACTOR (HIF)

An essential factor in understanding the hypoxic response of cells is the regulation of hypoxic response proteins, such as hypoxic-inducible factor α (HIFα) (Wang, Jiang et al., 1995; Semenza, 1998). HIF consist of two subunits, the oxygen sensitive α unit and the oxygen insensitive β unit (Wang, Jiang et al., 1995). In cells cultured under normoxic conditions (atmospheric oxygen), HIFα levels are regulated by a proteosomal degradation. In the presence of oxygen, prolyl-hydroxylase 2 (PHD 2) hydroxylates the alpha subunit of the HIF compound, these residues are termed the oxygen sensitive degradation domains (Jaakkola, 2001; Berra, 2003). Hydroxylation of the HIFα subunit ensures binding to the von Hippel–Lindau VHL protein which renders the complex susceptible to ubiquitylation proteosomal degradation and thus inactivation (Kallio, 1999; Maxwell, 1999). As hydroxylation requires the presence of oxygen, in low oxygen conditions PHD is unable to hydroxylate HIFα (Ward, 2008). The result is a dimerization of HIFα with HIFβ and the formation of a stable complex which is capable of transcriptional activity by the binding to a hypoxic response element of hypoxic response genes. Hypoxia response elements (HRE) contain a HIF-1 attachment position with a sequence of 5’-RCGTG-3’ which encodes for several genes including VEGF, glucose transporters and glycolytic enzymes (Forsyth, Jiang et al., Semanza, Jiang et al., Seagroves, Ryan et al., 2001). Thus the role of HIF as a key hypoxic responsive protein
may prove essential to the revascularisation and survival of cells during the islet transplantation process (Stokes., Cheng et al., 2013).

1.7.3 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND ANGIOGENESIS

Angiogenesis is defined as the formation of new capillaries from active blood vessels in an effort to supply nutrients to cells (Owen, Ryan et al., 2003). The role of angiogenesis in the development of diseases such as tumour development has long been investigated (Lichtenbeld, van Dam-Mieras et al., 1996; Cao, 2005; Morales-Guitierrez, Abad-Barahona et al., 2011). In response to hypoxia, HIFα signals several genes, one of which has been identified as vascular endothelial growth factor A or VEGF-A (Forsythe, Liang et al 1996). VEGF-A is a renowned regulator of angiogenesis and proliferation of endothelial cells with several isoforms forming from the alternative splicing of just one gene (Shima, Kuroki et al., 1996; Ferrara, 2003). Although there are several different members of the VEGF family of proteins, it is the VEGF-A protein which is known to have a vascularisation effect in the pancreas (Inoue, 2002). Following transplantation, islets are thought to produce VEGF-A to promote revascularization (Brissova 2006). Production of VEGF-A might be able to assist in islet revascularisation and thus increase β-cell survival following transplantation.
Figure 1. 3 Schematic representation of the AMPK/mTOR/S6K pathway
A shift in the AMP:ATP ratio can result in an increase in the phosphorylation of AMP activated protein kinase and a resulting increase in the membrane translocation of glucose transporters, GLUT. Activation of this protein also results in the inactivation of the mammalian target of rapamycin mTOR pathway and as a result the reduction of ribosomal S6 kinase and 5’ UTR translation.

1.7.4 5’ ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE (AMPK)
AMP-activated protein kinase acts as an energy sensor in the cell (Carling, Mayer et al., 2011). During times of low ATP generation, concomitant increase in AMP levels results in the activation of AMPK (Pan and Hardie, 2002; Carling, Sanders et al., 2008). AMPK is a heterotrimeric compound which consists of three key subunits. The first is a catalytic subunit, AMPKα, responsible for the enzymatic activity of the protein while AMPKβ and AMPKγ are the regulatory subunits (Stapleton, 1996; Kahn, Alquier et al., 2005). AMPK is activated by binding of AMP to the AMPKγ subunit which causes a conformational change to the kinase (Sanders, 2007). This change is thought to allow phosphorylation of the threonine-172 site on the α subunit of AMPK (Jeyabalak, Shah
et al., 2012). Tumour suppressor kinase-liver kinase B or LKB1, calmodulin dependent kinase kinase CaMKK and transformation growth factor-β-activated kinase TAK1 are three specific kinases that have been identified upstream of AMPK and are thought to be responsible for phosphorylation of the α subunit (Steinberg and Kemp, 2009; Jeyabalan, Shah et al., 2012). The binding of AMP to the γ subunit of AMPK is also believed to inhibit dephosphorylation of the α subunit (Sanders, 2007). Combined, each of these markers may contribute to the regulation and response of AMP activated protein kinase to various stimuli. As previously mentioned, AMPK is a key modulator of energy homeostasis in the cell and as such is responsible for conserving energy use during times of stress (Hardie, 2011). AMPK activation is coupled with a direct inhibition of mTOR, the mammalian target of rapamycin, and this reduces protein synthesis during times of energy reduction in an attempt to preserve ATP consumption (Shaw, 2009). AMPK is also associated with increased regulation of glucose transporters upon activation by AMP and thus may use this to increase ATP generation (Hallows, 2005).

1.7.5 MAMMALIAN TARGET OF RAPAMYCIN (MTOR)

mTOR, the mammalian target of rapamycin, is a serine/threonine kinase which controls cell growth and survival (Wullschleger, Loewith et al., 2006). Structurally, mTOR has two distinct complexes, mTORC1 and mTORC2 (Wullschleger, Loewith et al., 2006). mTORC1 or mammalian target of rapamycin complex 1 is the rapamycin sensitive complex consisting of five components; mTOR, regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with sec13 protein 8 (mLST8), proline-rich AKT substrate 40kDa (PRAS40) the DEP-domain-containing mTOR-interacting protein
(DEPTOR) (Hara, Maruki et al., 2002; Guertin, Stevens et al., 2006; Thedieck, Polak et al., 2007; Laplante and Sabatini, 2009). Upstream mTORC1 can be activated or inactivated in response to several different stimuli. Increased PI3K/Akt/mTOR pathway stimulation is associated with poor prognosis in many cancers due to the increased cell proliferation associated with the stimulation of this pathway (Gao, Zhang et al., 2003; Meng, Xia et al., 2006; Johnson, Gulhati et al., 2010). Increased activation of mTOR results in the enhanced phosphorylation of the key proliferation factor ribosomal S6 kinase which results in an increase in cell proliferation (Isotani, Hara et al., 1999; Meng, Xia et al., 2006). AMPK activation works to inhibit the activity of mTORC1 via the TSC1/TSC2 complex while PI3K/Akt activation leads to an increase in activity of mTORC1 (Hay and Sonenberg, 2004; Shaw, 2009). Depletion of ATP can lead to a concomitant increase in AMP, this in turn will lead to AMPK activation (Carling, Sanders et al., 2008). Activation of AMPK can lead to a further decrease in mTOR/S6K pathway and reduced cell proliferation (Naoki, Chiharu et al., 2003).
Hypoxia may result in the inactivation of phosphatidylinositol 3-kinase. This results in downstream inactivation of Akt/protein kinase B and the mammalian target of rapamycin mTOR. A reduction in expression of ribosomal S6 kinase and 5’ UTR translation is observed following inhibition of mTOR.

1.7.6 PHOSPHATIDYLINOSITOL 3-KINASE (PI3K)

Phosphatidylinositol 3-kinase family of lipid kinases have a central role in controlling cell cycle progression and angiogenesis (Gao, Flynn et al., 2004; Skinner, Zheng et al., 2004; Fang, Ding et al., 2007). PI3K compounds are characterised by their ability to phosphorylate inositol phospholipids in the generation of second messengers (Vara, Casado et al. 2004). Phosphorylation of the inositol ring of phosphatidylinositol (PtdIns) generates membrane lipids such as, PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (Toker, 2000). PI3K is an active upstream regulator of mTOR and thus in several studies the activation of the mTOR pathway is also dependent upon PI3K activity.
(Brunn, 1996, Sekulic, Hudson et al., 2000; Hay and Sonenberg, 2004). In cancer cells, the regulation of S6 kinase by PI3 Kinase is an important controller of cell growth (Meng, Xia et al., 2006). While the regulation of PI3K is varied amongst different cell lines in response to low oxygen, an increase in PI3K and subsequent mTOR/S6K protein expression is associated with an increase in cell proliferation during hypoxia (Humar, Kiefer et al., 2002; Gerasimovskaya, Tucker et al., 2005).

1.7.7 AKT, PROTEIN KINASE B

Akt, also known as protein kinase B (PKB) is a serine/threonine kinase and under circumstances where the lipid kinase PI3K is activated, serial phosphorylation steps results in the production of a second messenger, PIP₃, which in turn leads to the translocation of the Akt protein to the cell membrane (Andjelkovic, Alessi et al., 1997). Once at the cell membrane, Akt is activated by the phosphatidylinositol-dependent protein kinase-1 (PDK-1) at the activation loop threonine 308, Thr³⁰⁸, residue (Alessi, James et al., 1997). A further phosphorylation step is required for the activation of Akt as mTORC2/rictor is documented to phosphorylate the “hydrophobic motif” HM serine residue serine⁴⁷³ of Akt (Hresko and Mueckler, 2005; Sarbassov, Guertin et al., 2005).

The PI3K/Akt/S6K pathway is associated with cell growth and proliferation as a reduction in Akt is observed during times of low proliferation (Manning, Tee et al., 2002; Skeen, Bhaskar et al., 2006). Hypoxia differentially regulates Akt and thus the regulation of Akt in response to hypoxia is highly dependent upon the cells investigated (Obara, Utsugisawa et al., 2002; Gerasimovskaya, Tucker et al., 2005).
1.7.8 RIBOSOMAL S6 KINASE

The 70kDa ribosomal S6 kinases are extensively studied serine/threonine kinases which control several cellular functions including cell proliferation and are a key component of the translation process (Dufner and Thomas, 1999; Liu, Cash et al., 2006). The S6 kinase protein is a component of the 40s ribosomal subunit and has two recognized isoforms, S6K1 (S6K α) and S6K2 (S6Kβ) (Gout, Minami et al., 1998). The phosphorylation of S6 by p70S6k results in an increase in translation of mRNA containing the polypurine tract in the 5’ untranslated region (Jefferies, Fumagalli et al., 1997). Several upstream mediators have been implicated in the activation of S6K, most notably mammalian target of rapamycin (Jefferies, Fumagalli et al., 1997; Schalm and Blenis, 2002). S6 kinase has long been investigated as a target of hypoxic stress and has been shown to reduce energy consuming pathways of the cells, such as protein translation, as a mechanism of energy preservation (Liu, Cash et al., 2006). Respectively, hypoxic tumours are associated with an increase in the S6K gene which is associated with the increased cell proliferation observed in hypoxic tumours (Nozawa, Watanabe et al., 2007; Ismail, 2012).

1.7.9 NUCLEAR FACTOR KAPPA B

The transcription factor NFκB controls several cellular processes such as apoptosis, cell growth and inflammation (Grimm, Bauer et al., 1996; Guttriddge, Albanese et al., 1999). NFκB is activated in response to various stimuli including growth factors, death receptors and ischaemic stress amongst many others (Baldwin, Azizkhan et al., 1991; Koong, Chen et al., 1994; Gabriel, Justicia et al., 1999). During times of inactivation, NFκB remains in the cytoplasm in a dimerised state with the inhibitor of κB complex,
IκB (Baeuerle and Baltimore, 1988). Following activation via an external stimulus, IκB is phosphorylated and degraded by ubiquitylation and proteosomal degradation (Link, Kerr et al., 1992). This renders the κB unit active and thus translocates to the nucleus where it binds to DNA and regulates the transcription of target genes (Chen, Huang et al., 1998; Wan, Anderson et al., 2007). The NFκB family consists of five important proteins, p65/RelA, p75/c-Rel, RelB, p50/p105, and p52/p100 each of which are involved in DNA binding and gene transcription (Coleman, Kunsch et al., 1993; Chen, Huang et al., 1998; Wan, Anderson et al., 2007).

1.8 Laboratory investigations of Diabetes

Due to the complexities of gaining human in vitro models of diabetes for research, several rodent cell lines are currently used by researchers in the area of diabetes research. These include the INS-1 and BRIN-BD11 cells lines derived from rats and the MIN6 cell line derived from mice. The choice of cell line is dependent upon the culture conditions and timescales involved within the research. INS-1E and BRIN-BD11 were not used for this research as routine culture of these cells requires media containing higher levels of glucose than would be physiologically experienced by a healthy β-cell (Asfari, Janjic et al., 1992; McClenaghan, Barnett et al., 1996). This research used a murine in vitro model of diabetes, the MIN6 mouse insulinoma transgenic β-cell (Ishihara, Asano et al., 1993). We chose to use the MIN6 β-cell line as this laboratory routinely cultures these cells at physiological glucose concentrations and we can grow MIN6 cells for up to five days before reaching contact inhibition. These culture conditions and timescales are essential to the enquiries of this research.
1.9 Aims

Beta-cell transplantation may provide us with a potential cure for type 1 diabetes. However, the number of people who actually receive the benefits from beta-cell transplantation is low. Hypoxia, or low oxygen concentration, has been identified as one of the key problems in the transplantation process. One of the main problems with the investigation of hypoxia and the effect of such on the β-cell is the provision of a low oxygen environment in which to perform such investigations. This study used a hypoxic chamber, which mimics the low oxygen environment during transplantation, and allowed us to understand the clinical implications of hypoxia on beta cell function for the first time.

This research investigated four key areas which we believe may provide the answers that islet transplantation studies require to explain why the transplantation primary outcomes have been falling short.

1) We began by investigating the effect of hypoxia on the viability of the MIN6 β-cell.

2) Then we endeavoured to identify the intracellular pathway adopted by the β-cell following exposure to hypoxia in an attempt to elucidate the markers controlling β-cell viability during transplantation.

3) We then investigated the ability of the β-cell to revascularise during the transplantation process by investigating the regulation of key revascularisation markers by the β-cell.
4) Finally, we attempted to identify the glucose-stimulated insulin secretory response of these cells during exposure to hypoxia.

It is hoped that by identifying the specific role of hypoxia on the insulin-producing β-cell of the pancreas that this research will outline some of the problems experienced by the β-cell post-isolation. We believe that understanding these essential issues will better the outcomes associated with islet transplantation and ultimately lead to people with type 1 diabetes living an insulin independent life.
Chapter Two: Materials and Methods

2.2 Materials

Cell culture media, foetal bovine serum (FBS) and trypsin were purchased from PAA, UK. Western blotting material purchased from Amersham, Biosciences UK. PDX-1 antibody was a gift from Professor Chris Wright, Vanderbilt University, Nashville, Tennessee, USA. GLUT2, pAkt, pS6K, PI3K, NK-κB and pAMPKα antibodies were from Abcam, UK. Bradford protein assay reagent and SYBR Green was purchased from Bio-Rad Laboratories, UK. BrdU components were purchased from DAKO, UK. Reverse transcription kit was purchased from Invitrogen, UK. All other chemicals were purchased from Sigma, UK. Consumables were purchased from Fisher Scientific, UK.

2.2 Methods

2.2.1 CULTURE CONDITIONS

Cells were subcultured when approximately 80% confluent. Cells were grown in T75 flasks. Once confluent, media was removed and cells washed with 10ml phosphate buffered saline (PBS). To dislodge cells from the flask, 2ml of 0.05% w/v trypsin/0.5mM EDTA solution was added to each flask and incubated for approximately 5 minutes at 37°C. Once cells were dislodged, approximately 8ml of media was used to neutralise the trypsin solution and the total contents from the T75 flask was transferred to a 25ml universal tube. All cell lines were centrifuged at 500g for 5 minutes. Media
was removed and the pellet of cells re-suspended in 1ml of media. From this, cells were counted using a haemocytometer and then seeded for experimentation.

2.2.2 CELL LINES

MIN6 mouse β-cells (passage 27-34) were cultured in DMEM 5mM glucose containing 2mM L-glutamine and supplemented with 10% foetal bovine serum and 100 IU/ml Penicillin and Streptomycin 100μg/ml. MIN6 cells are a known and vastly used β-cell line in the study of diabetes and glucose stimulated insulin secretion and for this reason were chosen in this study (Isihara, 1993).

2.2.3 CULTURE CONDITIONS

Normoxic culture conditions were set to 21% oxygen, 5% carbon dioxide and cells were cultured at 37°C. Hypoxic culture conditions were set to 1% oxygen, 5% carbon dioxide and 94% nitrogen. Unless stated otherwise, cells were routinely cultured in normoxic conditions. Hypoxic glove box is detailed on Figure 2.1.

![Figure 2.1: Picture of Hypoxic Glove Box from COY laboratories.](image)

Hypoxic glove box used in these studies has the facility to control cells under an oxygen controlled environment and also encompasses a temperature, humidity and carbon dioxide control options.
2.2.4 MTT VIABILITY ASSAY:

MTT is reduced by mitochondrial enzymes to an insoluble crystal which is dissolved by a detergent and read by a spectrophotometer at 570nm. Following specified experimental conditions, media was removed from all cells and cells were washed with phosphate buffered saline (PBS). 1ml of MTT solution 0.5mg/ml was added to each well in a 6-well plate and incubated for 1 hour for MIN6 cells. Following this, MTT solution was aspirated and 1ml DMSO (dimethyl sulfoxide) solution added to each well to dissolve the crystals. DMSO was left on each well for 5 minutes to allow dissolution of crystals. A 200µl sample from each well was transferred in duplicate to a 96-well plate and read at 570nm spectrophotometer from Multiscan Plate Reader, Thermo Scientific®. A DMSO control was also transferred to a well in the 96-well plate as a background control reading.

2.2.5 QUANTIFICATION OF CELL NUMBER BY HAEMOCYTOMETER

Haemocytometer count was used to optimise an equal seeding density for cell viability, cell counting and H/PI experiments. In experiments which required cell number to be determined post experimentation, 200µl of trypsin was placed on cells in each well of a 6-well plate. Following incubation in 37°C cells were re-suspended in 800µl of media in aliquots of 200µl to ensure all cells were removed from the base of each well. Cells solution was then transferred to an eppendorf and cells were pelleted by centrifugation (500g for 5 minutes) and re-suspended in media. Cell number was calculated using a haemocytometer.
2.2.6 HOECHST PROPIDIUM IODIDE (HPI)

The Hoechst propidium iodide stain identifies cellular apoptosis and necrosis. This technique uses Hoechst fluorescent dye to stain chromatin of apoptotic cells a brighter blue than the chromatin of healthy cells which stain a light blue (Latt and Stetten, 1976). The pink fluorescence of propidium iodide stains the DNA of necrotic cells and this is based on the inability of propidium iodide to traverse the cell membrane in healthy cells and to readily stain the DNA of dead cells (Riccardi and Nicoletti, 2006). A solution containing 5mg/ml Hoechst and 1mg/ml propidium iodide was prepared in cell culture media. Following cellular exposure to experimental conditions, media was removed from each well in 6-well plates and all wells were washed with PBS. A 200µl solution of HPI mixture was added to each well and incubated for 5 minutes in foil and at 37°C. In a dark area, and under fluorescence microscopy, viable cells were detected from apoptotic and necrotic cells. Cells were observed using an Axiovert 25 microscope equipped with fluorescence and digital camera.

2.2.7 PREPARATION OF WHOLE CELL, NUCLEAR AND CYTOPLASMIC EXTRACTS:

Cells were seeded in 10cm cell culture plates. Upon extraction, cells were washed with phosphate buffered saline (PBS) and 1ml PBS was added to each plate. Cells were scraped from the base of the plate and centrifuged for 5 minutes. The supernatant was removed and the pellet re-suspended in 200µl buffer A (10mM HEPES pH 7.9; 10mM KCl, 0.1mM EDTA pH 8.0, EGTA pH 8.0, 1mM DTT, 1 X protease inhibitor cocktail in water) and incubated on ice for 15 minutes.
Whole cell: 12.5µl of 10% Triton X added to buffer A solution and incubated on ice for 30 minutes. Cells were then centrifuged for 60 seconds. Supernatant was removed and snap frozen in liquid nitrogen.

Cytoplasmic: 12.5µl of 10% NP-40 added to buffer A and solution vortexed for 30 seconds. Cells are then centrifuged for 45 seconds. Supernatant was removed and snap frozen in liquid nitrogen.

Nuclear: Following cytoplasmic extraction, pellet was re-suspended in 50µl buffer C (20mM HEPES pH 7.9; 400mM NaCl, 1mM EDTA pH 8.0, 1mM EGTA pH 8.0, 1mM DTT, 5% glycerol, 1 X protease inhibitor cocktail in water) and incubated shaking for 1 hour at 4°C. Cells were centrifuged for 30 seconds and supernatant was removed and snap frozen in liquid nitrogen.

2.2.8 **BRADFORD ASSAY**

Bradford assay was used to determine the amount of protein in a given sample. Protein assay dye (BIO-RAD) was used at a concentration of 4:1 water:dye. Firstly a standard curve was determined by using bovine serum albumin, BSA, at various known concentrations i.e. 0, 1, 2, 4, 6, 8, 10, 16 and 20µg in 1ml of protein assay dye solution. In order to determine the concentration of protein in each sample, 3µl of each was added to 1ml protein assay dye. Following inversion of each cuvette, the absorbance of each sample was measured using a spectrophotometer (Eppendorf Biophotometer®, 8.5mm) at 595nm.
2.2.9 SDS-PAGE & WESTERN BLOT ANALYSIS

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique was used to separate proteins from a given sample according to mobility on a polyacrylamide gel. Following protein extraction and protein Bradford assay, an equal amount of sample was prepared and made up in a 1:1 SDS buffer solution (0.1% bromophenol blue, 1.25M Sucrose, 1M Tris-HCl pH 6.8, 20% sodium dodecyl sulphate and 10% β-mercaptoethanol). SDS acts as a detergent and thus denatures the protein and donates a negative charge. All SDS-PAGE, in this study, use a 10% polyacrylamide gel. Resolving gel was composed of 30% acrylamide/bis, 1.5M Tris-HCl pH 8.8, 10% SDS, 10% APS (ammonium persulphate) and TEMED. The Stack gel was made from 30% acrylamide/bis, 1M Tris-HCl pH 6.8, 10% SDS, 10% APS and TEMED. A molecular weight marker was incorporated in the first lane of the gel to assist in identifying the approximate size of the proteins on the gel. Samples for PDX-1, GLUT2, pS6k, S6K, pAkt, pAMPKα, NF-κB and PI3K analysis were run for 1 hour at 150V in running buffer (0.3% Tris base, 1.44% glycine and 0.1% SDS).
<table>
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<th>Volume</th>
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<td>Water</td>
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<tr>
<td>30 % Acrylamide/Bis mix</td>
<td>3.3ml</td>
</tr>
<tr>
<td>1.5M Tris-Hcl pH 8.8</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% Sodium Dodecyl Sulfate</td>
<td>100µl</td>
</tr>
<tr>
<td>10 Ammonium Persulphate</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4µl</td>
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**Table 2.1:** Reagents used in 10% SDS-PAGE resolving gel

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<th>Reagent</th>
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<tr>
<td>Water</td>
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<tr>
<td>30 % Acrylamide/Bis mix</td>
<td>830µl</td>
</tr>
<tr>
<td>1.5M Tris-Hcl pH 8.8</td>
<td>630µl</td>
</tr>
<tr>
<td>10% Sodium Dodecyl Sulfate</td>
<td>50µl</td>
</tr>
<tr>
<td>10 Ammonium Persulphate</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
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</tbody>
</table>

**Table 2.2:** Reagents used in 10% SDS-PAGE stacking gel
Once the samples are separated on the SDS-PAGE gel, the gel contents were then transferred to a PVDF membrane. Transfer was performed using the TRANS-BLOT® semi-dry blotting apparatus. Transfer was set up for Western blotting by soaking one sheet of thick filter paper in transfer buffer (0.3% Tris-base, 1.44% glycine). All air bubbles were removed after placing on the transfer apparatus. A small strip of PVDF membrane, drenched in methanol, was placed in the filter paper and the recently run SDS-PAGE gel placed directly on PVDF membrane. This was also performed carefully to ensure no air bubbles were present. Finally, another layer of filter paper was soaked in transfer buffer and placed in top of the gel. All air bubbles were removed from the filter paper before transferring for 30 minutes, 15V with a current limit of 200mA for small proteins <80 kDa. For larger proteins >80 kDa transfer was continued for one hour. After transfer, membranes were blocked in 50ml of a 10% dried milk solution for non-phosphorylated proteins, or 5% bovine serum albumin for phosphorylated proteins, for 60 minutes with agitation. Following this, the membrane was washed in wash buffer (200mM Tris-HCl pH 7.6, 14.4% NaCl, 2.5ml Tween 20) for 3 X 5 minutes.

For immunodetection, the membrane was then placed in rotation with primary antibody overnight at 4°C. Concentration of primary antibody varied depending on the antibody being used. PDX-1, α-tubulin, PI3K, NFκB and GLUT2 antibody was used at a concentration of 1:5000. pS6k, pAkt and pAMPKα antibodies were used at a concentration of 1:1000. Following this, the membrane was thoroughly washed, 2 X rinse, 6 X 5 minutes wash in 1 X wash buffer, before 1 hour incubation in secondary HRP linked (horse radish peroxidase) anti-rabbit or anti-mouse 1:5000 antibody at room temperature. After 1 hour incubation, the membrane was washed 2 X rinse, 6 X 5
minutes wash in 1 X wash buffer. Membranes were developed using ECL plus detection kit. ECL or enhanced chemiluminescence is a detection system used in Western blotting to recognise immobilized proteins. The HRP-linked secondary antibody catalyzes the oxidation of luminol in the presence of hydrogen peroxide. Oxidation of luminol leads to the emission of light at 425nm which is detected by X-ray film making it possible to detect the position of proteins on a membrane by visualisation. Molecular weight comparison with the molecular weight marker identifies the specific protein of interest.

2.2.10 BRDU 5-BROMODEOXYURIDINE

The identification of proliferating cells within a given culture was performed by immunohistochemical staining. Under culture conditions, BrdU is added to media and is incorporated into the newly synthesised DNA of the S-phase cells and can thus provide an estimate for the fraction of cells in S-phase at a given time. Antibodies against BrdU recognize only BrdU incorporated during DNA synthesis.

Cells were seeded onto coverslips and allowed to attach and grow for 24 hours. During specified times during experimentation, 35µM BrdU was added to cells and allowed to be up-taken by cycling cells. At the end of experimentation, media was removed from cells and cells washed with PBS. Cells were fixed directly onto coverslips using 70% ethanol for 20 minutes at room temp. Following this, ethanol was removed and cells permeabilised using 4M HCL for 20 minutes at room temp. HCl was removed and coverslips washed twice with PBS/T. The endogenous peroxidase sites were then blocked by the addition a solution of 0.3% H₂O₂ + 0.3% normal serum in PBS, for 5 minutes at room temp. Cells were again washed twice with PBS/T. A humid chamber was manufactured using 10cm petri dishes, 2 sheets of filter paper and parafilm wrap.
Coverslips were transferred into the humid chamber and primary antibody at a concentration of 1:50 added to each coverslip and incubated 18-24 hours at room temperature. Following incubation, cells were washed in 3 changes of phosphate buffered saline/0.5% tween20 PBS/T. Excess solution was removed and coverslips placed back into the humid chamber. Secondary antibody, 1:200, was added to all coverslips and incubated at room temperature for 1 hour. Wash in 3 changes of PBS/T place the coverslips into an individual well in a 6-well plate. A DAB solution from DAKO was used to stain all target nucleic acids using a peroxide based immunohistochemical stain. DAB solution was added until all target antigens were stained brown. For MIN6 cells this time was 5-10 seconds. DAB solution was removed and each coverslip covered with haematoxylin (Mayers) for 60 seconds, until all nuclei were stained blue. Cells were dehydrated according to the following protocol; 1 x 95% ethanol for 10 seconds; 1 x 100% ethanol for 10 seconds; 1 x histoclear for 10 seconds. All excess solution was drained off and coverslips were mounted on larger coverslips using permanent mountant. Slides were viewed using a light microscope.

In order to calculate the total percentage of proliferating cells a total of 1000 cells per coverslip must be counted and percentage proliferating cells calculated as follows number of positive cells/ total number cells x 100 = % proliferating cells.

2.2.11 INSULIN SECRETION ELISA

The Mercodia Mouse Insulin ELISA was performed to quantify the amount of insulin secreted from MIN6 cells. In these experimentations, cells were seeded in 10cm petri dishes and experiment initiated once cells reached 50% confluency. MIN6 cells were cultured in 5mM basal glucose. Under different experimentation conditions cells were
cultured in 0mM glucose for one hour. Following a washing step, cells were then
cultured in 25mM glucose for one hour. Media was removed at all steps of
experimentation for analysis.

In order to perform an insulin ELISA 10µl of calibrators and unknown samples were
added to each well and each well labelled on 96-well plate lid. 100µl of 1 x enzyme
conjugate was added to each well and plate incubated on plate shaker for two hours at
room temperature. Wells were washed six times with 350µl of 1 x wash buffer solution.
200µl of substrate TMB was added to each well and incubated for 15 minutes at room
temperature. 50µl of stop solution was added to each well and the plate incubated on a
shaker for five minutes. The plate was read at 450nm on Multiscan Plate Reader,
Thermo Scientific®.

2.2.12 RNA EXTRACTION

RNA was extracted from cells according to the manufacturer’s protocol using
GenElute™ Mammalian Total RNA Miniprep Kit from Sigma Aldrich. Briefly, cells
were seeded and treated as according to the stated experimental conditions. Cells were
then harvested by scraping. Pelleted cells were re-suspended in lysis solution and
filtered until a pure RNA extract of approximately 50µl was collected. This extract was
either used immediately, frozen at -20°C or snap frozen for later use and stored in -
80°C.

2.2.13 DNASE TREATMENT

RNA extracts were treated with DNase to remove any genomic DNA contamination
from the RNA extracts. 1µl of DNase enzyme, 1µl of DNase buffer and 8µl of RNA
sample were added to a tube at 37°C for 30 minutes. 1µl of stop buffer was added to stop DNase reaction and the reaction tube was incubated at 65°C for 10 minutes.

### 2.2.14 RNA QUANTIFICATION/NANODROP

RNA quantification was performed using the Nanodrop 2000 from ThermoScientific® at 260nm. This method of RNA quantification also determined purity of the RNA sample using the 260/280 ratio. An ideal ratio of sample purity is 1.9-2.1. Any sample outside of this range was excluded from the experiment.

### 2.2.15 REVERSE TRANSCRIPTION

Reverse transcription is a method which converts single stranded RNA into double stranded cDNA which can then be analysed by polymer-chain reaction and is a key step in the analyses of mRNA. A reverse-transcription and cDNA synthesis kit from Invitrogen contained all the products required for cDNA synthesis. A mix of 1µl 50µM Oligo(dT), 2µl 10mM dNTP mix and 5µg RNA sample and 4µl DEPC water was denatured by incubating at 65°C for 5 minutes. A master mix was prepared for all reaction tubes in total. Each reaction tube required 4 µl 5 X cDNA synthesis buffer, 1 µl 0.1M DTT, 1µl RNaseOUT (40U/ µl), and 1 µl DEPC water. Cloned AMV was omitted for the negative samples. 7 µl of master mix and 1 µl of DEPC water were added per tube of negative samples. 1 µl cloned AMV was added to each positive sample tubes. Tubes were incubated at 55°C for 45 minutes followed by 85°C for 5 minutes. These samples were used for PCR.
2.2.16 REAL-TIME POLYMERASE-CHAIN REACTION

PCR amplification of DNA can be performed on reverse transcribed RNA samples using the Rotor-Gene Q from Qiagen. SYBR Green binds to DNA and is used to quantify the amount of DNA in a PCR reaction. Each PCR vessel was made up with 12.5µl SYBR Green, 2µl 5’ primer, 2µl 3’ primer, 2µl reverse transcribed reaction and 6.5µl DEPC water. Each sample was run in triplicate. PCR was concomitantly performed for negative samples. This allowed us to exclude contamination and ensure amplification was just from reverse transcribed samples. Contamination products, primer dimers and negative amplification products were analysed using the melt curve.

2.2.17 REAL-TIME PCR CALCULATION

Calculation of relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ calculation which assumes equal efficiencies when efficiency is 100% (+/- 5%) as described by Livak et al (Livak and Schmittgen, 2001). The calculation is deemed to predict relative gene expression, as opposed to absolute gene expression, as an unknown quantity of gene of interest and housekeeping gene are serially diluted to provide a standard curve, from which the efficiency is determined. From this efficiency we perform the below calculation which provides a ratio of treated (hypoxia) versus untreated (normoxia) Briefly:

\[
\begin{align*}
(1) \quad & \Delta Ct = \text{Gene of Interest (i.e. \textit{vegf})} - \text{Housekeeping Gene (i.e. \textit{\(\beta\)-actin})} \\
(2) \quad & \Delta \Delta Ct = \Delta Ct \text{ (treated)} - \Delta Ct \text{ (untreated)} \\
(3) \quad & 2^{-\Delta \Delta Ct} = 2^{-\left(\Delta Ct \text{ (treated)} - \Delta Ct \text{ (Untreated)}\right)}
\end{align*}
\]

Real-time PCR conditions were as follows:
**GLUT2**

*glut2F* (mouse forward)

CGGTGGGACTTGTGCTGCTGG

*glut2R* (mouse reverse)

CTCTGAAGACGCCAGGAATTCCAT

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**β-ACTIN**

*β-actin* (mouse forward)

TTCTGGGGCATGGAGTCCTGT

*β-actin* (mouse reverse)

CGCCTAGAAGCATTTGCGGTG

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<tr>
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**HIF-1α**

*hif-1α* (mouse forward)

TGCTTGGGTGCTGATTTGTGA

*hif-1α* (mouse reverse)

GGTCAGATGATCAGAGTCCA

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**VEGF**

*vegf* (mouse forward)

AGCCAGAAAATCACTGTGAGC

*vegf* (mouse reverse)

GCTTGTCACTCAGCAAGTACG

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INSULIN

*insulin* (mouse forward)

TCCGCTACAATCAAAAACCAT

*insulin* (mouse reverse)

GCTGGGTAGTGTTGGTCTA

<table>
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2.2.18 DATA & STATISTICAL ANALYSIS:

All data is normally distributed. For this reason, all data analysis was performed using a paired t-test, unless otherwise stated. Significance was detected at a p-value of < 0.05 and denoted a * symbol. All data < 0.01 was denoted a ** symbol.
Chapter Three: β-cell Viability in Hypoxia

3.1 Introduction

Type 1 diabetes is an autoimmune disease of the endocrine pancreas. In this disease, autoimmunity results in the destruction of the insulin-producing β-cells of the islets of Langerhans. The only current widely available treatment for type 1 diabetes is insulin replacement therapy which involves the injection of exogenous insulin to replace the depleted stores. Whilst this treatment helps in the management of the disease, existing insulin therapies to treat type 1 diabetes do not mimic the glucose-stimulated insulin secretion of a healthy β-cell (Shamoon, 1993; The Diabetes Control and Complications Trial Research Group, 1997). Islet transplantation has been investigated as a treatment option and a potential cure for type 1 diabetes. This process involved the transplantation of pancreatic islets from a cadaveric donor to a diabetic recipient (Boker, 2001). In this procedure, the endocrine islet of Langerhans were isolated using Liberase; a combination enzymatic product containing collagenase I and II (Olack, Swanson et al., 1999). These islets were transplanted into the hepatic portal vein of the recipient with type one diabetes (Owen, Ryan et al., 2003; Shapiro, Ricordi et al., 2006). This resulted in short term insulin-independency. Thus transplantation can provide a potential cure for type 1 diabetes. However, this procedure does not currently offer life-long insulin-independence and the number of successful transplantations are low (Gaglia, 2005). The Edmonton protocol currently only offers 80% insulin-independence at year one and this is reduced over the next five years to approximately 10% (Ryan, Paty et al., 2005). Despite these figures, the use of the Edmonton protocol does provide proof of principle
that islets can successfully be transplanted into the hepatic portal vein of the recipient with type 1 diabetes.

One important problem identified with islet transplantation was the removal of islet vasculature during the transplantation process (Mattsson, 2002). Once isolated, islets were removed from a normoxic environment which is supplied by a normal vasculature to a hypoxic environment of low oxygen without vasculature. Once transplanted to the recipient, revascularisation has been reported to take several days with more recent investigations identifying that revascularisation may never be similar to that of the islet prior to transplantation (Mendola, Conget et al., 1997; Henriksnas, Lau et al., 2012). Thus, β-cells were exposed to a low oxygen concentration for a period of time post isolation, which has been identified as a key factor in the decrease in β-cell survival during transplantation (Zhang, 2004). As islet transplantation offers the only potential cure for type 1 diabetes, the process of islet transplantation needs optimisation in order to improve the health outcomes and prognosis for type 1 diabetes patients.

The aim of this research was to investigate the effect of low oxygen directly on the β-cell and by doing so construct a definitive answer detailing the processes experienced by the β-cell during islet transplantation. A hypoxic environment was designed using a hypoxic glove box. The MIN6 mouse transgenic β-cell line was used for all experiments. The aim of such was to mimic the low oxygen environment experienced by the β-cells following devascularisation during the transplantation process. The investigations began by looking at the viability of β-cells in low oxygen.
3.2 Viability of β-cells in Hypoxia

In order to gain a better understanding of post-transplant β-cell viability we investigated the effects of low oxygen, hypoxia, on MIN6 pancreatic β-cell viability in cell culture conditions. We hypothesised that low oxygen concentration would decrease cellular viability. Cells were seeded at 200,000 cells per well in a 6-well plate. These cells were cultured in normoxic (21% oxygen) or hypoxic conditions (1% oxygen) for 8 hours. Viability was examined by MTT assay at 2 hour, 4 hour and 8 hour time points. It is clear from the result in Figure 3.1 that a low oxygen concentration had a detrimental effect on the viability of β-cells. Cell viability was reduced by 31% following 8 hours incubation in 1% oxygen \(p=0.004\). This difference in cell viability at the 8 hour time point was significant. Normoxic cells continued to grow while viability of hypoxic cells was significantly reduced.

As the period of revascularization may take several days, as documented by Mendola et al (1997), the viability in MIN6 β-cells over a longer period of time was examined. Cells were incubated in normoxia and hypoxia over 72 hours. Viability was based on mitochondrial activity and detection of viability was by MTT assay. Viability assay was performed at 0 hours, 24 hours, 48 hours and 72 hours on normoxic and hypoxic cells. The results are displayed in Figure 3.2. Cell viability was decreased by approximately 37% after 24 hours, and significantly decreased by 76% after 48 hours and by 79% following 72 hours, \(p=0.03\) and 0.01 respectively. These results indicate that exposure of β-cells to hypoxic conditions can have a detrimental effect over time on cell viability.
MIN6 β-cell were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen or 21% oxygen over a period of 8 hours. Cell viability was determined at 2 hours, 4 hours and at 8 hours. Viability was examined by MTT assay. Results shown are representative of three separate experiments, error bar values represent mean +/- standard error. Significant difference in viability between normoxic and hypoxic cells is evident from 8 hour timepoint, **$p=0.01$. 

**Figure 3. 1 MTT assay of MIN6 cells exposed to 1% oxygen over 8 hours**
MIN6 β-cell were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen or 21% oxygen over a 72 hour period. Cell viability was determined for normoxic and hypoxic cells at 0 hours, 24 hours, 48 hours and 72 hours. Viability was detected by MTT assay. Results shown represent three separate experiments. Error bar values represent mean +/- standard error. *p<0.05, **p<0.01. A significant difference in viability of cells at 48 hours and 72 hours was observed.
3.3 Growth of β-cells in Hypoxia

As cell viability, as measured by mitochondrial activity, was low in β-cells following exposure to 1% oxygen, this research next investigated the effect of hypoxia on cell number. Cells were seeded at 200,000 cells per well in a 6-well plate and cultured in normoxic or hypoxic conditions for 0 hours, 24 hours, 48 hours or 72 hours. Cells were then removed and counted using a haemocytometer. From the results detailed in Figure 3.3, it is clear that cell number was significantly lower in the cells exposed to hypoxic conditions compared with the normoxic samples. Cell number was decreased by 45% at 48 hours, \( p=0.01 \), and decreased by 33% at 72 hours, \( p=0.01 \). This poses the question as to whether MIN6 β-cells are experiencing hypoxic induced β-cell death upon exposure to low oxygen.

3.4 Hoescht Propidium Iodide Staining of MIN6 β-cells in Normoxia and Hypoxia

The effect of hypoxia in the induction of β-cell death was examined. In order to investigate whether β-cell viability was reduced by either apoptosis or necrosis, a Hoescht propidium iodide (HPI) stain was performed on β-cells at 24 hours, 48 hours and 72 hours following exposure to 1% and 21% oxygen. This experiment used the Hoechst propidium iodide stain to establish viable cells, from apoptotic and necrotic cells. HPI is a technique which uses fluorescence to stain the chromatin of apoptotic cells a brighter blue than the chromatin of healthy cells which stain a light blue. Propidium iodide stains necrotic cells a bright pink.
Figure 3.3 MIN6 β-cell number following exposure to 1% oxygen over 72 hours.
MIN6 β-cell were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen or 21% oxygen over a 72 hour period. Cell number was determined for normoxic and hypoxic cells at 0 hours, 24 hours, 48 hours and 72 hours by haemocytometer count. Results shown are representative of three separate experiments, error bar values represent mean +/- standard error. **p<0.01. A significant difference in the number of cells present was observed at 48 hours and 72 hours.
MIN6 cells were seeded at 200,000 cells per well in 6-well plates and exposed to normoxic or hypoxic conditions. A solution of Hoechst/propidium iodide was prepared in cell culture media. These results are shown in Figure 3.4. These pictures indicate that, relative to the normoxic controls, β-cells were neither apoptosing nor necrosing during the hypoxic time course. It was also noted whilst performing this stain that the morphology of the MIN6 β-cells did not differ between normoxic and hypoxic cells, even when cell number is evidently increased in normoxic cells and unchanged in hypoxic samples.

3.5 Morphology of MIN6 β-cells in Normoxia and Hypoxia by light microscopy

Cell morphology of MIN6 β-cells following exposure to 21% and 1% oxygen for 72 hours was examined. Cells were seeded at 200,000 cells per well in a 6-well plate and incubated at either 1% or 21% oxygen. Cell morphology was determined by light microscopy at 24 hours, 48 hours and 72 hours. The results are displayed in Figure 3.5. These pictures further indicate that MIN6 β-cells grow and increase in number in 21% oxygen over 72 hours. MIN6 cells in 1% oxygen do not appear to grow in hypoxia over 72 hours. The morphology of these cells remains unchanged in hypoxia when compared to the morphology of the cells grown in normoxia.
MIN6 β-cell were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose 24 hours. Cells were exposed to 1% oxygen or 21% oxygen over a 72 hour period. HPI stain was performed for normoxic and hypoxic cells at 24 hours, 48 hours and 72 hours. Cells were visualised under fluorescent microscopy. Images are taken at x25 magnification and results are representative of three separate experiments. Images were represented in twelve separate fields. All cells were viable and stained light blue, even following incubation in 1% oxygen for 24 hours (B), 48 hours (D) and 72 hours (F).
Figure 3.5 Morphology imagery of MIN6 cells exposed to 21% and 1% oxygen for 72 hours.

MIN6 β-cells were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen or 21% oxygen over a 72 hour period. Light Microscopy was performed for normoxic and hypoxic cells at 24 hours, 48 hours and 72 hours. Results are representative of three separate experiments in twelve individual fields. Cell morphology appears unchanged in cells exposed to hypoxia compared with the MIN6 cells in normoxia.
3.6 Cell Quiescence and Oxygen Re-stimulation

From the HPI data and the viability results over 72 hours, it was clear that while cell number was lower in MIN6 β-cells exposed to 1% oxygen, this was not due to cell death. This then poses the question; does hypoxia induce cell senescence or quiescence in β-cells? Senescence and quiescence can be defined as withdrawal from the cell cycle. Cell quiescence is reversible upon re-stimulation with appropriate stimuli to promote proliferation. Senescence is irreversible, and is identified by a termination in cell division (Sedivy, 1998). In order to investigate these results further, MIN6 β-cells were exposed to 1% oxygen for 24 hours and re-stimulated with 21% oxygen for a further 24 hours. Cell viability was determined by MTT assay at 0 hours and 24 hours hypoxia. Following 24 hours in hypoxia, cells were exposed to 21% oxygen for 24 hours and viability tested using MTT assay. At each stage of the experiment, a 0 hour, 24 hours and a 48 hour normoxic control was also viability tested using MTT assay to demonstrate normal cell growth. The results are displayed in Figure 3.6. From these results it is evident that β-cells had an ability to re-grow in 21% oxygen following stunted growth under exposure to 1% oxygen for 24 hours, \( p=0.01 \). Thus, it appears that hypoxia induces quiescence in MIN6 β-cells following 24 hours exposure. This experiment was replicated for cells exposed to 1% oxygen for 48 hours. Cell viability was determined using MTT assay at 0 hours, 48 hours hypoxia. Cells were then exposed to 21% oxygen for a further 24 hours and viability was analysed using MTT assay. At each stage of the experiment, a 0 hours, 24 hours and a 72 hours normoxic control was also viability tested using MTT assay to validate normoxic cell growth. The results are
shown on Figure 3.7 MIN6 β-cells exposed to 21% oxygen for 24 hours following 
exposure to 1% oxygen for 48 hours showed a significant increase in growth, p<0.05.

3.7 Nuclear Factor kappa B

This research continued by investigating the role of NFκB on the regulation of the 
hypoxic response in MIN6 cells. NFκB is a cytoplasmic protein which in response to 
hypoxia becomes activated and translocates to the nucleus to act as a transcription factor 
(Grimm, Bauer et al., 1996). NFκB has several functions within the cell, one of these 
being the control of cell growth (Guttridge, Albanese et al., 1999; Brantley, Chen et al., 
2001). As increased expression of NFκB was associated with increased cell 
proliferation this study hypothesised that during times of hypoxic stress, MIN6 cells 
functioned to reduce NFκB expression and thus reduce β-cell growth. A Western blot 
experiment was designed to investigate the nuclear expression of active NFκB in MIN6 
β-cells following 24 hours, 48 hours and 72 hours of hypoxia. The results are detailed 
on Figure 3.8. From these results it was clear the NFκB was significantly reduced after 
72 hours of hypoxia, p<0.05, relative to the normoxic control at 0 hours of hypoxia. 
There was no difference in NFκB expression in MIN6 cells at 24 hours or 48 hours of 
hypoxia relative to the normoxic control. Thus the reduced MIN6 proliferation observed 
at 24 hours and 48 hours of hypoxia is not as a result of reduced NFκB expression. 
Thus, another method of cell proliferation control must be operating within these cells 
during hypoxia.
MIN6 re-stimulation with 21% oxygen following 24hrs exposure to 1% oxygen.

MIN6 cells were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen for 24 hours. Cell viability was determined for normoxic and hypoxic cells at 0 hours and 24 hours. Following 24 hours exposure to 1% oxygen, cells were exposed to 21% oxygen for a further 24 hours and cell viability determined using the MTT assay. Results shown are representative of three separate experiments, error bars values represent mean +/- standard error. Cells exposed to 24 hours of 1% oxygen followed by 24 hours of 21% oxygen had a significantly greater viability compared with cells exposed to 1% oxygen for 24 hours, **p<0.01.
Figure 3.7 MIN6 re-stimulation with 21% oxygen following 48hrs exposure to 1% oxygen.

MIN6 cells were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen for 48 hours. Cell viability was determined for normoxic and hypoxic cells at 0 hours and 48 hours. Following 48 hours exposure to 1% oxygen, cells were exposed to 21% oxygen for a further 24 hours and cell viability determined using the MTT assay. Results shown were reproduced in three separate experiments. Error bars values represent mean +/- standard error. Cells exposed to 1% oxygen for 48 hours followed by 24 hours of 21% oxygen had a significantly greater viability compared with cells exposed to 1% oxygen for 24 hours, *p<0.05.
Figure 3.8 NFκB expression in MIN6 cells exposed to hypoxia over 72 hours.
MIN6 cells were exposed to 1% oxygen over 72 hours. 10µg of nuclear extract was run on 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in NFκB antibody overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti-rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. Error bar values represent mean +/- standard error. Results are representative of three separate experiments. Panel A represents data from western blotting. (i) demonstrates NFκB expression and (ii) shows lamin protein loading control. Panel B shows the densitometry of NFκB protein expression is significantly reduced, *p<0.05.
3.8 5-bromodeoxyuridine, BrdU

In order to investigate whether cells are capable of transit through the cell cycle during hypoxia, an experiment was designed to investigate the number of cells in the S-phase of the cell cycle using a thymidine analogue, bromodeoxyuridine or BrdU. During the S-phase of the cell cycle BrdU becomes incorporated into the newly synthesised DNA in place of thymidine. This experiment was designed using BrdU specific antibodies to detect the presence of BrdU in MIN6 cells cultured in normoxic and hypoxic conditions for 48 hours. It was hypothesised that MIN6 cells have a reduced uptake of BrdU during exposure to low oxygen. The experimental conditions were divided into 4 time points. At time point 0 hours, 35µM BrdU was added to normoxic and hypoxic cell culture conditions for 48 hours. At time point 48 hours, 35µM BrdU was added to normoxic and hypoxic cell culture conditions for one hour. The aim of these time points was to fully understand the number of cells which have undergone DNA replication during hypoxia relative to the number of cells replicating in normoxia throughout the 48 hours of hypoxia and also after 48 hours of hypoxia and normoxia. The results are demonstrated in Figure 3.9 and Figure 3.10.

The findings from our study of BrdU incorporation in MIN6 cells throughout 48 hours exposure to hypoxia identified that MIN6 cells undergo S-phase DNA synthesis at some point during exposure to hypoxia. This is represented by the fact that the number of cells turning over throughout the 48 hours of hypoxia is not significantly different to the number of cells turning over in normoxia.
Figure 3.9 BrdU staining of MIN6 cell exposed to normoxic or hypoxic conditions over 48 hours.
MIN6 cells were seeded at 200,000 cells per well on cover slips and exposed to 1% Oxygen or 21% Oxygen for 48 hours. Panels A and B represent cells pulsed with BrdU for 1 hour at 48 hours. Panels C and D represent cells pulsed with BrdU for 48 hours. Panels A and C represent normoxic cells and panels B and D represent hypoxic cells. Results are representative of three separate experiments in twenty individual fields. There was no difference between the number of cells staining positive for BrdU in normoxia when compared with cells stained in hypoxia for the 48 hour experimental period. A reduction in the incorporation of BrdU was observed in panel B (hypoxia) relative to panel A (normoxia).
Figure 3.10 Cell count of BrdU stained MIN6 cells exposed to normoxic or hypoxic conditions over 48 hours.
MIN6 cells were seeded at 200,000 cells per well on cover slips and exposed to 1% Oxygen or 21% Oxygen for 48 hours. The number of BrdU positive stained cells was counted relative to the total number of cells in a field. The percentage of BrdU positive cells were then calculated as a percentage of total cell number. Cell count is representative of three separate experiments containing twenty fields. A significant difference in the percentage of cells incorporated with BrdU after 48 hours of hypoxia was observed, *p=0.03.
Our findings from the observations of BrdU incorporation following 48 hours of hypoxia depict a different finding. A significant reduction in MIN6 cell number was observed in the S-phase of the cell cycle after 48 hour exposure to hypoxia as measured by a one-tailed t-test relative to cells in normoxia, $p=0.03$.

### 3.9 Discussion

The aim of this study was to investigate the viability of MIN6 β-cells in low oxygen as a clinically reflective model of the environment experienced by the β-cells during islet transplantation. Initial experiments involved the use of a MTT assay to detect mitochondrial activity in a hypoxic environment. These experiments demonstrated a reduction in viability of MIN6 β-cells in hypoxia when compared to the viability of β-cells in normoxia (figure 3.1) and (figure 3.2). This reduced viability was further confirmed by cell counts using the haemocytometer cell count method (figure 3.3). From these results it was clear that β-cell number and growth was reduced in hypoxia.

In an attempt to identify whether these cells were undergoing apoptosis or necrosis in response to hypoxia, a Hoescht propidium iodide stain was performed on cells in normoxic conditions and hypoxic conditions. HPI staining of dead and live cells showed that there was no increase in cell death in those cells exposed to 1% oxygen when compared with cells exposed to 21% oxygen (figure 3.4). Cell morphology of MIN6 β-cells did not appear any different between cells in normoxia and hypoxia. Also from our morphology studies it was clear that cell population was increased in a normoxic environment while cell population remained approximately the same in hypoxia throughout the 72 hour timescale (figure 3.5).
We next investigated the oxygen dependent turnover of MIN6 β-cells. In order to test this, an experiment was performed in which the re-stimulation of β-cells following a period of hypoxia with 21% oxygen was investigated. This assessment identified that the β-cells had an ability to increase viability and re-start growing upon stimulation with 21% oxygen conditions following 24 hours and 48 hours in hypoxia (figure 3.6) and (figure 3.7). Interestingly, these results identified that the turnover of MIN6 β-cells was oxygen dependent. These results also identify a definite need to maintain oxygen supply to the β-cell during the process of islet transplantation.

Investigations continued by determining the regulation of NFκB in hypoxia by MIN6 β-cells. NFκB is a documented regulator of cell proliferation and has also been connected with the hypoxic response of cells (Koong, Chen et al., 1994). Here, we identified that although NFκB remained unchanged at 24 hours and 48 hours of hypoxia, the regulation of this protein was reduced following 72 hours exposure to low oxygen (figure 3.8). However, this decrease in NFκB at 72 hours of hypoxia could not account for the decreased proliferation of MIN6 cells at 24 hours and 48 hours of hypoxia. Therefore it was concluded that another mechanism of β-cell growth control must be adapted by MIN6 cells during hypoxia.

The method of incorporation of BrdU into cell DNA during the S-phase of the cell cycle was utilised to assess the ability of MIN6 cells to turnover at 48 hours of hypoxia (figure 3.9) and (figure 3.10). While the incorporation of BrdU was significantly reduced in cells pulsed with BrdU at 48 hours of hypoxia, cells pulsed with BrdU for
the entire 48 hours of hypoxia demonstrated an almost 100% turnover rate which was the same as the observation in normoxic cells. This result was interesting as MIN6 cell number and cell viability was reduced in hypoxic conditions. From these results, it would appear that MIN6 cells have the ability to transit through the cell cycle during hypoxia. This transit, however, may be protracted in MIN6 cells during exposure to 1% oxygen. Hypoxia has been previously documented to slow the rate of transition through the cell cycle, (Liang, Ma et al., 2012), as opposed to a complete cell cycle arrest. Based on the BrdU results obtained here, it would appear that it is an increase in cell cycle transit time which is responsible for the decrease in viable cell number observed from the results in figure 3.1 and figure 3.2. This transit time was decreased as observed in figure 3.6 and figure 3.7 upon stimulation of cells with oxygen. Thus, we can conclude that oxygen is essential to the maintenance of optimal cell cycle proliferation rate. Although the experiments performed here were performed in an immortalised mouse transgenic cell line, the importance of these finding relative to human β-cell turnover could be far more significant. It has been shown that human β-cell turnover rate is slower than the turnover rate observed by cell lines, with approximately only 1% of islet β-cells proliferating at any one time. Taking the above observations on protracted β-cell turnover into consideration, the amount of human β-cells turning over in vivo during the process of islet transplantation may actually be much lower than 1%.

Several conclusions can be made from the initial results of this research. Firstly, while a low oxygen environment did not lead to β-cell death it did have a detrimental effect on the growth of the β-cell by reducing the ability of the β-cell to proliferate at the same rate as expected from β-cells in normoxia. Secondly, this detrimental effect on β-cell
viability was reversed upon stimulation with 21% oxygen. Thus, we conclude that the proliferation of the β-cell is oxygen dependent and this period of stunted growth observed during hypoxia can be reversed. This reversal, however, is also oxygen dependent requiring of the β-cell to increase oxygen supply sufficient to survive and regrow after hypoxia. From the results in the oxygen re-stimulation in figure 3.6 and figure 3.7 we can also conclude that earlier re-stimulation with 21% oxygen after 24 hours of hypoxia yielder a greater increase in β-cell viability relative to the re-stimulation with 21% oxygen following 48 hours of hypoxia. These results identify that early revascularisation of these cells is optimum in order to better the β-cell viability outcomes following isolation. These results also show that prolonged exposure to hypoxia may convert the cells from a quiescent state where cells can be recovered to a senescent state which ultimately results in β-cell death.

Reflecting clinically to the field of islet transplantation, these results demonstrated that the reduced long term success of islet transplantation may be explained by restricted β-cell growth immediately following isolation. Two very important questions must now be addressed. The first, what is the molecular pathway which β-cells adapt during exposure to hypoxia? By answering this question, the essential pathway of proliferation and adaption to hypoxia may be elucidated. The second critical issue which must be investigated is the revascularisation of β-cells during hypoxia and the regulation of key markers to aid revascularisation. As clearly observed in this study, ensuring a constant supply of oxygen to the β-cell is essential to cell growth. Thus identifying whether β-cells increase oxygen supply in response to low oxygen is essential to the understanding
of cell survival post transplantation. This research will now delve further in the molecular pathway regulating β-cell growth.
Chapter Four: Molecular Pathway in Hypoxia

4.1 Introduction

This research, to date, has described the reduction in viability of β-cells when exposed to hypoxia. In an attempt to understand why a reduction in viability is observed but not a subsequent increase in cell death, we next aimed to understand the molecular adaptation of MIN6 cells during exposure to hypoxia.

4.2 Ribosomal S6 Kinase, S6K

The mitogen-induced S6 kinase controls translation of genes consisting of a 5’TOP start site. Such genes are known modulators of proteins essential to cellular proliferation (Dufner and Thomas, 1999). S6 kinase has specifically been associated with the effect of hypoxia on cell proliferation; being increased during times of increased cell growth and decreased during times of low cell proliferation (Lui, Cash et al., 2006). In an attempt to understand the molecular pathway adapted by MIN6 cells during hypoxic stress, we investigated the effect of hypoxia on the phosphorylation of S6K at threonine\textsuperscript{389} over 72 hours in MIN6 β-cells. A western blot experiment was designed to separate proteins on a 10% SDS-PAGE gel. The protein expression of phospho-S6K at normoxia (0hrs hypoxia), 24 hours, 48 hours and 72 hours of 1% oxygen was assessed by densitometry. The results are detailed on Figure 4.1. These results indicate that phosphorylation of S6K was reduced during hypoxia by approximately 60%. The decrease in MIN6 phosphorylation of S6 kinase is significant at 24 hours, 48 hours and
72 hours of hypoxia, $p=0.03$, 0.03 and 0.04 respectively, relative to the normoxic control. This decrease in phosphorylation remained constant throughout the hypoxic state. The effect of the phosphorylation of S6 kinase on the regulation of β-cell growth during hypoxia may prove to be essential to the transplantation process. By identifying upstream regulators of pS6K in hypoxia, we aimed to increase the phosphorylation of this protein and following this, identify whether this may increase the proliferation of these cells in hypoxia.

4.3 Phosphatidylinositol 3-kinase (PI3K)

It was clear from previous investigation that MIN6 cells had a significant reduction in the phosphorylation of the ribosomal protein S6 under hypoxic stress. In order to fully understand the molecular pathway adapted by MIN6 β-cells in response to low oxygen, we next aimed to identify the upstream biomarkers which controlled the phosphorylation of S6 kinase protein during hypoxia. S6 kinase has been an acknowledged downstream marker of mTOR (mammalian target of rapamycin) activity and a controller of proliferation (Schalm and Blenis, 2002). A number of upstream signalling pathways are recognized to control mTOR regulation. Phosphatidylinositol 3-kinase, PI3K, is one of the most commonly documented upstream activators of S6 kinase (Sekulic, Hudson et al., 2000). Upon activation by growth factors or mitogens, PI3 Kinase functions to phosphorylate PIP$_2$ to PIP$_3$, which in turn results in the phosphorylation of the serine residue on Akt. This pathway is highlighted in Figure 4.2. The results of such are the activation of the mTOR complex and the subsequent phosphorylation of S6 kinase.
MIN6 cells were exposed to 1% oxygen over 72 hours. 10µg of cytoplasmic cell extract was separated by 10% SDS-PAGE and analysed by western blot using a specific phospho- S6 kinase antibody. Panel A represents data from western blotting. (i) displays pS6K protein expression and (ii) shows α-tubulin protein loading control. Panel B illustrates the densitometry of pS6K relative to α-tubulin. These results were reproduced in at least three separate experiments. Error bar values represent mean +/- standard error. The phosphorylated portion of the S6 kinase protein was reduced by approximately 60% in MIN6 cells exposed to hypoxia, *p<0.05.
Numerous investigations into cellular growth in tumorous hypoxic environments have identified a marked increase in activity of the PI3K/Akt/mTOR pathway and subsequent increase in cell growth (Elfiky, Aziz et al., 2011). With this in mind, the absence of stimulation may result in a decrease in PI3K protein and a reduction in downstream phosphorylation. For this reason, the regulation of PI3K in hypoxia was investigated.

Experimentation was performed using a PI3K-p83 regulatory subunit specific antibody in hypoxia for 72 hours. Cells were cultured in hypoxia for 72 hours and cells extracted at 0 hours, 24 hours, 48 hours and 72 hours. Whole cell protein was extracted according the protocol detailed in chapter two. Western blotting was performed to investigate the PI3K p83 levels in MIN6 cells exposed to hypoxia. The results are detailed on Figure 4.3. From these results it is clear that PI3K levels remained unchanged in MIN6 cells throughout 72 hours of hypoxia. From these initial investigations we were able to identify that the regulation of PI3K p83 was not significantly changed in response to hypoxia at 24 hours, 48 hours and 72 hours relative to the expression of PI3K under normoxic conditions.

4.4 Protein Kinase B (Akt)

Upstream activation of mTOR can be dependent upon the phosphorylation of Akt (Link, Kerr et al. 1992). Initial phosphorylation of Akt occurs at the threonine\textsuperscript{308} residue by PDK1 (Alessi, James et al., 1997). In order to investigate the effect of hypoxia on the regulation of Akt in MIN6 cells, an experiment was designed to investigate the response
Figure 4.2 Schematic representation of the PI3K/Akt/mTOR/S6K pathway

Hypoxia may result in the inactivation of phosphatidylinositol 3-kinase. This results in downstream inactivation of Akt/protein kinase B and the mammalian target of rapamycin (mTOR). A reduction in expression of ribosomal S6 kinase (S6K) and 5’ UTR translation is observed following inhibition of mTOR.
MIN6 cells were exposed to 1% oxygen over 72 hours. 10µg of whole cell extract was run on 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in PI3K-p83 antibody overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. Panel A represents data from western blotting. (i) demonstrates PI3K expression and (ii) shows α- tubulin protein loading control. Panel B shows the densitometry of PI3K relative to α-tubulin. Error bar values represent mean +/- standard error. Figure is representative of three separate experiments. PI3K protein expression did not significantly change in MIN6 β-cells during hypoxia, p>0.05.
to 72 hours of hypoxia in MIN6 cells. MIN6 cells were incubated in hypoxia for 24 hours, 48 hours and 72 hours and whole protein extracted. 20µg of whole cell protein was separated on 10% SDS-PAGE gel and transferred onto a PVDF membrane. Western blot analysis was performed using an antibody specific to the phosphorylated Akt threonine 308 residue. The results are detailed on Figure 4.4. From the densitometry data it is clear that there was no significant difference in the phosphorylation of Akt (thr 308) at 24 hours, 48 hours and 72 hours of hypoxia, $p=0.74$, 0.54 and 0.15 respectively, relative to the regulation of pAkt thr$^{308}$ in normoxia. Thus, it can be concluded that Akt thr$^{308}$ is not phosphorylated, and thus not regulated, in MIN6 β-cells exposed to hypoxia.

The phosphorylation of the serine$^{473}$ residue is essential to the activity of the Akt protein and is believed to be phosphorylated by the mTORC2 subunit of mTOR (Sarbassov, Guertin et al., 2005). In order to investigate the phosphorylation of Akt ser$^{473}$ an experiment was designed to determine protein expression at 24 hours, 48 hours and 72 hours in MIN6 cells exposed to hypoxia. 20µg of whole cell protein was separated on 10% SDS-PAGE gel and transferred via semi-dry transfer onto a PVDF membrane. Western blot analysis was performed using an antibody specific to the phosphorylated Akt serine$^{473}$ residue. The results are detailed on Figure 4.5. Panel B demonstrates the densitometry data. It is clear that there was no significant difference in the phosphorylation of Akt serine$^{473}$ at 24 hours, 48 hours and 72 hours of hypoxia, $p=0.18$, 0.62 and 0.73 respectively, relative to the regulation of Akt serine$^{473}$ at normoxia.
MIN6 cells were exposed to 1% oxygen over 72 hours. 20µg of whole cell extract was run on 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in pAkt antibody overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. Panel A represents data from western blotting. (i) demonstrates pAkt expression and (ii) shows Akt protein loading control. Panel B shows the densitometry of pAkt relative to total Akt. Results are from three separate experiments. Error bar values represent mean +/- standard error. The levels of phosphorylated Akt\textsuperscript{thr308} did not significantly change in MIN6 β-cells during hypoxia, p>0.05.

Figure 4.4 pAkt\textsuperscript{thr308} expression in MIN6 cells exposed to hypoxia over 72 hours.
Figure 4.5  pAkt$^{\text{ser473}}$ expression in MIN6 cells exposed to hypoxia over 72 hours. MIN6 cells were exposed to 1% oxygen over 72 hours. 20 μg of whole cell extract was run on 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in pAkt antibody overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. Panel A represents data from western blotting. (i) demonstrates pAkt expression and (ii) shows Akt protein loading control. Panel B shows the densitometry of pAkt relative to Akt. Figure is representative of three separate experiments. Error bar values represent mean +/- standard deviation. Levels of phosphorylated Akt$^{\text{ser473}}$ did not significantly change in MIN6 β-cells during hypoxia, $p>0.05.$
4.5 GLUT2

GLUT2 is an essential glucose transporter expressed by β-cells to aid glucose transport across the lipid bilayer of the cell membrane. Several publications have indicated that an increase in glucose transporters in hypoxia may have an essential role in the metabolic shift from oxidative phosphorylation to glycolysis in an attempt to increase glucose uptake to the cell (Hochachka, Buck et al., 1006; Ouiddir, Planes et al., 1999). In normal oxygen conditions, oxygen is used as an ATP generator and yields 38 ATP molecules per glucose molecule (Brahimi-Horn, Cliche et al., 2007). Under low oxygen conditions, cells switch to glycolysis as a method of generating ATP, glycolysis yields 2 ATP molecules per glucose molecule (Brahimi-Horn, 2007). Thus we hypothesised that in order to maintain an active metabolic process within the cell, MIN6 cells must function to increase glucose uptake in order to yield a greater amount of ATP. In order to investigate this, the regulation of glut2 transporters in MIN6 β-cells over 72 hours of exposure to 1% oxygen was investigated. A polymerase-chain reaction PCR experiment was performed to investigate the transcription of the glut2 gene in response to hypoxia in MIN6 pancreatic β-cells over 72 hours. MIN6 cells were exposed to hypoxia over 72 hours and RNA extracted at 0 hours (normoxia), 24 hours, 48 hours and 72 hours. RNA was DNase treated and reverse transcribed to cDNA. Real-time PCR analysis was performed using primers specific for glut2. cDNA for both glut2 gene of interest and β-actin housekeeping gene were serial diluted to give a standard curve, which was then utilised to determine the efficiency of the real-time PCR reaction. An efficiency of plus or minus 5% of 100% was accepted. The relative mRNA expression was calculated using the delta delta Ct calculation.
Figure 4.6 Expression of *glut2* gene in MIN6 cells exposed to hypoxia over 72 hours.

MIN6 cells were seeded into dishes exposed to 1% oxygen over 72 hours. Cells were extracted at 0 hours (normoxia), 24 hours, 48 hours and 72 hours of hypoxia and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for *glut2* and *β-actin*. Figure is representative of three separate experiments. Error bar values represent mean +/- standard error. The relative amount of gene present was calculated using the delta-delta Ct formula. From the results details above, a significant increase on *glut2* gene expression was visible following 72 hours exposure to 1% oxygen, *p*<0.01.
The results are displayed on Figure 4.6. The expression of \textit{glut2} increased gradually throughout the 72 hours of hypoxia. There was no significant difference in \textit{glut2} gene expression at 24 hours and 48 hours of hypoxia relative to normoxic control, \( p=0.19 \) and 0.10 respectively, although a gradual increase is clearly visible at 48 hours. Following 72 hours of exposure to hypoxia it is clear that MIN6 cells do significantly increase \textit{glut2} expression by 1.43 fold, \( p<0.01 \). Thus, when exposed to hypoxia MIN6 \( \beta \)-cells gradually increased the transcription of glucose transporters as a mechanism to switch to a glycolytic mechanism of ATP production.

In order to further evaluate the expression of glucose transporters in MIN6 \( \beta \)-cells exposed to 1\% oxygen for 72 hours, a western blot experiment was designed to investigate the GLUT2 protein expression. As \textit{glut2} mRNA was observed to increase significantly at 72 hours, this research investigated the translation from mRNA to protein within the \( \beta \)-cell while exposed to hypoxia. MIN6 cells were exposed to 1\% oxygen for 72 hours and whole cell protein extracted at 0 hours, 24 hours, 48 hours and 72 hours as described in chapter 2. 10\( \mu \)g of protein was separated on a 10\% SDS-PAGE gel and transferred to PVDF membrane. Western blot analysis was performed using an antibody specific to the GLUT2 protein. In Figure 4.7 it is clear from the bands indicated in the western blot that GLUT2 protein expression was unchanged by MIN6 \( \beta \)-cells following exposure to 1\% oxygen. From the densitometry data on Figure 4.7 there was no significant increase in GLUT2 protein expression at 24 hours, 48 hours or 72 hours exposure to hypoxia relative to expression at normoxia, \( p=0.24, 0.31 \).
MIN6 β-cells were exposed to 1% oxygen over 72 hours. 10µg of whole cell extract was separated on a 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in GLUT2 overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. Panel A represents data from western blotting. (i) demonstrates GLUT2 expression and (ii) shows β-actin protein loading control. Panel B shows the densitometry of GLUT2 relative to β-actin. Results detailed on figure 4.7 are representative of three separate experiments. Error bar values represent mean +/- standard error. GLUT2 protein expression did not significantly change in MIN6 β-cells during exposure to hypoxia, p>0.05.
and 0.49 respectively. Thus it can be concluded that MIN6 cells were able to increase the transcription of glucose transporters in response to hypoxia. However this increase in gene expression did not result in increased protein levels. ATP is commonly described as “the energy currency of the cell” and readily abundant in a fully functioning cell. The ratio of AMP:ATP or ADP:ATP functions to recognise periods of low energy production and switch to a method of increased ATP production. Hypoxia has previously been documented to be one of the key triggers of ATP depletion. This resulting depletion has been documented to lead to an increased expression of AMP activated protein kinase (Kudo, Barr et al., 1995). From the literature, the role of glucose regulation during times of hypoxia is essential. It has previously been reported that as cells undergo hypoxic stress, a shift in metabolism from oxidative phosphorylation to glycolysis as a method to increase ATP production and energy output was observed (Pan and Hardie, 2002; Carling, Sanders et al., 2008). From the experiments detailed above, it was observed that MIN6 β-cells did not function to increase glucose metabolism and thus potentially may result in decreased ATP production as was required by cells during hypoxia. As previous investigations in this research have identified, β-cell growth was reduced in hypoxia. As GLUT2 protein was unchanged, and thus a switch to ATP production not observed, it was hypothesised that the β-cell was functioning to conserve energy when exposed to hypoxia as a mechanism of survival. In order to extrapolate this further, a key energy sensor of the cell was investigated, AMP-activated protein kinase.
4.6 Adenosine monophosphate-activated protein kinase (AMPK)

We next investigated the regulation of AMP kinase (AMPK), an upstream marker of mTOR activity and S6 kinase phosphorylation. During hypoxia, AMPK increases the uptake of glucose and may also effect phosphorylation of S6K as demonstrated on the schematic in Figure 4.8. Previous studies involving AMPK and S6K have shown a negative correlation between AMPK activation by AICAR and concomitant S6K protein expression (Bolster, Crozier et al., 2002).

During low ATP generation, increased AMP levels activate the catalytic α subunit on the AMPK protein and specifically the phosphorylation of threonine\(^\text{172}\) (Carling, Clarke et al., 1989). The role of such activation is to reduce energy consumption and to confer protection to the hypoxic treated cell as has been previously documented by Borger et al (Borger, Gavrilescu et al., 2008). One of the most energy consuming mechanisms within a cell is the translation of mRNA into protein. Thus, this increase in AMPK may result in a decrease mRNA translation and protein synthesis. Taking all this into consideration, western blotting was performed to analyse AMPK-α protein expression levels within the β-cell over 72 hours of hypoxia. Cells were extracted at 0 hours, 24 hours, 48 hours and 72 hours of exposure to 1% oxygen. A western blot experiment was performed to study the regulation of phosphorylation of AMPKα in MIN6 cells exposed to hypoxia. Cytoplasmic proteins were extracted and separated on a 10% SDS-PAGE gel before proteins were transferred to PVDF membrane for
overnight probing with primary antibody specific for phosphorylated AMPKα. The results are detailed on **Figure 4.9**.

**Figure 4.8 Schematic representation of the AMPK/mTOR/S6K pathway.**
A shift in the AMP:ATP ratio can result in an increase in the phosphorylation of AMP activated protein kinase and a resulting increase in the membrane translocation of glucose transporters, GLUT. Activation of this protein also results in the inactivation of the mammalian target of rapamycin mTOR pathway and as a result the reduction of ribosomal S6 kinase and 5’ UTR translation.
MIN6 cells were exposed to 1% oxygen over 72 hours. 20µg of cytoplasmic cell extract was separated on 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in pAMPKα antibody overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. Panel A represents data from western blotting. (i) demonstrates pAMPK expression and (ii) shows α-tubulin protein loading control. Panel B shows the densitometry of pAMPK relative to α-tubulin. Results represent data from three separate experiments. Error bar values represent mean +/- standard error. pAMPK protein expression was increased in MIN6 cells exposed to 1% oxygen. This increase was significant at 24 hours and 48 hours, *p=0.03 and 0.04 respectively, relative to the expression of pAMPK in normoxic conditions.
The phosphorylation of AMPK was increased in MIN6 cells in response to 1% oxygen at 24 hours and 48 hours. This increase was significant as denoted by the \( p \)-values, \( p=0.03 \) and 0.04 respectively. A slight increase in AMPK expression was observed at 72 hours of hypoxia, however this increase was not significantly greater than AMPK expression in normoxia, \( p=0.054 \). Thus, MIN6 cells may function to switch off protein translation in response to low oxygen and this may provide explanation for the reduced phosphorylation of S6 kinase.

In order to further investigate this pathway, the direct effect of AMPK activation on the phosphorylation of S6 kinase was important to define. An experiment was designed to inhibit the AMPK protein using dorsomorphin (Compound C), a known AMPK inhibitor. Compound C 2.5\( \mu \)M was added to MIN6 cells with concomitant exposure to 1% oxygen in an attempt to investigate the phosphorylation of S6 kinase in cells treated with compound C. The timescales were kept the same as the previous experiments. MIN6 cells were exposed to hypoxia without concomitant treatment with compound C as experimental controls. Cells were extracted at 0 hours, 24 hours, 48 hours and 72 hours of hypoxia. A western blot experiment was performed by separating cytoplasmic proteins on a 10% SDS-PAGE gel. Proteins were then transferred to a PVDF membrane and membranes were probed using antibodies specific to the phospho-S6 kinase protein. Results are detailed in Figure 4.10. From these results it was clear that the AMPK inhibitor did have a significant effect on the up-regulation of S6 kinase at 24 hours of hypoxia, \( p<0.01 \). This increase was approximately 3 fold greater than the expression of S6 kinase at 24 hours of hypoxia without concomitant treatment with an AMPK
Figure 4.10 pS6K expression in MIN6 cells exposed to hypoxia over 72 hours with and without incubation with 2.5µM AMPK inhibitor Compound C.

MIN6 cells were exposed to 1% oxygen over 72 hours. 20µg of cytoplasmic cell extract was separated on 10% SDS-PAGE for 1 hour. Proteins were transferred to PVDF membrane and incubated in pS6K antibody overnight at 4°C. Primary antibody was probed with HRP-conjugated secondary anti rabbit antibody and the membrane was developed using ECL detection reagent and visualised by autoradiography. The inhibition of pAMPK was also investigated by membrane incubation in primary antibody overnight at 4°C. Panel A represents data from western blotting. (i) demonstrates pS6K expression and panel (ii) shows α-tubulin protein loading control. Panel (iii) shows pAMPK inhibition by AMPK inhibitor at 2.5µM concentration. Panel B shows the densitometry of pS6K relative to α-tubulin. Results represent data from three separate experiments. pS6K protein expression was increased in MIN6 cells exposed to 1% oxygen over at 24 hours incubation with 2.5 µM Compound C. This increase was significant at 24 hours, *p<0.01.
inhibitor. Interestingly, the inhibition of AMPK at 48 hours and 72 hours of hypoxia did not have any significant effect on the regulation of S6 kinase relative to S6 kinase phosphorylation at 48 hours and 72 hours of hypoxia without AMPK inhibitor $p=0.11$ and 0.15 respectively. A significant difference was observed between S6 kinase regulation at normoxic conditions compared with hypoxic regulation of s6 kinase with AMPK inhibitor treatment at 24 hours, $p=0.04$. There was no observed difference between the regulation of S6 kinase at normoxia compared with S6 kinase phosphorylation in hypoxic samples at 48 hours and 72 hours hypoxia with AMPK inhibitor treatment, $p=0.09$ and 0.92 respectively.

In order to identify whether the inhibition of AMPK had any effect on the viability of MIN6 cells in hypoxia, a MTT viability assay was performed. Cells were seeded at 200,000 cells per well and left for 24 hours incubation in normoxia until attached to plate surface. 2.5µM AMPK inhibitor compound C was added to cells in hypoxia and MTT assay performed at 0 hours, 24 hours, 48 hours and 72 hours of hypoxia. As AMPK inhibitor compound was originally dissolved in filter sterilised DMSO before experimentation, a DMSO control was also included in this experiment to ensure the DMSO compound was not responsible for any effects identified with the AMPK inhibitor. A 1% oxygen control viability assay was also performed. From this experiment demonstrated on Figure 4.11 it was concluded that AMPK inhibitor compound does not have a significant beneficial effect on the viability of MIN6 cells exposed to hypoxia at 24 hours, 48 hours and 72 hours, $p=0.25, 0.45, 0.35$ respectively.
Figure 4.11 MTT assay of MIN6 β-cells exposed to 1% oxygen 72 hours and incubated with 2.5µM AMPK inhibitor compound C. MIN6 β-cells were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose 24 hours. Cells were exposed to 1% oxygen over a period of 72 hours. Cells were treated with AMPK inhibitor compound C or DMSO control. Cell viability was determined at 0 hours, 24 hours, 48 hours and 72 hours. Viability was detected by MTT assay. Results shown are representative of at least three separate experiments, error bar values represent mean +/- standard error. Cell viability was not significantly affected by the addition of 2.5µM AMPK inhibitor Compound C.
4.7 Discussion

This chapter of investigations focused specifically on the regulation of key molecular markers in MIN6 β-cells during exposure to hypoxia. The aim was to identify the biomarkers of interest controlling MIN6 β-cell proliferation and by doing so try to firstly, develop a better understanding of the response of the β-cell to low oxygen and secondly, try to intervene in this experience in order to improve the outcomes for the β-cell during islet transplantation. As initial investigations previously identified, MIN6 cell viability was reduced in hypoxia but this reduction was not as a result of β-cell death.

The initial investigation into the molecular pathway adapted by MIN6 β-cells in hypoxia involved the phosphorylation of the proliferation marker S6 kinase at the threonine site. From the results detailed in (figure 4.1), there was a significant reduction in pS6K in cells exposed to 1% oxygen compared with normoxia. S6 kinase is a known protein involved in regulation of cell cycle progression and cell growth and is regulated in response to hypoxia (Liu, Cash et al., 2006). For this reason, this research continued by investigating the potential upstream pathways which may be responsive to hypoxia and thus may be responsible for this decrease in S6 kinase phosphorylation and β-cell growth.

The first hypoxic response pathway investigated was the PI3K/Akt/mTOR/S6K pathway. It was hypothesised that reduced mitogenic stimulation of the PI3K protein would result in a downstream cascade reduction in key proteins involved in regulating
the growth of pancreatic β-cells in hypoxia. The regulation of the PI3K p83 subunit was unchanged throughout the 72 hours of hypoxia (figure 4.3). Investigating this pathway further, two key phosphorylation sites on the Akt protein were also investigated, threonine$^{308}$ and serine$^{473}$. From the results detailed on (figure 4.4) and (figure 4.5) it was clear that the regulation of Akt was unchanged throughout hypoxia. Thus, our initial conclusions from this section of analysis were that the PI3K/Akt pathway did not regulate the mTOR/S6K pathway in β-cells exposed to hypoxia.

As several investigations have previously identified, the switch by cells exposed to hypoxia to glycolysis as a method of survival is key to the hypoxic response. In respect of this finding, this research investigated the key glycolytic response glucose transport protein within the β-cell known as GLUT2. From the results indicated in (figure 4.6) and (figure 4.7), it is clear that there is some discrepancy in the regulation of glut2 between gene transcription and protein translation. While protein expression was unchanged throughout the hypoxic response, it was observed that on an mRNA level glut2 does increase to significance at 72 hours. As the up-regulation of GLUT2 would potentially signify a switch in ATP generation from oxidative phosphorylation to glycolysis, we conclude at this point that MIN6 cells are not producing ATP from AMP or ADP. Thus, there should be an increase in AMP levels in MIN6 cells when exposed to hypoxia. As a result this research identified that MIN6 cells exposed to hypoxia function to increase AMP protein kinase as seen on (figure 4.9). This result interpreted alongside the glut2 mRNA increase indicated that MIN6 cells do try to increase ATP by the transcription of genes responsible for glycolysis. The reduction of protein
translation, as indicated by a reduction in pS6K, may explain the reduced GLUT2 protein expression observed in MIN6 cells.

A negative correlation between AMPK and S6K has previously been documented (Bolster, Crozier et al., 2002). This research aimed to identify whether the decrease in S6 kinase was as a result of increased AMPK activation. From the results in (figure 4.10) it is clear that by the addition of AMPK inhibitor compound C to the cell culture system it was possible to increase S6 kinase at 24 hours of exposure to hypoxia. While AMPK inhibitor compound C did inhibit the phosphorylation of AMPKα during MIN6 cells 72 hour exposure to hypoxia, this inhibition only resulted in increased phosphorylation of S6 kinase at 24 hours. An increase in phospo-S6K was not observed at 48 hours and 72 hours of hypoxia. We thus hypothesised that the mTOR/S6K pathway in MIN6 cells exposed to hypoxia must receive additional inhibitory signals from alternative upstream proteins.

As viability was reduced in hypoxia and is of concern during the transplantation process, in (figure 4.11) this research identified that the addition of AMPK inhibitor compound C did not result in a significant increase in viability in cells exposed to hypoxia at 24 hours, 48 hours or 72 hours compared with cells in hypoxia without inhibitor compound C. From these results it is clear that while MIN6 cells, with the addition of AMPK inhibitor compound C, function to increase the phosphorylation of S6 kinase at 24 hours of hypoxia and this does not result in increased viability of these cells in hypoxia. This result may also further prove that S6K may not be the key regulatory protein controlling cell proliferation.
Several conclusions can be drawn from these results. PI3K/Akt/mTOR is not an active pathway of response in the MIN6 β-cells to low oxygen. At 24 hours of hypoxia it would appear that MIN6 cells switch off protein translation, being one of the most energy consuming pathways of the cell. This is evident from the results detailing a reduction in phospho-S6 kinase. At 48 hours of hypoxia MIN6 cells began to switch on glycolysis as is evident from the increase in *glut2* mRNA. Although this increase is not significant at 48 hours, this does become significant at 72 hours. The switch in ATP production may be as a result of the increase in AMPKα phosphorylation, as AMPK is a known positive regulator of glucose transporters and functions to switch on ATP production during times of reduced ATP.

The AMPK/mTOR/S6K pathway is active in MIN6 cells in response to low oxygen but it is not the only pathway which MIN6 cells regulate. Increased phosphorylation of S6 kinase at 24 hours provides sufficient proof that AMPK does regulate, in part, the MIN6 hypoxic response. However, as the AMPK inhibitor continues to inhibit AMPKα throughout the 72 hours of hypoxia, the phosphorylation of S6K returns to its pre-treatment reduced state.

It is clear at this point that the β-cell molecular response to hypoxia is a complex one which may involve several pathways, one of which has been identified as the AMPK/mTOR/S6K pathway. From this research, one thing that is clear is that β-cells require the up-regulation of AMPK to survive during hypoxia as a mechanism of energy control during a time of reduced energy output from the cell.
Irrespective of the pathway adopted by these cells, there is one certified conclusion which can be drawn from the results to date from both chapter three and chapter four- MIN6 β-cells response to low oxygen is energy dependent and completely reversible upon restimulation with 21% oxygen, (figure 3.6) and (figure 3.7). Using these essential results, this research will now continue by investigating the ability of the β-cell to revascularise when in hypoxia and, by doing so, create an increased oxygen supply from surrounding capillaries which may aid growth following pancreatic isolation during the transplantation process.
Chapter Five: Revascularisation and $\beta$-cell Function

5.1 Introduction

The aim of this research was to identify the problems, if any, associated with decreased oxygen supply to pancreatic $\beta$-cells during the transplantation process. As previously discussed, the published literature in this field has identified that the delay in revascularisation of pancreatic islets may take several days post transplantation (Vajkoczy, Menger et al., 1995; Carlsson, Fredrik et al., 2002; Mattsson, 2002). The first problem identified in chapter three was the reduced viability of pancreatic $\beta$-cells exposed to 1% oxygen relative to normal $\beta$-cell viability in 21% oxygen. It was also identified in this chapter that re-stimulation of these cells with 21% oxygen could stimulate re-growth following exposure to low oxygen. This chapter will now focus specifically on the ability of MIN6 $\beta$-cells to mimic the process of re-vascularisation in response to hypoxia with an aim to increase $\beta$-cell viability. One of the main markers of revascularisation known is the vascular-endothelial growth-factor, VEGF (Brissova et al., 2006). During times of hypoxic stress, VEGF is documented to increase oxygen supply by enhancing and maintaining vascularisation (Benjamin et al., 1997). The result is an increase in capillary circulation to the cell and thus increased oxygen delivery. The regulation of VEGF is itself a complex process but is shown to be highly dependent upon the transcription factor hypoxia-inducible factor, HIF (Forsythe, Jiang et al., 1996). As documented with VEGF, HIF is highly regulated in response to low oxygen tension (Wang et al., 1995). The degradation of HIF is dependent upon the binding of
oxygen to oxygen dependent degradation domains on the HIF 1α protein which eventually results in inactivation and degradation of HIF 1α (Huang et al, 1998). During times of low oxygen, the HIF 1α protein, which is located in the cytoplasm, is not degraded and is instead translocated to the nucleus where it signals the transcription of several revascularisation genes, the most extensively known to be VEGF (Brissova et al, 2006).

5.2 Revascularisation of the β-cell

5.2.1 HIF

In order to investigate the effect of hypoxia on the MIN6 pancreatic β-cells, MIN6 cells were exposed to hypoxia over 72 hours and RNA extracted at 0 hours, 24 hours, 48 hours and 72 hours. RNA was DNase treated and reverse transcribed to cDNA. Real-time PCR analysis was performed using primers specific for hif-1α. cDNA for each gene was serially diluted to give a standard curve, which was then used to determine the efficiency of the real-time PCR reaction. An efficiency of plus or minus 5% of 100% was accepted and the relative mRNA expression determined using the delta delta Ct calculation as detailed in chapter 2. Results are shown on Figure 5.1. From these results it was clear the MIN6 pancreatic β-cells reduced hif-1α gene transcription by 72.57% at 24 hours hypoxia and by 31.95% at 48 hours of hypoxia, $p=0.02$ and $p=0.06$ respectively. Thus, a significant reduction in hif-1α mRNA by MIN6 cells was evident following 24 hours exposure of cells to hypoxia.
Figure 5.1 The effect of 1% oxygen on hif 1α gene expression over 72 hours exposure to hypoxia.
MIN6 cells were seeded into dishes exposed to 1% oxygen over 72 hours. Cells were harvested at 0 hours (normoxia), 24 hours, 48 hours and 72 hours of hypoxia and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for hif 1α and β-actin and the data represented on figure 5.1 represents the ratio of hif 1α relative to β-actin in each sample. Graph represents data from three separate experiments. Error bar values represent mean +/- standard error. The relative amount of mRNA present was calculated using the delta-delta Ct formula. A significant decrease in hif-1α mRNA levels was observed at 24 hours hypoxia, *p<0.05.
5.2.2 VEGF

In an attempt to further investigate the effect of low oxygen tension on the regulation of angiogenesis in MIN6 β-cells, an experiment was designed to analyse the ability of MIN6 cells to regulate the transcription of the *vegf* gene during hypoxia. MIN6 cells were exposed to 1% oxygen for 72 hours and RNA extracted at 0 hours, 24 hours, 48 hours and 72 hours. Following DNase treatment of the extracts, samples were reverse transcribed to cDNA and real-time PCR analysis completed using primers specific to the mouse *vegf* gene. cDNA for target gene and housekeeping gene were serial diluted to give a standard curve, which was used to calculate the efficiency of the real-time PCR reaction. The results are shown on Figure 5.2. MIN6 pancreatic β-cells appeared to increase the transcription of the *vegf* gene in response to hypoxic stress. There was approximately a 2 fold increase in gene transcription at 24 hours, \(p=0.03\), and this was reduced to 72% increase in gene transcription at 48 hours, \(p=0.04\). At 72 hours of hypoxia, the MIN6 β-cells had no significant increase or decrease in *vegf* gene transcription relative to the normoxic control at 0 hours of hypoxia, \(p=0.82\).

The ability of β-cells to mount a hypoxic response and increase angiogenesis in response to a low oxygen environment is essential to the survival of pancreatic islets post transplantation. As the results detailed in Figures 5.1 and Figure 5.2 demonstrated, in response to low oxygen stress, MIN6 cells did not mount a sustained hypoxic response. While these cells did increase *vegf* gene transcription at 24 hours, this response diminished over the first 72 hours following exposure to hypoxia while the regulation of the *hif-1α* gene transcript was reduced as an immediate response to hypoxia. As this 72 hours period had been previously documented to be the most critical
MIN6 cells were seeded into dishes exposed to 1% oxygen over 72 hours. Cells were harvested at 0 hours, 24 hours, 48 hours and 72 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for *vegf* and *β-actin* and the data represented the ratio of *vegf* relative to *β-actin* in each sample. Results represent experiments performed in triplicate. Error bars represent mean +/- standard error. The relative amount of mRNA present was calculated using the delta-delta Ct formula. A significant increase in *vegf* gene transcription was observed at 24 hours and 48 hours, *p*<0.05.

**Figure 5.2 The effect of 1% oxygen on *vegf* gene expression over 72 hours exposure to hypoxia.**
in the post isolation survival of pancreatic β-cells, it is imperative that the β-cell has an ability to increase revascularisation markers in response to low oxygen and mount a sustained hypoxic response throughout these 72 hours.

5.3 GLP-1 and Hypoxia

For this reason, this research enlisted the use of glucagon-like peptide-1, GLP-1, as a potential therapeutic agent which may up-regulate key revascularisation genes in an attempt to improve the outcomes for the β-cell during transplantation. GLP-1 is an incretin hormone which is used in the treatment of type 2 diabetes and has previously been shown to increase the secretion of VEGF in endothelial progenitor cells (Xiao-Yun, Chao-Hui et al., 2011). Separate investigations identified a similar stimulation of HIF-1α by GLP-1 in INS-1 pancreatic β-cell line (Van de Velde, Hogan et al., 2011). Exendin-4, a GLP-1 analogue, also offers protective effects from the oxidative stress associated with hypoxia on the pancreatic β-cell (Padmasekar, Lingwal et al., 2013; Wang, Qi et al 2013).

5.3.1 GLP-1 & HIF

In order to investigate the effect of GLP-1 on hif-1α gene transcription in MIN6 cells exposed to hypoxic conditions, MIN6 cells were cultured in 1% oxygen and 21% oxygen for 72 hours with or without the addition of 10nM GLP-1. RNA was extracted at 0 hours, 24 hours, 48 hours and 72 hours. Following extraction, RNA was DNase treated and reverse transcribed using Bio-Rad iscript cDNA synthesis kit. Real-time PCR analysis was performed using primers specific for hif-1α. The ability of
Figure 5.3 The regulation of *hif-1a* gene expression in MIN6 β-cells exposed to 1% or 21% oxygen in the presence of 10nM GLP-1.

MIN6 cells were seeded into dishes exposed to 21% Oxygen and 1% oxygen over 72 hours with/without the presence of 10nM GLP-1. Cells were harvested at 0 hours, 24 hours, 48 hours and 72 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for *hif-1α* and β-actin and the data represented the ratio of *hif-1α* relative to β-actin in each sample. Results represent experiments performed in triplicate. Error bar values represent mean +/- standard error. The relative amount of gene present was calculated using the delta-delta Ct formula as detailed in chapter two. 10nM GLP-1 has no significant effect on the regulation of *hif-1α* in response to hypoxia relative to the MIN6 β-cell response without GLP-1 at 24 hours and 48 hours. At 72 hours of hypoxia a significant decrease in *hif-1α* gene expression was observed in treated cells compared with untreated hypoxic samples, *p*=0.02
GLP-1 to regulate \textit{hif-1\(\alpha\)} gene expression in response to low oxygen was not significantly different from \(\beta\)-cell regulation of \textit{hif-1\(\alpha\)} in response to 1\% oxygen without incubation with GLP-1 at 24 hour and 48 hours hypoxia, \(p=0.60\) and \(p=0.27\). At 72 hours of hypoxia, MIN6 \(\beta\)-cells had a greater ability to increase \textit{hif-1\(\alpha\)} without the presence of GLP-1 \(p=0.02\). From the results on \textbf{Figure 5.3}, it was evident that 10nM GLP-1 had little effect on the regulation of \textit{hif-1\(\alpha\)} in MIN6 \(\beta\)-cells under hypoxic conditions.

\textbf{5.3.2 GLP-1 AND VEGF}

The regulation of \textit{vegf} by GLP-1 in MIN6 \(\beta\)-cells in response to low oxygen was investigated by incubating MIN6 cells with 10nM GLP-1 for 72 hours. Cells were cultured in 1\% oxygen and 21\% oxygen for 72 hours with or without the addition of 10nM GLP-1. RNA was extracted at 0 hours, 24 hours, 48 hours and 72 hours. Following this RNA was DNase treated and reverse transcribed using Bio-Rad \textit{iscript} cDNA synthesis kit. cDNA for each gene was serial diluted to give a standard curve, which was then used to determine the efficiency of the real-time PCR reaction. Real-time PCR analysis was performed using primers specific for \textit{vegf}. From the results detailed in \textbf{Figure 5.4}, it was evident that 10nM GLP-1 did have a positive effect, although not significant, \(p=0.08\) on \textit{vegf} gene transcription at 72 hours incubation in hypoxia compared with gene transcription at 0 hours. However, relative to the amount of \textit{vegf} transcription under hypoxic conditions without GLP-1, this was not significant \(p=0.27\) and thus this increase may be attributable to the general response of \(\beta\)-cells to hypoxia. There was no significance between the amount of \textit{vegf} mRNA expressed in
Figure 5.4 The regulation of the vegf gene expression in MIN6 β-cells exposed to 1% or 21% oxygen in the presence of 10nM GLP-1.
MIN6 cells were seeded into dishes exposed to 21% Oxygen and 1% oxygen over 72 hours with/without the presence of 10nM GLP-1. Cells were harvested at 0 hours, 24 hours, 48 hours and 72 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for vegf and β-actin and the data represented the ratio of vegf relative to β-actin in each sample. Results represent three separate experiments. Error bars represent mean +/- standard error. The relative amount of mRNA present was calculated using the delta-delta Ct formula as detailed in chapter two. From the above results, GLP-1 did increase vegf gene transcription at 72 hours of hypoxia, however this increase was not significant, p>0.05.
MIN6 cells at 24 hours and 48 hours of hypoxia with incubation with GLP-1 relative to normoxia, \( p = 0.13 \) and 0.14 respectively. Thus it can be concluded that, while the treatment of MIN6 \( \beta \)-cells with 10nM GLP-1 does increase \( \text{vegf} \) mRNA at 72 hours of hypoxia, the effects are not significantly greater than normal mRNA regulation by MIN6 cells in response to hypoxia. Thus, GLP-1 as a therapeutic agent in the induction of revascularisation markers, does not offer a beneficial effect to the MIN6 cells following 72 hours of hypoxia relative to cells cultured in hypoxia without GLP-1.

### 5.3.3 GLP-1 AND BETA-CELL VIABILITY

Another key problem documented with MIN6 \( \beta \)-cells exposed to hypoxia was the reduction in \( \beta \)-cell viability, as documented in chapter three. As GLP-1 is a therapeutic agent used in the treatment of diabetes with published positive effects on \( \beta \)-cell proliferation (Buteau et al, 2003; Xin-Yu et al., 2013), this research investigated the effect of GLP-1 on MIN6 pancreatic \( \beta \)-cell proliferation during hypoxic stress. The effect of GLP-1 on the viability of MIN6 \( \beta \)-cells in hypoxia was investigated using an MTT viability assay. Cells were seeded at 200,000 cells per well and incubated at 21% oxygen at 37°C for 24 hours. At 0 hours the assay began by assessing mitochondrial activity before incubation at 1% oxygen for 72 hours. MTT assay was again performed at 24 hours, 48 hours and 72 hours for cells incubated with or without 10nM GLP-1. From the results shown on Figure 5.5, it is clear that GLP-1 has neither negative nor positive effect on \( \beta \)-cell viability during hypoxia as there is no significant difference between viability at 24 hours, 48 hours or 72 hours of hypoxia with or in the absence of 10nM GLP-1, \( p = 0.70, 0.31 \) and 0.98 respectively.
Figure 5.5 MTT assay of MIN6 β-cells exposed to 1% oxygen over 72 hours with or without 10nM GLP-1/10nM GLP-1 activity verification.

A. MIN6 β-cells were seeded at 200,000 cells per well in 6-well plates and incubated in DMEM low glucose 24 hours. Cells were exposed to 1% oxygen over a 72 hour period with or without the incubation with 10nM GLP-1. Cell viability was determined for hypoxic cells at 0 hours, 24 hours, 48 hours and 72 hours. Viability was detected by MTT assay. Results shown represent three separate experiments. Error bar values represent mean +/- standard error. No significant difference was observed between treated and untreated cells throughout the hypoxic timescale.

B. MIN6 cells treated with 10nM GLP-1 for 24 hours demonstrated an increase in PDX-1 protein expression.

(i) PDX-1 → 42 kDa

(ii) Lamin → 66 kDa

GLP-1 - +
In order to investigate whether the results observed with GLP-1 were due to an inactive GLP-1 compound, a simple western blot experiment was performed to detect regulation of the PDX-1 protein. As GLP-1 is a known activator of PDX-1, an active GLP-1 compound would increase the regulation of this protein within 24 hours. Thus, MIN6 cells were cultured in normoxic conditions and when 50 % confluent cells were treated with 10nM GLP-1 for 24 hours. A treated and an untreated sample were used for this experiment. Nuclear extracts were removed and a western blot experiment performed using antibodies specific for PDX-1. From the experiment detailed on Figure 5.5 it can be seen that PDX-1 protein increases in response to 10nM GLP-1 treatment for 24 hours. We concluded that GLP-1 is active.

5.3.4 GLP-1 AND S6 KINASE

The effect of GLP-1 on the phosphorylation of S6 kinase was investigated. The GLP-1 analogue liraglutide is a known positive regulator of S6 kinase (Miao Xin-Yu et al, 2013). It was previously identified that during times of hypoxic stress, the phosphorylation of S6 kinase was reduced. In an attempt to increase the phosphorylation of S6 kinase, MIN6 cells were incubated with 10nM GLP-1 for 24 hours, 48 hours and 72 hours hypoxia and cytoplasmic protein extracted as according to the protocol detailed in chapter two. 10µg of protein was separated on an SDS-PAGE gel and transferred onto a PVDF membrane. Western blot analysis was performed using an antibody specific to the phospho-S6 kinase protein. The results are displayed on Figure 5.6. From these results it is evident that 10nM GLP-1 had no effect on the regulation of S6K in hypoxia. The phosphorylation of S6 kinase was significantly
Figure 5.6 Phosphorylation of S6K in MIN6 β-cells exposed to hypoxia over 72 hours and treated with or without 10nM GLP-1.

MIN6 cells were exposed to 1% oxygen over 72 hours. 10µg of cytoplasmic cell extract was separated by 10% SDS-PAGE and analysed by western blot using a specific phospho-S6 kinase antibody. Panel A represents data from western blotting. (i) displays pS6K protein expression and (ii) shows α-tubulin protein loading control. Panel B illustrates the densitometry of pS6K relative to α-tubulin. These results were representative of three separate experiments. Error bar values represent mean +/- standard error. The phosphorylated portion of the S6 kinase protein significantly reduced in MIN6 cells exposed to hypoxia, *p<0.05. The addition of 10nM GLP-1 does not increase phosphorylation of S6 kinase in 1% oxygen.
decreased at 24 hours, 48 hours and 72 hours of hypoxia in cells left untreated compared with normoxic control at 0 hours, \( p = 0.02, 0.02 \) and 0.03 respectively. Cells treated with GLP-1 during hypoxia also demonstrated a significant decrease in phosphorylated S6 kinase at 24 hours, 48 hours and 72 hours, \( p = 0.03, 0.002 \) and 0.02 respectively. At 24 hours, 48 hours and 72 hours there was no significant difference in the phosphorylation of S6 kinase between treated and untreated samples, \( p = 0.32, 0.86 \) and 0.84 respectively.

GLP-1 showed no benefit on the effect of hypoxia on MIN6 β-cells. 10nM GLP-1 did not increase the revascularisation markers required to increase oxygen supply such as hif 1α and vegf in MIN6 cells during hypoxic stress. The addition of 10nM GLP-1 to MIN6 β-cells did not increase β-cell viability or phosphorylated S6 kinase during hypoxia.

### 5.4 Rosiglitazone and Hypoxia

For the above reasons, this research next investigated the use of the thiazolidinedione Rosiglitazone as a therapeutic agent with potential benefits on the MIN6 cell during hypoxia. As previously reported by this laboratory in 2011, Rosiglitazone increases the transcription of vegf mRNA in MIN6 β-cells (Ferris, Marriott et al., 2011). Although currently Rosiglitazone is unlicensed in the treatment of diabetes due to cardiovascular negative effects, it was hypothesised that Rosiglitazone may have a positive effect on pancreatic cells and the ability of Rosiglitazone to increase vegf mRNA may prove vital in the protection of β-cells during the transplantation process.
5.4.1 ROSIGLITAZONE AND HIF

Initial investigations using this drug investigated the effect of Rosiglitazone on the regulation of the hif 1α gene over 72 hours of exposure to hypoxia. Cells were exposed to 1% oxygen over 72 hours with or without the presence of 10μM Rosiglitazone. RNA extracts were removed at 0 hours, 24 hours, 48 hours and 72 hours of hypoxia and reverse transcribed. Real time PCR analysis was performed for the hif 1α gene using primers specific for hif 1α. cDNA for the gene of interest and housekeeping gene were serial diluted to give a standard curve, which was then used to determine the efficiency of the real-time PCR reaction. From these results it is clear that Rosiglitazone is a positive regulator of the hif 1α gene as evident from the results detailed on Figure 5.7. At 24 hours of hypoxia there was no significant increase in hif 1α gene expression between cells exposed to hypoxia compared with cells exposed to hypoxia and treated with Rosiglitazone, \( p=0.13 \). The amount of hif 1-α gene expressed in normoxic cells treated with Rosiglitazone was not significantly greater than the amount in hypoxic cells treated with Rosiglitazone, \( p=0.27 \). At 48 hours of hypoxia, the results indicated that there was a significant increase in hif1-α gene expression in hypoxic cells treated with Rosiglitazone relative to the MIN6 cells in normoxia at 0 hours, \( p=0.008 \). At this timepoint there was even a significantly greater amount of hif 1α expressed in hypoxic cells treated with Rosiglitazone compared with normoxic cell treated with Rosiglitazone, \( p=0.04 \). At 72 hours of hypoxia, there was also a significant increase in hif1-α gene expression in MIN6 cells treated with Rosiglitazone compared with MIN6 cells in hypoxia and untreated, \( p=0.001 \). There was no significant difference between
the expression of hif 1α between normoxic and hypoxic cells treated with Rosiglitazone at the 72 hour time point, \(p=0.47\).

5.4.2 ROSIGLITAZONE AND VEGF

In order to further investigate the effect of Rosiglitazone on the regulation of angiogenesis within the β-cell, a real-time PCR analysis experiment was performed looking at the regulation of vegf over 72 hours of hypoxia. MIN6 cells were exposed to 10µM Rosiglitazone and RNA was extracted at 0 hours, 24 hours, 48 hours and 72 hours. RNA was reverse transcribed to cDNA and real-time PCR analysis performed with primers specific to the vegf gene. cDNA for each gene was serial diluted to give a standard curve, which was then used to determine the efficiency of the real-time PCR reaction.

From the results detailed in Figure 5.8 it can be seen that vegf gene transcription is increased gradually over 72 hours and is increased to approximately 3 times greater at 72 hours relative to the normoxic 0 hours control. Incubation of MIN6 cells in 1% oxygen with Rosiglitazone caused an increase in vegf gene expression at 24 hours, 48 hours and 72 hours, relative to normoxic samples at 0 hours. This increase was significant at 72 hours, \(p=0.04\). There was a 1.75 fold increase in vegf at 72 hours which was significantly greater than the amount of vegf transcribed by MIN6 cells at 72 hours in hypoxia without Rosiglitazone, \(p=0.038\).
Figure 5.7 The regulation of hif-1α gene expression in MIN6 β-cells exposed to hypoxia in the presence of 10µM Rosiglitazone.

MIN6 cells were seeded into dishes exposed to 21% Oxygen and 1% oxygen over 72 hours with/without the presence of 10µM Rosiglitazone. Cells were harvested at 0 hours, 24 hours, 48 hours and 72 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for hif 1-a and β-actin and the data represented the ratio of hif-1α relative to β-actin in each sample. Results represent three separate experiments. The relative amount of gene present was calculated using the delta-delta Ct formula as detailed in chapter two. 10 µM Rosiglitazone has no significant effect on the regulation of hif-1α at 24 hours of hypoxia compared with MIN6 cells in hypoxia without treatment, \( p > 0.05 \). Error bar values represent mean +/- standard error. A significant increase in hif 1-a gene expression was seen at 48 hours and 72 hours of hypoxia, \( **p = 0.01 \) respectively, in cell treated with Rosiglitazone compared with cells left untreated. A significant increase in hif 1a gene expression was observed between cells treated with Rosiglitazone in hypoxia relative to gene expression in normoxic cells treated with Rosiglitazone, \( *p = 0.05 \).
5.4.3 ROSIGLITAZONE AND BETA-CELL VIABILITY

From these initial Rosiglitazone results, Rosiglitazone would appear to have a positive effect on the key revascularisation genes in MIN6 cells during exposure to hypoxia, increasing the transcription of \textit{hif} and \textit{vegf}. With this in mind, the effect of Rosiglitazone on the viability of MIN6 \(\beta\)-cells over 72 hours of hypoxia was investigated. An experiment was performed using MIN6 \(\beta\)-cells exposed to hypoxia over 72 hours and incubated either in the presence or absence of 10\(\mu\)M Rosiglitazone. An MTT assay was performed at 0 hours, 24 hours, 48 hours and 72 hours. The results in Figure 5.9 demonstrate that Rosiglitazone had no effect on the viability of MIN6 \(\beta\)-cells during hypoxia. There was no significant difference between the viability of MIN6 cells in hypoxia cultured with 10\(\mu\)M Rosiglitazone directly compared with the viability of MIN6 cells cultured in hypoxia without Rosiglitazone at 24 hours, 48 hours and 72 hours, \(p=0.38, 0.62\) and 0.90 respectively. While this research did not demonstrate an increase in MIN6 \(\beta\)-cell viability upon incubation with Rosiglitazone, a negative effect on \(\beta\)-cell viability was also not observed.

5.4.4 ROSIGLITAZONE AND S6 KINASE

In order to further investigate the effect of Rosiglitazone on MIN6 \(\beta\)-cells during hypoxia, an experiment was performed to investigate the effect of 10\(\mu\)M Rosiglitazone on the proliferation marker pS6 kinase in MIN6 \(\beta\)-cells exposed to hypoxia over 72 hours.
Figure 5.8 The effect of 10µM Rosiglitazone on vegf gene expression in MIN6 cells cultured in 21% and 1% oxygen over 72 hours.

MIN6 cells were seeded into dishes exposed to 1% oxygen over 72 hours. Cells were harvested at 0 hours, 24 hours, 48 hours and 72 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for vegf and β-actin and the data represented the ratio of vegf relative to β-actin in each sample. Results are representative of three separate experiments. Error bars represent mean +/- standard error. The relative amount of gene present was calculated using the delta-delta Ct formula as detailed in chapter two. The difference in vegf gene transcription at 72 hours incubation with Rosiglitazone was significantly greater than the expression of vegf in MIN6 cells exposed to hypoxia without Rosiglitazone, *p<0.05.
Figure 5.9 MTT assay of MIN6 β-cells exposed to 1% oxygen over 72 hours with or without 10µM Rosiglitazone.

MIN6 β-cells were seeded at 200,000 cells per well in 6-well plates and incubated at 37°C in DMEM low glucose for 24 hours. Cells were exposed to 1% oxygen over a 72 hours with or without incubation with 10µM Rosiglitazone. Cell viability was determined for cells at 0 hours, 24 hours, 48 hours and 72 hours. Viability was detected by MTT assay. Results shown represent three separate experiments. Error bar values represent mean +/- standard error. No significant difference was observed between treated and untreated cells throughout the hypoxia, $p>0.05$. 
It has previously been identified that hypoxia reduces the phosphorylation of this protein which may control the proliferation of these cells in low oxygen. Western blot analysis was performed to detect cellular protein levels. From the results shown on Figure 5.10 it can be seen that Rosiglitazone had no effect on the phosphorylation of S6K in MIN6 β-cells. Phosphorylation of S6 kinase was significantly reduced at 24 hours, 48 hours and 72 hours of hypoxia in untreated samples compared with normoxic control, \( p = 0.04, 0.03 \) and 0.01 respectively. Cells treated with Rosiglitazone during hypoxic stress also demonstrated a significant decrease in phosphorylated S6 kinase at 24 hours, 48 hours and 72 hours, \( p = 0.01, 0.002 \) and 0.04 correspondingly. At 24 hours, 48 hours and 72 hours there was no significant difference in the phosphorylation of S6 kinase between treated and untreated samples, \( p = 0.35, 0.27 \) and 0.14 respectively.

In conclusion, with respect to Rosiglitazone, this drug had a positive effect on the regulation of revascularisation markers in MIN6 cells exposed to low oxygen. This drug, however, does not have any distinct effect on the viability of these cells nor the regulation of S6 kinase during hypoxia.

The consequence of such a decrease in oxygen on the functionality of the β-cell has yet to be investigated. As this may replicate the low oxygen environment experienced by β-cells during islet transplantation, the necessity to further investigate the potential risk is essential. β-cell function is imperative to the future success with islet transplantation and in an attempt to determine the effect of hypoxia on β-cell function, key regulatory signals were assessed. In order to identify the effect of hypoxia on β-cell function, the functional properties of the β-cell were analysed in hypoxic and normoxic cells.
Figure 5.10 Phosphorylation of S6K in MIN6 β-cells exposed to hypoxia over 72 hours and treated with or without 10µM Rosiglitazone.

MIN6 cells were exposed to 1% oxygen over 72 hours. 10µg of cytoplasmic cell extract was separated by 10% SDS-PAGE and analysed by western blot using a specific phospho-S6 kinase antibody. Panel A represents data from western blotting. (i) displays pS6K protein expression and (ii) shows α-tubulin protein loading control. Panel B illustrates the densitometry of pS6K relative to α-tubulin. These results were representative of three separate experiments. Error bars represent mean +/- standard error. The phosphorylated portion of the S6 kinase protein was significantly reduced in all samples exposed to hypoxia, *p<0.05. The addition of 10µM Rosiglitazone did not have an effect on the phosphorylation of S6 kinase in hypoxia.
5.5 Beta-cell function in hypoxia

5.5.1 PDX-1

PDX-1, or pancreatic duodenal homobox-1, is a transcription activator which is essential to β-cell development and function. PDX-1 signals the insulin gene response and is essential to the glucose-induced insulin response of the β-cell. Thus, PDX-1 controls one of the main functions of the pancreatic β-cell. Defects in PDX-1 are associated with diabetes and pancreatic disorders. In order to investigate the effect of low oxygen on the expression of PDX-1, a western blot experiment was performed to assess the protein expression of PDX-1 in MIN6 pancreatic β-cells at 0 hours (normoxia), 24 hours, 48 hours and 72 hours of 1% oxygen. The results are displayed in Figure 5.11.

From the densitometry data, there was no significant difference between the amount of PDX-1 protein at 24 hours, 48 hours and 72 hours of hypoxia relative to normoxia, \( p=0.28, 0.18 \) and 0.11 respectively. From these results, it is clear that PDX-1 protein expression is unchanged in MIN6 β-cells in response to low oxygen. This is a positive finding as any defect in PDX-1 signalling could result in decreased β-cell function.
Figure 5.11 Regulation of PDX-1 protein in MIN6 cells exposed to hypoxia for 72 hours.
MIN6 cells were exposed to 1% oxygen over 72 hours. 2µg of nuclear cell extract was separated by 10% SDS-PAGE and analysed by western blot using a specific PDX-1 antibody. Panel A represents data from western blotting. (i) displays PDX-1 protein expression and (ii) shows lamin protein loading control. Panel B illustrates the densitometry of PDX-1 relative to lamin. These results were reproduced in at least three separate experiments. Error bar values represent mean +/- standard error. There was no difference in the regulation of PDX-1 in MIN6 β-cells throughout the 72 hours of hypoxia, p>0.05.
5.5.2 GLUCOSE-STIMULATED INSULIN-RESPONSE

5.5.2.1 Glucose-stimulated insulin gene transcription

The role of the β-cell is to recognise high circulating levels of glucose and in response increase insulin gene transcription and release insulin from intracellular vesicles within the β-cell. We sought to investigate this response of the β-cell in a hypoxic environment relative to the normoxic environment. A glucose-stimulated insulin gene transcription experiment was performed using real-time PCR analysis of the regulation of the insulin gene transcript in response to the high level of glucose. Under normoxic conditions, MIN6 β-cells were grown until 50% confluent in 5mM glucose. At 0 hours of the experiment, basal glucose cells grown at 5mM glucose were harvested and RNA extracted. Non-harvested cells were exposed to 0mM glucose containing serum for 48 hours to insure insulin synthesis was completely switched off. Following this, cells were then stimulated with 25mM glucose for 24 hours. Under hypoxic conditions for 24 hours, cells were grown until 50% confluent and at 0 hours the cells exposed to basal glucose (5mM glucose) were harvested and RNA extracted. Cells were cultured in 0mM glucose media supplemented with serum for 48 hours in order to switch off insulin synthesis. In order to test the glucose stimulated insulin gene transcription response of the β-cell during 24 hours of hypoxia, cells were moved to a hypoxic environment following 24 hours of exposure to 0mM glucose and the further 24 hours required to switch off insulin synthesis was performed in a hypoxic environment. At 24 hours of hypoxia, cells were washed with PBS and 25mM glucose media added to MIN6 β-cells for 24 hours. RNA was extracted for all cells and DNase treated. Extracts were reverse transcribed using the Bio Rad iscript cDNA synthesis kit. Real time PCR analysis for normoxic extracts and hypoxic extracts was performed using primers
specific for the mouse insulin gene transcript. cDNA for each gene, gene of interest and housekeeping, were serially diluted to give a standard curve, which was then used to determine the efficiency of the real-time PCR reaction. Results are shown on Figure 5.12. From these results it is clear that MIN6 cells exposed to hypoxia did not have the same insulin response to high glucose as MIN6 cells cultured in normoxic conditions. The increase of insulin gene transcription in response to 25mM glucose was significantly greater in MIN6 cells cultured under normoxic conditions compared with cells cultured in hypoxia, \( p=0.02 \). insulin mRNA showed a significant increase in normoxic cells from 0mM glucose to 25mM glucose, \( p=0.02 \). In hypoxia, MIN6 cells exposed to 1% oxygen for 24 hours showed no significant increase in insulin response from 0mM glucose to 25mM glucose, \( p=0.50 \). These results indicated that while exposed to 24 hours of hypoxia, MIN6 \( \beta \)-cells did not maintain the initial glucose-stimulated insulin gene transcription seen with MIN6 \( \beta \)-cells cultured in normoxic conditions and stimulated with 25mM glucose.

In order to further investigate the effect of 48 hours of hypoxia on glucose stimulated insulin gene transcription response from MIN6 pancreatic \( \beta \)-cells, MIN6 cells were cultured in normoxia in 5mM glucose until approximately 50% confluent. At 0 hours, basal insulin response cells were harvested. All cells were then washed with PBS and 0mM media supplemented with foetal bovine serum added to all cells immediately before incubation in 1% oxygen for 48 hours. At time 48 hours, 0mM glucose insulin response cells were harvested and 25mM glucose response cells were washed with PBS and 25mM glucose added to cells for 24 hours. RNA was extracted from all cells and all extracts were DNase treated. Extracts were reverse transcribed and real-time
PCR analysis performed for using primers specific for the insulin mouse gene. cDNA for each gene was serially diluted to give a standard curve, which was then used to determine the efficiency of the real-time PCR reaction. From the results detailed on Figure 5.13 it is clear that MIN6 cells have a diminished response to glucose when cultured in hypoxia for 48 hours. Cells cultured in normoxic conditions and treated with 25mM glucose have a significantly greater amount of insulin gene transcription compared with the cells cultured in 1% oxygen in response to high glucose, \( p = 0.03 \). The insulin mRNA response of MIN6 cells cultured in normoxia showed a significant increase in from 0mM glucose to 25mM glucose, \( p = 0.02 \). There was no significant increase in insulin gene transcription from 0mM glucose to 25mM glucose in cells cultured in hypoxia, \( p = 0.43 \). These results indicated that MIN6 cells cultured in hypoxia for 48 hours has a similarly diminished response to high glucose as the cells cultured in hypoxia for 24 hours. From these results it is clear that low oxygen has an negative effect on the glucose simulated insulin gene transcription in MIN6 \( \beta \)-cells over 24 hours and 48 hours.
MIN6 cells were seeded into dishes and cultured in basal glucose concentration of 5mM for 24 hours. Basal insulin cells were harvested at timepoint 0 hours. In order to fully switch off insulin synthesis at timepoint 0 hours, 24 hours before cells were incubated in hypoxia, cells were incubated in 0mM glucose and then incubated in 1% oxygen for 24 hours bringing the total time in 0mM glucose to 48 hours and the length of time in low oxygen to 24 hours. At 24 hours in low oxygen, cells were stimulated with 25mM glucose for a further 24 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for insulin and \( \beta \)-actin and the data represented the ratio of insulin relative to \( \beta \)-actin in each sample. Results are representative of three separate experiments. Error bar values represent mean +/- standard error. The relative amount of gene present was calculated using the delta-delta Ct formula. There was no significant increase in the transcription of the insulin gene in response to 25mM glucose in cells exposed to hypoxia for 24 hours, \( p>0.05 \). The amount of insulin gene transcribed under normoxic conditions was significantly greater than the in hypoxic conditions, \( *p=0.02 \).
Figure 5.13 Insulin gene transcription in response to 25mM glucose in hypoxia and normoxia at 48 hours.

MIN6 cells were seeded into dishes and cultured in basal glucose concentration of 5mM for 24 hours. Basal insulin cells were harvested at timepoint 0 hours. In order to fully switch off insulin synthesis at timepoint 0 hours, cells were incubated in 0mM glucose and incubated in 1% oxygen for 48 hours. At 48 hours in low oxygen, cells were stimulated with 25mM glucose for a further 24 hours and RNA extracted. Following DNase treatment, RNA was reverse transcribed. Real-time PCR was performed for insulin and β-actin and the data represented the ratio of insulin comparative to β-actin in each sample. All experiments were repeated in triplicate. The relative amount of gene present was calculated using the delta-delta Ct formula. There was no significant increase in the transcription of the insulin gene in response to 25mM glucose in cells exposed to hypoxia for 24 hours, P>0.05. Error bar values represent mean +/- standard error. The amount of insulin gene transcribed in response to 25mM glucose under normoxic conditions was significantly greater than the in hypoxic conditions, *p=0.03.
5.5.2.2 Glucose-stimulated insulin secretion

In an attempt to further analyse the glucose-stimulated insulin protein secretion in MIN6 cells, an insulin ELISA was performed on the media extracts from MIN6 cells exposed to 24 hours of hypoxia. MIN6 cells were seeded in dishes and when 50% confluent media was removed from basal cells. MIN6 cells were placed into hypoxic conditions for 24 hours. At 24 hours cells were washed with PBS and 0mM glucose media supplemented with foetal bovine serum added to each plate for one hour. After one hour, media was removed from all of the cells and cells were washed with PBS. 25mM glucose media was added to cells for one hour. All media was held in reserve at each stage of the experiment. The protocol for the insulin ELISA experiments are detailed in chapter 2. From the results detailed on Figure 5.14 and Figure 5.15 it is clear that the secretion of insulin from MIN6 pancreatic β-cells was impaired in a hypoxic environment, relative to the secretion of insulin from MIN6 cells in a normoxic environment. From these results on Figure 5.14, the amount of insulin secreted from MIN6 β-cells in normoxia was greater than the cells cultured under hypoxic conditions, $p=0.03$.

As previous experiments have demonstrated, the number of MIN6 β-cells in hypoxia is lower than the number of cells in normoxia. For this reason, the results from the previous experiment in Figure 5.14 were manipulated to demonstrate glucose response of MIN6 β-cells relative to response at 0mM glucose. This allowed investigations to focus more specifically on β-cell function irrespective of cell number. These results are detailed on Figure 5.15. Here, it was evident that the insulin response of MIN6 cells
A glucose-stimulated insulin secretion experiment was designed to investigate the percentage of insulin secretion from MIN6 β-cells. Cells were seeded in 5mM DMEM media and at timepoint 0 hours the samples were incubated in normoxia and hypoxia for 24 hours. At timepoint 24 hours, basal insulin secretion media was collected from each sample. Cells were incubated in 0mM glucose media for 1 hour and this media was removed. Cells were then incubated at 25mM glucose media for one hour and media removed. Results above represent two separate experiments. Error bars represent +/- standard deviation. A significant difference in the amount of insulin secreted at 24 hours of hypoxia was observed between normoxic and hypoxic samples, *p=0.03.

These results were based on two readings and therefore caution was taken when evaluating these results.
Figure 5.15 Insulin secretion in response to 25mM glucose in hypoxia and normoxia at 24 hours.

A glucose-stimulated insulin secretion experiment was designed to investigate the percentage of insulin secretion from MIN6 β-cells. Cells were seeded in 5mM DMEM media and at timepoint 0 hours the samples were incubated in normoxia and hypoxia for 24 hours. At timepoint 24 hours, basal insulin secretion media was collected from each sample. Cells were incubated in 0mm glucose media for 1 hour and this media was removed. Cells were then incubated at 25mM glucose media for one hour and media removed. Results above represent two separate experiments. Error bars represent +/- standard deviation. There was a significant increase in the amount of insulin protein secreted from the β-cell in response to 25mM glucose in normoxic cells compared to hypoxic cells, *p=0.04.

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2 These results were based on two readings and therefore caution was taken when evaluating these results.
cultured in normoxia is significantly greater than the glucose response of cells in hypoxia, \( p=0.04 \). This experiment warrants further investigation as this has only been repeated in this study in duplicate. The effect of hypoxia on the secretion of insulin from MIN6 cells at 48 hours of hypoxia was also not investigated here.

5.6 Discussion

In chapter three, we reported an increase in \( \beta \)-cell growth in MIN6 cells exposed to hypoxia and recovered in normoxia. From these results it was deemed essential to inspect the angiogenic properties of the \( \beta \)-cell during the transplantation process. An experiment was designed to investigate the ability of MIN6 cells to mount a hypoxic response and increase the transcription of key revascularisation markers, HIF and VEGF, in a bid to stimulate \( \beta \)-cell growth post transplantation via an increase in oxygen delivery to the cell. While MIN6 \( \beta \)-cells were capable of increasing the VEGF revascularisation marker initially upon exposure to hypoxic stress (figure 5.2), the reduction in \( vegf \) regulation at 72 hours may be a cause for concern. These results indicate that there is a reduction in angiogenesis on day three following isolation. We need to maintain a sustained revascularisation response from the \( \beta \)-cell throughout the post isolation hypoxic period if we want successful outcomes with islet transplantation.

These results also indicate that MIN6 cells were also unable to regulate the transcription factor HIF 1\( \alpha \) in hypoxia (figure 5.1). HIF is a basic helix-loop-helix protein which, under conditions of low oxygen, is activated to translocate into the nucleus and initiate gene transcription of angiogenesis proteins such as VEGF. These initial investigations
are both interesting and controversial. It would appear that in hypoxic conditions, MIN6 cells do not rely upon HIF 1α to control the regulation of VEGF transcription. Thus, based on these initial results, it was concluded that the regulation of VEGF is via a HIF independent pathway. Although several theories have been presented in the alternative regulation of VEGF, very few theories have actually been readily proven. In osteoblasts, the regulation of VEGF has been closely linked with upstream SAPK/JNK pathway (Tokuda, Hirade et al., 2003). In an investigation into the HIF-1-independent regulation of VEGF in the Hep3B cell line, investigators used siRNA for HIF-1 knockdown were able to express a hypoxic induced increase in VEGF regulation independent of HIF-1 (Choi, Park et al., 2011). Similar investigations using HIF-1α knockdown in mice have demonstrated an increase in VEGF production in the absence of HIF which may be attributable to the interleukin-8, (IL)-8, proangiogenic cytokine (Mizukami, Jo et al., 2005). Of interest, research in the field of oncology associated the induction of mitogen-activated protein kinase or MAPK with the induction of angiogenesis via the (IL)-8 and VEGF pathway, independent of HIF (Bancroft et al, 2001). Thus, it can be conceived that several pathways responsive to hypoxia may be responsible for the regulation of VEGF. From the results displayed in this chapter, MIN6 β-cells express VEGF in response to hypoxia via a HIF-independent pathway and the role of which requires further investigation.

An investigation by Treins et al (2006) on the effect of 5-aminoimidazole-4-carboxamide-riboside (AICAR), an AMPK agonist, on the regulation of HIF-1α, VEGF and pS6K identified some interesting findings (Treins, Murdaca et al, 2006). Using the ARPE-19 retinal epithelial cell, Treins and colleagues observed a positive correlation in
the induction of AMPK by AICAR with VEGF. Interestingly, the induction of AMPK resulted in the reduction in HIF-1α, a trend which has been similarly shown in MIN6 β-cells in this study. Thus, the increased regulation of AMPK by MIN6 cells exposed to hypoxia may prove to be a significant player in the hypoxic response of β-cells.

In an attempt to stimulate revascularisation markers in β-cells, MIN6 cells were incubated with 10nM GLP-1 whilst being exposed to 1% oxygen over a 72 hour period. The results established that 10nM GLP-1 had little effect on the revascularisation of pancreatic β-cells (figure 5.3, figure 5.4). Recent investigations using GLP-1 as a treatment in the regulation of the revascularisation marker VEGF in HUVEC cells identified that at a concentration <200mM GLP-1 had little effect on revascularisation (Aronis, Chamberland et al., 2012). This use of supra-physiological concentration of GLP-1 might account for the little revascularisation response seen in our MIN6 cells in response to the therapeutic concentration of 10nM.

Although previous publications have observed an increase in proliferation and in the phosphorylation of S6 kinase in cells treated with the GLP-1 analogue liraglutide, similar results was not observed here (figure 5.5, figure 5.6) (Miao Xin-Yu, Gu Zhao-Yan et al., 2013). One possible explanation for the lack of GLP-1 response observed in this chapter might be explained by the findings of previous experimentations from chapter four. In chapter four we observed an increase in phosphorylated AMP kinase in response to low oxygen concentration. AMP kinase is increased as a result of the decreased ATP production from β-cells when oxidative phosphorylation is reduced during times of hypoxia. Adenylation cyclase is responsible for the conversion of ATP to
cyclic AMP (cAMP) which is a key second messenger in the signalling of GLP-1 (Drucker, Philippe et al., 1987). We hypothesise that during times of hypoxic stress this reduction in ATP production may be detrimental to the second messenger activity required by GLP-1. Thus, GLP-1 activity may be diminished in β-cells exposed to hypoxia.

Following these initial results with GLP-1, we next investigated the use of 10µM Rosiglitazone on the induction of revascularisation markers over a 72 hour period in hypoxia. Rosiglitazone showed a significant increase in angiogenesis factors in cells exposed to hypoxia, by increasing both VEGF and HIF 1α throughout the 72 hours period of exposure to hypoxia (figure 5.7, figure 5.8). With respect to VEGF a significant increase in gene transcription was observed at 72 hours of exposure to hypoxia which was in contradiction to the findings from hypoxia alone. A gradual increase in VEGF gene transcription in MIN6 β-cells over 72 hours in hypoxia indicates a sustained revascularisation response from cells incubated with Rosiglitazone in hypoxia. Of even more interest, the results from HIF gene transcription following treatment with Rosiglitazone indicate that Rosiglitazone is a positive regulator of HIF 1α response during hypoxia. The role of HIF in the protection of the β-cell and the restoration of β-cell function during hypoxia may prove to be important.

No effect was observed upon treatment of MIN6 cells with Rosiglitazone on the viability of MIN6 cells (figure 5.9) nor had it any effect on the regulation of phosphorylated S6K (figure 5.10) during hypoxia. However, it is believed that the viability results detailed above should be extrapolated with caution. As previous experimental data concluded from chapter three, following culture in a hypoxic chamber
for 24 hours and 48 hours, the MIN6 β-cell can commence normal proliferation again post exposure to hypoxia and returned to a normoxic environment. When cultured in a cell culture system such as a hypoxic chamber, MIN6 cells have no physical ability to increase oxygen transport as might be achieved by β-cells in a physiological system in the hepatic portal vein. Thus, the viability results for Rosiglitazone are dependent upon β-cells activating a hypoxic response and increasing revascularisation. By doing so in a physiological system, β-cells may indeed increase viability purely by the increased oxygen delivery.

As GLP-1 had no significant effect on revascularisation, it was concluded here that GLP-1 is not the agent of choice in the treatment of MIN6 cells during the transplantation process. Rosiglitazone, however, does have a significant positive effect on the regulation of vascularisation genes and for this reason could potentially, in a physiological environment, provide sufficient oxygen to aid β-cell growth. From these viability results we have also demonstrated that Rosiglitazone does not have a negative effect on the viability of MIN6 cells in hypoxia (figure 5.9). These results do provide sufficient evidence that Rosiglitazone may be the therapeutic agent of choice for culture with pancreatic islets post devascularisation and pre infusion into the hepatic portal vein. Rosiglitazone is a known positive regulator of β-cell function thus is an excellent agent to protect β-cells during the transplantation process while also increasing oxygen supply to the pancreatic islet.

Whilst the β-cell function data in this chapter are in the preliminary stages, they do offer an interesting insight into the detrimental effect of hypoxia on the function of the pancreatic β-cell. We investigated the glucose-stimulated insulin-secretion of MIN6 β-
cells whilst exposed to hypoxia in order to identify whether the fundamental function of the β-cell was compromised. PCR analysis of the insulin gene transcript following 24 hours exposure to hypoxia showed a decrease in the insulin gene transcript in response to high glucose (figure 5.12), which is in direct paradox to the response of the MIN6 cells cultured in a normoxic environment. This decrease in response is replicated in MIN6 cells cultured in hypoxia for 48 hours and stimulated with 25mM glucose (figure 5.13). An insulin secretion ELISA detailed the secretion of insulin from the MIN6 β-cell in response to 25mM glucose. From these preliminary results it is clear that there was a diminished insulin response from the β-cells to high glucose during 24 hours of hypoxia (figure 5.14, figure 5.15). This research did not investigate the response of the MIN6 cell to 25mM glucose following re-stimulation with 21% oxygen. From the results above we can draw very significant conclusions. Removal of oxygen from the β-cell is detrimental to glucose-stimulated insulin-secretion from both transcript and protein perspective. Thus, it would appear that following isolation and for the first three-five days post transplantation, the β-cell is potentially not a functioning β-cell as regards insulin secreting ability in response to glucose.

As PDX-1 is an essential transcription factor for the secretion of insulin from pancreatic β-cells, we investigated whether the expression of this protein would be changed in a low oxygen environment. Here, we have identified that PDX-1 expression, in basal glucose concentration, remains unchanged throughout the 72 hours of hypoxia exposure (figure 5.11). Thus, our initial investigations on β-cell function and hypoxia concluded from this result that PDX-1 protein is unaffected by low circulating oxygen levels. Also, as the PDX-1 protein remained unchanged during hypoxia it can also be
concluded that there is no mutation or defect in the PDX-1 gene or protein and thus this cannot be contributing to the diminished response of the β-cells to high glucose during hypoxia. Likewise, from chapter four, the levels of GLUT2 were unchanged during 72 hours of hypoxia. Therefore, as GLUT2 is essential to glucose signalling in the β-cell, GLUT2 down regulation in hypoxia cannot be accredited with the diminished insulin response.

Reflecting on the molecular results from chapter four, one possible theory which may explain the reduction in insulin mRNA and protein in response to glucose might be explained by the increase in AMP activated protein kinase. Investigations using the AMPK agonist 5-aminoimidazole-4-carboxamide-riboside (AICAR) observed a decreased glucose-stimulated insulin-secretion response of β-cells following activation of AMPK in both cell lines and rat islets and even was responsible for decreased insulin vesicle movement to the cell membrane for release (Salt, Johnson et al., 1998; Tsuboi, daSilva et al., 2003). During times of low oxygen MIN6 cells have been shown to phosphorylate AMPKα and thus this may be responsible for the diminished insulin response to high circulating levels of glucose during hypoxic stress.

Another interesting finding, which was observed from our revascularisation investigations, might also provide some insight into this diminished β-cell response. Studies looking at the role of HIF 1 in type 2 diabetes identified a reduced β-cell response in cells with a decreased expression of this key transcription factor (Cheng, Ho et al., 2010). While initially this result appears interesting, it must also be considered that in these findings, a reduction in glucose transporters was also observed and may account for the link between HIF and glucose response. We did not observe any
diminished regulation of GLUT2 protein and thus these results identify that reduced HIF 1 is not the cause of diminished insulin release from MIN6 β-cells in hypoxia.

It is clear from these results that further experimentation is required in order to better understand the effect of hypoxia on β-cell function and post hypoxic glucose-stimulated insulin-secretion in an attempt better justify the need for earlier revascularisation of pancreatic β-cells. These findings do, however, elucidate several key problems experienced by the pancreatic β-cell post isolation. Firstly, the revascularisation markers necessary to increase revascularisation to the pancreatic β-cell are not readily regulated in MIN6 cells upon exposure to hypoxia. Secondly, this reduced angiogenic response is accompanied by a reduced glucose-stimulated insulin-response from the MIN6 cell. We have identified a potential therapeutic agent in Rosiglitazone which has a significantly positive effect on the up-regulation of key revascularisation proteins which may aid to increase oxygen supply to the β-cell. This, in turn, should result in increased β-cell viability post transplantation.
Chapter Six: Conclusions and Future Work

6.1 Aim of research

The process of islet transplantation has been compromised by several factors, one of which has been documented to be low oxygen levels (Carlsson, Liss et al., 1998). The isolation process of pancreatic islets involves the removal of surrounding vasculature by the digestion of the exocrine pancreas by liberase (Olack, Swanson et al., 1999). The resulting decrease in oxygen supply has been identified as a potentially significant factor in β-cell survival in transplant patients (Carlsson, Liss et al., 1998; Itoh, Iwahashi et al., 2011; Wang, Khan et al., 2013). As the number of people remaining insulin-independent post transplantation is low, we sought to determine, for the first time, what affect a low oxygen environment would have on the β-cell. The timescales used in this thesis were clinically reflective of the three day period post isolation for transplantation. This study focussed on the viability, growth, function and revascularisation of MIN6 β-cells over 72 hours of hypoxia (1% oxygen).

This research contributes to the existing literature in the following ways:

i. For the first time, the impact of hypoxia on the viability of MIN6 β-cells has been established and the importance of re-stimulating these cells with normoxia (21% oxygen) on viability was identified.

ii. We are the first to culture MIN6 β-cells at physiological glucose concentrations in hypoxic conditions.
iii. We postulate, for the first time, that diminished β-cell response in hypoxia may not be as a result of β-cell death rather it may be as a result of cell cycle protraction during hypoxia.

iv. The role of AMPK in the β-cell response to hypoxia was first identified here.

v. This research is the first of its kind to postulate that β-cell hypoxic response at physiological glucose may not involve the up-regulation of hypoxia-inducible factor, HIF.

vi. Finally, we implicate a beneficial role for Rosiglitazone in the pre-culture of islets following isolation and pre-infusion.

This chapter will now conclude as follows;

Firstly, it will summarise the main findings of this thesis taking into consideration the limitations of our research. Then it will divide these findings into three separate time points and by doing so construct an image of the day by day changes in the molecular adaptation of the β-cell to hypoxia.

Secondly, it will try to explain the findings relating to β-cell function with an emphasis on what the role these results from our time point investigations might have on β-cell function in hypoxia.

Finally, this chapter will conclude by outlining the future directions of this research and identifying the implications of this work.
6.2 Summary of main findings

This thesis identified that a reduction in β-cell viability and growth during hypoxia was not as a result of apoptosis or necrosis. We also demonstrated that although viability was reduced in low oxygen, this could be reversed upon stimulation of MIN6 cells with 21% oxygen post hypoxia exposure. Reflecting upon the findings from our BrdU study, it would appear that MIN6 cells do proliferate in low oxygen but this rate of proliferation is lower in hypoxia. This indicates that oxygen may have an important role to play in β-cell rate of proliferation and transition through the cell cycle.

In an effort to identify the intracellular pathways of significance in the MIN6 β-cell response to hypoxia, we discovered that the ribosomal S6 kinase protein was reduced throughout the 72 hour hypoxic response. Investigating this further, we were able to directly link the regulation of S6K to the adenosine monophosphate activated protein kinase (AMPK) at 24 hours of hypoxia by the inhibition of the AMPK protein. Inhibition of this protein at 48 hours and 72 hours of hypoxia had no effect on the regulation of S6K, implying that another response pathway must be adapted by the β-cell to survive hypoxic stress. Other investigations of the PI3K/Akt pathway showed that MIN6 cells demonstrate no change in the regulation of these proteins and thus it was concluded that this pathway is not adapted by the β-cell. Interestingly, AMPK was increased throughout the β-cell response to hypoxia. It was concluded that AMP:ATP ratio was reduced which resulted in the activation of this essential survival response. As AMPK is an energy sensor of the cell which functions to shut down energy consuming pathways, we hypothesised that the key survival mechanism of the β-cell was a slower
proliferation rate, as identified by BrdU staining and reduced 5’cap protein translation as identified by S6K phosphorylation.

The potential increase in AMP and the subsequent decrease in ATP were not identified in this thesis and is thus one of the key limitations of this finding. The ideal method for AMP and ATP identification is by using the luciferase assay as a quantitative method of ATP detection. This method is based on the reaction of luciferin with ATP and oxygen to produce oxyluciferin and light. As oxygen is a key molecule in this reaction and our studies are all performed in a hypoxic environment a false negative could not be ruled out by performing this experiment. Thus we hypothesise a decrease in ATP production but this decrease is not specifically identified in this study.

We continued our investigation by looking at the regulation of angiogenesis by the β-cell in low oxygen. We identified that VEGF regulation by the β-cell was independent of HIF 1α in hypoxia. We also demonstrated that Rosiglitazone was a positive regulator of these essential revascularisation proteins in MIN6 β-cells during hypoxia. This resulted in a sustained increase in HIF 1α and VEGF gene transcription over 72 hours, which is potentially necessary to increase oxygen supply to the β-cell and stimulate growth post transplantation.

As our investigations were performed in a closed cell culture system, using a hypoxic glove box, the precise effect of Rosiglitazone on the revascularisation of MIN6 β-cells and viability cannot be identified as physiologically, these cells cannot activate endothelial signalling. Thus the results obtained from our Rosiglitazone studies are
limited as our results are observational and warrant further investigation in a physiological system.

In our final investigation in this thesis, we identified a significantly diminished glucose-stimulated insulin-response of the MIN6 β-cell during hypoxia. As an essential property of β-cell function, we concluded that β-cell function was diminished whilst exposed to low oxygen over 48 hours. Due to the complexity of monolayer culture over more than 5 days we were unable to perform glucose-stimulated insulin gene transcription studies in MIN6 cells re-stimulated with 21% oxygen as MIN6 cells would require being cultured for more than 5 days. Further investigations using the MIN6 pseudoislet culture system would allow longer incubation periods relative to monolayer culture (Chowdhury, Satagopam et al., 2013).

6.3 Growth, viability and revascularisation in hypoxia;

24 hours

Following 24 hours of exposure to hypoxia, it was observed that MIN6 β-cells respond to low circulating levels of oxygen by restricting high energy consuming functions of the cell such as growth (figure 3.1, figure 3.2, figure 3.3) and protein synthesis (figure 4.1). At this timepoint, MIN6 β-cells also increased energy preservation pathways such as AMP kinase and tried to restore ATP production properties of the MIN6 cells (figure 4.9). As AMP kinase is an upstream activator of glucose transporters, these findings
proved to be interesting. It would appear that the energy restoration process occurs at a slow rate as no increase in glucose transporters was observed at 24 hours of hypoxia (figure 4.6, figure 4.7) as would have been anticipated following an increase in AMP kinase. Direct inhibition of the AMPK/mTOR/S6K pathway at the 24 hour timepoint resulted in an increased phosphorylation of S6K, but this inhibition did not result in an increase in β-cell growth during hypoxia. Also of interest, this increase in S6 kinase following AMPK inhibition was only evident at 24 hours of concomitant exposure to hypoxia (figure 4.10). Thus, it would appear that upon exposure to low oxygen, MIN6 β-cells have more than one upstream pathway of activation. It may also mean that restoration of normal β-cell properties during hypoxia may be even more difficult than previously envisaged.

The role of revascularisation during times of low oxygen is crucial to the survival of the β-cell. From observations of β-cell re-growth upon re-oxygenation (figure 3.6), it was deemed imperative to investigate the ability of the MIN6 β-cell to increase oxygen supply by the signalling of key revascularisation markers. HIF and VEGF regulation within the β-cell demonstrated unusual results (figure 5.1) and (figure 5.2). While VEGF mRNA expression was increased at 24 hours in response to hypoxia, a corresponding increase in HIF 1α mRNA levels was not observed. Thus, it can be concluded that at 24 hours of exposure to hypoxia, MIN6 cells increased the transcription of VEGF in a HIF independent manner. This result is paradoxical to many of the hypoxia related publications which identify HIF as a major transcription factor for VEGF (Forsythe, Jiang et al., 1996). This phenomenon may be explained by the increased AMPK phosphorylation in response to hypoxia. Similar results to the findings
identified in this thesis were also observed by Treins et al (Treins, Murdaca et al. 2006). This study also reported a decrease in S6 kinase phosphorylation when cells were exposed to an AMPK activating agent similar to our results in this investigation.

A schematic summation of these findings at 24 hours is demonstrated on **Figure 6.1**.

![Figure 6.1 Schematic demonstrating MIN6 response to hypoxia at 24 hours.](image)
MIN6 cells exposed to hypoxia over 24 hours demonstrated a significant increase in vegf mRNA. We have hypothesised that this increase may be as a result of an increased AMPK phosphorylation. S6 kinase and hif 1α are both down regulated in response to MIN6 exposure to hypoxia.

### 6.4 Growth, viability and revascularisation in hypoxia;

#### 48 hours

Beta-cell viability at 48 hours of hypoxia demonstrated similar results to the findings at 24 hours of hypoxia (figure 3.2, figure 3.3). Growth and viability of MIN6 cells at 48
hours is significantly reduced compared to the growth and viability of these cells exposed to normoxic conditions at 48 hours. From the HPI staining results it was also clear that these cells are not dying by apoptosis or necrosis when exposed to hypoxia (figure 3.4). Also, similar to 24 hours results, a reduction in the phosphorylation of S6 kinase was observed alongside an increase in AMP activated protein kinase regulation (figure 4.1) and (figure 4.9). Thus, MIN6 cells are still in a state of energy preservation at 48 hours of hypoxia. Also of interest, the regulation of glycolytic marker GLUT2 remained unchanged whereas GLUT2 mRNA expression increased in response to 48 hours of hypoxia, although this increase was not to significant levels. An increase in gene expression does indicate a hypoxic response of the cell to low oxygen. The inability of this change in gene expression to have any effect on protein levels indicates that the process of translation from gene to protein is either slowed or inhibited during hypoxia. Interestingly, inhibition of AMPK does not result in increased S6K at 48 hours of hypoxia as was observed at 24 hours (figure 4.10). This is evidence to suggest that while protein translation and cell growth was reduced at 48 hours of hypoxia, this reduction was not as a response to AMPK. Thus, it can be concluded that another pathway of regulation must be responsible for the cellular response to hypoxia observed in MIN6 cells at 48 hours of hypoxia.

Interesting cell growth results from the 48 hours timepoint were presented in the form of our BrdU staining for S-phase synthesis (figure 3.9) and (figure 3.10). This investigation hypothesised that, based on our original MTT assay and HPI results, the amount of cells in the S-phase of the cell cycle would be reduced. However, our results do not demonstrate this. Firstly, cells which were pulsed with BrdU for the entire 48
hours of exposure to hypoxia demonstrated a similar uptake of BrdU relative to the uptake in normoxic cells pulsed with BrdU for 48 hours. This implied that all MIN6 β-cells, whether in hypoxia or normoxia, were able to undergo S-phase synthesis during the 48 hour experimental period. This is possibly as a result of dissolved oxygen content in the media which would have enabled initial cell transit through the cell cycle. The second investigation using BrdU allowed cells to be cultured in normoxia and hypoxia for 48 hours and, following this 48 hour period, pulsed with BrdU for one hour. The aim here was to investigate what percentage of cells, if any, are cycling through the cell cycle following exposure to 48 hours of hypoxia. From the results obtained we can see that there was a significant reduction in S-phase synthesis in MIN6 β-cells exposed to hypoxia for 48 hours, although cell transit was not completely stalled. Thus, the transit of the cell through the cell cycle was reduced following exposure to hypoxia demonstrating that cell cycle transit time must be dependent upon oxygen.

Following 48 hours of exposure to hypoxia, MIN6 β-cells were able to increase viability upon re-stimulation with 21% oxygen (figure 3.7). This proved that these cells were able to respond to higher levels of oxygen by increasing viability following exposure to low oxygen for 48 hours. With respect to β-cell generated revascularisation markers, in response to 48 hours of hypoxia, we observed an approximate increase in HIF mRNA expression of 50% relative to the regulation of HIF mRNA at 24 hours. This increase in HIF mRNA was still approximately 20% lower than the regulation of HIF in normoxia (figure 5.1). Regulation of VEGF was significantly increased similar to the 24 hour response (figure 5.2). From this, we may draw similar conclusion as from our 24 hour result. The regulation of VEGF by MIN6 β-cells is independent of the transcription
factor HIF. We hypothesise here that the regulation of VEGF in MIN6 β-cells exposed to hypoxia may be as a result of increased regulation of AMPK in these cells.

A schematic summation of these findings at 48 hours is demonstrated on Figure 6.2.

**Figure 6.2 Schematic demonstrating MIN6 response to hypoxia at 48 hours.**
MIN6 cells exposed to hypoxia over 48 hours demonstrated a significant increase in vegf mRNA. An increase in AMPKα phosphorylation was also observed and thus we hypothesise that a direct link may exist between the regulation of AMPK and downstream VEGF. An increase in glut2 mRNA was also observed at 48 hours, however this increase was not significant. S6 kinase and hif 1α were reduced in MIN6 cells exposed to hypoxia for 48 hours.
6.5 Growth, viability and revascularisation in hypoxia; 72 hours

Similar markers of viability, growth and angiogenesis were investigated in MIN6 cells exposed to hypoxia for 72 hours. Analogous with the results observed at 24 hours and 48 hours of hypoxia, viability and growth remained reduced in 1% oxygen at 72 hours compared with viability and growth in normoxia (figure 3.2) and (figure 3.3). Cell death was also undetected at 72 hours (figure 3.4). S6 kinase was reduced, as was observed in both 24 hours and 48 hours time points. Thus it can be concluded that cell growth and proliferation is significantly reduced throughout the hypoxic response of the β-cell. AMP activated protein kinase was increased relative to normoxic control however when compared with the regulation of AMPK observed at 24 hours and 48 hours, it was observed that there was a decrease in the regulation of AMPK at 72 hours of hypoxia (figure 4.9). The expression of GLUT2 mRNA was significantly greater at 72 hours of hypoxia relative to normoxic control at 0 hours exposure to low oxygen (figure 4.6). This further indicated that MIN6 β-cells were responding to hypoxia by increasing glycolytic genes necessary for the production of ATP, a key energy component within the cell. This increase in mRNA was not reflected by an increase in GLUT2 protein (figure 4.7). Thus we can conclude that either the process of glycolytic response is slowed in hypoxia or the process of protein translation is reduced.

The regulation of HIF was reduced at 72 hours of hypoxia relative to normoxic control, however, relative to the observations of HIF at 24 hours and 48 hours of hypoxia, HIF
mRNA does appear to be increasing (figure 5.1). Paradoxically, the regulation of VEGF was decreased at 72 hours relative to the increase in VEGF regulation at 24 hours and 48 hours of hypoxia. At 72 hours of hypoxia, VEGF levels are similar to normoxic control (figure 5.2). As has already been postulated, the regulation of VEGF may be as a direct result of AMP kinase activation in response to low ATP generation. As AMPK is decreased at 72 hours of hypoxia this decrease corresponds with a decrease in VEGF mRNA at 72 hours.

A schematic summation of these findings at 74 hours is demonstrated on Figure 6.3.

Figure 6.3 Schematic demonstrating MIN6 response to hypoxia at 72 hours. MIN6 cells exposed to hypoxia over 72 hours demonstrated a significant increase in glut2 mRNA. NFκB and phosphorylated S6 kinase were significantly reduced at 72 hours. The regulation of hif 1α was reduced however this reduction was not significant. In this schematic we hypothesise that there is a link between the regulation of AMPK
and VEGF. At 72 hours of hypoxia, a significant reduction in AMPKα phosphorylation was observed as was a reduction in vegf mRNA; both relative to the regulation of each marker at 24 hours and 48 hours of hypoxia.

6.6 Therapeutic compounds and hypoxia

A significant finding of this investigation has been the reduction in viability of MIN6 β-cells upon exposure to low oxygen with a subsequent increase in viability upon stimulation of these cells with 21% oxygen. In order to improve the viability response of the β-cell during transplantation, we treated MIN6 cells with therapeutically available anti diabetic compounds during exposure to low oxygen. The effect of therapeutically active treatment compounds on β-cell viability and proliferation was studied by the addition of GLP-1 and Rosiglitazone to MIN6 cells during exposure to hypoxia. MTT assay was used to determine viability by investigating mitochondrial activity. From the results in figure 5.5 and figure 5.9, it was clear that neither drug had any effect on viability. The regulation of the proliferation marker S6 kinase using either drug compound was unchanged relative to regulation under hypoxic conditions (figure 5.6) and (figure 5.10). It is believed that, upon consideration of the culture conditions utilised in these experimentations, the viability results using therapeutic agents must be interpreted carefully. As these cells are grown in a hypoxic chamber which is consistently kept at 1% oxygen, any increase in angiogenesis or oxygen-dependent viability would actually have required an increase in oxygen supply. This process is not achieved in these culture conditions and thus any potential for an increase in cell viability during exposure to hypoxia by a therapeutic agent cannot be fully determined under these experimental conditions.
Although β-cell viability was reduced in hypoxia, it was observed in chapter three that removing cells from a hypoxic environment and providing cells with an oxygen supply does increase β-cell viability (figure 3.6) and (figure 3.7). Regulation of angiogenesis markers by the MIN6 β-cell, as previously discussed, demonstrated some unusual findings with a reduction in HIF expression and, although an essential increase in VEGF was observed, this increase was not maintained (figure 5.1) and (figure 5.2). For this reason, it was deemed imperative to identify an optimal treatment regimen which would offer the β-cells an increased supply of oxygen during transplantation. The use of therapeutically available drug compounds were investigated and shown to have no negative effect on β-cell viability figure 5.5 and figure 5.9. In order to increase oxygen supply to the cell we investigated the ability of the β-cell to increase the key revascularisation markers HIF 1α and VEGF during hypoxia.

The first therapeutically available drug compound investigated was GLP-1. The use of GLP-1 as a compound capable of inducing revascularisation was investigated as GLP-1 has previously been identified to increase revascularisation of a number of other cell lines (Van de Velde, Hogan et al., 2011; Xiao-Yun, Zhao-Hui et al., 2011). From the results detailed in figure 5.3 and figure 5.4, it was clear that 10nM GLP-1 had no beneficial effect on the regulation of revascularisation markers, HIF 1α and VEGF in MIN6 cells. We used GLP-1 at a concentration of 10nM as is reflective of the therapeutic concentration used in culture conditions. It was observed in a previous investigation by Xiao-Yun et al, 2011 and Wang et al, 2013 that increased revascularisation in cells using GLP-1 required the use of GLP-1 at a much larger therapeutic dose as would be utilised in diabetic β-cell investigations. This might
explain why increased VEGF expression was observed by these investigators upon the addition of GLP-1.

Although Rosiglitazone has now been removed from the market as a treatment for type 2 diabetes owing to the increased incidence of heart failure in 15% of patients, it has previously been shown to be a safe drug in trials investigating islet function and transplantation (Paget, Murray et al., 2013). Previous investigations have also demonstrated that Rosiglitazone can up-regulate the revascularisation marker VEGF in MIN6 cell (Ferris, Marriott et al., 2011). Our investigations have demonstrated that Rosiglitazone not only up-regulates VEGF in MIN6 cells under hypoxic conditions but also increases the transcription of HIF 1α as demonstrated in (figure 5.8) and (figure 5.7) respectively. The role which HIF may play in the hypoxic response of cells may prove imperative to the hypoxic response of these cells. A schematic summation of these findings using Rosiglitazone is demonstrated on Figure 6.4.
MIN6 cells treated with 10µM Rosiglitazone demonstrated a significant increase in vegf and hif 1α gene transcription during exposure to hypoxia. Treatment with Rosiglitazone had no effect on the deleterious down regulation of S6 kinase observed in MIN6 cells exposed to hypoxia.

6.7 Hypoxia and β-cell function

The effect of hypoxia on β-cell function was also investigated. As the primary function of the β-cell is to respond to circulating levels of glucose by secreting insulin, we sought to identify this glucose response in MIN6 cells during 24 hours and 48 hours of hypoxia. From the results in chapter five, it is clear that glucose-stimulated insulin gene transcription was reduced in MIN6 cells at 24 hours and 48 hours of hypoxia relative to the normoxic response (figure 5.12) and (figure 5.13). The results at 24 hours are further confirmed using an insulin ELISA to determine insulin protein levels (figure 5.14) and (figure 5.15). The precise cause of this is unknown, although it could be postulated that an increase in AMPK may be responsible for this diminished response of the β-cell as
was observed by Salt et al, 1998 (Salt, Johnson et al., 1998). However, the direct effect of AMPK inhibition on the regulation of insulin gene expression and secretion in MIN6 cells during hypoxia was not investigated here.

6.8 Future work

The precise pathways adapted by the beta-cells throughout hypoxia warrants further investigation. We have identified and explained the role of AMPK in the regulation of S6K however we have also shown that phosphorylation of S6K remains reduced at 48 hours and 72 hours of hypoxia even following treatment with the AMPK inhibitor. The PI3K/Akt/mTOR/S6K pathway has also been investigated and has been identified not to be implicated in the hypoxic response of the β-cell. Other upstream markers of S6K regulation require investigation in an effort to understand not just what markers these cells regulate but also to help understand what processes the β-cell is experiencing due to hypoxic exposure.

This research also identified a concomitant increase in AMPK and VEGF in the absence of HIF. Several other investigations have identified that the regulation of VEGF may be via an AMPK dependent pathway and independent of HIF. In order to investigate this further, future research would need to identify a direct link between AMPK and VEGF, potentially via the use of an AMPK inhibitor as used here.

Our glucose-stimulated insulin-secretion studies require further investigation of insulin secretion at 48 hours and 72 hours of exposure to hypoxia. Also, with respect to
glucose-stimulated insulin-secretion, we hypothesised that a decrease in insulin secretion may be as a response to increased AMPK phosphorylation as has previously been identified. The use of an AMPK inhibitor in future studies could identify a direct link between AMPK regulation and glucose-stimulated insulin-secretion in MIN6 β-cells during exposure to hypoxia.

The function of the MIN6 β-cell according to these timescales in a monolayer culture system was difficult as confluency was usually reached on day five or day six of culture, which made any longer timescale studies difficult. The use of a MIN6 pseudoislet culture system would allow for longer incubation periods in hypoxia and normoxia and also allow for the investigation of the effect of hypoxia on a whole islet 3-D structure.

Finally, our revascularisation studies using Rosiglitazone as a therapeutic agent which can increase the regulation of revascularisation markers in β-cells during hypoxia can now be studied in a murine model of islet transplantation. Isolation of mouse islets followed by culture with Rosiglitazone and transplantation of these islets into diabetic mice may provide an exciting insight into a future therapy which may yield better results for islet transplantation.

6.9 Implications of this research

The race to discover the optimum therapeutic regimen which can be implemented into the islet transplantation protocol is well underway. We believe that the implications of the findings in this thesis could provide essential important information about the β-cell
experience during transplantation; findings which may better the future outcomes in optimising the transplantation protocol. This research is the first of its kind to identify a reduction in β-cell growth as opposed to previously described β-cell death as a cause for the reduction in primary outcomes observed during islet transplantation. We are also the first to identify that the solution to this problem of decreased proliferation was revascularisation of β-cells to increase oxygen supply. Now, islet research will require an optimum treatment regimen to increase revascularisation to these cells and thus increase β-cell proliferation.

We have also elucidated in this research that Rosiglitazone, a PPARγ agonist, does have a positive effect on β-cell revascularisation without a corresponding negative effect on β-cell viability. We believe from the results presented in this thesis that Rosiglitazone has a prominent role to play in the future of revascularisation of pancreatic islets and may provide one of the essential missing links in the current islet transplantation processes which are currently ongoing worldwide.

The identification of AMPK as an essential protein marker in the β-cell response to hypoxia points future researchers to a HIF independent method of revascularisation. HIF is defined as a hypoxia response protein which aids cellular response to hypoxia and may signal several downstream pathways of apoptosis, revascularisation and glycolysis (Forsythe, Jiang et al., 1996; Bruick, 2000; Akakura, Kobayashi et al., 2001; Seagroves, Ryan et al., 2001). Here we have identified that HIF regulation is reduced in MIN6 β-cells and thus whether this is part of the hypoxia survival mechanism of β-cells
remains unknown. The implication of reduced HIF signalling in β-cells may indeed be part of the cellular adaptation to hypoxia, as a response to AMPK.

In conclusion, the research area of islet transplantation and the role which hypoxia may play in determining sustained β-cell survival and function post transplantation is current, exciting and relevant. If future transplantations are to provide type 1 diabetic patients with an insulin-independent lifestyle then understanding the hypoxia mediated cellular response is imperative to long-term successful outcomes.
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Abstracts

2010:

Postgraduate Annual Research Presentation Day 2010

Title: The Protection of β-cells using GLP-1.

Aims: The aim of this work is to investigate what effect the therapeutic compound GLP-1 has on free fatty acid treated pancreatic β-cells.

Methods: Min6 β-cells were maintained in 5mM glucose media and Brin cells were maintained in RPMI media. Cells were treated with 0.5mM palmitate to induce cell death. GLP-1 was used at a concentration of 10nM and 50mM. Cell viability was detected using the MTT assay and the HPI stain.

Results: Palmitate, a saturated fatty acid, caused significant β-cell death in all pancreatic β-cell models of diabetes. The co-incubation of palmitate with GLP-1 did not alter the reduced viability seen by palmitate alone. HPI staining of each β-cell line indicated that apoptosis was clear in the presence of palmitate and no visible change in viability was seen when co-incubated with GLP-1.

Conclusions: GLP-1 10nM and 50nM has no protective ability against 0.5mM palmitate in Brin and MIN6 cells.

2011:

Postgraduate Annual Research Presentation Day 2011

Title: Role of HIF-1α in the Response of MIN6 β-cells to Hypoxia.

Aims: The aim of this study is to mimic the low oxygen environment experienced in vitro during islet transplantation. We will investigate the effect of low oxygen on β-cell function and aim to establish the effect of low oxygen on cell viability and HIF-1α protein expression.

Methods: MIN6 β-cells were maintained in 5mM glucose DMEM and 21% oxygen. HEPG2 liver cells were maintained in MEM and 21% oxygen. Viability was assessed by MTT assay and cell number was determined at 0, 24, 48 and 72hr time points. MIN6 cells were seeded in 2-chamber slides and exposed to either 21% oxygen or 1% oxygen for 8 hours. HEPG2 cells were seeded in chamber slides and exposed to 21% oxygen or 1% oxygen for 24 hours. A primary HIF 1α antibody and secondary TRITC anti-mouse antibody were used.

Results: Initial viability results indicate that low oxygen can have a detrimental effect on the viability of β-cells when compared with the viability of cells grown in normoxic conditions. Hypoxia promotes the translocation of active HIF-1α to the nucleus of HEPG2 cells, which is a known cellular hypoxic response. This was not seen in the
MIN6 cells as HIF-1α is located in both the nucleus and the cytoplasm. The inability of MIN6 β-cells to adapt to a hypoxic environment may be due to a cellular failure to translocate HIF-1α to the nucleus and thus mediate a hypoxic response.

**Conclusions:** This study indicates that hypoxia may have a detrimental effect on the growth of β-cells. β-cells show little capacity to mount a hypoxic response and this is further indicated by the location of HIF-1α in the β-cell.

**2012:**

**Diabetes UK, Annual Profession Conference 2012.**

**Title:** Beta-cell Function During Islet Transplantation: Studies in a Clinically Reflective Model of Hypoxia.

**Aims:** Beta-cell survival is low following islet transplantation and may be linked to a delay in revascularisation of donor cells. This decrease in oxygen supply is termed hypoxia, the result of which is detrimental to beta-cell survival. We sought to investigate post-transplant beta-cell viability and function by investigating the effects of low oxygen on MIN6 pancreatic beta-cells.

**Methods:** MIN6 beta-cells were exposed to 1% or 21% oxygen over a period of 72 hours. Viability was assessed by MTT assay and cell number was determined at 0, 24, 48 and 72hr time points. Western blot was performed to determine the PDX-1 and GLUT2 protein levels and RT PCR determined *pdx1* and *glut2* gene expression by MIN6 cells following exposure to 1% oxygen over various time points.

**Results:** Cell viability was reduced at 48 and 72hrs (P=0.03 and 0.01 respectively) and cell numbers in hypoxia were lower than in normoxia. GLUT2 protein was upregulated in response to low oxygen and *Glut2* gene expression was increased. Western blot analysis of PDX-1 expression over 72hrs showed no change in basal PDX-1 protein in hypoxia. RT PCR showed a decrease in *pdx-1* gene expression in 1% oxygen.

**Conclusion:** We have established and validated a novel model system to study the effects of hypoxia on pancreatic beta-cell function. Our initial studies indicate that beta-cells exposed to hypoxia have restricted growth and may enter a hypoxia-induced quiescence phase of the cell cycle. Our system provides a unique opportunity to begin to delineate the cell signalling and cell cycling events controlling beta cell function in the acute period of tissue hypoxia immediately following islet transplantation.
investigate post-transplant beta-cell viability, growth and function by investigating the effects of low oxygen on MIN6 pancreatic beta-cells.

**Methods:** MIN6 beta-cells were exposed to 1% or 21% oxygen over a period of 72 hours. Viability was assessed by MTT assay and cell number was determined by haemocytometer count at 0, 24, 48 and 72hr time points. Western blot was performed to determine the pS6K protein levels.

**Results:** Cell viability was reduced at 48 and 72hrs (P=0.03 and 0.01 respectively) as detected by MTT assay. Cell numbers in hypoxia were also lower than in normoxia. Western blot analysis of phosphorylated S6 kinase protein showed a decrease in pS6K expression in cells exposed to hypoxia.

**Conclusion:** We have established a new model system to study the potential effects of hypoxia on beta-cell function. These initial findings indicate that beta-cells exposed to hypoxia have restricted growth and may enter a hypoxia-induced quiescence phase of the cell cycle. Our system provides a unique opportunity to begin to understand the cell signalling and cell cycling events controlling beta cell function in the immediate period of tissue hypoxia following islet isolation and pre transplantation.

2013:

**Diabetes UK, Annual Profession Conference 2013.**

**Title:** Adaptive Response of Beta-Cells to Hypoxia; Regulation of Cell Survival and Growth

**Aims:** Beta-cell survival is compromised following islet transplantation and this may be linked to a delay in revascularisation of the graft. This causes hypoxia, which has been linked to decreased beta-cell viability. We investigated the molecular adaptation of MIN6 beta-cells to a hypoxic environment.

**Methods:** MIN6 beta-cells were exposed to 1% (hypoxic) or 21% (normoxic) oxygen over a period of 72 hours. Cell viability was assessed by MTT assay and western blotting was performed to monitor pS6K, PI3K, pAkt and pAMPK protein expression throughout the 72 hour study period. Dorsomorphin (AMPK inhibitor) was used as a control for hypoxic conditions.

**Results:** Cell viability was reduced at 48 and 72hrs (P=0.03 and 0.01 respectively). Western blot analysis showed a decrease in expression of pS6K protein in cells exposed to hypoxia. PI3K and pAkt protein expression was unchanged in hypoxia while AMPK protein expression was increased. The reduced expression of pS6K in hypoxia was reversed following dorsomorphin treatment. MIN6 β-cell viability was also increased following culture with dorsomorphin.

**Conclusion:** We found that beta-cells exposed to hypoxia demonstrated restricted growth confirmed by a reduced expression of the proliferation marker S6K. AMPK expression was increased following exposure to 1% oxygen. These results demonstrated that S6K expression and MIN6 beta-cell viability was increased by dorsomorphin in hypoxic conditions. This study showed that hypoxia-induced growth was associated with changes in AMPK expression which may be a key regulatory pathway in the hypoxia response of the beta-cell.
Publications
