The Role of SPARC in β Cells and Diabetes

Claire Louise Ryall

A thesis submitted in partial fulfilment of the requirements of Kingston University for the degree of Doctor of Philosophy

Kingston University, School of Life Sciences

October 2013

Declaration

I declare that the work reported in this thesis is entirely my own and has been carried out at Kingston University and King's College London.

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Claire Louise Ryall

Abstract

Diabetes mellitus was responsible for 4.8 million deaths worldwide in 2012. Cardiovascular diseases arising from the condition are responsible for half of all diabetes related deaths. The loss of insulin due to pancreatic β cell death can be restored by replacement with transplanted islets from a donor. However, β cell replacement is limited by the number of donors and the limited capacity of the β cell to proliferate. Attempts to stimulate β cell proliferation and improve islet transplantation methods are successful in the short term, however preventing β cell death after transplant and inducing growth with continued long-lasting insulin secretion is still the aim to prevent hypoglycaemia in patients with diabetes. Elucidating the mechanisms required for β cell survival and proliferation would allow successful restoration of blood glucose regulation.

Secreted Protein Acidic and Rich in Cysteine (SPARC) is secreted by stromal cells such as fibroblasts and endothelial cells and binds collagen. In adults it is expressed in tissues that have a high rate of proliferation and during tissue remodelling. Experimentally, SPARC has been found to prevent cell growth *in vitro* in certain cell types, as well as promoting cell rounding and detachment.

The aims of this study were to determine whether SPARC had inhibitory effects on β cell growth and signalling as tested in the cell line INS-1, and whether this translated to islet cells tested *ex vivo*. SPARC was found to be sparsely located within islets to stromal cell types and peri-islet basement membranes. The expression of SPARC was higher in a 4 week pancreas compared to 12 week pancreas ($p = 0.012$) and was higher in a pre-diabetic model than in control pancreas ($p = 0.008$). This suggests the involvement of SPARC in early development of the pancreatic islets and indicates that SPARC may be important in the islet during the progression of diabetes. SPARC prevented IGF-1-induced

INS-1 proliferation ($p < 0.001$) and islet survival ($p = 0.045$). Presence of a stellate cell line PS-1 in co-culture with islets was also found necessary for their survival ($p = 0.010$). The interference of SPARC with the growth-factor response was also demonstrated by the prevention of signalling protein Akt and ERK 1/2 activation in both INS-1 cells and islets.

This study established that SPARC had a detrimental effect on β cell lines and dispersed islet cells and was over-expressed in diabetic pancreas. SPARC as a regulator of β cell growth may be promising as a target for diabetes therapy.

Acknowledgements

First and foremost I would like to thank my Director of Studies Dr. Natasha Hill for her supervision throughout the project. Her guidance, support and help have been invaluable. I would like to thank Professor Tony Walker for his training in various techniques and his guidance without which I would have been lost. I am grateful to Dr. Charlotte Edling and Dr. David Mackintosh for their support as secondary supervisors. Special thanks go to the researchers at King's College London, Aileen, Peter, James and Chloe for their invaluable advice and training in islet isolation techniques. I would like to thank Dr. Ruth Kirk for training in immunohistochemistry techniques and equipment.

The technical staff Jayne, Gurm, Lindsey and Michael deserve a special mention for their help and expertise as well as the provision of chemicals and equipment at short notice. To my fellow researchers, not naming all but especially Saira, Jo, Niousha, Lauren, Nick, Delphine and Tam, I am forever grateful for making these four years easier, keeping me sane with hugs and tea. Thanks also go to Diabetes UK and the Biomedical and Pharmaceutical Sciences Group at Kingston University for funding this project.

I would also very much like to thank all my friends and family, especially Mum and Dad, for their support throughout the project and helping me get through the rough times. Without my friends and family this would have been impossible, and I am grateful even if you never understood what I was doing. Thank you for your patience and understanding, I am pleased that I will have more time to spend with you all now.

Finally I would like to dedicate this thesis to my grandparents, Sheila and Les, both of whom sadly passed away while I was working on this project.

"The mind is not a vessel to be filled, but a fire to be kindled." -Plutarch

Table of Contents

Abstract i
Acknowledgements iii
Table of Contents iv
List of Figures vii
List of Tables ix
List of Abbreviations x
CHAPTER 1: Introduction 1
1.1 Background to diabetes 1
1.2 Anatomy of the pancreas 3
1.3 Normal β cell function 6
1.4 Type 1 diabetes mellitus 8
1.5 Type 2 diabetes mellitus 18
1.6 Gestational diabetes 23
1.7 Problems with current treatments for diabetes 23
1.8 Transplantation 25
1.9 Islet transplantation 28
1.9.1 Improvements in islet isolation and transplantation 33
1.10 Regenerating pancreatic β cells 38
1.11 Alternative sources of β cells 40
1.12 SPARC 44
1.12.1 Structure of SPARC 44
1.12.2 SPARC expression 45
1.12.3 SPARC interactions 46
1.12.4 SPARC and cancer 48
1.12.5 SPARC function 49
1.12.6 The role of SPARC in diabetes 51
1.13 Growth factors 53
1.13.1 Growth factors and the β cell 53
1.14 SPARC and growth factors 57
1.15 Aims 58
CHAPTER 2: Materials and Methods 62
2.1 Animals 62
2.2 Reagents and Antibodies 63
2.3 Cell culture 64

2.3.1 Cell lines and media 64
2.3.2 Sub-culturing 64
2.3.3 Trypan blue dye exclusion assay 65
2.3.4 Cryopreservation of cell lines 65
2.4 Islet isolation 66
2.5 Preparation of reagents 66
2.6 Fluorescence immunohistochemistry 67
2.7 Immunocytochemistry 68
2.8 Immunohistochemistry (SPARC or FSP-1) 69
2.9 Islet insulin scoring 70
2.10 SPARC expression (western blotting) 71
2.10.1 Preparation of buffers 71
2.10.2 Preparation of cell lysates 72
2.10.3 Quantification of protein 72
2.10.4 Preparation of the gel and protein separation by SDS-PAGE 73
2.10.5 Western blot 73
2.11 ERK and Akt signalling 74
2.11.1 Experimental procedure and preparation of cell lysates 74
2.12 Cell proliferation assay 75
2.13 Live/Dead islet assay including SPARC siRNA knockdown 76
2.14 Statistics 77
CHAPTER 3: Results 79
3.1 Confirmation of INS-1 as a β cell line 79
3.2 Expression of SPARC 81
3.2.1 SPARC and FSP-1 are expressed in PS-1 cells, but not INS-1 cells 81
3.2.2 SPARC is expressed early in islets of mice, and expression is increased in a diabetes-susceptible strain 85
3.2.3 SPARC is located in the same areas as FSP-1 in pancreas sections 88
3.3 Islet insulin scoring 90
3.3.1 No difference in islet area in SPARC knockout mice 90
3.3.2 No difference in islet size in SPARC knockout mice 92
3.4 Intracellular kinase signalling 96
3.4.1 IGF-1 and HGF induce activation of signalling proteins Akt and ERK 1/2 in a transient manner 96
3.4.2. The optimal concentration of growth factor for stimulation of ERK and Akt signalling in INS-1 Cells was 20 ng/ml 99
3.4.3 SPARC inhibits IGF-1-mediated kinase signalling in INS-1 cells 101

3.4.4 SPARC inhibits HGF-induced Akt phosphorylation in INS-1 cells 103
3.4.5 HGF induces ERK 1/2 signalling in intact islets 105
3.4.6 SPARC had no effect on HGF-induced signalling in intact islets 107
3.4.7 SPARC inhibits HGF induced signalling in dispersed islets 108
3.5 Proliferation of a β cell line 109
3.5.1 Optimisation of seeding density 109
3.5.2 Varying the concentration of FBS in starvation media 110
3.5.3 200 ng/ml IGF-1 in media containing 0.5% serum is optimal to induce INS-1 cell proliferation 114
3.5.4 SPARC inhibits IGF-1 induced cell growth 117
3.5.5 SPARC in 0.5% serum reduces protein content 118
3.6 Islet live/dead assay 119
3.6.1 Development of islet survival assay; islets induced to survive or perish after a 2 day treatment 119
3.6.2 Islets survive with the addition of IGF-1 121
3.6.3 SPARC prevents IGF-1-induced survival of islets 122
3.6.4 PS-1 secreted SPARC is necessary for islet survival 124
CHAPTER 4: Discussion 127
4.1 Expression 128
4.2 Signalling 135
4.3 Proliferation and survival 140
4.4 Roles for SPARC 149
4.4 Conclusions 151
4.5 Future work 151
References 155
Appendix 1 I
Appendix 2 IV
Appendix 3 VII

List of Figures

Figure 1.1. Anatomy of the pancreas 3

Figure 1.2. Composition of the islets of Langerhans 6

Figure 1.3. Events leading to the release of insulin from the β cell 7

Figure 1.4. Factors contributing to type 1 diabetes 10

Figure 1.5. Pancreatic islet transplantation 29

Figure 1.6. Examples of islet encapsulation and embedding within scaffolds. 37

Figure 1.7. The domains of SPARC 45

Figure 1.8. Intracellular growth factor signalling 54

Figure 3.1. Expression of insulin in INS-1 cells and islets 80

Figure 3.2. SPARC is expressed by PS-1 cells 82

Figure 3.3. FSP-1 is expressed by PS-1 cells 84

Figure 3.4. Higher expression of SPARC in NOD islets than control islets 87

Figure 3.5. SPARC is strongly expressed by stellate cells as well as a minority cell population in islets 89

Figure 3.6. Example of section images from one B6 (wild type) mouse pancreas 91

Figure 3.7. SPARC knockout mice show no differences in islet area to wild type mice 93

Figure 3.8. In females, SPARC knockout mice show no differences in islet area to wild type mice 95

Figure 3.9. Stimulation of intracellular signalling by growth factors 98

Figure 3.10. Optimisation of treatment times and concentration of growth factors 100

Figure 3.11. Reduction of IGF-1 induced INS-1 signalling by SPARC 102

Figure 3.12. SPARC reduced HGF induced Akt signalling 104

Figure 3.13. HGF induced ERK signalling in intact islets 106

Figure 3.14. No effect of SPARC on signalling in intact islets 107

Figure 3.15. SPARC inhibits HGF-induced signalling in dispersed islets 108

Figure 3.16. Reduced seeding density allows for increased rate of proliferation 110

Figure 3.17. Mid-range FBS is optimal for cell growth and detecting changes when using growth factors 113

Figure 3.18. IGF-1 stimulated optimal increase in growth in 0.5% serum-containing media 116

Figure 3.19. SPARC reduces IGF-1 induced INS-1 growth....117

Figure 3.20. SPARC in 0.5% serum reduces INS-1 growth....118

Figure 3.21. Induced death in dispersed islets after a 2 day treatment....120

Figure 3.22. IGF-1 induces cell survival in dispersed islets....121

Figure 3.23. Exogenous SPARC prevents IGF-1-induced islet survival....123

Figure 3.24. PS-1 secreted SPARC is necessary for islet survival....126

Figure A2.1. Secondary control for texas red antibody....IV

Figure A2.2. SPARC secondary controls are negative....V

Figure A2.3. SPARC and FSP-1 secondary controls are negative....VI

List of Tables

Table 2.1. Commercial antibodies used for immunohistochemistry and western blotting..... 63
Table A1. List of reagents.....I
Table A2. List of disposables.....II
Table A3. List of equipment.....III

List of Abbreviations

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BM-40	Basement Membrane 40
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD3/CD4/CD8	Cluster of Differentiation 3/4/8
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular-signal Regulated Kinase
FBS	Foetal Bovine Serum
FDA	Fluorescein Diacetate
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FSP-1	Fibroblast Specific Protein
FTO	Fat mass and Obesity-associated
GADA	Glutamic Acid Decarboxylase
GFP	Green Fluorescent Protein
GLP-1	Glucagon-like Peptide 1
GLUT2/4	Glucose Transporter 2/4
GWA	Genome-Wide Association
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
IA2	Islet Antigen 2
IAA	Islet Autoantibody

IAPP	Islet Amyloid Polypeptide
IBMIR	Instant Blood Mediated Inflammatory Reaction
ICA	Islet Cell Antigen
ICR (mice)	Institute for Cancer Research
IFG	Impaired Fasting Glycaemia
IFN	Interferon
IGF	Insulin-like Growth Factor
IGF1R	Insulin-like Growth Factor 1 Receptor
IGF2R	Insulin-like Growth Factor 2 Receptor
IGFBP	Insulin-like Growth Factor Binding Protein
IGT	Impaired Glucose Tolerance
IHC	Immunohistochemistry
IL	Interleukin
ILK	Integrin Linked Kinase
INS-1	Insulin-Secreting Cell Line
ISL1	Insulin Enhancer Binding Protein 1
JNK	c-Jun N-terminal Kinases
K^{ATP}	ATP-sensitive potassium channels
LADA	Latent Autoimmune Diabetes of Adulthood
LDL	Low Density Lipoprotein
MAPK	Mitogen Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
MODY	Maturity Onset Diabetes of the Young
MSC	Mesenchymal Stromal Cell

NF-κB	Nuclear Factor kappa-light-chain-Enhancer of Activated B Cells
NO	Nitric Oxide
NOD	Non-Obese Diabetic
NOS	Nitric Oxide Synthase
PDGF	Platelet Derived Growth Factor
PET	Polyethylene Terephthalate
PI	Propidium Iodide
PI3-Kinase	Phosphatidylinositide 3-Kinase
PP cells	Pancreatic Polypeptide Cells
PS-1	Pancreatic Stellate Cells
RNA	Ribonucleic Acid
SCID	Severe Combined Immunodeficiency
SEM	Standard Error of the Mean
SPARC	Secreted Protein, Acidic and Rich in Cysteine
SPK	Simultaneous Pancreas-Kidney
SRB	Sulphorhodamine B
STZ	Streptozotocin
TCA	Trichloroacetic Acid
TCF7L2	Transcription Factor 7-like 2
TGF-β	Transforming Growth Factor-β
TNF	Tumour Necrosis Factor
TRITC	Tetramethylrhodamine Isothiocyanate
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
WB	Western Blotting

CHAPTER 1: Introduction

1.1 Background to diabetes

It is estimated that 347 million people worldwide suffer from diabetes mellitus, which includes type 1, type 2 and gestational diabetes (Danaei *et al.*, 2011). In 2012, 4.8 million people died and $471 billion was spent as a result of diabetes (Idf.org, 2012). In the same year, the diagnosed prevalence in the UK was 3 million, though many more cases are thought to go undiagnosed. Globally, diabetes is expected to affect 522 million people by 2030 (Diabetes.org.uk, 2012), at which time it is projected to be the 7th leading cause of death (Mathers and Loncar, 2006). Cardiovascular disease, including heart disease and stroke, is the most common cause of death in diabetes patients being responsible for half of all diabetes related deaths (Morrish *et al.*, 2001). Other deaths resulting from diabetes are renal diseases (14.3%), cancer (malignant neoplasms, 13%) and death due to diabetes itself (3.3%) which include diabetic ketoacidosis and hypoglycaemia due to insulin overdose (Morrish *et al.*, 2001).

Latent Autoimmune Diabetes of Adulthood (LADA), otherwise known as type 1.5 diabetes, is not always diagnosed correctly at first and may be mistaken for type 2 diabetes since it presents later in life and patients still produce their own insulin. However insulin levels decrease, albeit slowly, and autoantibodies (explained below) are present, as typically seen in type 1 diabetes. The combination of qualities from both type 1 and 2 diabetes led to this particular form being named type 1.5 diabetes.

Rarer forms of diabetes include the conditions impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG), both of which comprise pre-diabetes (an early condition associated with obesity that usually develops into type 2 diabetes). Other forms include

Maturity Onset Diabetes of the Young (MODY), an inherited form of diabetes caused by β cell dysfunction, and pancreoprivic diabetes in which the individual has no functioning pancreas due to a medical condition or surgical removal (Gavin *et al.*, 2003). Diabetes *insipidus* is also a condition causing excessive urine excretion, although this is not related to high blood glucose levels (Robertson, 1995).

A number of other conditions related to the pancreas can also result in β cell loss with diabetes occurring as a side effect of the underlying condition. These include pancreatitis, cancer and exocrine pancreatic insufficiency. Pancreatitis is the inflammation of the pancreas and can present itself in the acute and chronic forms. This inflammation of the pancreas results from pancreatic enzymes, especially trypsin, being activated too early and before release into the intestine. The acute form begins suddenly and lasts days, whereas the chronic form can last for years. It is usually caused by gallstones and alcohol (Mitchell *et al.*, 2003).

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the USA and Europe (Dhayat *et al.*, 2011). Adenocarcinoma accounts for approximately 95% of the tumours, and results from tumours arising in the exocrine part of the pancreas. By the time of diagnosis, the carcinoma has usually metastasized and therefore pancreatic cancer has a poor prognosis (Haeno *et al.*, 2012, Tuveson and Neoptolemos, 2012).

Exocrine pancreatic insufficiency results from a loss of exocrine cells, leading to a reduced amount of digestive enzymes. It is found in patients already suffering with cystic fibrosis, Shwachman-Diamond syndrome and chronic pancreatitis (Leus *et al.*, 2000, Mitchell *et al.*, 2003, Dror and Freedman, 2002). Other lesser-known complications arising in the pancreas also exist. Inflammation and scarring caused by these diseases, especially pancreatitis, cancer and cystic fibrosis, may lead to the progressive loss of β cells and

hence diabetes may result. It is also possible for diabetes to lead to pancreatitis and cancer (CancerResearchUK.org, 2009).

1.2 Anatomy of the pancreas

The pancreas (Figure 1.1) is a multifunctional organ situated in the abdomen, important in the digestive and endocrine systems. It contains exocrine acinar cells that are responsible for secreting enzymes into the digestive system. The enzymes consist of amylases, lipases and proteases amongst others, and are secreted into the pancreatic duct which flows into the bile duct, and the digestive enzymes are released into the duodenum.

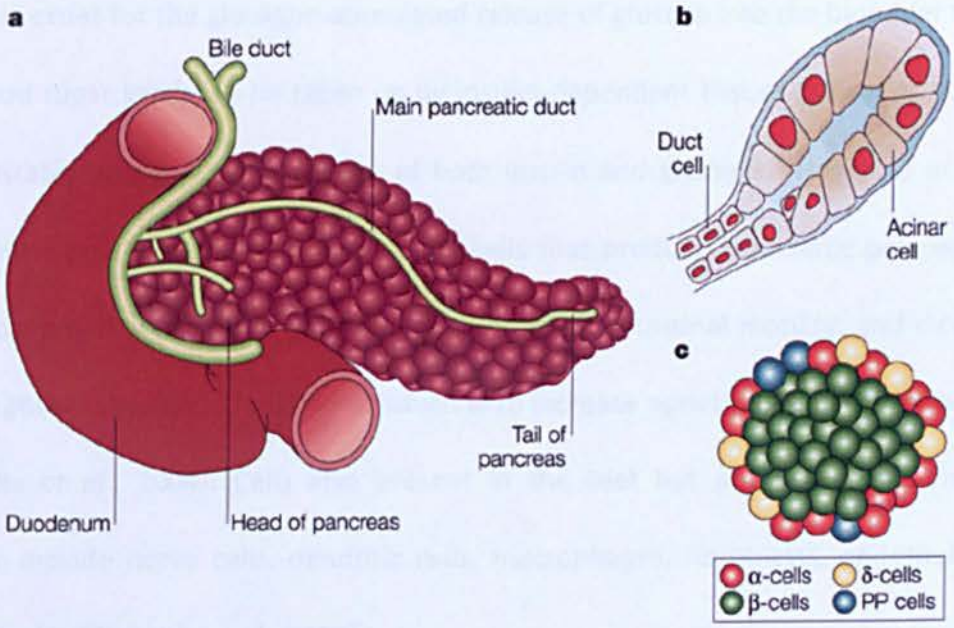


Figure 1.1. Anatomy of the pancreas. The pancreas, about 6 inches long, sits in the abdomen and consists of a head, body and tail (**a**). The pancreatic duct joins into the bile duct and delivers digestive enzymes from acini (**b**) into the duodenum. Islets of Langerhans (**c**) are dispersed throughout the pancreas and release hormones into the bloodstream. Edlund, 2002.

The endocrine component of the pancreas consists of scattered islets of Langerhans (named after the physician who discovered them in 1869), which make up 1-2% of the pancreas. The normal human pancreas contains approximately 1 million islets randomly situated amongst the exocrine tissue, connected by blood vessels (Figure 1.2). Despite only taking up 1-2% of the pancreatic mass, the islets take 5-10% of the pancreatic blood supply (Brissova and Powers, 2008).

The islets consist of glucagon-producing α cells and insulin-secreting β cells, both of which regulate blood glucose. The δ cells of the islet produce somatostatin to inhibit the release of other hormones (e.g. growth hormone and gastrointestinal hormones). To ensure proper glucose homeostasis, insulin and glucagon also regulate the release of each other in a paracrine fashion. Insulin inhibits the release of glucagon when blood glucose concentration is high (Maruyama *et al.*, 1984). Glucagon stimulates insulin secretion from β cells, in order for the glucagon-stimulated release of glucose into the blood (at times of low blood sugar levels) to be taken up by insulin-dependent tissues (Kawai *et al.*, 1995). Somatostatin suppresses the release of both insulin and glucagon (Hagen *et al.*, 1994). The other types of islet cells are PP (or F) cells that produce pancreatic polypeptide to inhibit pancreatic secretions, gall bladder activity and intestinal motility, and ε cells that secrete ghrelin, the main function of which is to increase appetite (Beglinger *et al.*, 1984, Korbonits *et al.*, 2004). Cells also present in the islet but not possessing endocrine function include nerve cells, dendritic cells, macrophages, fibroblasts, endothelial cells and pericytes (Richards *et al.*, 2010).

The β cells are the sole producers of insulin in the body. Under normal circumstances, β cells undergo very low rates of proliferation, as do the other islet cell types (Teta *et al.*, 2005, Kassem *et al.*, 2000). The supply of insulin to the body therefore depends upon the long life span of the β cells (Cnop *et al.*, 2010). Humans have a relatively high rate of β cell

proliferation (3.2%) at the foetal stage (17 to 32 weeks of gestation), after which the rate of proliferation slows throughout the first few months of life to a rate of 0.13% after 6 months of age (Kassem *et al.*, 2000). Rates of apoptosis, however, fluctuate during the early phases of life. In the foetal stages and after 6 months of age, apoptosis is relatively low (0.6% and 0.08%, respectively) whereas during the peri-natal period (2 months before and after birth) there is a relatively high rate of β cell apoptosis (1.3%) (Kassem *et al.*, 2000). During the early postnatal period β cell proliferation and apoptosis may be responsible for the islet remodelling that occurs during pancreatic development in order to form the mature islet structure and remove β cells present in exocrine tissue. After this period, β cell proliferation and apoptosis slow to very low levels (0.13% and 0.08%, respectively) (Kassem *et al.*, 2000).

Distribution of cells within the islet varies between species. Mouse islets have a distinct architecture with a core of β cells surrounded by α cells at the periphery (Brissova *et al.*, 2005). Humans, however have a less distinct architecture, where α and β cells are interspersed throughout (Figure 1.2). Proportions of β cells in the islet also vary, with mouse and human islets containing means of 75% and 54% β cells, respectively. As well as the lower proportion of β cells in human islets, the percentage also varies considerably between islets, from 28% to 75% (Brissova *et al.*, 2005).

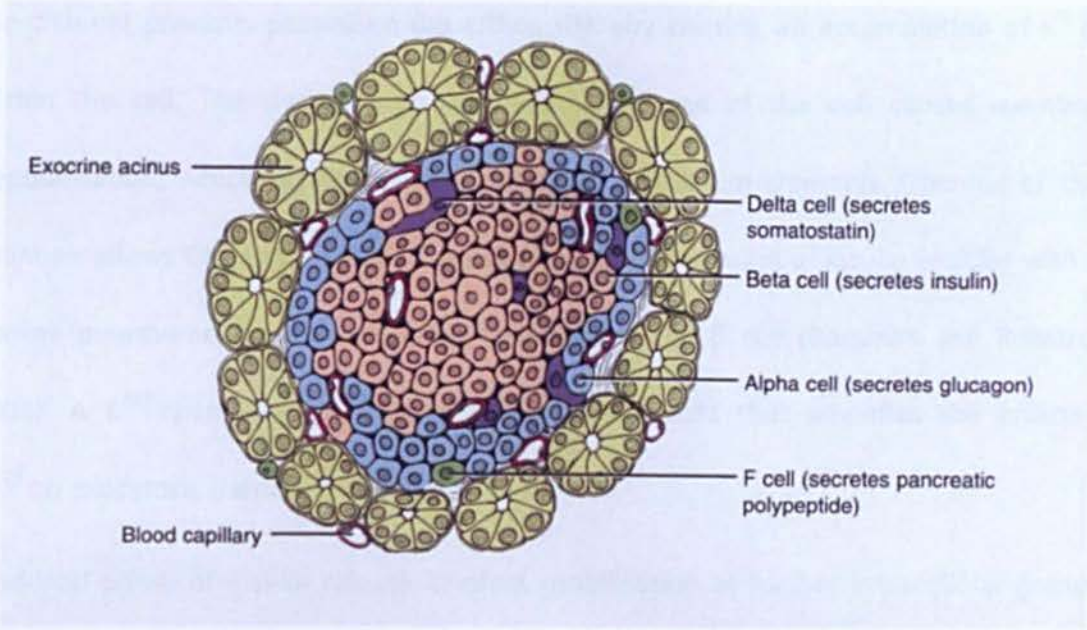


Figure 1.2. Composition of the islets of Langerhans. Exocrine cells surround the islet, consisting of α, β, δ, F (PP) and ε cells. Blood capilaries are dispersed throughout. http://web.campbell.edu/faculty/nemecz/308_lect/lect6/lect6.html

1.3 Normal β cell function

Links between the pancreas and diabetes were first discovered in 1889. In experimental animals, the whole pancreas was removed resulting in the development of severe diabetes mellitus. In 1922, research carried out by a group of 4 scientists and surgeons, Banting, Macleod, Best and Collip, resulted in the successful reversal of diabetes in a 14 year old boy. This was achieved through a series of injections of their pancreatic extract. They received a Nobel Prize for their research (Joshi *et al.*, 2007).

In response to high blood glucose concentrations, insulin is released from islet β cells into the bloodstream. A brief summary of the mechanics of this action is outlined in Figure 1.3. In periods of increased blood glucose (after a meal) the GLUT2 transporter transports glucose into the β cell. Metabolism of this glucose during glycolysis and the Krebs cycle produces adenosine triphosphate (ATP), therefore increasing the ratio of ATP to adenosine diphosphate (ADP) in the cell. The increased ATP/ADP ratio inhibits ATP-sensitive potassium channels (K^{ATP}) which are located at the cell membrane. Inhibition of

the channel prevents potassium ion efflux, thereby causing an accumulation of K^+ ions within the cell. The decrease in the negative charge of the cell causes membrane depolarisation, which activates voltage-dependent calcium channels. Opening of these channels allows Ca^{2+} ions to enter the cell and stimulate fusion of insulin vesicles with the plasma membrane, thereby releasing insulin from the β cell (Rorsman and Renstrom, 2003). A K^{ATP} channel-independent pathway also exists that amplifies the effects of Ca^{2+} on exocytosis (Henquin, 2000).

A second phase of insulin release involves mobilisation of further intracellular granules causing fusion with the membrane and release of insulin, as well as the additional steps of insulin biosynthesis, packaging into granules and transport to the plasma membrane for release (Rorsman and Renstrom, 2003).

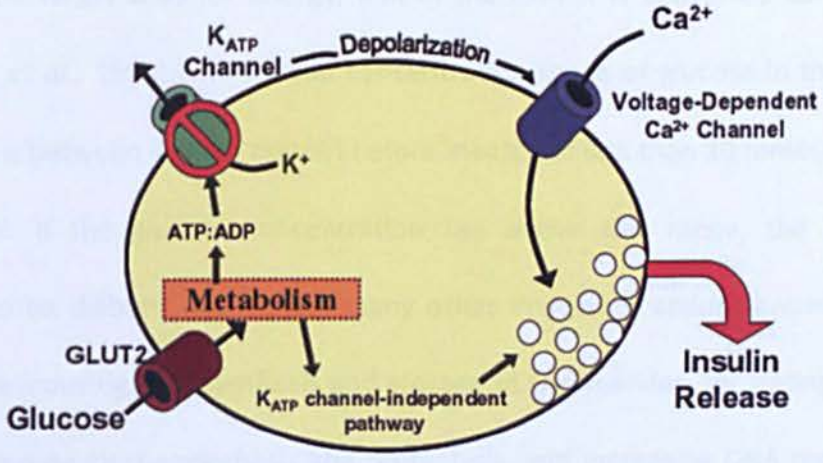


Figure 1.3. Events leading to the release of insulin from the β cell. The GLUT2 transporter transports glucose into the β cell where glucose metabolism produces an increase in the ATP/ADP ratio. This increase inhibits ATP-sensitive potassium channels on the plasma membrane, thereby sequestering K^+ ions within the cell and causing membrane depolarisation, activation of voltage-dependent Ca^{2+} channels and influx of extracellular calcium. High intracellular calcium levels causes insulin granules to fuse to the plasmas membrane and release their insulin.
http://www.betacellmetabolism.org/index_files/Page1204.htm

The mature structure of insulin consists of two polypeptide chains comprising 21 and 30 amino acid residues linked by disulphide bridges. The shorter chain also contains an intra-chain disulphide bridge. The formation of mature insulin starts with the translation of pre-pro-insulin in the endoplasmic reticulum. Pre-pro-insulin consists of one long chain containing an amino-terminal signal sequence. Processing involves the removal of the signal sequence and formation of the disulphide bonds giving pro-insulin. Disulphide bonds form and proteases cleave the middle portion, liberating mature active insulin and C-peptide (Halban, 1991).

Target cells for insulin include muscle and fat cells, as well as liver cells in which glucose is stored as glycogen. The binding of insulin to its receptor on target cells stimulates a signalling cascade that results in translocation of the glucose transporter GLUT4 to the membrane. The presence of GLUT4 at the membrane allows uptake of glucose from the blood into the target cells for energy, and in the liver it is converted to glycogen for storage (Klip *et al.*, 1990). The normal concentration range of glucose in the blood in a non-diabetic is between 4 and 7 mmol/l before meals and less than 10 mmol/l 90 minutes after a meal. If the glucose concentration lies above this range, the individual is considered to be diabetic. Insulin has many other important actions around the body. These include inducing lipid synthesis and storage of triglycerides, increasing amino acid uptake, decreasing gluconeogenesis and proteolysis, and increasing DNA replication and protein synthesis (Kimball *et al.*, 1994).

1.4 Type 1 diabetes mellitus

Type 1 (insulin-dependent, juvenile or childhood-onset) diabetes accounts for approximately 10% of patients with diabetes. It results from an inability of the β cells of

the pancreas to produce enough insulin to meet the requirements of the body for carbohydrate and fat metabolism. Type 1 can be classified further into type 1A diabetes, with an autoimmune cause, and type 1B diabetes, which is idiopathic. Individuals with type 1B do not show evidence of autoimmunity and test negative for autoantibodies (Jahromi and Eisenbarth, 2007).

Diagnosis of diabetes onset can include the detection of autoantibodies that react with endocrine cells of the islet (Knip, 2002, Wasserfall and Atkinson, 2006). Important autoantibodies include islet cell cytoplasmic antigen (ICA), insulin autoantibody (IAA), glutamic acid decarboxylase (GADA), and islet antigen 2 (IA2)/islet cell antigen (ICA)-512 autoantigen (IA2A), which bind to the β cell and target it for destruction (Figure 1.4) (Jahromi and Eisenbarth, 2007, Wasserfall and Atkinson, 2006). These islet autoantibodies can also be used in the prediction of developing diabetes in later life, especially in combination with other markers such as reduced first phase insulin secretion after glucose stimulation (Wasserfall and Atkinson, 2006).

Although type 1 diabetes is known to be an autoimmune disease, events that initiate the attack of T lymphocytes on the β cells are largely unknown. It is thought to be due to a combination of genetic and environmental factors. The genetic element to the disease is polygenic with the strongest genetic factor (for susceptibility and resistance) being the locus *IDDM1* (Major histocompatibility complex (MHC) class II) on chromosome 6 (Todd, 2010). In humans, the *MHC* locus is also known as human leukocyte antigen (HLA). *MHC II* genes encode cell surface receptors on antigen presenting cells, and most of the type 1 diabetes susceptibility loci encode products that function in the immune response, however the biological mechanism by which HLA and other loci confer diabetes susceptibility remains unknown. The pathway by which T cells are activated entails antigen presentation by the MHC II molecules on antigen presenting cells to

CD4 receptors on T-helper cells (Redondo *et al.*, 2001a, Todd, 2010). Antigen presentation occurs in the islets, as well as in the thymus and in peripheral lymphatic tissue such as the pancreatic lymph nodes (Figure 1.4). CD4+ T cells activate CD8+ cytotoxic T cells, the main effectors of islet β cell death in diabetes (Todd, 2010).

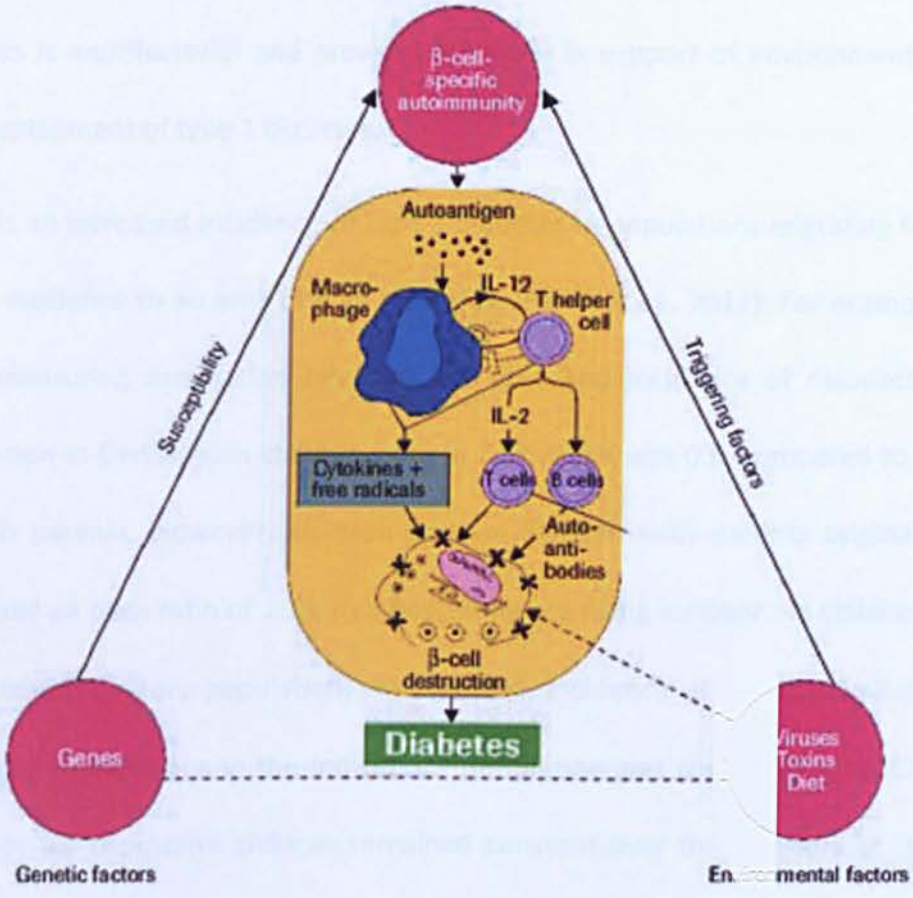


Figure 1.4. Factors contributing to type 1 diabetes. A schematic diagram shows how both environmental and genetic factors can initiate type 1 diabetes onset through β cell-specific autoimmunity processes which lead to the destruction of the pancreatic β-cell. An antigen presenting cell (e.g. macrophage) releases cytokines in response to autoantigens. Cytokines signal T-helper cells (which express CD4+) to activate B and T cells which then release autoantibodies that bind pancreatic β-cell and target it for destruction. Jahromi, 2013.

Although there are strong implications for a genetic link in type 1 diabetes, it is not entirely genetically determined and possession of these genes does not mean that an individual will develop diabetes. As shown in Figure 1.4, it is one of the contributing

factors towards susceptibility for β cell autoimmunity. There are studies showing that individuals with the susceptibility genes do not develop diabetes, for example, Knip *et al* discuss that less than 10% of individuals with the HLA susceptibility genes developed clinical diabetes (Knip *et al.*, 2005). A study in monozygotic twins from the United States and Great Britain found that if one twin has diabetes, only 25% of the non-diabetic co-twins later developed diabetes (Redondo *et al.*, 2001b). This demonstrates that type 1 diabetes is multifactorial and provides evidence in support of environmental factors in the development of type 1 diabetes.

There is an increased incidence of type 1 diabetes in populations migrating from an area of low incidence to an area of high incidence (Hjern *et al.*, 2012). For example, the odds ratio, measuring association between the area and incidence of diabetes for insulin medication in East African children born in East Africa was 0.5, compared to offspring of Swedish parents. However, children born in Sweden with parents originating in East Africa had an odds ratio of 1.29, demonstrating the rising incidence in childhood diabetes in the transmigratory population. In Bradford, incidence of diabetes in Asian children compared to incidence in the indigenous population was compared over 12 years. The incidence for the native children remained constant over the 12 years at 10.5/100,000 per year. However the incidence for the Asian children increased from 3.1/100,000 per year in the first three years of the study to 11.7/100,000 per year in the last three years. (Bodansky *et al.*, 1992). The increase in development of diabetes in the high incidence area strongly suggests environmental factors are responsible for diabetes development (Jahromi, 2013). Progression to clinical diabetes is thought to be the result of a combination of genetic predisposition as well as the influence from environmental factors (Knip *et al.*, 2005).

Many groups are still presenting evidence for or against the influence of viruses as an environmental trigger for onset of type 1 diabetes. Detection of the presence of viruses in pre-diabetic individuals, specifically enterovirus and especially coxsackieviruses has recently strengthened the association between viruses and the onset of diabetes. Coxsackievirus B1 is associated with an increased risk of diabetes susceptibility, shown by screening neutralising antibodies with sera from children with increased diabetes predictive autoantibodies (Laitinen *et al.*, 2013), however, the precise role of coxsackie viruses are still unknown. One recent study has shown that coxsackie B4 virus is able to stimulate the production of autoantibodies in pre-disposed individuals (with recent onset type 1 diabetes) by possessing antigens mimicking those of β cell antigens, thus stimulating the production of autoantibodies towards β cells (Bason *et al.*, 2013).

Despite the recent evidence, a causal role for virus-induced pathogenesis of diabetes is still difficult to prove. Data from animal models suggests a number of different mechanisms by which viruses can contribute to β cell death. These include the direct attack of the virus on the β cell, causing cytolysis. Dotta and colleagues demonstrated that coxsackie B4 virus is present in diabetic patients, and when isolated from infected pancreas and applied to β cells *in vitro*, the virus replicates, infects the β cell and reduces insulin secretion (Dotta *et al.*, 2007). However they did not show it was a cause of diabetes, only demonstrated its presence in the pancreas post-mortem, and only in three out of a total of six patients. Direct action on the β cells themselves is not in accordance with the pathogenesis of diabetes, since the mechanism of diabetes onset is thought to be through activation of CD4+ and CD8+ T cells, not through direct infection of the β cell. This research woud also need to be replicated *in vivo* with outcome of diabetes to be considered evidential proof (Dotta *et al.*, 2007).

Damaging the self tolerance of the immune system to the β cells without infecting β cells directly, as described above, is achieved by expression of viral antigens in the β cells, which may also increase expression of MHC antigens or cytokines. The bystander model suggests the presence of local inflammation caused by the presence of virus can cause tissue damage, releasing islet antigens and activating T cells (Seewaldt *et al.*, 2000, Horwitz *et al.*, 1998, Bason *et al.*, 2013).

Enteroviral infection in children with susceptibility to type 1 diabetes (either showing positive for autoantibodies, carrying the high risk HLA genotype, or with a first degree relative with diabetes) can increase the rate of progression of diabetes onset (Stene *et al.*, 2010). However it was specified in the study that the presence of enterovirus did not predict progression to type 1 diabetes. Of the children who developed diabetes, only three out of 36 were positive for viral infection (Coxsackie B), and no evidence of viral persistence (same strain in successive samples) was found. Occurrence of viral infection in individuals who did not develop diabetes (but who also had high risk markers autoantibodies and/or HLA genotype) was not clear in their report (Stene *et al.*, 2010).

Oikarinen and colleagues discovered greater amounts of enteroviral RNA in serum from diabetic children (5.1%) than in the non-diabetic control group (1.9%), and the highest infection period was in the six months prior to the first autoantibody positive serum sample (Oikarinen *et al.*, 2011). In all three of the studies, samples were taken only every 3-6 months therefore viral occurrence may have been underestimated.

The most recent study investigating whether viruses contribute to the triggering of diabetes concluded that a link was not found (Lee *et al.*, 2013). However there were many limitations to this study, including small sample set (24 children), only young children were investigated, the investigation into only rapid onset-diabetes (less than six months from first autoantibody detection to diabetes onset). This does not rule out that a virus

can be responsible for later or slower-onset diabetes. Another limitation of their study lies with sampling only every three months, leaving long periods during which viral infection may have been present for a short while and was therefore not detected in the samples for the study (Lee *et al.*, 2013). Evidence for a viral cause is therefore not conclusive, however it is likely that a virus could be responsible in some individuals, and if it is not the initiator of diabetes onset, it may accelerate it. It is important to remember that in these studies some patients may have viral resistance and heterogeneity between patients is not considered. Some patients in the group may have a form of diabetes that is triggered by a viral infection and other patients may not.

The hygiene hypothesis for inducing diabetes suggests that individuals with genetic predisposition are at increased risk of developing diabetes in germ-free environments. This means that upon exposure to certain bacterial infections, development of diabetes is reduced and the infection is protective (Wen *et al.*, 2008). This is supported by the occurrence of spontaneous diabetes in the non-obese diabetic (NOD) mouse. Incidence of diabetes is increased when the mice are raised under specific pathogen-free conditions (Leiter, 2001, King and Sarvetnick, 2011). There is also a reverse trend observed between infectious disease incidence and the incidence of autoimmune diseases worldwide, and development of diabetes is higher in children with the lowest level of infectious disease exposure (Bach and Chatenoud, 2012). However, to prove the link, it must be shown that suppressing infections in individuals increases diabetic incidence (Bach and Chatenoud, 2012).

Other evidence for environmental triggers of type 1 diabetes includes the findings that lack of vitamin D (due to low sunlight exposure and failure to provide supplements) is associated with increased incidence of diabetes. Genes involved in the metabolism of vitamin D are associated with a risk for type 1 diabetes (Cooper *et al.*, 2011). Incidence is

lower in regions close to the equator compared with regions further north or south of the equator, and meta-analysis has shown that vitamin D supplementation can reduce the risk of developing type 1 diabetes (Jahromi, 2013, Zipitis and Akobeng, 2008). However, a study conducted in the Russian Karelian and Finnish populations (regions of similar latitude), which have a six-fold difference in diabetes incidence, showed no difference in circulating vitamin D in these populations (Viskari *et al.*, 2006).

Another possible risk factor for type 1 diabetes is particular dietary components provided to babies and children. Much of the research into early diet has assessed increases in circulating islet autoantibodies rather than the onset of diabetes, so may overestimate the connection with development of diabetes. Triggers investigated all have evidence both for and against their involvement with increasing autoantibodies. One potential trigger is bovine insulin which is supplied to infants in cow's milk. Ingestion of bovine insulin increases circulating antibodies to bovine insulin, and these antibodies cross-react with human insulin (Vaarala *et al.*, 1999). Insulin is an autoantigen, and insulin autoantibodies, when found in combination with other autoantibodies show high risk of developing diabetes. In breast-fed children, human insulin was found to be less immunogenic and did not cause an increase in circulating antibodies (Vaarala *et al.*, 1999). Weaning to formula milk without bovine insulin reduces occurrence of autoantibodies in genetically high risk children up to three years of age (Vaarala, 2012). However since long term (more than three year) outcomes are not assessed, it is unknown if the bovine insulin is a trigger or just an accelerator (Jahromi, 2013, Vaarala, 2012). Other studies have reported that there is no link between bovine insulin and diabetes (EURODIAB, 2002).

Reports on breastfeeding are also inconclusive. Autoantibody prevalence was increased in children who were breast-fed for less than four months compared with those breast-fed

for longer (Holmberg *et al.*, 2007). It is difficult to deduce whether the increased autoantibodies present in early-weaned children is due to early exposure to cow's milk protein, or whether the breastfeeding itself confers a protective effect, regardless of the ingestion of cow's milk. In another report, breastfeeding was shown to reduce the risk of diabetes (EURODIAB, 2002), and others have found no link between the period of breastfeeding and signs of islet autoantibodies (Ziegler *et al.*, 2003, Virtanen *et al.*, 2006). Other antigens that could trigger the onset of diabetes include cereals. Gluten feeding at early age (first three months of life) increased the percentage of islet autoantibody frequency (Ziegler *et al.*, 2003), however other reports have found no link (Virtanen *et al.*, 2006).

The pathogenesis of diabetes causing deficiency in insulin production is usually brought about by autoimmune attack of T lymphocytes on the β cells of the islets. T lymphocytes are present at the time of diabetes onset (Jahromi and Eisenbarth, 2007), which occurs when β cell mass decreases to 10% of the normal amount, estimated from autopsy studies of β-cell mass in people with recent-onset type 1 diabetes (Matveyenko and Butler, 2008, Butler *et al.*, 2007). Cells of the immune system such as macrophages, T helper cells and cytotoxic T cells release cytokines such as interleukin (IL)-1, IL-6, interferon (IFN)-γ and tumour necrosis factor (TNF). These cytokines may also be released from the endocrine cells themselves, which has been found in patients with autoimmune disease. The target tissue itself may be responsible for its own decline due to expression of cytokines (Hill *et al.*, 2007). Release of the cytokines by cells of the immune system or by the endocrine cells initiates a cascade leading toward inhibition of insulin response in the β cell, and may even induce β cell lysis (Pukel *et al.*, 1988, Bendtzen *et al.*, 1986). Pro-inflammatory cytokines induce Fas-mediated β cell apoptosis. Anti-inflammatory cytokines such as IL-13 are able to prevent this occurring and are cytoprotective to islet

cells treated with pro-inflammatory cytokines, as well as islets treated with palmitate or undergoing serum withdrawal (Russell *et al.*, 2013).

Destruction of the β cells and failure to secrete insulin therefore prevent activation of insulin receptors on target cells. Consequently, glucose transporters do not locate to the plasma membrane and target organs cannot take in glucose from the bloodstream for utilisation, leaving cells without an energy source. The high blood glucose levels subsequently lead to high amounts of glucose in the urine, due to damaged kidney glomeruli. The liver responds by breaking down fat and proteins to use as a substitute respiratory substrate. The increase in ketone bodies and amino acids can significantly decrease the pH of the blood, known as ketoacidosis, which then leads on to osmotic diuresis, dehydration, and loss of electrolytes and eventually ketoacidosis can be fatal due to falling blood pressure and coma (Wolfsdorf *et al.*, 2009).

Symptoms recognised by the patient include an increased excretion of urine (polyuria), thirst (polydipsia), hunger (polyphagia), unexplained weight loss, numbness in the extremities, foot pain (disesthesias), fatigue, blurred vision, recurrent or severe infections, loss of consciousness or severe nausea and/or vomiting (as a result of ketoacidosis) or coma. The considerable cardiovascular risks associated with diabetes are a result of arterial damage, caused by a build up of excess sugar. The risks include heart disease, heart attack, stroke, retinopathy leading to visual impairment and total blindness, neuropathy, nephropathy, and peripheral vascular disease which potentially leads to gangrene and amputation (Beckman, 2002).

Current insulin therapy (discussed in more detail below) involves the use of insulin pumps or subcutaneous injection of insulin; administration is required at least once per day, depending on the type (rapid, intermediate or long-acting). Purified porcine and bovine

insulin used to be prescribed, however recombinant DNA technology now allows the production of human insulin (Joshi *et al.*, 2007).

1.5 Type 2 diabetes mellitus

Type 2 diabetes mellitus was previously known as insulin independent diabetes mellitus, or adult-onset diabetes, and is accountable for approximately 90% of diabetic cases. The four most common symptoms are thirst, increased urine excretion, tiredness and weight loss. Many symptoms experienced by type 1 patients are also present for type 2 patients although they are often less marked, hence the condition can go unnoticed for many years before diagnosis. By this time, complications may have already developed and cardiovascular diseases are already at high risk (Hu *et al.*, 2002).

Type 2 diabetes is exemplified by high blood glucose brought about by insulin resistance as well as relative insulin deficiency due to loss of β cell function. Insulin resistance occurs when target organs and tissues do not respond to the insulin that has been secreted into the blood stream by the islet β cells. The subsequent lack of glucose receptor translocation results in the failure of cells to transport glucose into muscle and fat cells for energy use, or into liver cells for storage. Insulin resistance leads to the progressive exhaustion of the β cell and increased apoptosis. Eventually, the altered insulin secretion leads to deficiency (Reaven, 1988, Donath *et al.*, 2005).

The genetic component to type 2 diabetes is polygenic with associated genes occurring at multiple different chromosomal sites (Horikawa *et al.*, 2000). More than 40 genes associated with increased risk of type 2 diabetes were discovered between 2006 and 2012 (Ashcroft and Rorsman, 2012). The discovery of inherited genetic regions in affected family members has allowed identification of susceptibility loci. Discoveries have been

made through linkage analyses, candidate gene association studies and genome-wide association (GWA) scans (Grant *et al.*, 2009).

The transcription factor 7-like 2 (TCF7L2) gene locus confers the strongest effect on type 2 diabetes (Sladek *et al.*, 2007, Turki *et al.*, 2013, Scott *et al.*, 2007). TCF7L2 regulates transcription of glucagon-like peptide 1 (GLP-1), which regulates insulin secretion (Zhang *et al.*, 2006). Other genes shown to be associated with diabetes are Calpain-10 (*CAPN10/NIDDM1*), a cysteine protease which may confer diabetes by its involvement in GLUT4 translocation, insulin exocytosis and mitochondrial function (Turner *et al.*, 2005, Bodhini *et al.*, 2011), and *kcnj11*, which encodes the protein Kir6.2, a subunit of the β cell ATP-sensitive potassium channel (Gloyn *et al.*, 2003, Scott *et al.*, 2007). As well as these, many other genes have been identified with links to type 2 diabetes, however the loci that have been identified only account for about 10% of the estimated genetic factors in type 2 diabetes (Herder and Roden, 2011).

Another interesting gene that may have a connection with diabetes is the *FTO* gene, which encodes the fat mass and obesity-associated protein. The *FTO* gene has been shown by GWA scans to be linked to obesity (Scuteri *et al.*, 2007). Obesity is a major predictor of future risk of type 2 diabetes (Abdullah *et al.*, 2010), and although there is currently no evidence that it is associated with type 2 diabetes susceptibility, *FTO* may be one of the molecular determinants linking obesity and type 2 diabetes (Meyre, 2012).

β cell apoptosis which contributes to type 2 diabetes may be a direct result of high glucose concentrations in the blood. Islets isolated from mice, when treated with high glucose concentrations (16.7 or 33.3 mM) show inhibited proliferation and induced apoptosis as measured by DNA fragmentation and release of mitochondrial cytochrome c (Maedler *et al.*, 2001, McKenzie *et al.*, 2010). Another factor that may be responsible for β cell death in diabetes is islet amyloid polypeptide (IAPP). IAPP is produced by β cells

along with insulin and is therefore increased during insulin resistance. IAPP produces islet amyloid deposits and is cytotoxic to β cells, initiating apoptosis (Jurgens *et al.*, 2011, Janson *et al.*, 1999).

Elevated levels of the pro-inflammatory cytokine TNF are strongly associated with type 2 diabetes (Mirza *et al.*, 2012). TNF prevents insulin receptor and insulin receptor substrate-1 function in target tissues and therefore explains the link between insulin resistance in obesity and diabetes (Hotamisligil and Spiegelman, 1994). TNF is able to induce apoptotic cell death, and may do in β cells, possibly through activation of the caspase pathway (Stephens *et al.*, 1999). However, much research has shown that TNF may actually be involved in protecting the β cell against death. The intracellular pathways induced by cytokines leading to β cell death are complex and in relation to type 2 diabetes is not completely understood (Thomas *et al.*, 2009).

Apoptosis can be caused by multiple pathways involving reactive oxygen species (ROS), IL-1β, activation of transcription of nitric oxide (NO) synthase (NOS) and subsequent production of NO. Oxidative stress due to increasing levels of ROS and NO is responsible for β cell dysfunction (Pitocco *et al.*, 2010). The activation of JNK and p38, as well as caspases causes DNA fragmentation and cell death. Hyperglycaemia leads to increased production of IL-1β by β cells (Donath *et al.*, 2005) and apoptosis can occur through activation of the Bcl-2 or Fas receptor apoptotic pathways (Maedler *et al.*, 2001, McKenzie *et al.*, 2010).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor involved in cytokine production and cell survival in response to cellular stress, cytokines, viral antigens, oxidised low density lipoprotein (LDL) and other signals. NF-κB is activated in the liver by obesity and a high fat diet, as well as hyperglycaemia and insulin resistance (Cai *et al.*, 2005). The downstream inflammatory cytokine production resulting

from NF-κB activation results in insulin resistance both in the liver and in systemic tissues (Cai *et al.*, 2005, Shoelson *et al.*, 2003).

Type 2 diabetes is associated with excess body weight and lack of physical activity, and risk is increased with high dietary saturated fat intake (Riserus *et al.*, 2009). Obesity is thought to be the main cause in genetically predisposed individuals. The prevalence of type 2 diabetes in children is on the rise, accounting for 8-45% of new cases depending on geographical location (Kaufman, 2002). This increased prevalence may be due to increased calorie and fat intake as well as a lack of physical activity (American Diabetes Association, 2000, D'Adamo and Caprio, 2011).

Obesity is accompanied with dyslipidaemia and increased circulating leptin and cytokine levels in the body. Saturated fatty acids, for example palmitate, induce β cell death and are therefore responsible for the reduction in β cell mass and reduced insulin secretion. Monounsaturated and polyunsaturated fatty acids have been shown to prevent saturated fatty acid-induced β cell death (Maedler *et al.*, 2003, Dhayal and Morgan, 2011). Low density lipoproteins which are also associated with obesity induce apoptosis of β cells, whereas high density lipoproteins have the opposite effect (Roehrich *et al.*, 2003).

Leptin, which is expressed primarily by adipose tissue, causes satiety. In response to a meal, leptin is secreted to reduce hunger and reduce fat storage. Leptin levels increase proportionally with increased adipose tissue mass (Skurk *et al.*, 2007). Leptin resistance in the hypothalamus means that individuals do not feel full and also causes further increases in adipose tissue mass and therefore obesity. The high circulating leptin levels correlate with high circulating insulin, and are known to be connected to insulin resistance (Mohiti *et al.*, 2009). Leptin has negative effects on β cells, and inhibits insulin secretion in islets (Roduit and Thorens, 1997). The rise in leptin levels in obesity presents the link by which obesity can initiate the onset of diabetes. Leptin induces release of IL-1β and decreases

expression of IL-1 receptor antagonist in human islets, both of these effects leading to impaired β cell function and apoptosis (Maedler *et al.*, 2004). Early administration of leptin accelerates the onset and progression of type 1 diabetes (as measured by high blood glucose) in female NOD mice. Administration of leptin leads to early islet inflammation by leukocytes and increased IFN-1 in spleen (Matarese *et al.*, 2002). This demonstrates a link between the pathogenesis of type 1 and type 2 diabetes.

In some cases, it is possible to control type 2 diabetes by managing diet and increasing physical exercise. In a diet and exercise program, 91% of patients with type 2 diabetes taking oral hypoglycaemic agents were off medication by the end of the program (Barnard *et al.*, 1982). In a more recent study, type 2 diabetes patients took part in a restricted diet consisting of just 600 calories per day for eight weeks. After just one week, fasting plasma glucose returned to normal. After eight weeks, hepatic triacylglycerol content dropped, first phase insulin response increased and insulin sensitivity returned to levels of that above control (Lim *et al.*, 2011). Exercise up-regulates GLUT4 transporters to the membranes of target tissues and increase glycogen storage (Ren *et al.*, 1994, Richter and Hargreaves, 2013). The beneficial effects of exercise are thought to be due to increased insulin sensitivity. In non-diabetics, it was demonstrated that exercise decreased basal fatty acid circulation. Fatty acids reduce the insulin sensitivity at target tissues. Exercise also improves insulin sensitivity to endogenous glucose and glucose uptake (Shojaee-Moradie *et al.*, 2007, Richter and Hargreaves, 2013).

In the absence of improvement by diet and exercise, medication is required, for example metformin (which suppresses glucose production in the liver), sulphonylureas or insulin (Turner *et al.*, 1999). Sulphonylureas act upon the SUR1 subunit of the K^{ATP} channel of the β cell, described above (Figure 1.3). The closing of the K^{ATP} channel (*via* the glucose pathway or by sulphonylureas) prevents efflux of potassium ions and therefore causes

depolarization of the membrane, and the subsequent steps leading to insulin release from the β cell (Figure 1.3) (Proks *et al.*, 2002). Metformin is the optimal first line drug, however in most instances one treatment is not enough and patients are usually prescribed a combination of medications, and ultimately, many patients will also need insulin therapy in order to maintain the control of glucose (Inzucchi *et al.*, 2012).

1.6 Gestational diabetes

Women without previously diagnosed diabetes can develop diabetes during pregnancy, usually in the third trimester. During pregnancy, there is an increased metabolic demand, and therefore an increased demand for insulin. Insulin resistance in target cells can also occur. This is thought to be caused by the inability of the insulin receptor signalling pathway to effectively promote GLUT4 receptor translocation to the membrane, which may be due to certain factors upregulated in pregnancy, such as placental lactogen or oestrogen, however the mechanism remains unknown (Rieck and Kaestner, 2010, Barros *et al.*, 2008). This leads to raised blood glucose levels. Women who have had gestational diabetes have at least seven-fold increased risk of developing type 2 diabetes later in life compared to women who have had a normoglycaemic pregnancy (Bellamy *et al.*, 2009).

1.7 Problems with current treatments for diabetes

Patients with any of the types of diabetes are at high risk of cardiovascular disease (described above) (Morrish *et al.*, 2001) and these risks remain even with the current treatments including insulin therapy (Aviles-Santa *et al.*, 2006, Gerstein, 2012). Type 1 and 2 diabetes patients may also overreact to high or low blood glucose results. The risk

of overmedicating on insulin can be fatal, firstly sending the patient into hypoglycaemic coma followed by death (Ben-Ami, 1999).

Type 1 diabetes (and some type 2) requires insulin supplementation, usually injected by patients. Blood sugar levels need to be monitored at regular intervals. Glucose monitoring itself poses an inconvenience for those unwilling to monitor, as well as the pain experienced. Apart from the aversion to needles and self-injecting, administration of insulin also poses issues for individuals too young or otherwise unable to inject themselves. Although insulin pumps require less self-monitoring, issues associated with them include pain and inconvenience, inaccuracy and for some patients they remain unsuccessful. In one study as an example, five out of 105 patients stopped insulin pump therapy in the programme. In the remaining patients, HbA1c levels and episodes of severe hypoglycaemia decreased, however there were still 11.4 hypoglycaemic events per 100 patient years. The quality of life measure for impact of diabetes on the patients fell indicating decreased impact after pump therapy, however the score for satisfaction with life, and worries about diabetes did not change (McMahon *et al.*, 2005).

Treatments for type 2 diabetes involve administration of oral medications. A number of these have been taken off the market due to safety concerns. Metformin, now used widely in the treatment of type 2 diabetes, replaced phenformin and buformin, which were withdrawn due to the risk of lactic acidosis (Fowler, 2007). Side effects of metformin include anorexia as well as gastrointestinal upset such as nausea, vomiting, and diarrhoea. The lactic acidosis risk is still present, however is very rare (Bouchoucha *et al.*, 2011).

First generation sulphonylureas such as acetohexamide have been discontinued due to their low safety profiles, and have been replaced by second generation sulphonylureas such as glyburide. However hypoglycaemia is a high risk when taking these drugs.

Glinides, similar to sulphonylureas but binding at a different site on the receptor, are much safer than sulphonylureas, however this comes with increased cost (Fowler, 2007). In the thiazolidinedione class of drugs (insulin sensitisers on fat cells), Avandia (rosiglitazone) use is restricted in the USA and suspended from the market in Europe due to increased risk of heart attack thought to be due to increased LDL (NHS.uk, 2010, Brackenridge *et al.*, 2009). The Food and Drug Administration recently requested a review and reanalysis of the risks associated with Avandia and it was voted that restrictions should be eased. It has become clear through the Avandia reviews that diabetes drugs were not assessed on their efficacy toward easing the cardiovascular risks and that in future these should be taken into consideration as well as efficacy toward lowering glycated haemoglobin (Hiatt *et al.*, 2013).

1.8 Transplantation

Whole pancreas transplantation was first used as a treatment for Type 1 diabetes in the 1960s in an attempt to regain normoglycaemia and minimize vascular complications. Whole pancreas transplants are usually performed in combination with kidney transplants, known as simultaneous pancreas-kidney transplant (SPK), since most pancreas transplant patients are also suffering from kidney failure. SPK transplants account for more than 72% of transplants (Richter *et al.*, 2011). Also possible is pancreas-after-kidney transplant (17%) during which a kidney is received from a living donor but the pancreas is from a deceased donor. Pancreas-alone transplant also occurs (7%), usually in patients who experience severe hypoglycaemic attacks without warning and have poorly controlled type 1 diabetes (Richter *et al.*, 2011).

In the first attempted pancreas transplantation, two patients underwent surgery to receive a pancreas and kidney (Kelly *et al.*, 1967). Evidence of insulin secretion was found, however successful function only lasted six days in one patient and two months in the other. Using current protocols, whole pancreas transplant regains normal blood glucose levels, increasing patient survival rates and without the need for administering insulin. However the transplanted pancreas typically only lasts for ten years with proper function (NHS.uk, 2013c). Ten years post-transplant, graft function for SPK, pancreas-after-kidney, and pancreas alone transplants are 68%, 46% and 39%, respectively (Gruessner *et al.*, 2012).

With whole pancreas transplant there still remains a high rate of surgical complications including infection and bleeding, and the requirement for lifelong immunosuppressive drugs (Larsen, 2004, Sutherland *et al.*, 1998). Typically patients are in the hospital for two to three weeks after the surgery (NHS.uk, 2013c). Approximately 300 people await pancreas transplant in the UK every year, and the average waiting time is one year (NHS.uk, 2013c). In the year to March 2012 in the UK, there were 409 pancreas donors but only 209 of these transplanted (NHS.uk, 2013a). It is clear that although pancreas transplantation successfully restores normoglycaemia for many, much improvement needs to be made in making full use of the available donors, reducing waiting times and the number of patients awaiting pancreas transplant.

A relatively new innovation for diabetes therapy is the development of an artificial pancreas specifically for endocrine function. Artificial pancreases are able to deliver insulin as well as glucagon in response to the glucose concentration in the blood (Bakhtiani *et al.*, 2013). They have closed-loop control combining continuous glucose monitoring with an insulin (and glucagon) pump. These do not contain any cells, but are able to monitor levels of glucose and have resulted in successful control of patients' blood

glucose levels without the constant need for self-monitoring by the patient, or injections (Bakhtiani *et al.*, 2013).

One bi-hormonal closed loop study achieved mean blood glucose levels lower than the target level set, indicating successful monitoring of the blood and release of satisfactory concentrations of insulin for the right length of time (El-Khatib *et al.*, 2010). Initially five of the eleven subjects had episodes of hypoglycaemia due to differing insulin absorption rates in patients, that upon adjustment of the algorithm was prevented and successful normoglycaemia was obtained. Matters of concern are the subcutaneous delivery of the hormones causing insulin accumulation at the site, higher than control level post-prandial blood glucose concentrations, and the short experimental period of just 27 hours. The study was also carried out in a clinical centre under resting conditions thus long-term efficacy of the device and response to activity and exercise are unknown (El-Khatib *et al.*, 2010).

Another limiting factor is the use of glucagon in pumps. Glucagon solutions can form amyloid fibrils and cannot be stored for long periods. Although solutions are being optimised, they must be stored at a higher pH (pH 10) than is required for use in the body (Ward *et al.*, 2010). Many studies continue to develop artificial pancreases with the use of insulin pump only. One study involving the use of closed-loop insulin administration demonstrated good glucose control throughout as well as during 30 min moderate exercise (Breton *et al.*, 2012). Overall improvement of the amount of time spent in the near-normoglycaemic range (3.9 - 10 mmol/l) was demonstrated, when compared to previous insulin pump results (prior to artificial pancreas experiment). The overnight blood glucose range was much improved, and the number of hypoglycaemic events dropped. Time spent in the tight glycaemic range (4.4 - 7.8 mmol/l) was not different, and some datasets were excluded from the analysis due to software or sensor failure,

extended post-prandial effect or differing metabolic characteristics. The study lasted 22 h therefore long-term use is unknown (Breton *et al.*, 2012).

The increase over the past decade in Smartphone use and constant connection to the internet has also been exploited for medical use. The use of an artificial pancreas that is connected to a Smartphone and 3G connection to a remote server allows monitoring by doctors to ensure the artificial pancreas is suitably regulating the patient's blood glucose without the need for doctor's appointments, thereby one step further in improving the quality of life of a diabetes sufferer (Bakhtiani *et al.*, 2013). This new technology has been tested in outpatient format, however still under close monitoring. The wearable artificial pancreas (pumping insulin only) produced good initial results, with avoidance of hypoglycaemia and major hyperglycaemia (Cobelli *et al.*, 2012). The study was only conducted in two subjects however, and still requires some user interaction with the pump, therefore is subject to human error as well as being viewed as a possible inconvenience by the patient. The phone itself could not be used for normal functions (phone calls, internet browsing) and on two occasions the communication box malfunctioned (Cobelli *et al.*, 2012). With some fine-tuning the outlook for artificial pancreas technology looks promising.

1.9 Islet transplantation

Islet transplantation is an experimental treatment in type 1 diabetes patients with the aims of decreasing the hypoglycaemic risk, improving glucose metabolism and reducing diabetic complications. It is less invasive than whole pancreas transplantation, allowing for shorter healing times (and therefore reduced hospital time) and a decreased risk of morbidity and mortality compared with whole organ transplant (Humar *et al.*, 2000,

Bucher *et al.*, 2004, Choudhary *et al.*, 2012). The idea of islet transplantation is to restore endogenous insulin secretion by the transplant of intact islets secreting insulin (Scharp *et al.*, 1990, Warnock *et al.*, 1991). The whole pancreas is isolated first from cadaveric donors, and the islets are separated from the exocrine portion and the rest of the pancreas. After purification, islets are perfused into the portal vein of the liver of the recipient (Figure 1.5). Situated in the portal vein, the islets have a generous blood supply, ensuring not only a good provision of oxygen, but also insulin released from islets has direct access to the circulation (Merani *et al.*, 2008). Other sites for implantation have been investigated, such as the kidney capsule, splenic capsule and peritoneal cavity, however the hepatic portal site is chosen by many since it is the least invasive, has a good blood and oxygen supply and regain of normoglycaemia is successful with the fewest amount of islets (Merani *et al.*, 2008).

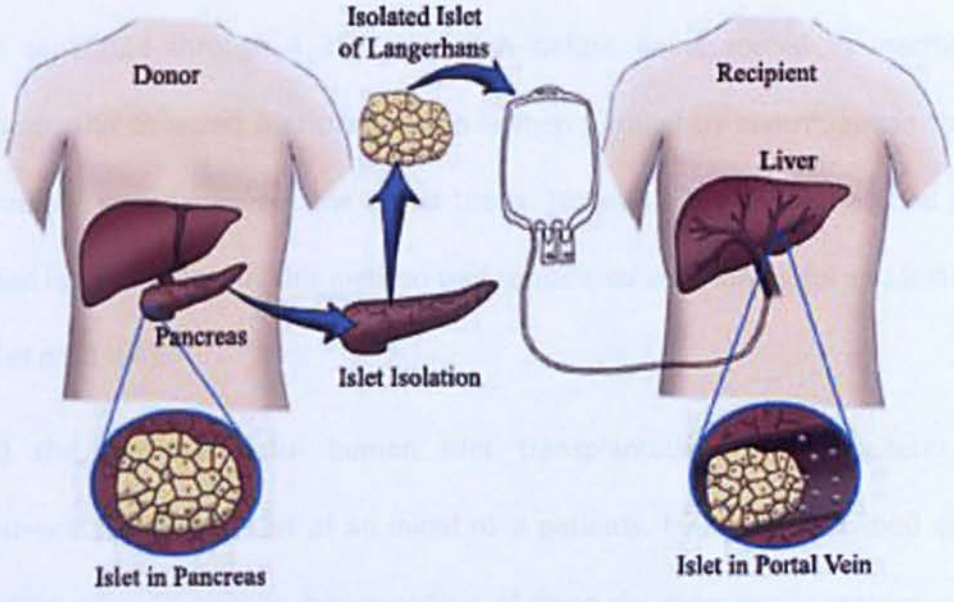


Figure 1.5. Pancreatic islet transplantation. Whole pancreas is removed from a deceased donor before separating out the islets of Langerhans from exocrine tissue. Purified islets are perfused into the portal vein of the recipient's liver. http://blog.itechtalk.com/2011/pancreatic-islet-transplantation/

Islet isolation was first investigated in animals in the 1960's, for the examination of the physiology and morphology of islets (Petersson, 1966). By the early 1970's, islet transplantation was found to cure diabetic symptoms in rodents and the procedures were being optimised with the use of collagenase and Ficoll density gradient (Kemp *et al.*, 1973, Scharp *et al.*, 1980, Gray *et al.*, 1984). Collagenase digests exocrine tissue, and Ficoll was used to separate the digested tissue from the islets. Despite these advances, human islets were more difficult to isolate, being from a larger pancreas and more fibrous (Gray *et al.*, 1984).

In 1988, Ricordi and colleagues developed a method by which islets could be isolated and purified, with high yield and minimal trauma to the islets (Ricordi *et al.*, 1988). Their method allows continuous collagenase digestion of the human pancreas to yield as many islets as possible, with the progressive collection of islets to prevent their complete digestion. Collagenase was injected into the pancreas *via* the bile duct followed by mechanical digestion at 37°C in a stainless steel chamber containing glass beads, and the solution separated through a 280 μm mesh before being cooled to inactivate the collagenase. The collected islet preparation is then purified by centrifugation through a Ficoll gradient to separate out the acinar tissue. Isolated islets showed a good glucose-stimulated insulin response. This method was considered very successful and is still in use (Ricordi *et al.*, 1988).

In 1990 the first successful human islet transplantation with long-term insulin independence took place. Out of an initial nine patients, five were described as insulin independent after six months, however four of these did occasionally require overnight insulin infusions, and none of the patients had normal insulin secretory kinetics (Tzakis *et al.*, 1990). A landmark study that advanced islet transplantation methods and thereby improved patient prognosis and lifestyle was a trial performed at the University of

Alberta, in Edmonton, Canada, a procedure that has become known as the Edmonton Protocol. In 1999, seven patients underwent islet transplantation with a modified glucocorticoid-free immunosuppressive regimen (Shapiro *et al.*, 2000). Immunosuppressive drugs such as tacrolimus and cyclosporine are necessary to suppress the recipient's immune system and prevent attack on the newly implanted islets, however, these drugs are diabetogenic. They reduce glucokinase activity, suppress insulin gene expression and inhibit insulin-stimulated glucose transport, and cyclosporine has been shown to induce apoptosis of β cells (Radu *et al.*, 2005, Drachenberg *et al.*, 1999, Ozbay *et al.*, 2011, Lopes *et al.*, 2013). By removing the toxic cyclosporine and using only low dose tacrolimus, in combination with other non-diabetogenic immunosuppressors, Shapiro and colleagues managed to successfully transplant islets into patients with initial good follow up (Shapiro *et al.*, 2000). A follow up of these patients between 4.4 and 14.9 months after transplant revealed good insulin production, no episodes of hypoglycaemia and requirement for exogenous insulin only during periods of minor illness. However, while 80% of patients remained insulin independent at one year post-transplant, the five year follow up showed islet graft function loss to the extent that only 10% remained insulin independent (Ryan *et al.*, 2005).

The longevity of islet function using this protocol was further investigated in an international study with several locations. Insulin independence after islet transplantation decreased from 44% of patients after one year to 14% at two years (Shapiro *et al.*, 2006). What must be taken into consideration when assessing transplantation success is that patients currently taken into this and other clinical transplantation trials are carefully selected to determine whether they are eligible to undergo the treatment. As it is currently not a routine treatment, only the patients with the most severe diabetic complications and risk of hypoglycaemic coma are selected. This indicates that in a

greater sample of patients whose diabetic complications are not as problematic, transplant procedures may have better rates of islet graft survival (Choudhary *et al.*, 2012).

It is clear that islet transplantation is on its way to becoming a widely used procedure. In the UK the first islet transplant procedure was performed in 2005 at King's College, London (King's College London, 2005), and in the year to March 2012, 30 islet transplants were performed (NHS.uk, 2013b). However, many obstacles still need to be overcome. A major limiting factor in the process is the availability of donor islets. On average approximately 10,000 human islet equivalents per kilogram of recipient body weight are required to achieve insulin independence (Shapiro *et al.*, 2000). The requirement of islets from two to three donors per recipient is far too great to be sustained as a system in practice. Procedures must also be quick and fresh islets must be used since cultured islets (even at 24 h post-transplant) perish and cryopreserved islets have reduced function compared to fresh islets (Warnock *et al.*, 1991, Thomas *et al.*, 1999). Islet survival is low after transplantation has occurred, which is partly due to disconnection from their vasculature. An unsuccessful revascularisation process results in islet death and failure of the graft (Jansson and Carlsson, 2002).

Islet transplantation still comes with the provisos of any normal allotransplant procedure. The donor and recipient blood types and tissue antigens must be matched, and the patient will need lifelong immunosuppression and multiple drugs. Despite the use of immunosuppressive drugs, only a small fraction of the islets transplanted to the liver engraft due to immune attack from the host. Apart from the β cell toxicity of immunosuppressive drugs (Drachenberg *et al.*, 1999, Avram *et al.*, 2008), they can also lead to weight gain, dyslipidemia, increased blood pressure and insulin resistance. Further

complications from immunosuppressive agents include low leukocyte count, increased infection risk and occurrences of malignancies such as skin cancer (Ryan *et al.*, 2004).

Another immune-mediated side effect of islet transplantation that requires monitoring is the triggering of an 'instant blood mediated inflammatory reaction' (IBMIR), caused by activation of the immune response when human islets are mixed with human blood from the host. The islets become surrounded by leukocytes, clotting is evident as well as a high platelet turnover (Bennet *et al.*, 2000b). Islets are damaged and the transplant graft fails, however improvements are being made *in vitro* by treating islets with combinations of factors that inhibit the reaction, such as heparin, soluble complement receptor-1 (an inhibitor of the complement activation cascade), NF-κB inhibitors (to inhibit the inflammatory response), and knockdown of tissue factor, a receptor necessary for the coagulation cascade (Luan and Iwata, 2013, Ma *et al.*, 2012, SoRelle *et al.*, 2013).

The necessity for toxic immunosuppressive therapy (despite being fine-tuned to prevent any diabetogenic effects) and a gradual decline in insulin independence rates over time (24% of patients remaining insulin-free over five years (CITRegistry.org, 2010)) exemplifies the much needed requirement for improvements in islet transplantation therapy in order for it to be effective, efficient, safe, economical and practical for widespread clinical use.

1.9.1 Improvements in islet isolation and transplantation

The improvements in islet transplantation since 1999 are documented by improved C-peptide levels, increased duration of insulin independence, the reduction of HbA1c levels and fewer hypoglycaemic episodes (Barton *et al.*, 2012). However improvements still need to be made in the isolation and transplantation procedures.

One area of consideration is the site of graft transplantation in the recipient. Even though the intraportal site is a popular one for implantation of the islets, it is not ideal since the islets are subject to high levels of the immunosuppressive drugs, which are toxic to β cells, as discussed above (Desai *et al.*, 2003). Accessibility is also better if the islets are implanted to intramuscular sites, as Witkowski and colleagues have shown (Witkowski *et al.*, 2009). They implanted cells in an intramuscular site, in an alginate matrix, to produce a microenvironment to increase islet survival rates. However they also impregnated the islets with vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), to promote intra-islet angiogenesis and prevent anoikis (apoptosis induced by incorrect extracellular matrix (ECM) attachment). Although they found improved glucose blood levels and neovascularisation, they found a higher rate of successful function in the implants also infused with the growth factors and less improvement in the grafts without these growth factors (Witkowski *et al.*, 2009). One major limitation of this study in promoting intramuscular sites for graft placement was that they did not implant islets into the intraportal site in the rats as a control. Thus improved graft function may be due to use of alginate microenvironment plus growth factors (Witkowski *et al.*, 2009).

Gels and matrices are frequently used in islet transplantation experiments, since they protect the islet cells from the immune system, maintain the three-dimensional structure and mimic the ECM, thereby improving graft survival for transplantation purposes. Collagen matrix forms a 3D scaffold that islets can be cultured in as an alternative to two-dimensional culture. Zhang and colleagues also cultured fibroblasts in the collagen matrix to see if they had any effect on the islets (Zhang *et al.*, 2012). Islets cultured in collagen matrix and fibroblast populated collagen matrices had reduced amyloid formation compared to the 2D culture, and around a 75% reduction in β cell apoptosis. The 3D matrices also had increased β cell to α cell ratios, increased insulin levels and insulin

response. Matrices with fibroblasts had higher insulin response, β/α cell ratio and a lower amyloid formation than in collagen matrix alone (Zhang *et al.*, 2012).

Another study investigated the use of fibrin gels, in combination with hepatocyte growth factor (HGF), fibroblast growth factor-4 (FGF4) and nicotinamide on the survival of human islets. Fibrin increased total DNA and insulin content of the islets, which was further increased in the presence of the growth factor cocktail (Beattie *et al.*, 2002). After transplantation of the cultured islets under the kidney capsule in mice, islets from the fibrin gels produced much larger grafts with good endocrine function compared to islets from free-floating culture. When islets were cultured in fibrin gels with the growth factor cocktail, proliferation was observed and was much higher than in islets cultured free-floating (with or without growth factors) and islets cultured in fibrin gels without growth factors (Beattie *et al.*, 2002).

Sheets of monolayer islet cells have been constructed and implanted subcutaneously in rats to allow islet-extracellular matrix interactions. This increases islet cell survival and function (Shimizu *et al.*, 2009). The single celled sheet allows for cell-cell contact. The monolayer sheet secreted insulin when transplanted subcutaneously, however, glucose levels in the recipient were not monitored and long-term survival of the islet sheets is unknown since the experiment was only conducted for seven days (Shimizu *et al.*, 2009).

Polymer scaffolds to contain the islets in the graft create a barrier to prevent attack on the islet cells from the immune system (Blomeier *et al.*, 2006). Scaffolds are transplanted into extra-hepatic sites, which avoids the negative influences of the liver and the IBMIR (Bennet *et al.*, 2000b). Scaffolds are biodegradable, and can be formed from collagen, fibrin and clotted plasma, which are eventually degraded, leaving the islets able to integrate with the host cells (Hiscox *et al.*, 2008).

A 3D polymer scaffold composed of biodegradable poly(DL-lactide-co-glycolide) acid was fabricated for housing human islets (Figure 1.6C-E). Experiments were conducted comparing the function of the islets when in the scaffold, a collagen I gel and in the collagen gel plus fibronectin and collagen IV. After ten days culture, islets incubated in the scaffold plus the collagen I gel, fibronectin and collagen IV produced a glucose-stimulated insulin response similar to freshly isolated islets (Daoud *et al.*, 2011). Islets cultured in a collagen I gel alone did not produce as good stimulation profiles as islets in culture with added fibronectin and collagen IV, or with all components in the scaffold, however insulin release was much improved over islets cultured in suspension. A true test of the significance of the scaffold would involve transplantation of islets within the biodegradable scaffolds into animal models and determination of function *in vivo*, since these islets were experimentally assessed *in vitro* (Daoud *et al.*, 2011).

Microencapsulation is another technique used to isolate the islets from the immune system and minimise the need for immunosuppressive drugs. Materials used for the microencapsulation gels are under much experimentation, for example the use of alginate/polyethylene glycol gels (Hall *et al.*, 2011). The biomaterials used for encapsulation allow permeation of glucose and insulin, but remain impermeable to lymphocytes and immunoglobulin molecules (Figure 1.6A). Successful transplants of microencapsulated islets into humans began in the early 1990's. One early study which used microencapsulated human islets transplanted into human diabetic recipients demonstrated a reduction in the amount of exogenous insulin required by type 2 diabetic and increase in serum C-peptide levels in a type 1 diabetic, however the islet grafts lasted a maximum of seven months (Calafiore, 1992).

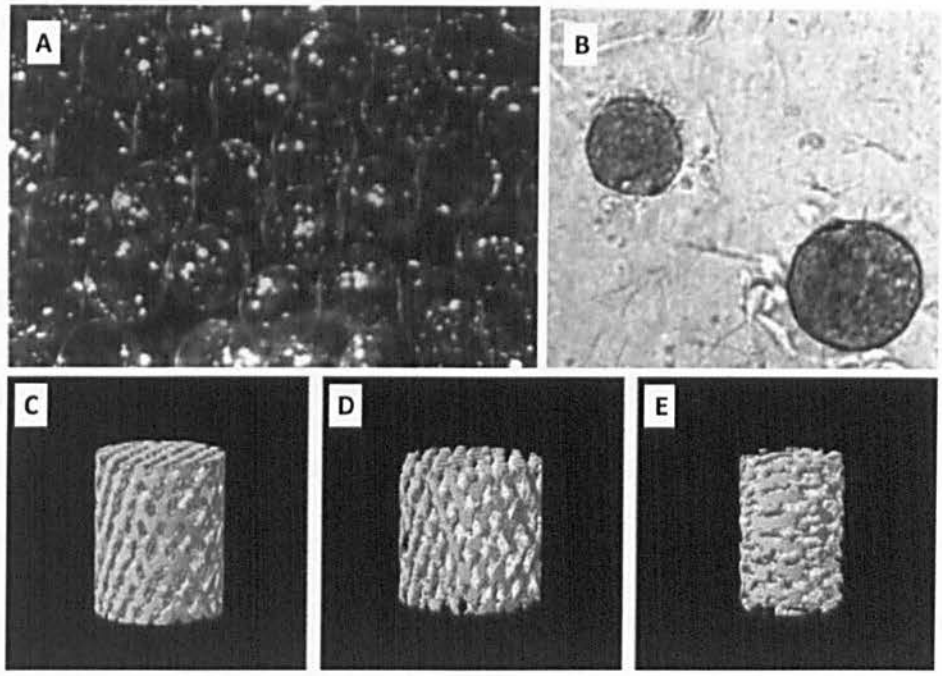


Figure 1.6. Examples of islet encapsulation and embedding within scaffolds. A. Photomicrograph of islet-containing microcapsules of alginic acid and aminoacidic polycations. **B.** Brightfield image of islets encapsulated in a silk hydrogel after 7 days (10 × magnification). **C-E.** Micro computed tomography images of 1.0 mm-diameter, 3.6 mm-thickness of scaffold sections immersed in water fabricated with layer spacing parameters of (**C**) 300 µm, (**D**) 400 µm, (**E**) 500 µm. Daoud *et al*, 2011; Davis *et al*, 2012; Calafiore, 1992.

A silk fibroin hydrogel was used as an islet encapsulation platform to co-encapsulate islets, the ECM proteins collagen IV and laminin, and mesenchymal stromal cells (MSCs; Figure 1.6B). Fibroin is strong and insoluble with tightly packed layers, which maintain or enhance islet function. Encapsulated islets with ECM proteins demonstrated good insulin stimulatory index and higher functional gene expression (e.g. insulin, glucagon) compared to non-encapsulated islets (Davis *et al.*, 2012). Islets encapsulated in silk with MSCs plus ECM proteins produced a 3.2-fold increase in the insulin stimulatory index compared to islets in silk alone. MSCs may enhance the response either through direct cell-cell contact or by producing trophic factors to the islets. They also secrete immunosuppressive factors, which prevent an inflammatory response (Davis *et al.*, 2012).

With increased mechanical support and higher seeding density, scaffolds and gel methodology have been found to maintain or improve islet viability and function.

Incubation with growth factors and ECM components and/or secondary stromal cells further enhances the islet function.

1.10 Regenerating pancreatic β cells

Methods of stimulating islet proliferation *in vitro*, or stimulation of remaining healthy islets *in vivo* are currently a major focus of diabetes research (Kayali *et al.*, 2007). Adult β cells are well differentiated and hence have a low mitotic rate, and are difficult to expand *in vitro,* in particular without loss of insulin secretion (Kayali *et al.*, 2007). However, under certain conditions such as pregnancy, obesity and islet inflammation, β cells increase their rate of growth *in vivo* (Sherry *et al.*, 2006, Marynissen *et al.*, 1983, Hull *et al.*, 2005).

The NOD mouse model is a spontaneous model of type 1 diabetes and inflammation is present in the islets (Leiter, 2001). In pre-diabetic NOD mice, before an increase in blood glucose concentration or a significant decrease in β cell mass is observed, β cell replication increases. This is thought to be due to the infiltrating cells of islet inflammation which are present in pre-diabetic mice, as demonstrated by experiments on CD3. The CD3 epitope is part of the T cell receptor on T lymphocytes and therefore involved in T cell immune response. Use of anti-CD3 antibodies inhibited the immune response, causing a decrease in β cell proliferation (Sherry *et al.*, 2006).

Increases in β cell mass have been associated with high fat feeding. In mice that were fed a high fat diet for one year, as well as the increase in body weight, a much larger islet and total β cell area was found when compared to control (low fat diet) mice (Hull *et al.*, 2005). This was due to an increase in β cell numbers rather than hypertrophy. However insulin response to glucose administration did not increase in proportion with the increased islet size, suggesting that although β cell proliferation is induced, the function

of these cells is not increased. This demonstrates the inadequate adaptation of the β cells during a high fat diet resulting in obesity (Hull *et al.*, 2005).

During pregnancy there is an increased metabolic demand. Target cells have a reduced sensitivity to insulin and therefore β cell secretion of insulin increases. The increased insulin demand leads to an increased rate of proliferation of islet cells (Rieck and Kaestner, 2010). In pregnant rats from mid-way through gestation until just prior to birth the islet cell mass increases, with a higher volume density of the β cells in particular. (Parsons *et al.*, 1992, Rieck and Kaestner, 2010, Marynissen *et al.*, 1983, Nieuwenhuizen *et al.*, 1997). This could be due to the stimulatory effects of placental lactogen, prolactin and growth hormone which have been shown *in vitro,* however are yet to be shown *in vivo* (Brelje *et al.*, 1993, Rieck and Kaestner, 2010).

Stimulating β cells or islets to undergo epithelial to mesenchymal transition (EMT) and therefore dedifferentiating has the potential to produce endocrine progenitor cells that may be more susceptible to proliferation. The expanded mass of progenitor cells could then be re-differentiated to β cells expressing insulin. Through lineage tracing and labelling of β cells with green fluorescent protein (GFP) it has been possible to demonstrate that human β cells can undergo EMT to produce progenitor cells that have the capacity to proliferate (Russ *et al.*, 2008). The insulin-negative cells derived from β cells were able to proliferate (shown by cell counting), with an approximate doubling time of seven days for a maximum of 16 population doublings before senescence (Russ *et al.*, 2008). Kayali and colleagues attempted to expand islets *in vitro* with two different protocols and tested whether cells undergo EMT, followed by expansion and then re-differentiation. However, insulin content was very low after the re-differentiation phase, and any cells that expressed mesenchymal markers may have meant the presence of contaminating cells (Kayali *et al.*, 2007).

There is evidence suggesting that proliferation of existing β cells is the major mechanism of increasing β cell mass in adults, rather than differentiation from a pluripotent precursor. A transgenic mouse system expressing diphtheria toxin in β cells only was used to induce β cell apoptosis (Nir *et al.*, 2007). After β cell specific ablation, β cell mass increases to normal levels, accompanied by a regain of normal insulin levels. This suggests that after diabetogenic injury β cells have the capacity to recover with full function. Studies of proliferation and apoptosis detected that the rate of apoptosis in recovering cells did not differ from control rates of apoptosis, however the rates of proliferation increased two- to three-fold, indicating that the recovery to normal β cell mass is due to an increase in proliferation rather than a decrease in apoptosis, and therefore not an altered life span of β cells (Nir *et al.*, 2007).

1.11 Alternative sources of β cells

As well as manipulating β cells themselves to induce proliferation, non-β cells may also be stimulated to differentiate to β cells and increase mass and solve the issue of the β cell shortfall. This may be done by the use of stem cell differentiation, induction of non-β cells to secrete insulin *via* mesenchymal-epithelial transition, and use of non-human islets as a substitute (Ramiya *et al.*, 2000, Sun *et al.*, 1996, Gershengorn *et al.*, 2004).

Xenotransplantation using porcine islets is so far successful in non-human primates. One group managed to achieve insulin independence in one non-human primate for 13 months (van der Windt *et al.*, 2009). Due to the shortage of human islets available for transplant, pig islets have been thought of as the next best option, especially considering there are benefits to using porcine islets over human islets. Pig islets are less susceptible to the diabetic autoimmune attack, do not accumulate amyloid and it is possible to

genetically modify the pig donors, so that islets from them have improved immunomodulatory and cytoprotective features (Koulmanda *et al.*, 2003, Potter *et al.*, 2010, Bennet *et al.*, 2000b). Immunomodulation techniques include treatment with inhibitors of NOS, which prevents the immune destruction of cells (Varotto *et al.*, 2006). Pig insulin also only differs from human insulin by 1 amino acid. The risk of transmitting infectious disease is higher in human allotransplantation than in porcine xenotransplantation. However, disadvantages include early graft loss and porcine endogenous retroviruses. Transplant rejection due to immunity against pig epitopes is a major drawback and the same major drawback applies to allotransplant also (Bennet *et al.*, 2000a).

Production of differentiated β cells (or islets) from undifferentiated stem cells are methods by which large masses of β cells may be generated (Assady *et al.*, 2001). Stem cells being used experimentally include embryonic, mesenchymal, bone marrow, cord blood and adipocyte-derived stem cells. One example of steering stem cells to a pancreatic fate is the study by D'Amour and colleagues, who managed to produce all types of pancreatic endocrine cells that produce insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin from human embryonic stem cells (D'Amour *et al.*, 2006). This was successful due to leading the embryonic stem cells through the natural developmental processes that pancreatic progenitor cells normally follow. This is a carefully timed stimulation of embryonic cells with specifically designed combinations of growth factors. Despite the success of these methods, long term glucose stimulation of insulin secretion still needs to be fine-tuned (Assady *et al.*, 2001). Major safety issues exist when stem cells are being used for treatment. Multiple replications before transplantation can bring about chromosome abnormalities which have the potential to cause cancer (Baker *et al.*, 2007, Spits *et al.*, 2008).

Thorel and colleagues demonstrated that after destruction of the present β cell mass in mice by 99.6%, the reprogramming of α cells increased the β cell mass (Thorel *et al.*, 2010). Heterologous regeneration from α cells to β cells was demonstrated by lineage tracing of adult α cells and almost 90% of the reprogrammed α cells continued to produce glucagon. The cells of β origin did not proliferate, and were diluted by β cells formed from α cell precursors. The mechanism of α cell reprogramming was unknown, but speculated to be activation of certain transcription factors necessary for insulin production. Although reprogramming is a potential method for increasing β cell mass, after 99.6% of β cell destruction only 10% of the original β cell mass was regained after ten months in mice, therefore stimulation of proliferation after reprogramming would be required (Thorel *et al.*, 2010).

As well as the reprogramming of other endocrine cells within the islet into β cells, stimulation of pancreatic exocrine cells to do the same has also been shown possible. The *in vivo* adenovirus delivery of transcription factors in the host environment successfully produced insulin-producing β cells. The streptozotocin-induced diabetic animals had increased glucose tolerance and higher levels of serum insulin after reprogramming when compared to control mice (Zhou *et al.*, 2008). Another cell type capable of being reprogrammed are hepatocytes. Liver and pancreas cells share a common progenitor, and researchers are investigating the use of liver cells as replacement β cells. Expression of specific genes at specific times determines cell fate, for example, the expression of a certain bone morphogenic protein (Bmp2b) determines a hepatocyte fate, rather than a pancreatic one (Chung *et al.*, 2008). Controlled expression of genes such as FGF, sonic hedgehog and the pancreatic duodenal homeobox transcription factor (pdx-1), as well as Bmp2b, determines a liver or pancreatic cell fate (Deutsch *et al.*, 2001, Chung *et al.*, 2008). Recently, reprogramming of liver cells to pancreatic cells has become possible,

both *in vitro* and *in vivo.* These cells were able to express insulin in response to glucose, and ameliorate hyperglycaemia (Liu *et al.*, 2013).

1.12 SPARC

The discovery of secreted protein acidic and rich in cysteine (SPARC) occurred in the early to mid 1980's, when several groups connected their findings for a protein labelled as either SPARC, osteonectin or Basement membrane 40 (BM-40; due to being found in basement membranes), with a molecular weight of 43 kDa (Sage *et al.*, 1984, Termine *et al.*, 1981, Mason *et al.*, 1986, Wrana *et al.*, 1988). Throughout the late 1980's, this protein was further characterised as a calcium-binding protein, and distribution in tissues was determined (Sage *et al.*, 1989a, Engel *et al.*, 1987).

1.12.1 Structure of SPARC

SPARC is a secreted glycoprotein that is a soluble, non-structural part of the ECM. There are two binding sites for Ca^{2+}, the acidic N-terminal domain, and two EF-hand motifs contained within the C-terminal domain (Figure 1.7) (Brekken and Sage, 2001, Engel *et al.*, 1987). SPARC has 7 disulphide bonds and is glycosylated during post-translational modification (Hohenester *et al.*, 1997). The gene encoding the protein is predicted to give a molecular weight of 33 kDa, and following glycosylation, results in a protein of around 43 kDa in most tissues.

The full SPARC protein has 303 amino acids, and has high sequence identity between species. Mature human SPARC shares 99%, 92%, 92%, 87% and 30% amino acid identity with cow, mouse, rat, chicken and *Drosophila* SPARC, respectively (Brekken and Sage, 2001). The human gene has been localised to chromosome 5q31-33.

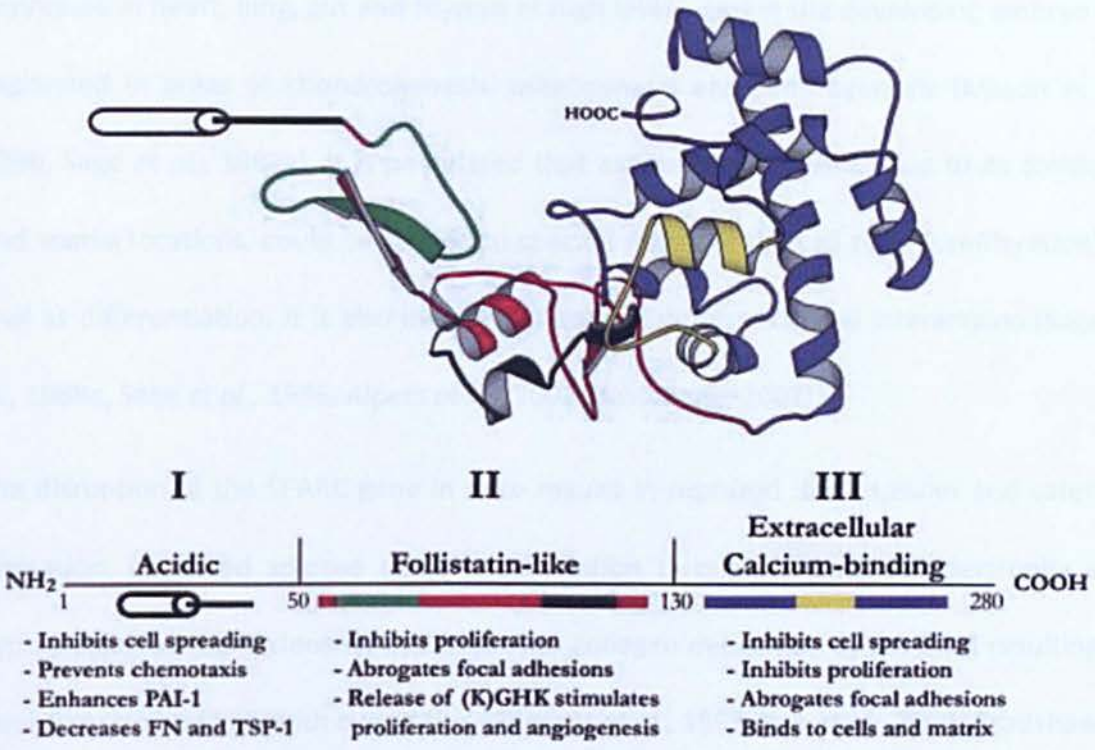


Figure 1.7. The domains of SPARC. The acidic N-terminal region of SPARC inhibits cell spreading, where as the follistatin-like domain inhibits proliferation. The C-terminal end contains two EF-hand domains that bind calcium ions. Cell binding demonstrated in binding assays to receptors and collagens. PAI-1, plasminogen activator inhibitor 1. FN, fibronectin. TSP-1, thrombospondin-1. (K)GHK, Protein sequence (lysine)-glycine-histidine-lysine (binds copper and stimulates angiogenesis). Brekken and Sage, 2001.

Since the domains of SPARC include residues identified for cell binding and inhibition of adhesion on one side of the protein and collagen binding regions on the other side, it is likely that SPARC acts at the surface of cells, and interacts with the ECM (Hohenester *et al.*, 1997).

1.12.2 SPARC expression

SPARC is expressed by stromal cells such as fibroblasts and endothelial cells (Wasi *et al.*, 1984, Zhou *et al.*, 2002, Sato *et al.*, 2003, Zung *et al.*, 1986, Wrana *et al.*, 1988). In the adult mouse, SPARC is expressed in gut, skin and glandular tissue which are tissues

associated with a high rate of replication (Sage *et al.*, 1989a). In foetal tissues it is expressed in heart, lung, gut and thymus at high levels, and in the developing embryo it is expressed in areas of chondrogenesis, osteogenesis and somitogenesis (Mason *et al.*, 1986, Sage *et al.*, 1989a). It is postulated that expression of SPARC, due to its temporal and spatial locations, could be related to specific stages of the cell cycle (proliferation) as well as differentiation. It is also involved in epithelial-mesenchymal interactions (Sage *et al.*, 1989a, Sage *et al.*, 1986, Alpers *et al.*, 2002, Basu *et al.*, 2001).

The disruption of the SPARC gene in mice results in ruptured lens capsules and cateract formation, increased adipose tissue accumulation through adipocyte hypertrophy and hyperplasia, low bone density, and improper collagen deposition in the ECM resulting in weak/lax skin and tails with curled tips (Gilmour *et al.*, 1998, Yan *et al.*, 2002, Bradshaw *et al.*, 2003a, Delany *et al.*, 2000, Bradshaw *et al.*, 2003b). All of these phenotypes are potentially the result of improper production and/or assembly of ECM, especially collagen processing. SPARC is also abundant in bone. SPARC-null mice display decreased bone formation, lower levels of osteoblasts and osteoclasts and impaired remodelling of bone (Delany *et al.*, 2000).

1.12.3 SPARC interactions

SPARC co-localises with collagen type I, and is involved in collagen processing and deposition for matrix formation (Harris *et al.*, 2011, Rentz *et al.*, 2007, Giudici *et al.*, 2008, Termine *et al.*, 1981). SPARC also binds collagens type II, III, IV and V (Sage *et al.*, 1989b, Maurer *et al.*, 1995, Giudici *et al.*, 2008). SPARC also binds to and induces integrin linked kinase (ILK) activation, through which cells are induced to a contractile state. It is this state of fibroblasts that allows the proper assembly of fibronectin matrix, and fibronectin

assembly precedes collagen accumulation. In SPARC-null fibroblasts the ability to generate fibronectin fibres is reduced (Barker *et al.*, 2005). SPARC has also been found to interact with integrins to regulate other cellular processes. Through interactions with integrin β1 heterodimers and activation of ILK, SPARC protects lens epithelial cells from stress-induced apoptosis (Weaver *et al.*, 2008). SPARC can also bind α5β1 integrin to prevent adipose stromal stem cells binding to ECM (Nie *et al.*, 2008). The binding of SPARC to integrins is important also in tumour progression. SPARC bound to α6β4 (otherwise known as β4 integrin) facilitates β4-mediated invasion of breast carcinoma cells. Integrin β4 also regulates the expression of SPARC (Gerson *et al.*, 2012).

As well as calcium ions, SPARC binds copper (Cu^{2+}), iron (Fe^{2+}), as well as albumin, laminins, thrombospondin and several growth factors (section 1.14) (Sage *et al.*, 1984, Sage *et al.*, 1989b, Vernon and Sage, 1989). Other components of the ECM that SPARC also binds include vitronectin and entactin (Brekken and Sage, 2001, Sweetwyne *et al.*, 2004). SPARC is cleaved by trypsin, chymotrypsin and plasmin (Sage *et al.*, 1984).

One known receptor for SPARC is stabilin-1, a large transmembrane receptor involved in endocytosis. Stabilin-1 is expressed by tissue macrophages and sinusoidal endothelial cells, for example in liver, spleen and lymph node (Kzhyshkowska *et al.*, 2006a). It may function in angiogenesis, lymphocyte homing, cell adhesion, or receptor scavenging. It is recycled between the cell membrane and the endosomes within the cell, indicating it functions as a scavenger receptor. Stabilin-1 facilitates endocytosis of bacteria and ligands such as low density lipoprotein. Stabilin-1 mediates the internalisation and delivery of SPARC to the endocytic pathway for lysosomal degradation. In alternatively activated macrophages, stabilin-1 mediates the uptake and targeting for degradation of SPARC, however the reason for this is unknown (Kzhyshkowska *et al.*, 2006b).

Vascular cell adhesion molecule 1 (VCAM-1) is a receptor on endothelial cells that is involved in the passage of leukocytes from the blood stream into the surrounding tissue (diapedesis). Kelly and colleagues discovered that VCAM-1 binds SPARC, and that presence of SPARC is necessary for transmigration of leukocytes (Kelly *et al.*, 2007). SPARC induces actin cytoskeletal rearrangements and intercellular gaps (Goldblum *et al.*, 1994), thus SPARC produced by leukocytes could be the mechanism by which leukocytes trigger their transit through vessel walls. SPARC therefore has multiple binding partners and mediates multiple downstream effects.

1.12.4 SPARC and cancer

SPARC is over-expressed in many diseases and carcinomas, in the cells of the tumour or in the stroma surrounding the cancerous cells, and has contrasting effects on tumour cells (Chlenski and Cohn, 2010). SPARC is up-regulated in meningiomas, oesophageal carcinoma, and the high levels of SPARC in osteosarcoma and cervical cancer correlate with tumour growth *in vitro* and *in vivo* (Chen *et al.*, 2012, Rempel *et al.*, 1999, Porte *et al.*, 1998, Dalla-Torre *et al.*, 2006). In breast cancer cells SPARC promotes motility and invasion, and high levels of SPARC in melanoma induce EMT and contribute to tumour progression and invasion (Briggs *et al.*, 2002, Ledda *et al.*, 1997, Robert *et al.*, 2006). In gliomas, SPARC has been found to promote the survival of glioma cells by preventing apoptosis and promoting invasion, but delaying the tumour growth (Schultz *et al.*, 2002, Shi *et al.*, 2004).

SPARC also has inhibitory effects on tumours. Over-expression of SPARC by transfection of the gene into human embryonic kidney cell lines inhibits tumour growth (Chlenski *et al.*, 2006). SPARC is expressed at low levels in ovarian carcinoma cells, and upon

transfection of SPARC cDNA into the carcinoma cells it suppresses tumour growth (Mok *et al.*, 1996). High levels of SPARC are detected in colorectal cancer, however this high level is associated with a positive outcome and better patient survival rates (Chew *et al.*, 2011). Moreover, SPARC-null mice exhibit enhanced tumour growth, however this may be due to facilitated metastasis due to improper matrix formation in these mice (Brekken *et al.*, 2003, Puolakkainen *et al.*, 2004).

SPARC expression is upregulated in pancreatic cancer, and can be used as a biomarker for the condition (Von Hoff *et al.*, 2006). SPARC is currently being tested as a target for the treatment of pancreatic cancer. A chemotherapy drug paclitaxel inhibits cell division. The formulation *nab*-paclitaxel (an albumin bound form of paclitaxel) successfully results in tumour regression. Since SPARC binds albumin, the albumin-bound paclitaxel is able to access the peri-tumoural stroma (where SPARC is expressed highly) and prevent spread of cancer. This is being tested in animals but also in stage I/II clinical trials and a stage III trial has been initiated (Desai *et al.*, 2008, Von Hoff *et al.*, 2011).

1.12.5 SPARC function

The role of SPARC is incompletely understood but it is important in development, as demonstrated by the localisation of SPARC protein expression in the developing embryo compared to the adult. In early stages of *Xenopus* development, if SPARC antibodies are injected into the embryos, malformed *Xenopus* embryos form with distorted embryonic axes (bent and shortened) and lack of eye pigment associated with deformities in development. These resulted from disorganised myotome patterns, and loss of the segmental boundaries (Purcell *et al.*, 1993). In the nematode *Caenorhabditis elegans* SPARC shares approximately 38% protein identity with human and mouse. Over-

expression of endogenous SPARC in *C. elegans* produces deformed embryos, and paralysis in the adult worm (Schwarzbauer and Spencer, 1993). Normal levels of SPARC are required at all stages of development to maintain body morphology and muscle function. Since SPARC is associated with the basement membrane in nematodes as well as mammals, deposition of excess SPARC in the nematode may prevent muscle attachment to the basement membrane. During contraction, attachment sites are not strong enough and paralysis is evident (Schwarzbauer and Spencer, 1993).

SPARC is known to have inhibitory effects on cells. It can prevent proliferation, function, adhesion and migration in murine and bovine cells (Francki *et al.*, 2003, Sage *et al.*, 1989b, Funk and Sage, 1991). The addition of exogenous SPARC to endothelial cells causes cell rounding, rapid partial detachment of the confluent monolayer, inhibition of spreading of newly plated cells and induction of apoptosis *in vitro* (Sage *et al.*, 1989b). SPARC can reduce [^{3}H]thymidine incorporation in bovine aortic endothelial cells, and by doing so restrict the cells in G_1 phase of the cell cycle and prevent entry into S phase (Funk and Sage, 1991). SPARC may directly increase levels of the cell cycle inhibitors p21 and p27 (Francki *et al.*, 2003).

When mesangial cells, fibroblasts and smooth muscle cells were isolated from SPARC-null mice, the cells displayed increased rates of proliferation compared to wild type cells. Upon addition of recombinant SPARC to these cells, proliferation was slowed. Wild type cells with endogenous expression of SPARC showed decreased presence of cyclin A within the cells, demonstrating the inhibitory effects of SPARC on cell cycle progression (Bradshaw *et al.*, 1999).

SPARC acts as an inhibitor of angiogenesis, which has been demonstrated through inhibition of VEGF and bFGF stimulation (Chlenski *et al.*, 2006), and also through decreased fibronectin and thrombospondin-1 mRNA levels, after purified SPARC was

added to aortic endothelial cells in culture (Lane *et al.*, 1992). In certain tumours SPARC is thought to inhibit growth primarily through inhibition of angiogenesis (Chlenski *et al.*, 2010).

1.12.6 The role of SPARC in diabetes

Recent evidence indicates the involvement of SPARC in obesity and type 2 diabetes. SPARC expression in adipocytes was increased in mouse models of obesity and upon high fat feeding in mice and rats (Li *et al.*, 2002, Tartare-Deckert *et al.*, 2001, Shen *et al.*, 2013) which was replicated in human studies (Kos *et al.*, 2009). SPARC levels in plasma were higher in type 2 diabetes patients than control subjects, and this correlated with increased body mass index (BMI), blood lipids and plasma insulin levels (Wu *et al.*, 2011). Leptin as well as insulin can increase SPARC expression in adipocytes causing increased SPARC secretion (Tartare-Deckert *et al.*, 2001, Kos *et al.*, 2009).

High circulating leptin is correlated with insulin resistance (Mohiti *et al.*, 2009). SPARC plays a critical role in obesity-induced insulin resistance in adipocytes and correlates with the homeostasis model assessment of insulin resistance (Kos *et al.*, 2009). Over-expression of SPARC decreases GLUT4 in adipocytes of rats fed a high fat diet, inducing insulin resistance (Shen *et al.*, 2013). However SPARC has been found to increase GLUT4 expression in myocytes (Song *et al.*, 2010). SPARC increases inflammatory cytokine production in adipocytes, specifically TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) which can contribute to insulin resistance (Song *et al.*, 2010). The influence of SPARC in insulin resistance and type 2 diabetes may arise from its role in the regulation of collagen assembly and ECM remodelling. In adipose tissue SPARC is thought to increase fibrosis by increased deposition of insoluble collagen fibres. As the adipose tissue

becomes increasingly fibrotic in obesity, accumulation of triglycerides in the plasma may result in their deposition in liver or muscle, predisposing the tissue to insulin resistance. SPARC may therefore play a role in the link between obesity and diabetes mellitus (Kos and Wilding, 2010).

Recently, SPARC mRNA was found expressed at detectable levels in pancreatic islets, albeit low levels, at less than one tenth of the expression levels found in adipose tissue (Harries *et al.*, 2013). The location of SPARC within particular cells of the islet has not been determined however. SPARC mRNA expression is lower in human islets from patients with type 2 diabetes compared to control subjects, however the amount of SPARC protein has not been determined in diabetic or control subjects. There is no evidence of correlation between SPARC expression in islets and basal plasma insulin levels (Harries *et al.*, 2013), however as mentioned above, higher SPARC in plasma is correlated with high plasma insulin levels (Wu *et al.*, 2011), indicating that SPARC is context-dependent.

Also linked to diabetes but not specifically related to the islets, SPARC may have involvement in the kidney during diabetic nephropathy. In diabetic rats, there is a decrease of SPARC in the kidney, as shown by northern blot analysis of mRNA (Gilbert *et al.*, 1995). However, increased SPARC gene expression occurred in the intima and adventitia of mesenteric vessels after 1, 3, and 32 weeks of diabetes (Jandeleit-Dahm *et al.*, 2000). SPARC inhibits mesangial cell proliferation and may contribute to the progression of diabetic nephropathy (Francki *et al.*, 2003, Kanauchi *et al.*, 2000).

There is very little known about the effect of SPARC on islets and β cells in particular, and whether the presence or absence of SPARC has any role in diabetic progression. Establishing any effect of SPARC on β cells may give greater insight into the pathogenesis of diabetes and the mechanisms of graft death after transplant.

1.13 Growth factors

Growth factors play critical roles in the development and morphogenesis of tissues and regulate almost all cell activities such as proliferation, differentiation, development, inflammation, apoptosis, cell mobility and migration. Growth factors bind receptors on the cell surface of the β cell and trigger tyrosine-kinase activity of the receptor (Figure 1.8). Multiple downstream signalling pathways can be activated, two of which are the extracellular-signal regulated kinase (ERK) 1/2 (p44/p42 mitogen activated protein kinases) pathway, and the Akt-Phosphatidylinositide 3-kinase (PI3-kinase) pathway. When activated by phosphorylation, ERK 1/2 regulate growth factor responsive targets in the cytosol and also translocate to the nucleus where they activate specific transcription factors involved in mitosis (Chen *et al.*, 1992, Seger and Krebs, 1995). Activation of Akt *via* PI3-kinase promotes cell survival and prevents apoptosis (Dudek *et al.*, 1997, Kennedy *et al.*, 1997). Akt is also the mediator of insulin-induced glucose transport and glycogen synthesis (Ueki *et al.*, 1998).

1.13.1 Growth factors and the β cell

Pancreatic growth factors responsible for the growth and survival of islet cells may be targeted during the autoimmune destruction of β cells. Growth factors have been studied in great detail regarding the treatment of diabetes (Garcia-Ocana *et al.*, 2001). Several factors, such as growth hormone, prolactin, placental lactogen, insulin-like growth factors (IGFs) and hepatocyte growth factor (HGF) have been found to induce pancreatic islet expansion, decrease cell death or enhance function, as described above (Fiaschi-Taesch *et al.*, 2008, Hugl *et al.*, 1998, Gahr *et al.*, 2002, Billestrup and Nielsen, 1991, Nielsen *et al.*, 1989, Hayek *et al.*, 1995).

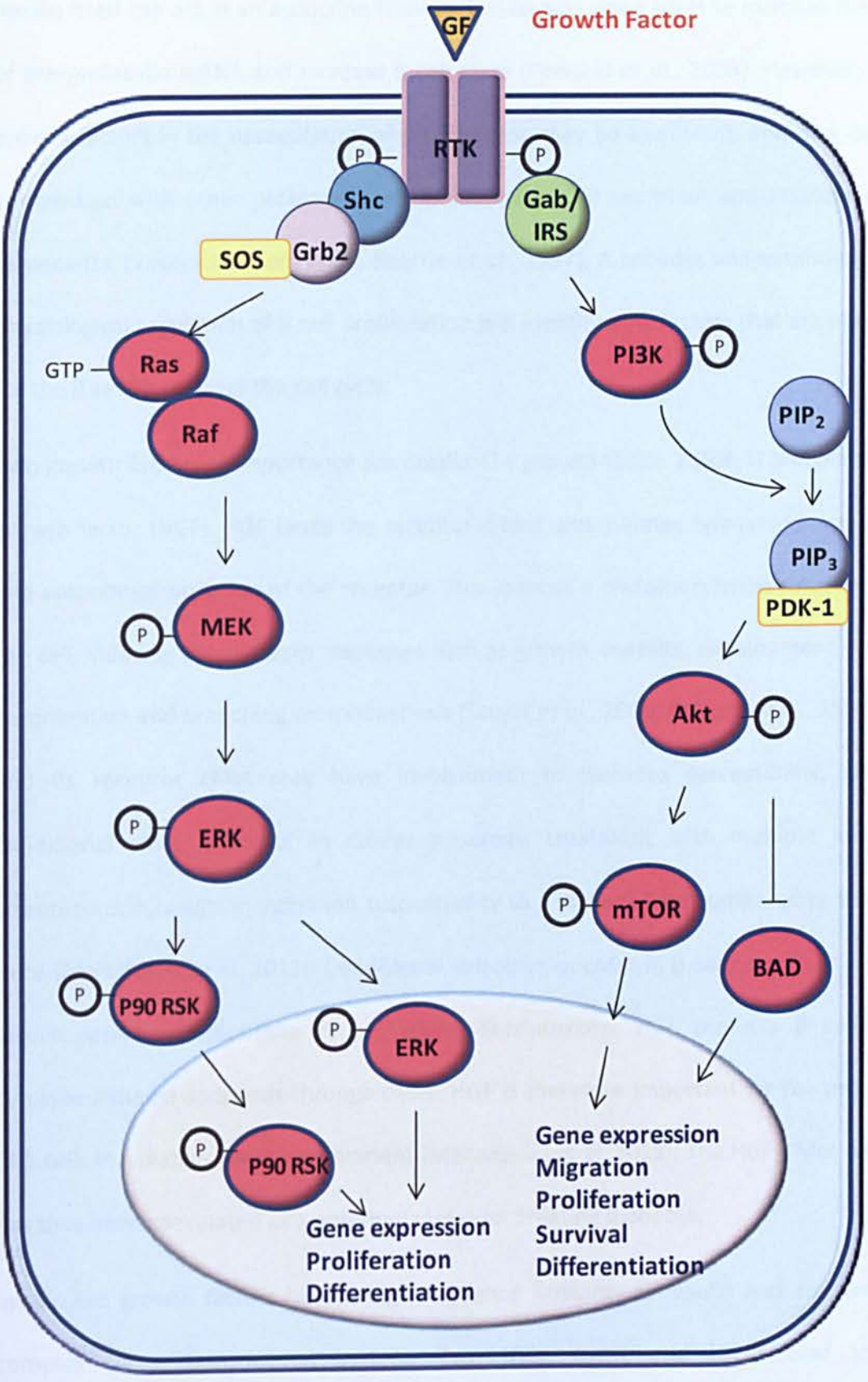


Figure 1.8. Intracellular growth factor signalling. Upon binding of growth factor to a receptor tyrosine kinase (RTK), autophosphorylation occurs, resulting in the 2 subunits becoming phosphorylated. Sequences of kinase phosphorylation lead to the activation of many pathways, including Ras/Raf/MEK/ERK and IRS/PI3K/Akt pathways. Translocation of proteins such as ERK into the nucleus results in transcription and gene expression leading to proliferation, differentiation, survival and migration.

Insulin itself can act in an autocrine fashion. Insulin acts upon islets to increase the levels of pre-proinsulin mRNA and increase β cell mass (Persaud *et al.*, 2008). However, use of growth factors in the upregulation of proliferation may be inefficient, and may occur in conjunction with other problems such as loss of insulin secretion and accelerated cell senescence (Vasavada *et al.*, 2006, Beattie *et al.*, 1997). A broader understanding of the physiological regulation of β cell proliferation will identify new factors that are important for the β cell to re-enter the cell cycle.

Two growth factors of importance are insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF). HGF binds the receptor c-Met and initiates tyrosine kinase activity and autophosphorylation of the receptor. This induces a phosphorylation cascade within the cell, inducing downstream responses such as growth, motility, development, survival, regeneration and branching morphogenesis (Stuart *et al.*, 2000, Bottaro *et al.*, 1991). HGF and its receptor cMet may have involvement in diabetes susceptibility, since in conditional cMet knockout in mouse pancreas, treatment with multiple low dose streptozotocin results in increased susceptibility to a diabetic fate compared to wild type mice (Mellado-Gil et al. 2011). Conditional knockout of cMet in β cells results in reduced insulin secretion (Roccisana *et al.*, 2005). Furthermore, HGF protects β cells from cytokine-induced apoptosis through cMet. HGF is therefore important for the protection of β cells in a diabetogenic environment (Mellado-Gil et al. 2011). The HGF/cMet pathway has thus been speculated as a potential target for treating diabetes.

Insulin-like growth factors have a high sequence similarity to insulin and are part of a complex signal transduction system. Two IGFs, IGF-1 and IGF-2 bind to two transmembrane IGF receptors, IGF1R and IGF2R. Insulin-like growth factor binding proteins have high affinity toward IGF-1 and IGF-2 and circulate in the plasma. There are 6 binding proteins, IGFBP-1 to IGFBP-6. Binding to IGFBPs prolongs the half-life of the IGFs.

The function of both IGFs is to induce cell growth (Reddan and Dziedzic, 1982), however IGF-2 (somatomedin A) is expressed primarily during the early developmental stages in gestation (Sara *et al.*, 1981).

IGF-1, also known as somatomedin C, is released in response to growth hormone in liver cells and in target tissue (Roberts *et al.*, 1986) and is a primary mediator of the effects of growth hormone. IGF-1 mainly binds to IGF1R, although it also binds the insulin receptor (with 10-fold less activation). The IGF1R is a tyrosine kinase receptor. Autophosphorylation of tyrosines in the receptor stimulate a protein phosphorylation cascade that results in growth and survival in almost all cells of the body.

IGF-1 and HGF induce beneficial effects in pancreatic β cells including function (insulin secretion), growth, DNA synthesis and survival, *via* the signal transduction pathways described in Figure 1.8 (Elghazi *et al.*, 2007, Fiaschi-Taesch *et al.*, 2008, Hugl *et al.*, 1998, Gahr *et al.*, 2002, Frodin *et al.*, 1995, Dickson *et al.*, 2001, Briaud *et al.*, 2003, Zhang *et al.*, 1998, Giannoukakis *et al.*, 2000, Garcia-Ocana *et al.*, 2003, Liu *et al.*, 2002). HGF acts as a β cell mitogen *in vitro* or *ex vivo* (Gahr *et al.*, 2002, Beattie *et al.*, 1997, Beattie *et al.*, 1996). HGF over-expression improves insulin content, reduces β cell apoptosis and improves glucose tolerance in streptozotocin (STZ)-induced diabetic severe combined immunodeficiency (SCID) islets *in vivo* and in β cells *in vitro* (Garcia-Ocana *et al.*, 2003). As discussed above, successful islet grafts with good endocrine function were made by growing islets in fibrin gels and a combination of HGF, FGF4 and nicotinamide, and proliferation was observed, three-fold over free floating islets (Beattie *et al.*, 2002). IGF-1 enhances β cell mitogenesis, induces DNA synthesis in β cells *in vitro* and can prevent interleukin-1β-induced β cell apoptosis in human islets (Hugl *et al.*, 1998, Zhang *et al.*, 1998, Giannoukakis *et al.*, 2000).

1.14 SPARC and growth factors

Previous experiments have shown that SPARC may be involved in modulating growth factor signalling pathways by interaction (direct or indirect) with various growth factor receptors. Cytokine and growth factor binding was first demonstrated by interactions with transforming growth factor (TGF)-β. TGF-β is a cytokine that can halt proliferation and can induce differentiation and apoptosis (Wolf *et al.*, 1992, Takehara *et al.*, 1987, Schuster and Krieglstein, 2002). Secretion of SPARC is induced by stimulating cells with TGF-β, and TGF-β expression can similarly be stimulated with SPARC, shown in cranial bone cells, foetal lung fibroblasts and epithelial cells. This demonstrates the interplay between SPARC and TGF-β in order to inhibit proliferation and induce epithelial damage (Wrana *et al.*, 1988, Shibata and Ishiyama, 2013, Schiemann *et al.*, 2003).

SPARC inhibits vascular endothelial growth factor (VEGF)-stimulated DNA synthesis in microvascular endothelial cells. Ligand binging assays were used to demonstrate that SPARC can bind VEGF, and this binding prevents association of VEGF with its cell surface receptors (Kupprion *et al.*, 1998). SPARC also binds specific dimeric forms of platelet-derived growth factor (PDGF) to inhibit the binding of PDGF to its receptor on human dermal fibroblasts (Raines *et al.*, 1992), and it inhibits basic fibroblast growth factor (bFGF)-induced migration of endothelial cells (Chlenski *et al.*, 2006).

SPARC can also regulate IGF signalling. SPARC inhibits IGF-1-induced proliferation of mesangial cells, as shown by a 75% reduction in [^{3}H]thymidine incorporation (Francki *et al.*, 2003). SPARC-null mice expressed increased levels of IGF-1, IGF-2 and the receptor, IGF1R, and upon addition of SPARC to both wild type and SPARC-null mesangial cells IGF-1, IGF-2 and IGF1R mRNA levels were decreased (Francki *et al.*, 2003). SPARC can also reduce IGF-1 induced ERK 1 phosphorylation in mesangial cells by more than 300%; this was not due to the binding of SPARC to IGF-1 (Francki *et al.*, 2003). This ability of SPARC

to modulate growth factor receptor signalling resulting in altered proliferation and DNA synthesis may be of potential use in promoting β cell expansion/regeneration. The little evidence available suggests that SPARC is expressed at low levels in the islet, which is not upregulated in diabetes. However plasma levels of SPARC are increased during diabetes and therefore circulating SPARC may modulate cytokine/growth factor binding in islets.

HGF and IGF-1 protect β cells from cytokine induced apoptosis and induce β cell mitogenesis, and moreover HGF receptor knockout mice have increased susceptibility to diabetes. SPARC has been shown to interact with IGFs and IGF1R, however currently there is no evidence for interaction of SPARC with HGF. There are implications for targeting HGF and/or cMet for diabetes treatment so this is one avenue that may be of interest in investigating the effect of SPARC on β cells. Elucidating the interplay between SPARC and growth factors in the context of diabetes, islets and the β cell in particular could further our understanding of β cell death and diabetes progression, and lead us to improved islet graft function after transplant. If SPARC has involvements in stimulating the β cell to re-enter the cell cycle or dedifferentiate this could lead to a promising future for diabetes therapy.

1.15 Aims

Type 1 and type 2 diabetes result in β cell apoptosis. Current protocols for improving β cell survival after attack by the immune system are limited in their success. Increasing the rate of β cell proliferation and preventing damage to the β cell would allow development in procedures for preventing β cell loss *in situ* and improving graft survival after transplant. A broader understanding of the physiological regulation of β cell proliferation

and death will identify novel factors that are important for the β cell to survive and re-enter the cell cycle.

SPARC has clear roles in moderating cell growth, survival, migration and adhesion in various cell types. SPARC binds to and modulates signalling by growth factors, prevents cells entering the S phase of the cell cycle and induces apoptosis. In patients with diabetes, the circulating concentration of SPARC is increased. However evidence showing SPARC interactions with β cells is lacking. If it can be proven that SPARC has modifying effects on β cells, and especially if it has cell inhibitory effects as shown on other cell types, this has huge implications for the future of diabetes therapy and islet graft procedures.

One aim of this study was to determine if SPARC was expressed in or around the β cells of the islets. If SPARC is expressed in the islets at an early stage of development it may suggest involvement in β cell maturity. If SPARC expression is up- or down-regulated in models of diabetes this may infer roles in diabetes onset. The location of SPARC expression in the pancreas of NOD mouse would help determine whether SPARC is important in the early stages of diabetes.

Looking at the influence of SPARC on islet size would determine if it has any large influence on proliferation of cell types within the islet, or destruction of peri-islet membranes. The investigation of islet size in SPARC knockout mice would help us to determine the scale to which SPARC is important in this. If SPARC does have an impact on islet size it could be targeted in order to increase islet mass *ex vivo*.

Growth factors influence all aspects of cell behaviour, including proliferation and survival. Use of growth factors in the upregulation of proliferation may be inefficient, and may coincide with other problems such as loss of insulin secretion, and accelerated cell

senescence (Vasavada *et al.*, 2006). SPARC not only has inhibitory effects on cells through growth factor interaction, but beneficial effects of SPARC on cells have also been found.

These experiments aim to determine whether SPARC interacts with growth factor signalling in β cells and islets, and to ascertain if it is beneficial or inhibitory. This ability of SPARC to modulate growth factor receptor signalling is of potential use in promoting β cell expansion/regeneration. To find out if SPARC influences growth factor binding to β cells, the insulin-secreting cell line (INS-1) was used. INS-1 is a β cell line that exhibits beneficial responses such as proliferation, in response to glucose and growth factors, and has a higher mitotic index than other β cell lines (Hugl *et al.*, 1998, Frodin *et al.*, 1995, Dickson *et al.*, 2001, Briaud *et al.*, 2003, Asfari *et al.*, 1992). INS-1 cells may be used experimentally as an initial indicator of *in vitro* pancreatic β cell response, therefore were chosen for study. The aim in INS-1 cells was to assess growth factor signalling and proliferation after SPARC treatment. Primary islets from mice were used to assess the impact of SPARC on growth factor signalling in a more physiologically relevant system, as well as the effect of SPARC on survival of islets *ex vivo* when they are cultured in extreme conditions unsuitable for their continued existence.

Islet β cells require a functioning ECM containing collagens, laminin, fibronectin, growth factors and other stimulatory components to provide support, adhesion, induce survival and activate intracellular pathways (Stendahl *et al.*, 2009, Wang and Rosenberg, 1999, Daoud *et al.*, 2010, Jalili *et al.*, 2011). Therefore another aim was to culture islets within a more physiological environment to give us greater insight into the role of SPARC, since it is a secreted protein residing in the ECM and influences proper ECM maturation (Barker *et al.*, 2005).

To provide an environment where islets can be co-cultured with a SPARC-secreting cell line, human pancreatic stellate (PS-1) cells were used. The PS-1 cell line was developed

originally to study the effect of co-culturing stellate cells with pancreatic cancer cells (Froeling *et al.*, 2009). Here PS-1 cells were used to generate SPARC *in vitro*, as it is a SPARC-secreting cell line. It was found to inhibit cancer cell growth (Froeling *et al.*, 2009), thereby our hypothesis is that SPARC produced from these cells may have an influence on growth of surrounding cells.

The potential for SPARC to be involved in β-cell signalling, proliferation and survival was thus assessed permitting any potential for SPARC in therapeutic interventions for diabetes patients to be determined.

CHAPTER 2: Materials and Methods

2.1 Animals

Adult female or male outbred (ICR) mice 21-25 g (8 weeks of age), and female C57BL/6 (B6) mice at 4 or 12 weeks of age, were obtained from Harlan, Bicester, UK. Pancreatic tissue for immunohistochemistry was kindly provided by Professor Nora Sarvetnick, The Scripps Research Institute, CA, USA. All animal procedures were undertaken in accordance with the UK Home Office Regulations.

2.2 Reagents and Antibodies

A list of commercial antibodies used is found in Table 2.1. A list of reagents and equipment with supplier information is provided in Appendix 1.

Table 2.1. Commercial antibodies used for immunohistochemistry and western blotting.

Primary antibodies	Method	Raised in	Catalogue code	Supplier
anti-insulin	IHC[1]	Guinea-pig	IS002	DAKO
anti-FSP-1	IHC	Rabbit	07-2274	Millipore, UK
anti-SPARC	IHC	Goat	AF942	R & D Systems, UK
anti-β-tubulin	WB[1]	Rabbit	2128	New England Biolabs, UK
anti-SPARC	WB/IHC	Rabbit	SC-25574	Santa Cruz (Insight Biotechnology), UK
anti-phospho-Akt	WB	Rabbit	9271	New England Biolabs, UK
anti-pan-Akt	WB	Rabbit	4691	New England Biolabs, UK
anti-phospho-p44/p42 MAPK	WB	Rabbit	4377	New England Biolabs, UK
anti-total p44/p42 MAPK	WB	Rabbit	9102	New England Biolabs, UK
Secondary antibodies	**Label**	**Raised in**	**Catalogue code**	**Supplier**
anti-goat	FITC[2]	Rabbit	FI-5000	Vector Labs, UK
anti-goat	TRITC[2]	Rabbit	T-7028	Sigma-Aldrich, UK
anti-goat	NL637 (red)	Donkey	NL002	R & D Systems, UK
anti-guinea pig	Texas Red	Goat	TI-7000	Vector Labs, UK
anti-guinea pig	TRITC	Rabbit	Ab50627	Abcam, UK
anti-guinea pig	FITC	Goat	Ab97154	Abcam, UK
anti-rabbit	FITC	Goat	F0382	Sigma-Aldrich, UK
anti-rabbit	NL493 (green)	Donkey	NL006	R & D Systems, UK
anti-rabbit	Alexa-488	Goat	A11008	Invitrogen, UK
anti-rabbit	Cy3	Goat	A10520	Invitrogen, UK
anti-goat	Biotin	Horse	BA-9500	Vector Labs, UK
anti-guinea pig	Biotin	Goat	BA-7000	Vector Labs, UK
anti-rabbit	Biotin	Goat	BA-1000	Vector Labs, UK
anti-rabbit	Horseradish peroxidise	Goat	7074	New England Biolabs, UK

[1]Method: IHC Immunohistochemistry. WB Western blotting. [2]Label: TRITC Tetramethylrhodamine isothiocyanate. FITC Fluorescein isothiocyanate.

2.3 Cell culture

2.3.1 Cell lines and media

The PS-1 cell line was kindly provided by Mr Hemant Kocher, and the INS-1 cell line provided by Dr. Charlotte Edling, both at Barts and The London School of Medicine and Dentistry, Queen Mary University of London, UK. The rat insulin-secreting β cell line, INS-1 cells were cultured in RPMI-1640 media containing 11.1 mM glucose and 2 mM glutamine, supplemented with 10% foetal bovine serum (FBS; heat inactivated at 56°C for 1 h), 0.05 mM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. INS-1 cells were maintained in a 37°C, 5% CO_2 environment and sub-cultured every 3-4 days and were used within 20 passages.

The human pancreatic stellate cell line, PS-1 was maintained in DMEM (high glucose): Ham's F12 media (1:1) supplemented with 10% FBS, 1 µg/ml puromycin, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. PS-1 cells were maintained in a 37°C, 5% CO_2 environment, subcultured every 2-3 days and used within 10 passages.

2.3.2 Sub-culturing

Cells were cultured in medium (75 cm^2) or small (25 cm^2) filter-capped flasks. When the cells were near confluent (approximately 70-80%), cells were washed in Dulbecco's phosphate buffered saline (PBS; without Ca^{2+}, Mg^{2+}), followed by incubation with 0.05% Trypsin-EDTA solution until cells detached from the flask. Trypsin was deactivated with the addition of cell culture medium containing 10% FBS, and transferred to 25 ml universal tubes. Suspensions were centrifuged at 1000 rpm for 4 min, the supernatant

was discarded and cell pellets were resuspended in fresh cell culture medium before seeding into flasks at the required density.

2.3.3 Trypan blue dye exclusion assay

For cell counting, cells were trypsinised as described in section 2.3.2 and resuspended in culture medium. Aliquots of cell suspension were transferred to microfuge tubes and diluted with an equal volume of 0.2% trypan blue solution. 20 µl was transferred to the chamber of a Neubauer haemocytometer. To obtain a viable cell count, non-stained cells were counted in the 4 large corner squares of the haemocytometer. Cell counts were averaged (to obtain a mean for one corner square), adjusted for trypan blue dilution factor converted to cells per ml as shown below. Replicate aliquots were counted and the average taken.

$$\text{Cell concentration (cells/ml)} = \left(\frac{\text{cell count}}{4}\right) \times 2 \times 1\text{x}10^4$$

2.3.4 Cryopreservation of cell lines

For long term storage, cell lines were grown in 75 cm^2 flasks. Cell lines were trypsinised as in section 2.3.2 and first resuspended in medium with high FBS (RPMI, or DMEM/Ham's F12 as appropriate, containing 40% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin). Freezing medium consisting of 20% DMSO (in RPMI, or DMEM/Ham's F12 as appropriate) was added slowly to the cell suspension to give final concentrations of 20% FBS and 10% DMSO, and the cell suspension was aliquotted into 1 ml cryovials (cells from one T75 flask were aliquotted into 4 cryovials). Cells were cooled slowly in an

isopropanol chamber at -80°C overnight. The following day cells were transferred to liquid nitrogen for long term storage.

For thawing of cell lines, vials were removed from liquid nitrogen and thawed quickly in a 37°C water bath. Cells from each vial were transferred to 5 ml pre-warmed culture medium. The following day, the medium in each flask was removed and replaced with fresh medium.

2.4 Islet isolation

Islets were isolated from 5-8 ICR mice using collagenase digestion followed by separation using a density gradient. Mice were sacrificed by cervical dislocation, and a laparotomy was performed. After clamping of the ampulla of Vater, approximately 2 ml collagenase (1 mg/ml in minimal essential medium) was injected into the pancreas *via* the common bile duct and the pancreas was removed. Tubes containing up to 3 pancreases were incubated in a stationary water bath for 10 min at 37°C. The islets were separated by a density gradient (Histopaque-1077) and centrifuged at 1170 *g* for 25 min. After washing, islets were handpicked and cultured overnight in RPMI-1640 plus 10% FBS, penicillin/streptomycin.

2.5 Preparation of reagents

Recombinant SPARC (50 µg, Cat. 941-SP) was dissolved in 500 µl sterile PBS to make a stock 100 µg/ml solution. Recombinant insulin-like growth factor-1 (IGF-1; Cat. 4326-RG) was received as 50 µg and dissolved in 500 µl sterile PBS to make a stock 100 µg/ml solution. Recombinant hepatocyte growth factor (HGF; Cat. 2207-HG/CF) was received as a 25 µg powder and dissolved in 250 µl sterile PBS to make a stock 100 µg/ml solution. All

were supplied from R & D Systems, UK. All stocks of SPARC, IGF-1 and HGF were further diluted to 20 µg/ml aliquots for freezing.

2.6 Fluorescence immunohistochemistry

Pancreas sections: Whole pancreas was removed from C57BL/6 mice (hereafter referred to as B6) and fixed in 10% neutral buffered formalin for 24 h, then histologically processed through a series of 70%-100% alcohol solutions before clearing in Histoclear, and embedding in paraffin wax. Sections (5 µm) of pancreas were cut and mounted on poly-lysine coated slides. Slides were dewaxed in Histoclear and rehydrated through a series of 100%-70% alcohol baths.

Cells: Coverslips were coated in poly-lysine before use. Coverslips were sterilised in 70% ethanol for 1 h, washed thoroughly in sterilised water and allowed to dry completely. Poly(D)lysine hydrobromide was diluted to 0.1 mg/ml in sterile water. Coverslips were coated with poly(D)lysine for 1 h, then were washed in sterile water and allowed to dry. Coverslips were stored sealed in a Petri dish at 4°C until use. INS-1 cells were grown on poly-lysine coated coverslips and after attachment (overnight), were washed in PBS and fixed with cold methanol for 10 min at 4°C.

Pancreas sections and cells on coverslips were incubated with 0.3% Triton-X-100 in PBS (PBS-Triton) at room temperature for 20 min. Blocking was performed in 10% normal horse serum, diluted in PBS-Triton for 1.5 h at room temperature. Anti-insulin primary antibody in PBS-Triton was applied to cells and pancreas sections at 1/200 dilution and left at 4°C overnight. After a series of washes (PBS-Triton), sections were exposed to Texas Red anti-guinea pig secondary antibody (1/200) for 1.5 h at room temperature. After washing, coverslips were mounted to slides using SlowFade mounting medium plus

DAPI fluorescent nuclear stain. Coverslips were sealed onto slides and stored at 4°C. A Leica TCS SP2 AOBS confocal laser scanning microscope was used to visualise fluorescence, using a 63X oil immersion objective and images were captured with Leica confocal software. Series of z scans were captured and a single scan image was reported.

2.7 Immunocytochemistry

INS-1 and PS-1 cells were grown on poly-lysine coated coverslips (see section 2.6) and allowed to attach overnight. Cells were washed in PBS and fixed with cold methanol for 10 min at 4°C. After rinsing thoroughly in PBS, cells were stored at 4°C in PBS until needed. Coverslips were incubated with PBS-Triton at room temperature for 20 min. Blocking was performed in 10% normal horse serum, diluted in PBS-Triton for 1.5 h. Coverslips were incubated with anti-SPARC primary antibody (1 in 25) anti-FSP-1 antibody (1 in 200) or anti-insulin (1 in 200), all diluted in blocking solution, at 4°C overnight followed by room temperature for 1.5 h. After a series of washes (PBS-Triton), sections were exposed to anti-goat, anti-rabbit or anti-guinea pig secondary antibody conjugated to biotin (1 in 400) for 1.5 h at room temperature. 1% hydrogen peroxide was used for blocking of endogenous peroxidise activity, followed by exposure to avidin-biotin complex (ABC) reagent. Bound antibody was then visualised upon addition of diaminobenzidine (DAB). Sections were treated with haematoxylin before mounting with Di-N-Butyl Phthalate in Xylene (DPX). Slides were visualised on a Nikon Eclipse 80i microscope and Nikon Digital Sight DS-U1 camera, using NIS elements software.

2.8 Immunohistochemistry (SPARC or FSP-1)

Whole pancreas was removed from 4 or 12 week old female B6 control and NOD mice. Preparation of tissue was performed as described in section 2.6. After PBS-Triton treatment, sections for SPARC and FSP-1 labelling were subject to proteinase K treatment (50 μg/ml, 20 min at 37°C). Blocking was performed as before, and sections were incubated with primary antibody (goat anti-SPARC at 1/25, or rabbit anti-FSP-1 at 1/100, diluted in blocking solution) at ambient temperature overnight. Positive areas were visualized with biotin-conjugated secondary antibody to goat or rabbit IgG (1/200, diluted in PBS-Triton), and use of the peroxide, ABC and DAB steps as described in section 2.7. Pancreas sections incubated with secondary antibody only served as controls. Sections were counterstained with haematoxylin, dehydrated through 70-100% alcohol, cleared with Histoclear, and mounted with DPX. Slides were visualised on a Nikon Eclipse 80i microscope and Nikon Digital Sight DS-U1 camera, using NIS elements software, and digital images of sections were used for ImageJ analysis (Rasband, 1997-2012).

For analysis of SPARC-stained area, the amount of staining within the islet area (including the peri-islet basement membrane) was quantified in μm^2, by using the thresholding tool to highlight brown areas. Any brown areas outside of the islet boundary, and areas of insulitis (area of high haematoxylin nuclear staining, due to invading cells of the immune system) were excluded from the analysis. The area taken up by the entire islet was also quantified in this way by outlining the visible islet area (recognition of the paler islet area by eye), deletion of all areas outside of the islet (including insulitis) and thresholding of the entire islet. SPARC-stained area was quantified as a percentage of the total islet area.

2.9 Islet insulin scoring

SPARC knockout and age-matched B6 control mouse pancreas tissues were kindly provided through collaboration with Professor Karlsson at Lund University, Sweden. SPARC knockout had previously been confirmed in this mouse colony by PCR analysis of DNA, Northern blotting of RNA and western blotting of protein (Siva *et al.*, 2012).

Wild type B6 mouse pancreas and SPARC knockout pancreas tissue (provided in 10% neutral buffered formalin) were processed as described in section 2.6. After the blocking step, incubation with primary antibody (guinea pig anti-insulin at 1/400, in blocking solution) was performed at 4°C overnight. Positive areas were visualized as described in section 2.7, using a biotin-conjugated secondary antibody to guinea pig IgG (1/200). Pancreas sections incubated with secondary antibody only served as controls. Sections were counterstained with haematoxylin, dehydrated through 70-100% alcohol, cleared with Histoclear, and mounted with DPX.

Five pancreases were analysed for each mouse type (SPARC knockout and wild type control). Six sections were analysed per pancreas (per mouse), sections were chosen approximately 250 μm apart in the pancreas (minimum 200 μm ensured). In a blind study (mouse type unknown to analyser), whole pancreas sections were visualised using Nikon Eclipse 80i microscope and Nikon Digital Sight DS-U1 camera, using NIS elements software and digital images of the entire pancreas were progressively captured at 40x magnification and then overlayed to produce a whole pancreas image using Dia software (Macke, 2012). Insulin (islet) stained areas and total pancreas section area were quantified using ImageJ software (Rasband, 1997-2012).

2.10 SPARC expression (western blotting)

2.10.1 Preparation of buffers

Solutions made for polyacrylamide gel electrophoresis (PAGE) and western blotting:

SDS sample buffer (5X): 312 mM Tris HCl (pH 6.8), with 10% (v/v) sodium dodecyl sulphate (SDS), 1% (v/v) 2 mercaptoethanol, 25% (v/v) glycerol, 0.015% (v/v) bromophenol blue. Aliquots were stored frozen until use.

Resolving gel (12%): The following quantities were mixed to make 10 ml gel: 3.75 ml 1 M Tris, pH 8.8, 4 ml Protogel (supplied as a 30% acrylamide solution), 50 µl (w/v) 20% SDS, 12.5 µl Tetramethylethylenediamine (TEMED), 300 µl 10% (w/v) ammonium persulphate (APS; made within 2 weeks of use) and made up to 10 ml with water.

Stacking gel: The following quantities were mixed to make 5 ml gel: 625 µl 1 M Tris, pH 6.8, 900 µl Protogel (30%), 25 µl 20% (w/v) SDS, 12.5 µl TEMED, 150 µl 10% (w/v) APS, made to 5 ml with water.

Running buffer (10X): 30 g Tris, 145g glycine and 10 g SDS dissolved in 1 l water and pH adjusted to 8.3. For a 1X working solution, 100 ml was diluted into 900 ml water.

Transfer buffer (1X): 3 g Tris and 15 g glycine dissolved in 200 ml methanol and made up to 1 l with water.

Tris buffered saline (TBS; 10X): 18.2 g Tris and 87 g sodium chloride were dissolved in 1 l water and pH adjusted to 8.0.

TBS-Tween (1X): 100 ml of 10 X TBS was diluted to 1 l with water and 1 ml Tween 20 (polysorbate 20) added.

2.10.2 Preparation of cell lysates

Cells: INS-1 (1 x 10^6) and PS-1 (5 x 10^5) cells were seeded into T25 flasks and incubated in media containing reduced serum overnight (in 0.5% FBS), media was removed and concentrated in a Vivapore concentrator to approximately 100-fold. After rinsing in PBS, cells were lysed in RIPA buffer.

Islets: 50 islets from male ICR mice were serum-starved (1% FBS) overnight before media was concentrated in a Vivapore concentrator to approximately 40 X. After rinsing in PBS, islets were lysed in RIPA buffer.

SDS buffer was added to the lysates to a final concentration of 1X, and the samples heated to 95°C for three minutes.

2.10.3 Quantification of protein

Bovine serum albumin (BSA) was made up to 10 µg/µl in water, and from this a 2 µg/µl stock was made. BSA standards ranging from 0 – 4 µg/µl were made, diluted in RIPA lysis buffer. Triplicate 5 µl aliquots of BSA standards were pipetted into clear-bottomed 96-well plates, to give protein amounts ranging from 0 – 20 µg. Duplicate 5 µl aliquots of cell or islet lysates in RIPA buffer were pipetted into clean wells. 250 µl of Bradford reagent (Coomassie Brilliant Blue) added to each well, and the plate carefully shaken. Absorbance was read at 595 nm using a FLUOstar Optima plate reader with FLUOstar Optima software. A standard curve was plotted from the absorbance readings of the BSA standards. The equation of the line was used to calculate the amount of protein in each sample, and from the known volume aliquotted into the plate, the protein concentration of each sample was determined.

2.10.4 Preparation of the gel and protein separation by SDS-PAGE

Ingredients for the resolving gel were mixed in a universal tube and poured in between the glass plates of a Fisherbrand gel running unit. Isopropanol was layered over the top to ensure a level edge. Once set, isopropanol was poured off and the stacking gel was layered on top and a 12 well comb was set into the gel. Once gels were set, the comb was removed and 1X running buffer was poured into the top and bottom compartments of the gel unit. Equal volumes of INS-1 or islet lysates were loaded into the wells of a 12% polyacrylamide gel. Electrophoresis was performed at 130 V for approximately 1.5 h.

2.10.5 Western blot

Proteins were electro-transferred onto 0.45 μm nitrocellulose membrane by semi-dry transfer at 15 V for 90 min, using either a Hoefer or Fisherbrand semi-dry transfer unit. Transfer of proteins was checked by Ponceau S staining. After removal of Ponceau S by washing in TBS-Tween, membranes were blocked in 5% non-fat milk in TBS-Tween for 45 min. Primary antibody incubations were carried out at 4°C or room temperature overnight, with anti-SPARC (1:1000), and anti-β-tubulin (1:3000) for equal protein loading. Primary antibodies were diluted in a solution of 1% bovine serum albumin (BSA) in TBS-Tween. Non-specific binding was removed by washing in TBS-Tween. Membranes were then incubated in horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2500) for 1-2 h at room temperature. Proteins were visualised with the addition of Supersignal West Pico chemiluminescent substrate using a GeneGnome imaging system with GeneSnap software. Quantitation of band intensity was carried out using GeneTools software (semi-quantitative analysis) (Syngene, 2008).

2.11 ERK and Akt signalling

2.11.1 Experimental procedure and preparation of cell lysates

INS-1 Cells: Cells were plated in 48-well plates at a density of 1.2 x 10^5 cells per well and allowed to attach for 24 h before starving overnight (RPMI 1640 medium containing 0.5% FBS). Cells were treated with SPARC (0.5 – 2.5 μg/ml for 2 h) followed by growth factor (IGF-1 or HGF). Cells were harvested at the indicated time points by first washing in ice-cold PBS and lysing in cold RIPA buffer containing HALT protease and phosphatase inhibitors. SDS sample buffer was added to each sample before heating to 95°C for three min.

Islets: The day after isolation, islets were picked into groups of equal amounts and sizes of islets (intact islets, approximately 130), and cultured for 1 h in RMPI-1640 with 1% FBS. Groups of islets were then treated with SPARC (2.5 or 20 μg/ml) for 1 h, before treatment with HGF or IGF-1 (200 ng/ml) for 7.5 min before lysing in RIPA buffer on ice for 30 min.

Use of dispersed islets: islets were centrifuged and incubated in Accutase at 37°C with gentle pipetting for approximately 10 min. After dispersal, equal volumes of the cell suspension were distributed into tubes to contain 150 islets per treatment. After starvation (1% FBS for 1 h), cells were treated with SPARC (2.5 or 20 μg/ml) for 1 h before HGF treatment (20 ng/ml, 7.5 min). After centrifugation cells were lysed in RIPA buffer. SDS buffer (5X) was added to tubes containing treated islets (lysed intact or dispersed) and boiled for 10 min. Protease and phosphatase inhibitors were added before storing at -80°C.

Proteins were separated by SDS-PAGE as described previously, in section 2.10.4, using a 12% gel. Proteins were transferred to nitrocellulose membrane by western blot as described in section 2.10.5, with the following modifications:

Primary antibody incubations were carried out at 4°C overnight, with anti-phospho-Akt (1:500-1:1000), anti-phospho-p44/p42 MAPK (1:1000) and anti-β-tubulin (1:1000-1:10000). After visualisation, the membrane was stripped using Restore stripping buffer for 3 h at room temperature. The blots were probed for anti-pan-Akt (1:1000) and anti-total p44/p42 MAPK (1:1000) and visualised as described previously in section 2.10.5.

2.12 Cell proliferation assay

The effect of SPARC on the growth of the INS-1 β cell line was investigated using a colorimetric assay with sulphorhodamine B (SRB). INS-1 cells were plated at a density of 5000 cells/well in 96-well plates in culture medium. After attachment (overnight incubation), media was replaced with RPMI containing a reduced amount of FBS (detailed in figure legends). The following day, cells were pre-treated with SPARC (2 h, 2.5 or 20 μg/ml) followed by growth factor (IGF-1 or HGF, concentration detailed in figure legends) and incubated at 37°C for 2 or 4 days. Separate plates of cells were fixed on day 0 (day of treatment), and days 2 and 4 after treatment. Trichloroacetic acid (TCA) was added to the plate to a final concentration of 10% (100 μl per well). After 1 h at 4°C, cells were washed 5 times with tap water and left to air dry. Cells were incubated with 100 μl SRB (0.4% in 1% acetic acid) for 30 min at room temperature. Cells were thoroughly washed with 1% acetic acid and left to air dry. For analysis of protein content, plates were all analysed on the same day. The SRB was solubilised with addition of 10 mM Tris base (pH 10, 100 μl per well) and the absorbance was read at 565 nm on an Epoch Microplate Spectrophotometer (n = 6, unless otherwise specified). To calculate the total protein content, readings taken on days 2 and 4 were divided by the day 0 (untreated cells) mean to give a fold change in absorbance from day 0.

In some instances, experimental results were combined, but to allow for slight differences in amount of cell growth on different days, data was converted to a percentage of the control rate of growth. Each treatment absorbance reading was divided by the reading for the control on the same plate, multiplied by 100 to give percentage, and all data for one treatment were averaged.

2.13 Live/Dead islet assay including SPARC siRNA knockdown

After isolation and overnight recovery, islets from male or female ICR mice were dispersed with accutase (10 min) and seeded onto poly-lysine coated coverslips (see section 2.6 for preparation) in 6-well plates in media without serum to allow attachment (40 min). 30 dispersed islets were used per coverslip. After recovery in media containing 10% serum (2 h) dispersed islets were treated with media containing 10% serum, 0% serum, or 0% serum plus combinations of SPARC (20 μg/ml) and IGF-1 (200 ng/ml). After 2 days coverslips were removed from the plates and fluorescein diacetate (FDA) and propidium iodide (PI) stains were used to assess live/dead status of individual cells using fluorescence microscopy. 3 μl FDA (5 mg/ml in DMSO) and 2 μl PI (2 mg/ml in PBS) were diluted in 1 ml PBS (working concentrations 15 μg/ml and 4 μg/ml, respectively) and mixed thoroughly. Coverslips containing islets were placed in a dish and 100 μl FDA/PI mix was added and left for 3 min in the dark. Fluorescing green (live) and red (dead) cells were viewed on a Nikon Eclipse 80i microscope and images were overlayed in ImageJ software. Results show the mean pooled from 4 fields from 3 separate experiments from a total of 6 mice. Live cells (green) were calculated as a percentage of total cells visible as either green or red using ImageJ software (Rasband, 1997-2012).

For islet co-culture with stellate cells, PS-1 cells had SPARC siRNA or control siRNA treatment the day prior to dispersal and seeding of islets onto coverslips. SPARC siRNA oligonucleotides (ON-TARGETplus Human SPARC siRNA) were first made to a concentration of 150 nM with HiPerFect in media without serum, before addition of 1 x 10^5 PS-1 cells to each tube (final concentration of siRNA, 30 nM). After mixing, PS-1 cells with or without siRNA oligos were plated in 24-well plates and left overnight. For co-culture, PS-1 cells were trypsinised from the plate and seeded into 1 µm-pore PET membrane inserts resting above the dispersed islets in a 6-well plate. The co-culture was left in media without serum (0% media) for 2 days before visualising as before.

After visualisation of the coverslips, knockdown of SPARC protein was confirmed (3 days post-transfection). The media was removed from each of the wells and placed in microfuge tubes. PS-1 cells in the inserts were washed in PBS, and lysed in 50 µl RIPA buffer. Lysates were transferred to microfuge tubes. SDS buffer (5X) was added to tubes containing media or cell lysates and boiled for 10 min.

Proteins were separated by SDS-PAGE as described previously, in section 2.10.4, using a 12% gel. Proteins were transferred to nitrocellulose membrane by western blot as described in section 2.10.5, using anti-SPARC (1:700) and anti-β-tubulin (1:2500) primary antibodies.

2.14 Statistics

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 18 (SPSS UK Ltd, Woking, UK). Statistical difference was assessed by either the unpaired two-tailed

Student's t test, or analysis of variance (ANOVA) with the least significant difference post-hoc analysis, where appropriate. A p value ≤ 0.05 was considered significant.

CHAPTER 3: Results

3.1 Confirmation of INS-1 as a β cell line

The INS-1 cell line was used in this study to determine β cell responses during *in vitro* experimentation. It is derived from an insulinoma and has a higher mitotic index than other β cell lines and therefore is suitable for testing proliferation. In order to confirm that the INS-1 cell line used produced insulin, the cells were fixed onto coverslips and immunostained with an anti-insulin antibody, followed by either fluorescent labelling or the DAB technique to produce an area of brown stain where insulin was present. Presence of insulin in these cells was determined by the appearance of brown DAB staining that was absent in secondary-only controls (Figure 3.1A compared to 3.1B), and also the presence of fluorescent red staining in the images of the cell line (Figure 3.1 C,D) as well as in islet areas in control mouse pancreas sections (Figure 3.1 E,F). In fixed cells and sections that were immunostained with the secondary Texas red antibody only, no fluorescent red stain was detected, indicating the specificity of secondary antibody to the insulin primary (shown in appendix figure A2.1). In INS-1 cells that were positive for insulin, expression was spread evenly throughout the cytoplasm, and as shown on images depicting the red insulin channel only, insulin was not detected in the nucleus (Figure 3.1 D). As shown in Figure 3.1, while insulin was not detected in every INS-1 cell using fluorescence microscopy, it was detected in all cells using DAB staining, although fainter in some than others.

In ten week old B6 mouse control pancreas, fluorescently labelled insulin is restricted to only few areas within the section, determined as islets due to their shape, the fact that the insulin stain is not present in the nuclei, and also that insulin is detected throughout the islet although not in every single cell, as would be expected due to the multiple cell

types present in islets (Figure 3.1 E,F). Secondary-only controls did not show any red staining in sections (appendix figure A2.1).

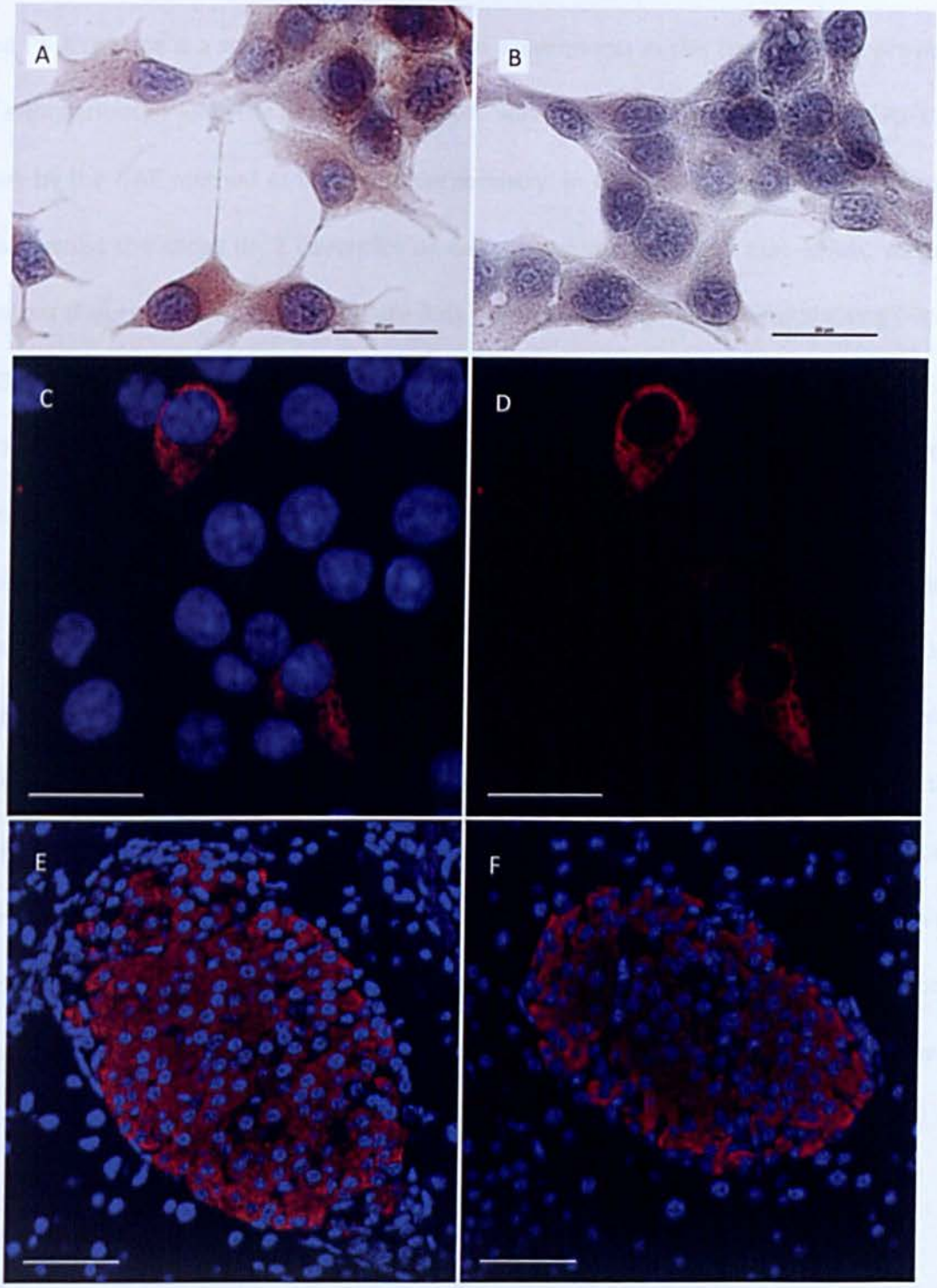


Figure 3.1. Expression of insulin in INS-1 cells and islets. INS-1 cells fixed on coverslips (**A-D**) or control B6 pancreas sections (5 µm; **E,F**) were immunostained for insulin. **A**: Visualised with a biotin-conjugated secondary antibody and the ABC-DAB method. **B:** Secondary only control. **C-F**: Visualised with a Texas red-conjugated secondary antibody. SlowFade plus DAPI was used to mount slides and stain nuclei. **D** shows red channel only (Texas red). Slides were imaged on a confocal microscope. Representative of multiple images taken from 2 slides. Scale bar: 20 µm for INS-1 cells; 50 µm for pancreas sections.

3.2 Expression of SPARC

3.2.1 SPARC and FSP-1 are expressed in PS-1 cells, but not INS-1 cells

The PS-1 cell line is a stellate cell line, used in experiments in this thesis for the provision of endogenous SPARC. The presence of SPARC was determined in both INS-1 and PS-1 cell lines by the DAB method of immunocytochemistry. In INS-1 cells, the absence of brown stain across the slides (in 2 coverslips of cells examined) indicated that SPARC was not present (Figure 3.2 A,C). The secondary-only control was also negative for staining (Figure 3.2E). In PS-1 cells, brown staining was found indicating the presence of SPARC (Figure 3.2 B,D). Staining was weak using this method, however the clear difference in the negative secondary-only control by comparison (Figure 3.2F) indicates that SPARC is present in PS-1 cells. A diffuse SPARC staining could be detected throughout the cytoplasm. In addition, occasional 'spot-like' areas of staining were sometimes observed, that were not detected in the control (Figure 3.2F). Using the method here it was not possible to determine whether SPARC was present in the nucleus. There were 'spot-like' areas of staining in the regions of the nucleus, however since the image is not a single plane but a projection from all levels of the cell, staining that may appear in the nucleus could be due to staining in the cytoplasm behind or in front of it. Immunofluorescence techniques and confocal microscope analysis were attempted, in both cell lines and pancreas sections, however the method was unsuccessful.

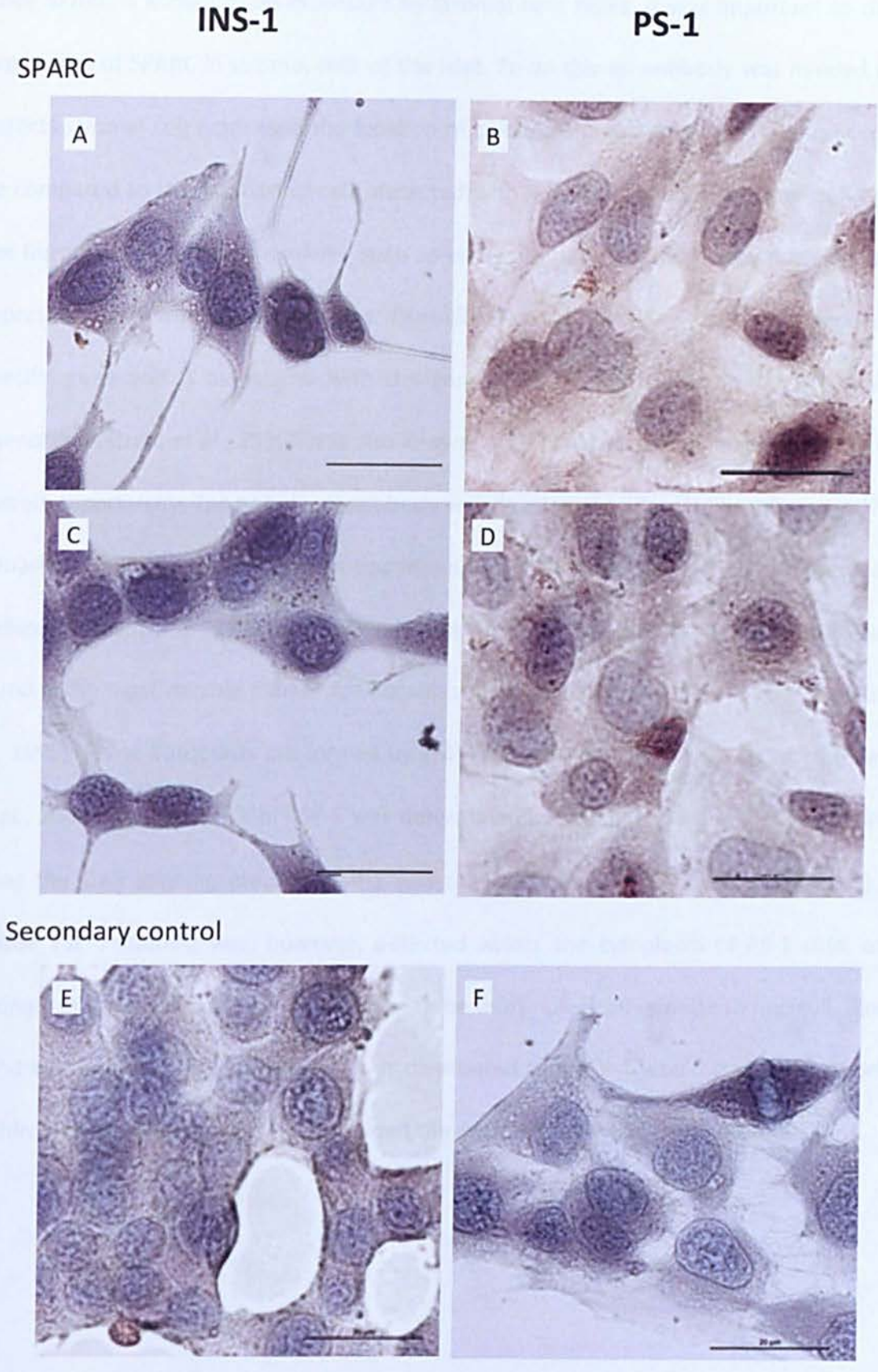


Figure 3.2. SPARC is expressed by PS-1 cells. Fixed INS-1 (**A,C,E**) and PS-1 (**B,D,F**) cells were immunostained for SPARC with biotin-conjugated secondary antibody (**A-D**), or secondary only antibody, as a control (**E,F**). Positive areas was visualised using the ABC/DAB method producing a brown colour. Representative of multiple images taken of cells from 2 coverslips. Bar = 20 μm.

Since SPARC is known to be expressed by stromal cells types, it was important to check expression of SPARC in stromal cells of the islet. To do this an antibody was needed that detects stromal cell types, and the location of cell types detected by this antibody could be compared to the location of cells detected with a SPARC antibody. Stromal cell types like fibroblasts have many markers, such as vimentin, however these may not always be expressed by fibroblasts at all times. Fibroblast specific protein (FSP-1) is a fibroblast specific gene and is associated with the conversion of epithelial cells to a fibroblast phenotype (Strutz *et al.*, 1995). It is also known as S100A4 protein, belonging to the S100 protein superfamily. The polyclonal antibody was developed to detect fibroblasts, and it is a marker of fibrogenesis and cells undergoing EMT (Rossini *et al.*, 2005, Zeisberg and Neilson, 2009, Iwano *et al.*, 2002). This marker has been used by other researchers who found it the most reliable marker for fibroblast phenotypes (Iwano *et al.*, 2002, Strutz *et al.*, 1995). Some fibroblasts are formed by EMT, and express FSP-1 while doing so (Iwano *et al.*, 2002). The presence of FSP-1 was determined in both the INS-1 and PS-1 cell lines using the DAB staining method. FSP-1 was not detected in INS-1 cells (Figure 3.3). A diffuse FSP-1 staining was, however, detected across the cytoplasm of PS-1 cells, and strongly in areas of the nuclei that appear to be morphologically similar to nucleoli. Since FSP-1 was expressed by PS-1 cells, it was considered that FSP-1 would stain stromal cells within islets of pancreas sections, provided the antibody worked in both methods.

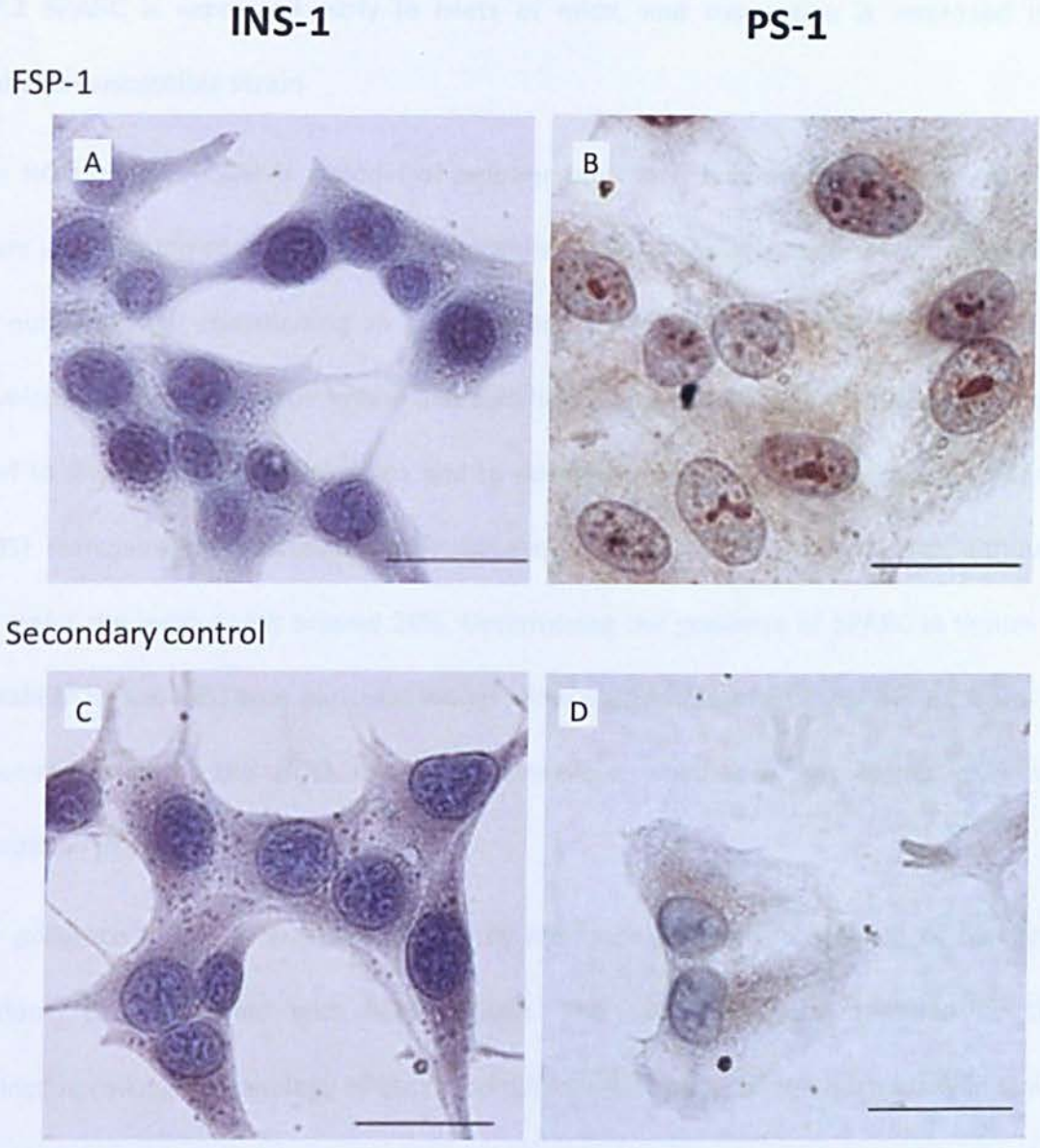


Figure 3.3. FSP-1 is expressed by PS-1 cells. Fixed INS-1 (**A,C**) and PS-1 (**B,D**) cells were immunostained for FSP-1 with biotin-conjugated secondary antibody (**A,B**) or secondary only antibody, as a control (**C,D**). Positive areas was visualised using the ABC/DAB method producing a brown colour. Representative of multiple images taken of cells from 2 coverslips. Bar = 20 µm.

3.2.2 SPARC is expressed early in islets of mice, and expression is increased in a diabetes-susceptible strain

The NOD mouse model is a model of autoimmunity that was developed more than 30 years ago (Makino *et al.*, 1980). It has polygenic inheritance of diabetes, with more than 20 non-MHC loci contributing to the diabetic susceptibility (Ikegami *et al.*, 2004). It develops spontaneous autoimmune diabetes that is similar to type 1 diabetes and widely used to study the onset of diabetes and to develop therapies (Anderson and Bluestone, 2005). Females spontaneously develop diabetes in approximately 80% of cases, although for males the incidence is around 20%. Determining the presence of SPARC in tissues of female NOD and wild type pancreas would allow elucidation of whether SPARC is up- or down-regulated in the NOD strain and therefore whether it has higher or lower expression in a pre-diabetic state.

The presence of SPARC was determined by immunohistochemical staining of pancreas sections, counterstained with haematoxylin. The islet area was identified by the distinctive cellular morphology of islets and differential uptake of the haematoxylin stain. Islets have a roughly circular or oval shape that is lighter in appearance compared to the darkly stained surrounding acinar tissue, which takes up more of the haematoxylin stain. The acinar cells constantly secrete digestive enzymes and the abundant RNA in the endoplasmic reticulum stains with haematoxylin as well as the nucleus.

The SPARC-stained area was quantified with ImageJ analysis, using the thresholding tool to create a binary image from which the stained area could be calculated. Only staining within islets was measured. Staining in the acinar tissue, peri-islet ductal and vascular areas as well as in areas of insulitis within the islets was excluded. Insulitis was identified as regions of concentrated nuclear staining (due to infiltrating lymphocytes) adjacent to

the islet, as pictured in Figure 3.4A (image h). The SPARC-stained regions were calculated as a percentage of the total islet area. Secondary-only controls were performed to check antibody specificity (shown in Appendix Figure A2.2).

Immunostaining for SPARC in pancreas sections of mice revealed that SPARC was present in the islets of both B6 wild type and NOD pancreas (Figure 3.4 A). In B6 control mice, the area of SPARC staining within islets was greatest at 4 weeks of age (Figure 3.4 B), and by 12 weeks of age, the SPARC area had significantly decreased ($p = 0.012$, $n = 6$), and very few SPARC-positive cells were observed. SPARC is present around the basement membrane of islets as well as in scattered cells within the islet, and many of the SPARC-positive cells had an elongated shape. The overall staining pattern is very different to that observed with an insulin antibody (see above), suggesting that SPARC is not expressed in the β cells of the islet.

As shown in Figure 3.4 B, there is a noticeable difference in SPARC staining between the two strains of mice. The SPARC-stained area within islets is greater in NOD mice compared to age-matched control islets, at both 4 and 12 weeks of age ($p = 0.008$ at 4 weeks, $p = 0.001$ at 12 weeks, $n = 6$). This increase in NOD islets occurs both within the islet, and in peri-islet membrane areas.

As already mentioned, the SPARC stained area decreased in B6 control islets from 4 to 12 weeks of age, and a small decrease in mean SPARC staining was also observed in NOD islets from 4 weeks to 12 weeks of age, although this was not statistically significant. SPARC staining is therefore greater within the islets of diabetes-susceptible NOD mice than in resistant B6 mice, and the reduction in expression with age is less pronounced.

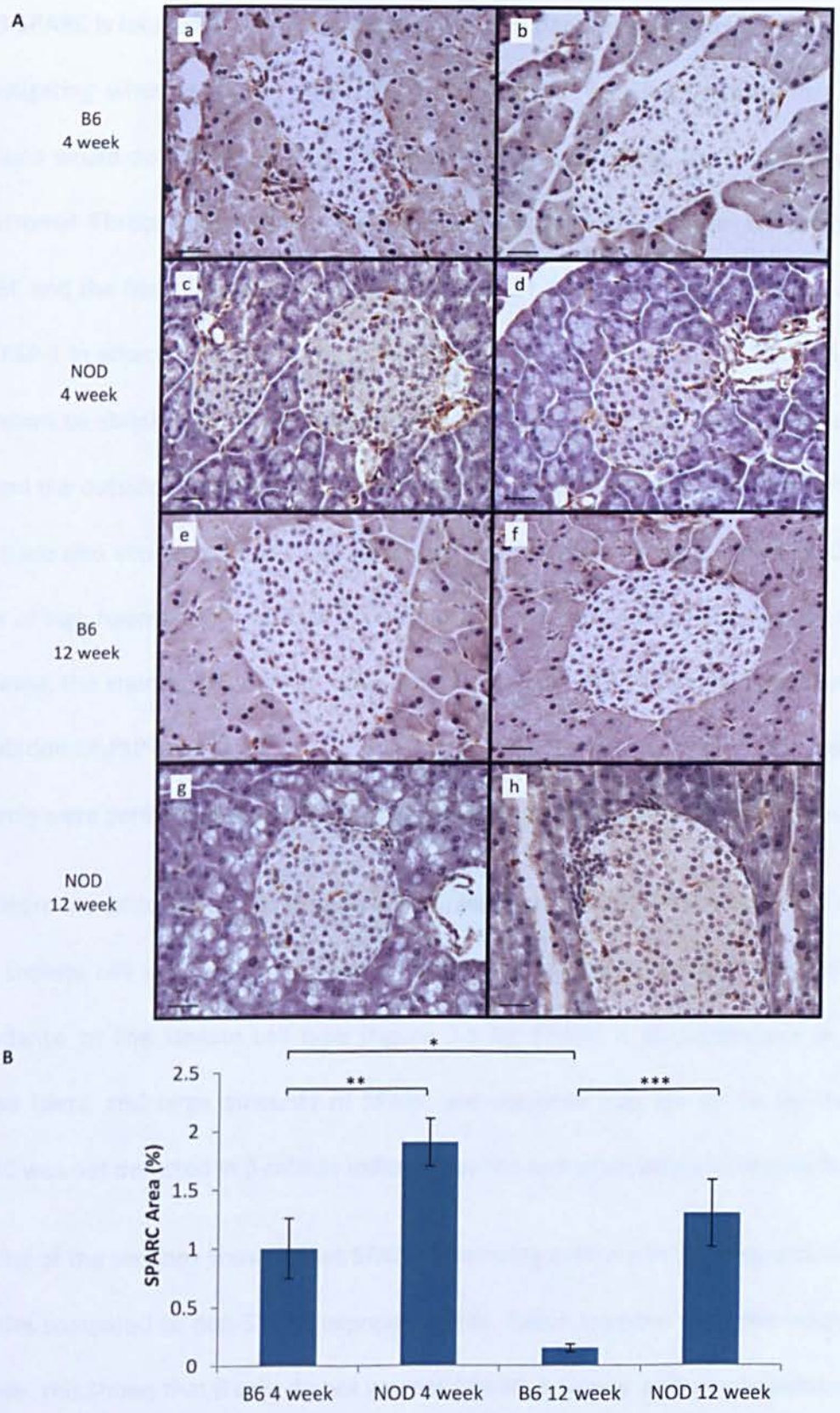


Figure 3.4. Higher expression of SPARC in NOD islets than control islets. Sections (5 µm) of mouse pancreas were subjected to treatment with anti-SPARC (1 in 25) antibody followed by biotin-conjugated secondary antibody. Positive areas were visualised using the ABC/DAB method producing a brown colour. Representative images shown of sections from at least 3 different mice. **A.** SPARC staining in 4 and 12 week old pancreas from NOD or control mice. SPARC is present in basement membranes and scattered throughout islets, and is more abundant in NOD pancreas. Scale bar = 20 µm. **B.** ImageJ analysis of SPARC stained islet areas represented as a percentage of total islet area within each section. Areas of islet infiltration as well as peri-ductal and peri-vascular staining were excluded from the analysis. Bars represent mean ± SEM; n = 6, from 3 individual mice of each type. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

3.2.3 SPARC is located in the same areas as FSP-1 in pancreas sections

Investigating whether SPARC and FSP-1 exhibit similar staining patterns in pancreatic sections would determine whether the primary source of SPARC within islets is likely to be stromal fibroblast-like cells. Adjacent 5 μm sections of pancreas were stained for SPARC and the fibroblast specific protein marker, FSP-1 in islets. The expression of SPARC and FSP-1 in adjacent islet sections of NOD mice indicate similarities of SPARC and FSP-1 as shown by staining in the same areas of the islet (Figure 3.5 A). This is seen especially around the outside of the islet, where the peri-islet basement membrane lies. SPARC and FSP-1 are also abundant in areas of inflammation, as shown in the 12 week NOD section (area of high haematoxylin nuclear staining, due to invading cells of the immune system). However, the staining with SPARC does not fully overlap FSP-1 staining, suggesting that a population of FSP-1 negative cells within islets may also express SPARC. Secondary-only controls were performed to confirm antibody specificity (shown in Appendix Figure A2.3).

To determine which cell types secrete SPARC, western blot analysis of the INS-1 cell line, PS-1 stellate cell line and islets revealed that SPARC is expressed and secreted in high abundance by the stellate cell type (Figure 3.5 B). SPARC is also expressed in cells of mouse islets, and large amounts of SPARC are secreted into the media by the islets. SPARC was not detected in β cells as indicated by the lack of an immunoreactive band.

Staining of the sections showed that SPARC expressing cells are in low proportions within the islet compared to non-SPARC expressing cells. Taken together with the western blot analysis, this shows that β cells do not express SPARC, however a small population of cells within the islets do, which include stromal cell types.

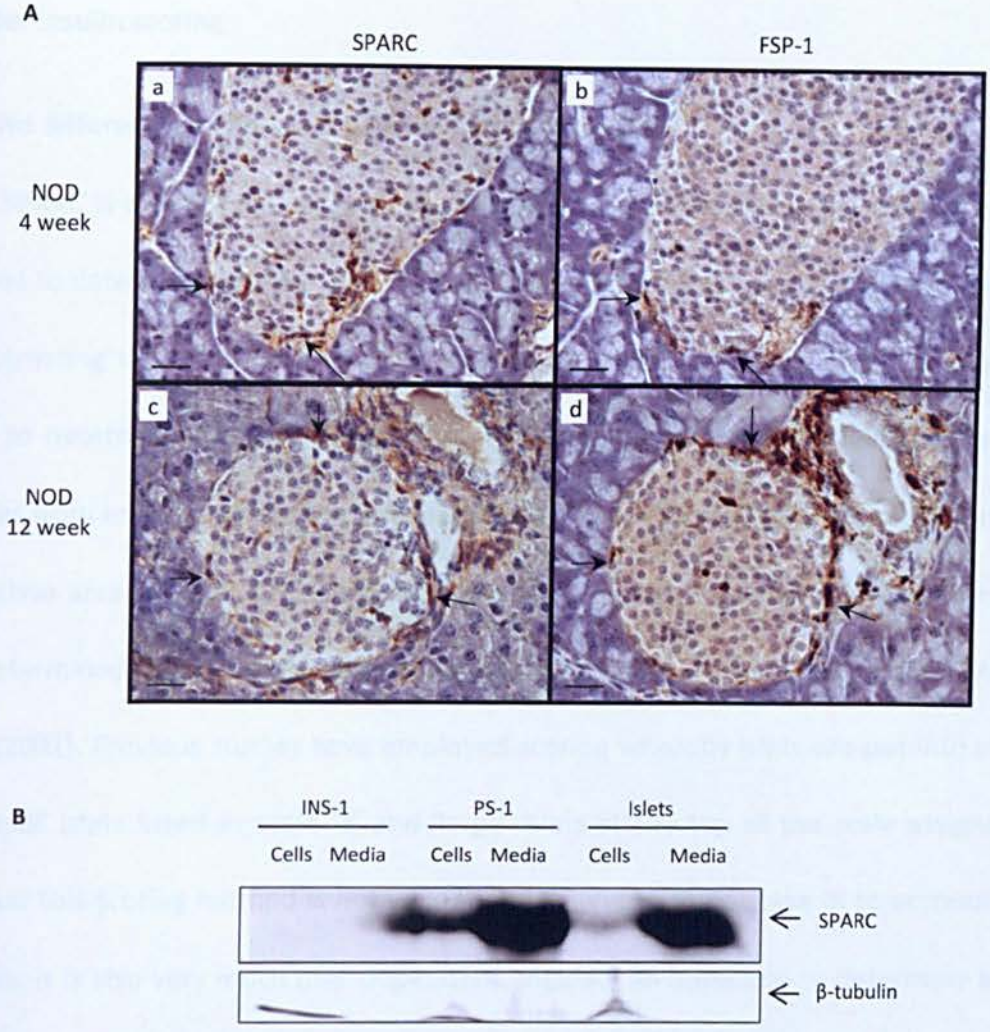


Figure 3.5. SPARC is strongly expressed by stellate cells as well as a minority cell population in islets. A. Sections (5 μm) of mouse pancreas were subjected to treatment with anti-SPARC (1 in 25) or anti-FSP-1 (1 in 100) antibody followed by biotin-conjugated secondary antibody. Positive areas were visualised using the ABC/DAB method producing a brown colour. Representative images shown of sections from at least 3 different mice. SPARC (left panels) and FSP-1 (right panels) staining in 4 and 12 week adjacent NOD sections. SPARC and FSP-1 show similarities in areas expressed (arrowheads). Scale bar = 20 μm. **B.** Media from INS-1 and PS-1 cells, and intact islets (groups of 50 islets) were analysed by western blot and probed with antibodies to SPARC and β-tubulin. Since β-tubulin is only present within cells, it is not found present in the media from the cell culture.

3.3 Islet insulin scoring

3.3.1 No difference in islet area in SPARC knockout mice

Since SPARC is an extracellular protein and expressed in basement membranes, and the aim was to determine whether SPARC has an influence on β cell and islet growth, it would be interesting to gauge whether the presence of SPARC influences islet size *in vivo*. In order to determine whether SPARC had any effect on islet area, SPARC-null pancreas samples from mice were collected and sectioned along with control samples (littermates). To analyse area of islets (area of β cells) and pancreas sections, the insulin stained area was determined along with the area of section, similar to a study done by Bernal-Mizrachi *et al.* (2001). Previous studies have employed scoring whereby islets are put into classes, i.e. 'small' islets listed as scale '1' and 'large' islets at the top of the scale assigned '5'. However this scoring method is not very accurate, and does not take in to account small changes. It is also very much user dependent. Instead, an approach to determine area in μm^2 was adopted, for both the β cell area and the pancreas section area.

Sections of pancreas were picked with enough distance between sections (250 μm) to avoid analysing two sections of the same islet. Six sections across each pancreas were imaged in order to rule out any apparent anomalies that may have resulted from only imaging one section per animal. Size and distribution of islets vary throughout the pancreas. Islet areas are larger in the tail than the head, and more islets are present in the head (Saito *et al.*, 1978). Upon obtaining the fixed pancreas samples, it was unclear which were head and which were tail ends of the pancreas, therefore it was best to average any results found across all pancreas sections from one animal. Entire sections were pieced together from individual images in Dia software (Figure 3.6). Overlapping areas in the images were used to accurately reconstruct the section.

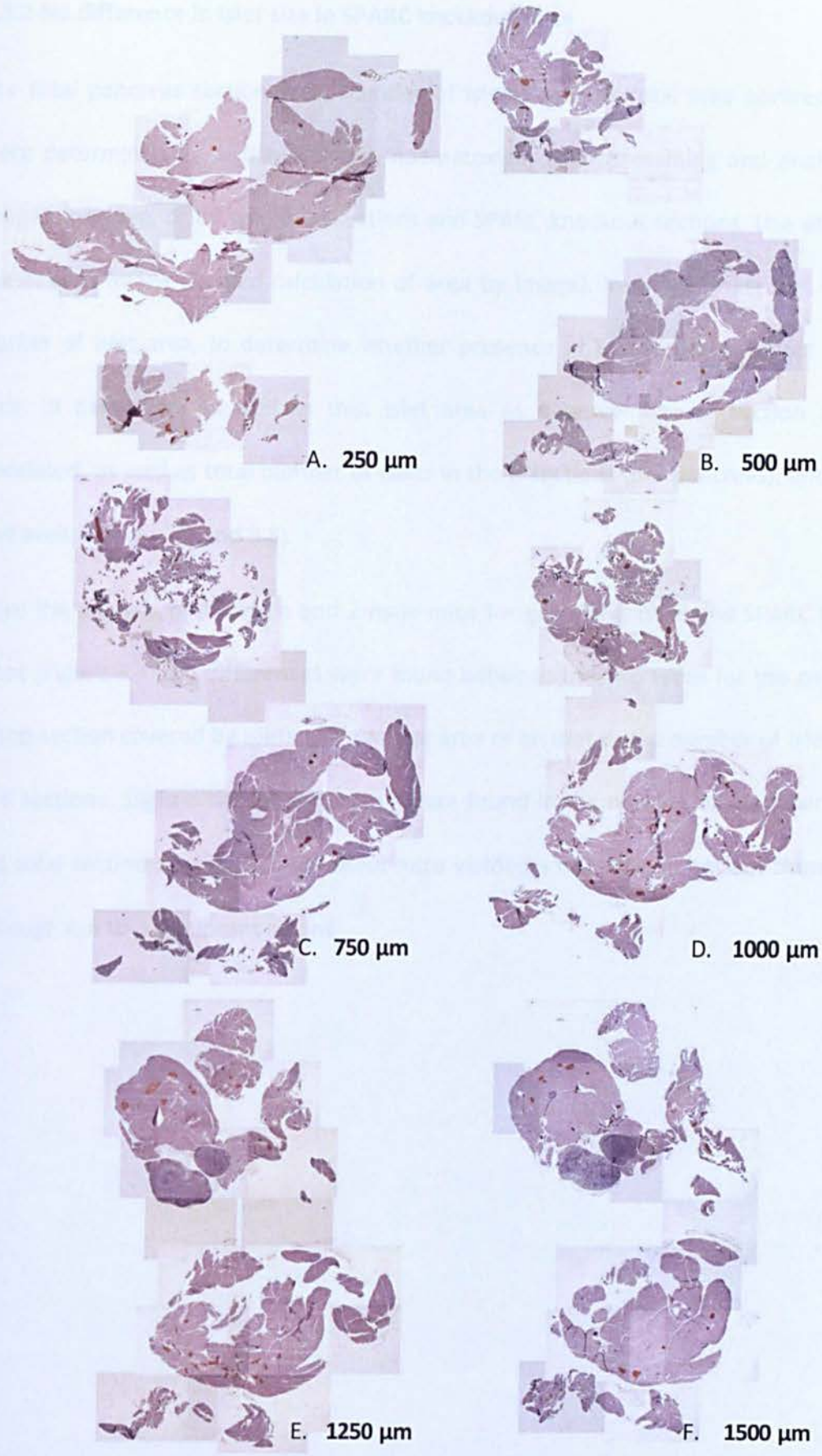


Figure 3.6. Example of section images from one B6 (wild type) mouse pancreas. Images of insulin-immunostained sections were pieced together in Dia software. 6 sections were imaged per pancreas, 250 µm apart. First section is taken 250 µm from edge of embedded pancreas and subsequent numbering in µm specifies distance through the pancreas.

3.3.2 No difference in islet size in SPARC knockout mice

The total pancreas section area, number of islets, and the total area covered by islets were determined by insulin staining, haematoxylin counter-staining and analysis using ImageJ software, of B6 wild type sections and SPARC knockout sections. Use of the scale bar in each image allowed calculation of area by ImageJ. Insulin staining was used as a marker of islet area, to determine whether presence of SPARC has an effect on β cell area, in particular. As well as this, islet area as a percentage of section area was calculated, as well as total number of islets in the 6 sections (per pancreas), and average islet area (Figures 3.7 and 3.8).

From the analysis of 3 female and 2 male mice for each of control and SPARC knockout types (Figure 3.7), no differences were found between the two types for the percentage of the section covered by islets, the average area of an islet or the number of islets across all 6 sections. Slight differences in mean were found in the number of islets per section, and total section area. SPARC knockout mice yielded a decrease in both of these factors although not to a significant extent.

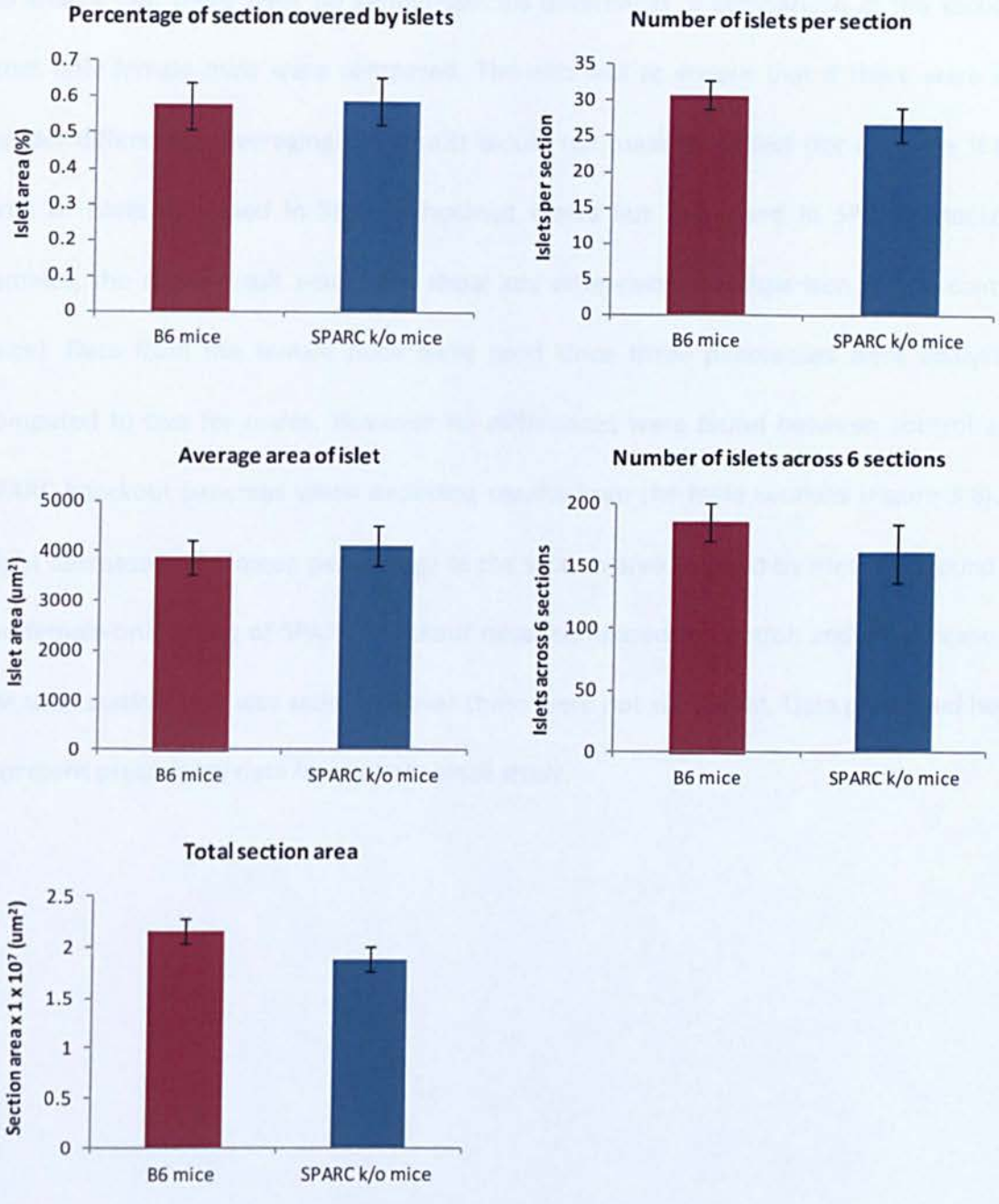


Figure 3.7. SPARC knockout mice show no differences in islet area to wild type mice. Control (labelled B6) and SPARC knockout mouse pancreas sections were immunostained for insulin and imaged. Analysis of insulin stained areas, and total section areas were used to calculate islet area as percentage of section, average islet numbers, average islet area, total number of islets per pancreas (in 6 sections), and total section area. Bars represent mean ± SEM of a total of 30 sections (6 sections from 5 pancreases). Two male and three female mice were used per type (control or SPARC knockout).

To ensure that there were no gender-specific differences, a comparison of the sections from only female mice were compared. The aim was to ensure that if there were any gender differences, averaging the results would not mask the effect (for example, if the area of islets increased in SPARC knockout males but decreased in SPARC knockout females, the mean result would not show any difference in comparison to the control mice). Data from the female mice were used since three pancreases were analysed, compared to two for males. However no differences were found between control and SPARC knockout pancreas when excluding results from the male sections (Figure 3.8). A slight decrease in the mean percentage of the section area covered by islets was found in the female-only group of SPARC knockout mice, compared to control, and an increase in the total section area was seen, however these were not significant. Data presented here represent preliminary data from a very small study.

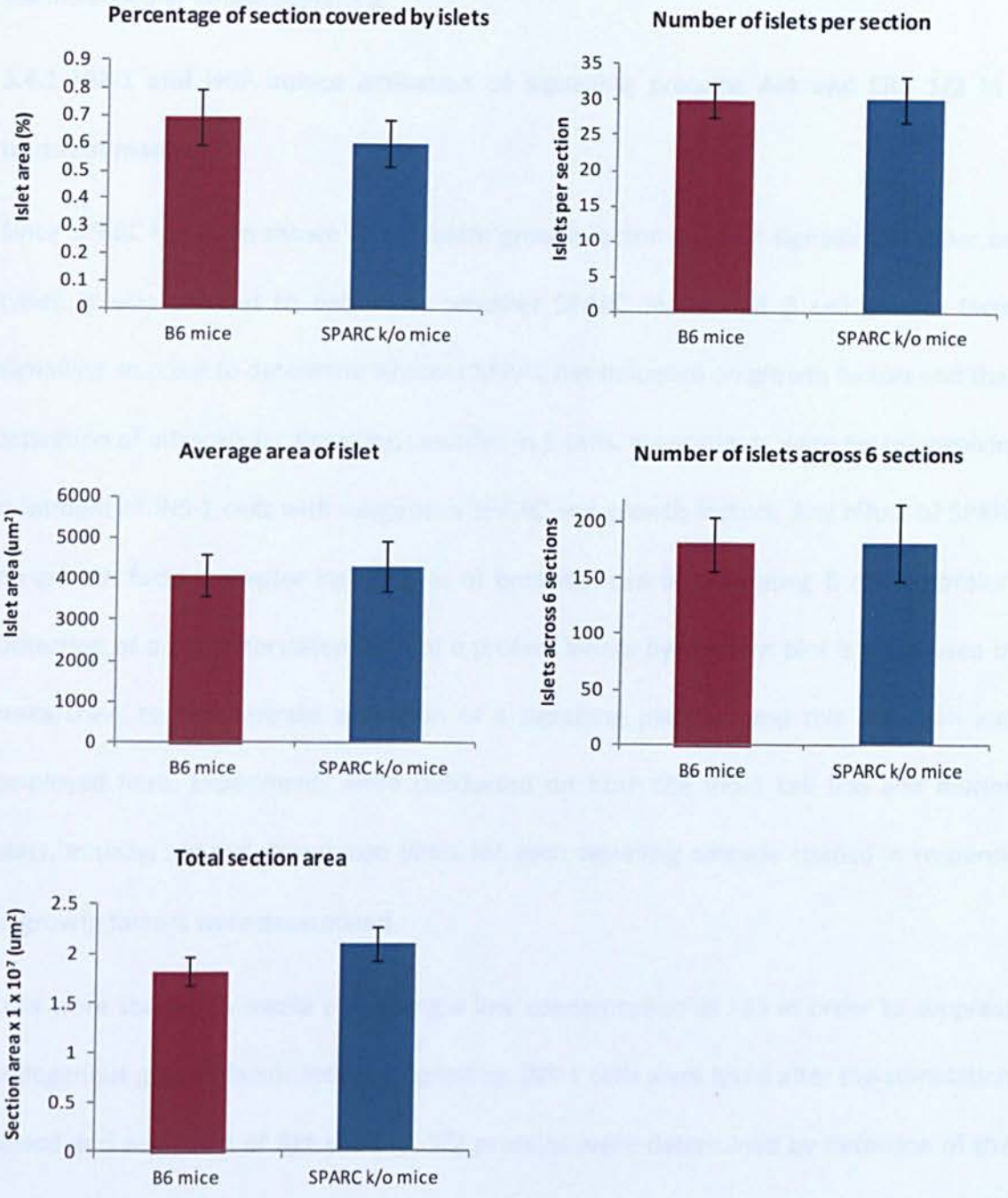


Figure 3.8. In females, SPARC knockout mice show no differences in islet area to wild type mice. Female control (labelled B6) and SPARC knockout mouse pancreas sections were immunostained for insulin and imaged. Analysis of insulin stained areas, and total section areas were used to calculate islet area as percentage of section, average islet numbers, average islet area, total number of islets per pancreas (in 6 sections), and total section area. Bars represent mean ± SEM of a total of 18 sections (6 sections from 3 pancreases). Three female mice were used per type (control or SPARC knockout).

3.4 Intracellular kinase signalling

3.4.1 IGF-1 and HGF induce activation of signalling proteins Akt and ERK 1/2 in a transient manner

Since SPARC has been shown to modulate growth factor receptor signalling in other cell types, it was decided to determine whether SPARC modulated β cell growth factor signalling. In order to determine whether SPARC has influence on growth factors and their activation of intracellular signalling cascades in β cells, experiments were set up involving treatment of INS-1 cells with exogenous SPARC and growth factors. Any effect of SPARC on growth factor receptor signalling is of potential use in promoting β cell expansion. Detection of a phosphorylated form of a protein kinase by western blot is often used by researchers to demonstrate activation of a signalling pathway and this approach was employed here. Experiments were conducted on both the INS-1 cell line and murine islets. Initially, optimal stimulation times for each signalling cascade studied in response to growth factors were determined.

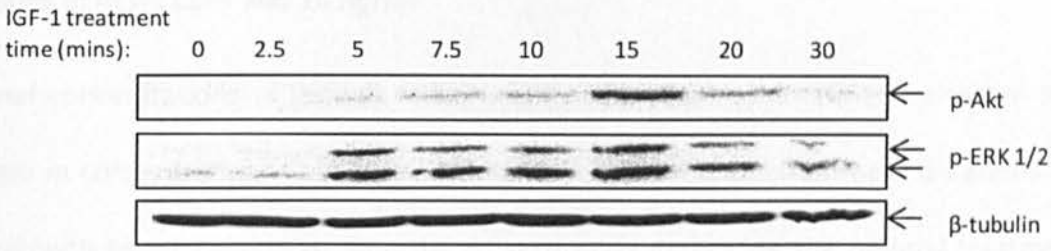
Cells were starved in media containing a low concentration of FBS in order to suppress endogenous growth factor-induced signalling. INS-1 cells were lysed after the stimulation period and activation of Akt and ERK 1/2 proteins were determined by detection of the phosphorylated forms by appropriate antibodies using western blot techniques.

Optimal stimulation times for kinase activation were determined by treatment with 20 ng/ml IGF-1 or HGF for varying times between 2.5 and 30 min before lysis. Basal levels are indicated in the left lane of the blot. IGF-1 upregulated Akt phosphorylation, which was first noticeable after 7.5 min and a large increase was present 15 min after IGF-1 treatment (Figure 3.9 A). Phosphorylation was transient, and had almost returned to basal levels after 30 min stimulation. Relative ERK phosphorylation after IGF-1 incubation

was very similar between the two ERK isoforms, and showed transient phosphorylation of which the maximal phosphorylation time varied between experiments but was found to be between 5 and 15 min. Phosphorylation began to return to basal levels at 30 min.

Similarly, after HGF treatment, phosphorylation of the ERK 1 and ERK 2 isoforms were upregulated at early time points. Stimulation of ERK 1/2 phosphorylation was very quick, with strong immunoreactivity evident at 2.5 min (Figure 3.9 B). Activation of ERK 1/2 remained strong between 2.5 and 7.5 min but declined toward basal levels after 10 min treatment. HGF treatment did not consistently increase the phosphorylation level of Akt from basal levels.

A



B

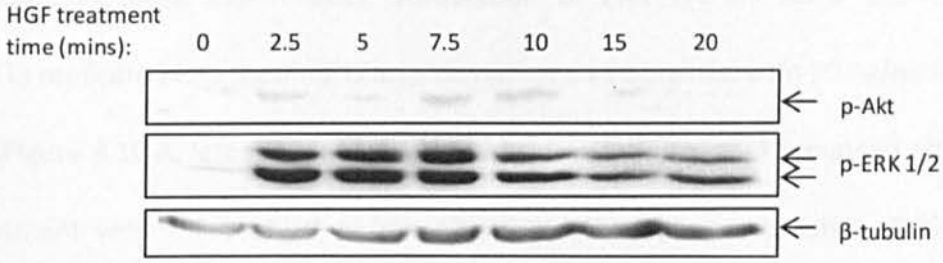


Figure 3.9. Stimulation of intracellular signalling by growth factors. INS-1 cells were plated at 1.2 x 10^5 cells/well in 48-well plates, cells were starved overnight prior to treatment with 20 ng/ml growth factor for the times indicated before lysis. Samples were run on a 10% polyacrylamide gel before western blot transfer. Blots were incubated with primary antibodies raised against phosphorylated Akt, phosphorylated ERK 1/2 and β-tubulin. Results are representative of 2 separate experiments. Band intensities were standardised to β-tubulin band intensities and the control. **A.** Phosphorylated Akt and ERK 1/2 after IGF-1 treatment. **B.** Phosphorylated Akt and ERK 1/2 after HGF treatment.

3.4.2. The optimal concentration of growth factor for stimulation of ERK and Akt signalling in INS-1 Cells was 20 ng/ml

Optimal concentrations of growth factor were determined by treatment with ten-fold changes in concentrations (2, 20 and 200 ng/ml). Two different treatment durations for each growth factor were used, in order to accurately determine the optimal treatment combination. In these experiments, stimulation of ERK 1/2 by IGF-1 was minimal, however in replicate blots maximal phosphorylation was obtained with 20 ng/ml IGF-1 for 7.5 min (Figure 3.10 A, left side of blot). Akt phosphorylation was pronounced after a 7.5 min treatment with IGF-1 at 20 ng/ml. Although a higher concentration (200 ng/ml) increased the band intensity at 7.5 min, and the period of stimulation (a strong band is detected at 15 min, right hand side of blot), 20 ng/ml was selected for subsequent experiments in order to detect any increases or decreases in Akt phosphorylation with the addition of SPARC. If the stimulation is too strong, SPARC effects (either increasing or decreasing amount of phosphorylation) may not be seen.

For treatment with HGF, since phosphorylation of ERK 1/2 appeared early, the two time periods of 2.5 and 7.5 min were chosen for treatment. For all combinations of treatment time and concentration of HGF used, there was very little change in Akt phosphorylation when compared to the basal rate in unstimulated (0 min) cells, consistent with the previous experiment (Figure 3.10 B). Conversely, phosphorylation of ERK 1/2 was strong, the greatest changes occurring after 7.5 min treatment (right hand side of blot). As with IGF-1, 20 ng/ml HGF was decided sufficient for subsequent experiments.

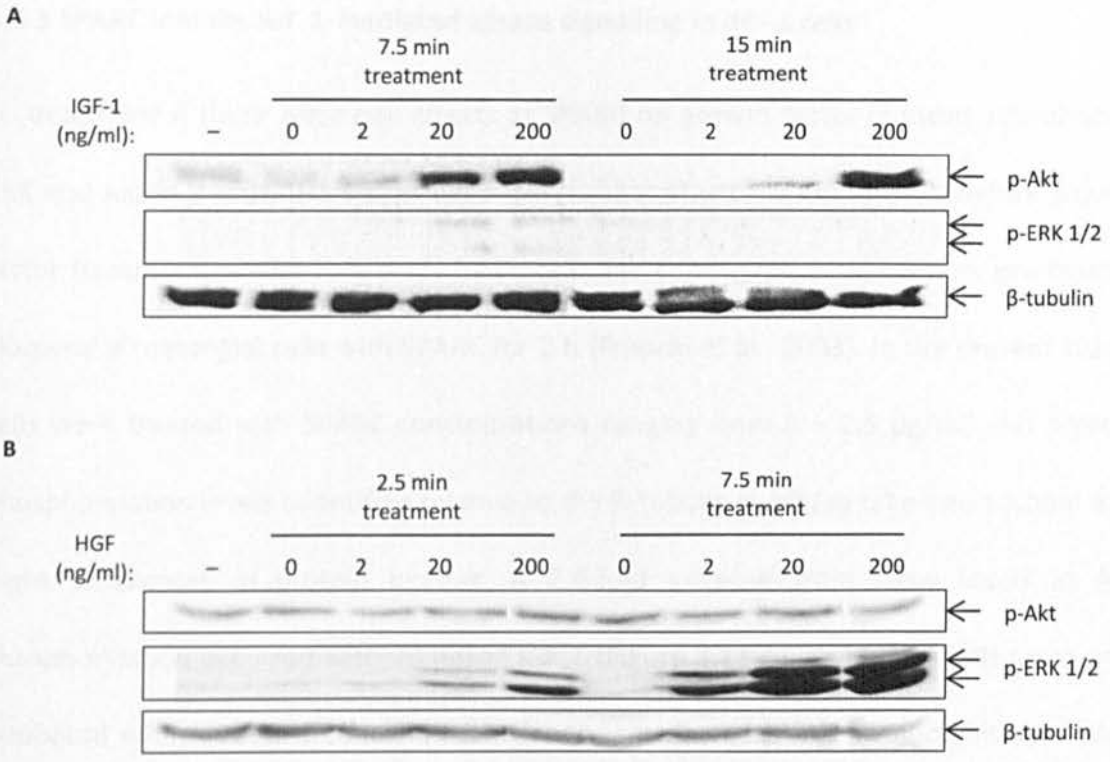


Figure 3.10. Optimisation of treatment times and concentration of growth factors. INS-1 cells were plated at 1.2 x 10^5 cells/well in 48-well plates, cells were starved overnight prior to treatment with growth factor (2, 20 or 200 ng/ml) for either 2.5, 7.5 or 15 minutes before lysis. Samples were run on a 10% polyacrylamide gel before western blot transfer. Blots were incubated with primary antibodies raised against phosphorylated Akt, phosphorylated ERK 1/2 and β-tubulin. Results are representative of 2 separate experiments. **A.** Phosphorylated Akt and ERK 1/2 after IGF-1 treatment. **B.** Phosphorylated Akt and ERK 1/2 after HGF treatment.

3.4.3 SPARC inhibits IGF-1-mediated kinase signalling in INS-1 cells

To determine if there were any effects of SPARC on growth factor-induced activation of ERK and Akt in β cells, INS-1 cells were pre-incubated with SPARC for 2 h before growth factor treatment, similar to a study by Francki and colleagues, in which they pre-treated glomerular mesangial cells with SPARC for 2 h (Francki *et al.*, 2003). In the present study, cells were treated with SPARC concentrations ranging from 0 – 2.5 μg/ml, and protein phosphorylation levels quantified relative to the β-tubulin levels, to take into account any slight differences in protein loading. A 2.6-fold increase from basal levels in Akt phosphorylation occurred with 20 ng/ml IGF-1 (Figure 3.11 A), and when cells were pre-incubated with SPARC, a concentration-dependent decrease in phosphorylation of Akt was observed. At 0.1 μg/ml SPARC no inhibition of IGF-1 stimulated Akt phosphorylation was observed, but a progressive inhibition of IGF-1-induced Akt phosphorylation was seen with increasing concentrations of SPARC. The 2.6-fold increase in Akt phosphorylation with IGF-1 was almost completely attenuated at the highest SPARC concentration used.

ERK 1/2 protein phosphorylation levels were quantified relative to the β-tubulin levels. IGF-1 induced a 2.5-fold increase in ERK activation (Figure 3.11 B), and pre-treatment with SPARC before IGF-1 exposure resulted in inhibition of activation. At the highest concentration of SPARC (2.5 μg/ml), ERK signalling was reduced to below that of basal levels, a 0.9-fold change.

In both blots, addition of SPARC alone to INS-1 cells resulted in levels of phosphorylated forms of Akt and ERK 1/2 being comparable to that found in unstimulated cells at all SPARC concentrations.

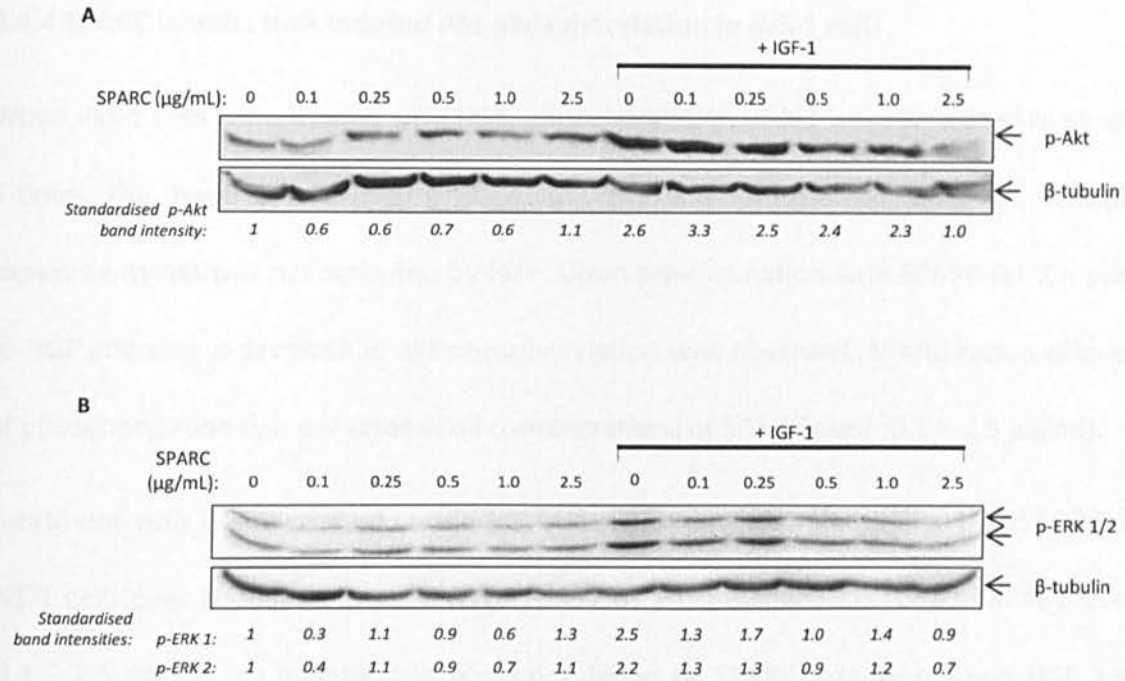


Figure 3.11. Reduction of IGF-1 induced INS-1 signalling by SPARC. Western blot analysis of INS-1 cell lysates after SPARC and IGF-1 treatment (20 ng/ml; 7.5 minutes). Each blot is representative of 3 independent experiments. Band intensities were standardised to β-tubulin band intensities and the control. p-Akt, p-ERK 1 and p-ERK 2 standardisation shown. **A.** Concentration-dependent decrease in IGF-1-induced phosphorylated Akt after SPARC treatment. **B.** Decrease in IGF-1-induced phosphorylated ERK 1/2 after SPARC treatment.

3.4.4 SPARC inhibits HGF-induced Akt phosphorylation in INS-1 cells

When INS-1 cells were treated with HGF, phosphorylation of Akt was stimulated to almost 3-times the basal levels (Figure 3.12 A). This was unexpected since in previous experiments Akt was not activated by HGF. Upon pre-incubation with SPARC for 2 h prior to HGF addition, a decrease in Akt phosphorylation was observed. SPARC reduced levels of phosphorylation to basal rates at all concentrations of SPARC used (0.1 – 2.5 μg/ml).

Treatment with HGF increased considerably levels of phosphorylated ERK 1 and ERK 2 in INS-1 cells over 20-fold from basal (Figure 3.12 B). At the concentrations of SPARC used (0.1 – 2.5 μg/ml), no inhibition of phosphorylation by SPARC was seen when HGF and SPARC were used in combination. As before, SPARC did not affect levels of Akt or ERK 1/2 when used alone.

These findings in INS-1 cells demonstrate the inhibitory action of SPARC on β cell signalling. SPARC inhibits IGF-1-induced Akt and ERK 1/2 phosphorylation, and inhibits HGF-induced Akt phosphorylation in cells.

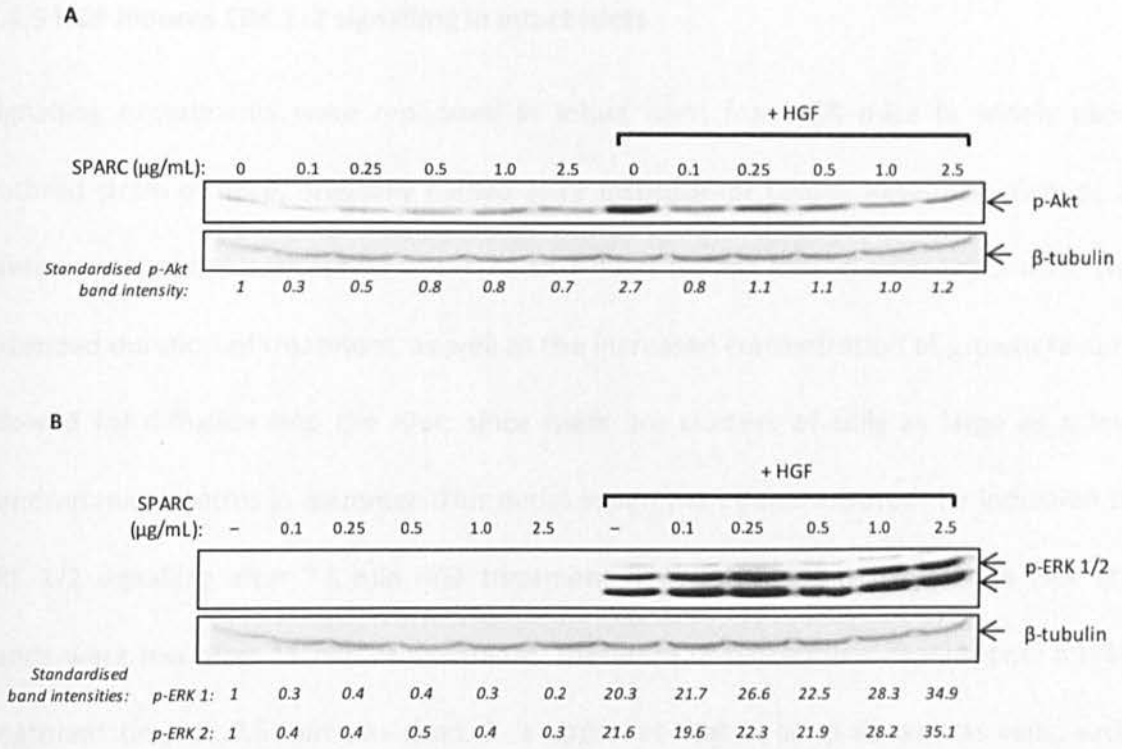


Figure 3.12. SPARC reduced HGF induced Akt signalling. Western blot analysis of INS-1 cell lysates after SPARC and HGF treatment (20 ng/ml; 7.5 minutes). Each blot is representative of 3 independent experiments. Band intensities were standardised to β-tubulin band intensities and the control. p-Akt, p-ERK 1 and p-ERK 2 standardisation shown. **A.** Decrease in phosphorylated Akt after SPARC treatment. **B.** SPARC had no effect on phosphorylated ERK 1/2 induced by HGF.

3.4.5 HGF induces ERK 1/2 signalling in intact islets

Signalling experiments were replicated in intact islets from ICR mice (a widely used, outbred strain of mice, originally named after Institute for Cancer Research). Groups of islets were treated with either IGF-1 or HGF (200 ng/ml) for 7.5 min or 15 min. The extended duration of treatment, as well as the increased concentration of growth factors allowed for diffusion into the islet, since islets are clusters of cells as large as a few hundred micrometres in diameter. This initial experiment demonstrated the induction of ERK 1/2 signalling after 7.5 min HGF treatment (Figure 3.13). Intensity of the ERK 1/2 bands were less after 15 min of treatment, therefore in subsequent experiments a HGF treatment time of 7.5 min was used. It is apparent that in islets as well as cells, such signalling occurs within 7.5 min, but is transient, declining after 15 min. In islets, the β-tubulin antibody produced very inconsistent bands on the blot, therefore pan-Akt and total-ERK 1/2 antibodies were used to demonstrate equal protein loading. The anti-total ERK 1/2 antibody used is reported to have less affinity for total ERK 2 than ERK 1, hence the faint total ERK 2 bands. The total ERK 1 bands were used to assess protein loading.

In this instance, bands across the right hand side of the blot appeared to fade, either due to unequal protein loading, or inefficient transfer of protein on this side of the blot. Further experiments were therefore performed to confirm any effect of SPARC on the stimulation of Akt or ERK 1/2 found in this blot.

It appears that Akt signalling is affected by growth factors and/or SPARC (Figure 3.13). However these results were inconsistent with repeat and subsequent experiments. SPARC (2.5 μg/ml) with HGF appeared to increase Akt phosphorylation, however this result was not consistent and occurred in 50% of the blots (n of 8, see Figure 3.14 also).

Although in this initial experiment islets were treated with IGF-1, no robust stimulation of either Akt or ERK 1/2 was found after five further attempts at using IGF-1 at both time points. IGF-1 had little or no impact on Akt or ERK 1/2 signalling, therefore no further experiments on islets were performed with IGF-1.

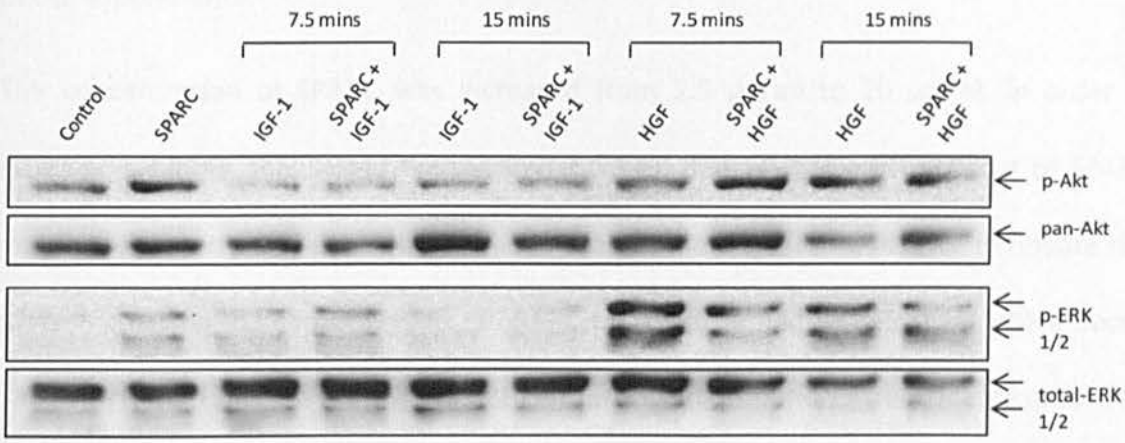


Figure 3.13. HGF induced ERK signalling in intact islets. Western blot analysis of lysates from intact islet after SPARC (2.5 μg/ml; 2 hours) and growth factor treatment (200 ng/ml). Pan-Akt and total-ERK antibodies were used to assess even protein loading. The growth factor treatment time was optimal at 7.5 minutes for HGF.

3.4.6 SPARC had no effect on HGF-induced signalling in intact islets

Further to the initial experiment in Figure 3.13, experiments were conducted to ascertain whether the effect of SPARC on signalling was robust. HGF (200 ng/ml, 7.5 min) induced strong phosphorylation of ERK 1/2 however pre-incubation with 2.5 µg/ml SPARC had no effect on HGF-mediated ERK activation (Figure 3.14 A). As mentioned before, SPARC plus HGF induced Akt phosphorylation, although this was not consistent and occurred in 50% of the experiments.

The concentration of SPARC was increased from 2.5 µg/ml to 20 µg/ml, in order to ascertain whether this would induce any changes, due to increased amount of SPARC molecules present, and potentially an increased amount of SPARC able to permeate the islets. Increasing the concentration of SPARC to 20 µg/ml had no effect on HGF-induced phosphorylation of ERK 1/2 (Figure 3.14 B), producing a band pattern identical to that when 2.5 µg/ml SPARC was used.

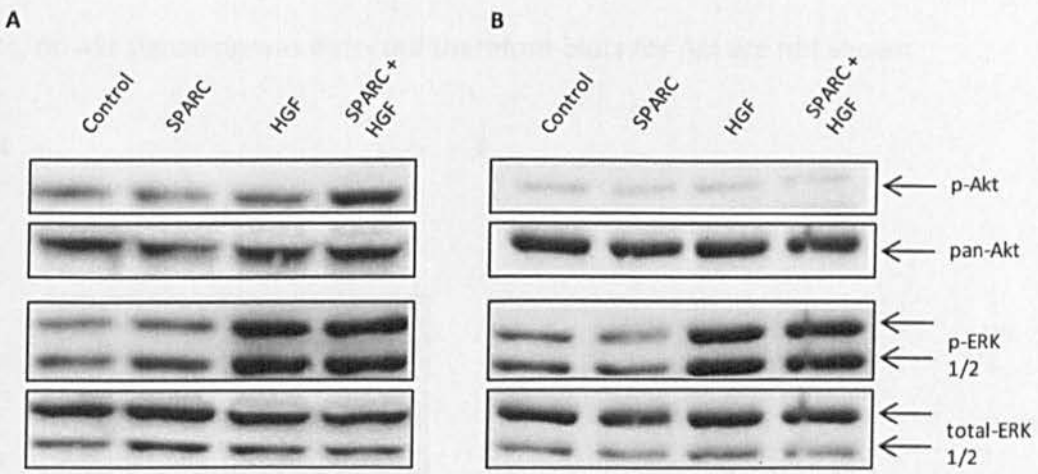


Figure 3.14. No effect of SPARC on signalling in intact islets. Groups of intact islets were pre-treated with SPARC at 2.5 µg/ml (**A**) or 20 µg/ml (**B**) for 2 hours before treatment with HGF at 200 ng/ml for 7.5 minutes. Western blot analysis of phosphorylated Akt and ERK 1/2. Membranes were stripped and reprobed for total Akt and ERK 1/2. Representative of several individual experiments (**A**: n = 4; **B**: n = 2).

3.4.7 SPARC inhibits HGF induced signalling in dispersed islets

The use of intact islets poses the problem of diffusion of molecules into and throughout the islet. In the previous experiments, HGF stimulated signalling (Figure 3.14), however the lack of SPARC response may be due to the use of islets that remain intact. It was considered that dispersal of islets into a single cell suspension (and allowing for attachment) before treatment may overcome this and thus islet-dispersed cells were prepared. The HGF concentration was reduced to 20 ng/ml, since with single dispersed cells diffusion poses less of a problem. 20 ng/ml HGF was optimal in the INS-1 cells, so the concentration was used in the dispersed islet experiments. For the same reason, the pre-incubation time with SPARC was reduced to 1 h. HGF induced stimulation of ERK 1/2 phosphorylation, however no response by SPARC was detected when used at 2.5 µg/ml (Figure 3.15 A). However, when the concentration of SPARC was increased to 20 µg/ml, phosphorylated ERK 1/2 levels decreased to basal levels (Figure 3.15 B). In all experiments, treatment with SPARC alone did not affect ERK phosphorylation. Bands detected were of comparable intensity to basal levels of phosphorylation. In dispersed islets, no Akt signalling was detected therefore blots for Akt are not shown.

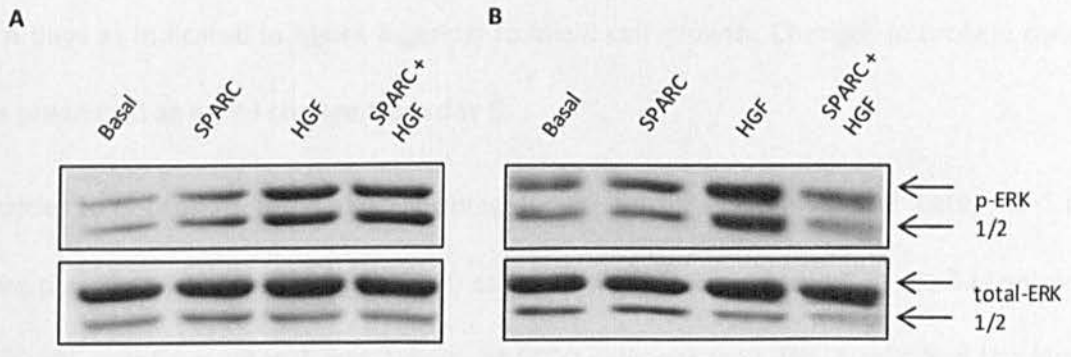


Figure 3.15. SPARC inhibits HGF-induced signalling in dispersed islets. Groups of dispersed islets were pre-treated with SPARC at 2.5 µg/ml (**A**) or 20 µg/ml (**B**) for 1 hour before treatment with HGF at 20 ng/ml for 7.5 minutes. Western blot analysis of phosphorylated Akt and ERK 1/2. Membranes were stripped and reprobed for total Akt and ERK 1/2. Representative of three individual experiments (n = 3). No phosphorylation of Akt was seen in dispersed islets in any treatment.

3.5 Proliferation of a β cell line

3.5.1 Optimisation of seeding density

Determining the effects of SPARC on β cell proliferation could be of importance in expanding β cells for use in diabetes patients. The growth factors IGF-1 and HGF were chosen in this study as initiators of β cell activity because they are regulators of β cell growth *in vitro* and *in vivo*. SPARC has been shown here to have an inhibitory effect on IGF-1 induced phosphorylation of Akt and ERK and HGF-induced phosphorylation of Akt in INS-1 cells, and also its inhibitory effect on HGF-induced phosphorylation of ERK in dispersed islets. Since SPARC modulates growth factor signalling, it was important to determine whether SPARC could affect proliferation, a downstream outcome of IGF-1 and HGF receptor signalling.

The extent of proliferation of cells can be determined by the total protein content increase, over a period of time. SRB, a red fluorescent dye, binds protein and can therefore be used as an indicator of total cellular protein and thereby determine proliferation rates, as described by Skehan *et al.* (1990). Protein content was measured on the day after cell starvation (as a day 0 measure) and then after set durations (after 2 or 4 days as indicated in figure legends) to allow cell growth. Changes in protein content are presented as a fold change from day 0.

In order to determine the correct seeding density for assays in a 96-well plate, INS-1 cells were plated at 5000, 10000 and 15000 cells per well, serum-'starved' in media containing 0.5% FBS and fixed after 2 and 4 days. At 5000 cells per well, INS-1 cells had the largest increase in total protein after 2 and 4 days (Figure 3.16). The increase in protein content using 5000 cells/well was significantly different from the increase found when using 15000 cells/well at day 2 ($p = < 0.001$, $n = 12$) and at day 4 ($p = < 0.001$, $n = 12$). On day 4,

cells seeded at 10000 or 15000 cells/well had a total increase of 1.9 times and 1.3 times that on day 0, respectively, whereas cells seeded at 5000/well had increased 2.8 fold after 4 days. The optimal seeding density was therefore determined as 5000 cells per well, and in subsequent experiments, cells were seeded at this density to allow any changes in cell growth in response to treatments to be observed.

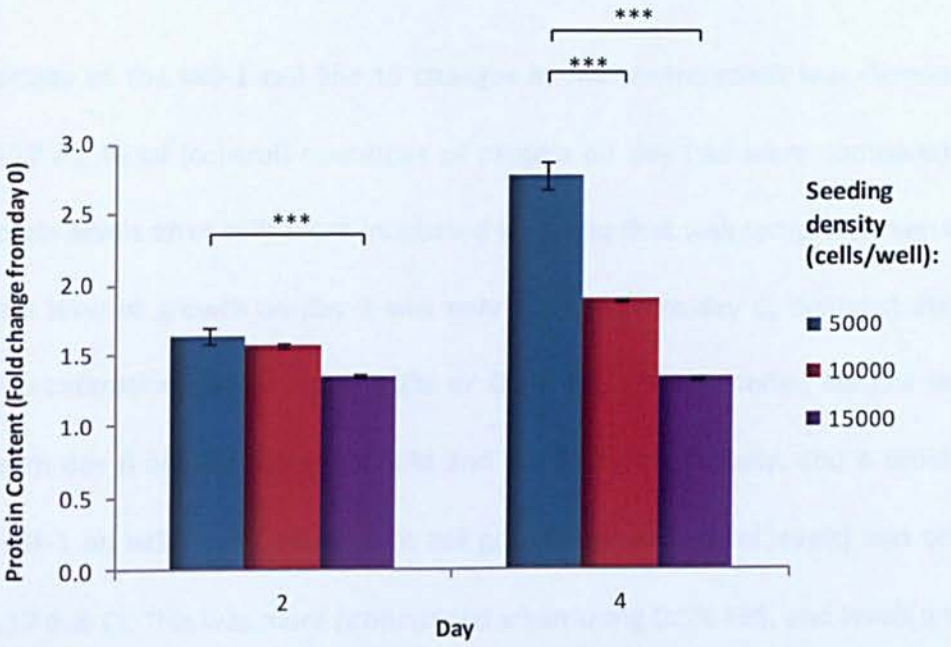


Figure 3.16. Reduced seeding density allows for increased rate of proliferation. INS-1 cells were seeded between 5 x 10^3 and 1.5 x 10^4 per well in 96-well plates. After attachment, cells were starved in 0.5% serum media and fixed after 2 and 4 days. Sulphorhodamine B dye was used to determine total protein content. Bars indicate mean ± SEM. *** $p \leq 0.001$ between groups

3.5.2 Varying the concentration of FBS in starvation media

Using the right amount of FBS to supplement the media was a balance between starving the cells from any growth factors that may be present in the FBS (which, when trying to observe effects of exogenous growth factors, would interfere with the results), and having enough serum present for continued cell growth.

It was determined that when seeding at 5000 cells per well, fixing cells (precipitation with TCA treatment) on day 2 after treatment was sufficient to observe changes in cell growth when growth factors were used. After 4 days, greater increases in cell proliferation and restrictions of well area meant that cells became over populated and cell death, identified as clumps of floating cells, was observed. Etoposide (10 μM) was used in these experiments to verify that a cytotoxic agent reduces cell growth in this assay, and in the case here, causes cell death (a decrease in SRB content from day 0).

The sensitivity of the INS-1 cell line to changes in the environment was demonstrated (Figure 3.17 A). Basal (control) quantities of protein on day two were compared to the day 0 protein levels after cells were incubated in media that was completely serum-free. The control level of growth on day 2 was only 1.1 fold from day 0, demonstrating very little cell proliferation. When using 0.1% or 0.5% FBS in the media, control levels of growth from day 0 are increased 1.4 fold and 2.1 fold, respectively, and a proliferative effect of IGF-1 on INS-1 cells (increase in cell growth above control levels) was observed (Figure 3.17 B & C). This was more pronounced when using 0.5% FBS, and levels are more significantly increased from controls with a 20% increase in growth rate ($p = 0.001$, $n = 6$). IGF-1 stimulated a 2.6-fold change in growth compared to day 0.

Using either 2% or 5% FBS in media resulted in high rates of control cell growth (2.6 and 2.5-fold over 2 days, respectively; Figure 3.17 E & F). It is evident therefore that any proliferative molecules within the serum interfere with the results, masking any possible exogenous growth factor effects.

To determine if extra glucose in the media provided beneficial effects to the experiment, the glucose concentration was doubled to 22.2 mM in the 0.5% FBS-containing media. This had an inhibitory effect on the proliferative activity of IGF-1 (Figure 3.17 D, in comparison with 3.17 C), possibly due to the β cell inherent properties of glucose-

stimulated insulin release, rather than using glucose for proliferative activity. Therefore glucose levels were kept at 11.1 mM for subsequent experiments.

HGF did not have any effect on INS-1 cell growth in these experiments. All total protein measurements made were compared to basal levels of growth.

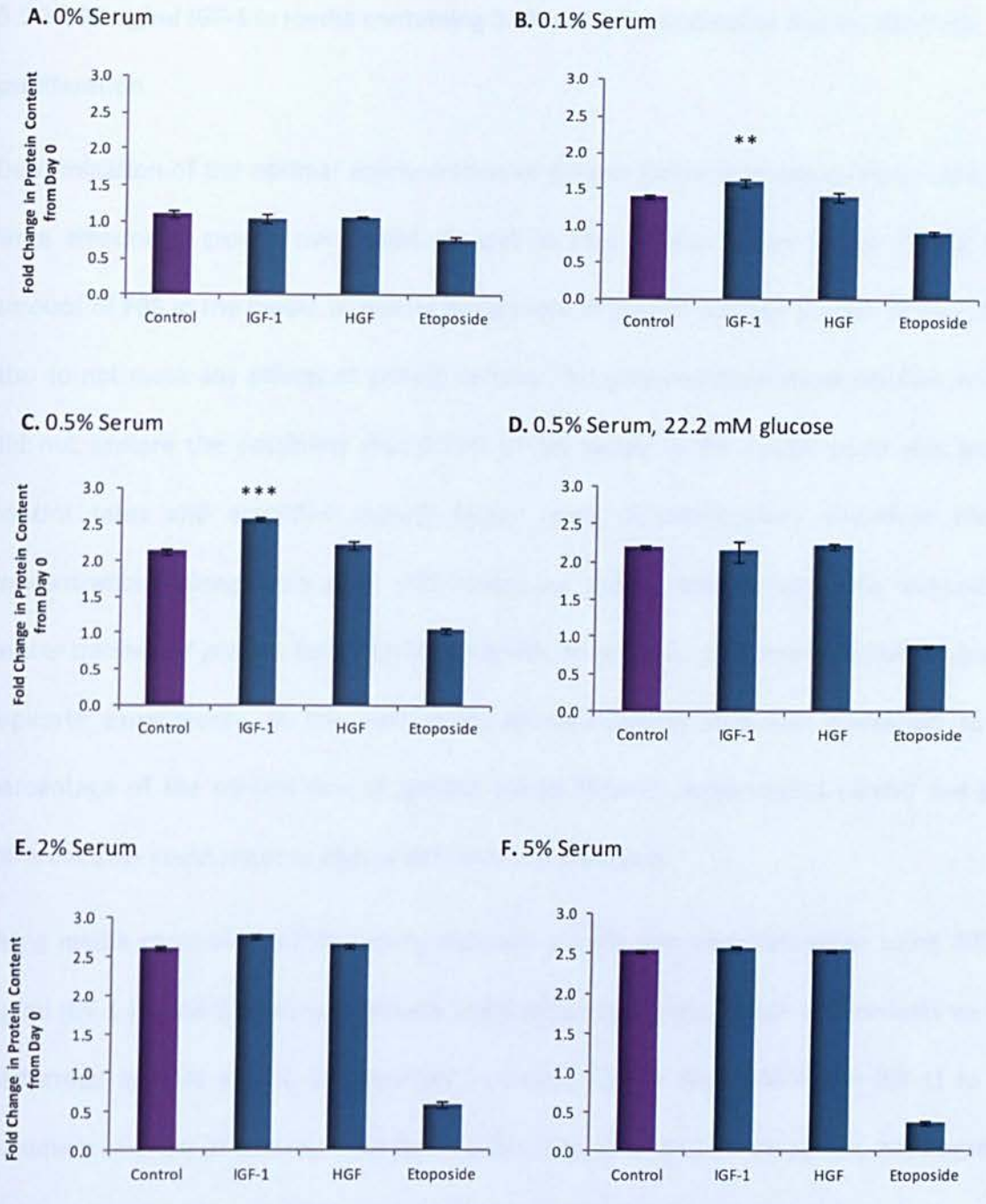


Figure 3.17. Mid-range FBS is optimal for cell growth and detecting changes when using growth factors. INS-1 cells seeded at 5000 cells/well in 96-well plates. After attachment, media was removed and replaced with media containing varying amounts of serum, or with double glucose level (22.2 mM). The following day cells were treated with IGF-1 or HGF (200 ng/ml) in the appropriate media and cell protein content analysed after 2 days, using sulphorhodamine B total protein dye. Results represent the mean ± SEM of n = 6 from the same culture. ** $p \leq 0.01$, *** $p \leq 0.001$ compared to the control data set.

3.5.3 200 ng/ml IGF-1 in media containing 0.5% serum is optimal to induce INS-1 cell proliferation

Determination of the optimal concentration of growth factor was necessary to obtain a large amount of growth over basal. As well as this, studies aimed at fine-tuning the amount of FBS in the media to ensure basal rates of growth without growth factors, but also to not mask any effects of growth factors. The previous experiment (section 3.5.2) did not explore the possibility that 0.25% or 1% serum in the media could give good control rates and amplified growth factor rates of proliferation, therefore these concentrations along with 0.5% FBS-containing media were used, with increasing concentrations of growth factor (2-2000 ng/ml). In order to pool results obtained from replicate experiments, in the next series of experiments data was converted to a percentage of the control rate of growth (since different experiments carried out on different days could result in slightly different fold changes).

Using media containing 0.25% serum, maximal growth was observed when using 2000 ng/ml IGF-1 (Figure 3.18 A) with growth 118% of control levels. When experiments were performed in 0.5% serum, proliferation increased further (by 2000 ng/ml IGF-1) to a maximum of 123% of control levels ($p = < 0.001$; Figure 3.18 C). In 1% serum, 2000 ng/ml IGF-1 stimulated growth 112% of control (Figure 3.18 E). This verified that 0.5% serum was the optimal concentration to observe maximal effects of IGF-1 on proliferation, giving the largest percentage increase above control levels of growth.

At this serum level (0.5%), treatment with 2 ng/ml IGF-1 did not significantly influence proliferation of INS-1 cells (Figure 3.18 C), however 20 ng/ml and 200 ng/ml IGF-1 increased cell growth by 113% ($p = < 0.001$) and 123% respectively ($p = < 0.001$). Further

increase in IGF-1 concentration to 2000 ng/ml did not stimulate cell growth more than 200 ng/ml IGF-1.

As previously shown (Figure 3.17), treatment with HGF did not stimulate cell growth above control in these experiments also, at any serum levels tested (Figures 3.18 B, D and F). Maximal stimulation occurred using 20 ng/ml HGF in 0.5% FBS, in which cell growth was 107% of basal, which was not a significant change (Figure 3.18 D). Therefore no further cell growth experiments were performed using HGF.

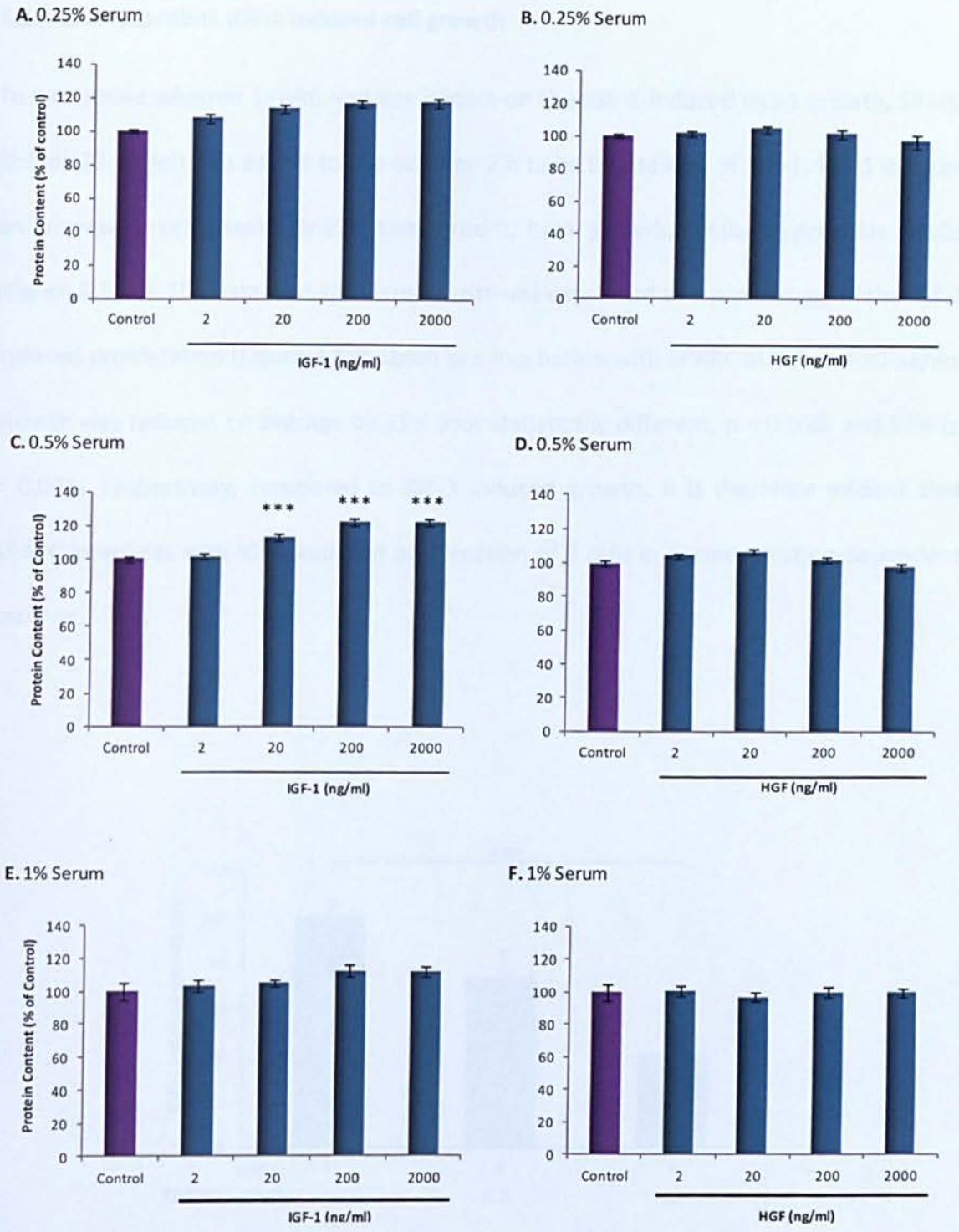


Figure 3.18. IGF-1 stimulated optimal increase in growth in 0.5% serum-containing media. INS-1 cells seeded at 5000 cells/well were treated with IGF-1 (**A, C, E**) or HGF (**B, D, F**) in media with 0.25%, 0.5% or 1% serum, and cell protein content analysed after 2 days. For **A-D**, results are pooled from 3 independent experiments and are expressed as mean ± SEM (n = 18). For **E & F**, results represent the mean ± SEM of n = 6 from the same culture. ***p <0.001 compared with control data set.

3.5.4 SPARC inhibits IGF-1 induced cell growth

To determine whether SPARC had any impact on the IGF-1-induced INS-1 growth, SPARC (2.5 or 20 µg/ml) was added to the cells for 2 h prior to addition of IGF-1. IGF-1 induced an increase in cell growth by 23% compared to basal growth, similar to previous results (Figure 3.18 C). The data for SPARC-treatment was expressed as a percentage of the IGF-1 induced proliferation (Figure 3.19). Upon pre-incubation with SPARC at 2.5 and 20 µg/ml, growth was reduced on average by 25% (not statistically different, $p = 0.058$) and 59% ($p < 0.001$), respectively, compared to IGF-1 induced growth. It is therefore evident that SPARC interferes with IGF-1-induced proliferation of β cells in a concentration-dependent manner.

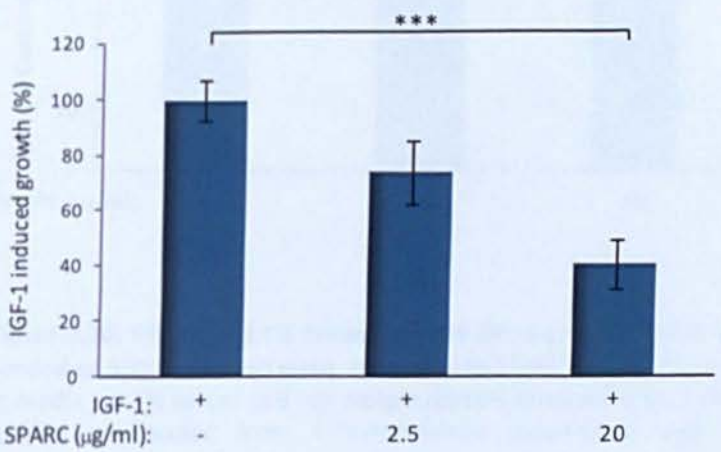


Figure 3.19. SPARC reduces IGF-1 induced INS-1 growth. INS-1 cells seeded at 5000 cells/well were treated with IGF-1 (200 ng/ml) and SPARC in media + 0.5% serum and cell protein content analysed after 2 days. Results are expressed as mean percentage of IGF-1 induced growth ± SEM (n = 24, pooled from 4 independent experiments). ***p ≤0.001.

3.5.5 SPARC in 0.5% serum reduces protein content

In the 2 day INS-1 proliferation assay, growth of cells treated with SPARC at 20 µg/ml was reduced by 11% when compared to controls ($p < 0.001$). Using 2.5 µg/ml SPARC, protein content was 98% that of control cells. This indicates SPARC had a slight, but significant effect on INS-1 proliferation (Figure 3.20). The ability of SPARC to reduce growth in these conditions may be due to the presence of 0.5% serum in the media, which contains a small amount of growth factors.

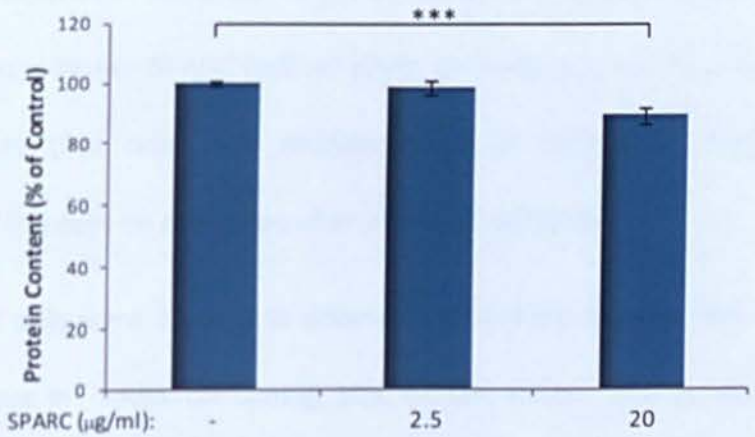


Figure 3.20. SPARC in 0.5% serum reduces INS-1 growth. INS-1 cells seeded at 5000 cells/well were treated with SPARC (2.5 or 20 µg/ml) in media + 0.5% serum and cell protein content analysed after 2 days. Results are pooled from 4 independent experiments and are expressed as mean ± SEM (n = 24). *** $p \leq 0.001$ compared with control data set.

3.6 Islet live/dead assay

3.6.1 Development of islet survival assay; islets induced to survive or perish after a 2 day treatment

The INS-1 cell line is an immortalised cell line capable of mitosis, and therefore suitable for proliferation experiments. But it is important to confirm findings using primary islet tissue to obtain more physiologically relevant data. However, using primary islets brings new challenges. Due to their characteristics, β cells in primary islets exhibit a very low mitotic rate, hence allowing the progression of diabetes. The SRB proliferation assay would not detect proliferation of primary islet cells. A survival assay was therefore developed to determine the effect of SPARC on growth factor induced islet survival. In order to assess whether SPARC had an effect on islets *ex vivo*, fluorescent markers of enzyme activity (live cells) and necrotic/apoptotic cells were used to determine proportions of live cells on coverslips after inducing cell death.

Dispersed islet cells were allowed to adhere to coverslips. Initially, cells were incubated for 1 or 2 days in media containing 10% or 0% serum, and in attempt to induce glucotoxicity, with 10% serum plus 33.3 mM glucose. Example microscope images showing green (live) and red (dead) cells as well as the overlay are shown in Figure 3.21 A. The percentage of living cells after 1 day of incubation was 70%, which did not vary between the treatment groups (Figure 3.21 B). After a further day of incubation, cell death increased in all treatments (Figure 3.21 C), although in the 0% serum treatment group and with high glucose, considerably greater cell death had occurred with 14% ($p = 0.001$) and 15% ($p = 0.002$) live cells respectively, compared to the cells in 10% serum (43% live). Subsequent experiments were therefore performed for 2 days, and since 0%

serum and high glucose in the media each gave comparable results, it was deemed suitable to use 0% serum as a way of inducing cell death in following experiments.

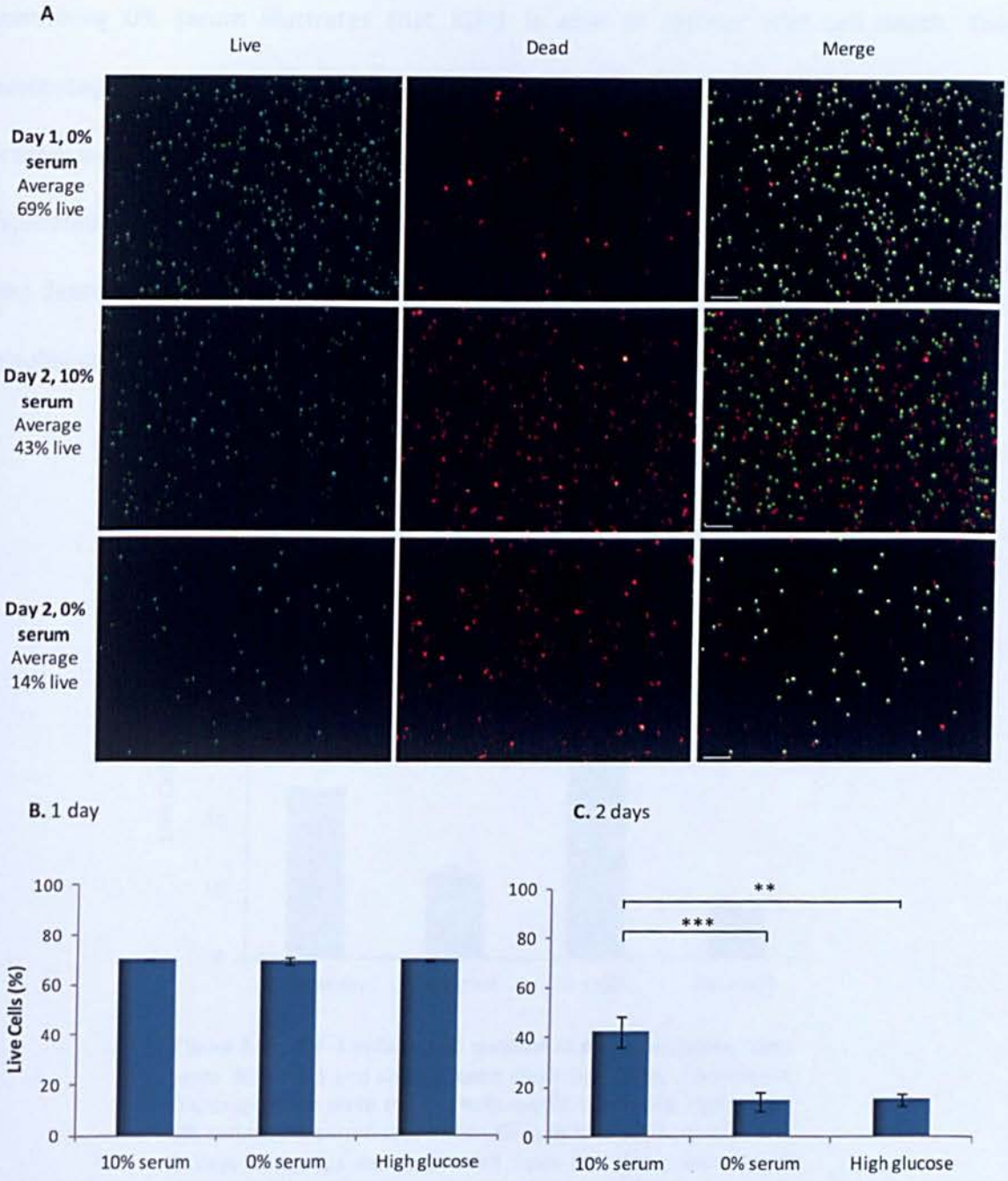


Figure 3.21. Induced death in dispersed islets after a 2 day treatment.
Islets were dispersed and seeded onto coverslips. After attachment, dispersed islets were treated with media containing 10% serum, 0% serum or 10% serum plus high glucose (33.3 mM). After 1 or 2 days, coverslips were removed from the plates and FDA/PI stains were used to assess live/dead status of individual cells using fluorescence microscopy. **A.** Representative images of live (green) and dead (red) dispersed islets adhered to coverslips when stained with FDA or PI. Bar = 200 µm. **B&C.** Graphical representation of the percentage of live islet cells as shown by FDA/PI staining. Treatments for 1 (**B**) or 2 (**C**) days. Live cells were calculated as a percentage of total cells visible as either green or red. Islets pooled from 4 ICR mice. Results shown are mean ± SEM (n = at least 4 per treatment), from several images taken from 1 coverslip. ** $p \leq 0.01$, *** $p \leq 0.001$ compared with control data set.

3.6.2 Islets survive with the addition of IGF-1

A 2-day assay with the addition of either IGF-1 or HGF (200 ng/ml) to the media containing 0% serum illustrates that IGF-1 is able to recover islet cell death. The percentage of live cells increased from 12% (in 0% serum) to 35% ($p = 0.001$) in the presence of exogenous IGF-1 (Figure 3.22). IGF-1 allowed cell survival above that of cells incubated in media containing 10% serum (25% living cells). HGF was not able to prevent islet death, showing only 7% live cells at the time of analysis. Therefore HGF was not used in subsequent experiments in this assay.

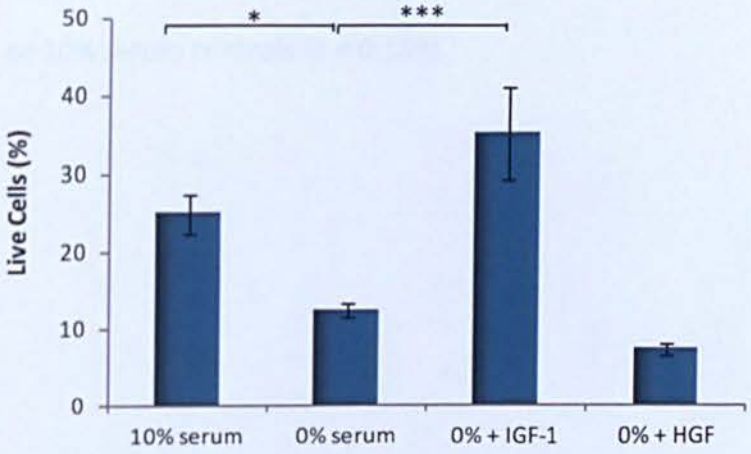


Figure 3.22. IGF-1 induces cell survival in dispersed islets. Islets were dispersed and seeded onto coverslips. After attachment, dispersed islets were treated with media containing 10% serum, 0% serum, 0% serum plus either IGF-1 or HGF (200 ng/ml). After 2 days coverslips were removed from the plates and FDA/PI stains were used to assess live/dead status of individual cells using fluorescence microscopy. Dead cells were calculated as a percentage of total cells visible as either green or red. Islets pooled from 4 ICR mice. Results shown are mean ± standard deviation. Each bar represents a mean of several images taken from 1-2 coverslips (n = at least 4 per treatment). * $p \leq 0.05$, *** $p \leq 0.001$

3.6.3 SPARC prevents IGF-1-induced survival of islets

Experiments were next performed to establish whether SPARC had any impact on the recovery of live cells induced by IGF-1. Dispersed islet cells on coverslips were treated with SPARC (20 μg/ml, in 0% serum) and IGF-1, and left for 2 days as performed previously.

During these experiments, the mean percentage of live cells was 52% in 10% serum, and 38% in 0% serum (Figure 3.23). This decrease in cell survival was counteracted by the addition of IGF-1, when live cells increased to 54% ($p = < 0.001$). SPARC had a detrimental effect on this IGF-1-induced survival, since cells subjected to treatment with SPARC and IGF-1 together showed decreased survival to 46% living cells ($p = 0.045$). When SPARC was used alone, the percentage of live cells was 46%, not significantly different from the 0% ($p = 0.106$), or 10% serum controls ($p = 0.159$).

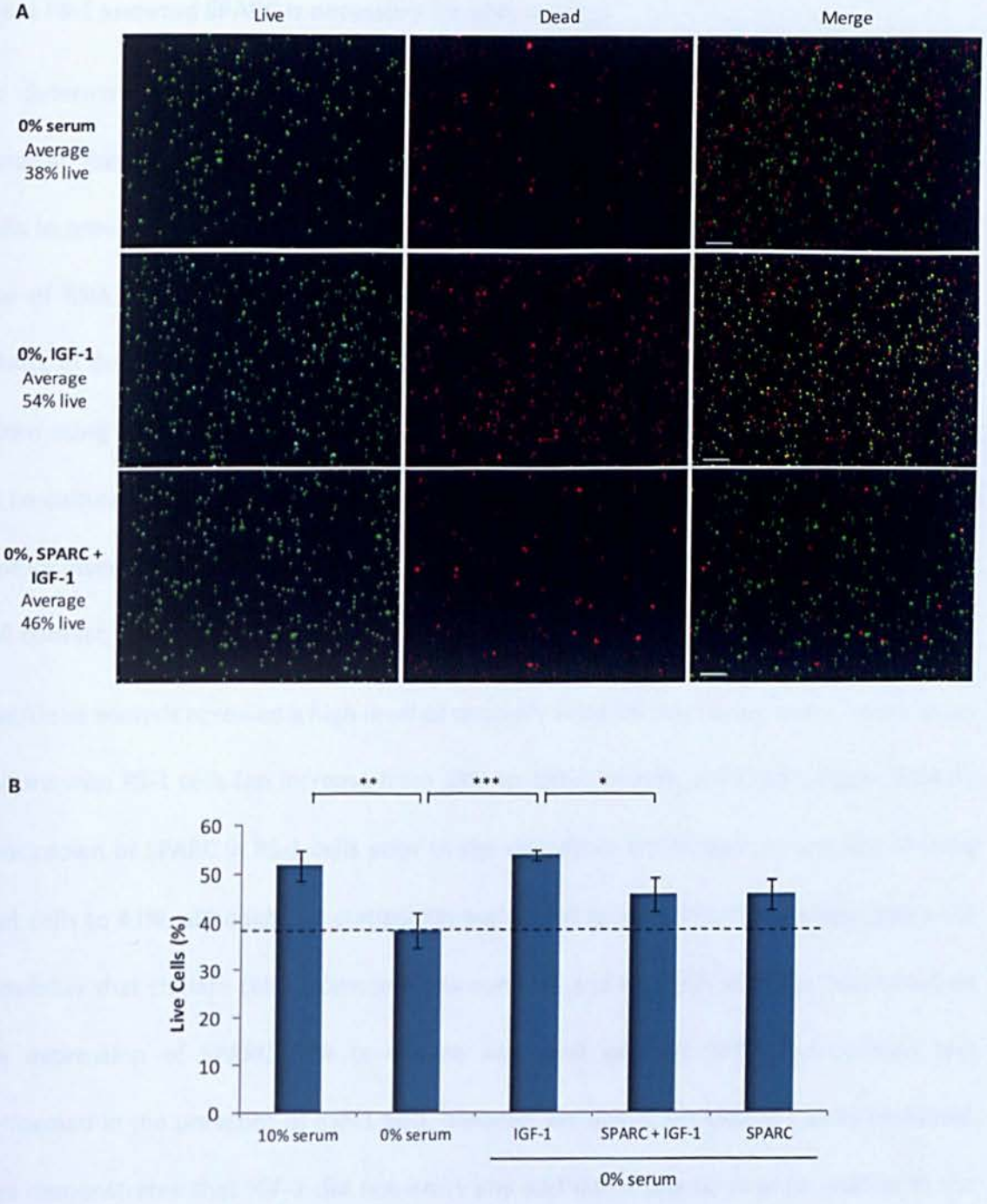


Figure 3.23. Exogenous SPARC prevents IGF-1-induced islet survival. Male or female islets were used in separate experiments, results combined. Islets were dispersed and seeded onto coverslips. After attachment, dispersed islets were treated with media containing 10% serum, 0% serum or 0% serum plus IGF-1 (200 ng/ml), SPARC (20 µg/ml) or both. After 2 days, coverslips were removed from the plates and FDA/PI stains were used to assess live/dead status of individual cells using fluorescence microscopy. **A.** Representative images of live (green) and dead (red) dispersed islets adhered to coverslips when stained with FDA or PI. Bar = 200 µm. **B.** Graphical representation of the percentage of dead islet cells as shown by FDA/PI staining. Results are mean ± SEM; data pooled from 3 independent experiments using a total of 6 mice (n = at least 19 per treatment). *$p \leq 0.05$,** $p \leq 0.01$, *** $p \leq 0.001$

3.6.4 PS-1 secreted SPARC is necessary for islet survival

To determine if secreted factors from fibroblast-like cells have an effect on islet cell survival, the PS-1 cell line was used in order to provide a source of SPARC. Using stellate cells to provide SPARC is a novel method to examine islet survival, when in co-culture. The use of RNA interference (RNAi) allowed the determination of whether knocking down SPARC in the PS-1 cells had any effect on islet cell death. SPARC expression was knocked down using SPARC small interfering RNA oligonucleotides from these cells one day prior to co-culture with islets (83% knockdown, see below). The PS-1 cells were seeded in cell culture inserts placed above the dispersed islets. The co-culture does not allow direct cell-cell contact, thus effects seen are due to molecules secreted by the PS-1 cells.

Live/dead analysis revealed a high level of recovery from the 0% serum levels, when in co-culture with PS-1 cells (an increase from 38% to 48% live cells, $p = 0.010$; Figure 3.24 A). Knockdown of SPARC in PS-1 cells prior to the co-culture decreased the amount of living islet cells to 41%, although not statistically significant ($p = 0.077$). This demonstrates the possibility that stellate cells promote β cell survival, and that this effect is dependent on the expression of SPARC. The co-culture with and without SPARC knockdown was performed in the presence of IGF-1 also, however no significant changes were observed. This demonstrates that IGF-1 did not exert any additional pro-survival properties in the presence of PS-1 stellate cells.

After live/dead fluorescence analysis, media from the culture was removed, and PS-1 cells were lysed. The presence of SPARC was analysed by western blot (Figure 3.24 B), which demonstrated the knockdown of SPARC protein in both the cells and the media. Image analysis of all blots from the experiments showed an average SPARC knockdown of 83% in cells, 93% in media (Figure 3.24 C).

These results demonstrate the ability of recombinant SPARC to prevent IGF-1 induced islet survival and that PS-1 cells are also necessary for islet survival.

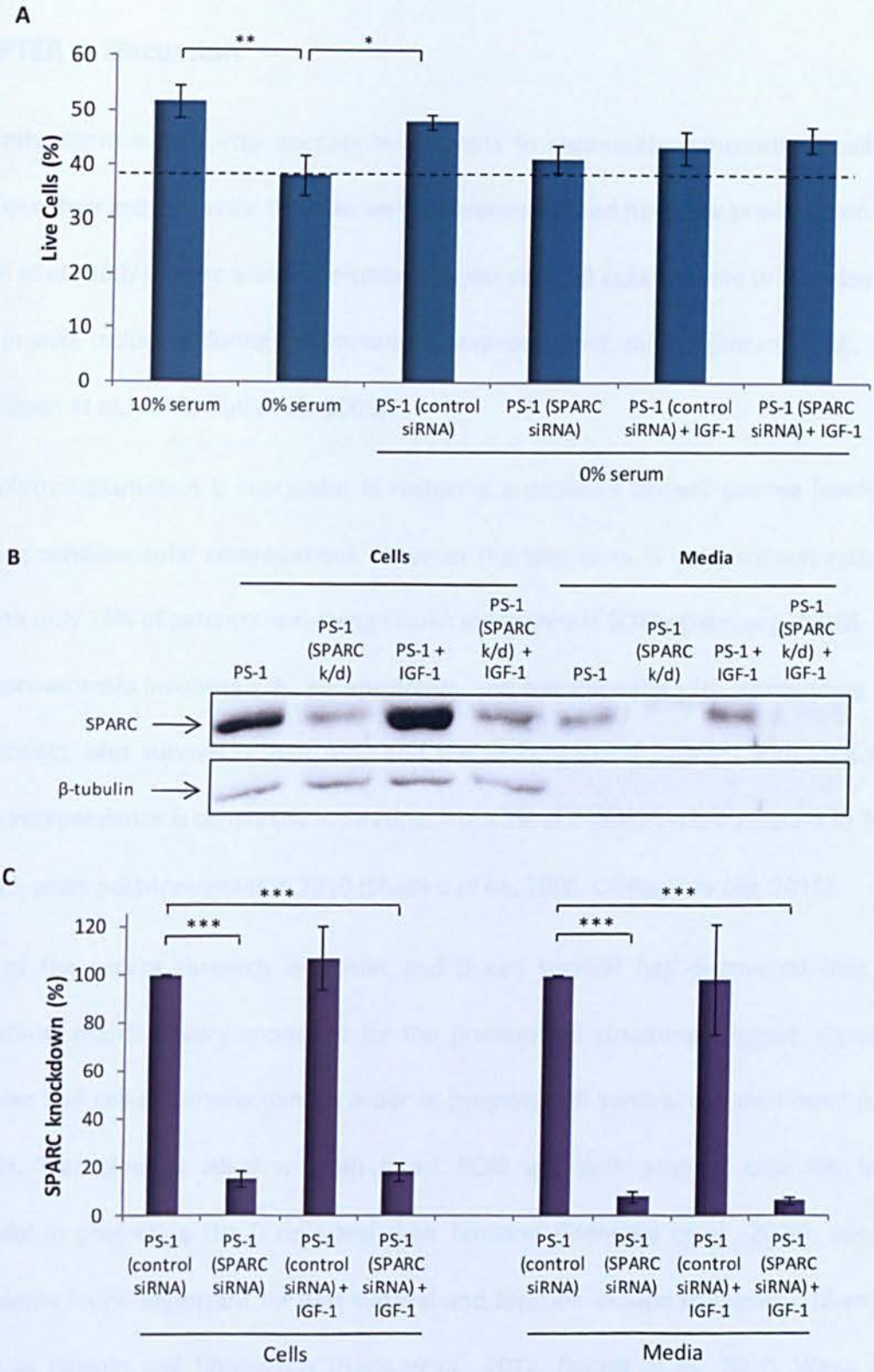


Figure 3.24. PS-1 secreted SPARC is necessary for islet survival. Dispersed islets on coverslips were in co-culture with PS-1 cells for 2 days, some of which SPARC had previously been knocked down by siRNA. **A.** Percentage (mean ± SEM) of live islet cells as shown by FDA/PI staining. Data pooled from 3 independent experiments from a total of 6 mice (n = at least 19 per treatment). *$p \leq 0.05$,** $p \leq 0.01$. **B.** Representative western blot of SPARC protein in cells and media from the PS-1 cell line in the co-culture set up. Successful knockdown of SPARC from PS-1 cells treated with SPARC siRNA oligonucleotides (SPARC k/d). **C.** Graphical representation of combined western blot analysis showing SPARC knockdown (mean ± SEM) in cell lysates and media. Mean knockdown was 83% in cells, 93% in media (n = 13). *** $p < 0.001$

CHAPTER 4: Discussion

Currently, there is very little success in attempts to regenerate pancreatic β cells and stimulate their growth, since they are well differentiated and have low proliferation rates (Kayali *et al.*, 2007). There are circumstances under which β cells are able to increase their mass *in vivo*, including during inflammation, pregnancy and obesity (Sherry *et al.*, 2006, Marynissen *et al.*, 1983, Hull *et al.*, 2005).

Islet allotransplantation is successful in restoring a patient's normal glucose levels and reducing cardiovascular complications, however the long term (5 year) success rates are low with only 24% of patients remaining insulin independent (CITRegistry.org, 2010). With the improvements involving gels, encapsulation, and transplanting with surrounding ECM components, islet survival is improving and the percentage of patients with long term insulin independence is constantly increasing, from 3% at 3 years-post-transplant in 2006, to 24% 5 years post-transplant in 2010 (Shapiro *et al.*, 2006, CITRegistry.org, 2010).

Much of the recent research into islet and β cell survival has discovered that the extracellular matrix is very important for the provision of structural support, signalling molecules and cell-cell interactions in order to promote cell survival and continued β cell function. Transplanting islets with an intact ECM and with stromal cells has been successful in protecting the β cells and their function (Stendahl *et al.*, 2009). Specific components found important for islet survival and function include collagens I, III and IV as well as laminin and fibronectin (Davis *et al.*, 2012, Daoud *et al.*, 2010, Wang and Rosenberg, 1999, Nagata *et al.*, 2002). However the role of SPARC has not previously been investigated.

The structural and non-structural components of the extracellular matrix work together in regulating the cellular environment. SPARC is a non-structural element to the matrix,

involved in biological processes including angiogenesis, inflammation, fibrosis, wound healing and tumourigenesis (Roberts, 2011). SPARC could be a necessary extracellular regulatory molecule important in β cell proliferation and function, however a precise role for SPARC in islet survival has not been previously examined.

4.1 Expression

In order to be able to perform experiments investigating the actions of SPARC on β cells, it was necessary to source a cell line that expressed insulin and therefore had proper β cell function. Expression of insulin revealed that the INS-1 cell line was suitable to use as a β cell line, since it was found to be expressed in the cytoplasm, with a void where the nucleus is located. Using fluorescence microscopy the insulin expression pattern demonstrated that it was not present in all of the cells at the time of fixing. Since insulin was detected in every cell using the DAB method, but not in the fluorescently labelled slides, it is thought that it is a consequence of the fluorescent labelling method. Other labelling issues were found with the fluorescence based method (no SPARC or FSP-1 staining was observed, as described below), therefore suggesting limited permeability of the cells to these antibodies. Other reasons for this expression pattern include intermittent expression. A sub-population of the cells may have lost the ability to produce insulin, for example during cellular senescence. High passage β cell lines have decreased insulin expression (Cheng *et al.*, 2012). It is also possible that cells are at different stages of insulin secretion, since cells were in media containing glucose at the required amount for insulin release, cells that have exhausted all stores may be undergoing insulin synthesis at the time of fixation. Given that insulin was observed in all cells by using the DAB method, it is likely that the method of fluorescent staining is the issue, perhaps in terms of antigen/antibody specificity.

Expression of insulin in the pancreas was sparsely distributed with few areas containing stained cells spread throughout the entire section, restricted to large rounded or oval shaped structures of the islets. In the islets, staining was not in all cells, apparent from the 'voids' of staining in the centre of the structures, due to the other cell types found in the islets.

SPARC was found to be expressed in PS-1 cells, and to be secreted by PS-1 cells into media. This confirms in pancreatic stellate cells what others have found in various fibroblast types, including pancreatic stellate cells also, by using western blot or pulse-chase labelling methods to show expression of SPARC (Wasi *et al.*, 1984, Zhou *et al.*, 2002, Mantoni *et al.*, 2008). In the present study SPARC expression was found in the cytoplasm of PS-1 cells, although it was difficult to tell whether SPARC was present in the nucleus, due to unsuccessful fluorescent labelling. Using a confocal laser scanning microscope with fluorescently labelled SPARC would have given the advantage of being able to scan one plane through the cell, cutting across the nucleus, and consequently determining if SPARC is present in the nucleus. If SPARC was found in the nucleus, this may suggest it also acts as a regulator of transcription for other genes. However, for the experiments conducted here, subcellular localisation of SPARC was not essential. In islets, SPARC is expressed within certain islet cells, and a large amount of SPARC was found to be secreted, as shown in the concentrated media. SPARC was not found in β cells with any of the techniques used. This agrees with the staining of pancreas sections for SPARC, since it is only expressed in a few cells within islets, not to the extent that coverage of β cells is shown by an insulin stain. Absence of SPARC in β cells suggests that it is limited to expression in regulatory cells of the ECM in islets.

FSP-1 staining was observed in PS-1 cells, most strongly within the nucleus. This disagrees with results previously found by Strutz *et al.* (1995) who showed staining of FSP-1 in

cytoplasm of fibroblasts. However, others have found FSP-1 expression in both the cytoplasm and nucleus (Kikuchi *et al.*, 2006, Ryan *et al.*, 2003). Here, it was not important to determine which part of the cell the antibody bound, but to demonstrate the fibroblast-like stromal nature of the PS-1 cells, therefore deeming them suitable for use in assays. Since INS-1 cells did not express FSP-1, FSP-1 could be used as a marker of stromal cell types in pancreas sections, without the concern that FSP-1 would also bind β cells.

Microscopy issues led to difficulty in detection of secreted proteins in cell lines, especially insulin and SPARC, an extracellular protein, since once secreted these proteins cannot be detected due to being removed with the media and lost in the washing steps of the immunocytochemistry protocol.

Staining of mouse pancreas sections showed that SPARC is expressed in elongated cell types, consistent with a stromal or fibroblast cell shape and including endothelial cells of blood vessels. SPARC is expressed around the edges of islets (peri-islet area), in the area where ECM (containing basement membrane components) and stromal cells have been found (Irving-Rodgers *et al.*, 2008). As shown in adjacent sections, SPARC and FSP-1 are located in similar, if not the same, islet areas, giving further evidence that SPARC is expressed by stromal cell types in the islet. However the areas stained by SPARC and FSP-1 do not completely overlap. SPARC may be expressed by other cell types that do not express FSP-1, such as endothelial cells. Fluorescence microscopy for SPARC and FSP-1 proteins in INS-1 and PS-1 cells, as well as in pancreas sections was attempted. This procedure was unsuccessful, possibly due to unsuccessful antibody-antigen binding in the procedure, or a lack of an amplification step (as is present in the ABC method). Permeability issues of the primary/secondary antibodies may have prevented this technique from working. Attempts using different SPARC primary antibodies, as well as

several different secondary antibodies to detect the SPARC and FSP-1 primaries were unsuccessful.

Detection of SPARC in the islets of NOD mice may give insight into the role of SPARC in a pre-diabetic islet environment. In NOD mice, islets still consist of a majority of β cells at 6 weeks of age (pre-diabetic), however by 40 weeks only a trace of β cells remain (Kharouta *et al.*, 2009). Wild type C57Bl/6 and NOD sections were used here and tested for the presence of SPARC.

The upregulation of SPARC in NOD islets compared to B6 control islets suggests that SPARC may be involved in early tissue remodelling of the pancreas that has been implicated in diabetes development. The infiltration of immune cells begins at around 3-4 weeks of age in the NOD mouse (Jansen *et al.*, 1994, Rosmalen *et al.*, 2000), which is when SPARC expression is higher. Areas of inflammation were excluded from the analysis, therefore minimising the inclusion of any SPARC expressed by the infiltrating cells. However single immune cells that have invaded the islet would be included. Further experiments involving macrophage or dendritic cell markers would inform as to whether SPARC is co-expressed with infiltrating immune cells. The peri-islet basement membrane (consisting of perlecan, laminins, collagen type IV α1 and α2, and nidogens), in which SPARC was found, is destroyed in the NOD mouse during leukocyte infiltration, which marks the onset of destructive insulitis (Irving-Rodgers *et al.*, 2008). High expression of SPARC at this time suggests the involvement of SPARC in the reorganisation of the islet ECM when insulitis, cell rearrangements and high rates of β cell apoptosis are occurring during the development of diabetes.

In control pancreas SPARC was expressed early with a decline in expression at 12 weeks, which implies a role for SPARC in the early stages of post-natal development. There was also a decrease in expression in NOD mice from 4 to 12 weeks, however this result was

not statistically significant. The loss of SPARC expression in control and NOD pancreas sections at 12 weeks of age may be attributed to the down-regulation of SPARC protein expression and secretion after the early weeks of development are completed, since expression of SPARC decreases after the initial stages of development (Sage *et al.*, 1989a). In NOD mice, the reduction could be attributed to loss of cell types expressing SPARC, and loss of basement membrane integrity as the insulitis develops, as stated above, since SPARC is expressed in basement membranes. The disparity between the increase in infiltrating immune cells expressing SPARC and loss of SPARC from islet cells may have given rise to the non-significant result.

It has been shown here that SPARC is expressed by stromal cells in the islet that surround or are interspersed with β cells. During development of the pancreas and islets of Langerhans, SPARC is present, in all probability to facilitate cell movements and ECM restructuring through the regulation of cell growth and survival. We hypothesise that SPARC is expressed during the onset of diabetes to regulate cell growth and is involved in ECM rearrangements during inflammatory cell infiltration.

To determine if knocking out the SPARC gene in mice made a difference to islet size, it was decided to use an insulin scoring method on pancreas sections. This was an alternative to analysing total islet area, due to excluding cell types in the islet that are not β cells. By doing this it was possible to see any effect on insulin-stained area, and therefore β cell area. The results did not show any differences in islet or pancreas section area, number of islets, islet area as a percentage of section area, or average islet area when comparing SPARC knockout mice to control mice. Slight (but not significant) differences were found in the number of islets per section and total section area. In SPARC knockout sections, the number of islets and total section area were both decreased, therefore indicating that presence of SPARC may be required for a larger islet

number, and larger section area. The percentage area covered by all the islets in the section was unchanged, meaning that the same area (as a percentage) was covered by a lower number of islets in knockout mice. Since the section area was decreased, and islet size has not changed, the determining factor is section (pancreas) size. Thus it would appear that SPARC knockout mice have smaller pancreas size than control mice, however to accurately quantify this, the whole pancreas, before fixing would need to be weighed and measured. Further experiments with significance would need to be performed to determine if SPARC is necessary for pancreas growth.

In order to determine whether any significant differences were suppressed by the use of both male and female sections, results from the females only (more female sections were analysed than male) were evaluated. For the female only comparison, the number of islets and area per islet remained the same between wild type and knockout sections. The total section area was decreased in the female control mice, compared to the male plus female analysis. This meant that the SPARC knockout section area in females appeared to increase, producing a decreased percentage area covered by islets (not significant). The decrease in wild type section area when analysing females only is not a result of the presence or absence of SPARC, but only infers the sex differences in mouse pancreas size.

Overall, no significant differences in this analysis were found between wild type control and SPARC knockout pancreas sections. This could be due to lack of sensitivity of the assay in trying to measure small changes in a small study. A large effect would have to be caused to change the sizes of islets, and it is apparent that SPARC does not induce such large changes. Occurrence of change in islet area may be present in SPARC knockout mice, however it is too small to be measurable by the techniques used here, and may happen at an earlier age than in this study (sections from mouse used here were over 10 weeks of age).

The use of sections from different ages of mice would have allowed determination of the effect of SPARC on islet size at an earlier or later age. Since the sections used (over 10 weeks of age) were past the early developmental stages (3-4 weeks) in which SPARC might act, discussed above, it is possible that any differences in islet size caused by the lack of endogenous SPARC may occur at these early stages only, and these developmental differences may have been missed. Due to the multitude of growth factors and regulators of cell growth present in the cell environment, the effect of SPARC may be shadowed by the redundancy of these factors.

Here total area (determined using the total area covered and the number of islets counted) was also used as a measurement, and not individual islet areas. Recording individual islet areas would be a very time consuming process, considering over 1700 islets were counted in this study. For the same reason other parameters such as the diameter or circumference of islets were not analysed. Measuring the diameter would have been misleading, since some islets possess elongated shapes, and the diameter is uncertain. The circumference of islets is also misleading, not specific for β cells and would have given data for all cells within the islet.

A major limitation of this method is that only six sections per pancreas are analysed. Analysis of entire pancreas would be very time consuming, however it would allow the absolute determination of total number of islets in the pancreas, as well as getting total islet and pancreas area. The issue with the method used here is that small islets analysed in the section may just be the edge of a large islet. The possibility of analysing the same islet is hugely minimised due to analysing a section every 250 μm, since the largest islets are typically smaller than this. Only 15% of mouse islets are greater than 250 μm (Bertera *et al.*, 2012). However a previous study analysed islets in this way, and chose sections 200 μm apart (Bernal-Mizrachi *et al.*, 2001), therefore analysis of sections 250 μm apart

should be more than sufficient. Analysis of different sides of the same islet was not an issue for this study, since it was not imperative to analyse completely individual islets. If analysing the entire pancreas then it would be crucial to analyse each islet only once.

While no change in islet size in SPARC deficient mice was observed in these experiments, the technique used and the small size of the study make it unlikely that relatively small changes in islet size would be detected.

4.2 Signalling

IGF-1 and HGF have previously been found to stimulate β cell growth, however they can be inefficient at promoting cell growth, cause loss of insulin secretion and may cause accelerated cell senescence (Fiaschi-Taesch *et al.*, 2008, Hugl *et al.*, 1998, Gahr *et al.*, 2002, Vasavada *et al.*, 2006, Beattie *et al.*, 1997). Determining causes of this unresponsiveness *in vitro* may help to understand the relationships of β cells with the surrounding environment. SPARC may be a regulator of growth factor signalling, and thereby enhance or inhibit growth of β cells.

The activation of the Akt and ERK 1/2 signalling cascades in the β cell line, INS-1, were optimal upon IGF-1 and HGF stimulation at 20 ng/ml for 7.5 minutes, consistent with other studies in which this growth factor concentration was used (Vasavada *et al.*, 2007, Francki *et al.*, 2003). Activation was transient, and in the case of HGF stimulation of ERK 1/2, was strong as well as robust, producing strong immunoreactive bands in every experiment.

Akt appears to be very transiently phosphorylated, and this activation is not robust. In some of the blots where HGF was used, phosphorylated Akt appeared either not at all, or at low levels throughout (basal level of Akt phosphorylation in all lanes). Any activation

was easily prevented with the addition of SPARC, even at lower concentrations of 0.1 μg/ml. Others have found good stimulation of Akt by HGF especially in protecting the cytokine-induced inactivation of phosphorylated Akt (Mellado-Gil *et al.*, 2011). Also, IGF-1 does stimulate activation of Akt in β cells even in low glucose concentrations (Briaud *et al.*, 2003, Assmann *et al.*, 2009). For ERK 1/2 also, stimulation appeared to be weak with IGF-1. However, ERK response to IGF-1 has been found to be minimal in both INS-1 cells and islets (Briaud *et al.*, 2003). In early optimisation experiments, phosphorylated ERK 1/2 after IGF-1 treatment is clearly stimulated, and the Akt response was stronger in the earlier experiments but this was not seen in the later experiments, or with islets. The INS-1 cell differences may be explained by differences in the cell environment. Experiments performed over several weeks may be due to cell changes in the cell line, different passage numbers may express a slightly different array of proteins, including growth factor receptors, and therefore become less responsive.

For combined growth factor and SPARC experiments, a 2 hour pre-incubation time with SPARC was used, consistent with a study by Francki and colleagues in which they also examined ERK 1/2 phosphorylation (Francki *et al.*, 2003). The results demonstrate that SPARC intervenes in the growth factor signalling pathway, in a β cell line and in intact islets, to inhibit phosphorylation (activation) of these cascades. SPARC has an inhibitory effect on the growth factor response in INS-1 cells, preventing IGF-1 induced phosphorylation of Akt and ERK in a dose-dependent manner, and preventing HGF-induced stimulation of Akt at all concentrations of SPARC used. Concentrations of SPARC used (up to 2.5 μg/ml) did not have any effect on HGF-induced ERK signalling. The differential regulation of SPARC on HGF signalling could suggest a mechanism by which SPARC is able to regulate the cMet receptor downstream pathway, and that SPARC has no interaction in the ERK stimulation but acts upon Akt activation. An alternative reason is

that the induction of ERK activation by HGF is such a robust reaction that SPARC up to 2.5 μg/ml is not sufficient to prevent this signalling.

In intact islets, a similar effect to the INS-1 response to HGF was seen with a strong ERK 1/2 activation. However after dispersal of islets, 20 μg/ml SPARC prevented HGF (20 ng/ml) induced phosphorylation of ERK 1/2, indicating the requirement for SPARC to access the inner cells by dispersal, allowing access to more surface area of the cells. No effect on Akt or ERK 1/2 signalling was evident when SPARC was used alone (without addition of exogenous growth factors). SPARC only acts on the signalling pathways if growth factors are present, therefore meaning that it either interferes with the growth factor, or receptor, or that the signalling cascade must first be activated in order for SPARC to exert its suppressive action. SPARC has an important role in the regulation of growth factors, and as mentioned previously, interacts with many different growth factors and may prevent cell differentiation, proliferation and gene transcription, and prevent activation of the anti-apoptotic signals, resulting in cell death. It has been previously shown with VEGF and PDGF that SPARC can bind directly to the growth factor and prevent association with the receptor (Kupprion *et al.*, 1998, Raines *et al.*, 1992), although this is not the case with IGF-1 (Francki *et al.*, 2003). SPARC may bind IGF-1-binding proteins, if it does not bind its receptor or IGF-1 itself. Otherwise, it could interact downstream of IGF-1-receptor pathway. There is currently no other research on SPARC interaction with HGF, therefore further binding studies would be required to determine whether SPARC is able to bind HGF, or the cMet receptor. It is possible that SPARC has specific interactions with different growth factors, and does not bind universally to them.

SPARC may bind different receptors and act upon growth factor signalling without interacting with the growth factor or growth factor receptor itself. SPARC interacts with integrin-linked kinase and modulates ECM organisation (Barker *et al.*, 2005). It has been

shown to inhibit integrin-induced attachment of tumour cells to the ECM (Said *et al.*, 2007). Integrin-mediated cell anchorage can regulate the efficiency of signalling from tyrosine kinase signalling to ERK and integrin signalling within cells can overlap or synergise with those of growth factors, producing a collaborative response to activate MAP kinases (Miyamoto *et al.*, 1996). Therefore in the studies presented here, SPARC may even act upon integrins and their linked kinases to intervene in growth factor signalling pathways.

SPARC could be internalised through different mechanisms unrelated to the growth factor receptors into the β cell and act upon the cytosolic portion of the growth factor receptors. So far, internalisation of SPARC has only been demonstrated with the Stabilin-1 receptor, which targets SPARC for degradation (Kzhyshkowska *et al.*, 2006b), and VCAM-1, which modulates leukocyte trafficking (Kelly *et al.*, 2007), therefore are unlikely to explain the responses observed in this study. Since SPARC was found to modulate growth factor signalling in a negative fashion in β cells, this raises questions about targeting SPARC for improving β cell regeneration in patients with diabetes, or during the transplantation process, to increase β cell mass and prevent cell death.

Intact islets pose a diffusion problem for SPARC, which prior dispersal of the islets overcomes, to allow SPARC to have an effect. The recombinant SPARC used here has a mass of 34 kDa, and the HGF has a mass of 53.4 kDa (α chain) plus 26 kDa (β chain), therefore the diffusion issue with SPARC is not due to mass. Different diffusion rates of these molecules could be the reason, possibly down to polarities of the molecules and interactions with cell types. Interaction of SPARC with cells in the outer islet area only and incomplete distribution throughout the islet may prevent SPARC action upon β cells. The outer islet in mice is known to consist of more α cell types (Kharouta *et al.*, 2009) but

could also include any residual basement membrane or ECM proteins, which may degrade SPARC or bind SPARC, preventing its movement. Use of longer incubation times with SPARC may improve diffusion of SPARC to the cell surfaces to interact with growth factors, especially for the intact islet/dispersed islet situations, but also in cells.

Islets consist of β cells as well as other cell types such as α (glucagon), δ (somatostatin) PP (or γ, pancreatic polypeptide) and ε (ghrelin). It is possible that signalling results are due to action on the non-β cells of the islet. It is reported that in mouse islets, β cells make up the vast majority (mean of 75% reported) of cells within the islet (Brissova *et al.*, 2005) and therefore effects observed are likely to be from the β cells.

Combined SPARC (2.5 μg/ml) and HGF treatment increased Akt phosphorylation in 50% of the experiments. However, as discussed above, phosphorylated Akt is transiently expressed and inconsistent, therefore it is likely that the stimulation seen in some instances using a combination of SPARC and HGF may not actually be caused by these factors, but only a fluctuation in the basal levels in the cell line.

Another factor to consider in these experiments is the differential activation of Akt or ERK using different growth factors. The different activation profiles of these growth factors could be due to the species difference in growth factors used. Recombinant IGF-1 from rat induces pathways in the INS-1 cell line originating from rat, but was not able to induce signalling in islets, however it does stimulate survival in dispersed islets. Recombinant mouse HGF induces signalling in both the cell line and in islets from mice, however as demonstrated in section 3.5, is unable to induce proliferation of the rat INS-1 cell line. Since it induces signalling in rat cells, this suggests it is able to initiate kinase phosphorylation, however may not be effective enough to sustain increased cell growth.

Since rat IGF-1 stimulates islet survival, and mouse HGF stimulates signalling in rat cells, the species differences are most likely not responsible for non-response, especially considering that between rat and mouse there is a 99% homology of IGF-1 and >91% homology of HGF. Cross species use of growth factors and cells has been shown before, for example, human IGF-1 with murine cells (Francki *et al.*, 2003). Growth factors elicit different cellular responses, and are context dependent. Stimulation of identical or overlapping kinase signalling does not necessarily lead to identical gene transcription (Sweeney *et al.*, 2001).

4.3 Proliferation and survival

Antagonists of cell-beneficial growth factor responses are potential targets for inhibition, with the intention of preventing β cell apoptosis and enhancing proliferation. SPARC is able to intervene in growth factor signalling cascades in various cell types (Kupprion *et al.*, 1998, Raines *et al.*, 1992, Chlenski *et al.*, 2006, Francki *et al.*, 2003), leading to the inhibition of proliferation and cell cycle progression (Francki *et al.*, 2003, Schiemann *et al.*, 2003, Funk and Sage, 1991) but also the prevention of apoptosis in some cases, particularly cancer cells (Shi *et al.*, 2004), indicating that SPARC actions are context dependent. After establishing that SPARC prevents signal activation in β cells, the present study examined whether it could inhibit proliferation in β cells.

The development of the SRB assay allowed growth of INS-1 cells to be determined in the right conditions. The correct seeding density allowed enough growth to calculate differences. Seeding too high, and therefore covering a lot of the well surface area, would not allow for much expansion in cell numbers before confluence was reached and a plateau in cell growth occurred. The correct amount of foetal bovine serum in the media

also needed to be determined. If the serum level was too low (less than 0.1%), the sensitive INS-1 cell line would not proliferate (with or without growth factor). If the serum levels were too high, cell growth was too rapid, and no effect of exogenous growth factor was seen. Cytokines and growth factors in the serum stimulate cell growth, and would have masked any effects on increased protein content produced by exogenous IGF-1 or HGF in these assays.

Addition of glucose to an amount equal to that already present in media, therefore doubling the glucose concentration to 22.2 mM, did not have any effect on INS-1 growth in comparison with the assay undertaken in normal amount of glucose (11.1 mM), when comparing control cell growth before addition of growth factor. As well as absence of growth stimulation, high glucose prevented the beneficial effect of IGF-1 on growth of the cells. Glucose is used by the β cell not only for growth, but as its biological function determines, it takes up glucose to stimulate insulin release. The lack of response may also be due to glucotoxicity, since β cells are very sensitive to glucose. Concentrations of glucose between 2 and 10 mM have been reported to be optimal for beneficial *in vitro* β cell effects, and concentrations over 10 mM are deleterious for β cell function and survival (Bensellam *et al.*, 2012). High glucose concentrations lead to the stimulation of pathways resulting in altered β cell differentiation and survival, as well as loss of insulin secretion. These processes include oxidative stress, endoplasmic reticulum stress, formation of advanced glycation end products and hypoxia (Bensellam *et al.*, 2012). Although the cell culture media used here contains 11.1 mM glucose, this is only slightly over the 10 mM limit reported here, so unlikely to be in the detrimental range, however doubling of the concentration to 22.2 mM is in the range causing glucotoxicity.

HGF did not stimulate cell growth. It is possible that HGF is not able to stimulate growth of INS-1 cells, however others have found HGF to stimulate INS-1 proliferation, using ^{3}H-

thymidine incorporation techniques (Gahr *et al.*, 2002). In their experiments, 1.375 nM HGF was used, so in this study, experiments in which 200 ng/ml HGF equates to 2.52 nM, and 2000 ng/ml is therefore 25.5 nM, an effect should have been observed. Stimulation of growth was also performed for 24 hours, thus these assays at 48 hours would have shown some positive result. This suggests the conditions were not right for induction of proliferation by HGF, and that possibly the HGF had degraded, either before use, or in the media, despite fresh HGF being used. It may also be due to species differences, with rat cells not responding to the mouse HGF, as described previously. However the ^{3}H-thymidine incorporation method may not directly translate to large changes in cell protein content, as measured here. Thymidine incorporation is a sensitive technique, measured on the single cell level, however for any change to be noticed in the technique presented here large numbers of cell divisions must occur. Since proliferation was detected after IGF-1 treatment, it may be likely that HGF only induces low levels of proliferation that are below the limits of detection using the SRB assay.

In the optimised experimental conditions for IGF-1, cells were treated with combinations of this growth factor and SPARC. SPARC acts in a concentration-dependent manner to inhibit INS-1 proliferation. At 20 μg/ml SPARC, IGF-1-induced INS-1 proliferation was inhibited by 59%. Acting without additional growth factors, SPARC alone had a slight inhibitory effect on INS-1 cells, reducing 2 day basal growth by 11%. This may be due to the actions of SPARC on the minimal amounts of growth factors present in the media, since these experiments were conducted with 0.5% serum. These results have great implications for SPARC as a moderator of β cell growth, suggesting that SPARC is potent enough to interact with the minimal amounts of growth factors or other components present in 0.5% serum in order to prevent their proliferative activity.

We wanted to replicate the proliferation experiments in a system more physiologically representative than an immortalised cell line, by using islets purified from mouse pancreas. Since β cells of the islet proliferate very slowly, long-term (months) assessment using bromodeoxyuridine assays are usually employed to measure rates of turnover which are as little as 1 in 1,400 cells replicating per day (Teta *et al.*, 2005). Other methods used include flow cytometry analysis of propidium iodide-stained cells to determine cell cycle stages (Guo *et al.*, 2011). However the aim of experiments here were to determine end result, *i.e.* the effect of SPARC on survival rather than whether it induced a halt in the cell cycle. For short-term assessment optimisation of an islet survival assay was necessary. Attempts were made to measure ATP amount in islets, however this gave inconsistent results (data not shown). A fluorescence assay measuring live and dead cells within the islet was therefore developed. This was first tested on intact islets in dishes, however it was apparent that the live and dead stains did not permeate to the middle of the islets (data not shown). To overcome this, dispersal of islets was necessary, and encouraging attachment onto coverslips allowed them to be visualised.

Attempts to induce cell death included incubation in media containing 0% serum as well as high amounts (33.3 mM) of glucose to induce glucotoxicity (Maedler *et al.*, 2002). After 1 day of treatment, the percentage of live cells using media containing 10% serum was 70%, and this did not change with no serum, or with high glucose, showing that dispersed islets are able to survive for 24 hours in these unfavourable conditions. After 2 days, however, cells without serum and in high glucose decreased the percentage of live cells by 28%, indicating that 2 days is required to induce cell death in these conditions.

Experiments were continued with the use of 0% serum to induce cell death, rather than high glucose, for practical purposes and also avoiding over complication of the experiments which high glucose may cause. Cell growth and survival induced by growth

factors requires the presence of moderate amounts of glucose and glucose is required by β cells for insulin production and release. However at the concentration used glucotoxicity would be induced, opposing these effects. Factors contributing to either cell survival or cell death could lead to inexplicable results from the experiments.

IGF-1 was successful at restoring cell survival from the 0% serum environment, demonstrating the potent effects it has on proliferation and prevention of apoptosis in these β cells. The concentration used (200 ng/ml, equivalent to 22.7 nM) was higher than used for signalling purposes in both the proliferation and survival experiments. It was also higher than has been used in other studies using either IGF-1 or HGF at 20 ng/ml to stimulate cell growth (Francki *et al.*, 2003, Vasavada *et al.*, 2006). A high concentration of IGF-1 was used in the present study to maximise effectiveness.

Variations in the control (10% serum) amount of growth between experiments were thought to be due to the experiments being conducted on different days, as well as the use of islets from different sets of animals. HGF did not stimulate cell survival, which is similar to results observed in the INS-1 proliferation experiments. It was expected that the mouse HGF would have stimulated an inhibition of apoptosis, considering the islets are of mouse origin also, however as described above it may depend on the reliability of the exogenous HGF added to the cells. When cMet is knocked out from β cells apoptosis occurs (Mellado-Gil *et al.*, 2011). Adenoviral delivery of HGF to islets improves graft survival and function when transplanted to diabetic mice (Fiaschi-Taesch *et al.*, 2008). Although these studies were set up differently and do not rely on recombinant HGF delivery to islets, it does show that HGF signalling is able to prevent islet cell apoptosis and improve islet function (insulin secretion). Because of this, it would suggest that it is the recombinant HGF that does not stimulate a response in the islet experiments

presented here. This may be due to degradation or low concentration. Future studies could involve the use of a different HGF, or altering the concentrations used.

Upon addition of recombinant SPARC in combination with IGF-1, SPARC prevented the recovery of the islet cells that IGF-1 induced. The inhibition of IGF-1-induced survival in dispersed islets by SPARC is consistent with the result of inhibition of INS-1 proliferation demonstrated *in vitro*, thus strengthening the rationale that SPARC, when provided to the cells exogenously, works to interact with growth factors and prevent their beneficial actions upon cells. This is also supported by the result showing that SPARC used alone had no significant effect on the proportion of live cells. In order to confirm the effect of SPARC on growth factor intervention, it would have been optimal to determine the effects of SPARC on more than one growth factor, however since no response was found by HGF this was not possible in this instance, and was not explored further.

SPARC is a secreted protein residing in the extracellular matrix and has influence in proper ECM maturation (Barker *et al.*, 2005), therefore experiments incorporating elements of the matrix allow a more physiological environment to be emulated. During islet transplantation, it has been noted that incomplete digestion of the pancreas is better for survival since it leaves some ECM intact with the islets (Thomas *et al.*, 1999). The co-culture environment used in the present study allows the secretion of SPARC by PS-1 cells into the culture to act upon islets. PS-1 cells, a stromal cell type, may also secrete the wide variety of extracellular components that pancreatic stellate cells express, such as collagens and fibronectin, and have been shown to express when in culture (Apte *et al.*, 1998, Bachem *et al.*, 1998, Bachem *et al.*, 2005).

When inserts were used to culture the PS-1 cells above the dispersed islet cells, the islets exhibited a good recovery from being cultured without serum. The percentage of cell death was almost recovered to control (10% serum) levels. In pancreatic cancer,

pancreatic stellate cells enhance tumour cell proliferation and migration resulting in metastasis (Erkan *et al.*, 2012, Hwang *et al.*, 2008, Vonlaufen *et al.*, 2008). The result here was expected since PS-1 cells secrete many factors conducive to cell survival, such as growth factors and structural components such as collagen. The presence of collagen (in particular type 1 collagen) in the ECM is necessary as a 3 dimensional scaffold in islet structure and anchoring (Jalili *et al.*, 2011, Wang and Rosenberg, 1999, Stendahl *et al.*, 2009). Fibronectin is important in cell anchoring and proper collagen deposition (Sottile *et al.*, 1998, Sottile *et al.*, 2007). Collagen matrix improves human islet survival and function, and moreover, fibroblast-populated collagen matrix (found to secrete growth factors and fibronectin) enhances islet survival, function (glucose-stimulated insulin secretion) and reduces the formation of islet amyloid aggregates (Jalili *et al.*, 2011, Zhang *et al.*, 2012). It is this provision of ECM components, including collagen and fibronectin, which may be the reason why increased islet survival was observed when PS-1 cells are present in co-culture experiments. The expression of collagen by PS-1 cells was not determined in experiments here, however pancreatic stellate cells have been shown previously to express collagens, including type I, III, as well as fibronectin, laminin and α-smooth muscle actin (Bachem *et al.*, 1998).

SPARC gene expression was silenced in PS-1 cells prior to co-culture, and knock down of the SPARC protein confirmed. After co-culture, the results showed no significant effect on islet cells, when compared to either the cells in 0% serum, or with PS-1 co-culture using control siRNA. Although not significant, a drop in the mean survival of islet cells from the control siRNA result (a decrease of 71% of the PS-1 induced survival) was observed, indicating that SPARC might be secreted from the PS-1 cells and is necessary for islet survival. Further experiments incorporating a more extensive study would be required to verify this result. Since the addition of recombinant SPARC to islets had the opposite

effect, it is possible that SPARC secreted from PS-1 cells may modulate factors in the environment rather than directly on the islets themselves to induce survival. SPARC may also act upon the PS-1 cells themselves to help them promote islet survival indirectly. It is likely that PS-1 derived SPARC has differences to recombinant SPARC. Although they are both human derived and both show a band at 43 kDa on western blot, there are probable differences in processing with different glycosylations and splice variants.

Other factors produced in the PS-1 co-culture environment may be the reason for the opposing actions of PS-1 derived SPARC and recombinant SPARC, since they are not present in the culture without PS-1 cells. SPARC has high affinity for type 1 collagen, regulates procollagen processing and collagen fibrillogenesis and allows the proper deposition of collagen within tissues (Giudici *et al.*, 2008, Termine *et al.*, 1981, Chavez-Munoz *et al.*, 2012, Rentz *et al.*, 2007). SPARC also binds and regulates deposition of fibronectin, important in cell adhesion and anchoring cells to collagen (Sangaletti *et al.*, 2008). If there is a requirement for SPARC to be present for the proper functioning of collagen, fibronectin or other structural components, this could explain why the number of dead cells increased in this system, since β cell lines in culture rely on proper collagen deposition for improved adhesion, spreading, cell viability, proliferation, and a decrease in apoptosis (Krishnamurthy *et al.*, 2008, Weber *et al.*, 2008). It is possible that in the co-culture, SPARC does not have a direct effect on islets, but in fact, is necessary for proper collagen formation, however further work would need to be done to confirm the presence of collagens (and fibronectin, laminin) and ensure proper fibrillogenesis of these.

SPARC is important for collagen regulation in the periodontal ligament (PDL) (Trombetta and Bradshaw, 2010). In young PDL, high levels of SPARC expression are found, which decreases as it develops. In the SPARC-null mice, fewer collagen fibres and reduced fibre

thickness were found. The presence of SPARC is important in collagen deposition. In a SPARC-null environment collagen bound to collagen receptors favoured phagocytosis of the collagen and/or increased degradation by pericellular collagenase activity (Rentz *et al.*, 2007). SPARC does not target collagen for degradation, but is necessary for the proper laying down of collagen in ECM construction. This property of SPARC has been found to prevent pancreatic tumour growth. In SPARC null mice, decreased collagen deposition and fibre formation results in decreased tumour cell apoptosis and faster tumour growth that in an environment where SPARC is present (Puolakkainen *et al.*, 2004).

Differences in cell responses to SPARC may also be explained by receptors for SPARC that are currently undiscovered. Another non-structural matricellular protein, thrombospondin-1, is known to have context-dependent opposing effects, which was later discovered to be due to different receptor types causing activation of different cell signalling pathways. Different signals may be required to activate each receptor, and activation of many receptors may have a negative effect on the transduction pathway (Roberts, 2011).

The IGF-1 in the co-culture (with PS-1 cells with either the control or the SPARC siRNA) did not exert any effect on the islet cells, most likely due to being masked by growth factors produced from the PS-1 cells themselves. In a complex co-culture system it is difficult to determine how exactly the co-culture benefits islets. Davis *et al.* (2012) encapsulated islets with mesenchymal stromal cells and discussed the possibility that addition of trophic factors or immunosuppressive agents (decreasing inflammation and immunogenicity) secreted by stromal cells may be the reason for enhanced insulin secretion. The secretion of growth factors and fibronectin by fibroblasts may be the reason PS-1 cells induce β cell survival, and may also be the reason why enhanced survival rates were not seen when adding IGF-1 to the PS-1 culture environment.

4.4 Roles for SPARC

SPARC was found to be located in areas corresponding to stromal cells in islet sections, and SPARC was more highly expressed in wild type pancreas in earlier stages of development, rather than later. SPARC was also more highly expressed in the NOD mouse pancreas compared to wild type controls, suggesting that SPARC may be involved in susceptibility to diabetes.

It is possible that if the chromosomal position of the SPARC gene is near to diabetes susceptibility genes they may both be regulated and the expression of SPARC may contribute to onset of diabetes. For type 1 diabetes, there are more than 40 genetic loci discovered, with the strongest genetic factor, MHC class II lying on chromosome 6 (Noble and Erlich, 2012). Human SPARC is localised to chromosome 5q31-33. The nearest genes found to have an involvement in diabetes include a locus on chromosome 5q that contains a gene that contributes to the development of T2D in non-obese Icelanders, and 5q11-q13, which confers susceptibility for type 1 diabetes in Scandinavians. This was thought to be insulin enhancer binding protein 1 (ISL1), a transcription factor important during the development of the pancreas (Reynisdottir *et al.*, 2003, Holm *et al.*, 2004). It is possible that SPARC and diabetes genes, in close proximity, may be regulated by a common factor, giving susceptibility to type 1 or type 2 diabetes and inducing over-expression of SPARC, however this is highly speculative. Although most diabetes susceptibility genes are assumed to exert their effect in the immune system and not in the islet, the gene ISL1 and SPARC may both be involved in pancreas development and contribute to type 1 diabetes onset.

The expression of SPARC at early age, and higher expression in NOD mice suggest the inhibition of cellular growth at a turning point in the time of diabetes progression, and

from what is already known about SPARC, it does this to promote rearrangements in the tissue architecture. SPARC binds to VCAM-1 on endothelial cells, and is essential in leukocyte transmigration (Kelly *et al.*, 2007), thus SPARC could facilitate the infiltration of inflammatory cells which lead to the progression of insulitis and diabetes. The targeting and inhibition of SPARC may therefore slow this progression.

Certain properties of SPARC strongly suggest a role for SPARC in the adhesion state of cells during their movement through the ECM. These properties are the effect of SPARC on cell migration, location in the extracellular matrix, involvement in collagen binding and its expression during early developmental stages as well as during states of tissue regeneration and remodelling (Sage *et al.*, 1989a, Sage *et al.*, 1989b). SPARC may play a role in preventing the strong adhesion state of cells, only allowing an intermediate state of adhesion where focal adhesions and stress fibres are not formed (Murphy-Ullrich, 2001). The β cells of the islet may undergo de-attachment when under attack by cells of the immune system during onset of diabetes. If this is the case SPARC may have some involvement in β cell mobilisation and possibly β cell death.

Stabilin-1 is a receptor for SPARC and targets SPARC for degradation. It is expressed only by cell types specialized for clearance, tolerance induction, and modulation of tissue remodelling, such as macrophages (Kzhyshkowska *et al.*, 2006b). Macrophages can resolve inflammation and promote angiogenesis. This could be due to the internalisation of SPARC by stabilin-1, thereby removing it from the extracellular environment, ensuring the amount of SPARC present is at the required concentration for the tissue's current needs. In a diabetic environment a failure of this regulation of SPARC could lead to further disruption of the β cell environment and cause β cell loss.

Expression of SPARC may allow proper collagen and matrix formation for the survival of islets (although data presented here was not statistically significant). In environments

without fibroblast type cells, exogenous SPARC has inhibitory effects on growth factor-induced β cell proliferation and signalling. SPARC modulated growth factor signalling in a negative fashion, inhibiting activation of Akt and ERK kinase pathways by the growth factors IGF-1 and HGF. SPARC inhibits IGF-1 induced proliferation of INS-1 cells, and SPARC prevents IGF-1 induced islet survival.

4.4 Conclusions

SPARC acts upon β cells preventing growth factor-induced signalling resulting in inhibited proliferation and survival. It may be possible to promote the survival of β cells prior to, during, or after the transplantation procedure for diabetic patients by co-culturing the implants with stromal cells beneficial for cell growth and survival, however with controlled SPARC expression. It has been demonstrated that stromal cells promote survival in islets. Further studies will be required to ascertain whether SPARC secreted by stromal cells has a beneficial effect. However it seems that SPARC has a detrimental effect on β cell lines and dispersed islet cells, expressed early in diabetic progression thus targeting SPARC and preventing its actions may be a promising tool in future diabetes therapy.

4.5 Future work

The studies conducted here show that although SPARC can inhibit some growth factor-induced proliferation and signalling, there is a requirement for further investigation into whether SPARC can inhibit the action of HGF, by resolving the issues found by the non-response to HGF in the proliferation and survival assays.

Elucidating the point of the growth factor signalling pathways in which SPARC acts by using inhibitors to specific signalling kinases, and use of other growth factors altogether would give us a better understanding of how SPARC interacts with the β cell. Altering the pre-incubation time with SPARC, would also give more information about how SPARC interacts with growth factors. Studies with integrins would allow us to demonstrate interaction between integrin and growth factor pathways in β cells. We could also optimise use of fluorescent antibodies and determine if SPARC enters the β cell, or remains extracellular to the cell. If SPARC enters the β cell, we could determine if it enters the nucleus, which may mean that it acts directly on transcription factors (although the results demonstrate it acts more upstream in the signalling pathway).

In the islet survival assay with PS-1 co-culture in which SPARC had been knocked down, it would be interesting to determine the effects upon addition of recombinant SPARC into the culture system. This could reinforce the prevention of survival and may even induce a further drop in live cells, since recombinant SPARC prevents survival. The alternate outcome, since endogenous PS-1 SPARC expression is possibly necessary for islet survival, would mean an increase in survival rate due to the combination of other factors and structural components secreted by PS-1 cells necessary for islet survival. It would be interesting to determine the effects on islet survival in the presence of other extracellular matrix components such as collagen, and determine whether addition of exogenous SPARC increases or decreases islet survival.

The effect of SPARC on the PS-1 cells themselves was not examined. If SPARC has a beneficial effect on PS-1 cells, and indirectly influences the secretion of more SPARC (through the increase in the PS-1 cell population), this may be detrimental to islets. SPARC may have a negative feedback on PS-1 cells, thus reducing secretion of more SPARC over time. Experiments measuring amounts of SPARC in media over time in the co-culture set

up along with proliferation experiments on PS-1 cells may allow us to determine whether SPARC has influence on its own secretion. In a diabetic environment, SPARC positively regulating itself could accelerate the onset of diabetes.

The use of antibodies could inform more about presence and function of SPARC. In the culture system, if SPARC antibodies were added to the culture in which endogenous SPARC plays a role, in order to prevent the action of endogenous SPARC, we could then determine whether SPARC in that environment does have a beneficial effect. Antibodies to collagen or fibronectin, or perhaps use of the relevant proteases might also tell us more information. However since the proteases that degrade extracellular matrix components (matrix metalloproteinases; MMPs) also have affinity for SPARC as a substrate, e.g. MMP3 (Sage *et al.*, 2003), this needs to be taken into consideration.

Experiments were conducted to determine the proliferation, signalling and survival of β cells, however we did not take into account any altering of β cell function. In order to draw reasonable conclusions from these studies on the use of SPARC for targeting purposes, the β cell secretion of insulin, and specifically, secretion in response to higher glucose levels must be tested after SPARC treatment. Long-lasting insulin secretion (in response to a glucose stimulus) is a priority when considering *in vivo* experimentation of β cells and islet transplantation.

A significant series of experiments would involve *in vivo* testing of our hypotheses. If SPARC prevents growth and survival in β cells as shown, knockdown of SPARC by retrovirus injection at sites of diabetic onset (sites of leukocyte infiltration) in NOD mice would promote the survival of β cells, and this could be used during transplantation procedures also. Use of co-transplantation with PS-1 cells would be conducive, especially including studies involving PS-1 cells not expressing SPARC. If endogenous SPARC is required for islet survival, ways in which we could increase endogenous SPARC production

in stromal cell co-transplantation (constitutive expression, or high proportions of stromal cells transplanted), or inhibiting its degradation (inhibition of certain MMPs) would be valuable to promote β cell survival and potentially have the capacity to delay diabetic onset.

Therapeutic possibilities of SPARC could include *ex vivo* gene transfer of downregulators of SPARC. Use of an adenovirus vector to transfer genes for inhibitors of SPARC (for example, antibodies), or disrupting the promoter region of SPARC or mutating the SPARC gene would decrease the amount of SPARC transcribed. If regulators of SPARC gene transcription are discovered, these could be targeted for the same outcome, leading to a decrease in SPARC expression. Antisense therapy by silencing the RNA is also an option, although this is often short-lived and improvements in duration are required for it to be an effective treatment. With targeted gene therapy expression of SPARC can be decreased. Use of this in human islets prior to transplant may be particularly useful in improving islet transplant performance and reducing the number of islets required for individuals with Type 1 diabetes. Improvement in insulin secretion and regulation of blood glucose levels without concern for hypoglycaemic episodes leads to huge improvements in the quality of life for someone with diabetes.

References

Abdullah, A., Peeters, A., De Courten, M. & Stoelwinder, J. (2010). The magnitude of association between overweight and obesity and the risk of diabetes: A meta-analysis of prospective cohort studies. *Diabetes Res Clin Pract,* **89,** 309-319.

Alpers, C. E., Hudkins, K. L., Segerer, S., Sage, E. H., Pichler, R., Couser, W. G., Johnson, R. J. & Bassuk, J. A. (2002). Localization of SPARC in developing, mature, and chronically injured human allograft kidneys. *Kidney Int,* **62,** 2073-86.

American Diabetes Association (2000). Type 2 diabetes in children and adolescents. *Diabetes Care,* **23,** 381-389.

Anderson, M. S. & Bluestone, J. A. (2005). The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol,* **23,** 447-85.

Apte, M. V., Haber, P. S., Applegate, T. L., Norton, I. D., Mccaughan, G. W., Korsten, M. A., Pirola, R. C. & Wilson, J. S. (1998). Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut,* **43,** 128-133.

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A. & Wollheim, C. B. (1992). Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology,* **130,** 167-78.

Ashcroft, F. M. & Rorsman, P. (2012). Diabetes Mellitus and the β Cell: The Last Ten Years. *Cell,* **148,** 1160-1171.

Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. L. & Tzukerman, M. (2001). Insulin Production by Human Embryonic Stem Cells. *Diabetes,* **50,** 1691-1697.

Assmann, A., Ueki, K., Winnay, J. N., Kadowaki, T. & Kulkarni, R. N. (2009). Glucose effects on beta-cell growth and survival require activation of insulin receptors and insulin receptor substrate 2. *Mol Cell Biol,* **29,** 3219-28.

Aviles-Santa, L., Salinas, K., Adams-Huet, B. & Raskin, P. (2006). Insulin therapy, glycemic control, and cardiovascular risk factors in young Latin Americans with type 2 diabetes mellitus. *J Investig Med,* **54,** 20-31.

Avram, D., Ranta, F., Hennige, A. M., Berchtold, S., Hopp, S., Haring, H. U., Lang, F. & Ullrich, S. (2008). IGF-1 protects against dexamethasone-induced cell death in insulin secreting INS-1 cells independent of AKT/PKB phosphorylation. *Cell Physiol Biochem,* **21,** 455-62.

Bach, J. F. & Chatenoud, L. (2012). The hygiene hypothesis: an explanation for the increased frequency of insulin-dependent diabetes. *Cold Spring Harb Perspect Med,* **2,** a007799.

Bachem, M. G., Schneider, E., Gross, H., Weidenbach, H., Schmid, R. M., Menke, A., Siech, M., Beger, H., Grunert, A. & Adler, G. (1998). Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology,* **115,** 421-32.

Bachem, M. G., Schunemann, M., Ramadani, M., Siech, M., Beger, H., Buck, A., Zhou, S., Schmid-Kotsas, A. & Adler, G. (2005). Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology,* **128,** 907-21.

Baker, D. E., Harrison, N. J., Maltby, E., Smith, K., Moore, H. D., Shaw, P. J., Heath, P. R., Holden, H. & Andrews, P. W. (2007). Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol,* **25,** 207-15.

Bakhtiani, P. A., Zhao, L. M., El Youssef, J., Castle, J. R. & Ward, W. K. (2013). A review of artificial pancreas technologies with an emphasis on bi-hormonal therapy. *Diabetes Obes Metab.*

Barker, T. H., Baneyx, G., Cardo-Vila, M., Workman, G. A., Weaver, M., Menon, P. M., Dedhar, S., Rempel, S. A., Arap, W., Pasqualini, R., Vogel, V. & Sage, E. H. (2005). SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity. *J Biol Chem,* **280,** 36483-93.

Barnard, R. J., Lattimore, L., Holly, R. G., Cherny, S. & Pritikin, N. (1982). Response of Non-insulin-dependent Diabetic Patients to an Intensive Program of Diet and Exercise. *Diabetes Care,* **5,** 370-374.

Barros, R. P., Morani, A., Moriscot, A. & Machado, U. F. (2008). Insulin resistance of pregnancy involves estrogen-induced repression of muscle GLUT4. *Mol Cell Endocrinol,* **295,** 24-31.

Barton, F. B., Rickels, M. R., Alejandro, R., Hering, B. J., Wease, S., Naziruddin, B., Oberholzer, J., Odorico, J. S., Garfinkel, M. R., Levy, M., Pattou, F., Berney, T., Secchi, A., Messinger, S., Senior, P. A., Maffi, P., Posselt, A., Stock, P. G., Kaufman, D. B., Luo, X., Kandeel, F., Cagliero, E., Turgeon, N. A., Witkowski, P., Naji, A., O'connell, P. J., Greenbaum, C., Kudva, Y. C., Brayman, K. L., Aull, M. J., Larsen, C., Kay, T. W., Fernandez, L. A., Vantyghem, M. C., Bellin, M. & Shapiro, A. M. (2012). Improvement in outcomes of clinical islet transplantation: 1999-2010. *Diabetes Care,* **35,** 1436-45.

Bason, C., Lorini, R., Lunardi, C., Dolcino, M., Giannattasio, A., D'annunzio, G., Rigo, A., Pedemonte, N., Corrocher, R. & Puccetti, A. (2013). In Type 1 Diabetes a Subset of Anti-Coxsackievirus B4 Antibodies Recognize Autoantigens and Induce Apoptosis of Pancreatic Beta Cells. *PLoS One,* **8,** e57729.

Basu, A., Kligman, L. H., Samulewicz, S. J. & Howe, C. C. (2001). Impaired wound healing in mice deficient in a matricellular protein SPARC (osteonectin, BM-40). *BMC Cell Biol,* **2,** 15.

Beattie, G. M., Cirulli, V., Lopez, A. D. & Hayek, A. (1997). Ex vivo expansion of human pancreatic endocrine cells. *J Clin Endocrinol Metab,* **82,** 1852-6.

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

Beattie, G. M., Rubin, J. S., Mally, M. I., Otonkoski, T. & Hayek, A. (1996). Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes,* **45,** 1223-8.

Beckman, J. A., Creager, M. A., Libby, P. (2002). Diabetes and atherosclerosis: Epidemiology, pathophysiology, and management. *JAMA,* **287,** 2570-2581.

Beglinger, C., Taylor, I. L., Grossman, M. I. & Solomon, T. E. (1984). Pancreatic polypeptide inhibits exocrine pancreatic responses to six stimulants. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* **246,** G286-G291.

Bellamy, L., Casas, J. P., Hingorani, A. D. & Williams, D. (2009). Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet,* **373,** 1773-9.

Ben-Ami, H., Nagachandran, P., Mendelson, A., Edoute, Y. (1999). Drug-induced hypoglycemic coma in 102 diabetic patients. *Archives of Internal Medicine,* **159,** 281-284.

Bendtzen, K., Mandrup-Poulsen, T., Nerup, J., Nielsen, J. H., Dinarello, C. A. & Svenson, M. (1986). Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. *Science,* **232,** 1545-7.

Bennet, W., Bjorkland, A., Sundberg, B., Davies, H., Liu, J., Holgersson, J. & Korsgren, O. (2000a). A comparison of fetal and adult porcine islets with regard to Gal alpha (1,3)Gal expression and the role of human immunoglobulins and complement in islet cell cytotoxicity. *Transplantation,* **69,** 1711-7.

Bennet, W., Groth, C. G., Larsson, R., Nilsson, B. & Korsgren, O. (2000b). Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci,* **105,** 125-33.

Benseliam, M., Laybutt, D. R. & Jonas, J. C. (2012). The molecular mechanisms of pancreatic beta-cell glucotoxicity: recent findings and future research directions. *Mol Cell Endocrinol,* **364,** 1-27.

Bernal-Mizrachi, E., Wen, W., Stahlhut, S., Welling, C. M. & Permutt, M. A. (2001). Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest,* **108,** 1631-8.

Bertera, S., Balamurugan, A. N., Bottino, R., He, J. & Trucco, M. (2012). Increased yield and improved transplantation outcome of mouse islets with bovine serum albumin. *J Transplant,* **2012,** 856386.

Billestrup, N. & Nielsen, J. H. (1991). The stimulatory effect of growth hormone, prolactin, and placental lactogen on beta-cell proliferation is not mediated by insulin-like growth factor-I. *Endocrinology,* **129,** 883-8.

Blomeier, H., Zhang, X., Rives, C., Brissova, M., Hughes, E., Baker, M., Powers, A. C., Kaufman, D. B., Shea, L. D. & Lowe, W. L., Jr. (2006). Polymer scaffolds as synthetic microenvironments for extrahepatic islet transplantation. *Transplantation,* **82,** 452-9.

Bodansky, H. J., Staines, A., Stephenson, C., Haigh, D. & Cartwright, R. (1992). Evidence for an environmental effect in the aetiology of insulin dependent diabetes in a transmigratory population. *BMJ,* **304,** 1020-1022.

Bodhini, D., Radha, V., Ghosh, S., Sanapala, K. R., Majumder, P. P., Rao, M. R. & Mohan, V. (2011). Association of calpain 10 gene polymorphisms with type 2 diabetes mellitus in Southern Indians. *Metabolism,* **60,** 681-8.

Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Vande Woude, G. F. & Aaronson, S. A. (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science,* **251,** 802-4.

Bouchoucha, M., Uzzan, B. & Cohen, R. (2011). Metformin and digestive disorders. *Diabetes & Metabolism,* **37,** 90-96.

Brackenridge, A. L., Jackson, N., Jefferson, W., Stolinski, M., Shojaee-Moradie, F., Hovorka, R., Umpleby, A. M. & Russell-Jones, D. (2009). Effects of rosiglitazone and pioglitazone on lipoprotein metabolism in patients with Type 2 diabetes and normal lipids. *Diabet Med,* **26,** 532-9.

Bradshaw, A. D., Francki, A., Motamed, K., Howe, C. & Sage, E. H. (1999). Primary mesenchymal cells isolated from SPARC-null mice exhibit altered morphology and rates of proliferation. *Mol Biol Cell,* **10,** 1569-79.

Bradshaw, A. D., Graves, D. C., Motamed, K. & Sage, E. H. (2003a). SPARC-null mice exhibit increased adiposity without significant differences in overall body weight. *Proc Natl Acad Sci U S A,* **100,** 6045-50.

Bradshaw, A. D., Puolakkainen, P., Dasgupta, J., Davidson, J. M., Wight, T. N. & Helene Sage, E. (2003b). SPARC-null mice display abnormalities in the dermis characterized by decreased collagen fibril diameter and reduced tensile strength. *J Invest Dermatol,* **120,** 949-55.

Brekken, R. A., Puolakkainen, P., Graves, D. C., Workman, G., Lubkin, S. R. & Sage, E. H. (2003). Enhanced growth of tumors in SPARC null mice is associated with changes in the ECM. *J Clin Invest,* **111,** 487-95.

Brekken, R. A. & Sage, E. H. (2001). SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol,* **19,** 816-27.

Brelje, T. C., Scharp, D. W., Lacy, P. E., Ogren, L., Talamantes, F., Robertson, M., Friesen, H. G. & Sorenson, R. L. (1993). Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology,* **132,** 879-87.

Breton, M., Farret, A., Bruttomesso, D., Anderson, S., Magni, L., Patek, S., Dalla Man, C., Place, J., Demartini, S., Del Favero, S., Toffanin, C., Hughes-Karvetski, C., Dassau, E., Zisser, H., Doyle, F. J., 3rd, De Nicolao, G., Avogaro, A., Cobelli, C., Renard, E. & Kovatchev, B. (2012). Fully integrated artificial pancreas in type 1 diabetes: modular closed-loop glucose control maintains near normoglycemia. *Diabetes,* **61,** 2230-7.

Briaud, I., Lingohr, M. K., Dickson, L. M., Wrede, C. E. & Rhodes, C. J. (2003). Differential activation mechanisms of Erk-1/2 and p70(S6K) by glucose in pancreatic beta-cells. *Diabetes,* **52,** 974-83.

Briggs, J., Chamboredon, S., Castellazzi, M., Kerry, J. A. & Bos, T. J. (2002). Transcriptional upregulation of SPARC, in response to c-Jun overexpression, contributes to increased motility and invasion of MCF7 breast cancer cells. *Oncogene,* **21,** 7077-91.

Brissova, M., Fowler, M. J., Nicholson, W. E., Chu, A., Hirshberg, B., Harlan, D. M. & Powers, A. C. (2005). Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem,* **53,** 1087-97.

Brissova, M. & Powers, A. C. (2008). Revascularization of transplanted islets: can it be improved? *Diabetes,* **57,** 2269-71.

Bucher, P., Mathe, Z., Bosco, D., Becker, C., Kessler, L., Greget, M., Benhamou, P. Y., Andres, A., Oberholzer, J., Buhler, L., Morel, P. & Berney, T. (2004). Morbidity associated with intraportal islet transplantation. *Transplant Proc,* **36,** 1119-20.

Butler, A. E., Galasso, R., Meier, J. J., Basu, R., Rizza, R. A. & Butler, P. C. (2007). Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. *Diabetologia,* **50,** 2323-31.

Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J. & Shoelson, S. E. (2005). Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med,* **11,** 183-90.

Calafiore, R. (1992). Transplantation of microencapsulated pancreatic human islets for therapy of diabetes mellitus. A preliminary report. *ASAIO J,* **38,** 34-7.

Cancerresearchuk.Org. (2009). *Pancreatic cancer Key Facts : Cancer Research UK* [Online]. Available: http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/pancreatic-cancer/#incidence [Accessed 08/04/2013].

Chavez-Munoz, C., Hartwell, R., Jalili, R. B., Jafarnejad, S. M., Lai, A., Nabai, L., Ghaffari, A., Hojabrpour, P., Kanaan, N., Duronio, V., Guns, E., Cherkasov, A. & Ghahary, A. (2012). SPARC/SFN interaction, suppresses type I collagen in dermal fibroblasts. *J Cell Biochem,* **113,** 2622-32.

Chen, J., Shi, D., Liu, X., Fang, S., Zhang, J. & Zhao, Y. (2012). Targeting SPARC by lentivirus-mediated RNA interference inhibits cervical cancer cell growth and metastasis. *BMC Cancer,* **12,** 464.

Chen, R. H., Sarnecki, C. & Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol Cell Biol,* **12,** 915-27.

Cheng, K., Delghingaro-Augusto, V., Nolan, C. J., Turner, N., Hallahan, N., Andrikopoulos, S. & Gunton, J. E. (2012). High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. *PLoS One,* **7,** e40868.

Chew, A., Salama, P., Robbshaw, A., Klopcic, B., Zeps, N., Platell, C. & Lawrance, I. C. (2011). SPARC, FOXP3, CD8 and CD45 correlation with disease recurrence and long-term disease-free survival in colorectal cancer. *PLoS One,* **6,** e22047.

Chlenski, A. & Cohn, S. L. (2010). Modulation of matrix remodeling by SPARC in neoplastic progression. *Semin Cell Dev Biol,* **21,** 55-65.

Chlenski, A., Guerrero, L. J., Peddinti, R., Spitz, J. A., Leonhardt, P. T., Yang, Q., Tian, Y., Salwen, H. R. & Cohn, S. L. (2010). Anti-angiogenic SPARC peptides inhibit progression of neuroblastoma tumors. *Mol Cancer,* **9,** 138.

Chlenski, A., Liu, S., Guerrero, L. J., Yang, Q., Tian, Y., Salwen, H. R., Zage, P. & Cohn, S. L. (2006). SPARC expression is associated with impaired tumor growth, inhibited angiogenesis and changes in the extracellular matrix. *Int J Cancer,* **118,** 310-6.

Choudhary, P., Parrott, N. R., Birtles, L. & Rutter, M. K. (2012). Islet cell transplantation: current status in the UK. *Practical Diabetes,* **29,** 280-285.

Chung, W. S., Shin, C. H. & Stainier, D. Y. (2008). Bmp2 signaling regulates the hepatic versus pancreatic fate decision. *Dev Cell,* **15,** 738-48.

Citregistry.Org. (2010). *CITR Reports 2010* [Online]. Available: http://www.citregistry.org/ [Accessed 01/05/2013].

Cnop, M., Hughes, S. J., Igoillo-Esteve, M., Hoppa, M. B., Sayyed, F., Van De Laar, L., Gunter, J. H., De Koning, E. J., Walls, G. V., Gray, D. W., Johnson, P. R., Hansen, B. C., Morris, J. F., Pipeleers-Marichal, M., Cnop, I. & Clark, A. (2010). The long lifespan and low turnover of human islet beta cells estimated by mathematical modelling of lipofuscin accumulation. *Diabetologia,* **53,** 321-30.

Cobelli, C., Renard, E., Kovatchev, B. P., Keith-Hynes, P., Ben Brahim, N., Place, J., Del Favero, S., Breton, M., Farret, A., Bruttomesso, D., Dassau, E., Zisser, H., Doyle, F. J., 3rd, Patek, S. D. & Avogaro, A. (2012). Pilot studies of wearable outpatient artificial pancreas in type 1 diabetes. *Diabetes Care,* **35,** e65-7.

Cooper, J. D., Smyth, D. J., Walker, N. M., Stevens, H., Burren, O. S., Wallace, C., Greissl, C., Ramos-Lopez, E., Hyppönen, E., Dunger, D. B., Spector, T. D., Ouwehand, W. H., Wang, T. J., Badenhoop, K. & Todd, J. A. (2011). Inherited Variation in Vitamin D Genes Is Associated With Predisposition to Autoimmune Disease Type 1 Diabetes. *Diabetes,* **60,** 1624-1631.

D'amour, K. A., Bang, A. G., Eliazer, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K. & Baetge, E. E. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol,* **24,** 1392-401.

D'adamo, E. & Caprio, S. (2011). Type 2 Diabetes in Youth: Epidemiology and Pathophysiology. *Diabetes Care,* **34,** S161-S165.

Dalla-Torre, C. A., Yoshimoto, M., Lee, C. H., Joshua, A. M., De Toledo, S. R., Petrilli, A. S., Andrade, J. A., Chilton-Macneill, S., Zielenska, M. & Squire, J. A. (2006). Effects of THBS3, SPARC and SPP1 expression on biological behavior and survival in patients with osteosarcoma. *BMC Cancer,* **6,** 237.

Danaei, G., Finucane, M. M., Lu, Y., Singh, G. M., Cowan, M. J., Paciorek, C. J., Lin, J. K., Farzadfar, F., Khang, Y. H., Stevens, G. A., Rao, M., Ali, M. K., Riley, L. M., Robinson, C. A. & Ezzati, M. (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet,* **378,** 31-40.

Daoud, J., Petropavlovskaia, M., Rosenberg, L. & Tabrizian, M. (2010). The effect of extracellular matrix components on the preservation of human islet function in vitro. *Biomaterials,* **31,** 1676-82.

Daoud, J. T., Petropavlovskaia, M. S., Patapas, J. M., Degrandpre, C. E., Diraddo, R. W., Rosenberg, L. & Tabrizian, M. (2011). Long-term in vitro human pancreatic islet culture using three-dimensional microfabricated scaffolds. *Biomaterials,* **32,** 1536-42.

Davis, N. E., Beenken-Rothkopf, L. N., Mirsoian, A., Kojic, N., Kaplan, D. L., Barron, A. E. & Fontaine, M. J. (2012). Enhanced function of pancreatic islets co-encapsulated with ECM proteins and mesenchymal stromal cells in a silk hydrogel. *Biomaterials,* **33,** 6691-7.

Delany, A. M., Amling, M., Priemel, M., Howe, C., Baron, R. & Canalis, E. (2000). Osteopenia and decreased bone formation in osteonectin-deficient mice. *J Clin Invest,* **105,** 1325.

Desai, N. M., Goss, J. A., Deng, S., Wolf, B. A., Markmann, E., Palanjian, M., Shock, A. P., Feliciano, S., Brunicardi, F. C., Barker, C. F., Naji, A. & Markmann, J. F. (2003). Elevated portal vein drug levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? *Transplantation,* **76,** 1623-5.

Desai, N. P., Trieu, V., Hwang, L. Y., Wu, R., Soon-Shiong, P. & Gradishar, W. J. (2008). Improved effectiveness of nanoparticle albumin-bound (nab) paclitaxel versus polysorbate-based docetaxel in multiple xenografts as a function of HER2 and SPARC status. *Anticancer Drugs,* **19,** 899-909.

Deutsch, G., Jung, J., Zheng, M., Lora, J. & Zaret, K. S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development,* **128,** 871-81.

Dhayal, S. & Morgan, N. G. (2011). Pharmacological characterization of the cytoprotective effects of polyunsaturated fatty acids in insulin-secreting BRIN-BD11 cells. *Br J Pharmacol,* **162,** 1340-50.

Dhayat, S., Mardin, W. A., Mees, S. T. & Haier, J. (2011). Epigenetic markers for chemosensitivity and chemoresistance in pancreatic cancer--a review. *Int J Cancer,* **129,** 1031-41.

Diabetes.Org.Uk. (2012). *Diabetes prevalence 2012 (March 2013) - Diabetes UK* [Online]. Available: http://www.diabetes.org.uk/Professionals/Publications-reports-and-resources/Reports-statistics-and-case-studies/Reports/Diabetes-prevalence-2012-March-2013/ [Accessed 22/03/2013].

Dickson, L. M., Lingohr, M. K., Mccuaig, J., Hugl, S. R., Snow, L., Kahn, B. B., Myers, M. G., Jr. & Rhodes, C. J. (2001). Differential activation of protein kinase B and p70(S6)K by glucose and insulin-like growth factor 1 in pancreatic beta-cells (INS-1). *J Biol Chem,* **276,** 21110-20.

Donath, M. Y., Ehses, J. A., Maedler, K., Schumann, D. M., Ellingsgaard, H., Eppler, E. & Reinecke, M. (2005). Mechanisms of beta-cell death in type 2 diabetes. *Diabetes,* **54 Suppl 2,** S108-13.

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

Drachenberg, C. B., Klassen, D. K., Weir, M. R., Wiland, A., Fink, J. C., Bartlett, S. T., Cangro, C. B., Blahut, S. & Papadimitriou, J. C. (1999). Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation,* **68,** 396-402.

Dror, Y. & Freedman, M. H. (2002). Shwachman–Diamond Syndrome. *British Journal of Haematology,* **118,** 701-713.

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R. & Greenberg, M. E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science,* **275,** 661-5.

Edlund, H. (2002). Pancreatic organogenesis--developmental mechanisms and implications for therapy. *Nat Rev Genet,* **3,** 524-32.

El-Khatib, F. H., Russell, S. J., Nathan, D. M., Sutherlin, R. G. & Damiano, E. R. (2010). A bihormonal closed-loop artificial pancreas for type 1 diabetes. *Sci Transl Med,* **2,** 27ra27.

Elghazi, L., Rachdi, L., Weiss, A. J., Cras-Meneur, C. & Bernal-Mizrachi, E. (2007). Regulation of beta-cell mass and function by the Akt/protein kinase B signalling pathway. *Diabetes Obes Metab,* **9 Suppl 2,** 147-57.

Engel, J., Taylor, W., Paulsson, M., Sage, H. & Hogan, B. (1987). Calcium binding domains and calcium-induced conformational transition of SPARC/BM-40/osteonectin, an extracellular glycoprotein expressed in mineralized and nonmineralized tissues. *Biochemistry,* **26,** 6958-65.

Erkan, M., Adler, G., Apte, M. V., Bachem, M. G., Buchholz, M., Detlefsen, S., Esposito, I., Friess, H., Gress, T. M., Habisch, H. J., Hwang, R. F., Jaster, R., Kleeff, J., Kloppel, G., Kordes, C., Logsdon, C. D., Masamune, A., Michalski, C. W., Oh, J., Phillips, P. A., Pinzani, M., Reiser-Erkan, C., Tsukamoto, H. & Wilson, J. (2012). StellaTUM: current consensus and discussion on pancreatic stellate cell research. *Gut,* **61,** 172-8.

Eurodiab, S. S. G. (2002). Rapid early growth is associated with increased risk of childhood type 1 diabetes in various European populations. *Diabetes Care,* **25,** 1755-60.

Flaschi-Taesch, N. M., Berman, D. M., Sicari, B. M., Takane, K. K., Garcia-Ocana, A., Ricordi, C., Kenyon, N. S. & Stewart, A. F. (2008). Hepatocyte growth factor enhances engraftment and function of nonhuman primate islets. *Diabetes,* **57,** 2745-54.

Fowler, M. J. (2007). Diabetes Treatment, Part 2: Oral Agents for Glycemic Management. *Clinical Diabetes,* **25,** 131-134.

Francki, A., Motamed, K., Mcclure, T. D., Kaya, M., Murri, C., Blake, D. J., Carbon, J. G. & Sage, E. H. (2003). SPARC regulates cell cycle progression in mesangial cells via its inhibition of IGF-dependent signaling. *J Cell Biochem,* **88,** 802-11.

Frodin, M., Sekine, N., Roche, E., Filloux, C., Prentki, M., Wollheim, C. B. & Van Obberghen, E. (1995). Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting beta-cell line, INS-1. *J Biol Chem,* **270,** 7882-9.

Froeling, F. E., Mirza, T. A., Feakins, R. M., Seedhar, A., Elia, G., Hart, I. R. & Kocher, H. M. (2009). Organotypic culture model of pancreatic cancer demonstrates that stromal cells modulate E-cadherin, beta-catenin, and Ezrin expression in tumor cells. *Am J Pathol,* **175,** 636-48.

Funk, S. E. & Sage, E. H. (1991). The Ca2(+)-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. *Proc Natl Acad Sci U S A,* **88,** 2648-52.

Gahr, S., Merger, M., Bollheimer, L. C., Hammerschmied, C. G., Scholmerich, J. & Hugl, S. R. (2002). Hepatocyte growth factor stimulates proliferation of pancreatic beta-cells particularly in the presence of subphysiological glucose concentrations. *J Mol Endocrinol,* **28,** 99-110.

Garcia-Ocana, A., Takane, K. K., Reddy, V. T., Lopez-Talavera, J. C., Vasavada, R. C. & Stewart, A. F. (2003). Adenovirus-mediated hepatocyte growth factor expression in mouse islets improves pancreatic islet transplant performance and reduces beta cell death. *J Biol Chem,* **278,** 343-51.

Garcia-Ocana, A., Vasavada, R. C., Takane, K. K., Cebrian, A., Lopez-Talavera, J. C. & Stewart, A. F. (2001). Using beta-cell growth factors to enhance human pancreatic Islet transplantation. *J Clin Endocrinol Metab,* **86,** 984-8.

Gavin, J. R. I., Alberti, K. G. M. M., Davidson, M. B., Defronzo, R. A., Drash, A., Gabbe, S. G., Genuth, S., Harris, M. I., Kahn, R., Keen, H., Knowler, W. C., Lebovitz, H., Maclaren, N. K., Palmer, J. P., Raskin, P., Rizza, R. A. & Stern, M. P. (2003). Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care,* **26,** S5-S20.

Gershengorn, M. C., Hardikar, A. A., Wei, C., Geras-Raaka, E., Marcus-Samuels, B. & Raaka, B. M. (2004). Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. *Science,* **306,** 2261-4.

Gerson, K. D., Shearstone, J. R., Maddula, V. S., Seligmann, B. E. & Mercurio, A. M. (2012). Integrin beta4 regulates SPARC protein to promote invasion. *J Biol Chem,* **287,** 9835-44.

Gerstein, H. C., Bosch, J., Dagenais, G.R. *Et Al* (2012). Basal Insulin and Cardiovascular and Other Outcomes in Dysglycemia. *New England Journal of Medicine,* **367,** 319-328.

Giannoukakis, N., Mi, Z., Rudert, W. A., Gambotto, A., Trucco, M. & Robbins, P. (2000). Prevention of beta cell dysfunction and apoptosis activation in human islets by adenoviral gene transfer of the insulin-like growth factor I. *Gene Ther,* **7,** 2015-22.

Gilbert, R. E., Mcnally, P. G., Cox, A., Dziadek, M., Rumble, J., Cooper, M. E. & Jerums, G. (1995). SPARC gene expression is reduced in early diabetes-related kidney growth. *Kidney Int,* **48,** 1216-25.

Gilmour, D. T., Lyon, G. J., Carlton, M. B., Sanes, J. R., Cunningham, J. M., Anderson, J. R., Hogan, B. L., Evans, M. J. & Colledge, W. H. (1998). Mice deficient for the secreted glycoprotein SPARC/osteonectin/BM40 develop normally but show severe age-onset cataract formation and disruption of the lens. *EMBO J,* **17,** 1860-70.

Giudici, C., Raynal, N., Wiedemann, H., Cabral, W. A., Marini, J. C., Timpl, R., Bachinger, H. P., Farndale, R. W., Sasaki, T. & Tenni, R. (2008). Mapping of SPARC/BM-40/osteonectin-binding sites on fibrillar collagens. *J Biol Chem,* **283,** 19551-60.

Gloyn, A. L., Weedon, M. N., Owen, K. R., Turner, M. J., Knight, B. A., Hitman, G., Walker, M., Levy, J. C., Sampson, M., Halford, S., Mccarthy, M. I., Hattersley, A. T. & Frayling, T. M. (2003). Large-scale association studies of variants in genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) confirm that the KCNJ11 E23K variant is associated with type 2 diabetes. *Diabetes,* **52,** 568-72.

Goldblum, S. E., Ding, X., Funk, S. E. & Sage, E. H. (1994). SPARC (secreted protein acidic and rich in cysteine) regulates endothelial cell shape and barrier function. *Proc Natl Acad Sci U S A,* **91,** 3448-52.

Grant, R. W., Moore, A. F. & Florez, J. C. (2009). Genetic Architecture of Type 2 Diabetes: Recent Progress and Clinical Implications. *Diabetes Care,* **32,** 1107-1114.

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

Gruessner, A. C., Sutherland, D. E. & Gruessner, R. W. (2012). Long-term outcome after pancreas transplantation. *Curr Opin Organ Transplant,* **17,** 100-5.

Guo, T., Wang, W., Zhang, H., Liu, Y., Chen, P., Ma, K. & Zhou, C. (2011). ISL1 promotes pancreatic islet cell proliferation. *PLoS One,* **6,** e22387.

Haeno, H., Gonen, M., Davis, M. B., Herman, J. M., Iacobuzio-Donahue, C. A. & Michor, F. (2012). Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. *Cell,* **148,** 362-75.

Hagen, P. M. V., Krenning, E. P., Kwekkeboom, D. J., Reubi, J. C., Anker-Lugtenburg, V. P. J., Löwenberg, B. & Lamberts, S. W. J. (1994). Somatostatin and the immune and haematopoetic system; a review. *European Journal of Clinical Investigation,* **24,** 91-99.

Halban, P. A. (1991). Structural domains and molecular lifestyles of insulin and its precursors in the pancreatic Beta cell. *Diabetologia,* **34,** 767-778.

Hall, K. K., Gattas-Asfura, K. M. & Stabler, C. L. (2011). Microencapsulation of islets within alginate/poly(ethylene glycol) gels cross-linked via Staudinger ligation. *Acta Biomater,* **7,** 614-24.

Harries, L. W., Mcculloch, L. J., Holley, J. E., Rawling, T. J., Welters, H. J. & Kos, K. (2013). A role for SPARC in the moderation of human insulin secretion. *PLoS One,* **8,** e68253.

Harris, B. S., Zhang, Y., Card, L., Rivera, L. B., Brekken, R. A. & Bradshaw, A. D. (2011). SPARC regulates collagen interaction with cardiac fibroblast cell surfaces. *Am J Physiol Heart Circ Physiol,* **301,** H841-7.

Hayek, A., Beattie, G. M., Cirulli, V., Lopez, A. D., Ricordi, C. & Rubin, J. S. (1995). Growth factor/matrix-induced proliferation of human adult beta-cells. *Diabetes,* **44,** 1458-60.

Henquin, J. C. (2000). Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes,* **49,** 1751-60.

Herder, C. & Roden, M. (2011). Genetics of type 2 diabetes: pathophysiologic and clinical relevance. *Eur J Clin Invest,* **41,** 679-92.

Hiatt, W. R., Kaul, S. & Smith, R. J. (2013). The Cardiovascular Safety of Diabetes Drugs — Insights from the Rosiglitazone Experience. *New England Journal of Medicine,* **epub,** null.

Hill, N. J., Hultcrantz, M., Sarvetnick, N. & Flodstrom-Tullberg, M. (2007). The target tissue in autoimmunity--an influential niche. *Eur J Immunol,* **37,** 589-97.

Hiscox, A. M., Stone, A. L., Limesand, S., Hoying, J. B. & Williams, S. K. (2008). An islet-stabilizing implant constructed using a preformed vasculature. *Tissue Eng Part A,* **14,** 433-40.

Hjern, A., Soderstrom, U. & Aman, J. (2012). East Africans in Sweden have a high risk for type 1 diabetes. *Diabetes Care,* **35,** 597-8.

Hohenester, E., Maurer, P. & Timpl, R. (1997). Crystal structure of a pair of follistatin-like and EF-hand calcium-binding domains in BM-40. *EMBO J,* **16,** 3778-86.

Holm, P., Rydlander, B., Luthman, H. & Kockum, I. (2004). Interaction and association analysis of a type 1 diabetes susceptibility locus on chromosome 5q11-q13 and the 7q32 chromosomal region in Scandinavian families. *Diabetes,* **53,** 1584-91.

Holmberg, H., Wahlberg, J., Vaarala, O. & Ludvigsson, J. (2007). Short duration of breast-feeding as a risk-factor for beta-cell autoantibodies in 5-year-old children from the general population. *Br J Nutr,* **97,** 111-6.

Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E., Del Bosque-Plata, L., Oda, Y., Yoshiuchi, I., Colilla, S., Polonsky, K. S., Wei, S., Concannon, P., Iwasaki, N., Schulze, J., Baier, L. J., Bogardus, C., Groop, L., Boerwinkle, E., Hanis, C. L. & Bell, G. I. (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet,* **26,** 163-75.

Horwitz, M. S., Bradley, L. M., Harbertson, J., Krahl, T., Lee, J. & Sarvetnick, N. (1998). Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med,* **4,** 781-5.

Hotamisligil, G. S. & Spiegelman, B. M. (1994). Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes,* **43,** 1271-8.

Hu, F. B., Stampfer, M. J., Haffner, S. M., Solomon, C. G., Willett, W. C. & Manson, J. E. (2002). Elevated Risk of Cardiovascular Disease Prior to Clinical Diagnosis of Type 2 Diabetes. *Diabetes Care,* **25,** 1129-1134.

Hugl, S. R., White, M. F. & Rhodes, C. J. (1998). Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem,* **273,** 17771-9.

Hull, R. L., Kodama, K., Utzschneider, K. M., Carr, D. B., Prigeon, R. L. & Kahn, S. E. (2005). Dietary-fat-induced obesity in mice results in beta cell hyperplasia but not increased insulin release: evidence for specificity of impaired beta cell adaptation. *Diabetologia,* **48,** 1350-8.

Humar, A., Kandaswamy, R., Granger, D., Gruessner, R. W., Gruessner, A. C. & Sutherland, D. E. (2000). Decreased surgical risks of pancreas transplantation in the modern era. *Ann Surg,* **231,** 269-75.

Hwang, R. F., Moore, T., Arumugam, T., Ramachandran, V., Amos, K. D., Rivera, A., Ji, B., Evans, D. B. & Logsdon, C. D. (2008). Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res,* **68,** 918-26.

Idf.Org. (2012). *IDF Diabetes Atlas Update 2012 | International Diabetes Federation* [Online]. Available: http://www.idf.org/diabetesatlas/5e/Update2012 [Accessed 20/03/2013].

Ikegami, H., Fujisawa, T. & Ogihara, T. (2004). Mouse models of type 1 and type 2 diabetes derived from the same closed colony: genetic susceptibility shared between two types of diabetes. *ILAR J,* **45,** 268-77.

Inzucchi, S. E., Bergenstal, R. M., Buse, J. B., Diamant, M., Ferrannini, E., Nauck, M., Peters, A. L., Tsapas, A., Wender, R. & Matthews, D. R. (2012). Management of hyperglycaemia in type 2 diabetes: a patient-centered approach. Position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetologia,* **55,** 1577-96.

Irving-Rodgers, H. F., Ziolkowski, A. F., Parish, C. R., Sado, Y., Ninomiya, Y., Simeonovic, C. J. & Rodgers, R. J. (2008). Molecular composition of the peri-islet basement membrane in NOD mice: a barrier against destructive insulitis. *Diabetologia,* **51,** 1680-8.

Iwano, M., Plieth, D., Danoff, T. M., Xue, C., Okada, H. & Neilson, E. G. (2002). Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest,* **110,** 341-50.

Jahromi, M. M. (2013). Update of Type 1 Diabetes, Type 1 Diabetes. ESCHER, A. (Ed.) ISBN: 978-953-51-1017-0 InTech, DOI: 10.5772/55960 Available: http://www.intechopen.com/books/type-1-diabetes/update-of-type-1-diabetes

Jahromi, M. M. & Eisenbarth, G. S. (2007). Cellular and molecular pathogenesis of type 1A diabetes. *Cell Mol Life Sci,* **64,** 865-72.

Jalili, R. B., Moeen Rezakhanlou, A., Hosseini-Tabatabaei, A., Ao, Z., Warnock, G. L. & Ghahary, A. (2011). Fibroblast populated collagen matrix promotes islet survival and reduces the number of islets required for diabetes reversal. *J Cell Physiol,* **226,** 1813-9.

Jandeleit-Dahm, K., Rumble, J., Cox, A. J., Kelly, D. J., Dziadek, M., Cooper, M. E. & Gilbert, R. E. (2000). SPARC gene expression is increased in diabetes-related mesenteric vascular hypertrophy. *Microvasc Res,* **59,** 61-71.

Jansen, A., Homo-Delarche, F., Hooijkaas, H., Leenen, P. J., Dardenne, M. & Drexhage, H. A. (1994). Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice. *Diabetes,* **43,** 667-75.

Janson, J., Ashley, R. H., Harrison, D., Mcintyre, S. & Butler, P. C. (1999). The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. *Diabetes,* **48,** 491-8.

Jansson, L. & Carlsson, P. O. (2002). Graft vascular function after transplantation of pancreatic islets. *Diabetologia,* **45,** 749-63.

Joshi, S. R., Parikh, R. M. & Das, A. K. (2007). Insulin--history, biochemistry, physiology and pharmacology. *J Assoc Physicians India,* **55 Suppl,** 19-25.

Jurgens, C. A., Toukatly, M. N., Fligner, C. L., Udayasankar, J., Subramanian, S. L., Zraika, S., Aston-Mourney, K., Carr, D. B., Westermark, P., Westermark, G. T., Kahn, S. E. & Hull, R. L. (2011). beta-cell loss and beta-cell apoptosis in human type 2 diabetes are related to islet amyloid deposition. *Am J Pathol,* **178,** 2632-40.

Kanauchi, M., Nishioka, M. & Dohi, K. (2000). Secreted protein acidic and rich in cysteine (SPARC) in patients with diabetic nephropathy and tubulointerstitial injury. *Diabetologia,* **43,** 1076-7.

Kassem, S. A., Ariel, I., Thornton, P. S., Scheimberg, I. & Glaser, B. (2000). Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. *Diabetes,* **49,** 1325-33.

Kaufman, F. R. (2002). Type 2 Diabetes in Children and Young Adults: A "New Epidemic". *Clinical Diabetes,* **20,** 217-218.

Kawai, K., Yokota, C., Ohashi, S., Watanabe, Y. & Yamashita, K. (1995). Evidence that glucagon stimulates insulin secretion through its own receptor in rats. *Diabetologia,* **38,** 274-276.

Kayali, A. G., Flores, L. E., Lopez, A. D., Kutlu, B., Baetge, E., Kitamura, R., Hao, E., Beattie, G. M. & Hayek, A. (2007). Limited capacity of human adult islets expanded in vitro to redifferentiate into insulin-producing beta-cells. *Diabetes,* **56,** 703-8.

Kelly, K. A., Allport, J. R., Yu, A. M., Sinh, S., Sage, E. H., Gerszten, R. E. & Weissleder, R. (2007). SPARC is a VCAM-1 counter-ligand that mediates leukocyte transmigration. *J Leukoc Biol,* **81,** 748-56.

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

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

Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N. & Hay, N. (1997). The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev,* **11,** 701-13.

Kharouta, M., Miller, K., Kim, A., Wojcik, P., Kilimnik, G., Dey, A., Steiner, D. F. & Hara, M. (2009). No mantle formation in rodent islets -- the prototype of islet revisited. *Diabetes Res Clin Pract,* **85,** 252-7.

Kikuchi, N., Horiuchi, A., Osada, R., Imai, T., Wang, C., Chen, X. & Konishi, I. (2006). Nuclear expression of S100A4 is associated with aggressive behavior of epithelial ovarian carcinoma: an important autocrine/paracrine factor in tumor progression. *Cancer Sci,* **97,** 1061-9.

Kimball, S. R., Vary, T. C. & Jefferson, L. S. (1994). Regulation of protein synthesis by insulin. *Annu Rev Physiol,* **56,** 321-48.

King's College London. (2005). *About King's College London : News and What's On : King's College London* [Online]. Available: About King's College London : News and What's On : King's College London [Accessed 30/03/2013].

King, C. & Sarvetnick, N. (2011). The incidence of type-1 diabetes in NOD mice is modulated by restricted flora not germ-free conditions. *PLoS One,* **6,** e17049.

Klip, A., Ramlal, T., Bilan, P. J., Cartee, G. D., Gulve, E. A. & Holloszy, J. O. (1990). Recruitment of GLUT-4 glucose transporters by insulin in diabetic rat skeletal muscle. *Biochem Biophys Res Commun,* **172,** 728-36.

Knip, M. (2002). Can we predict type 1 diabetes in the general population? *Diabetes Care,* **25,** 623-5.

Knip, M., Veijola, R., Virtanen, S. M., Hyoty, H., Vaarala, O. & Akerblom, H. K. (2005). Environmental triggers and determinants of type 1 diabetes. *Diabetes,* **54 Suppl 2,** S125-36.

Korbonits, M., Goldstone, A. P., Gueorguiev, M. & Grossman, A. B. (2004). Ghrelin—a hormone with multiple functions. *Frontiers in Neuroendocrinology,* **25,** 27-68.

Kos, K. & Wilding, J. P. (2010). SPARC: a key player in the pathologies associated with obesity and diabetes. *Nat Rev Endocrinol,* **6,** 225-35.

Kos, K., Wong, S., Tan, B., Gummesson, A., Jernas, M., Franck, N., Kerrigan, D., Nystrom, F. H., Carlsson, L. M., Randeva, H. S., Pinkney, J. H. & Wilding, J. P. (2009). Regulation of the fibrosis and angiogenesis promoter SPARC/osteonectin in human adipose tissue by weight change, leptin, insulin, and glucose. *Diabetes,* **58,** 1780-8.

Koulmanda, M., Qipo, A., Smith, R. N. & Auchincloss, H., Jr. (2003). Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. *Xenotransplantation,* **10,** 178-84.

Krishnamurthy, M., Li, J., Al-Masri, M. & Wang, R. (2008). Expression and function of alphabeta1 integrins in pancretic beta (INS-1) cells. *J Cell Commun Signal,* **2,** 67-79.

Kupprion, C., Motamed, K. & Sage, E. H. (1998). SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. *J Biol Chem,* **273,** 29635-40.

Kzhyshkowska, J., Gratchev, A. & Goerdt, S. (2006a). Stabilin-1, a homeostatic scavenger receptor with multiple functions. *J Cell Mol Med,* **10,** 635-49.

Kzhyshkowska, J., Workman, G., Cardo-Vila, M., Arap, W., Pasqualini, R., Gratchev, A., Krusell, L., Goerdt, S. & Sage, E. H. (2006b). Novel function of alternatively activated macrophages: stabilin-1-mediated clearance of SPARC. *J Immunol,* **176,** 5825-32.

Laitinen, O. H., Honkanen, H., Pakkanen, O., Oikarinen, S., Hankaniemi, M. M., Huhtala, H., Ruokoranta, T., Lecouturier, V., André, P., Harju, R., Virtanen, S. M., Lehtonen, J., Almond, J. W., Simell, T., Simell, O., Ilonen, J., Veijola, R., Knip, M. & Hyöty, H. (2013). Coxsackievirus B1 Is Associated With Induction of β-Cell Autoimmunity That Portends Type 1 Diabetes. *Diabetes.*

Lane, T. F., Iruela-Arispe, M. L. & Sage, E. H. (1992). Regulation of gene expression by SPARC during angiogenesis in vitro. Changes in fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1. *J Biol Chem,* **267,** 16736-45.

Larsen, J. L. (2004). Pancreas transplantation: indications and consequences. *Endocr Rev,* **25,** 919-46.

Ledda, M. F., Adris, S., Bravo, A. I., Kairiyama, C., Bover, L., Chernajovsky, Y., Mordoh, J. & Podhajcer, O. L. (1997). Suppression of SPARC expression by antisense RNA abrogates the tumorigenicity of human melanoma cells. *Nat Med,* **3,** 171-6.

Lee, H. S., Briese, T., Winkler, C., Rewers, M., Bonifacio, E., Hyoty, H., Pflueger, M., Simell, O., She, J. X., Hagopian, W., Lernmark, Å., Akolkar, B., Krischer, J. P. & Ziegler, A. G. (2013). Next-generation sequencing for viruses in children with rapid-onset type 1 diabetes. *Diabetologia,* **1-7.**

Leiter, E. H. (2001). The NOD mouse: a model for insulin-dependent diabetes mellitus. *Curr Protoc Immunol,* **Chapter 15,** Unit 15 9.

Leus, J., Van Biervliet, S. & Robberecht, E. (2000). Detection and follow up of exocrine pancreatic insufficiency in cystic fibrosis: a review. *European Journal of Pediatrics,* **159,** 563-568.

Li, J., Yu, X., Pan, W. & Unger, R. H. (2002). Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. *Am J Physiol Endocrinol Metab,* **282,** E1334-41.

Lim, E. L., Hollingsworth, K. G., Aribisala, B. S., Chen, M. J., Mathers, J. C. & Taylor, R. (2011). Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. *Diabetologia,* **54,** 2506-14.

Liu, J., Liu, Y., Wang, H., Hao, H., Han, Q., Shen, J., Shi, J., Li, C., Mu, Y. & Han, W. (2013). Direct differentiation of hepatic stem-like WB cells into insulin-producing cells using small molecules. *Sci Rep,* **3,** 1185.

Liu, W., Chin-Chance, C., Lee, E. J. & Lowe, W. L., Jr. (2002). Activation of phosphatidylinositol 3-kinase contributes to insulin-like growth factor I-mediated inhibition of pancreatic beta-cell death. *Endocrinology,* **143,** 3802-12.

Lopes, P., Fuhrmann, A., Sereno, J., Pereira, M. J., Nunes, P., Pedro, J., Melao, A., Reis, F. & Carvalho, E. (2013). Effects of cyclosporine and sirolimus on insulin-stimulated glucose transport and glucose tolerance in a rat model. *Transplant Proc,* **45,** 1142-8.

Luan, N. & Iwata, H. (2013). Inhibition of instant blood-mediated inflammatory responses by co-immobilization of sCR1 and heparin on islets. *Biomaterials,* **34,** 5019-5024.

Ma, X., Ye, B., Gao, F., Liang, Q., Dong, Q., Liu, Y., Rong, P., Wang, W. & Yi, S. (2012). Tissue Factor Knockdown in Porcine Islets: An Effective Approach to Suppressing the Instant Blood-Mediated Inflammatory Reaction. *Cell Transplant,* **21,** 61-71.

Macke, S. 2012. Dia Diagram Editor. 0.97.2 ed. The Gnome Project.

Maedler, K., Oberholzer, J., Bucher, P., Spinas, G. A. & Donath, M. Y. (2003). Monounsaturated Fatty Acids Prevent the Deleterious Effects of Palmitate and High Glucose on Human Pancreatic β-Cell Turnover and Function. *Diabetes,* **52,** 726-733.

Maedler, K., Sergeev, P., Ehses, J. A., Mathe, Z., Bosco, D., Berney, T., Dayer, J. M., Reinecke, M., Halban, P. A. & Donath, M. Y. (2004). Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets. *Proc Natl Acad Sci U S A,* **101,** 8138-43.

Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H. I., Spinas, G. A., Kaiser, N., Halban, P. A. & Donath, M. Y. (2002). Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest,* **110,** 851-60.

Maedler, K., Spinas, G. A., Lehmann, R., Sergeev, P., Weber, M., Fontana, A., Kaiser, N. & Donath, M. Y. (2001). Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes,* **50,** 1683-90.

Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K. & Tochino, Y. (1980). Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu,* **29,** 1-13.

Mantoni, T. S., Schendel, R. R., Rodel, F., Niedobitek, G., Al-Assar, O., Masamune, A. & Brunner, T. B. (2008). Stromal SPARC expression and patient survival after chemoradiation for non-resectable pancreatic adenocarcinoma. *Cancer Biol Ther,* **7,** 1806-15.

Maruyama, H., Hisatomi, A., Orci, L., Grodsky, G. M. & Unger, R. H. (1984). Insulin within islets is a physiologic glucagon release inhibitor. *J Clin Invest,* **74,** 2296-9.

Marynissen, G., Aerts, L. & Van Assche, F. A. (1983). The endocrine pancreas during pregnancy and lactation in the rat. *J Dev Physiol,* **5,** 373-81.

Mason, I. J., Taylor, A., Williams, J. G., Sage, H. & Hogan, B. L. (1986). Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell 'culture shock' glycoprotein of Mr 43,000. *EMBO J,* **5,** 1465-72.

Matarese, G., Sanna, V., Lechler, R. I., Sarvetnick, N., Fontana, S., Zappacosta, S. & La Cava, A. (2002). Leptin accelerates autoimmune diabetes in female NOD mice. *Diabetes,* **51,** 1356-61.

Mathers, C. D. & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med,* **3,** e442.

Matveyenko, A. V. & Butler, P. C. (2008). Relationship between beta-cell mass and diabetes onset. *Diabetes Obes Metab,* **10 Suppl 4,** 23-31.

Maurer, P., Hohenadl, C., Hohenester, E., Gohring, W., Timpl, R. & Engel, J. (1995). The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV. *J Mol Biol,* **253,** 347-57.

Mckenzie, M. D., Jamieson, E., Jansen, E. S., Scott, C. L., Huang, D. C., Bouillet, P., Allison, J., Kay, T. W., Strasser, A. & Thomas, H. E. (2010). Glucose induces pancreatic islet cell apoptosis that requires the BH3-only proteins Bim and Puma and multi-BH domain protein Bax. *Diabetes,* **59,** 644-52.

Mcmahon, S. K., Airey, F. L., Marangou, D. A., Mcelwee, K. J., Carne, C. L., Clarey, A. J., Davis, E. A. & Jones, T. W. (2005). Insulin pump therapy in children and adolescents: improvements in key parameters of diabetes management including quality of life. *Diabetic Medicine,* **22,** 92-96.

Mellado-Gil, J., Rosa, T. C., Demirci, C., Gonzalez-Pertusa, J. A., Velazquez-Garcia, S., Ernst, S., Valle, S., Vasavada, R. C., Stewart, A. F., Alonso, L. C. & Garcia-Ocana, A. (2011). Disruption of hepatocyte growth factor/c-Met signaling enhances pancreatic beta-cell death and accelerates the onset of diabetes. *Diabetes,* **60,** 525-36.

Merani, S., Toso, C., Emamaullee, J. & Shapiro, A. M. (2008). Optimal implantation site for pancreatic islet transplantation. *Br J Surg,* **95,** 1449-61.

Meyre, D. (2012). Is FTO a type 2 diabetes susceptibility gene? *Diabetologia,* **55,** 873-6.

Mirza, S., Hossain, M., Mathews, C., Martinez, P., Pino, P., Gay, J. L., Rentfro, A., Mccormick, J. B. & Fisher-Hoch, S. P. (2012). Type 2-diabetes is associated with elevated levels of TNF-

alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. *Cytokine,* **57,** 136-42.

Mitchell, R. M., Byrne, M. F. & Baillie, J. (2003). Pancreatitis. *Lancet,* **361,** 1447-55.

Miyamoto, S., Teramoto, H., Gutkind, J. S. & Yamada, K. M. (1996). Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol,* **135,** 1633-42.

Mohiti, J., Talebi, F. & Afkhami-Ardekani, M. (2009). Circulation free leptin in diabetic patients and its correlation to insulin level. *Pak J Biol Sci,* **12,** 397-400.

Mok, S. C., Chan, W. Y., Wong, K. K., Muto, M. G. & Berkowitz, R. S. (1996). SPARC, an extracellular matrix protein with tumor-suppressing activity in human ovarian epithelial cells. *Oncogene,* **12,** 1895-901.

Morrish, N. J., Wang, S. L., Stevens, L. K., Fuller, J. H. & Keen, H. (2001). Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. *Diabetologia,* **44 Suppl 2,** S14-21.

Murphy-Ullrich, J. E. (2001). The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptive state? *J Clin Invest,* **107,** 785-90.

Nagata, N. A., Inoue, K. & Tabata, Y. (2002). Co-culture of extracellular matrix suppresses the cell death of rat pancreatic islets. *J Biomater Sci Polym Ed,* **13,** 579-90.

Nhs.Uk. (2010). *Avandia diabetes drug suspended* [Online]. Available: http://www.nhs.uk/news/2010/09September/Pages/rosiglitazone-avandia-drug-suspended-heart-risk.aspx [Accessed 24/05/2013].

Nhs.Uk. (2013a). *Organ Donation UK | Statisitcs | Pancreas* [Online]. Available: http://www.organdonation.nhs.uk/statistics/centre-specific_reports/pancreas/pdf/uk.pdf [Accessed 01/04/2013].

Nhs.Uk. (2013b). *Organ Donation UK | Statistics | Transplant Activity Report* [Online]. Available: http://www.organdonation.nhs.uk/statistics/transplant_activity_report/current_activity_reports/ukt/pancreas_activity.pdf [Accessed 01/04/2013].

Nhs.Uk. (2013c). *Pancreas Transplant - NHS Choices* [Online]. Available: http://www.nhs.uk/conditions/pancreastransplant/Pages/Introduction.aspx [Accessed 30/03/2013].

Nie, J., Chang, B., Traktuev, D. O., Sun, J., March, K., Chan, L., Sage, E. H., Pasqualini, R., Arap, W. & Kolonin, M. G. (2008). IFATS collection: Combinatorial peptides identify alpha5beta1 integrin as a receptor for the matricellular protein SPARC on adipose stromal cells. *Stem Cells,* **26,** 2735-45.

Nielsen, J. H., Linde, S., Welinder, B. S., Billestrup, N. & Madsen, O. D. (1989). Growth hormone is a growth factor for the differentiated pancreatic beta-cell. *Mol Endocrinol,* **3,** 165-73.

Nieuwenhuizen, A. G., Schuiling, G. A., Moes, H. & Koiter, T. R. (1997). Role of increased insulin demand in the adaptation of the endocrine pancreas to pregnancy. *Acta Physiol Scand,* **159,** 303-12.

Nir, T., Melton, D. A. & Dor, Y. (2007). Recovery from diabetes in mice by beta cell regeneration. *J Clin Invest,* **117,** 2553-61.

Noble, J. A. & Erlich, H. A. (2012). Genetics of type 1 diabetes. *Cold Spring Harb Perspect Med,* **2,** a007732.

Oikarinen, S., Martiskainen, M., Tauriainen, S., Huhtala, H., Ilonen, J., Veijola, R., Simell, O., Knip, M. & Hyoty, H. (2011). Enterovirus RNA in blood is linked to the development of type 1 diabetes. *Diabetes,* **60,** 276-9.

Ozbay, L. A., Smidt, K., Mortensen, D. M., Carstens, J., Jorgensen, K. A. & Rungby, J. (2011). Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells. *Br J Pharmacol,* **162,** 136-46.

Parsons, J. A., Brelje, T. C. & Sorenson, R. L. (1992). Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. *Endocrinology,* **130,** 1459-66.

Persaud, S. J., Muller, D. & Jones, P. M. (2008). Insulin signalling in islets. *Biochem Soc Trans,* **36,** 290-3.

Petersson, B. (1966). Isolation and characterization of different types of pancreatic islet cells in guinea-pigs. *Acta Endocrinol (Copenh),* **53,** 480-8.

Pitocco, D., Zaccardi, F., Di Stasio, E., Romitelli, F., Santini, S. A., Zuppi, C. & Ghirlanda, G. (2010). Oxidative stress, nitric oxide, and diabetes. *Rev Diabet Stud,* **7,** 15-25.

Porte, H., Triboulet, J. P., Kotelevets, L., Carrat, F., Prevot, S., Nordlinger, B., Digioia, Y., Wurtz, A., Comoglio, P., Gespach, C. & Chastre, E. (1998). Overexpression of stromelysin-3, BM-40/SPARC, and MET genes in human esophageal carcinoma: implications for prognosis. *Clin Cancer Res,* **4,** 1375-82.

Potter, K. J., Abedini, A., Marek, P., Klimek, A. M., Butterworth, S., Driscoll, M., Baker, R., Nilsson, M. R., Warnock, G. L., Oberholzer, J., Bertera, S., Trucco, M., Korbutt, G. S., Fraser, P. E., Raleigh, D. P. & Verchere, C. B. (2010). Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. *Proc Natl Acad Sci U S A,* **107,** 4305-10.

Proks, P., Reimann, F., Green, N., Gribble, F. & Ashcroft, F. (2002). Sulfonylurea Stimulation of Insulin Secretion. *Diabetes,* **51,** S368-S376.

Pukel, C., Baquerizo, H. & Rabinovitch, A. (1988). Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. *Diabetes,* **37,** 133-6.

Puolakkainen, P. A., Brekken, R. A., Muneer, S. & Sage, E. H. (2004). Enhanced growth of pancreatic tumors in SPARC-null mice is associated with decreased deposition of extracellular matrix and reduced tumor cell apoptosis. *Mol Cancer Res,* **2,** 215-24.

Purcell, L., Gruia-Gray, J., Scanga, S. & Ringuette, M. (1993). Developmental anomalies of Xenopus embryos following microinjection of SPARC antibodies. *J Exp Zool,* **265,** 153-64.

Radu, R. G., Fujimoto, S., Mukai, E., Takehiro, M., Shimono, D., Nabe, K., Shimodahira, M., Kominato, R., Aramaki, Y., Nishi, Y., Funakoshi, S., Yamada, Y. & Seino, Y. (2005). Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. *Am J Physiol Endocrinol Metab,* **288,** E365-71.

Raines, E. W., Lane, T. F., Iruela-Arispe, M. L., Ross, R. & Sage, E. H. (1992). The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. *Proc Natl Acad Sci U S A,* **89,** 1281-5.

Ramiya, V. K., Maraist, M., Arfors, K. E., Schatz, D. A., Peck, A. B. & Cornelius, J. G. (2000). Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med,* **6,** 278-82.

Rasband, W. S. 1997-2012. ImageJ. U.S. National Institutes of Health, Bethesda, Maryland, USA.

Reaven, G. M. (1988). Role of Insulin Resistance in Human Disease. *Diabetes,* **37,** 1595-1607.

Reddan, J. R. & Dziedzic, D. C. (1982). Insulin-like growth factors, IGF-1, IGF-2 and somatomedin C trigger cell proliferation in mammalian epithelial cells cultured in a serum-free medium. *Exp Cell Res,* **142,** 293-300.

Redondo, M. J., Fain, P. R. & Eisenbarth, G. S. (2001a). Genetics of Type 1A Diabetes. *Recent Prog Horm Res,* **56,** 69-90.

Redondo, M. J., Yu, L., Hawa, M., Mackenzie, T., Pyke, D. A., Eisenbarth, G. S. & Leslie, R. D. (2001b). Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. *Diabetologia,* **44,** 354-62.

Rempel, S. A., Ge, S. & Gutierrez, J. A. (1999). SPARC: a potential diagnostic marker of invasive meningiomas. *Clin Cancer Res,* **5,** 237-41.

Ren, J. M., Semenkovich, C. F., Gulve, E. A., Gao, J. & Holloszy, J. O. (1994). Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle. *J Biol Chem,* **269,** 14396-401.

Rentz, T. J., Poobalarahi, F., Bornstein, P., Sage, E. H. & Bradshaw, A. D. (2007). SPARC regulates processing of procollagen I and collagen fibrillogenesis in dermal fibroblasts. *J Biol Chem,* **282,** 22062-71.

Reynisdottir, I., Thorleifsson, G., Benediktsson, R., Sigurdsson, G., Emilsson, V., Einarsdottir, A. S., Hjorleifsdottir, E. E., Orlygsdottir, G. T., Bjornsdottir, G. T., Saemundsdottir, J., Halldorsson, S., Hrafnkelsdottir, S., Sigurjonsdottir, S. B., Steinsdottir, S., Martin, M., Kochan, J. P., Rhees, B. K., Grant, S. F., Frigge, M. L., Kong, A., Gudnason, V., Stefansson, K.

& Gulcher, J. R. (2003). Localization of a susceptibility gene for type 2 diabetes to chromosome 5q34-q35.2. *Am J Hum Genet,* **73,** 323-35.

Richards, O. C., Raines, S. M. & Attie, A. D. (2010). The Role of Blood Vessels, Endothelial Cells, and Vascular Pericytes in Insulin Secretion and Peripheral Insulin Action. *Endocr Rev,* **31,** 343-363.

Richter, A., Lerner, S. & Schroppel, B. (2011). Current state of combined kidney and pancreas transplantation. *Blood Purif,* **31,** 96-101.

Richter, E. A. & Hargreaves, M. (2013). Exercise, GLUT4, and Skeletal Muscle Glucose Uptake. *Physiological Reviews,* **93,** 993-1017.

Ricordi, C., Lacy, P. E., Finke, E. H., Olack, B. J. & Scharp, D. W. (1988). Automated method for isolation of human pancreatic islets. *Diabetes,* **37,** 413-20.

Rieck, S. & Kaestner, K. H. (2010). Expansion of β-cell mass in response to pregnancy. *Trends in Endocrinology & Metabolism,* **21,** 151-158.

Riserus, U., Willett, W. C. & Hu, F. B. (2009). Dietary fats and prevention of type 2 diabetes. *Prog Lipid Res,* **48,** 44-51.

Robert, G., Gaggioli, C., Bailet, O., Chavey, C., Abbe, P., Aberdam, E., Sabatie, E., Cano, A., Garcia De Herreros, A., Ballotti, R. & Tartare-Deckert, S. (2006). SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. *Cancer Res,* **66,** 7516-23.

Roberts, C. T., Jr., Brown, A. L., Graham, D. E., Seelig, S., Berry, S., Gabbay, K. H. & Rechler, M. M. (1986). Growth hormone regulates the abundance of insulin-like growth factor I RNA in adult rat liver. *J Biol Chem,* **261,** 10025-8.

Roberts, D. D. (2011). Emerging functions of matricellular proteins. *Cell Mol Life Sci,* **68,** 3133-6.

Robertson, G. L. (1995). Diabetes insipidus. *Endocrinol Metab Clin North Am,* **24,** 549-72.

Roccisana, J., Reddy, V., Vasavada, R. C., Gonzalez-Pertusa, J. A., Magnuson, M. A. & Garcia-Ocana, A. (2005). Targeted inactivation of hepatocyte growth factor receptor c-met in beta-cells leads to defective insulin secretion and GLUT-2 downregulation without alteration of beta-cell mass. *Diabetes,* **54,** 2090-102.

Roduit, R. & Thorens, B. (1997). Inhibition of glucose-induced insulin secretion by long-term preexposure of pancreatic islets to leptin. *FEBS Lett,* **415,** 179-82.

Roehrich, M. E., Mooser, V., Lenain, V., Herz, J., Nimpf, J., Azhar, S., Bideau, M., Capponi, A., Nicod, P., Haefliger, J. A. & Waeber, G. (2003). Insulin-secreting beta-cell dysfunction induced by human lipoproteins. *J Biol Chem,* **278,** 18368-75.

Rorsman, P. & Renstrom, E. (2003). Insulin granule dynamics in pancreatic beta cells. *Diabetologia,* **46,** 1029-45.

Rosmalen, J. G., Homo-Delarche, F., Durant, S., Kap, M., Leenen, P. J. & Drexhage, H. A. (2000). Islet abnormalities associated with an early influx of dendritic cells and macrophages in NOD and NODscid mice. *Lab Invest,* **80,** 769-77.

Rossini, M., Cheunsuchon, B., Donnert, E., Ma, L. J., Thomas, J. W., Neilson, E. G. & Fogo, A. B. (2005). Immunolocalization of fibroblast growth factor-1 (FGF-1), its receptor (FGFR-1), and fibroblast-specific protein-1 (FSP-1) in inflammatory renal disease. *Kidney Int,* **68,** 2621-8.

Russ, H. A., Bar, Y., Ravassard, P. & Efrat, S. (2008). In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. *Diabetes,* **57,** 1575-83.

Russell, M. A., Cooper, A. C., Dhayal, S. & Morgan, N. G. (2013). Differential effects of interleukin-13 and interleukin-6 on Jak/STAT signaling and cell viability in pancreatic beta-cells. *Islets,* **5.**

Ryan, D. G., Taliana, L., Sun, L., Wei, Z. G., Masur, S. K. & Lavker, R. M. (2003). Involvement of S100A4 in stromal fibroblasts of the regenerating cornea. *Invest Ophthalmol Vis Sci,* **44,** 4255-62.

Ryan, E. A., Paty, B. W., Senior, P. A., Bigam, D., Alfadhli, E., Kneteman, N. M., Lakey, J. R. & Shapiro, A. M. (2005). Five-year follow-up after clinical islet transplantation. *Diabetes,* **54,** 2060-9.

Ryan, E. A., Paty, B. W., Senior, P. A. & Shapiro, A. M. (2004). Risks and side effects of islet transplantation. *Curr Diab Rep,* **4,** 304-9.

Sage, E. H., Reed, M., Funk, S. E., Truong, T., Steadele, M., Puolakkainen, P., Maurice, D. H. & Bassuk, J. A. (2003). Cleavage of the matricellular protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis. *J Biol Chem,* **278,** 37849-57.

Sage, H., Johnson, C. & Bornstein, P. (1984). Characterization of a novel serum albumin-binding glycoprotein secreted by endothelial cells in culture. *J Biol Chem,* **259,** 3993-4007.

Sage, H., Tupper, J. & Bramson, R. (1986). Endothelial cell injury in vitro is associated with increased secretion of an Mr 43,000 glycoprotein ligand. *J Cell Physiol,* **127,** 373-87.

Sage, H., Vernon, R. B., Decker, J., Funk, S. & Iruela-Arispe, M. L. (1989a). Distribution of the calcium-binding protein SPARC in tissues of embryonic and adult mice. *J Histochem Cytochem,* **37,** 819-29.

Sage, H., Vernon, R. B., Funk, S. E., Everitt, E. A. & Angello, J. (1989b). SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca+2-dependent binding to the extracellular matrix. *J Cell Biol,* **109,** 341-56.

Said, N., Najwer, I. & Motamed, K. (2007). Secreted protein acidic and rich in cysteine (SPARC) inhibits integrin-mediated adhesion and growth factor-dependent survival signaling in ovarian cancer. *Am J Pathol,* **170,** 1054-63.

Saito, K., Iwama, N. & Takahashi, T. (1978). Morphometrical analysis on topographical difference in size distribution, number and volume of islets in the human pancreas. *Tohoku J Exp Med,* **124,** 177-86.

Sangaletti, S., Di Carlo, E., Gariboldi, S., Miotti, S., Cappetti, B., Parenza, M., Rumio, C., Brekken, R. A., Chiodoni, C. & Colombo, M. P. (2008). Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis. *Cancer Res,* **68,** 9050-9.

Sara, V. R., Hall, K., Rodeck, C. H. & Wetterberg, L. (1981). Human embryonic somatomedin. *Proc Natl Acad Sci U S A,* **78,** 3175-9.

Sato, N., Fukushima, N., Maehara, N., Matsubayashi, H., Koopmann, J., Su, G. H., Hruban, R. H. & Goggins, M. (2003). SPARC/osteonectin is a frequent target for aberrant methylation in pancreatic adenocarcinoma and a mediator of tumor-stromal interactions. *Oncogene,* **22,** 5021-30.

Scharp, D. W., Downing, R., Merrell, R. C. & Greider, M. (1980). Isolating the elusive islet. *Diabetes,* **29 Suppl 1,** 19-30.

Scharp, D. W., Lacy, P. E., Santiago, J. V., Mccullough, C. S., Weide, L. G., Falqui, L., Marchetti, P., Gingerich, R. L., Jaffe, A. S., Cryer, P. E. & Et Al. (1990). Insulin independence after islet transplantation into type I diabetic patient. *Diabetes,* **39,** 515-8.

Schiemann, B. J., Neil, J. R. & Schiemann, W. P. (2003). SPARC inhibits epithelial cell proliferation in part through stimulation of the transforming growth factor-beta-signaling system. *Mol Biol Cell,* **14,** 3977-88.

Schultz, C., Lemke, N., Ge, S., Golembieski, W. A. & Rempel, S. A. (2002). Secreted protein acidic and rich in cysteine promotes glioma invasion and delays tumor growth in vivo. *Cancer Res,* **62,** 6270-7.

Schuster, N. & Krieglstein, K. (2002). Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res,* **307,** 1-14.

Schwarzbauer, J. E. & Spencer, C. S. (1993). The Caenorhabditis elegans homologue of the extracellular calcium binding protein SPARC/osteonectin affects nematode body morphology and mobility. *Mol Biol Cell,* **4,** 941-52.

Scott, L. J., Mohlke, K. L., Bonnycastle, L. L., Willer, C. J., Li, Y., Duren, W. L., Erdos, M. R., Stringham, H. M., Chines, P. S., Jackson, A. U., Prokunina-Olsson, L., Ding, C. J., Swift, A. J., Narisu, N., Hu, T., Pruim, R., Xiao, R., Li, X. Y., Conneely, K. N., Riebow, N. L., Sprau, A. G., Tong, M., White, P. P., Hetrick, K. N., Barnhart, M. W., Bark, C. W., Goldstein, J. L., Watkins, L., Xiang, F., Saramies, J., Buchanan, T. A., Watanabe, R. M., Valle, T. T., Kinnunen, L., Abecasis, G. R., Pugh, E. W., Doheny, K. F., Bergman, R. N., Tuomilehto, J., Collins, F. S. & Boehnke, M. (2007). A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. *Science,* **316,** 1341-5.

Scuteri, A., Sanna, S., Chen, W.-M., Uda, M., Albai, G., Strait, J., Najjar, S., Nagaraja, R., Orrú, M., Usala, G., Dei, M., Lai, S., Maschio, A., Busonero, F., Mulas, A., Ehret, G. B., Fink, A. A., Weder, A. B., Cooper, R. S., Galan, P., Chakravarti, A., Schlessinger, D., Cao, A., Lakatta, E.

& Abecasis, G. R. (2007). Genome-Wide Association Scan Shows Genetic Variants in the *FTO* Gene Are Associated with Obesity-Related Traits. *PLoS Genet,* **3,** e115.

Seewaldt, S., Thomas, H. E., Ejrnaes, M., Christen, U., Wolfe, T., Rodrigo, E., Coon, B., Michelsen, B., Kay, T. W. & Von Herrath, M. G. (2000). Virus-induced autoimmune diabetes: most beta-cells die through inflammatory cytokines and not perforin from autoreactive (anti-viral) cytotoxic T-lymphocytes. *Diabetes,* **49,** 1801-1809.

Seger, R. & Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J,* **9,** 726-35.

Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbutt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M. & Rajotte, R. V. (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med,* **343,** 230-8.

Shapiro, A. M., Ricordi, C., Hering, B. J., Auchincloss, H., Lindblad, R., Robertson, R. P., Secchi, A., Brendel, M. D., Berney, T., Brennan, D. C., Cagliero, E., Alejandro, R., Ryan, E. A., Dimercurio, B., Morel, P., Polonsky, K. S., Reems, J. A., Bretzel, R. G., Bertuzzi, F., Froud, T., Kandaswamy, R., Sutherland, D. E., Eisenbarth, G., Segal, M., Preiksaitis, J., Korbutt, G. S., Barton, F. B., Viviano, L., Seyfert-Margolis, V., Bluestone, J. & Lakey, J. R. (2006). International trial of the Edmonton protocol for islet transplantation. *N Engl J Med,* **355,** 1318-30.

Shen, Y., Zhao, Y., Yuan, L., Yi, W., Zhao, R., Yi, Q. & Yong, T. (2013). SPARC is over-expressed in adipose tissues of diet-induced obese rats and causes insulin resistance in 3T3-L1 adipocytes. *Acta Histochemica.*

Sherry, N. A., Kushner, J. A., Glandt, M., Kitamura, T., Brillantes, A. M. & Herold, K. C. (2006). Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. *Diabetes,* **55,** 3238-45.

Shi, Q., Bao, S., Maxwell, J. A., Reese, E. D., Friedman, H. S., Bigner, D. D., Wang, X. F. & Rich, J. N. (2004). Secreted protein acidic, rich in cysteine (SPARC), mediates cellular survival of gliomas through AKT activation. *J Biol Chem,* **279,** 52200-9.

Shibata, S. & Ishiyama, J. (2013). Secreted protein acidic and rich in cysteine (SPARC) is upregulated by transforming growth factor (TGF)-beta and is required for TGF-beta-induced hydrogen peroxide production in fibroblasts. *Fibrogenesis Tissue Repair,* **6,** 6.

Shimizu, H., Ohashi, K., Utoh, R., Ise, K., Gotoh, M., Yamato, M. & Okano, T. (2009). Bioengineering of a functional sheet of islet cells for the treatment of diabetes mellitus. *Biomaterials,* **30,** 5943-9.

Shoelson, S. E., Lee, J. & Yuan, M. (2003). Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance. *Int J Obes Relat Metab Disord,* **27 Suppl 3,** S49-52.

Shojaee-Moradie, F., Baynes, K. C., Pentecost, C., Bell, J. D., Thomas, E. L., Jackson, N. C., Stolinski, M., Whyte, M., Lovell, D., Bowes, S. B., Gibney, J., Jones, R. H. & Umpleby, A. M. (2007). Exercise training reduces fatty acid availability and improves the insulin sensitivity of glucose metabolism. *Diabetologia,* **50,** 404-13.

Siva, K., Jaako, P., Miharada, K., Rorby, E., Ehinger, M., Karlsson, G. & Karlsson, S. (2012). SPARC is dispensable for murine hematopoiesis, despite its suspected pathophysiological role in 5q-myelodysplastic syndrome. *Leukemia,* **26,** 2416-9.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., Mcmahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. & Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst,* **82,** 1107-12.

Skurk, T., Alberti-Huber, C., Herder, C. & Hauner, H. (2007). Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab,* **92,** 1023-33.

Sladek, R., Rocheleau, G., Rung, J., Dina, C., Shen, L., Serre, D., Boutin, P., Vincent, D., Belisle, A., Hadjadj, S., Balkau, B., Heude, B., Charpentier, G., Hudson, T. J., Montpetit, A., Pshezhetsky, A. V., Prentki, M., Posner, B. I., Balding, D. J., Meyre, D., Polychronakos, C. & Froguel, P. (2007). A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature,* **445,** 881-5.

Song, H., Guan, Y., Zhang, L., Li, K. & Dong, C. (2010). SPARC interacts with AMPK and regulates GLUT4 expression. *Biochem Biophys Res Commun,* **396,** 961-966.

Sorelle, J. A., Itoh, T., Peng, H., Kanak, M. A., Sugimoto, K., Matsumoto, S., Levy, M. F., Lawrence, M. C. & Naziruddin, B. (2013). Withaferin A inhibits pro-inflammatory cytokine-induced damage to islets in culture and following transplantation. *Diabetologia,* **56,** 814-824.

Sottile, J., Hocking, D. C. & Swiatek, P. J. (1998). Fibronectin matrix assembly enhances adhesion-dependent cell growth. *J Cell Sci,* **111 (Pt 19),** 2933-43.

Sottile, J., Shi, F., Rublyevska, I., Chiang, H. Y., Lust, J. & Chandler, J. (2007). Fibronectin-dependent collagen I deposition modulates the cell response to fibronectin. *Am J Physiol Cell Physiol,* **293,** C1934-46.

Spits, C., Mateizel, I., Geens, M., Mertzanidou, A., Staessen, C., Vandeskelde, Y., Van Der Elst, J., Liebaers, I. & Sermon, K. (2008). Recurrent chromosomal abnormalities in human embryonic stem cells. *Nat Biotechnol,* **26,** 1361-3.

Stendahl, J. C., Kaufman, D. B. & Stupp, S. I. (2009). Extracellular matrix in pancreatic islets: relevance to scaffold design and transplantation. *Cell Transplant,* **18,** 1-12.

Stene, L. C., Oikarinen, S., Hyoty, H., Barriga, K. J., Norris, J. M., Klingensmith, G., Hutton, J. C., Erlich, H. A., Eisenbarth, G. S. & Rewers, M. (2010). Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). *Diabetes,* **59,** 3174-80.

Stephens, L. A., Thomas, H. E., Ming, L., Grell, M., Darwiche, R., Volodin, L. & Kay, T. W. (1999). Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. *Endocrinology,* **140,** 3219-27.

Strutz, F., Okada, H., Lo, C. W., Danoff, T., Carone, R. L., Tomaszewski, J. E. & Neilson, E. G. (1995). Identification and characterization of a fibroblast marker: FSP1. *J Cell Biol,* **130,** 393-405.

Stuart, K. A., Riordan, S. M., Lidder, S., Crostella, L., Williams, R. & Skouteris, G. G. (2000). Hepatocyte growth factor/scatter factor-induced intracellular signalling. *Int J Exp Pathol,* **81,** 17-30.

Sun, Y., Ma, X., Zhou, D., Vacek, I. & Sun, A. M. (1996). Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *J Clin Invest,* **98,** 1417-22.

Sutherland, D. E., Gruessner, A. C. & Gruessner, R. W. (1998). Pancreas transplantation: a review. *Transplant Proc,* **30,** 1940-3.

Sweeney, C., Fambrough, D., Huard, C., Diamonti, A. J., Lander, E. S., Cantley, L. C. & Carraway, K. L., 3rd (2001). Growth factor-specific signaling pathway stimulation and gene expression mediated by ErbB receptors. *J Biol Chem,* **276,** 22685-98.

Sweetwyne, M. T., Brekken, R. A., Workman, G., Bradshaw, A. D., Carbon, J., Siadak, A. W., Murri, C. & Sage, E. H. (2004). Functional analysis of the matricellular protein SPARC with novel monoclonal antibodies. *J Histochem Cytochem,* **52,** 723-33.

Syngene 2008. GeneTools. 4.00 ed. Synoptics Ltd.

Takehara, K., Leroy, E. C. & Grotendorst, G. R. (1987). TGF-β inhibition of endothelial cell proliferation: Alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. *Cell,* **49,** 415-422.

Tartare-Deckert, S., Chavey, C., Monthouel, M. N., Gautier, N. & Van Obberghen, E. (2001). The matricellular protein SPARC/osteonectin as a newly identified factor up-regulated in obesity. *J Biol Chem,* **276,** 22231-7.

Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., Mcgarvey, M. L. & Martin, G. R. (1981). Osteonectin, a bone-specific protein linking mineral to collagen. *Cell,* **26,** 99-105.

Teta, M., Long, S. Y., Wartschow, L. M., Rankin, M. M. & Kushner, J. A. (2005). Very slow turnover of beta-cells in aged adult mice. *Diabetes,* **54,** 2557-67.

Thomas, F. T., Contreras, J. L., Bilbao, G., Ricordi, C., Curiel, D. & Thomas, J. M. (1999). Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. *Surgery,* **126,** 299-304.

Thomas, H. E., Mckenzie, M. D., Angstetra, E., Campbell, P. D. & Kay, T. W. (2009). Beta cell apoptosis in diabetes. *Apoptosis,* **14,** 1389-404.

Thorel, F., Nepote, V., Avril, I., Kohno, K., Desgraz, R., Chera, S. & Herrera, P. L. (2010). Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature,* **464,** 1149-54.

Todd, J. A. (2010). Etiology of type 1 diabetes. *Immunity,* **32,** 457-67.

Trombetta, J. M. & Bradshaw, A. D. (2010). SPARC/osteonectin functions to maintain homeostasis of the collagenous extracellular matrix in the periodontal ligament. *J Histochem Cytochem,* **58,** 871-9.

Turki, A., Al-Zaben, G. S., Mtiraoui, N., Marmmuoch, H., Mahjoub, T. & Almawi, W. Y. (2013). Transcription factor-7-like 2 gene variants are strongly associated with type 2 diabetes in Tunisian Arab subjects. *Gene,* **513,** 244-248.

Turner, M. D., Cassell, P. G. & Hitman, G. A. (2005). Calpain-10: from genome search to function. *Diabetes Metab Res Rev,* **21,** 505-14.

Turner, R. C., Cull, C. A., Frighi, V. & Holman, R. R. (1999). Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group. *JAMA,* **281,** 2005-12.

Tuveson, D. A. & Neoptolemos, J. P. (2012). Understanding metastasis in pancreatic cancer: a call for new clinical approaches. *Cell,* **148,** 21-3.

Tzakis, A. G., Ricordi, C., Alejandro, R., Zeng, Y., Fung, J. J., Todo, S., Demetris, A. J., Mintz, D. H. & Starzl, T. E. (1990). Pancreatic islet transplantation after upper abdominal exenteration and liver replacement. *Lancet,* **336,** 402-5.

Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B. M., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y. & Kadowaki, T. (1998). Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *J Biol Chem,* **273,** 5315-22.

Vaarala, O., Ilonen, J, ,Ruohtula, T., Pesola, J., Virtanen, S.M., Harkonen, T., Koski, M., Kallioinen, H., Tossavainen, O., Poussa, T., Jarvenpaa, a-L., Komulainen, J., Lounamaa, R., Akerblom, H.K., Knip, M. (2012). Removal of bovine insulin from cow's milk formula and early initiation of beta-cell autoimmunity in the FINDIA pilot study. *Archives of Pediatrics & Adolescent Medicine,* **166,** 608-614.

Vaarala, O., Knip, M., Paronen, J., Hamalainen, A. M., Muona, P., Vaatainen, M., Ilonen, J., Simell, O. & Akerblom, H. K. (1999). Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. *Diabetes,* **48,** 1389-94.

Van Der Windt, D. J., Bottino, R., Casu, A., Campanile, N., Smetanka, C., He, J., Murase, N., Hara, H., Ball, S., Loveland, B. E., Ayares, D., Lakkis, F. G., Cooper, D. K. & Trucco, M. (2009). Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. *Am J Transplant,* **9,** 2716-26.

Varotto, S. M., Basso, S. M., Ermani, M., Bordin, D., Pizzuti, D. & Lumachi, F. (2006). Immunomodulation techniques in a pig-to-rat model of xenoislet transplantation to prolong graft survival. *In Vivo,* **20,** 857-60.

Vasavada, R. C., Gonzalez-Pertusa, J. A., Fujinaka, Y., Fiaschi-Taesch, N., Cozar-Castellano, I. & Garcia-Ocana, A. (2006). Growth factors and beta cell replication. *Int J Biochem Cell Biol,* **38,** 931-50.

Vasavada, R. C., Wang, L., Fujinaka, Y., Takane, K. K., Rosa, T. C., Mellado-Gil, J. M., Friedman, P. A. & Garcia-Ocana, A. (2007). Protein kinase C-zeta activation markedly enhances beta-cell proliferation: an essential role in growth factor mediated beta-cell mitogenesis. *Diabetes,* **56,** 2732-43.

Vernon, R. B. & Sage, H. (1989). The calcium-binding protein SPARC is secreted by Leydig and Sertoli cells of the adult mouse testis. *Biol Reprod,* **40,** 1329-40.

Virtanen, S. M., Kenward, M. G., Erkkola, M., Kautiainen, S., Kronberg-Kippila, C., Hakulinen, T., Ahonen, S., Uusitalo, L., Niinisto, S., Veijola, R., Simell, O., Ilonen, J. & Knip, M. (2006). Age at introduction of new foods and advanced beta cell autoimmunity in young children with HLA-conferred susceptibility to type 1 diabetes. *Diabetologia,* **49,** 1512-21.

Viskari, H., Kondrashova, A., Koskela, P., Knip, M. & Hyöty, H. (2006). Circulating Vitamin D Concentrations in Two Neighboring Populations With Markedly Different Incidence of Type 1 Diabetes. *Diabetes Care,* **29,** 1458-1459.

Von Hoff, D. D., Penny, R., Shack, S., Campbell, E., Taverna, D., Borad, M., Love, D., Trent, J. & Bittner, M. (2006). Frequency of potential therapeutic targets identified by immunohistochemistry (IHC) and DNA microarray (DMA) in tumors from patients who have progressed on multiple therapeutic agents. *Journal of Clinical Oncology,* **24,** (suppl):18s Abstract 3071.

Von Hoff, D. D., Ramanathan, R. K., Borad, M. J., Laheru, D. A., Smith, L. S., Wood, T. E., Korn, R. L., Desai, N., Trieu, V., Iglesias, J. L., Zhang, H., Soon-Shiong, P., Shi, T., Rajeshkumar, N. V., Maitra, A. & Hidalgo, M. (2011). Gemcitabine plus nab-paclitaxel is an active regimen in patients with advanced pancreatic cancer: a phase I/II trial. *J Clin Oncol,* **29,** 4548-54.

Vonlaufen, A., Joshi, S., Qu, C., Phillips, P. A., Xu, Z., Parker, N. R., Toi, C. S., Pirola, R. C., Wilson, J. S., Goldstein, D. & Apte, M. V. (2008). Pancreatic stellate cells: partners in crime with pancreatic cancer cells. *Cancer Res,* **68,** 2085-93.

Wang, R. N. & Rosenberg, L. (1999). Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *J Endocrinol,* **163,** 181-90.

Ward, W. K., Massoud, R. G., Szybala, C. J., Engle, J. M., El Youssef, J., Carroll, J. M., Roberts, C. T., Jr. & Dimarchi, R. D. (2010). In vitro and in vivo evaluation of native glucagon and glucagon analog (MAR-D28) during aging: lack of cytotoxicity and preservation of hyperglycemic effect. *J Diabetes Sci Technol,* **4,** 1311-21.

Warnock, G. L., Kneteman, N. M., Ryan, E., Seelis, R. E., Rabinovitch, A. & Rajotte, R. V. (1991). Normoglycaemia after transplantation of freshly isolated and cryopreserved pancreatic islets in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia,* **34,** 55-8.

Wasi, S., Otsuka, K., Yao, K. L., Tung, P. S., Aubin, J. E., Sodek, J. & Termine, J. D. (1984). An osteonectinlike protein in porcine periodontal ligament and its synthesis by periodontal ligament fibroblasts. *Can J Biochem Cell Biol,* **62,** 470-8.

Wasserfall, C. H. & Atkinson, M. A. (2006). Autoantibody markers for the diagnosis and prediction of type 1 diabetes. *Autoimmun Rev,* **5,** 424-8.

Weaver, M. S., Workman, G. & Sage, E. H. (2008). The copper binding domain of SPARC mediates cell survival in vitro via interaction with integrin beta1 and activation of integrin-linked kinase. *J Biol Chem,* **283,** 22826-37.

Weber, L. M., Hayda, K. N. & Anseth, K. S. (2008). Cell-matrix interactions improve beta-cell survival and insulin secretion in three-dimensional culture. *Tissue Eng Part A,* **14,** 1959-68.

Wen, L., Ley, R. E., Volchkov, P. Y., Stranges, P. B., Avanesyan, L., Stonebraker, A. C., Hu, C., Wong, F. S., Szot, G. L., Bluestone, J. A., Gordon, J. I. & Chervonsky, A. V. (2008). Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature,* **455,** 1109-13.

Witkowski, P., Sondermeijer, H., Hardy, M. A., Woodland, D. C., Lee, K., Bhagat, G., Witkowski, K., See, F., Rana, A., Maffei, A., Itescu, S. & Harris, P. E. (2009). Islet grafting and imaging in a bioengineered intramuscular space. *Transplantation,* **88,** 1065-74.

Wolf, G., Sharma, K., Chen, Y., Ericksen, M. & Ziyadeh, F. N. (1992). High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-beta. *Kidney Int,* **42,** 647-56.

Wolfsdorf, J., Craig, M. E., Daneman, D., Dunger, D., Edge, J., Lee, W., Rosenbloom, A., Sperling, M. & Hanas, R. (2009). Diabetic ketoacidosis in children and adolescents with diabetes. *Pediatr Diabetes,* **10 Suppl 12,** 118-33.

Wrana, J. L., Maeno, M., Hawrylyshyn, B., Yao, K. L., Domenicucci, C. & Sodek, J. (1988). Differential effects of transforming growth factor-beta on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. *J Cell Biol,* **106,** 915-24.

Wu, D., Li, L., Yang, M., Liu, H. & Yang, G. (2011). Elevated plasma levels of SPARC in patients with newly diagnosed type 2 diabetes mellitus. *Eur J Endocrinol,* **165,** 597-601.

Yan, Q., Clark, J. I., Wight, T. N. & Sage, E. H. (2002). Alterations in the lens capsule contribute to cataractogenesis in SPARC-null mice. *J Cell Sci,* **115,** 2747-56.

Zeisberg, M. & Neilson, E. G. (2009). Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest,* **119,** 1429-37.

Zhang, C., Qi, L., Hunter, D. J., Meigs, J. B., Manson, J. E., Van Dam, R. M. & Hu, F. B. (2006). Variant of transcription factor 7-like 2 (TCF7L2) gene and the risk of type 2 diabetes in large cohorts of U.S. women and men. *Diabetes,* **55,** 2645-8.

Zhang, Q., Berggren, P. O., Hansson, A. & Tally, M. (1998). Insulin-like growth factor-I-induced DNA synthesis in insulin-secreting cell line RINm5F is associated with phosphorylation of the insulin-like growth factor-I receptor and the insulin receptor substrate-2. *J Endocrinol,* **156,** 573-81.

Zhang, Y., Jalili, R. B., Warnock, G. L., Ao, Z., Marzban, L. & Ghahary, A. (2012). Three-dimensional scaffolds reduce islet amyloid formation and enhance survival and function of cultured human islets. *Am J Pathol,* **181,** 1296-305.

Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. & Melton, D. A. (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature,* **455,** 627-32.

Zhou, X., Tan, F. K., Reveille, J. D., Wallis, D., Milewicz, D. M., Ahn, C., Wang, A. & Arnett, F. C. (2002). Association of novel polymorphisms with the expression of SPARC in normal fibroblasts and with susceptibility to scleroderma. *Arthritis Rheum,* **46,** 2990-9.

Ziegler, A. G., Schmid, S., Huber, D., Hummel, M. & Bonifacio, E. (2003). Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. *JAMA,* **290,** 1721-8.

Zipitis, C. S. & Akobeng, A. K. (2008). Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis. *Archives of Disease in Childhood,* **93,** 512-517.

Zung, P., Domenicucci, C., Wasi, S., Kuwata, F. & Sodek, J. (1986). Osteonectin is a minor component of mineralized connective tissues in rat. *Biochem Cell Biol,* **64,** 356-62.

Appendix 1

Table A1: List of reagents

Item	Catalogue number	Supplier
2-mercaptoethanol	M3148	Sigma-Aldrich, UK
Accutase	A6964	Sigma-Aldrich, UK
Acetic Acid	A/0360/PB17	Fisher Scientific, UK
Ammonium persulphate	A3678	Sigma-Aldrich, UK
Avidin-Biotin Complex (ABC)	PK-4000	Vector Labs, UK
Bovine Serum Albumin (BSA)	A3294	Sigma-Aldrich, UK
Bradford Reagent	B6916	Scientific Lab Supplies, UK
Collagenase type XI	C7657	Sigma-Aldrich, UK
DAB reagent (ImmPACT)	SK-4105	Vector Labs, UK
DMEM	E15-883	PAA Labs, UK
DMSO	BP231-100	Fisher Scientific, UK
DPX Mountant (Di-N-Butyl Phthalate in Xylene)	44581	Sigma-Aldrich, UK
Ethanol	E/0650DF/17	Fisher Scientific, UK
Etoposide	E1383	Sigma-Aldrich, UK
Fluorescein Diacetate (FDA)	F7378	Sigma-Aldrich, UK, UK
Foetal Bovine Serum (FBS)	VX10270098	Fisher Scientific, UK
Glutamine	25030	Gibco (Fisher Scientific, UK)
Glycine	410225	Sigma-Aldrich, UK
Glycine (electrophoresis)	G8898	Sigma-Aldrich, UK
Ham's F12	E15-890	PAA Labs, UK
HEPES	H0887	Sigma-Aldrich, UK
Haematoxylin	GHS232	Sigma-Aldrich, UK
HiPerFect	301705	Qiagen, UK
Hydrogen Peroxide	H3410	Sigma-Aldrich, UK
Industrial Methylated Spirits	23684.360	VWR, UK
Isopropanol	P/7490/17	Fisher Scientific, UK
Methanol	20847.307	VWR, UK
Normal horse serum	S-2000	Vector Labs, UK
Nuclease-free water	AM9938	Ambion/Applied Biosystems, UK
ON-TARGETplus Human SPARC siRNA (5 nmol)	J-003710-10-0005	Thermo Scientific, UK
PageRuler Protein ladder	SM0671	Fermentas, UK
Penicillin/Streptomycin (for cells)	VX15140148	Gibco (Fisher Scientific, UK)
Penicillin/Streptomycin (for islets)	P4458	Sigma-Aldrich, UK
Phosphate Buffered Saline (Dulbecco's)(+Ca^{2+}, +Mg^{2+})	VX14040091	Gibco (Fisher Scientific, UK)
Phosphate Buffered Saline (Dulbecco's) (-Ca^{2+}, -Mg^{2+})	VX14190094	Gibco (Fisher Scientific, UK)

Phosphate Buffered Saline tablets	18912	Gibco (Fisher Scientific, UK)
Poly(D)lysine hydrobromide	P6407	Sigma-Aldrich, UK Aldrich, UK
Propidium Iodide (PI)	81845	Sigma-Aldrich, UK (Fluka)
Protease and phosphatase inhibitors	78440	Thermo Scientific, UK
Proteinase K	P2308	Sigma-Aldrich, UK
Protogel	ELR-210-020M	National Diagnostics, UK
Puromycin dihydrochloride	P8833	Sigma-Aldrich, UK
Restore Western Blot Stripping Buffer	21059	Pierce (Perbio) , UK
RIPA buffer	9806	New England Biolabs, UK
RPMI 1640 (islets)	R8758	Sigma-Aldrich, UK
RPMI 1640 (INS-1 cells)	VX21875034	Gibco (Fisher Scientific, UK)
SlowFade with DAPI	S36939	Invitrogen, UK
Sodium chloride	S7653	Sigma-Aldrich, UK
Sodium dodecyl sulphate	L3771	Sigma-Aldrich, UK
Sodium pyruvate	S8636	Sigma-Aldrich, UK
Sulphorhodamine B	S1402	Sigma-Aldrich, UK
Tetramethylethylenediamine (TEMED)	T9281	Sigma-Aldrich, UK
Trichhloroacetic acid (TCA)	T/2950/53	Fisher Scientific, UK
Tris (hydroxymethyl)aminomethane	25,285-9	Sigma-Aldrich, UK
Triton-X-100	T8787	Sigma-Aldrich, UK
Trypan blue	VX15250061 (10062742)	Fisher Scientific, UK
Trypsin/EDTA (0.05%)	VX25300054 (10194522)	Fisher Scientific, UK
Tween 20	P1379	Sigma-Aldrich, UK
Vectashield	H-1000	Vector Labs, UK
Vectastain ABC Kit	PK-4000	Vector Labs, UK
West pico chemiluminescent substrate (SuperSignal)	PN34080	Pierce (Fisher Scientific, UK)

Table A2: List of disposables

Item	Catalogue number	Supplier
6-well non-treated plates	TKT-220-006R	Fisher Scientific, UK
12-well non-treated plates	TKT-220-012W	Fisher Scientific, UK
24-well plates	PAA30024X	PAA, UK
48-well tissue culture plates	TKT-190-090H	Fisher Scientific, UK
96-well TC plates	TKT-521-070B	Corning, UK
Cell culture inserts	353102	BD Falcon, UK
Coverslips #1.5 (20x20)	FB58730	Fisher Scientific, UK
Coverslips 50 x 22mm	SD6776315	Fisher Scientific, UK

Cryogenic vials	CRY-960-251U	Nunc
Hybond ECL nitrocellulose membrane	RPN303D	GE Healthcare, UK
Microcentrifuge tubes	FB74061	Fisher Scientific, UK
Microscope slides, poly-lysine coated	12637976	Fisher Scientific, UK
Syringe end filters 0.2µm	FDP-620-030U	Fisher Scientific, UK
T25 filter capped flasks	TKT-130-170F	Fisher Scientific, UK
T75 filter capped flasks	TKT-130-210T	Fisher Scientific, UK
Universal tubes (aseptic)	FB51527	Fisher Scientific, UK
Vivapore Concentrator	VP2001	Vivascience (Sartorius UK)

Table A3: List of equipment

Item	**Model**	**Brand**
Centrifuge	Heraeus Megafuge 16	Thermo Scientific
Fisherbrand gel running unit (PAGE)	V10-CDC	Fisher Scientific
GeneGnome Bioimaging System	50000	Syngene Bioimaging
Inverted microscope	AE31 (101M)	Motic
Microcentrifuge	Eppendorf AG minispin	Eppendorf
Microplate reader (Bradford assay)	FLUOstar OPTIMA	BMG Labtech
Microplate reader (SRB assay)	Epoch Microplate Spectrophotometer	BioTek
Microscope (cell count)	Olympus BH-2	PBA microscopes
Microscope (IHC and fluorescence)	Nikon Eclipse 80i	Nikon
Microtome	RM2255	Leica
pH meter	inoLab pH 720	WTW
pH probe	Sentix 42	WTW
Semi-dry blotter	TE70X semi-dry transfer unit	Hoefer
Semi-dry blotter	V10-SDB	Fisherbrand
Tissue processor	Shandon Citadel 1000	Thermo Electron Corporation

Appendix 2

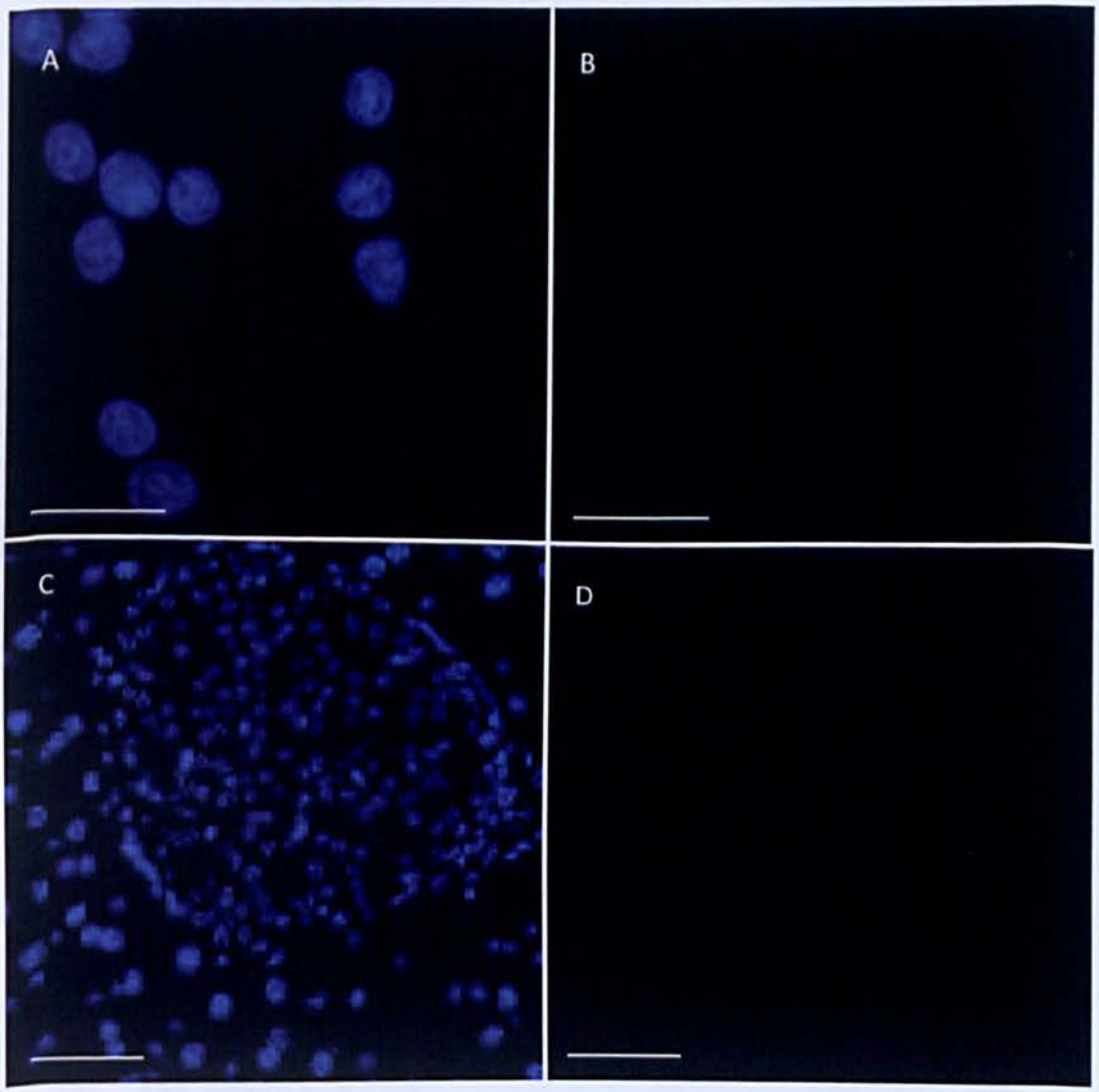


Figure A2.1. Secondary control for texas red antibody. INS-1 cells fixed on coverslips (**A,B**) or pancreas sections (5 µm; **C,D**) were treated with a Texas red goat anti-guinea pig secondary anti-body and mounted with SlowFade reagent with DAPI. **A&C**: Show blue channel only (DAPI). **B&D**: Show red channel only (Texas red). Slides were imaged on a confocal microscope. Scale bar: 20 µm for INS-1 cells; 50 µm for pancreas sections.

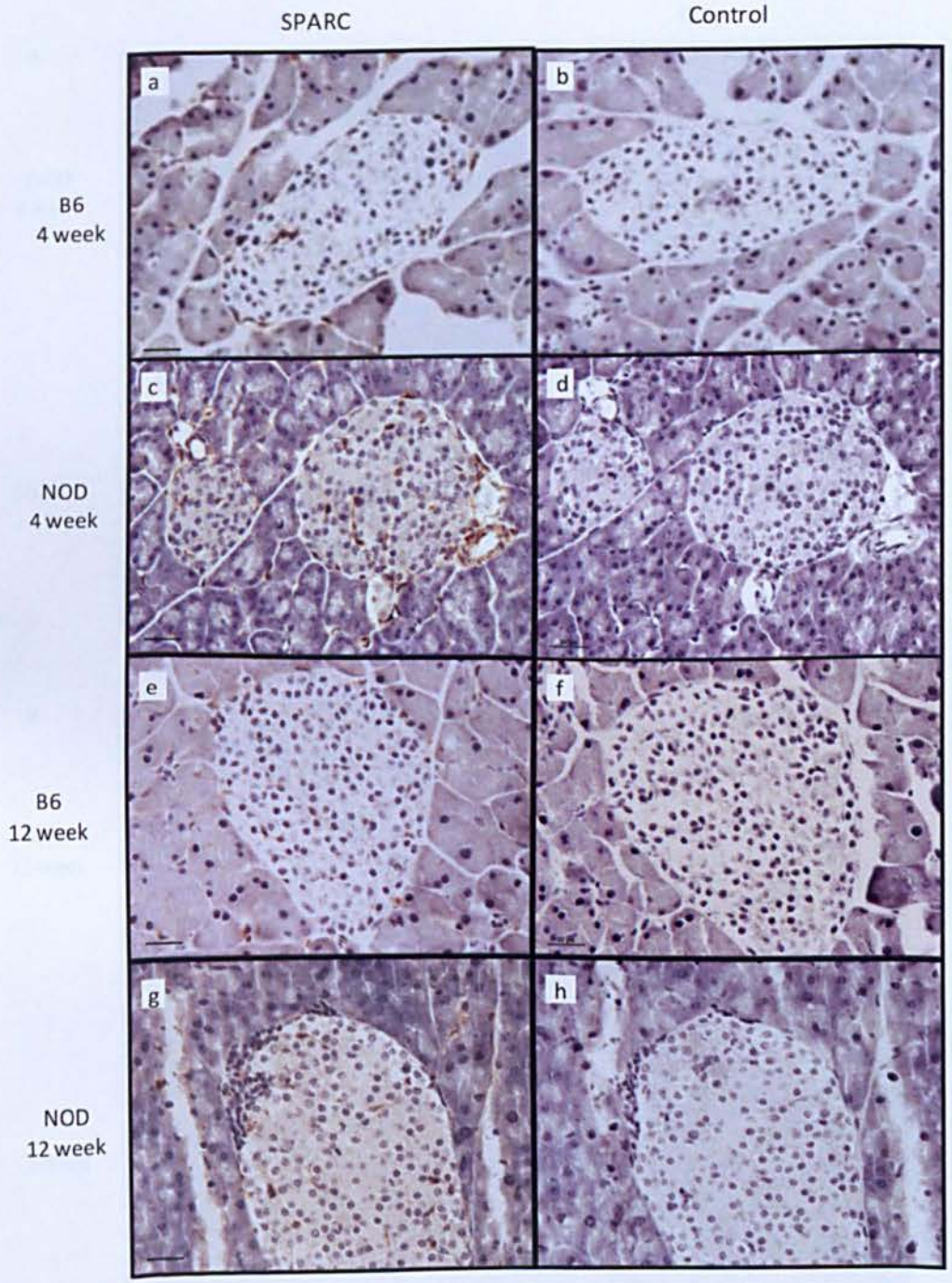


Figure A2.2. SPARC secondary controls are negative. Mouse pancreas sections (5 µm) from 4 and 12 week NOD or B6 mice were stained with anti-SPARC antibody (left panels) or without primary antibody (right panels) followed by biotin-conjugated secondary antibody. Positive areas were visualised using the ABC/DAB method producing a brown colour.

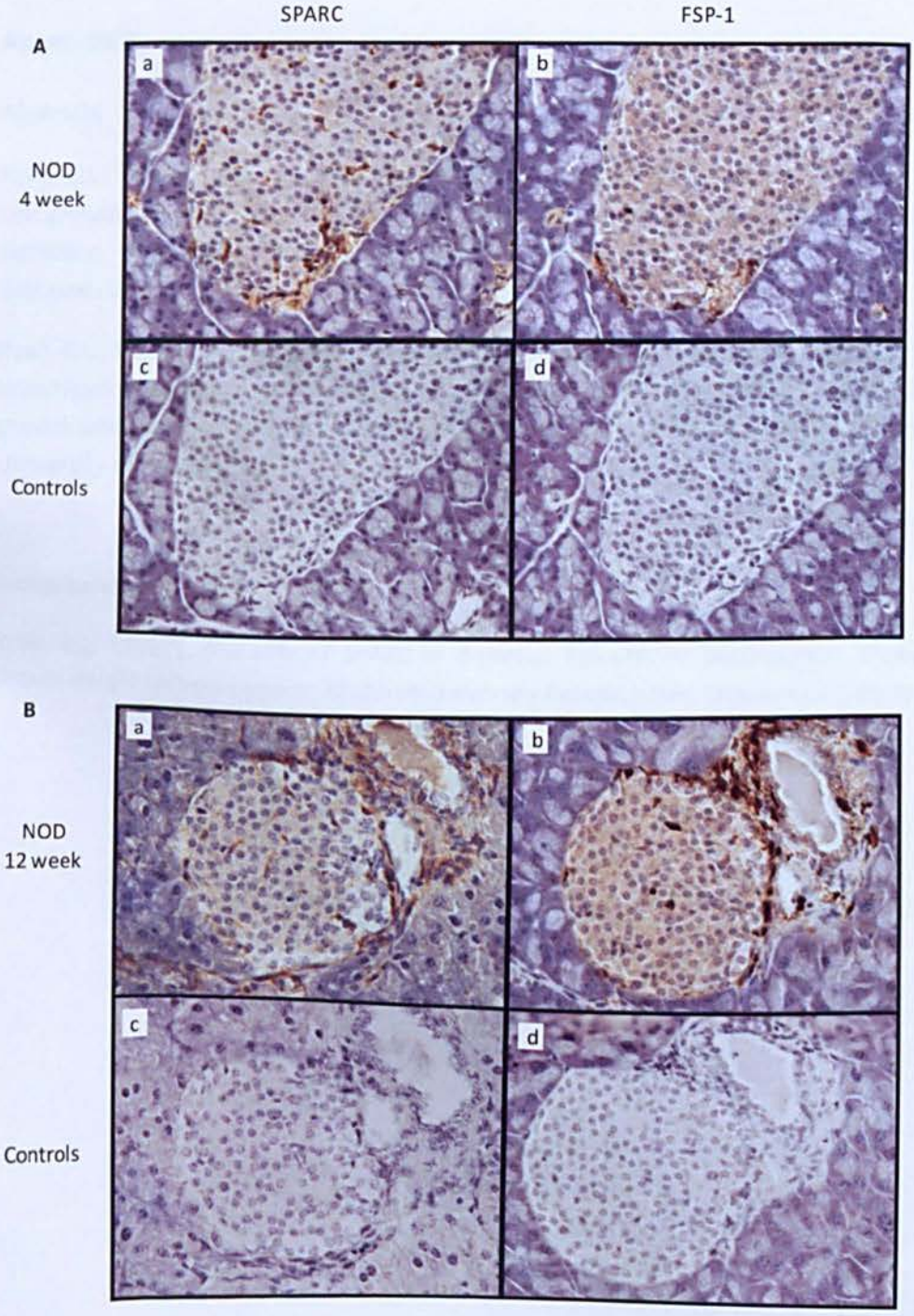


Figure A2.3. SPARC and FSP-1 secondary controls are negative. Adjacent sections (5 µm) of 4 (A) and 12 (B) week NOD mouse pancreas were stained with antibodies to SPARC (a) and FSP-1 (b) or without primary antibody (c&d) followed by biotin-conjugated secondary antibody. Positive areas were visualised using the ABC/DAB method producing a brown colour. Representative images are shown of sections from at least 3 different mice.

Appendix 3

Abstracts

Ryall, C.L., Walker, A.J., King, A.J., Jones, P.M., Mackintosh, D., Edling, C.E., Hill, N.J. (2012). Islet β cell growth: the role of secreted protein acidic and rich in cysteine (SPARC) in proliferation and signalling. Unpublished poster presentation at the *Diabetes UK Professional Conference 2012,* Glasgow, UK.

Ryall, C.L., Walker, A.J., King, A.J., Jones, P.M., Mackintosh, D., Edling, C.E., Hill, N.J. (2011). Investigation into the role of secreted protein, acidic and rich in cysteine (SPARC) in beta cell growth and signalling. Unpublished poster presentation at *St. George's Research Day*, St. George's University of London.

Presentations

Ryall, C.L. (2012). The role of SPARC in diabetes. PowerPoint presentation. Diabetes Research Group, King's College London. Kingston University Research Day, Kingston University London.