Using mesenchymal stromal cells to improve islet transplantation outcome

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Abstract

Pancreatic islet transplantation is an attractive treatment option for a subset of patients with Type 1 diabetes. However islet transplantation efficacy remains hampered by a number of factors, including the impaired quality of isolated islets available for transplantation and the loss of long-term islet function post transplantation. Harnessing the properties of multipotent mesenchymal stromal cells (MSCs), which can be derived from numerous clinically relevant post-natal tissues including adipose and pancreas, is currently under investigation for improving the survival of islet cells during culture and after transplantation. Amongst other beneficial properties, MSCs are known to provide a supportive micro-environmental niche via secretion of paracrine factors or deposition of extracellular matrix, making them excellent candidates to play the role of islet 'helper' cells for purposes of improving islet transplantation outcome.

Preliminary studies conducted within the group demonstrated that co-transplantation of islets and MSCs improve the survival and function of engrafted islets post-transplantation, leading to overall improved outcomes of islet grafts in mouse models of Type 1 diabetes. The aims of this thesis were to investigate the mechanisms through which MSCs were exerting beneficial effects on islet function *in vivo* using *in vitro* co-culture methods and then to develop these findings into potential pre-transplant islet culture protocols with the aim of improving the function of isolated human islets available for transplantation. After deriving and characterising adipose MSCs (adipMSCs) from mouse tissue, it was demonstrated that direct cell-cell contact, between mouse islets and MSCs co-cultured *in vitro* was necessary for enhancing islet insulin secretory function compared to islets cultured alone, whereas the trophic factors secreted by the MSCs alone were not able to positively affect islet function. Additional co-culture studies also showed that the extracellular matrix (ECM) deposited by adipMSCs alone was able to improve the insulin secretory function of co-cultured islets, but not to the full extent of when MSCs were present. Through adopting a direct contact monolayer co-culture configuration for human islets and MSCs, it was

also demonstrated that human adipMSCs improved the function of isolated human islets, more reproducibly than either human pancreatic MSCs (pMSCs) or human bone-marow MSCs (bmMSCs), supporting the use of adipMSCs in pre-transplant islet culture protocols. In summary adipMSCs enhanced the function of isolated islets through direct contact based mechanisms and warrant further investigation for use in clinical islet transplantation strategies, including the functional maintenance of isolated human islets in culture prior to transplantation.

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List of Abbreviations

AAgs	Autoantigens
adipMSC	Adipose-derived mesenchymal stromal cells
ADP	Adeonosine diphosphate
APC	Allophycocyanin
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
bmMSC	Bone marrow-derived mesenchymal stromal cells
BSA	Bovine serum albumin
C57BL/6	Inbred mouse strain C57 black 6
cAMP	Cyclic adenosine monophosphate
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD11b	Cluster of differentiation 11b
CD31	Cluster of differentiation 31
CD44	Cluster of differentiation 44
CD45	Cluster of differentiation 45
CD73	Cluster of differentiation 73
CD90	Cluster of differentiation 90
CD105	Cluster of differentiation 105
CITR	Collaborative islet transplant registry
CPD	Cumulative population doubling
CSII	Continuous subcutaneous insulin infusion
DC	Dendritic cell
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagles Medium
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
FBS	Fetal bovine serum

FFA	Free fatty acid
FITC	Fluorescein
GAG	Glycosaminoglycan
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GSIS	Glucose-stimulated insulin secretion
HGF	Hepatocyte growth factor
IAA	Insulin autoantibodies
ICA	Islet cell autoantigens
IL	Interleukin
IRS	Insulin receptor substrate
ISCT	International Society for Cellular Therapy
ITA	Islet transplant alone
kMSC	Kidney-derived mesenchymal stromal cells
MDI	Multiple daily injections
MMC	Mitomycin C
MSC	Mesenchymal stromal cell
NHS	National Health Service
PE	Phycoerythrin
PerCP	Perdinin chlorophyll
pMSC	Pancreatic-derived mesenchymal stromal cells
PTA	Pancreas transplant alone
RER	Rough endoplasmic reticulum
SC	Sertolli cell
Sca-1	Stem cell antigen-1
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF-β1	Transforming growth-factor beta 1
TW	Transwell

VEGF	Vascular endothelial growth factor

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Chapter 1 - General Introduction

1.1 Normal glucose homeostasis

Glucose is a vital source of energy for many tissues and cells of the body, and is the obligate metabolic fuel for the brain under physiological conditions. Glucose uptake by the brain is critical because alternative fuel substrates either circulate in low concentrations (e.g. ketones) or have limited transport across the blood-brain barrier (e.g. free fatty acids (FFAs)) (Siesjo, 1988). The brain does not synthesise or store glucose for more than a few minutes supply, and is therefore dependent upon a continuous supply of glucose from blood plasma. Glucose transporter (GLUT) molecules (GLUT1, 2, 3, and 8) are present permanently on brain cell membranes, to facilitate glucose uptake by the brain (Uldry and Thorens, 2004). Glucose in plasma comes either from dietary sources or as a result of either glycogen breakdown in the liver (glycogenolysis) or the formation of glucose in the liver and kidneys from other carbon compounds such as lactate, pyruvate and amino acids (gluconeogenesis). Glucose levels are tightly regulated by the body and average approximately 5mM throughout a 24 hour period. The maximal concentration does not usually exceed 9mM e.g. after meal ingestion or drop below 3mM e.g. after exercise (Rizza et al., 1980, Wahren et al., 1978). This narrow arterial plasma glucose range, defining normoglycaemia, is regulated through an intricate regulatory and counter-regulatory neuro-hormonal system, with two of the key hormones being insulin and glucagon. It is the role of the endocrine pancreas to regulate glucose homeostasis and metabolism through production of these key hormones.

The endocrine pancreas is composed of islets of Langerhans, which are clusters of cells dispersed amongst the acinar tissue of the exocrine pancreas. Two important cell types found within islets, for glucose regulation, are beta-cells (β-cells) cells and alpha-cells (α-cells) cells, which produce the hormones insulin and glucagon respectively. Insulin regulates glucose metabolism by direct and indirect actions. An increment in plasma glucose, e.g. caused by meal ingestion

(post-prandial state), will stimulate insulin release from β-cells to directly reduce blood glucose levels. Insulin accelerates the transport of glucose into target cells; tissues involved in glucose uptake include brain, liver, kidney, adipose tissue and muscle, with muscle cells being responsible for the majority (75-80%) of peripheral glucose uptake (DeFronzo, 2004). For adipose, muscle and kidney tissue, insulin is integral for glucose uptake. Through binding to insulin receptors in each tissue, insulin activates its signalling pathway which involves a complex cascade of protein kinases and phosphorylation of insulin receptor substrate (IRS) to activate GLUT4 molecules. These molecules then move to the cell surface and transport glucose into the cell. Like brain tissue, liver on the other hand uses GLUT molecules that are always present on the cell membrane and do not require insulin facilitation for glucose uptake (Uldry and Thorens, 2004). Once glucose enters the cell it is phosphorylated, which prevents its diffusion back out of the cell. Glucose is then either used in glycolytic pathways (both oxidative and non-oxidative) or converted to glycogen and stored in the liver. Although insulin does not increase glucose transport into the liver, it works through several other indirect mechanisms to lower blood glucose levels by promoting glycogen accumulation in the liver. These include the inhibition of liver phosphorylase (glycogenolysis enzyme) preventing the breakdown of stored glycogen and the stimulation of liver glycogen synthase activity.

Glucagon is the major counterpart to insulin in the regulation of plasma glucose. Glucagon release from α-cells is triggered by a lowering of blood glucose levels, e.g. caused by overnight fasting (post-absorptive state). It acts exclusively on the liver to accelerate the conversion of stored glycogen into glucose through the process of glycogenolysis in order to raise blood glucose levels. By binding to its receptors glucagon activates adenylate cyclase, which increases intracellular cyclic AMP (cAMP) levels and stimulates protein kinase A, phosphorylase kinase and phosphorylase activity to initiate glycogenolysis.

1.2 Regulation of insulin synthesis and secretion

Insulin is synthesised within the β-cells of the islets of Langerhans. In adult mammals, the insulin gene is expressed solely in pancreatic β-cells and gene transcription is regulated by specific hormones and nutrient secretagogues, with glucose being a prominent inducer. Insulin mRNA is translated as a single sequence precursor called preproinsulin in the rough endoplasmic reticulum (RER) of β-cells. Newly synthesised preproinsulin is rapidly cleaved to free proinsulin of its N-terminal signal peptide, during or shortly after its translocation across the RER membrane. Proinsulin is converted to insulin by the action of two prohormone-converting enzymes (PC1/3 and PC2) which become activated in the trans Golgi network. These enzymes excise pairs of basic amino acids to result in formation of an insulin molecule and a C-peptide chain (a 31 amino acid residue). Insulin is packaged and stored, along with excised proinsulin C-peptides in membrane bound cytoplasmic granules of the β-cell from where they are secreted in equimolar amounts in response to hyperglycaemic stimuli.

Insulin secretion is primarily triggered by glucose, but also involves other nutrients (e.g. FFAs and amino acids) and numerous neural and hormonal factors. Glucose-stimulated insulin secretion (GSIS) in islets is stimulated by glucose concentrations as low as 3-4mM in human islets and approximately 6-7mM in mouse islets, with half maximal stimulation achieved at approximately 6mM and 11mM in human and mouse islets respectively (Henquin et al., 2006, Hedeskov, 1980). GSIS requires the intracellular uptake and metabolism of glucose by the β-cell, leading to the subsequent activation of an intracellular messenger system (Fig. 1.1). Glucose transporter molecules, GLUT2 in rodents and GLUT1 and 3 in humans, (McCulloch et al., 2011, Devos et al., 1995) located on the plasma membrane of the β-cell facilitate glucose entry into the β-cell. Glucose metabolism is then initiated by phosphorylation of glucose to glucose-6-phosphate, a reaction that is catalysed by glucokinase. Metabolism of glucose increases the concentrations of ATP:ADP, allowing ATP to interact with ATP-dependent potassium channels and causing them to close. Closure of the

channels depolarises the plasma membrane potential, which in turn opens voltage gated calcium channels and allows the influx of extracellular calcium down its concentration gradient. Increases in the cytoplasmic calcium concentration causes activation of protein kinases and other calcium dependent transduction machinery, which ultimately leads to the exocytosis of insulin from secretory granules.

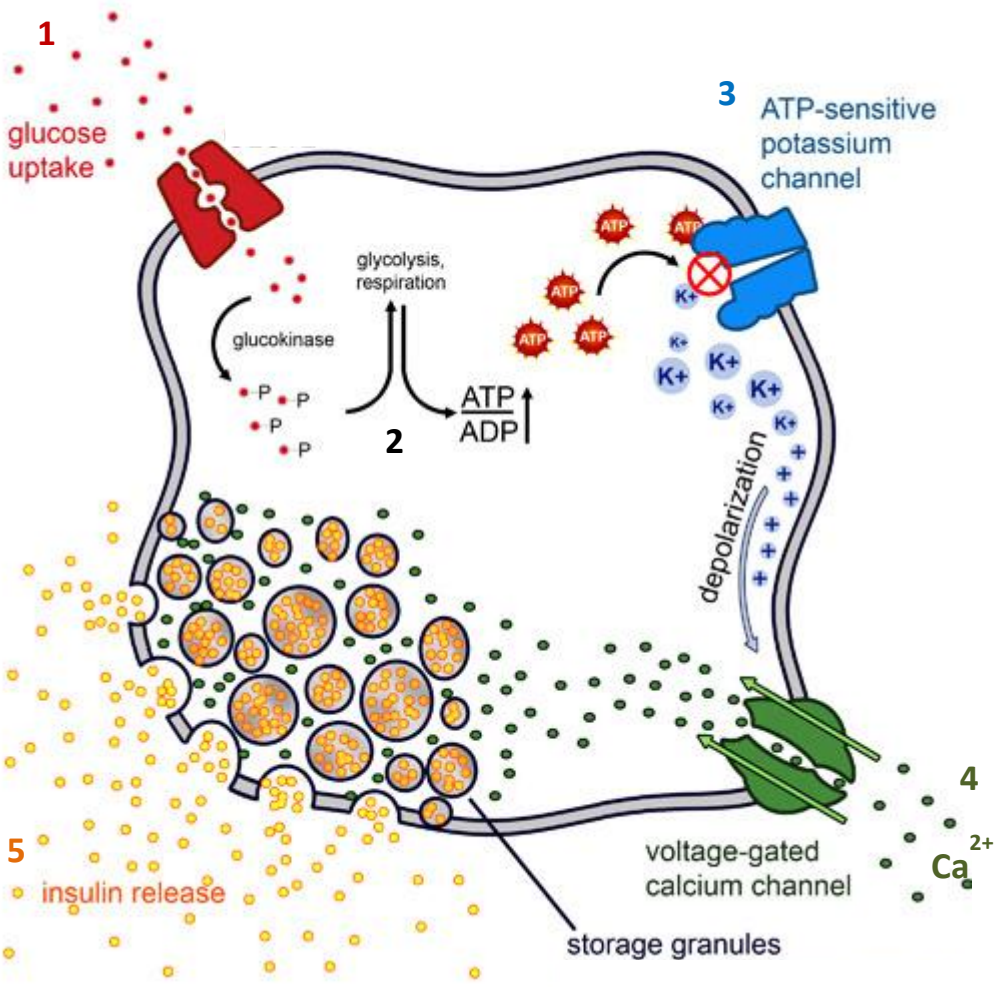

Figure 1.1: Schematic view of glucose-stimulated insulin secretion cascade. Glucose entry into the cell is facilitated by glucose transporters expressed on the plasma membrane of the β-cell (1). Glucose is metabolised (2) increasing ATP:ADP. ATP interaction with potassium channels causes their closure (3) and membrane depolarisation. This in turn opens voltage gated calcium channels (4) allowing an influx of calcium ions. Increased cytoplasmic calcium concentration ultimately leads to exocytosis of insulin from storage granules (5). Image adapted from beta cell biology consortium (www.betacell.org)

GSIS can be magnified by intestinal factors called incretins. Incretin hormones, including gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), are secreted by K and L cells respectively of the intestinal endocrine mucosa in response to elevated plasma glucose concentrations after meal ingestion. The hormones bind to their respective G-protein coupled receptors on the β-cell, increasing intracellular cAMP levels. Increases in cAMP cause an increase in cytoplasmic calcium concentration both directly by activating calcium channels and indirectly by activating protein kinase A which causes closure of potassium channels and depolarisation.

1.3 The pancreatic islet

The pancreas is structurally and functionally segregated into two parts, the exocrine (acinar) pancreas that forms most of the pancreatic mass, and the endocrine pancreas constituted of islets of Langerhans that account for only 1-2% of pancreatic mass. The exocrine pancreas consists of cells arranged in clusters known as acini and acts a source of digestive enzymes, such as amylase, trypsin and chymotrypsin, for digesting dietary fats and lipids. The islets of the endocrine pancreas consist of several types of cells, of which insulin-secreting β-cells, glucagon-releasing α-cells and somatostatin-secreting delta cells (δ-cells) are the most important. The cell clusters can range from 100-300μm in diameter and usually consist of approximately 2000-3000 cells. Much of our knowledge about islets of Langerhans and their function comes from work on rodent models. Human and rodent islets share many features, including their size; however they are not identical and differ both in cell type composition and cyto-architecture (Fig. 1.2). Human islets contain fewer insulin-secreting β-cells than rodent islets (50% vs. 70%) but contain more glucagon-secreting α-cells (40% vs. 18%) (Cabrera et al., 2006, Orci and Unger, 1975). In rodent islets the dominant β-cell can be found clustered at the core of the islet surrounded by a mantle of α and δ cells. In human islets the non-β cells line the islet capillaries located at the margin of the islet, as in rodents, but also penetrate the core and are directly juxtaposed to the β-cells (Bosco et al., 2010, Cabrera et al., 2006).

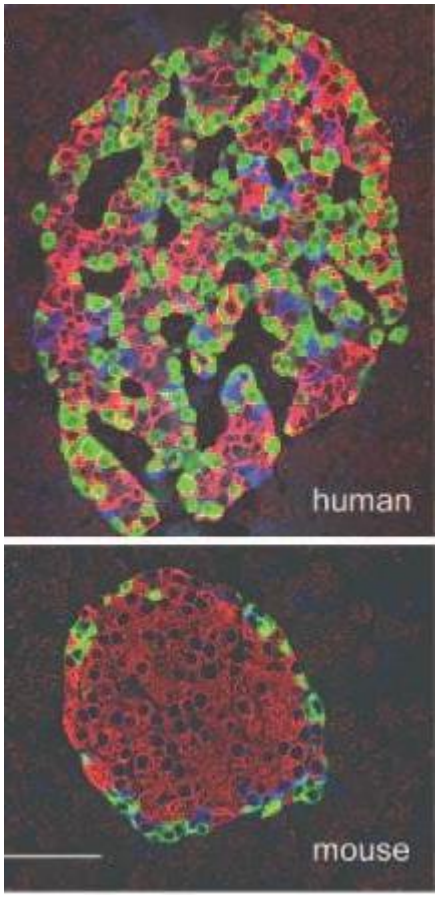

Figure 1.2: Human and mouse islets of Langerhans. Human islets (upper image) consist of α-cells (green), β-cells (red) and δ-cells (blue) intermingled throughout the endocrine cluster. Mouse islets on the other hand (lower image) have a more defined architecture with a β-cell core surrounded by a mantle of α-cells and δ-cells. Image taken from (Cabrera et al., 2006).

1.4 Diabetes Mellitus

As detailed above, the body's response to blood glucose requires a co-ordinated response from an array of mechanisms. Failure of any one component involved in insulin regulation, secretion, uptake or breakdown can lead to severe metabolic complications. Diabetes mellitus (DM) is a chronic metabolic disorder linked to the dysfunction and/or decreased mass of the pancreatic β-cell. Patients suffering from diabetes experience insulin deficiency or are unable to utilise the insulin that β-cells secrete effectively, which results in elevated blood glucose levels, a condition known as hyperglycaemia. Diabetes is diagnosed at fasting plasma glucose concentrations of ≥ 7.0mM (WHO, 1999). The disease

afflicts at least 382 million people worldwide at present, and based on estimations accounted for 6.8% of global all-cause mortality in 2010 (IDF, 2013). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. The classic symptoms of hyperglycaemia, referred to as the hyperglycaemic triad, are polyphagia - frequent hunger, especially pronounced hunger, polydipsia - frequent thirst, especially excessive thirst and polyuria - frequent urination, especially excessive urination. Other symptoms include blurred vision, fatigue and weight loss. Broadly speaking DM is classified into two major subgroups, both of which are characterised by hyperglycaemia; Type 1 diabetes (T1D; previously known as insulin-dependent, juvenile or childhood-onset) and Type 2 diabetes (T2D; formerly called non-insulin-dependent or adult-onset).

T1D accounts for 10-15% of all cases of diabetes (DUK, 2010) and is an autoimmune disease in which the body's immune system attacks and destroys the insulin producing β-cells. Although T1D can present at any age, it usually appears before the age of 40 and has an estimated prevalence in children in the UK of 1/700-1000 (DUK, 2010). The rate of β-cell destruction is quite variable, ranging from rapid in some individuals (mainly children and adolescents) to slowly progressive in others (mainly adults, sometimes referred to as latent autoimmune diabetes in adults (LADA)). Extensive β-cell destruction usually leads to an absolute insulin deficiency in patients (ADA, 2005), and requires exogenous insulin administration for survival.

T2D, the most prevalent category, often results from a relative insulin deficiency that is caused by a combination of factors. The relative deficiency arises through resistance to the action of insulin by fat, liver and muscle tissue and the inadequate insulin secretory response by the pancreatic β-cell to compensate for the impaired ability to use insulin. Often linked to lifestyle and obesity, T2D occurs most often in middle-aged and older patients, however the incidence of the disease in obese children and adolescents is increasing. It is common for T2D patients to suffer from only a degree of hyperglycaemia that is significant

enough to cause pathological and functional changes to various target tissues, but not provoke noticeable symptoms for a period of time; this can delay diagnosis until several years after onset. In some individuals adequate glycaemic control can be achieved with weight reduction, exercise and/or oral glucose lowering agents, meaning that at least initially and sometimes throughout their lifetime exogenous insulin is not required for survival.

1.4.1 Type 1 Diabetes

Type 1 Diabetes results from the specific immune-mediated destruction of the insulin producing β-cells in the pancreatic islets of Langerhans. At the time of clinical onset, approximately only 10% of normal β-cell mass remains (Gianani et al., 2010). The process of immune-mediated β-cell destruction is characterised by the recognition of β-cell proteins as self-antigens, called autoantigens (AAgs), by patients' own CD4+ and CD8+ T cells and/or autoantibodies produced by self-reactive B cells. Well-established AAgs include non-specific islet cell AAgs (ICA) (Bottazzo et al., 1974), insulin (Palmer et al., 1983), glutamic acid decarboxylase 65 (GAD65) (Baekkeskov et al., 1990) and insulinoma antigen-2 (IA-2). The appearance of diabetes-associated autoantibodies, insulin autoantibodies (IAAs), GAD autoantibodies and IA-2 autoantibodies are the first detectable sign of emerging β-cell autoimmunity and act as good predictive and diagnostic markers for the development of the disease (Knip, 2002). Since AAgs are the targets of immune responses in T1D, antigen specific immune reactions are believed to be involved in β-cell destruction. The exact steps involved in the process of β-cell destruction are still not clear, however the general process is believed to involve the generation of autoreactive CD4+ T cells in response to the presentation of AAGs by antigen presenting cells (APC) including macrophages, dendritic cells (DC) or B cells to naïve T cells in the pancreatic lymph nodes. It is then the activation of β-cell specific cytotoxic CD8+ T cells by these autoreactive CD4+ T cells that leads to the destruction of the β-cell (Stadinski et al., 2010, Yoon and Jun, 2005).

The underlying mechanisms contributing to the autoimmunity of T1D are not yet fully understood, but are believed to be an interplay between genetic susceptibility (polygenic) and environmental triggers. The most important genes contributing to disease susceptibility are located in the HLA class II loci *HLA-DRB1* and *HLA-DQB1* on the short arm of chromosome 6p21 (Ilonen et al., 2002). The function of these genes in terms of an immune response is well known since HLAs are a family of homologous proteins, which present antigenic peptides to both effector- and regulatory- T-cells (Polychronakos and Li, 2011) yet their specific contribution to the pathogenesis of type 1 diabetes remains unclear. A spectrum of risk is assigned to DR–DQ haplotypes from increased to protective. Common haplotypes that confer the highest risk of T1D are *DRB**301–*DQB**201 and *DRB**401–*DQA**301– *DQB**302, with over 90% of young-onset T1D cases carrying at least one copy of these haplotypes compared to approximately 20% of the general European-descent population. Conversely, strong protection is conferred by the *DQB**602 allele, which is carried by approximately 15% of Europeans but less than 1% of T1D sufferers (Erlich et al., 2008). Non-HLA genes are also implicated in T1D susceptibility. Polymorphisms in the insulin gene *INS* on chromosome 11p15.5 (Bell et al., 1984) and the protein tyrosine phosphatase, nonreceptor type 22 (lymphoid) *PTPN22* gene on chromosome 1p13 (Bottini et al., 2004) contribute most to T1D risk after HLA alleles, with INS conferring 10% of genetic susceptibility to T1D.

Several lines of evidence support the notion that additional non-genetic, environmental factors are also needed to trigger and potentiate β-cell destruction in genetically predisposed subjects including; 1) studies of T1D concordance rates in monozygotic twins ranges only between 13-50% (Redondo et al., 2008, Barnett et al., 1981, Kaprio et al., 1992) 2) <10% of individuals with HLA-conferred diabetes susceptibility progress to clinical disease (Knip, 2002), 3) in the last decade there has been a decrease in the proportion of newly diagnosed T1D patients carrying high-risk HLA genotypes and but an increase in the proportion of those with low-risk or even protective HLA genotypes (Hermann et al., 2003, Gillespie et al., 2004). The infectious agent believed to be

the strongest candidate for triggering autoimmunity is the enterovirus, with studies showing the timely appearance of both diabetes autoantibodies and enterovirus infections in newly diagnosed patients (Oikarinen et al., 2011). Other putative triggers include environmental toxins, or dietary factors such as exposure to bovine insulin in cow's milk from an early age (Vaarala et al., 1999, Vaarala et al., 2010). The traditional view that a single environmental trigger initiates autoimmunity in susceptible individuals has however been challenged by a more complex model, which proposes that multiple environmental influences could act to either promote or attenuate T1D during different stages of disease progression, with both the timing and quantity of exposure to the factors determining their effect (Atkinson and Eisenbarth, 2001).

1.5 Current treatments for T1D

1.5.1 Insulin Administration

The progressive destruction of beta-cells in individuals with T1D can ultimately lead to a complete deficiency of endogenous insulin. Daily exogenous insulin administration is therefore required by individuals for survival. It is currently the gold standard in treatment for diabetic patients; when used effectively it can provide adequate glycaemic control and lower the risk of both macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular (diabetic nephropathy, neuropathy and retinopathy) complications. Normoglycaemia is maintained in healthy individuals through both basal and prandial (bolus) endogenous insulin secretion. Basal insulin is secreted by the pancreas throughout the day (24 hr) at low levels (0.5-1.0 units/hour) in response to hepatic glucose output (Campbell et al., 1996), whilst the post-prandial phase (feeding-stimulated phase) of insulin secretion is intermittent in response to elevated glucose levels following nutrient ingestion (1.0 unit insulin/10g carbohydrate) (Galloway, 1993). The amount of bolus insulin secreted after feeding depends on the composition and size of the meal, and must act over a short period of time until normoglycaemia is established. In order for exogenous insulin therapy to mimic endogenous glycaemic control,

both short- and intermediate-acting insulin regimens are necessary. Recombinant human insulin was approved for pharmaceutical use in 1982, and accounts for the majority of insulin sold worldwide. The molecular structure of human insulin however restricts its ability to counter post-prandial hyperglycaemia, due to its low absorption rate into the bloodstream, leading to improper glucose management. Recombinant human insulin analogues have therefore been developed, which contain subtle deviations of the human insulin molecular structure to modify its pharmacokinetic and pharmacodynamic properties. Fast-acting and long-acting insulin analogues have been designed to offer individuals both flexibility and convenience in an attempt to achieve physiologically matched basal-bolus insulin secretion patterns with insulin injections. Metabolic variability between individuals is high however and therefore intensive insulin therapy, including multiple daily injections (MDI) or treatment with an insulin pump is required to keep blood glucose levels as close to the normal range as possible.

The use of insulin pumps for continuous subcutaneous insulin infusion (CSII) are becoming more popular with patients to provide a 24 hour preselected but adjustable basal rate of rapid-acting insulin, along with patient-activated mealtime bolus doses, and eliminate the need for periodic injections. The Diabetes Control and Complications Trial (DCCT) demonstrated that intensive therapy slows the rate of secondary complications of DM but at the expense of causing increased risk of iatrogenic hypoglycaemia (low blood glucose levels) (DCCT, 1993, Shannon et al., 2000). Although it is often claimed that CSII is less likely to cause severe hypoglycaemia than basal-bolus insulin regimens with MDI, meta-analysis of studies comparing the two treatments in patients with T1D was unable to show any significant benefit of CSII upon the rate of severe hypoglycaemia (Tamborlane et al., 2008, Fatourechi et al., 2009). A recent trial (the Hypo COMPaSS trial (Little et al., 2012)) also reported that equivalent outcomes were attained with conventional MDI regimens compared with CSII, although satisfaction was higher in CSII users (ADA, 2013). Development of an ‘artificial pancreas’, a fully automated closed loop system with a continuous

glucose monitor and insulin pump to deliver appropriate amounts of insulin at all times is, also under considerable investigation. The major concern surrounding this system is technology malfunction and the delivery of incorrect amounts of insulin, hence significant attention has been paid to the design of sensors, insulin delivery and control algorithms. Prototypes for such a device are currently being trialled in the UK, where participants used the artificial pancreas at night for four weeks at home without medical supervision (DUK, 2013). Promising results emerged for the device and patients reported improved physical, psychological and emotional benefits. However the technology remains early phase and requires significant further trialling before it can be offered as a routine treatment; at this stage it is only useful for providing glycaemic control at night.

Despite the improvements in efficacy, safety, mechanisms of delivery and patient flexibility and convenience, clinical challenges still remain with the use of insulin therapy for the treatment of T1D. The major goal of any treatment for T1D is to mimic the endogenous function of the β-cell. However, wide fluctuations in glucose levels and the risk of hypoglycaemic episodes, the latter not having ablated despite the progression of insulin therapy over the past two decades, (McCrimmon and Sherwin, 2010, Cryer, 2008), remain major barriers for intensive insulin therapy. The annual mortality rate of patients with insulin-induced inadvertent hypoglycaemia is estimated to be as high as 3–6% (Genuth et al., 2007, Patterson et al., 2007), with patients who report to suffer from severe hypoglycaemia being subject to a 3.4-fold higher risk of death (McCoy et al., 2012). Insulin has also been previously implicated as the most common drug to result in emergency room visits for adverse drug events (Budnitz et al., 2006). The ability of individuals to adhere to insulin therapy must not be overlooked either since it can be poor due to a number of factors, including age, fear of injections and type of delivery device (Davies et al., 2013). Therefore the only definitive, long-term treatment for individuals with T1D that can mimic glycaemic control to physiological standards without exposing patients to risk of severe hypoglycaemia at present is beta-cell replacement therapy.

1.5.2 Beta-cell replacement therapy

1.5.2.1 *Pancreas transplantation vs. Islet transplantation*

Replacement of beta-cell function by transplantation of endocrine tissue, whether it is whole organ pancreas transplantation or isolated pancreatic islet transplantation, is the most impactful mode of treatment available for patients with T1D to restore normoglycaemia and induce insulin independence. Nevertheless, pancreas transplants and islet transplants require patients to undergo lifelong immunosuppression to prevent rejection of the allogeneic-donor graft material. Immunosuppressive regimens used in association with the transplant procedures often cause severe and frequent side effects, including cancer, bleeding, infection and anaemia. In addition, the invasive nature of whole pancreas transplantation also carries with it significant morbidity and a small risk of mortality. Given these risks it is difficult to justify pancreas or islet transplants for individuals with well-controlled diabetes managed by intensive insulin therapy, and therefore beta-cell replacement is restricted to subgroups of T1D patients for whom the disorder poses major challenges.

The American Diabetes Association position statement (Amer Diabet, 2006) recommends β-cell replacement should be considered as a therapeutic alternative to continued insulin therapy in patients with T1D complicated by chronic kidney disease and who have had or are considering a kidney transplant; these patient could be eligible for a simultaneous pancreas-kidney transplant (SPK), simultaneous islet-kidney transplant (SIK), pancreas after kidney transplant (PAK) or islet after kidney transplant (IAK). An additional subgroup of T1D patients for whom chronic renal complications are absent can also be considered for either a pancreas transplant alone (PTA) or islet transplant alone (ITA). Patients falling into this subgroup exhibit i) highly labile metabolic control and experience repeated and unpredictable severe hypoglycaemic episodes or uncontrolled hyperglycemia culminating in ketoacidosis, or ii) severe and incapacitating clinical and emotional problems with exogenous insulin therapy, or iii) consistent failure of intensive insulin therapy to manage acute

complications. Within the UK, clinical beta-cell replacement therapy is considered an important treatment option for patients. The UK operates the world's first government funded clinical islet transplantation programme, in addition to a pancreas transplantation service. Treatment options are made available through the National Health Service (NHS) for subsets of patients with intractable hypoglycaemia or problematic diabetes control after kidney transplant (NHS, 2014). Where a patient is indicated for beta-cell replacement therapy, the choice between islet or pancreas transplantation is made upon a patient to patient basis and driven by factors including age, diabetic complications, general condition, and patient compliance/preference. For example, in the presence of known significant coronary disease, the patient might rather be subjected to islet transplantation than whole pancreas transplantation due to its less invasive nature; injection of isolated islets into the hepatic portal vein can be carried out in less than a day using only local anaesthetic coupled with fluoroscopic guidance. Both strategies should be regarded as tools for treating diabetes rather than competitors.

Pancreas transplantation is well established as the most effective treatment to restore normoglycaemia without the risk of iatrogenic hypoglycaemia, eliminate insulin-dependence and either prevent, reduce or halt secondary diabetic complications in patients with long-standing T1D (Robertson et al., 1994, Landgraf et al., 1991, Morel et al., 1991); approximately 30 000 procedures have been conducted in the past three decades (Pepper et al., 2013). Islet transplantation, although still largely regarded as an experimental procedure, also continues to improve and develop into an alternative, less invasive therapeutic strategy to achieve glycaemic control and abrogation of hypoglycaemia; over 850 islet transplants in total were reported to have taken place worldwide by the end of 2012 (CITR, 2014), a number which reflects the infancy of the procedure when compared to the number of pancreas transplantations. The overall graft function and long-term outcomes of islet transplants however remain below those of pancreas transplants. In the past decade, unadjusted graft survival rates, the criterion for which is usually

regarded as insulin independence, was 82% one year post-transplant and 58% after 5 years for PTA (Gruessner and Gruessner, 2012). The Collaborative Islet Transplant Registry (CITR), a registry established to monitor progress and safety of islet transplant procedures by using data from centres in the U.S, Canada, Europe and Australia, reported 3 year post transplant insulin independence rates for ITA of 44% in 2007-2010, an improvement however upon the rates of 27% achieved between 1999-2002 and 37% between 2003-2006 (Barton et al., 2012). The failure of ITA to achieve the durable long-term insulin independence rates of PTA is further exacerbated when it is taken into account that frequently one donor pancreas is not sufficient for islet transplantation, and multiple islet infusions can be necessary. However at this time, islet transplantation is a rapidly evolving technology, which holds significant potential advantages over whole gland transplants and strides are continually being taken to improve the successful outcome of the procedure.

1.5.2.2 *Islet transplantation*

The first successes of islet transplantation in patients with T1D were reported back in the early 1990s (Scharp et al., 1991), however reproducibility of these early results proved difficult. It was not until the development of the 'Edmonton protocol' by researchers at the University of Alberta in 2000, that a major breakthrough in the field was made (Shapiro et al., 2000). By using more potent immunosuppressive drugs, avoiding the use of diabetogenic glucocorticoid-based immunosuppressant inducers and improving the quality of human islet preparations the group were able to significantly advance the reproducibility and effectiveness of the procedure. A five-year follow up report for the original Edmonton results did however highlight the poor results for long-term insulin independence. Most recipients needed to return to moderate insulin administration 5 years post-infusion, though they no longer experienced recurrent hypoglycaemic events. Graft function in recipients was generally preserved beyond the loss of insulin independence, with 80% of patients maintaining C-peptide secretion at the 5 year time point (Ryan et al., 2005). Thus

the primary goal of islet transplant trials focused on a reduction in the incidence and severity of hypoglycaemic events and a reduction in exogenous insulin requirements rather than insulin independence. Nevertheless in the decade following the Edmonton milestone, further progress has been made in an attempt to improve the long-term status of insulin independence for islet graft recipients. Although the CITR reported 3 year post-infusion insulin independence rate of 44%, this is a collective figure representing only the centres which chose to report to the registry. At present, at least six single-centres worldwide have reported insulin independence rates of 50% or greater at the 5 year post-infusion mark through the use of improved immunosuppressive drugs, inflammatory inhibitors and apoptosis inhibitors (Shapiro, 2012, Bellin et al., 2012, Emamaullee et al., 2010, Berney et al., 2009). In addition to improving insulin independence rates, islet transplant centres have also paid much attention to reducing the need for using multiple pancreata for islet infusions to single-donor infusions to aid the problem of organ donor scarcity (Hering et al., 2005, Posselt et al., 2010)

Despite the impressive advances in islet transplantation over the past decade a broad spectrum of factors remains to hinder its success, especially with regards to the function of isolated islets both pre- and post-transplantation. The procurement of human islets pre-transplantation requires islet isolation from the native pancreas and increasingly their subsequent cultivation. Islets exist in a complex 3-dimensional (3D) microenvironment within the endocrine compartment of the pancreas, signalling with extracellular matrix (ECM) components and communicating with neighbouring cells; the destruction of this surrounding microenvironment and mechanical damage caused by the isolation procedure leaves the delicate micro-organs susceptible to devascularisation, apoptosis and hypoxia (Wang and Rosenberg, 1999, Rosenberg et al., 1999, Paraskevas et al., 2000). With the increasing need to culture islets short term prior to transplantation, allowing for factors such as islet quality control and recipient preparation, islet function becomes compromised even further. Although cultured islets for transplantation have been shown to have

immunologic advantages over freshly isolated islets for transplantation (Kuttler et al., 2002, Rabinovitch et al., 1982), islet culture is known to cause substantial loss of functional islet beta-cells (Rackham et al., 2013, Kin et al., 2008) and other islet cells such as endothelial cells (Nyqvist et al., 2005). Islet attrition and poor graft function post-transplantation also impacts significantly upon the successful outcome of ITA procedures. The recurrence of autoimmunity after islet-cell allo-transplantation leaves beta-cells prone to destruction. The Worcester Human Islet Transplantation Group reported the progressive loss of β-cells in a T1D recipient of two intrahepatic islet-cell allografts. Many glucagon positive cells were detected in a liver biopsy flowing transplantation, but in the absence of insulin positive cells (Sharma et al., 2006). The alloreactive immune response further contributes to a reduction in β-cell mass after the infusion of islets into the patient's portal circulation. It is estimated that greater than 50% of the transplanted islets are lost within hours post transplantation, which is in part due to the immediate blood mediated inflammatory reaction and complement coagulation cascade (Bennet et al., 2000, Bennet et al., 1999, Eich et al., 2007). The poor revascularisation of isolated islets post transplantation also results in the sub-optimal function of transplanted beta-cells. The vascular system of transplanted islets is markedly different from that of endogenous islets, manifested by their decreased vascular density, blood flow and partial pressure of oxygen (Jansson and Carlsson, 2002).

Undoubtedly, both the quality of human islet preparations available for transplantation and the longevity of post-transplant graft function are critical for successful clinical islet transplantation outcome. Devising strategies to maintain high-quality isolated human islets *in vitro* prior to transplantation and reducing islet attrition post transplantation will no doubt play an important role in enhancing clinical islet graft outcome.

1.6 Islet 'helper' cells for islet transplantation

A number of different approaches have been envisioned for the use of various helper cells in islet transplantation to improve transplantation outcome. These

include, the co-transplantation of helper cells with islets so that the helper cells can work locally to ensure islet engraftment and survival from the very early post-transplant stages, co-culture of the helper cells with the isolated islets to enhance their *in vitro* survival as well as provide appropriate culture conditions to keep the islets healthy prior to transplantation, or combined use of both approaches. In order to aid islet transplantation, the helper cells would need to possess a variety of characteristics including immunomodulatory or paracrine functions (Figure 1.3).

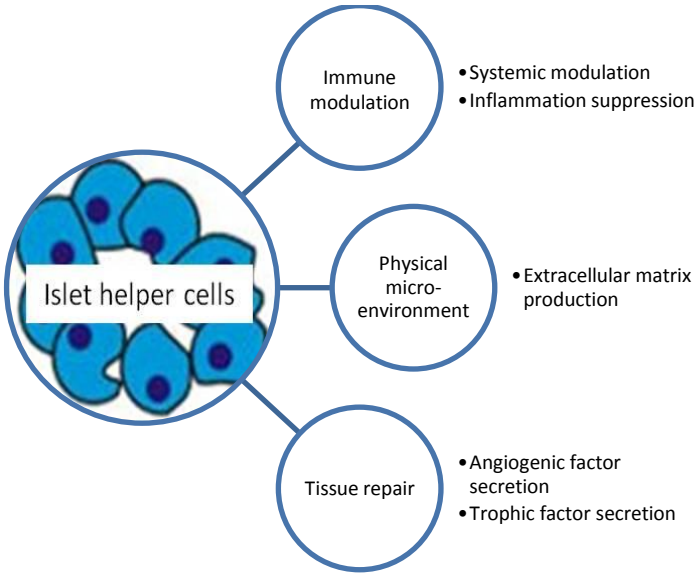

Figure 1.3: Key potential mechanisms of action for helper cells that could enhance islet transplantation outcome. Islet helper cells could aid islet transplantation through i) immune modulation via interaction with cells of both the innate and adaptive immune system, ii) providing a supportive microenvironmental niche by depositing extracellular matrix for isolated or transplanted cells or iii) promoting angiogenesis and vascularisation through paracrine signalling to aid islet engraftment.

The co-transplantation of islets with testicular Sertoli cells (SCs) is a commonly explored avenue, since the testis is considered an immune-privileged site, and SCs have been identified as key players for conferring this immune privilege (Selawry and Cameron, 1993). SCs have been used to prolong the survival of co-

transplanted allogeneic or xenogeneic cells by creating an ectopic immune-privileged environment. Evidence for both the auto- and allo-immunoprotective capabilities of SCs and the protection they can offer against xenogeneic rejection has been provided by numerous studies co-grafting islets with SCs in small animal models of T1D diabetes (Selawry and Cameron, 1993, Korbutt et al., 2000, Korbutt et al., 1997, Dufour et al., 2003). However the success of SC co-transplantation in higher mammals has had limited success to date, with xenotransplantation studies failing to result in fully functional islet grafts (Valdes-Gonzalez et al., 2005, Valdes-Gonzalez et al., 2007).

Another cell type popular in islet co-transplantation strategies is the endothelial progenitor cell (EPC). EPCs are known to aid neovascularisation in various pathophysiologic conditions (Asahara et al., 1997) and several groups have used them with the aim to enhance islet revascularisation and engraftment. However, the mechanism through which the EPCs aid islet revascularisation remains undetermined with some studies reporting the re-establishment of the islet vascular network (Oh et al., 2013, Quaranta et al., 2014), and others proposing paracrine activity mainly attributed to vascular endothelial growth factor (VEGF) production from the graft (Kang et al., 2012). Co-transplantation strategies with EPCs remain experimental to date, with investigations continuing to assess their potential as a therapeutic tool for enhanced post-transplantation graft revascularisation and survival.

Although SCs and EPCs hold promise for the clinical setting of islet transplantation, they only offer individual mechanisms of action to aid transplantation outcome. Combined strategies utilising endothelial cells and sertoli cells to synergistically enhance revascularisation and exert immunosuppressive effects have been devised (Li et al., 2013). However a single cell type which offers the full spectrum of benefits would be preferable over such a strategy. It is for this reason that multipotent mesenchymal stromal cells (MSCs) are currently the most extensively researched cell type for the clinical setting of islet transplantation, and the focus of this thesis. MSCs, defined as multipotent, adult stromal progenitors (Pittenger et al., 1999) found to reside in

most adult tissues and organs (da Silva Meirelles et al., 2006) have taken the field of cellular therapy by storm in recent years (Amado et al., 2005, Togel et al., 2005, Parr et al., 2007). The high profile nature of MSCs for cellular therapy has been attributed to a combination of key properties linked with the cells. MSCs act as multi-drug dispensers releasing large quantities of immunomodulatory and anti-inflammatory bio-factors, which have complex feedback mechanisms amongst many types of immune cells. The key immunomodulatory cytokines include prostaglandin 2, transforming growth factor-beta 1 (TGF-β1), nitric oxide (NO) and hepatocyte growth factor (HGF) alongside anti-inflammatory interleukin-4 (IL-4), IL-6 and IL-10 (Iyer and Rojas, 2008, Ren et al., 2008, Schinkothe et al., 2008). MSCs prevent proliferation and function of many inflammatory immune cells, including T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells (Spaggiari et al., 2009, Selmani et al., 2008), whilst inducing the proliferation of regulatory helper T cell populations (Maccario et al., 2005). The immunoregulatory capacity of MSCs is just one of their immune-related functions. They lack expression of class II major histocompatibility (MHC) proteins and co-stimulatory molecules (CD40, CD80 or CD86) on their cell surface conferring immune-privilege and in theory the potential to evade the immune system (Tse et al., 2003, Klyushnenkova et al., 2005). MSCs are also known secretors of a vast array of growth factors and chemokines which induce angiogenesis and vascularisation including vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (Caplan and Dennis, 2006). In addition to their paracrine functions, MSCs could also provide a physical niche for isolated or transplanted cells by acting as a stromal cell support system laying down extracellular matrix (ECM) for neighbouring cells (Gomez-Aristizabal et al., 2009). Finally the accessibility of MSCs and their potential to be expanded *in vitro* to clinically relevant numbers also makes them highly desirable helper cells for islet transplantation purposes.

1.6.1 MSCs and Islet Transplantation

In the context of islet transplantation, the properties of MSCs could be harnessed for *in vivo* co-transplantation to improve graft survival and function and/or for *in vitro* co-culture to prime freshly isolated islets prior to transplantation. One of the major goals of islet replacement therapy is to promote graft longevity and function post transplantation. This may not only improve long-term graft outcomes but also potentially reduce the numbers of islets required for transplantation, since high numbers are used to compensate for the significant β-cell death post transplantation. The beneficial properties of MSCs when utilised as a cell support system for transplanted islets, to enhance islet revascularisation, survival or function post transplantation has previously been explored. Studies investigating the co-transplantation of MSCs and islets, in animal models of type I diabetes, reported the prolonged survival and function of islet-MSC grafts and attributed the improvements to both the immunoregulatory actions of MSCs (Solari et al., 2009, Longoni et al., 2010), and the ability of MSCs to preserve islet morphology and enhance graft revascularisation (Ito et al., 2010, Figliuzzi et al., 2010), the latter being in accordance with findings previously reported by our own group (Rackham et al., 2011). We demonstrated that the rate of C57Bl/6 transplant recipients receiving islets and kidney-derived MSCs (kMSCs) reverting to normoglycaemia was more than double that of recipients receiving islet alone grafts. Graft retrieval 1 month post transplantation showed that islets co-transplanted with MSCs maintained a morphology closely resembling endogenous pancreatic islets, and superior vascular engraftment (Rackham et al., 2011). Co-culture studies have also been conducted *in vitro* to elucidate some of the beneficial interactions taking place between the islets and MSCs, especially the importance of MSC-derived trophic factors (Park et al., 2010, Park et al., 2009, Karaoz et al., 2010) and direct cell-cell contact (Jung et al., 2011, Luo et al., 2007), with evidence showing the effectiveness of both. Thus experimental studies support the potential of MSCs to be incorporated into clinical islet transplantation practices, especially with regards to co-transplantation strategies.

1.7 Aims and Objectives

Experimental studies conducted within the group to date, using mouse islets and kMSCs, demonstrated that co-transplantation strategies are effective at improving the outcome of islet transplantation. However kMSCs, although suitable for experimental co-transplantation studies beneath the renal capsule in mice, lack clinical relevance as they are not accessible in the human setting. The first aim of this thesis therefore was to move the project forward in a clinically relevant direction by generating a more translatable source of MSC. The second aim was to investigate the cellular interactions between islets and MSCs *in vivo* using controlled co-culture conditions *in vitro*. Following on from the co-culture studies, with the knowledge that islet isolation practices lead to mechanical damage and the destruction of the surrounding islet microenvironment, the final aim was to develop pre-transplant co-culture strategies *in vitro* to improve the quality of isolated human islets prior to transplantation. These aims, outlined further below, are encompassed by the overarching hypothesis of this thesis that 'Co-culture with MSCs will improve islet function and thus the outcome of islet transplantation'.

1. ***Derivation of clinically relevant MSCs***

Adipose tissue-derived MSCs are an attractive source of clinically relevant MSCs since they are easily available and accessible and can be harvested in abundance in humans from lipo-aspirate waste material. MSCs derived from pancreatic exocrine tissue, the by-product of islet isolations, are also an anatomically and clinically relevant MSC type in the context of manipulating islet function.

2. ***Interactions between islets and MSCs in vitro***

Both cell-cell communication and paracrine activity are believed to contribute to the beneficial effects of MSCs exerted over islets. The effects of various modes of direct and indirect contact co-culture upon mouse islet function were explored *in vitro*, as was the putative mechanism of islet-ECM (MSC-derived) interactions.

3. ***Develop pre-transplant co-culture strategy to improve quality of isolated human islets available for transplantation.***

The co-culture strategy which most effectively improved islet function, determined during mouse islet-MSC studies, was tested using human islets and MSCs. This was done not only to confirm any key observations made using mouse islets in human islets, but also in an effort to develop pre-transplant islet culture protocols using clinically relevant human tissue.

Chapter 2 - Materials and Methods

This chapter describes the general methods utilised for experiments conducted within this thesis. Methods specific to a particular results chapter are given in the relevant chapters.

2.1 Isolation of mouse islets of Langerhans

I am grateful to colleagues in the Diabetes research group for isolating the mouse islets used in the studies presented in this thesis. Briefly, male ICR or C57BL/6 mice (Charles River, Kent, UK) aged 8-12 weeks were used as pancreatic donors for islet procurement. Islets were isolated by collagenase digestion (from Clostridium histolyticum, type X1, Sigma-Aldrich, Poole, UK; 1mg/ml Modified Eagle's Medium (MEM; Sigma) followed by separation of islets from the exocrine pancreas using a density gradient (Histopaque-1077; Sigma). After washing with islet medium (RPMI-1640 (Sigma), 10% (vol./vol.) Fetal Bovine Serum (FBS; Sigma), 1% (vol./vol.) penicillin/streptomycin (pen-strep; Gibco, BRL, Gaithersburg, MD, USA) islets were hand-picked under a dissecting microscope into groups of defined numbers. Islets were incubated in islet medium at 37^0C, 5%CO_2 until use in *in vitro* co-culture studies.

2.2 Mesenchymal stromal cell culture

2.2.1 Subculture of MSCs

Following the initial derivation of murine MSCs, the cells were sub-cultured in order to expand cell numbers for purposes of cell characterisation and *in vitro* co-culture studies. MSCs are anchorage dependent cells grown in tissue culture vessels with finite growth surface areas. Within a cell culture flask there is usually three phases of growth: 1) cells acclimatise to their new environment (lag phase), 2) rapid cell growth (exponential phase), and 3) cell growth hits a plateau once the cell growing surface has been completely covered (confluence) (Martin, 1994). MSCs were dissociated from the tissue culture plastic prior to

reaching 100% confluency, to keep them in an exponential growth phase. To enable their expansion the cells were then either split into multiple new culture vessels and scaled out or re-seeded into larger culture vessels and scaled up, a process known as passaging. Passaging can be performed in a number of manners including manual cell scraping and enzymatic treatment, in these studies enzymatic dissociation using trypsin (Sigma) was employed for general MSC expansion. MSC medium was aspirated from the flasks and the cell monolayers rinsed with PBS to remove traces of serum contained in the medium, which would otherwise inhibit the action of trypsin. Pre-warmed (37^0C) 0.05% trypsin/0.02% EDTA was added to the flasks in volumes sufficient to cover the entire growth surface area (see Table 2.1) and rocked back and forth over the cell monolayer prior to incubation at 37^0C for 2-3 min. Following incubation, cell dislodgment was further encouraged by tapping the edge of the culture vessel in short sharp bursts. Trypsin was inactivated with the addition of FBS containing MSC medium (see Table 2.1) to the culture vessel and cells dissociated into a single cell suspension by trituration. A 10µl aliquot of cells was taken for cell counting and the remaining volume centrifuged at 400 x g for 3 min. The cell pellet was resuspended in fresh MSC medium (see Table 2.1) and reseeded into new culture vessels using split ratios of no greater than 1:6.

Vessel	Growth surface (cm^2)	Trypsin/EDTA volume (ml)	MSC medium volume for trypsin inactivation (ml)	MSC medium volume for re-seeding (ml)
6-well plate	9.6	0.5	2.5	3
T25	25	1	4	5
T75	75	3	7	15
T175	175	5	10	25

Table 2.1: Working volumes for tissue culture vessels

2.2.2 Counting of MSCs

During MSC subculture estimated cell counts were performed in order to gauge the cell numbers acquired through cell expansion, and for the design of *in vitro* co-culture studies. A 10 µl aliquot of trypsinised cell suspension was diluted 1:1 with 0.4% trypan blue (Sigma) and 10 µl of the diluted suspension loaded into the counting chamber of a Neubauer haemocytometer. The trypan blue dye selectively stained dead cells, since it could not cross the intact membranes of viable cells, thereby allowing total live cell counts to be performed. Under a light microscope (Coolpix, MDC lens, Nikon, Japan), the live cells in all four 4 x 4 squares of the counting chamber (i.e. A B C and D) (see Figure 2.1) were counted in a sinusoidal pattern and used to calculate the total number of live cells present in the cell suspension population. The equation used to calculate the cell total was as follows:

$$\boldsymbol{Total\ cell\ number\ (live) =}$$
$$\boldsymbol{Average\ cell\ count\ of\ all\ 4\ squares\ x\ dilution\ factor\ x\ volume\ of\ medium\ cells\ suspended\ in\ (ml) x\ 10^4}$$

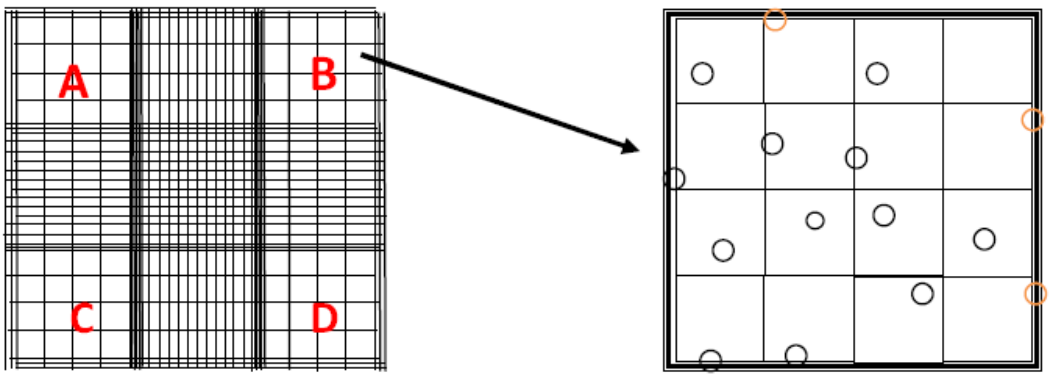

Figure 2.1: Cell counting using a haemocytometer: Left: Diagrammatic representation of a haemocytomer's counting chambers. Right: Single cells are counted in a sinusoidal pattern in each square. Cells shown in orange are not included in the count. A live cell count is performed, rather than a total cell count, by omitting any dead cells from the count (these cells looked dark blue and lacked a bright halo under the phase contrast microscope). (Image taken from Martin, 1994)

2.2.3 Cryopreservation of MSCs

During the MSC expansion process cells were cryopreserved at various stages of sub-culture to create a bank of MSC stocks at different passage numbers, thus preventing the need to expose the cells to prolonged culture. MSCs were trypsinised and counted, but rather than being resuspended in fresh MSC medium, the cells were resuspended at a density of 10^6 cells/ml in cryoprotectant containing FBS supplemented with dimethylsulphoxide (DMSO; 10% vol./vol.). One millilitre aliquots of cell suspension were distributed into cryotubes (Thermo Fisher, Roskilde, Denmark), stored in a Nalgene Mr Frosty freezing container (Sigma), and cooled at a rate of 1^0C/min in a -80^0C freezer overnight. Following controlled rate slow freezing, the cells were cryopreserved and stored in a suspended state in liquid nitrogen at -196^0C until future use.

2.2.4 Resuscitation of MSCs

The creation of a cryopreserved MSC bank allowed for the use of cells at consistent passage numbers in *in vitro* co-culture studies. When MSCs of a known passage number were required for experimental studies, appropriate vial(s) were retrieved from the liquid nitrogen tanks for thawing. The basic principle of successful cryopreservation and resuscitation requires a slow freeze and quick thaw. Since the MSCs were subjected to a slow freeze process during cryopreservation, they were thawed quickly by incubation in a 37°C water bath for up to 3 min. The 1 ml cell suspension was transferred from the cryotube to a 15 ml conical tube and 9ml pre-warmed (37^0C) MSC medium added to the tube in a drop-wise manner. The cell suspension was centrifuged at 400 x g for 3 min and the supernatant containing DMSO removed. The cell pellet was resuspended in MSC medium and seeded into a T25 or T75 culture vessel. MSCs were maintained in a tissue culture incubator at 37°C, 5% $C0_2$.

2.3 Measurements of mouse islet function *in vitro*

2.3.1 Measurement of insulin content

Following all modes of co-culture, islets were harvested for insulin content analysis. Up to 6 groups of 10 islets were picked from each condition, washed in PBS, and added to 200µl of acidified ethanol (absolute ethanol: deionised water: concentrated HCL in the ratio of 52:17:1). Samples were sonicated on ice (3x 5 second pulses, output 10-14) and insulin extracted overnight at 4°C. Samples were then stored at -20°C until insulin quantification by radioimmunoassay. Each sample was diluted between 1:22 – 1:1600 with borate buffer for the detection limits of the radioimmunoassay.

2.3.2 Measurement of basal and glucose-stimulated insulin secretion

Following all modes of co-culture, islets were analysed for their capacity to secrete insulin in static incubation. Islet medium was replaced with 2mM glucose Gey and Gey buffer, a bicarbonate- buffered physiologic salt solution containing 2 mM calcium chloride and 0.5 mg/mL bovine serum albumin, for each co-culture condition, and islets were incubated for 60 min to achieve basal insulin secretion levels. Ten groups of 3 islets were then hand-picked from each condition under a dissecting microscope, counted into 1.5 ml microcentrifuge tubes containing basal (2mM) or hyperstimulatory (20mM) levels of glucose in Gey and Gey buffer and incubated for 60min at 37^0C. At the end of the incubation period, the islets were centrifuged at 2,000 rpm, for 2 min, at 4˚C. The supernatant was collected and diluted 1:5-1:20 in borate buffer for detection limits of the radioimmunoassay. Samples were then stored at -20°C until insulin quantification by radioimmunoassay.

2.3.2.1 *Insulin secretion assay reagents*

- Borate Buffer

For 2L:

Reagent	Amount for 2L	Final concentration
Boric acid	16.5g	133mM
NaOH	5.4g	67.5mM
EDTA	7.4g	10mM

Table 2.2: Weight and concentration borate buffer components. All reagents were dissolved in approximately 1.8L of deionised water, before adjusting the pH to 8.0 with concentrated HCl. The volume was then made up to 2L with deionised water and BSA (100% w./v.) added before storing at 4°C ready to use.

- Gey and Gey Buffer 2x Stock Solution

For 1L:

Reagent	Amount (g)	Concentration (mM)
NaCl	6.50	222.0
$NaHCO_3$	2.27	54.0
KCl	0.37	9.92
$MgCl_2.6H_20$	0.21	2.06
$Na_2HP0_4.2H_20$	0.09	1.00
$MgSO_4.7H_20$	0.07	0.56
KH_2PO_4	0.03	0.44

Table 2.3: Weight and concentration of Gey and Gey buffer components (Gey and Gey 1936). All reagents were dissolved in approximately 800ml of deionised water and the volume made up to 1L with deionised water.

- Gey and Gey buffer working solution (2 and 20mM)

250ml of 2x Gey and Gey buffer stock solution was added to 250ml deionised water to make the working solution. 180mg D-glucose (Sigma) was added to make the glucose concentration of the buffer 2mM. The pH was adjusted to 7.4 using CO_2. 1ml of 1M $CaCl_2$ (Sigma) and 250mg bovine serum albumin (BSA;

Sigma) were added. To make a 20mM glucose working solution, 64.8mg D-glucose was added to 20ml of 2mM glucose Gey and Gey solution.

2.3.3 Insulin radioimmunoassay

The radioimmunoassay principle is based upon the competitive binding between radiolabelled insulin (I^{125}) and non-labelled insulin (present in samples for analysis) to their specific anti-insulin antibody (see Figure 2.2).

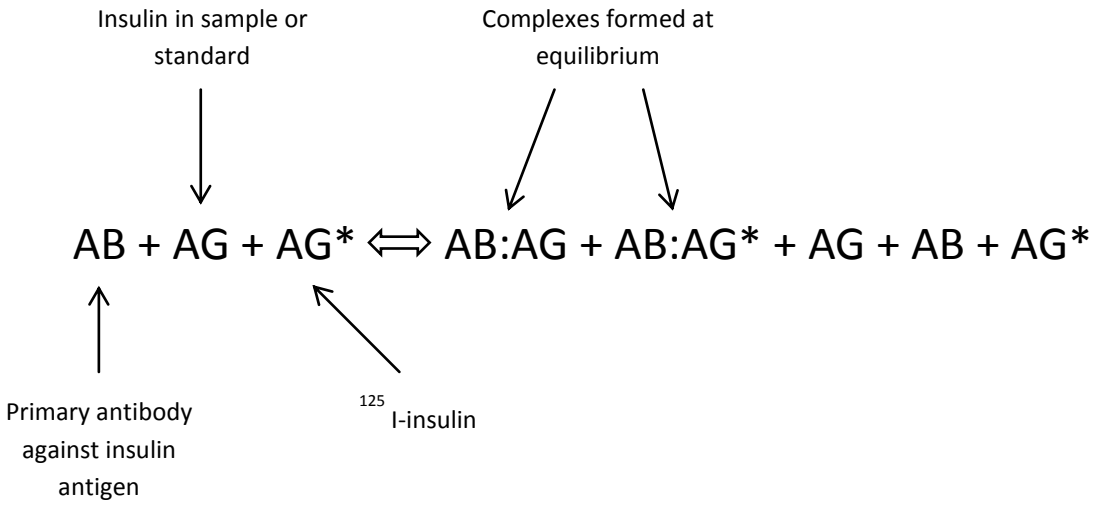

Figure 2.2: The principle reaction of the insulin radioimmunoassay

A standard curve was prepared in triplicate with known concentrations of insulin covering the range over which the assay is sensitive (0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5 and 10ng/ml) by performing serial dilutions of a 10ng/ml purified rat insulin stock with borate buffer.

Pre-diluted insulin content and insulin secretion samples collected post co-culture were defrosted and added to LP3 tubes (Greiner) prior to addition of ^{125}I-insulin tracer and anti-insulin antibody raised against bovine insulin in Hartley Guinea pigs (Jones et al., 1988).

The tracer was diluted in borate buffer at a concentration which would obtain approximately 8 000-10 000 cpm per assay tube. The amount of tracer needed was adjusted for each individual assay, due to radioisotopic decay. The final concentration of antibody used was 1:60 000.

A series of reference tubes were also prepared in triplicate, to determine binding of tracer in the absence of unlabelled insulin (maximum binding), binding of tracer in the absence of antibody (non-specific binding) and counts per minute of radio-labelled insulin added (totals).

Antibody, tracer, standards and samples were added to LP3 tubes in quantities outlined in Table 2.4. The standards were assayed in triplicate and samples in duplicate. The tubes were equilibrated at 4°C for 48 – 72 hr.

	Buffer (µl)	Antibody (µl)	Tracer (µl)	Standard (µl)	Sample (µl)
Non-specific binding (NSB)	200		100		
Maximum binding (Bo)	100	100	100		
Totals (T)			100		
Standards		100	100	100	
Samples		100	100		100

Table 2.4: Preparation of standards, reference tubes and samples for radioimmunoassay.

Following the incubation period, 1ml of precipitant (15% polyethylene glycol, PEG, 1mg/ml γ-globulin, 0.5µl/ml Tween 20) was added to all tubes, except the totals, to precipitate out the antibody-insulin complexes and the tubes centrifuged at 3000 x g for 15 min at 4°C. The supernatant was then aspirated to remove any unbound insulin and a γ-counter (WIZARD^2, Perkin Elmer, Waltham, USA) used to measure the radioactivity of the pellets (as counts per minute (cpm)).

2.3.3.1 *Insulin radioimmunoassay reagents:*

- PBS

95.5g Dulbecco's phosphate buffered saline was dissolved in 9L of deionised water. 3g sodium azide (0.03% w./v.) was added and the volume made up to 10L with deionised water and the solution kept at room temperature.

- 30% PEG

600g polyethylene glycol (PEG, MW 6000) was dissolved in 1L of deionised water and the volume made up to 2L. This solution was stored at 4°C.

- Precipitant

The 30% PEG solution was diluted 1:1 with PBS containing γ-globulin (final concentration 1mg/ml) and Tween 20 (final concentration 0.5µl/ml).

- Antibody

Anti-insulin antibody raised against bovine insulin in Hartley Guinea pigs (Persaud et al., 1989) was used at a final concentration of 1:60,000.

- ^{125}I-Insulin

The tracer was diluted in borate buffer at a concentration which would obtain approximately 10,000cpm per assay tube. The amount of tracer needed was determined for each individual assay, due to radioisotopic decay.

2.4 Statistical analysis

The statistical analyses were carried out using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA). All data are expressed as mean ± standard error of the mean. Within a given experiment, when two groups were compared, Students *t* test was used and for >2 groups analysis of variance (ANOVA) with a Bonferroni's or Dunnett's post hoc test was used. Where data was compared between experiments, for a given treatment, a paired t test was used where two groups per experiment were involved and a repeated measures ANOVA where >2 groups were involved. Results were considered significant at $p < 0.05$.

Chapter 3 - Isolation and characterisation of mouse and human MSCs

3.1 Introduction

Prior to investigating the use of mesenchymal stromal cells for islet transplantation, a note should be made about mesenchymal stem cells, which share the MSC acronym and are often confused with multipotent mesenchymal stromal cells. The term mesenchymal stem cell, or MSCs, is frequently used in present-day literature to reference the spindle-shaped, plastic adherent cell preparations commonly isolated from bone marrow (BM) and other tissues that are positive for several surface antigens (CD73, CD90 and CD105 - human; CD44, Sca-1, CD29 - mouse) whilst lacking hematopoeitic markers and that display trilineage differentiation potential towards osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006b, Boxall and Jones, 2012). However the original notion of mesenchymal stem cells dates back to the early studies of Friedenstein and colleagues in the late 1960s who identified genuine non-hematopoeitic stem cells within the bone marrow. Taking suspensions of dispersed BM cells, the group demonstrated that single cells within the suspension could form both clonally-derived fibroblastic colonies in monolayer culture *in vitro*, and also exhibit the capacity to self-renew and differentiate upon transplantation *in vivo* (Friedens.Aj et al., 1968, Friedenstein et al., 1976, Friedenstein et al., 1982). The term 'mesenchymal stem cell' and its acronym 'MSC' only came about when Caplan and his co-workers coined the term in the early 1990's to refer to adult stem cells in the bone marrow with osteo-chondrogenic differentiation potential (Caplan, 1991). During the last decade it has widely been reported that these 'mesenchymal stem cells' can be derived from sources extending far beyond the bone marrow stroma (Zuk et al., 2001, Sordi et al., 2010, Sabatini et al., 2005), with evidence that they exist in virtually all post natal organs and tissues (da Silva Meirelles et al., 2006). However, bona fide mesenchymal stem cell populations are rare and can only be truly characterised by stringent clonal assays demonstrating multilineage differentiation and self-renewal *in vitro* and most importantly *in vivo*. The cell

populations currently being derived from various adult tissues, including bone marrow, on the basis of adherence, culture-expansion, cell surface marker expression and *in vitro* differentiation potential alone, hence lacking stringent 'stem cell' characterisation, are in fact heterogeneous and likely to contain only a subset of true mesenchymal stem cells amongst numerous other mesenchymal progenitor and lineage-committed cells. As a consequence there have been numerous reports likening these tissue derived 'mesenchymal stem cells' to other stromal cells such as fibroblasts (Haniffa et al., 2007, Hematti, 2012). The International Society for Cellular Therapy (ISCT) attempted to clarify the terminology applied to these fibroblast-like plastic adherent cells *in vitro* and encouraged the scientific community to refer to them as 'multipotent mesenchymal stromal cells' (Dominici et al., 2006) rather than the misleading term of mesenchymal stem cells. However confusion in the field remains since the MSC acronym persists for both distinctions of cells. Within this thesis the acronym MSC will refer only to the adherent cell populations, derived from various adult tissues, which have not undergone rigorous stem cell characterisation, and are expected to contain a mix of mesenchymal stem cells and their progeny. Hence they should be regarded as multipotent mesenchymal stromal cells, and will be characterised in accordance to ISCT guidelines (Dominici et al., 2006) where applicable.

The adult tissues MSCs are isolated from must be considered carefully. Prior to the start of studies for this thesis, co-transplantation work had been conducted in the group using mouse kidney-derived MSCs (Rackham et al., 2011). The rationale for which was driven by the sub-renal capsular site of transplantation used in the mouse models of islet transplantation. The promising results from the above mentioned study resulted in kMSCs becoming the source of choice for the group. However, although suitable for preliminary experimental studies, deriving MSCs from kidney tissue bears little relevance in the clinical setting due to the lack of availability of human kidney tissue for such a procedure. Therefore as work continues in the group, investigating both the co-transplantation of MSCs and islets *in vivo* and co-culture *in vitro*, a transition must be made from using kMSCs

to more clinically relevant populations of MSCs, to ensure the work performed is translatable to the human setting. MSCs derived from bone marrow or adipose tissues are commonly considered the main sources of MSCs for clinical application. Since mesenchymal stem cells were originally discovered in the bone marrow (Friedens.Aj et al., 1968, Friedenstein et al., 1976, Friedenstein et al., 1982), initiating the whole mesenchymal stem/stromal cell field, bone marrow-derived MSCs (bmMSCs) gained the status of 'gold standard' MSC. More recently adipose-derived MSCs (adipMSCs) have attracted considerable attention since they can be accessed easily and harvested in abundance from the lipoaspirate waste material of liposuction procedures (Zuk et al., 2001). AdipMSCs and bmMSCs share many biological characteristics (Izadpanah et al., 2006), albeit minor differences in their immunomodulatory activities (Ivanova-Todorova et al., 2009), differentiation potential (De Ugarte et al., 2003, Huang et al., 2005) and immunophenotype (Pachon-Pena et al., 2011) have been reported. These differences may reflect variability in MSCs isolated from different tissue sources, or may simply be related to different isolation and culture protocols. Adipose tissue is fast becoming the preferred source of clinical MSCs over bone marrow. This is due not only to the minimally invasive nature of adipose tissue harvesting, compared to painful bone marrow harvest procedures, but also because of the superior yields and proliferative capacity of the MSCs isolated from adipose tissue (Kern et al., 2006, Peng et al., 2008). In the context of the current studies, an additional MSC source was considered, the exocrine pancreas. Exocrine pancreas digests are the by-product of clinical islet isolations. Sordi et al characterised adherent fibroblast-like cells, which had been reported to appear in cultures of human exocrine pancreas tissue as pancreatic MSCs (Sordi et al., 2010). This population of pancreatic MSCs (pMSCs) could hold great potential for the role of islet helper cells due to the anatomical relevance of their origin.

Prior to commencing investigations into the use of MSCs as islet helper cells, the aims of the studies presented in this chapter were to isolate and characterise clinically relevant populations of MSCS, primarily from adipose and exocrine pancreas tissue, in both mouse and human tissue. Where necessary however,

MSCs were commercially sourced for purposes of time saving. MSCs from both mouse and human tissue are necessary since, as stated in the overall aims and objectives of this thesis (Chapter 1), selected co-culture studies conducted *in vitro* with mouse MSCs and islets should be verified using clinically relevant human tissue.

3.2 Methods

3.2.1 Mouse mesenchymal stromal cell isolation and characterisation

3.2.1.1 *Isolation of adipose-derived mesenchymal stromal cells (adipMSCs)*

Testicular fat pads were harvested from male C57BL/6 mice, and chopped into 1-2 mm^3 pieces with sterile round edged scalpels. The adipose tissue was digested with collagenase type I (1mg/ml collagenase I (Sigma), Dulbecco's Modified Eagles Medium (DMEM; Sigma), 1% (vol./vol.) pen-strep) for 30min at 37^0C. The collagenase was diluted with an equal volume of standard MSC culture medium (DMEM supplemented with 1% (vol./vol.) pen-strep, 1% (vol./vol.) L-glutamine (Gibco) and 10% (vol./vol.) MSC-Qualified Fetal Bovine Serum (Gibco) and the resultant cell suspension passed through a 100µm cell strainer (SLS, Nottingham, UK) to remove any remaining large tissue clumps. The cell suspension was centrifuged at 400 x g for 10 min, the supernatant containing the abundance of mature adipocytes was discarded, and the cell pellet resuspended in MSC culture medium. Adipose digests were seeded into Nunclon $25cm^2$ tissue culture flasks (Thermo Fisher) and incubated overnight at 37^0C, 5% CO_2. Medium was changed after 24 hr to remove non-adherent cells and fat droplets, and then subsequently every 3 days. When cultures reached confluence, cells were trypsinised and subcultured for 4 passages (see Chapter 2, section 2.2.1) to enrich for adherent spindle shaped cells. MSC stocks were then expanded to passages 6-7 and then either cryopreserved (see Chapter 2, section 2.2.3) or characterised.

The process of murine adipMSC isolation was attempted using four independent batches of adipose tissue. Two of four derivations were successful and fully characterised (batch #A1 and batch #A2).

3.2.1.2 *Isolation of kidney-derived mesenchymal stromal cells (kMSCs)*

I am grateful to Pedro Chagestelles (Universida de Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil) and Chloe Rackham (King's College London) for isolating and characterising the kMSCs used in the studies presented in this thesis. Cryopreserved vials of kMSCs at passage 8 were available for the studies. Kidneys were harvested from male C57BL/6 mice and cut into small pieces. The fragments were digested with collagenase type I (1 mg/ml; Sigma) for 30 min at 37°C and then triturated with a 5ml seriological pipette. Cells were pelleted by centrifugation for 10 min at 400 × g at room temperature. After this, cells were resuspended in MSC culture medium and seeded in six-well Nunclon plates (Thermo Fisher) and incubated at 37°C in a humidified atmosphere containing 5% CO2. The medium was changed after 24 hr, with removal of non-adherent cells. When cultures reached confluence, cells were trypsinised and subcultured in new flasks, until spindle-shaped fibroblast like cells predominated in culture.

3.2.1.3 *Tri-lineage differentiation potential*

Characterisation of murine-derived MSCs was assessed in part by their differentiation potential towards adipogenic, osteogenic and chondrogenic lineages. Primary MSC cultures grown to passage 7 were used for directed differentiation studies under standard *in vitro* tissue culture conditions.

3.2.1.3.1 Osteogenic and Adipogenic differentiation

MSCs were seeded at 10^5 cells per well into 6-well Nunclon plates, and grown to 80-90% confluency for 2 days in MSC medium. The MSCs were then treated with either osteogenic or adipogenic differentiation medium for 14 days at 37^0C in a humidified 5% CO_2 incubator. MSC medium was supplemented with either 10 mM β-glycerophosphate (Sigma), 0.1 µM dexamethasone (Sigma), and 50 µM ascorbic acid (Sigma) to formulate osteogenic differentiation medium, or 2.5 µg/ml insulin (Sigma), 1 µM dexamethasone (Sigma), 5 µM rosiglitazone (Cambridge Bioscience, Cambridge, UK) and 100µM indomethacin (Sigma) to make up adipogenic differentiation medium. Medium changes were performed every 3-4 days.

Following the 14 day culture period, cell cultures treated with osteogenic differentiation medium were washed with distilled water and stained with alizarin red (2% (w./v.) alizarin red (Sigma), distilled water) for 30 min for the detection of extracellular mineralised calcium nodule deposition. Cell cultures treated with adipogenic medium were washed with PBS and stained with oil red o (0.2% (w./v.) oil red o (Sigma), isopropanol) for 30 min for the detection of intracellular lipid droplet formation.

3.2.1.3.2 Chondrogenic differentiation

MSCs were resuspended in MSC medium at 2 x 10^7cells/ml and seeded as single 20µl droplets per well of a 12-well Nunclon plate. Cells were allowed to adhere and form micromasses for 5 hours, prior to the addition of chondrogenic differentiation medium. Chondrogenic differentiation medium constituted 10ng/ml transforming growth factor β1 (TGF-β1; Miltenyi, Surrey, UK), 100µM ascorbic acid (Sigma), 1µM dexamethasone (Sigma), 0.1X ITS premix (Invitrogen, Paisley, UK) and 1.25µg/ml BSA (Sigma) in DMEM supplemented with 1% (vol./vol.) L-glutamine, 1% (vol./vol.) pen-strep and 2.5% (vol./vol.) FBS. Cells were maintained in this medium for 7 days at 37^0C in a humidified 5% CO2 incubator. Cells were then treated for a further 7 days in the absence of FBS. Medium changes were performed every 3-4 days. Following the 14 day culture period, micromasses were washed with PBS and stained with alcian blue (1% (w./v) alcian blue (Sigma), 3% (v./v.) acetic acid) for 1-2 hours for the detection of glycosaminoglycans (GAGs) deposition.

3.2.1.4 *Immunophenotyping by fluorescence activated cell sorting (FACs)*

Unlike human MSCs there is no general consensus on the panel of markers that should be expressed by mouse MSCs; key positive markers for human MSCs are believed to be CD73, CD90 and CD105 (Dominici et al., 2006) whereas CD44 and Sca-1 are considered more important for murine MSCs (Boxall and Jones, 2012).

Murine MSCs at passage 7-9 were analysed for a panel of cell surface markers by FACs. Cells were harvested by Accutase (Sigma) passaging. Accutase was the preferred enzymatic mode of passaging over trypsin since it releases cells from tissue culture plastic in a single cell manner and results in less cell clumping than trypsin; single cell suspensions are important to facilitate the passage of cells through the flow cytometer flow cell. MSCs were incubated with Accutase (1X Accutase enzymes in PBS) for 5 min at room temperature, after which the Accutase was diluted out with PBS. The cell harvest was centrifuged at 400 x g for 3 min and the cell pellet resuspended in PBS-EDTA (PBS Ca^{2+}/Mg^{2+} free (Sigma), 2mM EDTA (Sigma), 1% (vol./vol.) BSA). Cells were dispensed into 1.5ml microcentrifuge tubes (50 x 10^4 cells/tube) and incubated with the appropriate amount of antibody or isotype control to give a 1:20 final dilution for 30 min, 4^0C in the dark. Cell surface marker expression analysis was performed using anti-mouse antibodies for: CD90, CD73, CD105, Sca1, CD44, CD31, CD45 and CD11b (all supplied by BD Biosciences, Oxford, UK; see Table 3.1 for isotypes and fluorochrome conjugation). Unstained cells were used as a negative control. Cells were washed twice with PBS-EDTA to remove any unconjugated antibodies and then dispensed into 5ml polystyrene round bottom tubes (BD). MSCs were stored on ice and vortexed prior to processing with a FACSCanto II system (BD), set up to aquire 10 000 events (cells) per marker. Data collected were subsequently analysed using FlowJo software (Treestar, Ashland OR, USA).

ANTIBODY	IMUNNOGLOBULIN (Ig) ISOTYPE	FLUOROCHROME
CD90	IgG2b	PE
CD73	IgG2a	PE
CD105	IgG2a	PE
Sca-1	IgG2a	FITC
CD44	IgG2b	FITC
CD31	IgG2a	FITC
CD45	IgG2b	PE
CD11b	IgG2b	PE

Table 3.1: Antibodies used for immunophenotyping of murine-derived MSCs. A list of the cell surface markers used for FACs analysis, their corresponding isotypes and conjugated flurochromes. PE- phycoerythrin, FITC - fluorescein isothiocyanate.

3.2.2 Human mesenchymal stromal cell isolation and characterisation

3.2.2.1 *Isolation of pancreas-derived mesenchymal stromal cells (pMSCs)*

The exocrine fraction of donor pancreata procured for islet isolations were used for human pMSC isolations (all exocrine digests were kindly provided by Dr Guo Huang, King's College Hospital, London). Exocrine digest samples were received on ice in 50ml aliquots and pelleted. The pellets were washed in PBS and resuspended in MSC medium. Approximately $1/3^{rd}$-$1/6^{th}$ of the exocrine digest pellet was seeded into $75cm^2$ Nunclon tissue culture flasks and incubated overnight at 37^0C, 5% CO_2. Medium was changed after 24 hours to remove non-adherent cells and bulk debris, and then subsequently every 3 days to enrich for an adherent cell population. When cultures reached confluence, cells were trypsinised and subcultured (in the same manner as mouse MSCs, see Chapter 2 section, 2.2.1) in new flasks for at least a further 4 passages to enrich for adherent spindle-shaped cells. Cells were then either expanded (see section 3.2.2.2) and characterised or cryopreserved (in the same manner as mouse MSCs, see Chapter 2, section 2.2.3) and banked for use in co-culture studies.

The process of human pMSC isolation was attempted using four independent batches of pancreatic exocrine digest. Two of four derivations were successful and fully characterised (batch #P1 and batch #P2).

3.2.2.2 *Optimisation of culture medium for human pMSCs*

MSC medium, DMEM supplemented with 10% vol./vol. FBS, although suitable for the derivation of human pMSCs during early passages was not supportive of efficient human MSC expansion following their derivation. Commercially available low serum medium formulations, specialised for the expansion of human MSCs, were tested to optimise the culture of human pMSCs *in vitro*.

3.2.2.2.1 Human pMSC growth curves

Human pMSCs at passage 5 were seeded into 6-well Nunclon plates at a seeding density of 5000 cells/cm^2 in MSC medium and incubated for 24 hours to adhere in culture. Following the 24 hour incubation period, wells in triplicate were fed with either control medium (MSC medium), MesenPRO RS^{TM} (Gibco), or MSCGM (Lonza, Basel, Switzerland), the latter two being commercial low serum medium formulations supplemented with growth factors. pMSCs were cultured for 7 days, with full medium replacement on each well with the corresponding medium performed every 3-4 days. After the 7 day culture period, cells were harvested from the wells by trypsinisation and cell harvest counts performed (see Chapter 2, section 2.2.2). pMSCs were then reseeded (at passage 6) into 6-well Nunclon plates at a density of 5000cells/cm^2, triplicate wells fed with either control MesenPRO RS^{TM} or MSCGM medium formulations and cultured for 7 days. Cells were harvested and counted after the 7 day period and the whole process repeated until pMSCs were subcultured to passage 10. Cell count data was used to calculate pMSC population doublings (PD) at each passage and then the cumulative population doublings (CPD) over the duration of the entire culture period for all three medium formulations. Population doublings were calculated using the following formula:

$$Y = \frac{\log X}{\log 2}$$

where:

Y = Population doublings

X = Number of cell doublings = $\frac{Number\ of\ cells\ harvested}{Number\ of\ cells\ seeded}$

3.2.2.3 *Tri-lineage differentiation potential*

Tri-lineage differentiation potential was characterised by pMSC differentiation into osteoblasts, adipocytes and chondrocytes *in vitro*.

Human pMSCs (passage 6) at 80-90% confluency were treated with either osteogenic or adipogenic differentiation medium (see section 3.2.1.3.1 for medium compositions) for 28 days at 37^0C in a humidified 5% CO_2 incubator. Osteogenic differentiation was evaluated by alizarin red detection of mineralised calcium nodule deposition, whereas adipogenic differentiation was evaluated by oil red o staining of lipid droplet formation.

MSC differentiation was directed down the chondrogenic lineage using micromass cultures before addition of chondrogenic differentiation medium (see section 3.2.1.3.2 for medium composition). Cells were maintained in this medium for 7 days, followed by 21 days in medium without FBS. Chondrogenic differentiation was evaluated by alcian blue detection of glycosaminoglycans (GAGs) deposition.

3.2.2.4 *Immunophenotyping by FACs*

Human pMSCs at passage 6 were analysed for a panel of cell surface markers by FACs in accordance with ISCT guidelines ((Dominici, Le Blanc et al. 2006). Cells were harvested by trypsinisation and resuspended in PBS-EDTA. Cells were incubated with the appropriate amount of antibody or isotype control to give a 1:20 final dilution for 30 min, 4^0C in the dark. Surface marker expression analysis was performed using anti-human antibodies for: CD90, CD73, CD105, and CD14-/CD20-/CD34-/CD45 (Miltenyi Biotech, Surrey, UK; see Table 3.2 for isotypes and conjugated fluorochromes).Unstained cells were used as a negative control. Cells were washed twice with PBS-EDTA to remove any unconjugated antibodies and processed using a FACSCanto II system (BD), aquiring 10 000 events per marker. Data collected were subsequently analysed using FlowJo software (Treestar, Ashland OR, USA).

ANTIBODY	IMMUNOGLOBULIN (Ig) ISOTYPE	FLUOROCHROME
CD90	IgG1	FITC
CD73	IgG1	APC
CD105	IgG1	PE
CD14	IgG2a	PerCP
CD20	IgG1	PerCP
CD34	IgG2a	PerCP
CD45	IgG2a	PerCP

Table 3.2: Antibodies used for immunophenotyping of human-derived MSCs. A list of the cell surface markers used for FACs analysis, their corresponding isotypes and conjugated flurochromes. APC – allophycocyanin, PerCP - peridinin chlorophyll-A protein.

3.3 Results

3.3.1 Isolation and characterisation of mouse adipMSCs

3.3.1.1 *Derivation and maintenance of mouse adipMSCs in vitro*

Gonadal fat pads were excised from male C57Bl/6 mice and collagenase digested for the generation of mouse adipMSCs *in vitro*. The stromal fraction of the digested tissue was seeded into tissue culture flasks and definite spindle-shaped cells were present in culture three days post seeding (Fig. 3.1A). Heterogeneous populations of cells are known to reside in the stromal vascular fraction of adipose tissue (Pettersson et al., 1984) including endothelial cells, fibroblasts, lineage-committed progenitor cells and preadipocytes. A mix of cells remained 15 days into culture when cells were at P1 (Fig 3.1B). MSCs, known for their proliferative capacity *in vitro*, are able to outgrow other cells in culture and become the dominant cell population. It was not until after at least four weeks into culture, when cells were at P3, that cells with fibroblast-like morphology emerged to take over the culture (Fig 3.1C) and dominated it by P4 (Fig. 3.1D). Once MSCs were established in culture their maintenance or expansion was continued using standard DMEM culture medium supplemented with 10% vol./vol. FBS.

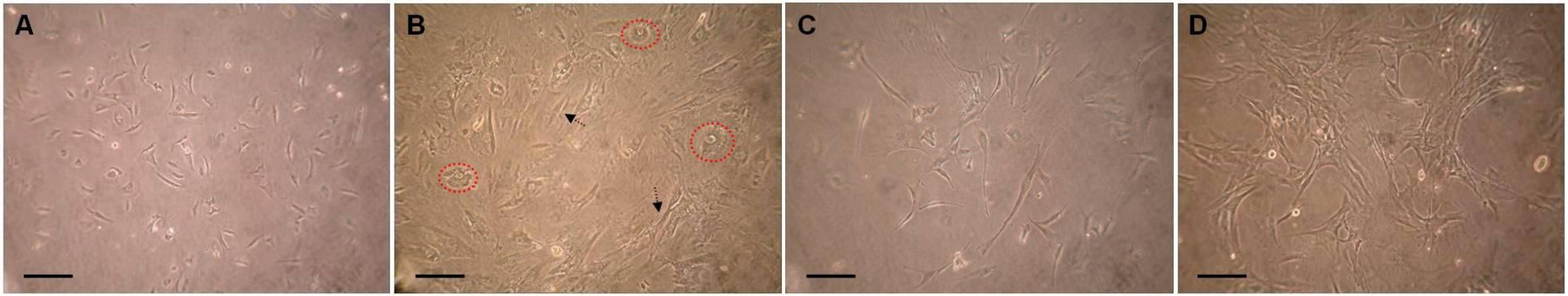

Figure 3.1: Derivation of mouse adipMSCs through serial passaging. Three days after initial seeding of the adipose tissue digest cells exhibiting a spindle shaped morphology (A) were present in culture (P0). The stromal vascular fraction of excised adipose tissue is a heterogeneous population of cells including mast cells, endothelial cells, pericytes, fibroblasts, and lineage-committed progenitor cells, or preadipocytes (Pettersson et al., 1984). A morphologically heterogenous mix of cells was still present in culture at P1 (B; red circles show cobblestone-like cells, black arrows show spindle-shaped cells). After 4 weeks (P3) fibroblast-like cells (C) emerged to overtake other cell types in culture until a population of cells with uniform spindle-shaped morphology (D) dominated at P4. Images representative of two independent adipMSCs isolations. Scale bar A-D; 100µm.

3.3.1.2 *Characterisation of mouse adipMSCs*

3.3.1.2.1 Trilineage differentiation potential of mouse adipMSCs

A key characteristic of MSCs is their ability to differentiate along osteogenic, chondrogenic or adipogenic lineages. kMSCs previously isolated and characterised within the group demonstrated this potential (Fig. 3.2). Therefore the newly isolated adipMSCs (two independent batches) were tested for their trilineage differentiation capacity using *in vitro* directed differentiation assays. Cultured MSCs (P7-P9) displayed characteristic spindle-shaped morphology in adherent culture prior to differentiation (Fig. 3.3A&E). Following treatment for 14 days with either osteogenic, chondrogenic or adipogenic differentiation supplements adipMSCs from both batches displayed differentiation potential along all three lineages. Mineralised calcium nodules deposited across the cell monolayer by bone-forming osteoblasts were visualised by punctuate alizarin red staining in MSC cultures treated with osteogenic medium (Fig 3.3B&F). Glycosaminoglycans (GAGs) secreted by cultured chondrocytes were detected in micromass cultures induced to adopt a chondrogenic fate (Fig 3.3C&G). Intracellular lipid droplets which accumulate in adipocytes were evident in MSCs treated with adipogenic medium as highlighted by oil red o staining (Fig 3.3D&H).

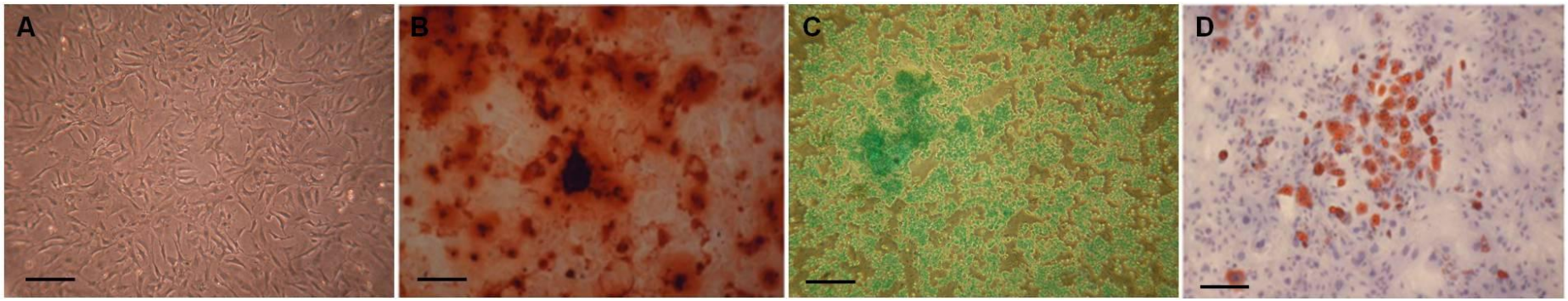

Figure 3.2: Trilineage differentiation potential of kMSCs, image modified from (Rackham et al., 2013). kMSCs displayed fibroblast-like morphology (A) under normal culture conditions. Exposure to osteogenic, chondrogenic or adipogenic differentiation supplements for 28 days induced trilineage differentiation *in vitro*. Mineralised calcium deposits (B) were detected by alizarin red staining on cell monolayers treated with osteogenic medium. GAG deposition (C) induced by micromass culture in chondrogenic medium was visualised by alcian blue staining. Intracellular lipid droplets (D) stained with oil red o dye were present in the cells that had been cultured in adipogenic differentiation medium. Scale bars A&C; 100µm, B&D; 25µm.

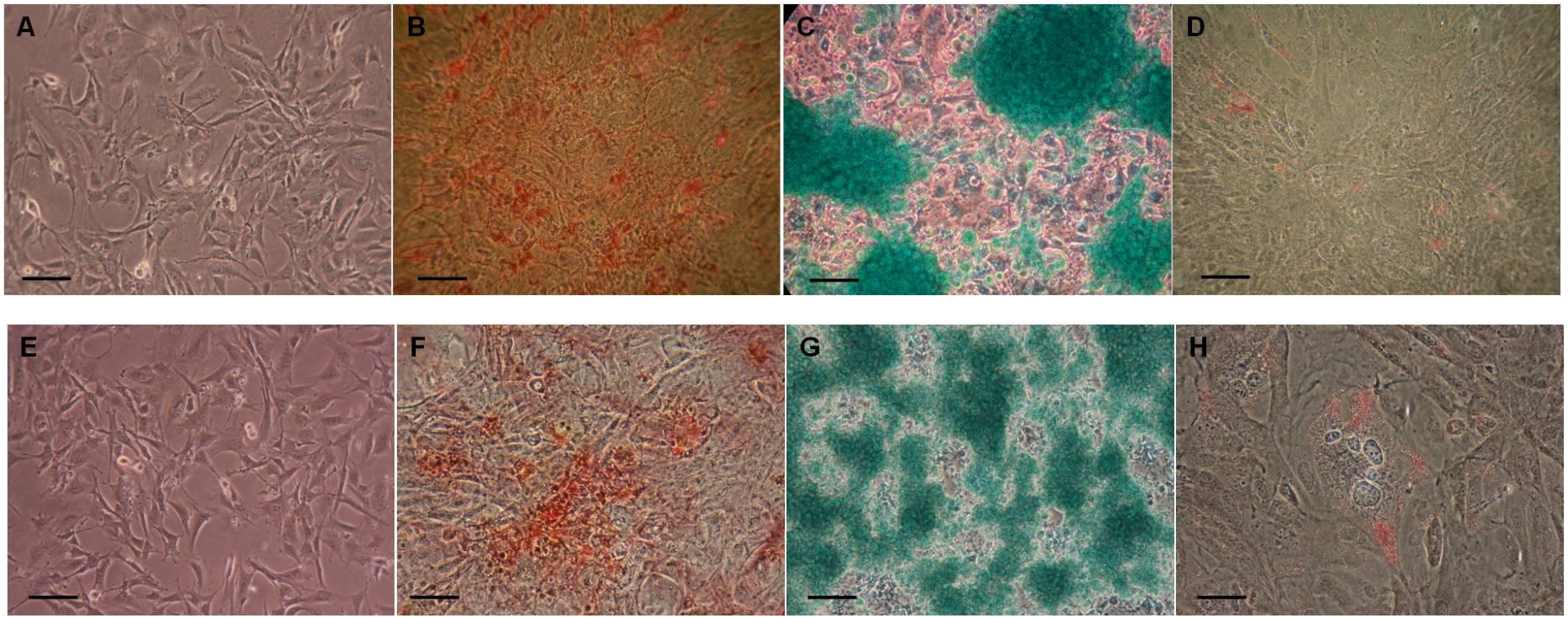

Figure 3.3: Trilineage differentiation potential of two independent batches of mouse adipMSCs (batch A1 (A-D), batch A2 (E-H)). Prior to differentiation both batches of mouse adipMSCs displayed characteristic spindle shaped morphology (A&E). Following 14 days treatment with osteogenic, chondrogenic or adipogenic differentiation supplements, both batches of adipMSCs demonstrated trilineage differentiation potential *in vitro*. Alizarin red staining highlighted punctuate mineral deposition across cell monolayers exposed to osteogenic medium (B&F). Alcian blue staining detected GAG deposition within cell micromasses directed towards a chondrogenic fate (C&G). Oil red O staining detected the formation of immature lipid droplets in MSCs treated with adipogenic medium (D&H). Each image representative of 3 wells. Scale bar A-G; 100µm, H; 50µm.

3.3.1.2.2 Immunophenotype of mouse adipMSCs

Unlike human MSCs, no consensus has been issued on the panel of cell surface markers expected to be expressed by mouse MSCs. Nevertheless, CD44 and Sca-1 are believed to be common markers of mouse MSCs, whilst the level of expression of key human MSC markers CD73, CD90 and CD105 by mouse MSCs is more varied (Boxall and Jones, 2012). kMSCs previously isolated and characterised within the group were tested for expression of all the above markers by flow cytometry (Fig. 3.4). The cells were found to strongly express CD44 and Sca-1, with moderate expression of CD73 and CD90. CD105 surface expression however was not detected. The newly isolated mouse adipMSCs (two independent batches) were all analysed for cell surface marker expression using the same panel of markers (Fig. 3.5 and Fig 3.6). Whilst the two batches of adipMSCs displayed a consistent immunophenotype, it differed from that of the kMSCs. The adipMSCs highly expressed CD44 and moderately expressed Sca-1 and CD73. However, neither CD90 nor CD105 cell surface expression was detected. All mouse MSCs, kMSCs and both batches of adipMSCs, lacked expression of CD11b, CD31 and CD45, which are markers of macrophages, endothelial cells and hematopoietic cells respectively.

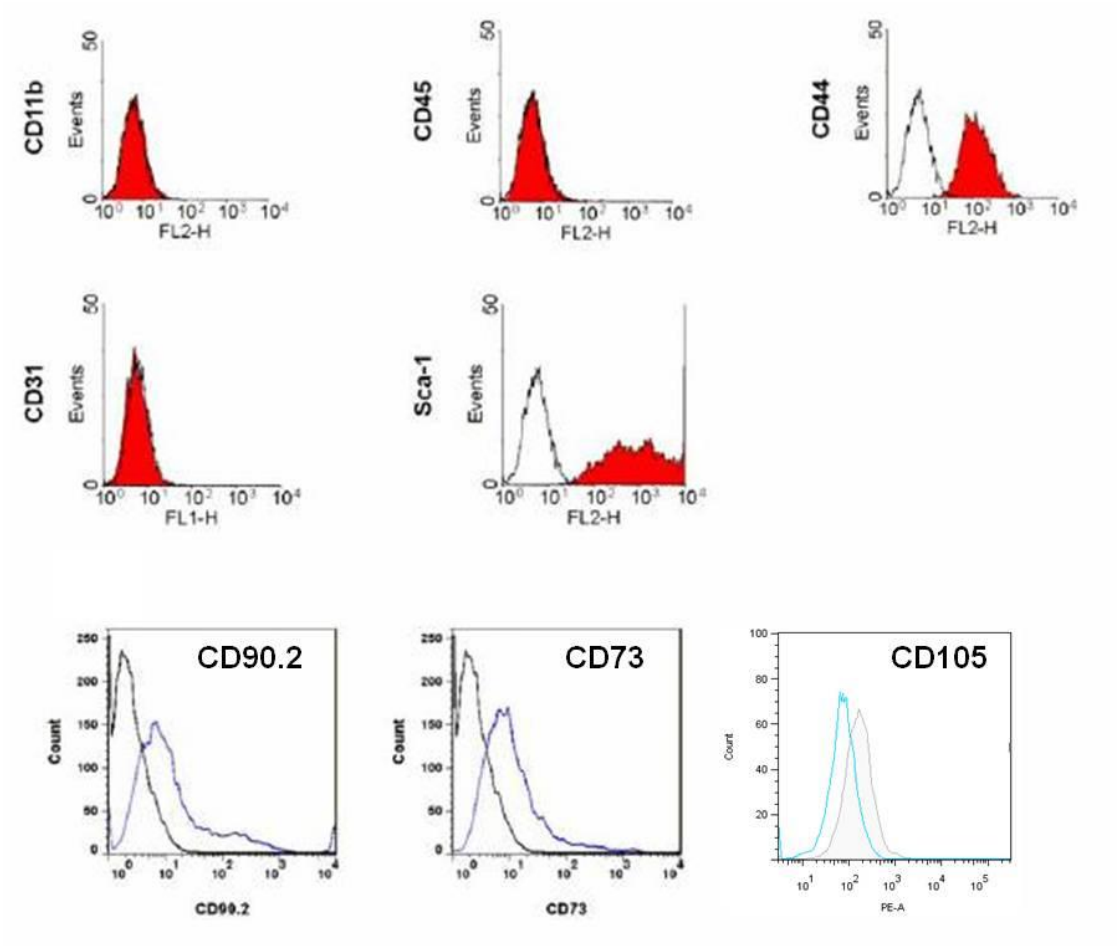

Figure 3.4: Immunophenotype of mouse kMSCs, (image modified from (Rackham et al., 2013)). kMSCs were assessed for the expression of a panel of cell surface markers associated with MSC phenotype, using flow cytometry. Negative isotype-matched controls shown by black or light grey peaks, marker expression shown by red or blue peaks Cells highly expressed mouse MSC markers CD44 and Sca-1, and moderately expressed human markers CD90 and CD73. The cells were negative for CD11b, CD31 and CD45, markers of macrophages, ECs and hematopoietic cells, respectively.

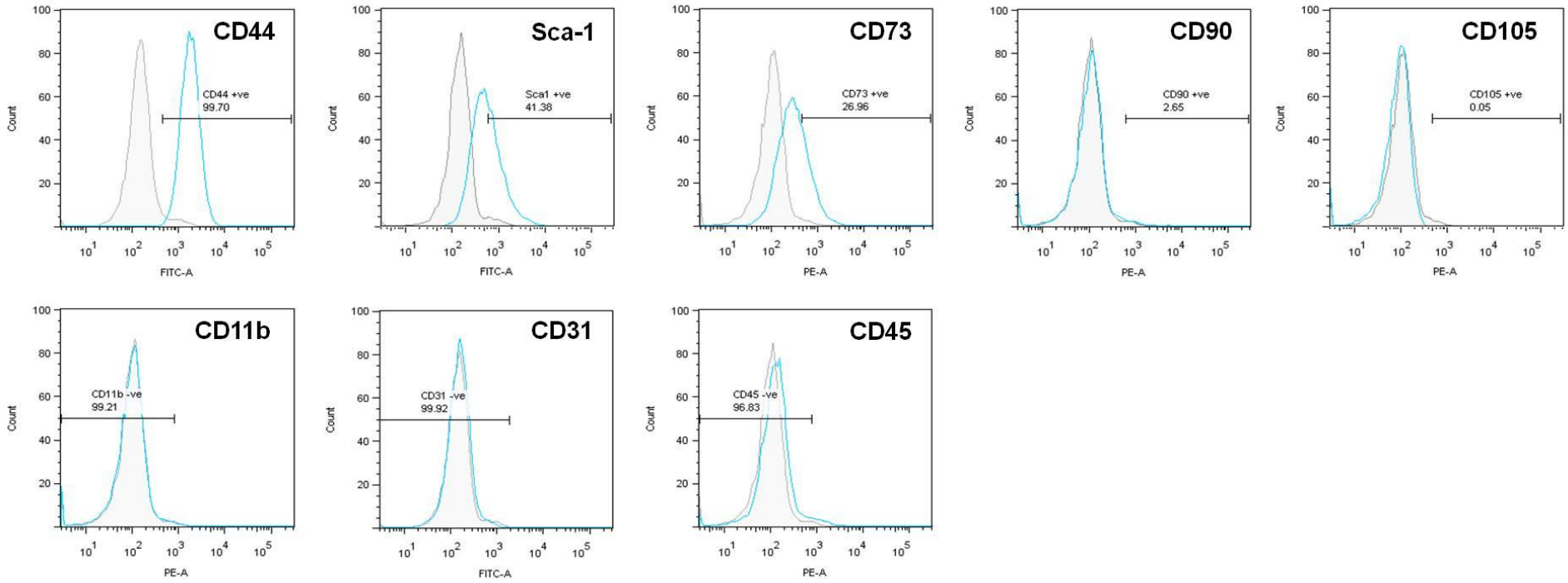

Figure 3.5: Immunophenotype of mouse adipMSCs (Batch A1). AdipMSCs were assessed for the expression of a panel of cell surface markers associated with MSC phenotype, using flow cytometry. Negative isotype-matched controls shown by light grey peaks, marker expression shown by blue peaks. Cells were positive for CD44 (>99%), Sca-1 (>41%) and CD73 (>26%), which are characteristic of murine MSCs. The stromal cells however did not express CD90 and CD105, which are commonly associated with human MSCs (Dominici et al., 2006a). Cells were also negative for CD11b, CD31 and CD45, which are markers of macrophages, endothelial cells and hematopoietic cells.

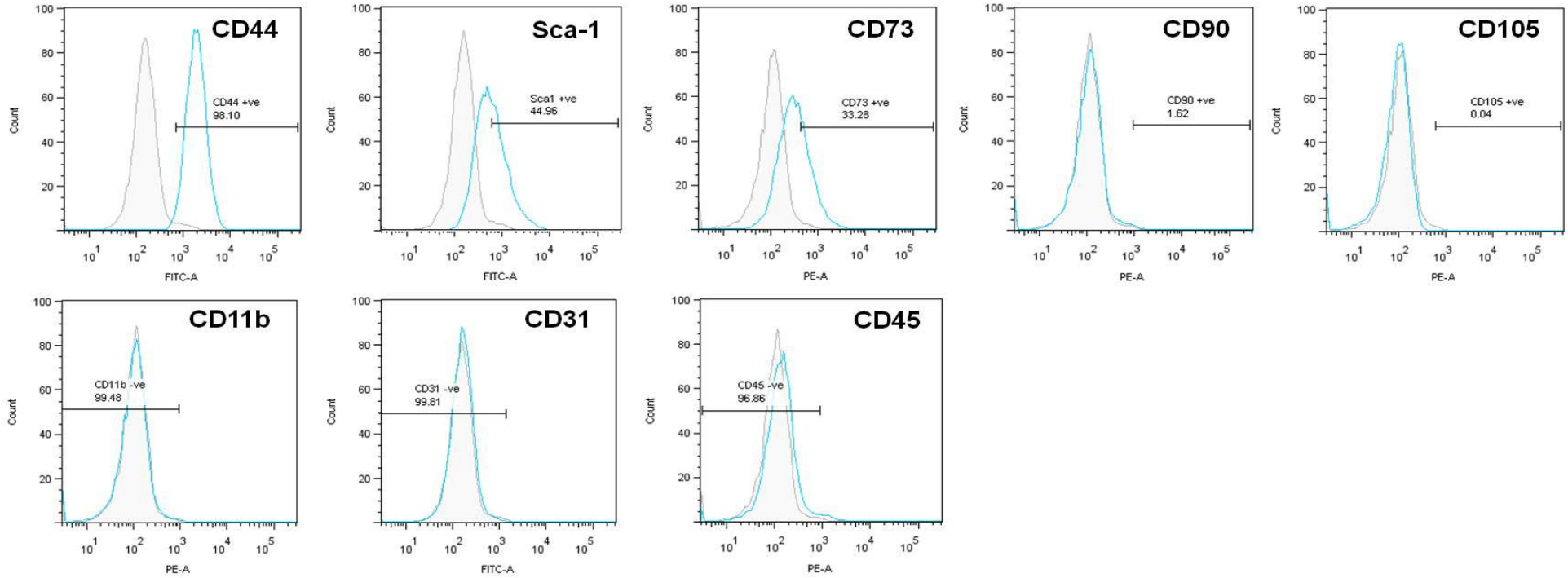

Figure 3.6: Immunophenoytpe of mouse adipMSCs (Batch A2). A second batch of adipMSCs was assessed for the expression of the same panel of cell surface markers associated with MSC phenotype as batch A1 (Figure 3.5), using flow cytometry. Negative isotype-matched controls shown by light grey peaks, marker expression shown by blue peaks. The cells were positive for CD44 (>98%), Sca-1 (>44%) and CD73 (>33%) to a comparable degree as batch A1. Similarly to batch A1, the murine adipMSCs did not express human MSC markers CD90 and CD105. Cells were also negative for CD11b, CD31 and CD45, which are markers of macrophages, endothelial cells and hematopoietic cells.

3.3.2 Isolation and characterisation of human pMSCs

3.3.2.1 *Derivation and maintenance of human pMSCs in vitro*

Pancreas exocrine digest, the by-product of clinical islet isolation, was harvested for the generation of human pMSCs *in vitro*. The exocrine tissue was seeded into tissue culture flasks and within 24 hours of seeding a heterogeneous mix of cells had adhered to the plastic surface. Both fibroblast-like cells (Fig. 3.7A) and cells with a flat 'cobblestone' morphology (Fig. 3.7B) were present at P0 after the 24 hour time point. This cell heterogeneity persisted in culture for two passages, 20 days after initial digest seeding. Cells exhibiting uniform spindle-shaped morphology (Fig. 3.7C) did not outgrow other cell types and dominate culture until the third cell passage, 28 days into culture.

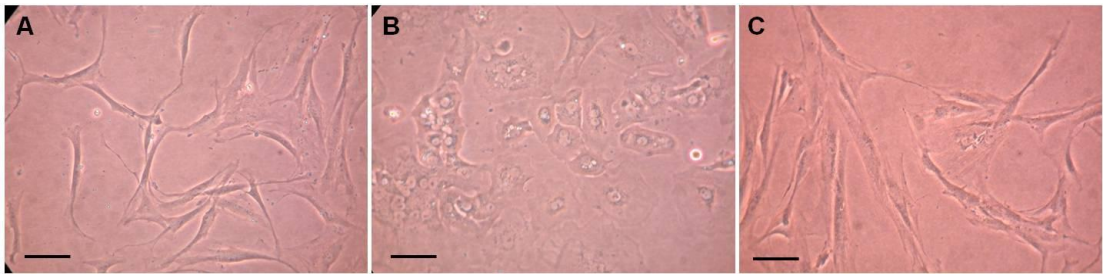

Figure 3.7: Derivation of human pMSCs through serial passaging. Heterogeneous cell populations were present in culture 24 hours post initial exocrine pancreas digest seeding (P0), including fibroblast-like cells (A) and 'cobblestone'-like cells (B). Cell heterogeneity remained after 2 passages 20 days into culture, with spindle shaped cells only dominating at P3 (C) 28 days into culture. Images representative of two independent pMSC isolations. Scale bar A-C; 100µm.

Through the process of deriving human pMSCs in culture using the same basic MSC medium as used for mouse MSCs, it was discovered that the standard formulation of DMEM supplemented with 10% vol./vol. FBS was not optimal for the maintenance and expansion of human cells *in vitro*. The growth curves of human pMSCs between P5-P10 cultured in two different commercially available low serum culture media designed especially for human MSCs, MesenPRO RS™ and MSCGM, versus standard DMEM culture medium were analysed to find a

culture medium best suited to human MSC expansion (Fig. 3.8). In general pMSCs cultured in either of the low serum formulations maintained a comparable, steady growth rate throughout the 32-day culture period. In comparison those cells cultured in 10% FBS experienced a decrease in growth rate around the 20 day mark. Low serum formulations also resulted in more rapid cell expansion, with cells experiencing a 35% ± 4% or 34% ± 6% increase in cumulative population doublings (CPD) after the 32-day culture period when cultured in MesenPRO RS™ or MSCGM respectively over those cultured in 10% FBS medium. Figure 3.9 shows the cell monolayer density of cells cultured in all 3 media after 11 days in culture. Denser monolayers of small compacted cells were observed in cultures using low serum media (Fig. 9B and Fig. 9C), compared to the sparser monolayer of larger cells cultured in 10% FBS medium (Fig. 9A), contributing to the enhanced CPD achieved by using low serum levels. Efficient human pMSC expansion was achieved using either MesenPRO RS™ or MSCGM; MesenPRO™ was selected for expanding pMSC stocks, as it was the most economically viable option.

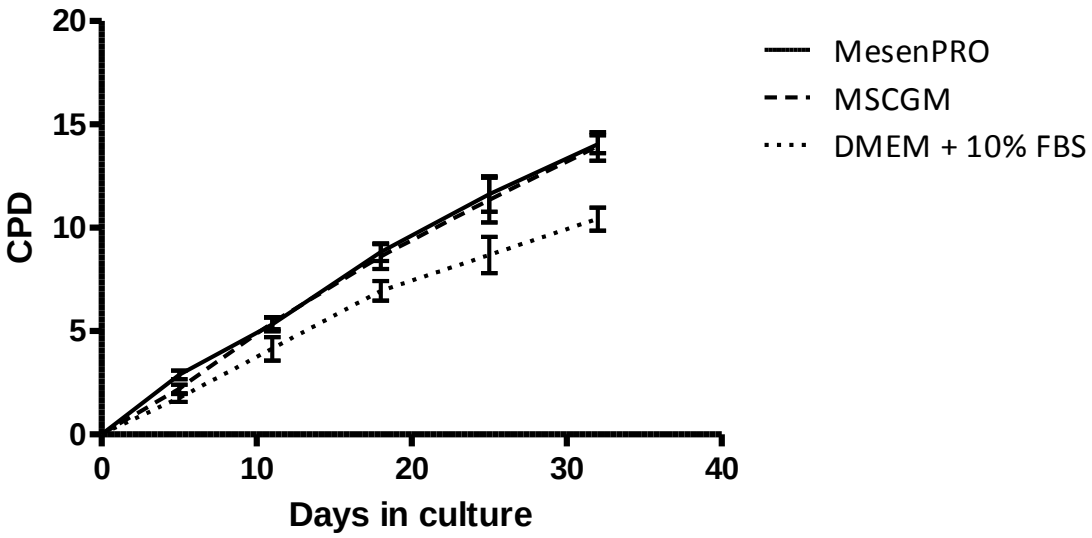

Figure 3.8: Comparative growth curves for human pMSCs cultured in low serum media formulations (MesenPRO RSTM and MSCGM) versus standard 10% FBS containing culture medium. pMSCs were cultured over a 32-day period between P5-P10 in standard 10% FBS containing DMEM medium or commercially available low serum formulation. Faster, more stable growth rates were achieved when were cells cultured in low serum formulations MesenPRO™ (solid black line) and MSCSGM (dashed black line) compared to cells cultured in DMEM medium with 10% FBS (dotted black line). Mean ± SD, n = 3 observations.

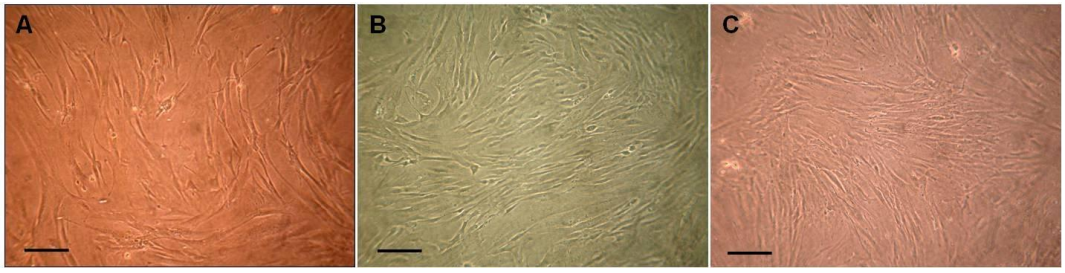

Figure 3.9: Cell monolayer density of human pMSCs cultured in low serum media formulations (MesenPRO RSTM and MSCGM) versus standard 10% FBS containing DMEM medium. On day 13 of culture cells, at P6, formed compacted, dense monolayers when cultivated in either MesenPRO RS^{TM} (Fig. 3.9B) or MSCGM (Fig. 3.9C) compared to the sparser monolayer of larger cells formed when exposed to 10% FBS (Fig. 3.9A). Each image representative of 3 wells. Scale bar; 100µm.

3.3.2.2 *Characterisation of human pMSCs*

3.3.2.2.1 Trilineage differentiation potential of human pMSCs

ISCT guidelines state that human MSCs must demonstrate trilineage differentiation potential (Dominici et al., 2006). Human pMSCs were exposed to osteogenic, chondrogenic and adipogenic *in vitro* differentiation assays to confirm their multipotency. Prior to differentiation, pMSCs in adherent culture exhibited classic spindle-shaped morphology (Fig. 3.10A&E). Following treatment with differentiation supplements for 28 days, two independent batches of pMSCs had the capacity to differentiate along each one of the three lineages. MSCs treated with osteogenic culture medium displayed osteoblast forming potential, with mineralised calcium nodules detected across the cell monolayer by alizarin red staining (Fig. 3.10B&F). GAGs deposited in micromass cultures directed towards a chondrogenic fate were visualised by alcian blue staining (Fig. 3.10C&G). The adipogenicity of pMSCs was also confirmed by oil red o staining of intracellular lipid droplets in cell monolayers exposed to adipogenic culture medium (Fig. 3.10D&H).

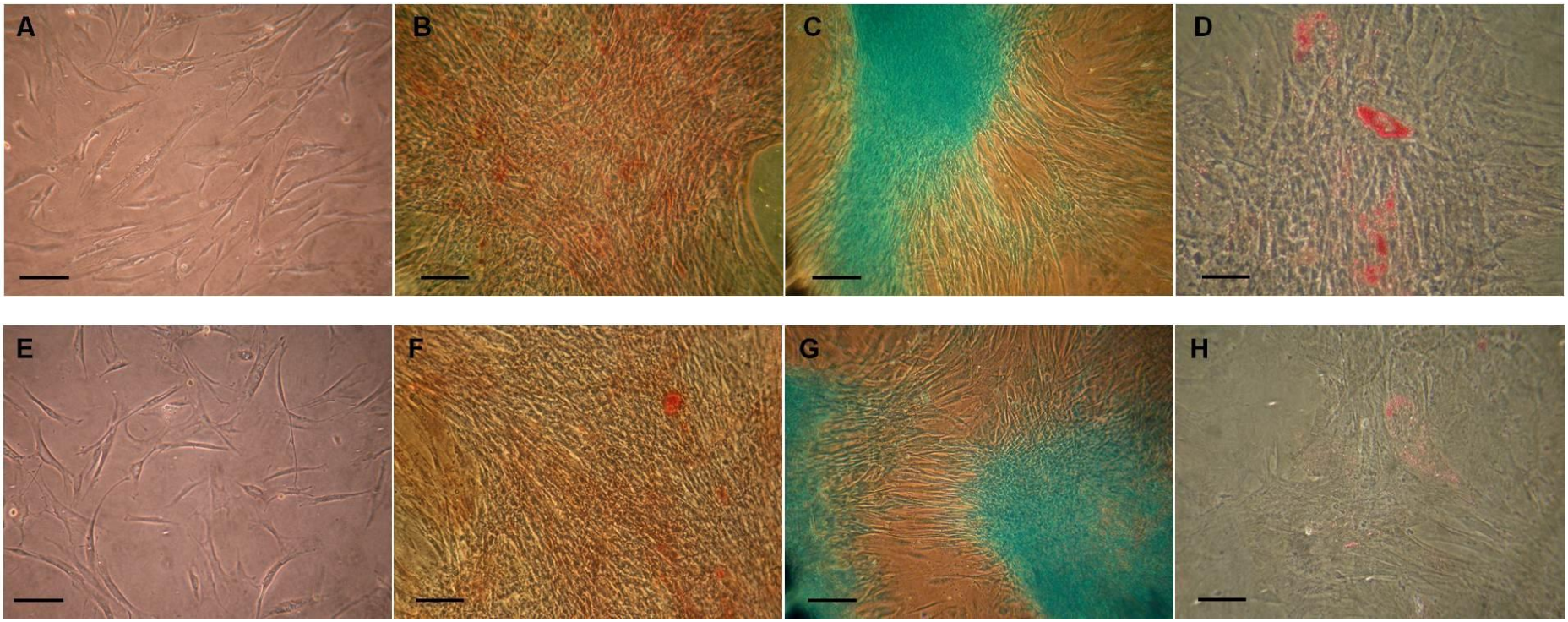

Figure 3.10: Trilineage differentiation potential of two independent batches of human pMSCs (batch P1 (A-D), batch P2 (E-H)). Prior to differentiation human pMSCs displayed characteristic spindle shaped morphology (A&E). Following 28 days treatment with osteogenic, chondrogenic or adipogenic differentiation supplements, pMSCs demonstrated trilineage differentiation potential *in vitro*. Alizarin red staining highlighted punctuate mineral deposition across cell monolayers exposed to osteogenic medium (B&F). Alcian blue staining detected GAG deposition within cell micromasses directed towards a chondrogenic fate (C&G). Oil red O staining detected the formation of lipid droplets in MSCs treated with adipogenic medium (D&H). Each image representative of 3 wells. Scale bar A-C and E-G; 100µm, D and H; 50µm.

3.3.2.2.2 Immunophenotype of human pMSCs

The ISCT guidelines for human MSCs also dictate that MSCs should express a panel of positive cell surfaces markers to include CD73, CD90 and CD105, whilst lacking expression of hematopoietic markers (Dominici et al., 2006). Two independent batches of pMSCs were tested for a panel of positive and negative cell surface markers by flow cytometry (Fig. 3.11 and 3.12). All three key markers CD73, CD90 and CD105 were detected at expression levels >99% on the pMSCs. Cell surface markers CD14, CD20, CD34 and CD45, markers of monocytes, B cells, and hematopoetic progenitors, were detected at levels <0.1% on the pMSCs.

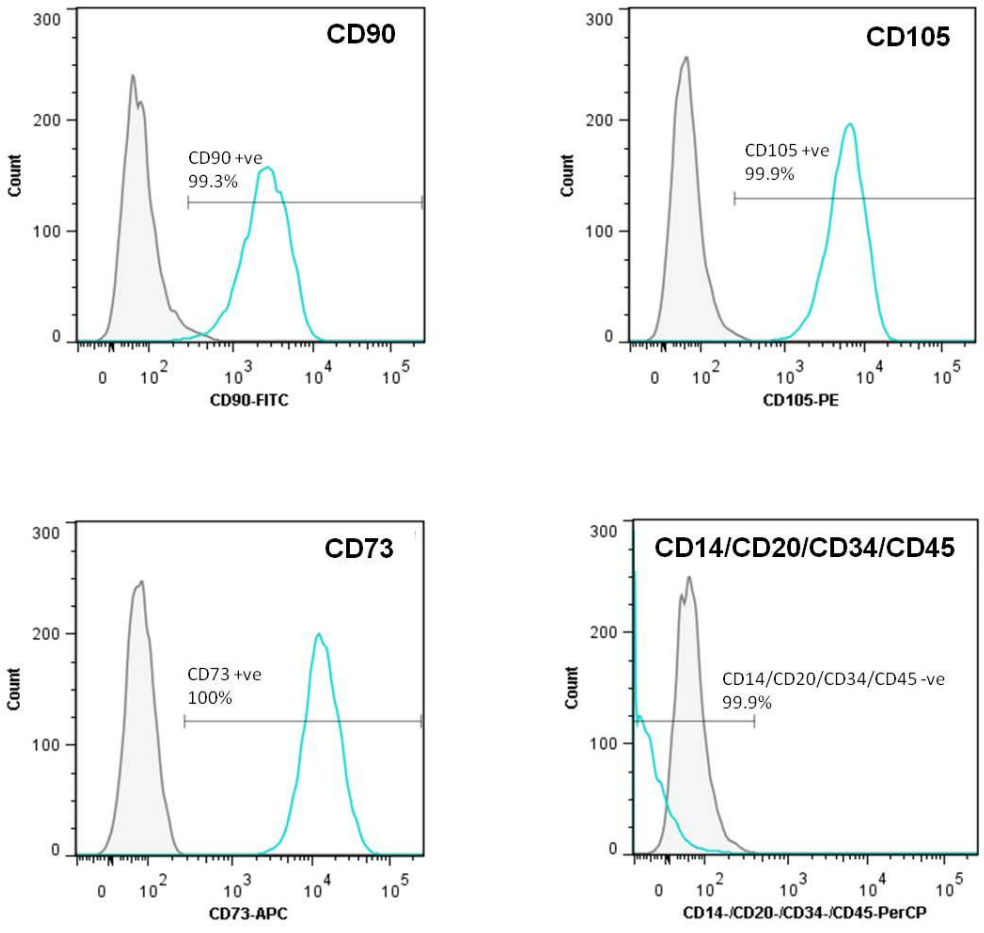

Figure. 3.11: Immunophenotype of human pMSCs (Batch P1). Cultured human pMSCs were analysed for expression of key cell surface markers associated with human MSCs, by flow cytometry. Negative isotype-matched controls shown by light grey peaks, marker expression shown by blue peaks. Cells highly expressed markers CD90, CD105 and CD73 (all > 99%). Expression of CD14, CD20, CD34 and CD45, markers of monocytes, B cells, and hematopoetic progenitors was absent.

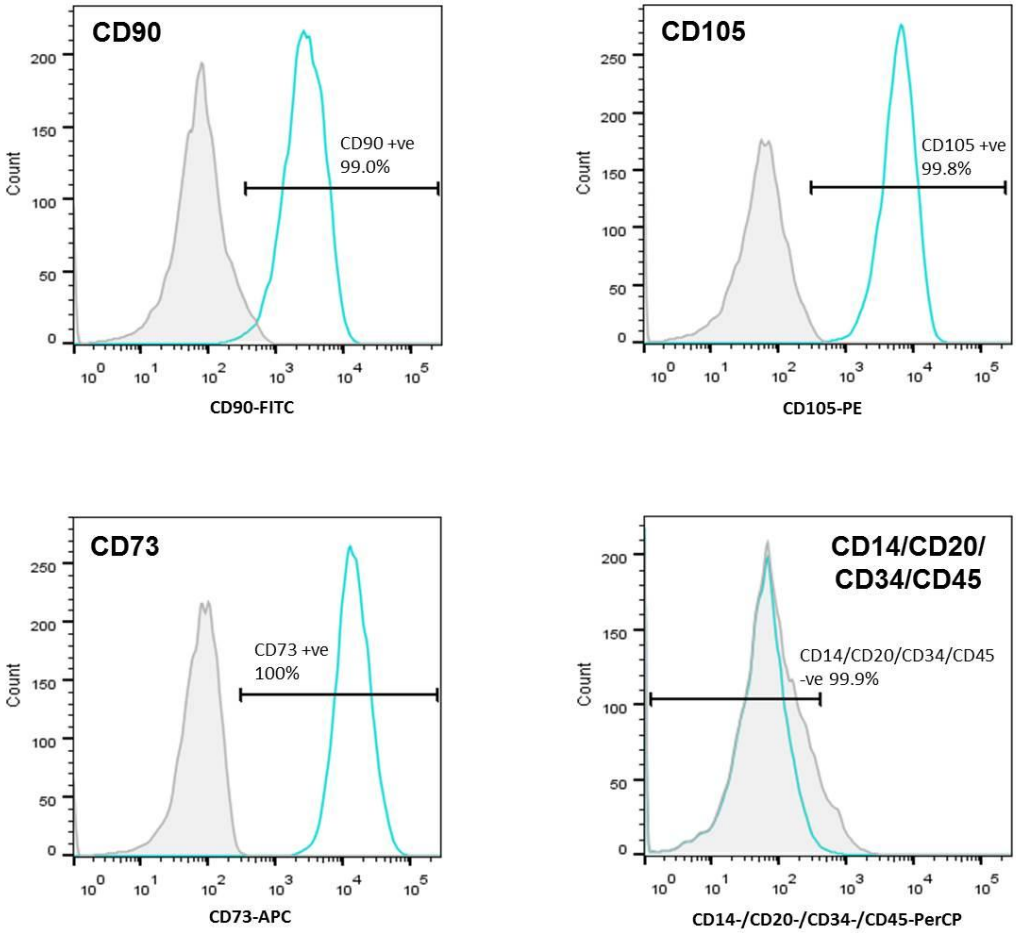

Figure. 3.12: Immunophenotype of human pMSCs (Batch P2). Cultured human pMSCs were analysed for expression of key cell surface markers associated with human MSCs, by flow cytometry. Negative isotype-matched controls shown by light grey peaks, marker expression shown by blue peaks. Cells highly expressed markers CD90, CD105 and CD73 (all > 99%). Expression of CD14, CD20, CD34 and CD45, markers of monocytes, B cells, and hematopoetic progenitors was absent.

3.4 Discussion

The versatility of MSCs for therapeutic applications has driven the significant increase in both fundamental and clinical research directed towards MSCs for cell therapy, in recent years. MSCs are believed to reside throughout the body, and to date have been isolated from virtually all organs (Zuk et al., 2001, Sordi et al., 2010, Sabatini et al., 2005, Da Silva et al., 2006). In order to harness the clinical potential of MSCs when considering them for cell therapy, the source tissue of choice should be easily available and accessible from human donors

(living or cadaveric). Two sources of MSCs considered clinically relevant for the current studies were adipose and exocrine pancreas tissue; multiple batches of both mouse adipMSCs and human pMSCs were successfully isolated and characterised.

Both MSC types were isolated using a traditional plastic adherence and enzymatic-based subculture method. Key observations made during the derivation process were 1) the heterogeneous cell morphologies appearing during the early passage stages, 2) the lengthy period of time required to achieve relatively homogenous MSC cultures and 3) the slow growth of human MSCs in standard 10% FBS containing MSC culture medium. Cobblestone-like cells were commonly observed in both mouse-adipose and human-pancreas early digest cultures; although not further characterised they displayed classic endothelial cell like morphology, a cell type known to exist in both the stromal fraction of adipose tissue (Pettersson et al., 1984) and exocrine fraction of pancreatic tissue (Klein et al., 2003). The simple basis of the plastic adherence and subculture method, is to reduce this initial cell heterogeneity by exploiting the subculture process. Cell cultures containing separate populations of cells can be crudely purified based on their differential growth rates and nutrient requirements (Seglen, 1976). MSCs known for their proliferative capacity outgrew other cell types when maintained in long-term culture. However for both mouse adipMSCs and human pMSCs this process took at least 1 month from initial seeding to emergence of MSC dominant cultures. This lengthy process further compounded the fact that only a 50% success rate was achieved for the derivation of both mouse adipMSCs and human pMSCs (as previously mentioned in sections *3.2.1.1* and *3.2.2.1* of this chapter). It was for this reason that the human adipMSCs required for subsequent studies in this thesis were sourced from commercial suppliers rather than generated in house, to save time. Fully characterised human adipMSCs isolated from lipoaspirate material were purchased from Gibco at passage 1 (Life Technologies). For completeness it would have also been desirable to have access to mouse pMSCs. Attempts were made to generate the cells by seeding either exocrine pancreas or whole

pancreas digests extracted from C57Bl/6 mice. However after 5 unsuccessful attempts it was deemed not possible using our standard isolation techniques. Since mouse pMSCs were not available for purchase, they had to be omitted from the functional studies. Human pMSCs, on the other hand, although successfully generated in house from human exocrine digest tissue, required culture medium optimisation for their expansion. Serum free medium has previously been shown to support the efficient expansion of human MSCs when compared to conventional 10% serum containing culture medium (Agata et al., 2009). The low serum containing medium formulations utilised in this study also suited human MSC expansion, with pMSCs displaying a higher proliferative capacity and compacted higher-density cell monolayers in culture compared to pMSCs cultured in conventional medium. A move towards low serum/serum free medium formulations not only benefits the efficient expansion of human MSCs, it also improves the clinical applicability of the cells. Human cells cultured in the presence of FBS are not desirable due to the risk of xenogenic viral contamination or immunological reaction against xenogenic serum antigens.

Following derivation of the human and mouse MSCs, their characterisation consisted of trilineage differentiation potential and immunophenotype analysis. The main findings observed when characterising primary MSC cultures in house were; 1) all MSCs (mouse kMSCs, adipMSCs and human pMSCs) demonstrated trilineage differentiation potential *in vitro* and 2) there were discrepencies in the immunophenotype of MSCs relating to both the tissue of origin (mouse kMSCs versus mouse adipMSCs) and species of origin (mouse MSCs did not express all three key human MSC cell surface markers). The trilinieage differentiation potential of the MSCs was assessed qualitatively *in vitro* using various stains to visualise markers of differentiation. This method, although excellent in detecting the presence of differentiation, is ineffective at quantifying the degree of cell conversion. It was not within the scope of this thesis to perform quantitative analysis of differention e.g. flow cytometry or colourimetric assays (Lee et al., 2004, Ab-Rahim et al., 2008). Without the use of such methods, in addition to the fact differentiation protocols were not always performed over the same

period of time, comparisons can not be drawn between the differentiation potential of each MSC type isolated. Within an MSC population however, it was observed that not all cells followed the induced differentiation pathway. MSCs generated in this study were not clonally derived, and the cell monolayers were expected to contain mixed populations of stem cells and their progeny, so divergent differentiation potential was not unexpected. Cells not appearing to have undergone conversion may have been lineage-committed cells with limited differentiation potential (Pittenger et al., 1999). However it could also have been the result of cells demonstrating a slower response to induction agents (Shigematsu and Pessin, 2001) or simply the loss of multi-lineage potential during *in vitro* culture (Javazon et al., 2004).

The discrepancies in the immunophenotype between MSC populations must be interpreted with caution. Phenotypically MSCs are generally accepted to be devoid of hematopoietic and endothelial markers, and only express a number of nonspecific markers. Variable expression of numerous cell surface adhesion molecules such as CD44, and CD49e and typically Sca-1, CD90 (Thy 1.1), CD105 (endoglin), CD73 (SH3 or SH4) and STRO-1 are often observed across species (Kern et al., 2006, Jin et al., 2013, Pittenger et al., 1999, da Silva Meirelles et al., 2006, Noort et al., 2012, Peister et al., 2004). However without knowledge of a definitive phenotype for MSCs, their specific identification by cell surface marker expression will always remain difficult to interpret. Nonetheless, marker expression remains a routine characterisation tool for newly isolated MSCs, and the variable expression of commonly observed markers between cell populations are often accounted for due to isolation methods, tissue and species of origin and culture conditions. As already shown in this study (sections *3.3.1.1 and 3.3.2.1*) the MSC isolation process is lengthy, and can take up to several weeks in culture following initial digest seeding. Thus the MSC populations are fundamentally a product of *in vitro* culture, so the cells are subjected to environmental conditions that are quite different from those found in their original anatomic location. *In vitro* culture does not protect the cells from toxic insults such as high oxygen and other stress conditions, thus increasing the

chance for accumulation of mutations. The latter may not necessarily cause transformation of the MSCs, but can generate cellular heterogeneity (Wagner et al., 2010). Indeed, MSCs that have been cultured extensively *in vitro*, exhibit changed surface molecule expression profiles (Wagner et al., 2008, Le Blanc et al., 2007). The impact of artificial conditions imposed upon cells may lead to the introduction of experimental artefacts, causing cell surface markers to be specific only in a particular context. This can introduce a degree of variability not only amongst MSCs from different tissues and species but also between cultured MSCs and their *in vivo* counterparts. However it cannot be excluded that MSC heterogeneity may also be a reflection of their *in vivo* repertoire. In this study species specific MSCs were isolated from different tissues (mouse kMSCs and mouse adipMSCs) and displayed differing cell surface marker expression. The *in vivo* identity of MSCs is often correlated with pericytes (Crisan et al., 2008, Meirelles et al., 2008), residing in perivascular niches, offering good reason for why MSCs can be isolated from most adult organs and tissues (da Silva Meirelles et al., 2006). According to this model, cues provided by the niche (cells, extracellular matrix, and signalling molecules) coordinate a gradual transition of the pericytes to progenitor and mature cell phenotypes which are tissue-specific (Kolf et al., 2007, Meirelles et al., 2008). It is therefore not too surprising that MSCs isolated from different niches show some phenotypical differences, as observed in the current study. This conclusion is supported by the observation that the phenotype of different batches of MSCs prepared from the same host tissue was very similar, consistent with the heterogeneity being between tissue niches rather than being introduced during the isolation and expansion process.

In summary various batches of MSCs generated from clinically relevant tissue sources were successfully isolated and characterised. All cells displayed multi-lineage potential and expressed cell surface markers commonly observed in the field of MSC research. These cells were subsequently utilised in *in vitro* co-culture studies to investigate their effects on isolated islet function as described in Chapters 4-6.

Chapter 4 - Co-culture of mouse islets with MSCs in direct contact configurations improves islet function *in vitro*

4.1 Introduction

Evidence is accumulating that MSCs may have therapeutic applications in a number of diseases, including diabetes. Recently there have been several studies reporting the benefit of co-transplanting MSCs with islets to improve graft efficacy in animal models of Type 1 diabetes. A study conducted by Ding et al. showed MSCs promoted islet allograft survival in streptozotocin (STZ)-induced diabetic mice (Ding et al., 2009). Mice receiving islet only grafts initially achieved normoglycaemia, but reverted to hyperglycaemia within 30–35 days due to graft rejection. On the other hand, mice receiving islets with MSCs maintained normoglycaemia for the long-term, a result attributed to the immunosuppressive actions of matrix metalloproteinases (MMPs) 2 and 9 secreted by the MSCs. Solari et al. utilised a rat model of STZ-induced diabetes to transplant a marginal mass of islets alone or islets with MSCs into the omental pouch and noted longer survival times for the MSC containing grafts (Solari et al., 2009). The suppression of immune or inflammatory responses by MSCs has also been shown by other groups (Longoni et al., 2010) (Fiorina et al., 2009).

Improved graft revascularisation has also been an important contributor to the beneficial effects of MSCs in islet co-transplantation (Ito et al., 2010) (Figliuzzi et al., 2010) (Sordi et al., 2010) (Berman et al., 2010) (Rackham et al., 2011). Ito et al. showed improvement in islet engraftment by co-infusion of MSCs and observed increased capillary density in the MSC infusion group compared to control (Ito et al., 2010). This indicated a potential role for MSC induced revascularisation in the promotion of islet graft survival. Co-transplantation of islets with MSCs was not only found to have a profound effect upon the vascular engraftment of islets by Rackham et al, but also upon islet morphology and organisation, with co-transplanted islets maintaining a morphology which closely resembled that of islets in the endogenous pancreas (Rackham et al., 2011). Improvement in islet engraftment following MSC treatment has also been shown

in a nonhuman primate model of diabetes, again revealing the efficacy of MSCs in aiding the engraftment of islets (Berman et al., 2010).

Overall, these *in vivo* studies encompassing both small and large animal models have focussed on the immunoregulatory and angiogenic capacity of the MSCs as the primary reasons for their beneficial effects on islet graft survival and function. Since the essential function of islets is to secrete hormones that are involved in the maintenance of glucose homeostasis, primarily insulin, one of the key objectives for the current studies was to assess whether MSCs had the capacity to directly enhance the function of islets, in terms of their insulin secretory function and insulin content levels, using controlled co-culture conditions *in vitro*. Direct contact co-culture systems were utilised to provide a microenvironment where MSCs could deliver direct signalling and physical contact to the islets, whilst at the same time acting as 'multidrug dispensers' (Caplan and Correa, 2011) secreting an array of bioactive factors. Two modes of direct contact co-culture were investigated. Islets were cultured either directly upon a monolayer of MSCs, the traditional manner for maintaining MSCs (Prockop, 1997), or in suspension with MSCs, which provided additional insight into the feasibility of forming islet-MSC composite structures via the adhesive capacity of the MSCs; structures that would be useful for the co-localised delivery of MSCs and islets in co-transplantation via the clinically relevant intraportal site. Both kMSCs, the MSC of choice for the group's previous studies, and adipMSCs, the clinically relevant MSC, were tested in this study.

4.2 Methods

4.2.1 Direct contact islet-MSC monolayer co-culture

4.2.1.1 *Experimental design*

Direct contact co-culture studies were designed to test the capacity of MSCs to improve islet function *in vitro*. The rationale behind the conditions selected for key co-culture variables is discussed below.

1. MSC number and density – The initial basis of the direct contact co-culture study was to culture isolated islets directly contacting a layer of MSCs. In order to ensure maximal contact between the islets and MSCs this required a confluent monolayer of MSCs coating the growth surface area of the co-culture vessel. 35mm petri dishes were sufficient for these small-scale studies, and from experience of routine MSC culture *in vitro*, it was known that 200 000 mouse MSCs formed approximately 80% confluent monolayers over the growth surface area of a 35mm petri dish.

2. Islet number – 35mm petri dishes are suitable for the culture of approximately 75 islets, as advised by experienced islet handlers in the group, to prevent overcrowding and clumping of the islets. The 200 000 MSCs to 75 islets ratio gave an approximate 1:1 cell ratio, based on the assumption that the average islet contains 2000-3000 cells. A similar islet:MSC ratio had also previously been shown to exert beneficial effects on islet function after transplantation below the kidney capsule *in vivo* by the group (Rackham et al., 2011).

3. Co-culture period – A 3 day co-culture period was selected, as in this time the MSCs are not expected to become over-confluent, a factor which can otherwise induce spontaneous MSC differentiation in culture.

4.2.1.2 *Direct contact islet-MSC monolayer co-culture system*

Two hundred thousand MSCs (kMSCs or adipMSCs) at passage 9 were seeded into 35 mm Nunclon petri dishes in MSC medium and incubated overnight to

adhere to the culture plastic. Twenty four hours after seeding the MSCs they were approximately 80% confluent and the medium was replaced with 3ml of islet medium. Islet medium was chosen as the medium of choice for co-culture, since it is suitable for islet maintenance and subsequent functional analysis, whilst its glucose concentration (11mM) is sufficient for actively proliferating MSCs. Defined numbers of fresh islets (75) were then added to each dish of MSCs and co-incubated for 3 days. Islets cultured alone for 3 days in islet medium served as a control group. A full medium replacement was performed for all control and co-culture groups on day 2.

4.2.2 Direct contact islet-MSC suspension co-culture

4.2.2.1 *Time-lapse imaging of islet-MSC suspension co-culture*

4.2.2.1.1 Islets and MSCs in co-suspension over 72 hour period

The Nikon Biostation incorporates an inverted microscope, incubation chamber and high-sensitivity camera into a single unit and was used to capture footage of 75 isolated mouse islets and 200 000 kMSCs seeded into a non-treated tissue culture petri dish in islet medium over 72 hours. 12 locations within the dish were selected, where the microscope's field of view captured at least one islet and any surrounding kMSCs at t = 0 hours and footage of each location was captured at 10 minute intervals over the 72 hour culture period.

4.2.2.1.2 Measurement of islet-MSC composite diameter over 72 hour period

Using time-lapse footage of islets and kMSCs in co-suspension for 72 hours, the diameter of 9 different islets captured by the Biostation was measured at 0 hours, 12 hours, 24 hours, 36 hours, 40 hours and 72 hours to assess any increase in islet size due to MSCs combining with the islets. The diameter of each islet was measured using the Nikon software measuring tool. The shortest axis of length was measured across each islet (use of the shortest axis omitted any obvious large MSC clusters attached to the islet edge or conglomerated islets

from the analysis) and the data used to calculate the average % increase in islet diameter as a percentage of basal diameter (i.e. islet diameter at t = 0 hours).

4.2.2.1.3 Fluorescent labelling of MSCs with QTracker 525 beads

To enable the tracking of MSCs, during suspension co-culture with islets, kMSCs were loaded with fluorescent Qtracker® nanocrystals (Invitrogen). Sub-cultured MSCs were seeded into the wells of a 6-well Nunclon plate at a density of 200 000 cells/well and incubated for 24 hours. Once cells had formed a confluent monolayer, Qtracker® labelling solution was freshly prepared (10nM Qtracker® component A, 0.5% (vol./vol.) PBS, DMEM) and 1ml dispensed per well of MSCs. The labelling solution and cells were incubated for 4-5 hours at 37^0C, 5% CO_2, to allow nanocrystal delivery into the cytoplasm of the kMSCs. Following the incubation period, cells were washed twice with MSC medium and trypsinised ready for suspension co-culture. Labelled cells could be visualised in the 405-485 excitation, 525 emission spectra of a Nikon Biostation microscope (Nikon).

4.2.2.1.4 Fluorescently-labelled MSCs and islets in co-suspension

The Nikon Biostation was used to capture the interactions between fluorescently labelled kMSCs and non-labelled islets in suspension co-culture. The Biostation captured time lapse footage of 75 isolated islets and 200 000 fluorescently tagged kMSCs seeded into a non-treated tissue culture petri dish and incubated for 48 hours in islet medium. 12 locations within the dish were selected, where the microscope's field of view captured at least one islet and any surrounding kMSCs at t = 0 hours and footage of each location was captured at 10 minute intervals over the 48 hour culture period.

4.2.2.2 *Direct contact islet-MSC suspension co-culture system*

Using the same islet:MSC ratio as decided for the direct contact monolayer co-culture system, 200 000 MSCs (kMSCs or adipMSCs) at passage 9 were seeded into non-treated tissue culture 35mm petri dishes (Greiner, Stonehouse, UK) in 3ml islet medium. Approximately 75 fresh islets were added directly to the dish

and the co-culture suspension was left to incubate for 3 days. Islets cultured alone in islet medium for 3 days served as a control group. A full medium replacement was performed for all control and co-culture groups on day 2.

4.2.3 Measurement of islet function in vitro

Assessment of islet function after all co-cultures was performed as described in Chapter 2, section 2.3 and quantified by radioimmunoassay as per Chapter 2, section 2.3.3.

4.3 Results

4.3.1 Direct contact islet-MSC monolayer co-culture

4.3.1.1 *Effects of monolayer co-culture on glucose-stimulated insulin secretion*

Preliminary co-culture studies were designed to test the capacity of MSCs to improve islet function, using controlled direct contact conditions *in vitro*. This was investigated using both kMSCs and the more clinically relevant adipMSCs. Islet function *in vitro* was assessed using a static basal (2mM) and glucose-stimulated (20mM) insulin secretion assay. Using a direct contact monolayer co-culture system, for a given population of islets both kMSCs (Fig. 4.1A-C) and adipMSCs (Fig. 4.2A-C) significantly enhanced glucose-induced insulin secretion at least 2-fold or 3-fold respectively over islets cultured alone. This result was consistently observed across three independent islet populations for both MSC types, and considered significant by repeated measures statistical analysis ($p<0.01$, n=3 repeats). Occasional increases in basal insulin secretion were observed for given islet populations co-cultured with kMSCs or adipMSCs (kMSCs – Fig. 4.1B, adipMSCs – Fig. 4.1B). However, the increases were not considered statistically significant ($p>0.2$, n=10 observations) nor detected across all islet populations.

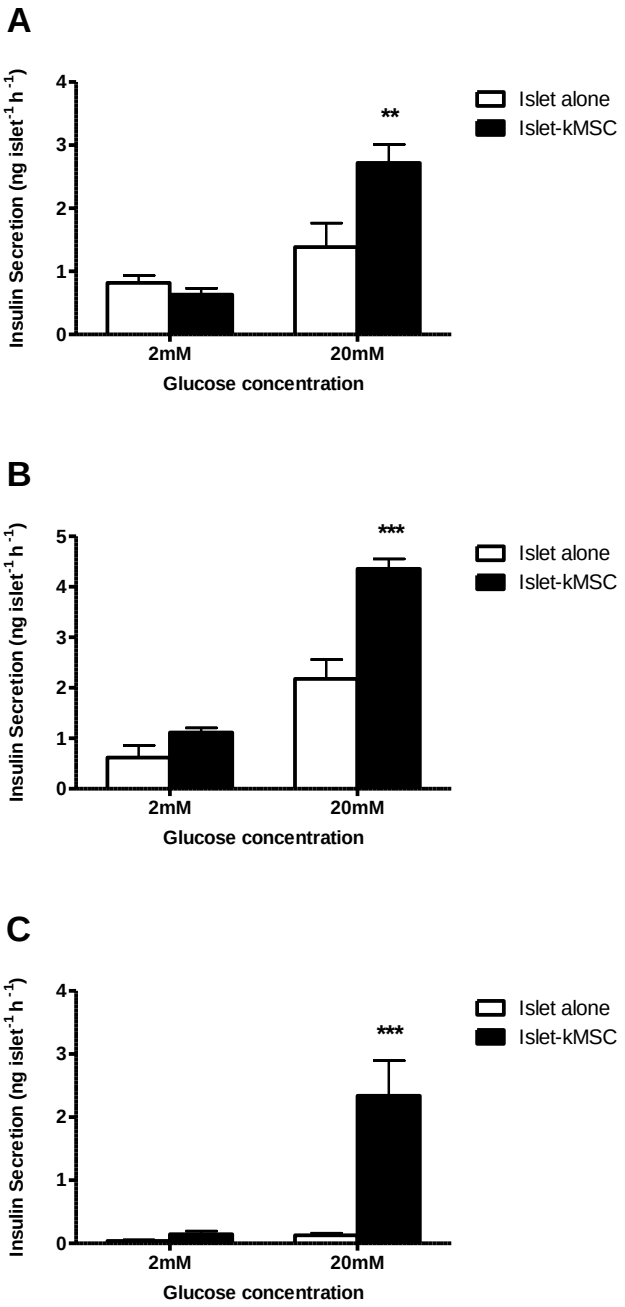

Figure 4.1: Glucose-stimulated insulin secretion *in vitro* of islets cultured alone vs. islets cultured directly upon a monolayer of kMSCs. In three independent experiments (A-C), insulin release at 2mM glucose (basal) and 20mM glucose (high), of groups of triplicate mouse islets previously cultured upon a monolayer of kMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, **p<0.01, ***p<0.001 vs. islet alone at the same glucose concentration. KMSCs consistently potentiated insulin secretion at stimulatory glucose levels across all three experimental repeats.

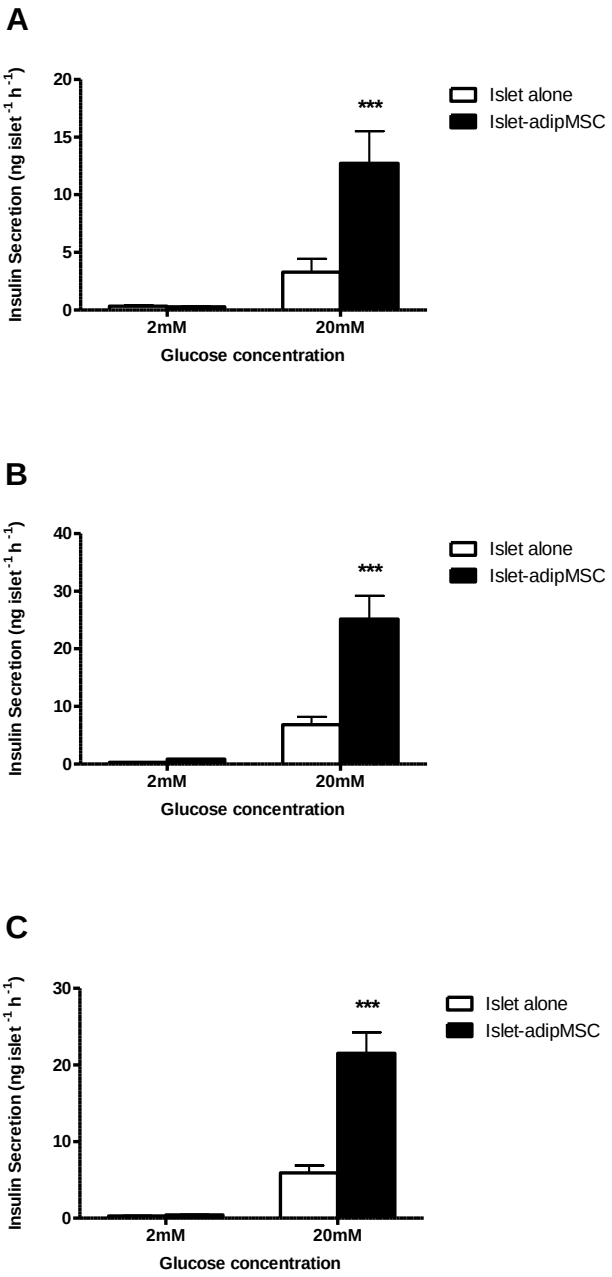

Figure 4.2: Glucose-stimulated insulin secretion *in vitro* of islets cultured alone vs. islets cultured directly upon a monolayer of adipMSCs. In three independent experiments (A-C), insulin release at 2mM glucose (basal) and 20mM glucose (high), of groups of triplicate mouse islets previously cultured upon a monolayer of adipMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, ***p<0.001 vs. islet alone at the same glucose concentration. AdipMSCs consistently potentiated insulin secretion at stimulatory glucose levels across all three experimental repeats.

4.3.1.2 *Monolayer co-culture effects on insulin content*

A second measure of islet function taken into account following direct contact monolayer co-culture was islet insulin content. When in direct contact, kMSCs were able to significantly elevate the insulin content levels of one population of islets (Fig. 4.3B), but a second population showing only a trend towards increased insulin (p=0.13 Fig. 4.3A) compared to islets cultured alone. AdipMSCs appeared to have no effect upon islet insulin content, an observation made across two independent islet populations (Fig. 4.4A-B).

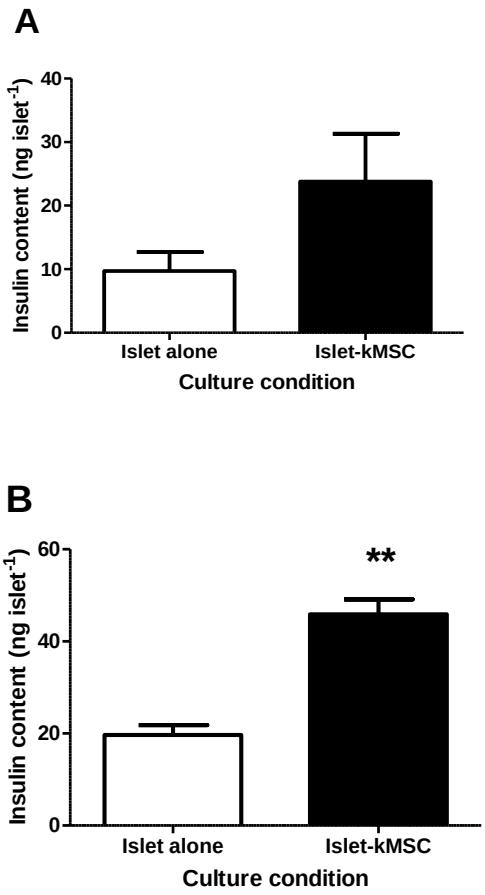

Figure 4.3: Insulin content *in vitro* of islets cultured alone vs. islets cultured directly upon a monolayer of kMSCs. In two independent experiments (A-B), insulin content of groups of 10 islets previously cultured upon a monolayer of kMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=3 observations, **p<0.01 vs. islets alone. N.b. these experiments use islets from the same batches of isolated islets used in the insulin secretory functional experiments A-B of Fig. 4.1. Islets cultured in the kMSC direct contact monolayer co-culture system showed elevated insulin levels compared to islets cultured alone.

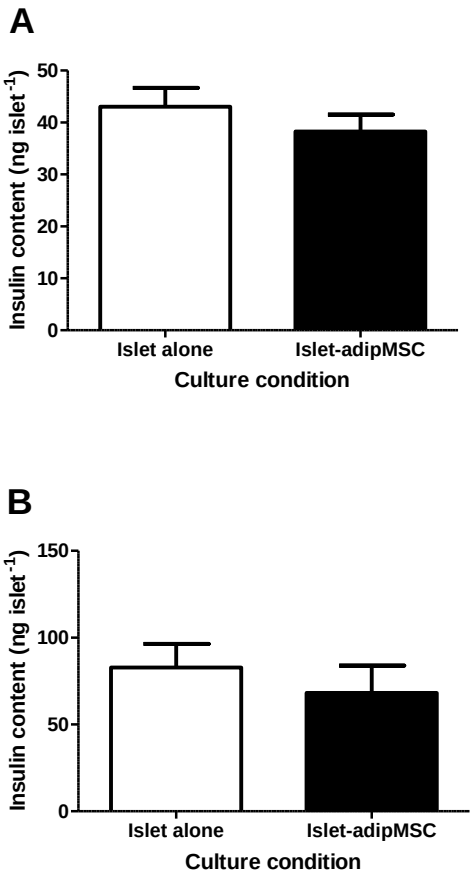

Figure 4.4: Insulin content *in vitro* of islets cultured alone vs. islets cultured directly upon a monolayer of adipMSCs. In two independent experiments (A-B), insulin content of groups of 10 islets previously cultured upon a monolayer of adipMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=3 observations. N.b. these experiments use islets from the same batches of isolated islets used in the insulin secretory functional experiments A-B of Fig. 4.2. No effect upon islet insulin content levels was observed when islets were cultured in an adipMSCs direct contact monolayer co-culture system compared to islets cultured alone.

4.3.2 Direct contact islet-MSC suspension co-culture

Having observed the distinct effects of direct contact co-culture upon islet insulin secretory function when employing a MSC monolayer, a second mode of direct contact co-culture was explored. Investigations into a suspension co-culture system were made, in part, to assess whether MSCs and islets would combine in suspension to form 'composite' structures, and whether these composite islets experienced the same beneficial functional status as MSC monolayer co-cultured islets.

4.3.2.1 *Composite formation studies*

The formation of islet-MSC composites is desirable for the co-localised delivery of islets and MSCs clinically via the intraportal vein. Operating with the same key co-culture variables as the direct contact monolayer studies, suspension co-culture was employed to investigate composite formation potential. Images captured using a Nikon Biostation, a compact cell incubation and monitoring system, demonstrated rapid interactions between 200 000 kMSCs co-suspended with 75 islets. The kMSCs migrated towards and adhered to the surface of the islets, although the interactions appeared largely to be at random. Typical footage of these interactions is displayed in a series of images in Figure 4.5. At 0 hours, the islet was surrounded by kMSCs in suspension (both single cells and cell clusters; Fig. 4.5A). Stills captured at 12 hours (Fig. 4.5B), 24 hours (Fig. 4.5C), 36 hours (Fig. 4.5D), 40 hours (Fig. 4.5E) and 72 hours (Fig. 4.5F) show the diameter of the islet increasing in a time-dependent manner, as the kMSCs attach. At both 40 hours and 72 hours, the most notable increase in islet diameter was due mainly to large kMSC clusters combining with the islet as opposed to single kMSCs in suspension integrating into the composite unit. kMSCs displayed great motility in suspension; they attached not only to the islets but also adhered to each other forming these large compact MSC clusters. Following the 72 hour co-culture period not all the kMSCs had combined with the neighbouring islet, as both small and large clusters of MSCs could still be observed in suspension (Fig. 4.5F).

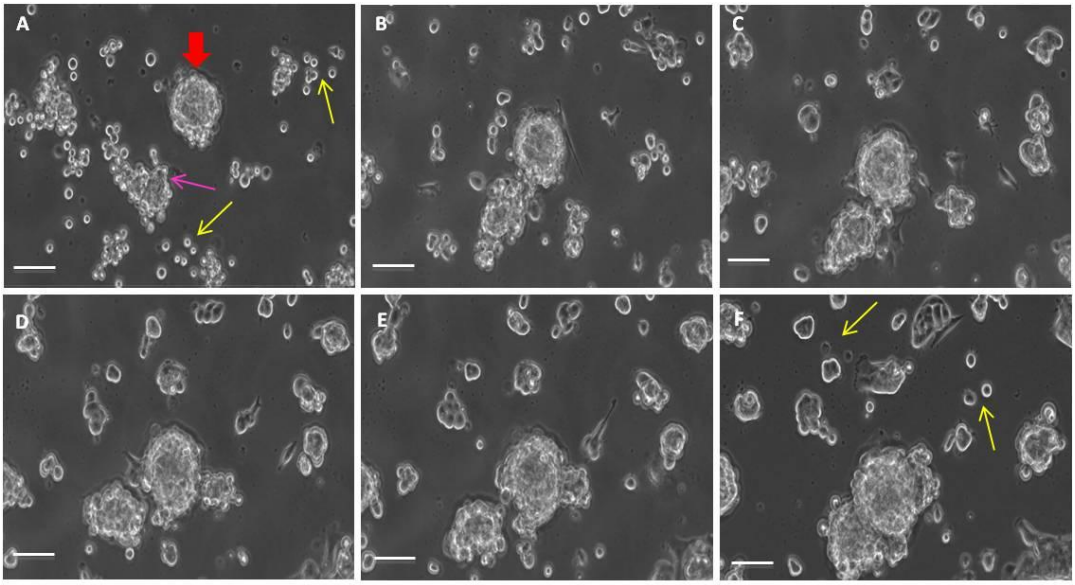

Figure 4.5: Time lapse footage of kMSCs and islets in suspension co-culture over a 72 hour time period. 75 islets and 200 000 kMSCs were co-suspended in 3ml of medium in a Nikon Biostation, a compact cell incubator and monitoring system, and recorded over a 72 hour period. A series of stills captured from the Biostation show a typical islet (red arrow) surrounded by MSCs (single cells – yellow arrows, cell cluster – pink arrow) in suspension at the beginning of the 72 hour co-culture period (A). The stills taken at 12 hours (B), 24 hours (C), 36 hours (D), 40 hours (E) and 72 hours (F) show some of the MSCs migrating towards the islet and combining with it to form an islet-MSC 'composite', highlighted by the observable time-dependent increase in islet diameter. After the 72 hour co-culture period however, it was observed that not all MSCs combined with the neighbouring islet and remained clustered in suspension (yellow arrows). Scale bar 100μm. The full video clip of kMSCs and islets in 72 hour co-culture suspension can be viewed in the supplementary data on the CD attached to the back of this thesis.

Using Biostation footage (such as that captured in Fig. 4.5) of 75 islets and 200 000 MSCs in co-suspension for 72 hours, the observed time-dependent increase in islet diameter due to MSC attachment was quantified using the Nikon Biostation software measuring tool. Data presented in Figure 4.6 shows a time-dependent increase in composite size between 0-36 hours, with composites reaching a maximal average percentage increase of 22 ± 3% after 36 hours of co-culture. Following this time point composite size remained relatively constant through to the end of the 72 hour co-culture period, suggesting the most active phase of composite formation occurred within the first 2 days of suspension co-culture.

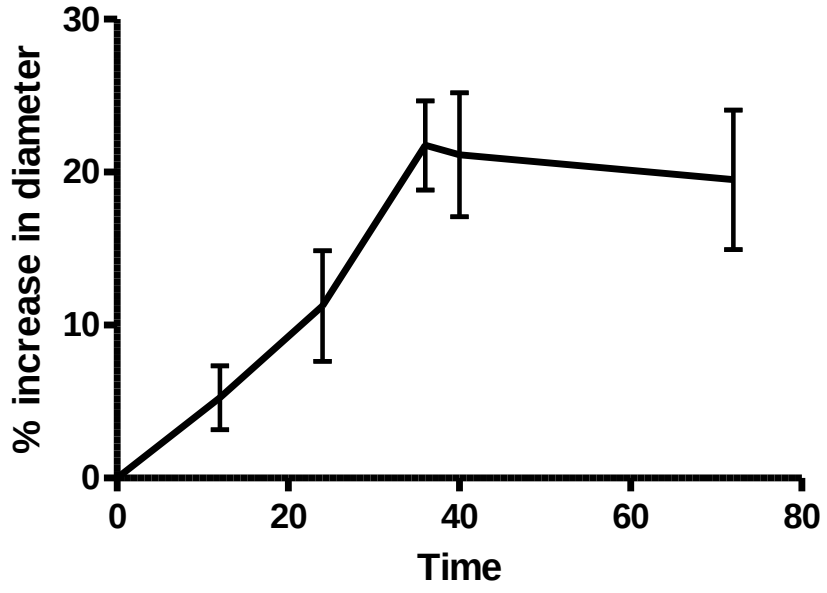

Figure 4.6: Average increase in islet-MSC composite size over a 72 hour co-suspension period. Using footage captured by the Biostation of 75 islets and 200 000 kMSCs in co-suspension (Figure 4.5 being a typical example of footage used) the change in islet diameter over the 72 hour period was tracked using the measuring tool on the Nikon Biostation. Average increase in diameter calculated as a percentage of basal diameter (i.e. diameter at 0 hours) is presented. A steady increase in islet diameter can be observed between 0-36 hours, followed by no further increase in size between 36-72 hours. Mean ± SEM are shown, n = 9 observations.

Having established a time period for the most active phase of composite formation, Biostation footage of 200 000 fluorescently labelled kMSCs and 75 islets in suspension co-culture was captured over 48 hours, in an attempt to visualise the location of MSCs in and around the composite structure. Images shown in Figure 4.7 indicated that MSCs migrated towards and adhered to the surface of the islets in a largely random manner within 2 hours and appeared to penetrate the islet structure by 8 hours.

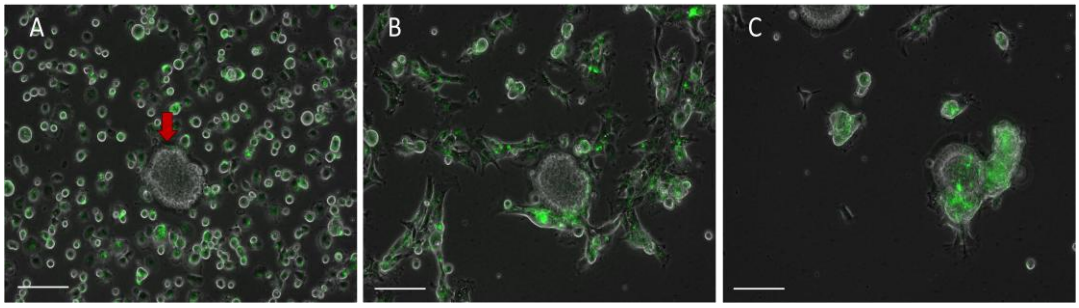

Figure 4.7: Coating of mouse islets with fluorescently-labelled MSCs (green) in suspension co-culture. 75 islets and 200 000 fluorescently labelled kMSCs were monitored over a 48 hour suspension co-culture period in order to visualise the migration of MSCs during the most active phase of composite formation. A single islet (red arrow) can be seen to be surrounded by MSCs (green) in single cell suspension at beginning of the co-culture period (A). MSCs appeared to adhere to the edge of islet, after 2 hours of culture (B). Following 8 hours of culture MSCs appeared to both penetrate the islet core and attach to islet perimeter (C) leading to the formation of MSC-islet composite structures. Scale bar 100µm. The full video clip of fluorescently labelled kMSCs and islets in 48 hour co-culture suspension can be viewed in the supplementary data on the CD attached to the back of this thesis.

4.3.2.2 *Effects of suspension co-culture on glucose-stimulated insulin secretion*

Following a 3 day suspension co-culture period, the insulin secretory function of both islet-kMSC composites and islet-adipMSC composites was assessed. The effects of both kMSCs and adipMSCs upon insulin secretion were variable. When kMSCs directly contacted islets in suspension culture, kMSCs had no effect upon insulin secretion at a basal glucose concentration, but significantly potentiated the insulin secretion of two independent islet populations at a stimulatory glucose concentration (Fig. 4.8A-B), between 2-3-fold over islets cultured alone. This result was not reproducible across all the islet populations tested (Fig. 4.8C), and not considered significant by repeated measure statistical analysis ($p>0.05$, $n=3$ repeats). AdipMSCs also had no effect upon the basal insulin secretion of islets, and variable effects at a hyperstimulatory glucose concentration. They significantly improved glucose-stimulated insulin secretion approximately 3-fold over control islets in one given population of islets (Fig. 4.9B), but had no effect upon the additional two islet populations tested (Fig. 4.9A&C).

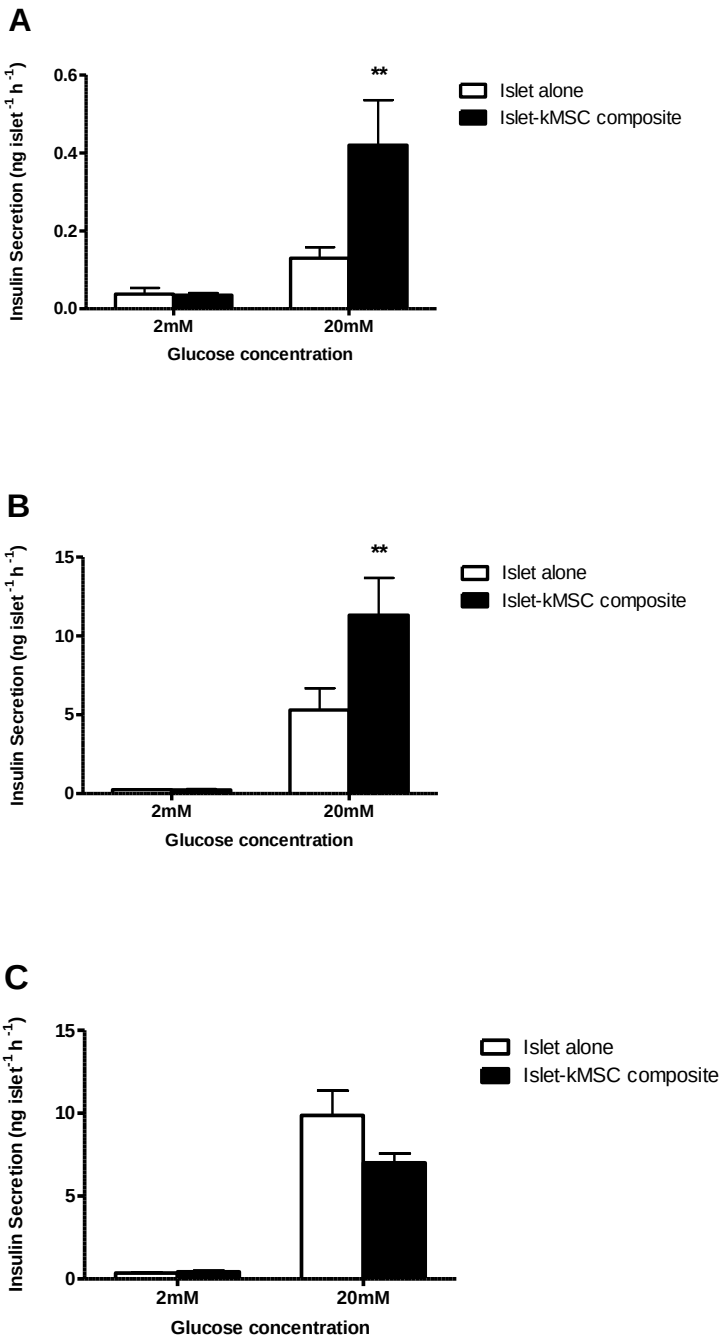

Figure 4.8: Glucose-stimulated insulin secretion *in vitro* of islets cultured alone vs. islet-kMSC composites cultured in suspension. In three independent experiments (A-C), insulin release at 2mM glucose (basal) and 20mM glucose (high), of groups of triplicate mouse islets previously cultured in suspension with kMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, **p<0.01 vs. islet alone at the same glucose concentration. When employing a direct contact suspension co-culture system, the effects of kMSCs upon islet insulin secretory function was variable. For 2 independent islet populations kMSCs potentiated insulin secretion at stimulatory glucose levels (A-B), but were not beneficial for the glucose-stimulated insulin secretion of a third batch of islets (C).

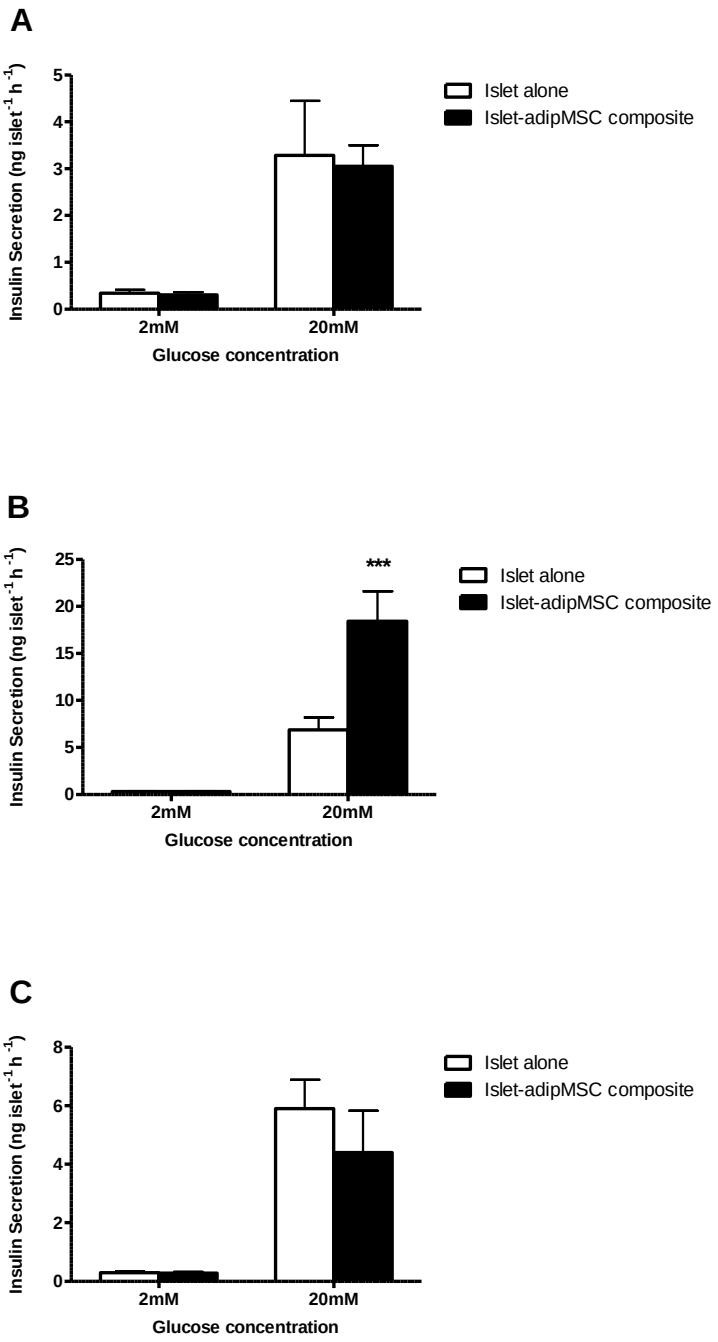

Figure 4.9: Glucose-stimulated insulin secretion *in vitro* of islets cultured alone vs. islet-adipMSC composites cultured in suspension. In three independent experiments (A-C), **i**nsulin release at 2mM glucose (basal) and 20mM glucose (high), of groups of triplicate mouse islets previously cultured in suspension with adipMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, ***p<0.001 vs. islet alone at the same glucose concentration. When employing a direct contact suspension co-culture system, the effects of adipMSCs upon islet insulin secretory function was variable. For a single population of islets adipMSCs potentiated insulin secretion at stimulatory glucose levels (B), but had no effect upon glucose-stimulated insulin secretion for two further batches (A&C).

4.3.2.3 *Effects of suspension co-culture on insulin content*

Utilising a direct contact suspension co-culture system, kMSCs did not affect islet insulin content levels following a 3 day culture period, as observed using two independent populations of islets (Fig. 4.10A-B). The same trend was observed across two islet populations when adipMSCs were employed in a suspension co-culture system (Fig. 4.11A-B).

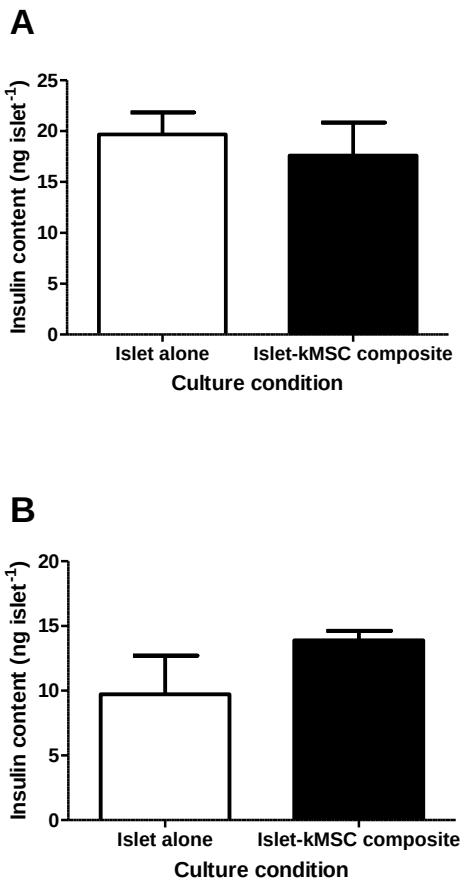

Figure 4.10: Insulin content *in vitro* of islets cultured alone vs. islet-kMSC composites cultured in suspension. In two independent experiments (A-B), insulin content of groups of 10 islets previously cultured in suspension with kMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=3 observations. No effects upon islet insulin content levels were observed when islets were cultured in suspension with kMSCs compared to islets cultured alone.

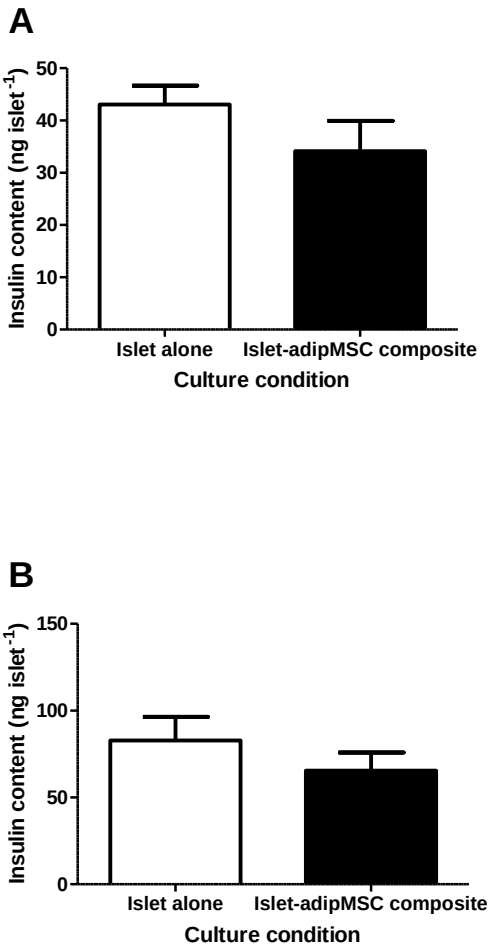

Figure 4.11: Insulin content *in vitro* of islets cultured alone vs. islet-adipMSC composites cultured in suspension. In two independent experiments (A-B), insulin content of groups of 10 of islets previously cultured in suspension with adipMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=3 observations. N.b. these experiments use islets from the same batches of isolated islets used in the insulin secretory functional experiments A-B of Fig. 4.9. No effects upon islet insulin content levels were observed when islets were cultured in suspension with adipMSCs compared to islets cultured alone.

4.4 Discussion

The beneficial effects of MSC co-transplantation with islets for the treatment of diabetic hyperglycaemia has widely been reported (Berman et al., 2010, Solari et al., 2009, Sordi et al., 2010, Figliuzzi et al., 2010, Ding et al., 2009, Ito et al., 2010, Rackham et al., 2011). Both the angiogenic and immunomodulatory capacity of the MSCs have frequently been described in the studies. In this study *in vitro* co-culture systems were used to address whether the MSCs, which have been identified as modulators of islet graft performance, had the capacity to directly enhance the insulin secretory function of islet β-cells. Initial co-culture studies confirmed that the insulin secretory function of islets cultured directly upon a monolayer of either kMSCs or adipMSCs was significantly enhanced compared to islets cultured alone. Within the direct contact monolayer configuration physical contact between MSCs and islets and MSC-derived trophic factors could potentially influence islet function, both of which have previously been found to support islets (Park et al., 2010, Park et al., 2009, Jung et al., 2011, Luo et al., 2007). Jung et al (2011) showed that rat islets cultured in contact with bone marrow-derived rat MSCs over a period of 4 weeks were able to maintain higher insulin secretion levels compared to islets cultured alone or indirectly with MSCs. Interestingly, the contact cultured islets also preserved their morphology and had the lowest frequency of fragmentation. Luo et al (2007), reported similar findings using human islets and bone marrow-derived MSCs, whereby contact cultured islets maintained their morphology and insulin secretion function in long term culture. This group also reported that control islets cultured alone had lost their configuration and attributed the significant loss of insulin secretion capacity from the 28 day-cultured islets to the leakage of dying β-cells and insulin from the fragmented structures. These reports differed from the present study in terms of cell source and culture period; nevertheless they suggest an important islet-protective effect of cell-cell contact between islets and MSCs.

There is also experimental evidence that MSC-derived soluble factors can exert beneficial effects on islets maintained *in vitro*. Park et al (2009; 2010) showed in

two independent studies that trophic derived molecules from MSCs enhanced the survival and function of isolated islets, finding in particular an up-regulation of the expression of anti-apoptotic molecules in the islets. These findings were made using indirect contact co-culture systems, to specifically assess the contribution of soluble bio-factors to improved islet function. In the direct contact co-culture setting MSC-secreted soluble factors will also be present, and may even be so at increased concentrations due to the localisation of the islets in relation to the MSCs.

The effects of kMSC and adipMSC monolayers upon islet insulin content were more ambiguous than those for insulin secretion. In two independent experiments for both MSC types, kMSCs appeared to elevate insulin content levels around two-fold over islets cultured alone, whereas no effect was observed when using adipose MSCs implying a possible tissue-dependent effect of the kMSCs. However when the actual insulin content levels of the islets used in the kMSC and adipMSC studies were taken into account, a more practical explanation may account for the observed differences. Figure 4.3 shows that the insulin content levels of the 3 day cultured control islets in the kMSC co-culture studies ranged between 10-20ng/islet, yet Figure 4.4 shows insulin content levels of approximately 40-70ng/islet for control islets used in the adipMSC co-culture studies. Within our research group it has been established that the insulin content levels of functionally viable cultured (up to 7 days) mouse islets are expected to range between 40-60ng/islet (Kerby et al., 2012, Vilches-Flores et al., 2013) and that the insulin content of isolated islets can decline with time in culture (Rackham et al., 2013). It may therefore be that the islets of the kMSC-based studies became compromised during isolation and/or culture and the MSCs had scope to act and reduce the loss of insulin content. On the other hand the islets used for the adipMSC-based studies didn't appear to be compromised during isolation/culture, so the MSCs acted only to maintain insulin content rather than improve it. This issue was further explored in studies using human islets (Chapter 6). Further experimental repeats beyond the two conducted for each MSC type in this study however, would be needed to confirm whether the

observed differences are related to kMSC tissue-dependent properties or the functionality of the islets.

A number of further observations can be made about the data presented. Firstly in the case of the kMSC-based experiments, even when islets may be functionally compromised MSCs were still able to reproducibly enhance their insulin secretory function. With regards to the adipMSC based studies, insulin secretory function was consistently and significantly enhanced across all experiments, where no effects upon insulin content levels were observed. This suggests that the improved insulin secretory function of the islets was not a direct consequence of elevated insulin content levels, and that MSCs may act through independent mechanisms to affect the processes. The biosynthesis and secretion of insulin are not obligatory coupled; studies have shown they can be dissociated under certain conditions, for example phosphodiesterase inhibitors such as isobutylmethylxanthine (IBMX) are able to potentiate GSIS by increasing islet cycle AMP levels, but do not significantly affect insulin biosynthesis (Ashcroft et al., 1978, Howell and Montague, 1973). Overall the findings of the direct contact monolayer co-culture studies may have important implications for the application of MSCs in the clinical setting of islet transplantation. Islets are metabolically active micro-organs which are susceptible to compromised function at any stage of the islet isolation/culture process. However co-culture with MSCs can prevent/reduce the gradual loss of insulin content and/or enhance the insulin secretory function of isolated islets, improving the quality of islets available for transplantation.

The second major objective of this chapter was to investigate direct contact interactions between islets and MSCs using suspension co-culture. Key motivation for using a suspension co-culture system was to assess the potential of MSCs to attach to the islets and form a composite unit to enable intervasculated implantation of islets and MSCs. MSCs, anchorage dependent cells by definition (Prockop, 1997), have an adhesive capacity so it was hypothesised that where adherence to the tissue culture plastic was not possible, adherence to neighbouring islets may take place instead. Composite

formation studies confirmed the ability of MSCs to not only attach to the islet surface (Figure 4.5) but also to appear to penetrate to the islet core (Figure 4.7), with maximal composite formation activity occurring between 0-36 hours (Figure 4.6). These findings are similar to those reported by other groups, who have previously used both MSCs and MSCs with endothelial cells to form islet-based composites (Duprez et al., 2011, Johansson et al., 2008). Duprez et al reported adherence of MSCs to islets within 1 hour of suspension co-culture, spreading of MSCs around and into the islets after 5 hours and no apparent improvement in the MSC coating after 24-48 hours. Interestingly an observation made both in the present study and by Duprez et al was the remainder of some MSCs in suspension which had not interacted with islets by the end of the co-culture period. This observation may be a key consideration when analysing the data presented on the functionality of the composites. The effects of the MSC incorporation upon islet insulin secretory function were variable when using either kMSCs or adipMSCs, whilst no improvements in islet insulin content were observed when either MSC type was used. Duprez et al reported no difference between the dynamic insulin secretion of MSC coated islets and control islets. Since not all MSCs appeared to contact or interact with islets during composite formation, the 'dose' of MSCs islets received was variable, which is likely to lead to inconsistencies in the level of MSC physical contact and MSC-derived trophic support received by the islets both within and between experiments.

A second fundamental factor which must be considered in the suspension co-culture system is the effects of being in suspension upon anchorage dependent MSCs. MSCs are conventionally cultured in a 2-dimensional (2D) monolayer (Meirelles and Nardi, 2003) where they actively secrete ECM molecules (Chen et al., 2007) essential for their survival, growth and division (Stupack et al., 2001). When cell-ECM interactions are disrupted apoptotic pathways are induced, a process termed anoikis (Howe et al., 1998). Anoikis of MSCs prevented from adhering *in vitro* has previously been reported (Feng et al., 2007), with significant increase in caspase 3 activity in suspended MSCs, a critical protease in the process of mammalian cell apoptosis, compared to MSCs cultured

conventionally. The secretory profile between 3 day-cultured MSCs maintained as monolayers or in suspension has also been shown to differ, with marked differences observed in the basal secretion of various growth factors and cytokines (Yeung et al., 2012). Therefore within the islet-MSC suspension co-culture system there may be major discrepancies in both the health and function of the MSCs as well as the level of islet-MSC interaction, contributing to inconsistent functionality of the composites. It should be noted that the composite formation studies presented herein were only preliminary investigations. Although deemed outside the scope of this thesis, future work could be done to optimise the formation and characterisation of composites *in vitro*, in order to improve the reproducibility of any functional benefits. This could include the use of continuous stirred tank reactors (CSTRs) for co-suspension, such as those commonly employed in microbial co-culture fermentations (Bader et al., 2010). Gentle agitation of islet-MSC co-suspensions would improve mixing rates in an effort to enhance MSC attachment to islets for potential reduction of non-adherence induced anoikis and more consistent MSC 'dosing'. Composites could then be characterised by the assessing degree of MSC integration. This could involve real-time PCR or flow cytometry cell sorting for the quantification of a marker found on MSCs and not islet cells following co-culture, to gain an idea of whether a certain threshold of MSC integration into the islet was required to achieve enhanced insulin secretory function.

In summary, the *in vitro* analysis presented demonstrates that MSCs have the capacity to directly enhance islet function. When maximal physical contact was allowed between MSCs and islets in a static 2D co-culture system i.e. islet co-culture with an MSC monolayer, the insulin secretory function of islets was consistently and significantly enhanced at stimulatory glucose levels only. Testing the functional response of islets co-cultured with MSC monolayers over a range of glucose concentrations would be an interesting future avenue to explore. It would allow assessment of whether the beneficial effects of MSCs were restricted to stimulatory glucose concentrations only, similar to those

effects exerted by gut derived incretins, in order to help decipher potential mechanisms through which MSCs may be acting.

The use of both kMSC and adipMSC monolayers for co-culture resulted in improved islet secretory function *in vitro*, suggesting that the stromal cells from both tissue sources have similar functional phenotypes. Taking into account this functional validation of mouse adipMSCs *in vitro*, all future studies using mouse islets for co-culture, utilised these clinically relevant MSCs only. The co-culture suspension studies presented in this study suggested there may be some potential in generating composite islet-MSC structures for the purpose of co-localised MSC and islet delivery in co-transplantations. However significant optimisation studies are required for the progression of the composite studies from this preliminary stage, which are outside the remit of this thesis. Future studies beyond this chapter therefore pursued the more reliable MSC monolayer co-culture system, and investigated potential broad mechanisms through which the MSCs may be acting (Chapter 5) and the reproducibility of the monolayer co-culture effect when using human MSCs and islets (Chapter 6).

Chapter 5 - The effects of indirect contact and direct contact MSC co-culture mechanisms on mouse islet function *in vitro*

5.1 Introduction

Direct contact co-culture studies using mouse islets and MSCs revealed the significant beneficial effects exerted by MSCs upon islet function *in vitro*. With this knowledge, the next step was to begin investigations into some of the potential mechanisms through which the MSCs were influencing islet function. MSCs are frequently reported to be valuable trophic mediators in tissue repair and regeneration (Caplan, 2007). A possible mechanism by which co-culturing islets with MSCs may improve islet function could be via the large spectrum of bioactive molecules they are known to secrete. *In vivo* co-transplantation studies conducted within the Diabetes Research Group showed the beneficial impacts of MSC co-transplantation in both syngeneic (Kerby et al., 2013) and microencapsulated, non-vascularised graft models of islet transplantation (Rackham et al., 2011). This suggests that trophic factors with the ability to influence islet function independently of effects associated with immunoregulation and angiogensis could have a role to play. The first aim of the studies described in this chapter was to utilise an indirect contact co-culture configuration in order to make an initial assessment of the role of trophic factors in MSC co-culture. Using the same co-culture variables, in terms of time course and cell density, as the direct contact co-culture studies of Chapter 4, a transwell co-culture system was developed to assess whether the broad spectrum of soluble factors secreted by MSCs alone could enhance islet function in the absence of islet-MSC physical contact *in vitro*.

The physical support provided by MSCs to isolated islets during direct contact co-culture *in vitro* may also be an important contributor to enhanced islet function. Physiologically, islets exist in a complex 3D microenvironment signalling with extracellular matrix components and communicating with neighbouring cells to promote β-cell differentiation and function. Adult islets are usually surrounded by an incomplete capsule consisting of a single layer of fibroblasts and the collagen

fibres they secrete (Stendahl et al., 2009). This capsule is closely associated with additional matrix proteins, such as laminins, known as the peri-insular basement membrane, which is almost completely lost to the mechanical and enzymatic stresses of islet isolation (Wang et al., 1999). In the interior of islets there is also a substantial amount of basement membrane associated with the pervading microvasculature, known as the perivascular basement membrane. Nikolova et al (2006) made the discovery that a subset of β-cells in the mouse pancreatic islet lack their own basement membrane and interact instead with matrix proteins deposited for them by local capillary endothelial cells (Nikolova et al., 2006).

Islet isolation and culture is known to result in a rapid and significant loss of intra-islet endothelial cells, which is not prevented by MSC contact co-culture (Rackham et al., 2013, Olsson et al., 2006, Parr et al., 1980). This also results in the loss of the associated peri-vascular basement membrane (Rosenberg et al., 1999, Wang and Rosenberg, 1999). Destruction of the ECM-based microenvironment and mechanical damage caused by the isolation procedure leaves the delicate micro-organs susceptible to devascularisation, apoptosis and hypoxia (Wang and Rosenberg, 1999, Rosenberg et al., 1999, Paraskevas et al., 2000). Numerous reports have highlighted the beneficial effects that extracellular matrix laid down *in vitro* by adherent cells such as fibroblasts (Jalili et al., 2011) or the rat carcinoma cell line 804G (Bosco et al., 2000, Hammar et al., 2004, Parnaud et al., 2009) have upon β-cell function in cultured islets, which may be a result of the reestablishment of appropriate cell-matrix interactions. Since MSCs are known to actively produce their own ECM in culture, constituted of collagens, fibronectin and laminins (Chen et al., 2007), it was hypothesised that MSC-derived ECM interactions with islet β-cells may contribute to the improved islet function observed during direct contact co-culture studies *in vitro*. Therefore the second aim of this chapter was to isolate MSC-deposited ECM from cultured MSCs and directly culture isolated mouse islets upon the matrix to assess whether MSC-derived ECM alone had the capacity to modulate islet function *in vitro*.

5.2 Methods

5.2.1 Indirect contact islet-MSC co-culture

5.2.1.1 *Transwell (TW) co-culture system*

Two hundred thousand MSCs at passage 9 were seeded into wells of a 6 well Nunclon plate in MSC medium and incubated overnight to adhere to culture plastic. Twenty four hours post cell seeding, medium was replaced with 3ml islet medium and a cell culture insert (1.0 µm pore size, PET membrane, Falcon, BD) placed into each well. Islets were placed into the insert in the upper compartment of each well (75 islets/insert) and cultures were left to co-incubate for 3 days. A schematic of the transwell design is shown in Figure 5.1. Control groups were set up where islets were placed into the inserts, but no MSCs were pre-seeded into the 6 well plates. A full medium replacement was performed for all control and co-culture groups on day 2.

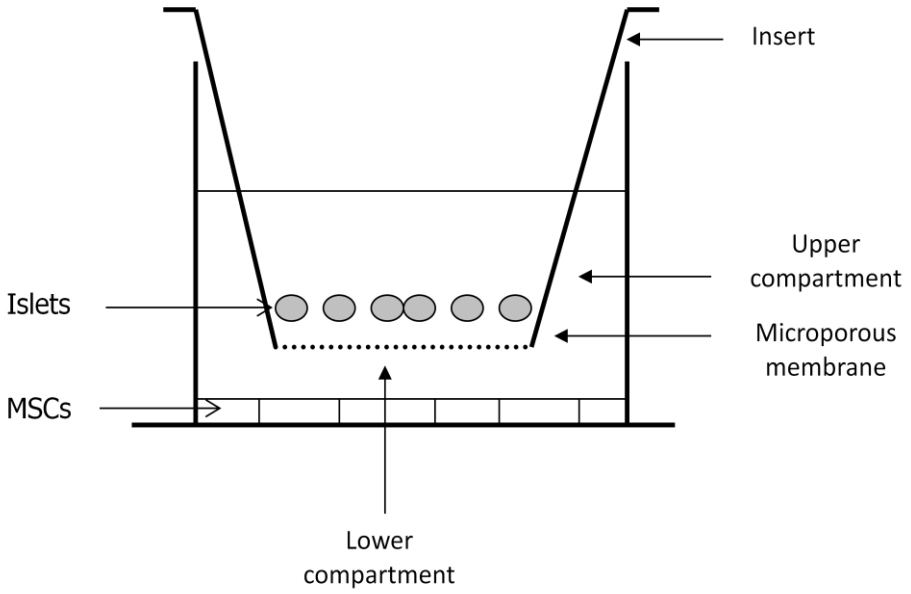

Figure 5.1: Schematic of MSC-islet transwell co-culture design. MSCs were seeded into the lower compartment of the well. Islets sit in the upper compartment of the well; direct cell-cell contact between the MSCs and islets is prevented by a PET microporous membrane, which stops the diffusion of soluble factors between the two compartments.

5.2.2 Islet-ECM co-culture

5.2.2.1 *Optimisation of MSC-derived ECM derivation*

MSC-derived ECM was prepared based on the optimisation of an existing published protocol (Chen et al., 2007). The protocol and others based on it since (Lin et al., 2012, Kim and Ma, 2012, Pei et al., 2011) included the following key steps for cultured MSC-derived ECM generation:

1. Seed 100 000 MSCs per well of a 6-well plate (6-wp).
2. Culture MSCs for **15 days**, supplementing the culture medium with 50µM ascorbic acid (a stimulant for collagen deposition) for the final 8 days.
3. After washing the MSC monolayer with PBS, decellularise the ECM with 0.5% Triton X-100 containing 20 mM NH4OH in PBS for **5 min** at 37°C.
4. Treat the freshly exposed ECM with DNase (100units/ml) for 1 hour at 37^{o}C.
5. Wash the ECM with PBS three times and store in PBS at 4^{o}C for up to 4 months.

Initial attempts of the protocol were trialled using mouse-derived adipMSCs. However problems were encountered with the variables highlighted in bold in steps 2 and 3.

Firstly, with regards to step 2, when MSCs were cultured for 15 days the monolayer became very dense and overgrown (Fig. 5.2A). Consequently when attempts were made to decellularise the monolayer using Triton-X-100 detergent to disrupt cell membranes, as per step 3, the dense monolayer appeared to 'pull up' any ECM it had deposited with it, clearing the well entirely of its contents. The 5 minute Triton X-100 incubation time suggested in step 3 was shortened to 2 min, 60 seconds or 30 s to try and prevent loss of ECM, however this resulted in either very uneven decellularisation throughout the well (following 30 second treatment) or the persistent clearing of all cellular and ECM material from the well (following 60 second or 2 min treatments). Since the overgrown nature of the MSC monolayer was the main cause of the problems experienced, two

different measures were attempted to combat this, i) mitomycin C (MMC) inactivation of the MSCs to prevent overgrowth over the 15 day culture period or ii) shortening of 15 day MSC culture period to length of time taken for MSCs to reach approximately 90% confluency, beyond which they would be deemed overgrown.

Mitomycin C, a DNA inter-strand cross-linking agent, was trialled for the mitotic inactivation of the actively dividing MSCs to prevent their overgrowth. MSCs (seeded at a density of 100 000 cells/well of 6-wp) were grown to 90% confluency over a 3-4 day period. Mitomycin C was then used at a final concentration of 10µg/ml over a 2 hour incubation period, as per protocol for the routine inactivation of mouse embryonic fibroblast (MEF) feeder layers for embryonic stem cell culture (Conner, 2001), to inactivate the MSCs for the remainder of the 15 day culture period. However, the MMC seemed to be toxic towards the cells; two days after MMC treatment MSC monolayers were left sparse, with numerous cells floating in suspension. Reducing the final concentration of MMC to 5µg/ml was also ineffective, because although toxicity towards the MSCs seemed to be reduced cell division wasn't fully inhibited and the monolayers once again became overgrown, as observed 4 days after MMC treatment (Fig. 5.2B).

Having limited success with mitotic inactivation, the second approach of cutting short the 15 day MSC culture period was tested. MSCs, seeded at a density of 100 000 cells/well of a 6-wp, were left in culture and their confluency monitored daily. After 3-4 days in culture the monolayers approached 90% confluency (Fig. 5.2C). These monolayers were then treated with Triton X-100 for decellularisation. The process of decellularisation was monitored closely microscopically, and the majority of cells appeared to lyse within 10 seconds of the detergents addition, negating the 5 minute incubation time originally suggested in step 3.

Based upon the optimisation steps trialled the previously published protocol required a two-fold modification. Both the MSC culture period and Triton X-100 decellularisation period required a significant reduction in duration. The fragile nature of the freshly exposed ECM was also observed during optimisation steps;

therefore a 24 hour 'settling' period where ECM was stored at 4^{o}C in PBS post decellularisation was introduced to reduce the possibility of ECM detachment during subsequent manipulation steps.

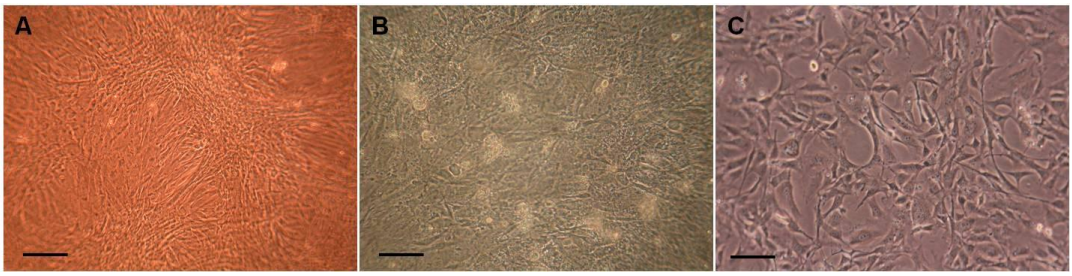

Figure 5.2: Optimisation of MSC-derived ECM generation. MSC monolayer culture prior to decellularisation for ECM exposure required optimisation. MSCs cultured for over a 15 day period (A) became dense and overgrown. MSC monolayers mitotically inactivated with MMC either experienced toxicity (final MMC concentration 10µg/ml; image not shown) or continued to over grow (final MMC concentration 5µg/ml; B). Reducing the MSC culture period to 3-4 days yielded healthy, approximately 90% confluent monolayers, and were most suited to the subsequent ECM manipulation steps. Scale bar A-C; 100µm.

5.2.2.2 *Optimised MSC-derived ECM derivation*

One hundred thousand adipMSCs at passage 9 were seeded into wells of a 6-well Nunclon plate and left to adhere overnight. Cells were cultured in MSC medium supplemented with 50µM ascorbic acid (Sigma) until they reached approximately 90% confluence (which usually took around 3-4 days); medium was completely replaced every 48 hours. The cell monolayer was decellularised to expose the MSC-deposited ECM by incubation with pre-warmed (37^{0}C) 0.5% (vol./vol.) Triton X-100 (Sigma) containing 20 mM NH_4OH (Sigma) in PBS for 10 seconds. The Triton X-100 solution was carefully diluted out with 3x PBS washes, and the ECM left to settle for 24 hr at 5^{0}C under PBS containing 1% (vol./vol.) pen-strep. The ECM was then treated with DNase (100 U/ml; Thermo Scientific, MA, USA.) for 1 hr at 37°C. The ECM was washed three times with PBS and either stored in 2ml of PBS containing 1% (vol./vol.) pen-strep, at 4°C until use in co-culture experiments up to 48 hours later, or stained with van Gieson dye to confirm the presence of ECM.

ECM deposits stained with van Gieson dye were washed with PBS and incubated with van Gieson (0.05% acid fuchsin (v./v.) in saturated picric acid (Sigma)) for 5 min for the detection of collagen deposits.

5.2.2.3 *Islet-ECM co-culture*

Approximately 75 fresh islets were added directly to wells of 6-well plates containing MSC-derived ECM in 3ml islet medium. The islet-ECM co-culture was incubated for 3 days. Islets cultured alone for 3 days in islet medium served as a control group. A full medium replacement was performed for all control and co-culture groups on day 2.

5.2.3 Measurement of islet function in vitro

Assessment of islet secretory function and insulin content after all co-cultures was performed as described in Chapter 2, section 2.3 and quantified by radioimmunoassay as per Chapter 2, section 2.3.3.

5.3 Results

5.3.1 Indirect contact islet-MSC co-culture

5.3.1.1 *Effects of transwell co-culture on glucose-stimulated insulin secretion*

Using the same key variables as the direct contact monolayer co-culture system, discussed in Chapter 4, section 4.2.1.1, an indirect contact transwell co-culture system was set up whereby cell-cell contact between islets and adipMSCs was prevented by a porous membrane that otherwise allowed transmission of any soluble factors present in the medium. When using an indirect contact co-culture system adipMSCs did not potentiate glucose-stimulated insulin secretion for any of the islet populations tested (Fig. 5.3A-C). They had a significantly detrimental impact upon the insulin secretory function of one islet population tested compared to islets cultured alone (Fig. 5.3A). The reproducibility of the non-beneficial effects of transwell co-culture were considered significant across the

three independent islet populations as tested by repeated measures statistical analysis ($p<0.05$, n=3 repeats).

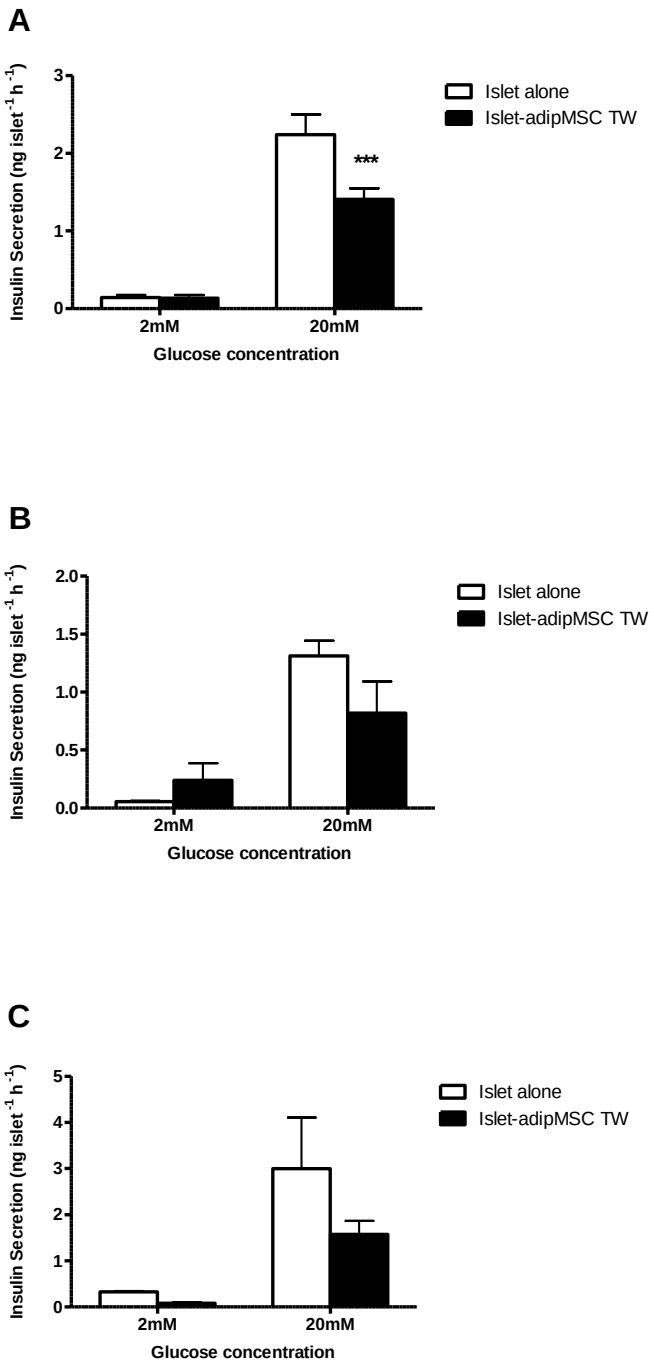

Figure 5.3: Glucose-stimulated insulin secretion *in vitro* of islets cultured alone vs. islets cultured indirectly adipMSCs in a transwell (TW) format. In three independent experiments (A-C), insulin release at 2mM glucose (basal) and 20mM glucose (high), of groups of triplicate mouse islets previously cultured indirectly with adipMSCs (black bars) or without MSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, ***p<0.001 vs. islet alone at the same glucose concentration. Using an indirect contact transwell co-culture system adipMSCs did not potentiate insulin secretion at stimulatory glucose levels, and in one population of islets caused a detrimental effect upon islet function (A).

5.3.1.2 *Effects of transwell co-culture on insulin content*

Co-culturing islets with adipMSCs via indirect contact had little effect upon the insulin content of islets, compared to islets cultured alone (Fig. 5.4A-C). Of the three islet populations analysed, one population of islets (Fig. 5.4A) treated with adipMSCs in a transwell format had a significantly lower level of insulin compared to control islets. However, this finding was not reproducible between the three islet populations tested and no significance was detected using paired statistical analysis ($p>0.05$, $n=3$ repeats). These three independent islet populations correspond to the same three islet populations used in the transwell co-culture insulin secretory analysis presented in Figure 5.3A-C.

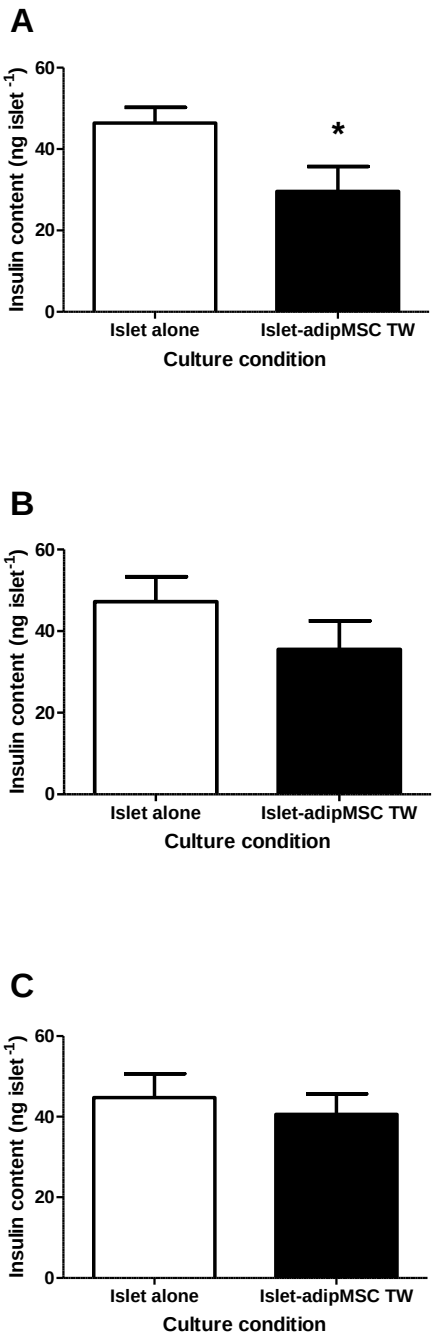

Figure 5.4: Insulin content *in vitro* of islets cultured alone vs. islets cultured indirectly with adipMSCs in a transwell format. In three independent experiments (A-C), insulin content of groups of 10 islets previously cultured indirectly with adipMSCs (black bars) or without adipMSCs (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=6 observations, *p<0.05 vs. islet alone at the same glucose concentration. N.b. these experiments use islets from the same batches of isolated islets used in the insulin secretory functional experiments A-C of Fig. 5.3. In general no effect upon insulin content was observed when islets were cultured indirectly with adipMSCs compared to islets cultured alone for the islet populations tested. For one population of islets the insulin content of adipMSC treated islets was considered significantly lower than control islets (A).

5.3.2 Islet-ECM direct contact co-culture

5.3.2.1 *MSC-derived ECM generation*

Extracellular matrix was generated from cultured adipMSCs *in vitro*. Once confluent monolayers (approximately 90%) of adipMSCs covered the growth surface of wells in a 6-well plate (Fig. 5.5A), Triton X-100 detergent was used to lyse cell membranes for decellularisation purposes. This exposed a 'cobblestone-like' monolayer residing beneath the original cell monolayer (Fig. 5.5B). Treatment of this cobblestone-like monolayer with DNase cleared any fragmented nuclear DNA remaining post decellularisation, to reveal a more web-like matrix attached to the tissue culture plastic (Fig. 5.5C). The exposed matrix was stained with van Gieson dye to confirm for the presence of collagens, major constituents of extracellular matrix. Positively stained collagen deposits could be seen throughout the web-like matrix (Fig. 5.5D), and the web-like matrix was detected throughout each well (Fig. 5.5E).

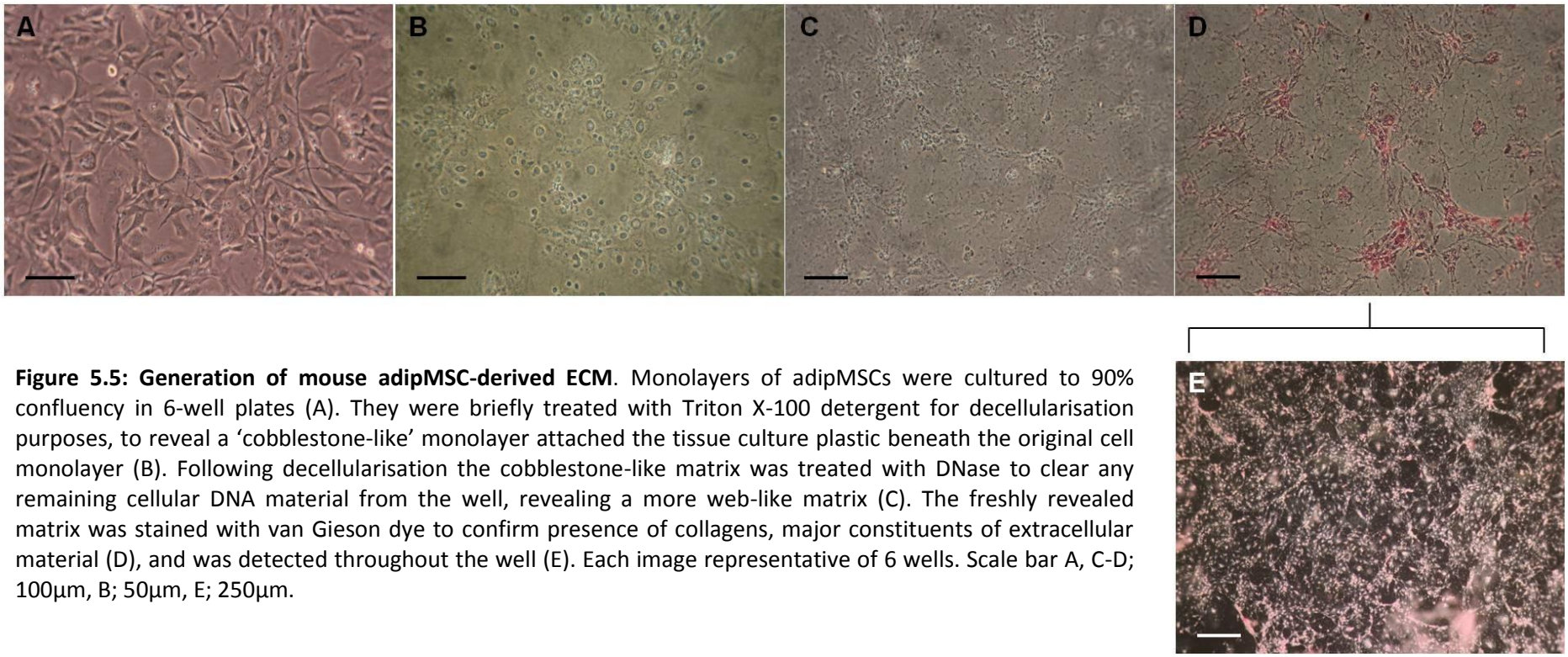

Figure 5.5: Generation of mouse adipMSC-derived ECM. Monolayers of adipMSCs were cultured to 90% confluency in 6-well plates (A). They were briefly treated with Triton X-100 detergent for decellularisation purposes, to reveal a 'cobblestone-like' monolayer attached the tissue culture plastic beneath the original cell monolayer (B). Following decellularisation the cobblestone-like matrix was treated with DNase to clear any remaining cellular DNA material from the well, revealing a more web-like matrix (C). The freshly revealed matrix was stained with van Gieson dye to confirm presence of collagens, major constituents of extracellular material (D), and was detected throughout the well (E). Each image representative of 6 wells. Scale bar A, C-D; 100µm, B; 50µm, E; 250µm.

5.3.2.2 *Effects of MSC-derived ECM co-culture on glucose-stimulated insulin secretion*

Freshly isolated islets were co-cultured with adipMSC-derived ECM to investigate the effects of ECM upon islet function (Fig. 5.6). ECM significantly enhanced glucose-stimulated insulin secretion in three of the four independent islet populations tested (Fig. 5.6A-B&D), approximately 1.5-2-fold over islets cultured alone. However in a fourth independent population of islets no effect of ECM was observed upon glucose-stimulated insulin secretion (Fig. 5.6C). The reproducibility of the beneficial effects of ECM across islet populations was considered statistically significant by repeated measures analysis ($p<0.05$; n=4 repeats).

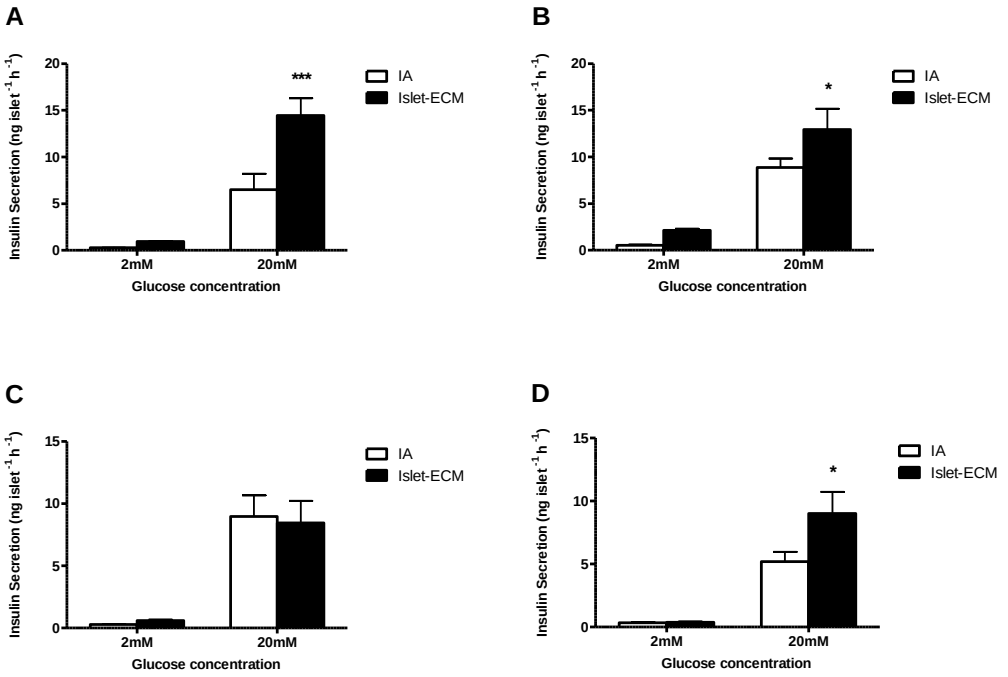

Figure 5.6: Glucose-stimulated insulin secretion *in vitro* of islets cultured alone vs. Islets cultured directly upon adipMSC-derived ECM. In four independent experiments (A-D), insulin release at 2mM glucose (basal) and 20mM glucose (high), of groups of triplicate mouse islets previously cultured in directly upon adipMSC-derived ECM (black bars) or alone (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, *p<0.05, ***p<0.001 vs. islets alone at the same glucose concentration. When islets directly contacted adipMSC-derived ECM, the effects of the ECM upon islet insulin secretory function was variable. For three of the four independent islet populations adipMSCs potentiated insulin secretion at stimulatory glucose levels (A, B, D), but had no effect on glucose-stimulated insulin secretion for one of the islet populations tested (C).

5.3.2.3 *Effects of MSC-derived ECM co-culture on insulin content*

Co-culture of freshly isolated islets with adipMSC-derived ECM did not affect the insulin content of the islets compared to islets cultured alone (Fig. 5.7). This finding was observed across 3 independent islet populations (Fig. 5.7A-C). These three independent islet populations correspond to the same three islet populations used in the ECM-islet co-culture insulin secretory analysis presented in Figure 5.6A-C.

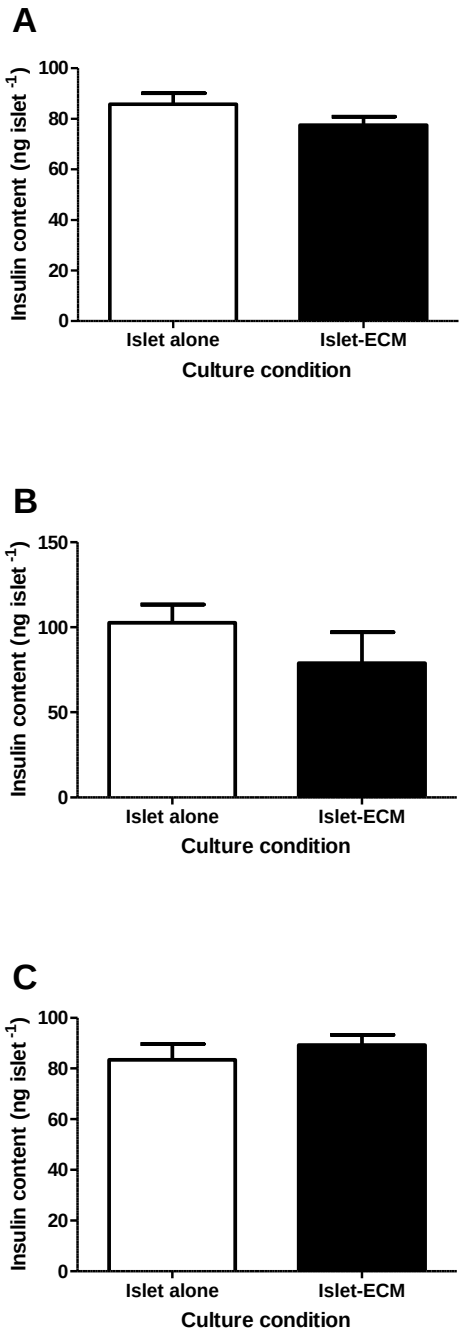

Figure 5.7: Insulin content *in vitro* of islets cultured alone vs. islets cultured directly upon adipMSC-derived ECM. In three independent experiments (A-C), insulin content of groups of 10 islets previously cultured directly upon adipMSCs-derived ECM (black bars) or alone (white bars) for 3 days was measured. For each experiment data are presented as mean ± SEM, n=6 observations. N.b. these experiments use islets from the same batches of isolated islets used in the insulin secretory functional experiments A-C of Fig. 5.6. No effect upon islet insulin content levels was observed when islets were cultured directly with adipMSC-derived ECM compared to islets cultured alone.

5.4 Discussion

The *in vivo* identity of MSCs has been correlated with pericytes (Meirelles et al., 2008), cells which are able to home to sites of injury and secrete a multitude of bioactive factors. The intrinsic trophic activity of MSCs has previously been reported to exert beneficial effects upon isolated islet function and survival during culture and after transplantation (Park et al., 2009, Park et al., 2010). However the *in vitro* analysis of the influence of adipMSC-derived soluble factors on islet function presented within this chapter were not able to confirm these findings. Using the same co-culture conditions as per Chapter 4 for the direct contact co-culture of islets and adipMSCs, when contact between the adipMSCs and islets was prevented by a porous membrane, soluble factors alone were not able to positively affect islet function in the same way that was observed by direct contact co-culture. This finding has also previously been reported by the group using mouse kMSCs (Rackham et al., 2013). It is difficult to comment on the discrepancies between the published findings and the results presented in this study since the source of MSCs and experimental protocols differ. MSCs derived from different tissues have been shown to have different secretory profiles (Yeung et al., 2012). The experimental design is also an important consideration since it would be useful to compare co-culture variables such as MSC density and islet:MSC ratios between studies when analysing the effects of soluble factors secreted into the culture medium. However although the published studies report the MSC numbers used in their studies, the number of islets and volume of medium used during co-culture were not provided. In the current study we maintained islet/MSC density and numbers between direct and indirect contact co-culture studies to avoid the confounding effects of changes in these variables between our studies.

Interestingly, for one population of islets used in the functional analysis studies, indirect contact co-culture resulted in a detrimental effect upon both the secretory function and insulin content levels of the isolated islets (Fig. 5.3A & Fig. 5.4A). It is unknown as to why this detrimental effect occurred, but this particular batch of islets may have deteriorated more in culture than other batches due to

exhaustion of the culture medium, which was commonly observed during both direct and indirect contact co-culture studies utilising actively proliferating MSCs.

Nevertheless, the cumulative findings of the direct contact and indirect contact co-culture studies of this thesis are in accordance with Jung et al (2011), who showed that contact co-culture with MSCs provided a microenvironment more supportive of islet function than the provision of MSC-derived soluble factors alone (Jung et al., 2011). However, the inability of MSC-derived trophic factors to support islet function in an indirect co-culture configuration does not rule out the importance of bioactive factors in co-culture. Direct islet-MSC contact co-culture may increase the concentration of MSC-secreted soluble factors available to the islet compared with using a transwell co-culture system. In the transwell configuration islets sit millimetres above an MSC monolayer in cell culture inserts. This may lead to the dilution below effective concentrations of soluble factors which may reach high local concentrations in direct contact co-culture systems. MSCs are also known to be dynamic secretors of bioactive factors, which respond to their local environment and activity status (Caplan, 2009). Soluble factors have been found to be differentially secreted by MSCs under direct contact and indirect contact co-culture conditions, as assessed by Jung et al (2011). Using cytokine array analysis they detected a 2-fold increase in monocyte chemotactic protein-1 (MCP-1) levels in transwell co-culture supernatants compared to contact co-culture supernatants, whereas vascular endothelial growth factor (VEGF) levels were 1.5-fold higher in the latter. Such factors may have contributed to the superior function of islets cultured directly on an MSC monolayer compared to islets cultured indirectly with MSCs using a transwell system, but it is clear that further studies are needed to elucidate specific soluble factor signalling mechanisms and their role in improving islet secretory function.

The failure to reproduce the significant benefits of MSC direct contact co-culture upon islet function via soluble factors alone suggested that alternative support mechanisms may be playing a role in the physical contact co-culture microenvironment. *In vitro* analysis confirmed that MSC-derived ECM had the capacity to enhance the insulin secretory function of isolated islets in culture

compared to islets cultured alone. ECM is an important component of the islet microenvironment. It is a dynamic complex of different proteins that serves as a cellular scaffold and plays significant roles in the regulation of cell behaviour and the maintenance of islet integrity (Lucasclerc et al., 1993). Destruction of the islet microenvironment occurring during islet isolation and the pre-transplant islet culture period, including the loss of peri-insular and peri-vascular basement membrane matrix proteins (Wang et al., 1999), subjects the islets to cellular stresses, which impairs β-cell function and survival (Rosenberg et al., 1999, Wang and Rosenberg, 1999). Studies using ECM derived from adherent cells such as fibroblasts and the 804G rat carcinoma cell line, to model the ECM environment, have shown that both laminin-5-rich 804G-derived ECM and fibronectin-rich fibroblast-derived ECM were able to promote the viability and glucose-stimulated insulin secretion of isolated islets (Bosco et al., 2000, Hammar et al., 2004, Parnaud et al., 2009, Jalili et al., 2011). These β-cell-matrix interactions have been associated with integrins (αβ heterodimeric integral membrane glycoproteins) expressed on the endocrine cells, which bind to extracellular matrix components such as collagens, fibronectin, and laminins (Bosco et al., 2000), providing a physical basis for cell adhesion and a platform for the transduction of biochemical signals both into and out of cells (Schwartz et al., 1995). Adult mouse islets express α3, β1 and β4 integrin subunits (Jiang et al., 2002), whilst it has been shown that mature human islets express α3, α5, αv, β1 and β5 integrin components (Wang et al., 1999, Virtanen et al., 2008). All αv integrins are believed to recognise the ECM component fibronection, as well as α5β1 and α3β1, whilst αvβ1 is known to bind collagen-IV, and integrins containing α3, β1 and β4 subunits adhere to laminin proteins (Kramer, 1994, Belkin and Stepp, 2000, Kaido et al., 2004, Ruoslahti, 1996). The molecular basis for the improved function/pro-survival signalling that emanates from ECM-integrin binding has been linked to several signal transduction components activated by integrins, including focal adhesion kinase (FAK) (Cai et al., 2012) Akt/protein kinase B (PKB), extracellular signal-regulated kinases (ERK) (Parnaud et al., 2009) and nuclear-factor kappa beta (NF-κB) signalling (Hammar et al., 2005).

The studies presented in this chapter only used a crude means of ECM characterisation, staining broadly for the presence of collagens. Further investigation is required into the detailed characterisation of MSC-derived ECM components and into the integrins/receptors expressed by islet cells through which they may interact to identify mechanisms through which MSC-derived ECM promotes insulin secretory function *in vitro*. At present the mechanisms of action remain unclear. A large portion of β-cells are on the interior of islets and will not have experienced direct contact with the peripheral ECM in this 2D experimental design. Therefore any benefits that may have originated from matrix restoration must have been transmitted indirectly, e.g. via intracellular gap junctions or other cell-cell mechanisms. Attachment of peripheral islet cells to ECM may also have benefited β-cells through the maintenance of islet architecture and preservation of intracellular relationships.

Interestingly, there is some evidence that MSC-derived ECM may also play an important role in the maintenance of islet grafts *in vivo*. Unpublished data from co-transplantation studies conducted within the group (Rackham et al) revealed that collagen fibres, staining positive for van Gieson, surrounded and infiltrated islets grafts co-transplanted with kMSCs under the kidney capsule of diabetic mice one month following transplantation (Fig.5.8C), which were absent in control islet alone grafts (Fig. 5.8B). One month following transplantation the presence of kMSCs in the MSC-bearing islet grafts was also very limited, as detected by alpha-smooth muscle actin (α-SMA) staining (Fig 5.8A). In the published report of this study the superior function of the islet grafts co-transplanted with kMSCs was attributed to the maintenance of islet morphology and improved graft revascularisation (Rackham et al., 2011). However, in light of the findings presented in this chapter it seems likely that MSC-deposited ECM, which remained in the absence of MSCs one month after transplantation, may have played a very important role in improving islet function.

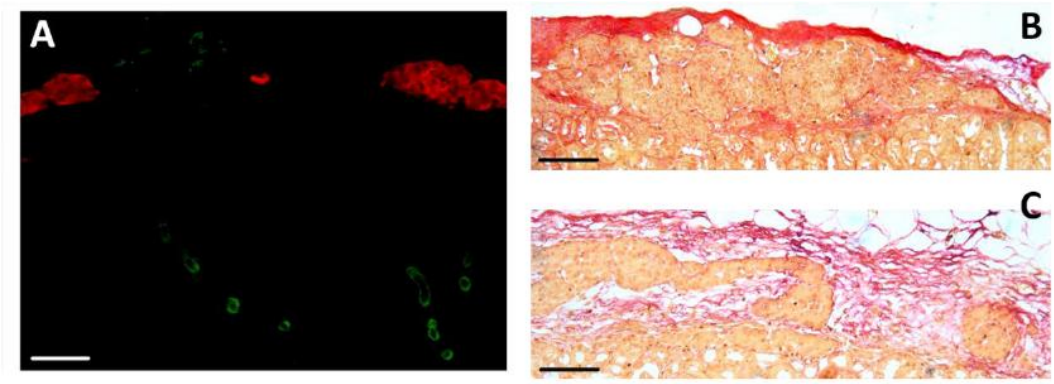

Figure 5.8: *In vivo* α-SMA and collagen staining detected in islet alone grafts vs. islets grafted with MSCs (image courtesy of Rackham et al). Representative sections of islet + MSC grafts one month after transplantation (A), showing insulin-positive (red) endocrine aggregates, with very few α-SMA-positive (green) cells present in the non-endocrine component of the graft. Islet alone grafts at one month stained with van Gieson (B) only contained a small number of collagen fibres at the graft periphery. However, an abundance of collagen fibres were detected in and around islet grafts co-transplanted with kMSCs (C). Scale bar 100µm.

In summary the findings of the present chapter, when considered alongside the results of Chapter 4, demonstrated that direct contact co-culture with MSCs has significant potential for supporting the function of isolated islets during culture, and was a more effective strategy than co-culturing islets with access only to MSC-derived soluble factors *in vitro*. When considering the action of MSCs and islets together *in vivo*, there is a prospective role for both MSC-secreted trophic factors and physical support. MSCs are known to be activated by cross-talk with their microenvironment (Prockop, 2009), and since the *in vivo* microenvironment is much more complex than the *in vitro* culture dish, the dynamic nature of MSCs is more likely to take effect in the co-transplantation setting. Evidence has also been presented in this study endorsing the role of MSC-derived ECM in the physical support network provided by MSCs, which islets may be interacting with both *in vitro* and *in vivo*. Interestingly *in vitro* analysis of the effects of adipMSC-derived ECM on the function of islets revealed a 1.5-2-fold increase in the glucose-stimulated insulin secretory function of ECM treated islets compared to islets cultured alone (see section 5.3.2.2). When this is compared to findings

presented in Chapter 4, which showed that direct contact monolayer culture with adipMSCs resulted in a 3-4-fold increase in the insulin secretory function of adipMSC treated islets compared to islets cultured alone (see section 4.3.1.1, Chapter 4), the intermediate effects of ECM alone are highlighted. This supports the notion that MSCs act as a dynamic support network for islets, and work via a broad spectrum of factors to exert their beneficial effects.

The overarching aim of these studies is to generate information to improve the outcomes of human islet transplantation. The studies using mouse islets and MSCs have demonstrated that the direct contact monolayer co-culture system was the most effective co-culture strategy and was assessed for its capacity to support the function of isolated human islets during the pre-transplant culture period (Chapter 6). Additional investigations were also made into the potential of using human MSC-derived ECM alone as an alternative cell-free support system (Chapter 6).

Chapter 6 - Direct contact co-culture of human islets with MSCs improves islet function *in vitro*

6.1 Introduction

The procurement of human islets pre-transplantation requires islet isolation from the native pancreas and often their subsequent maintenance in tissue culture *in vitro*. During the multi-hour isolation process, islets are exposed to mechanical stresses, hyperosmolarity and tissue-digesting enzymes. Isolated islets must then adapt to their new surroundings without the internal vascularisation and innervation that they had in the pancreas, as well as most or all of their native peripheral ECM. With the increasing need to culture islets prior to transplantation, to allow for factors such as islet quality control and recipient preparation, islet function becomes compromised even further. Although cultured islets have been shown to have immunologic advantages over freshly isolated islets for transplantation (Kuttler et al., 2002, Rabinovitch et al., 1982), islet culture is known to cause substantial loss of functional islet cell mass (Rackham et al., 2013, Kin et al., 2008) . Because of the detrimental effects of islet isolation and culture upon islet function pre-transplant, devising strategies to maintain high-quality isolated human islets *in vitro* prior to transplantation could play an important role in enhancing clinical islet graft outcome.

It has been shown previously in Chapter 4 that co-culturing mouse islets upon a monolayer of mouse kMSCs or adipMSCs improved islet insulin secretory function *in vitro*. The superior quality of these co-cultured islets has also been shown to improve the capacity of cultured islets to reverse hyperglycemia in diabetic mice *in vivo* (Rackham et al., 2013) (Rackham et al., in press). Having demonstrated the effectiveness of co-culturing islets upon MSC monolayers using mouse tissue, the aim of this study was to investigate whether these findings were translatable to clinically relevant human tissue. Previously, the favourable effects of islet and MSC co-culture for human islet function and survival have been reported in cultures where islets and MSCs were either physically separated (Park et al., 2009, Park et al., 2010) or co-cultured together in suspension (Yeung et al., 2012).

However, in studies for this thesis (Chapter 4 and 5) these two co-culture configurations did not produce the consistent beneficial effects of the direct contact monolayer co-culture system. This system was therefore chosen to investigate the effect of human MSCs upon human islet function *in vitro.* Based upon the hypothesis that restored contact between human islets and a supportive stromal layer would prevent/reduce the gradual decline of islet function in culture, direct contact co-culture with human MSCs was investigated in this study as a means to maintain the functional quality of cultured human islets *in vitro* prior to transplantation. Three types of clinically relevant human MSCs were used, adipMSCs to follow on from mouse MSC studies presented in this thesis (Chapter 4), pMSCs due to the anatomical nature of their origin and bmMSCs since they are utilised clinically in other pathologies e.g acute graft vs. host disease.

Finally, having identified a role for adipMSC-derived ECM in the support of islet function using mouse MSCs and islets (Chapter 5), additional investigations were performed in this study to assess the capacity of human adipMSC-derived ECM to preserve the function of cultured human islets *in vitro*.

6.2 Methods

6.2.1 Procurement of human islets

Human islets from ethically approved and next of kin consented cadaver pancreas donors were supplied for the studies presented in this thesis by the King's College Hospital Human islet isolation team. They were isolated according to protocols previously described by our group (Huang et al., 2004). Four independent batches of human islets received within 48 hr after harvest from cadaveric donors (70%-85% purity) were used.

6.2.2 Expansion of human mesenchymal stromal cells in culture

6.2.2.1 *Pancreas MSCs*

Cells (passage 4) were seeded at a density of 5000 cells per cm^2 of tissue culture plastic and left for 24 hours to adhere. MesenPRO RS^{TM} Medium was completely

replaced every 3 days, and MSCs passaged every 7th day for further expansion. Cells were not expanded beyond passage 6. Processes of trypsinisation, cell counting, cryopreservation and resuscitation for human pMSCs were all performed in the same manner as stated for murine MSCs (see Chapter 2, sections 2.2.1 – 2.2.4)

6.2.2.2 *Adipose MSCs and Bone Marrow MSCs*

Human adipMSCs and bmMSCs used in the studies presented in this thesis were obtained from commercial suppliers Invitrogen and Lonza respectively. The cryopreserved human MSCs (1 million cells/vial) were received at passage 0. Both adipMSCs and bmMSCs were expanded to passage 3 using the proprietary media specialised for their culture MesenPRO RS^{TM} and MSCGM respectively. Cells were seeded at a density of 5000 cells per cm^2 of tissue culture plastic and left for 24 hours to adhere. Medium was completely replaced every 3 days, and MSCs passaged every 7th day for further expansion. Processes of trypsinisation, cell counting, cryopreservation and resuscitation for human adipMSCs and bmMSCs were all performed in the same manner as stated for murine MSCs (see Chapter 2, sections 2.2.1 – 2.2.4).

6.2.3 Human islet-MSC direct contact co-culture

Human adipMSC, bmMSC and pMSC were seeded into 35 mm Nunclon petri dishes, 200 000 MSCs per dish, and cultured for 24 hr to form a confluent monolayer. MSCs were at passages 3-5 and cultured in MesenPRO RS^{TM}. For islet/MSC co-culture, 100 human islets were seeded directly onto the adipMSC, bmMSC and pMSC monolayers, and the culture medium switched to islet medium (RPMI-1640 supplemented with 10% (vol./vol.) FBS and 1% (vol./vol.) pen-strep). RPMI-1640 was selected as the islet medium of choice for co-culture over Connaught Medical Research Laboratories (CMRL) medium, which is typically used for human islet culture, since the glucose component of CMRL (5mM) was not deemed sufficient for the actively proliferating MSCs. The co-cultures were incubated for 4 days at 37^{0}C, 5% CO_2, with control groups of 100 islets alone in

RPMI-1640 medium seeded in non-treated 35 mm petri dishes run in parallel. Islets cultured on all three MSC types formed attachments with the MSCs and had to be retrieved by gentle flicking and pipetting for subsequent *in vitro* analysis.

6.2.4 Generation of human adipMSC-derived ECM

10^5 adipMSCs at passage 5 were seeded into wells of a 6-well Nunclon plate and left to adhere overnight. Cells were cultured in MesenPRO RS^{TM} medium supplemented with 50µM ascorbic acid (Sigma) until they reached approximately 90% confluence (approximately 6-7 days); medium was completely replaced every 48 hours. The cell monolayer was decellularised to expose the MSC deposited ECM by incubation with pre-warmed (37^0C) 0.5% (vol./vol.) Triton X-100 (Sigma) containing 20 mM NH_4OH (Sigma) in PBS for 10 seconds. The Triton X-100 solution was carefully diluted out with 3x PBS washes, and the ECM left to settle for 24 hr at 5^0C under PBS containing 1% (vol./vol.) pen-strep. The ECM was then treated with DNase (100 U/ml; Thermo Scientific, MA, USA.) for 1 hr at 37°C. The ECM was washed with PBS 3x and either stored in 2 ml of PBS containing 1% (vol./vol.) pen-strep, at 4°C until use in co-culture experiments or stained with van Gieson dye to confirm the presence of ECM. ECM deposits stained with van Gieson dye were washed with PBS and incubated with van Gieson (0.05% acid fuchsin (vol./vol.) in saturated picric acid (Sigma)) for 5min for the detection of collagen deposits.

6.2.5 Human islet-ECM co-culture

Approximately 75 human islets were added directly to wells of 6-well plates containing adipMSC-derived ECM in 3ml islet medium. Islet-ECM co-cultures were incubated for 4 days. Islets cultured alone for 4 days in either 35 mm Nunclon petri dishes or 35 mm non-treated tissue culture petri dishes served as control groups. A full medium replacement was performed for all control and co-culture groups on day 2.

6.2.6 Assessment of human islet function in vitro

Islets were harvested after co-culture and assessed for glucose-stimulated insulin secretion and insulin content. For two of the four independent batches of, a small proportion of islets were taken on day 0 (d0), prior to the initiation of co-culture studies, and assessed for insulin content.

Islets were pre-incubated for 1 hr in 2mM glucose Gey and Gey buffer. Ten groups of 5 islets from each culture treatment were transferred into 1.5-mL microcentrifuge tubes and incubated at 37^0C in Gey and Gey buffer supplemented with 2mM or 20mM glucose. After 1 hr, the islets were pelleted by centrifugation and samples of the incubation medium were stored at -20^0C until assayed for their insulin concentration using an in-house radioimmunoassay. Insulin secretion samples were diluted between 1:10 - 1:25 in borate buffer for detection limits of the radioimmunoassay. For the assessment of islet insulin content, islets were lysed in acidified ethanol sonicated and stored at -20^0C before radioimmunoassay. Insulin content samples were diluted between 1:200 – 1:1600 in borate buffer for detection limits of the radioimmunoassay.

6.2.6.1 *Insulin radioimmunoassay*

Insulin radioimmunoassay was performed in the same manner as for mouse islets, as described in Chapter 2, section 2.2.3.

6.3 Results

6.3.1 Effects of adipMSC monolayer co-culture on human islet function

6.3.1.1 *Effects of adipMSC monolayer co-culture on glucose-stimulated insulin secretion*

Results of co-culture with four independent batches of human islets revealed that human adipMSCs had no effect upon basal insulin secretion (Fig. 6.1A-D). The effects of adipMSCs upon islet insulin secretion in response to a maximum

stimulatory glucose concentration (20mM) varied between different islet populations. For one islet population no overall effect upon insulin secretion at stimulatory glucose levels was observed (Fig. 6.1A). However for the other three batches of islets, adipMSCs either significantly potentiated glucose stimulated insulin secretion approximately 2-fold/2.5-fold (Fig. 6.1C and Fig. 6.1D respectively) or showed a trend towards increased insulin secretion (Fig. 6.1B) over islets cultured alone. Analysis of the effects of adipMSCs upon human islets using repeated measures statistics, reported a degree of significance in the reproducibility of the beneficial effects of the MSCs across islet populations ($p<0.05$, n=4 repeats).

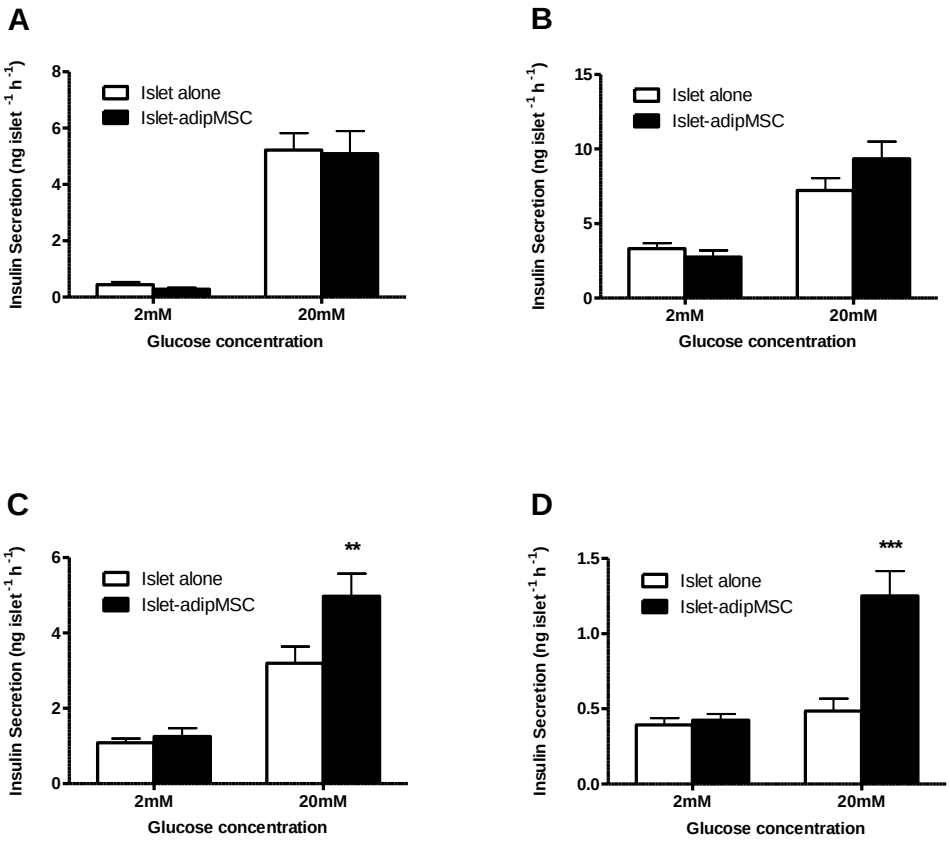

Figure 6.1: Glucose-stimulated insulin secretion *in vitro* of human islets cultured alone vs. human islets cultured directly upon a monolayer of adipMSCs. In four independent experiments (A-D) insulin release at 2mM glucose (basal) and 20mM glucose (stimulated) of groups of five islets previously cultured upon a monolayer of adipMSCs (black bars) or without MSCs (white bars) for 4 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, **p<0.01, ***p<0.001 vs. islet alone at the same glucose concentration. Across four independent batches of human islets adipMSCs had no effect upon the basal insulin secretion of human islets, and a variable effect upon glucose stimulated secretion. The latter ranged from no effect upon insulin secretory function (A) to a trend towards increasing insulin secretion (B) and finally significantly enhancing insulin secretion (C&D) compared to islets cultured alone.

6.3.1.2 *Effects of adipMSC monolayer co-culture on insulin content*

When maintained on a monolayer of adipMSCs for a 4 day co-culture period, the insulin content of human islets was significantly elevated, compared to islets cultured alone, as observed in four independent islet populations (Figure 6.2A-D). The result was considered significantly reproducible across islet populations by paired statistical analysis ($p<0.01$, n=4 repeats).

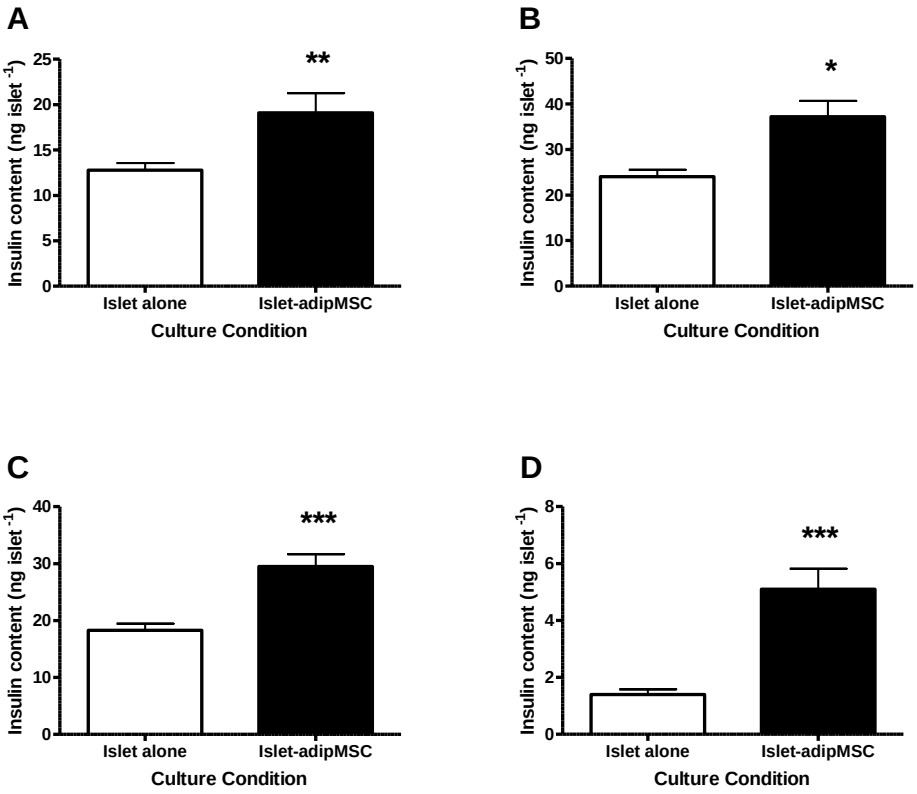

Figure 6.2: Insulin content in vitro of human islets cultured alone vs. human islets cultured directly upon a monolayer of adipMSCs. In four independent experiments (A-D), insulin content of groups of 10 islets previously cultured upon a monolayer of adipMSCs (black bars) or without MSCs (white bars) for 4 days was measured. For each experiment data are presented as mean ± SEM, n=20 observations, *p<0.05, **p<0.01, ***p<0.001 vs. islets alone. N.b. these experiments use islets from the same preparations of isolated islets used in the insulin secretory functional experiments A-D of Fig. 6.1. AdipMSCs consistently elevated the insulin content of co-cultured islets compared to islets cultured alone, across four independent batches of human islets.

6.3.2 Effects of pMSC monolayer co-culture on human islet function

6.3.2.1 *Effects of pMSC monolayer co-culture on glucose-stimulated insulin secretion*

In the context of manipulating islet function, pMSCs were considered a highly relevant candidate for co-culture, because of their anatomical location close to islets *in vivo*; hence their effects upon islet function *in vitro* were also investigated. Similarly to adipMSCs, no major effects of pMSCs upon basal insulin secretion were observed across all islet populations tested (Fig. 6.3A-D). However the effects of pMSCs upon glucose-stimulated insulin secretion for a given population of islets were broader ranging than those of adipMSCs. A detrimental effect upon insulin secretion at 20mM glucose was observed in one population of islets (Fig. 6.3A). However, in contrast they significantly enhanced glucose-stimulated insulin secretion (approximately 2-fold/5-fold, Fig. 6.3B and Fig. 6.3D respectively) over islets cultured alone for another two independent islet populations. In a fourth population of islets pMSC co-culture resulted in a slight improvement of insulin secretion at high glucose concentration (Fig. 6.3C). Repeated measures statistical analysis did not detect any significant reproducibility in the effects of pMSCs across human islets populations ($p>0.05$, $n=4$ repeats).

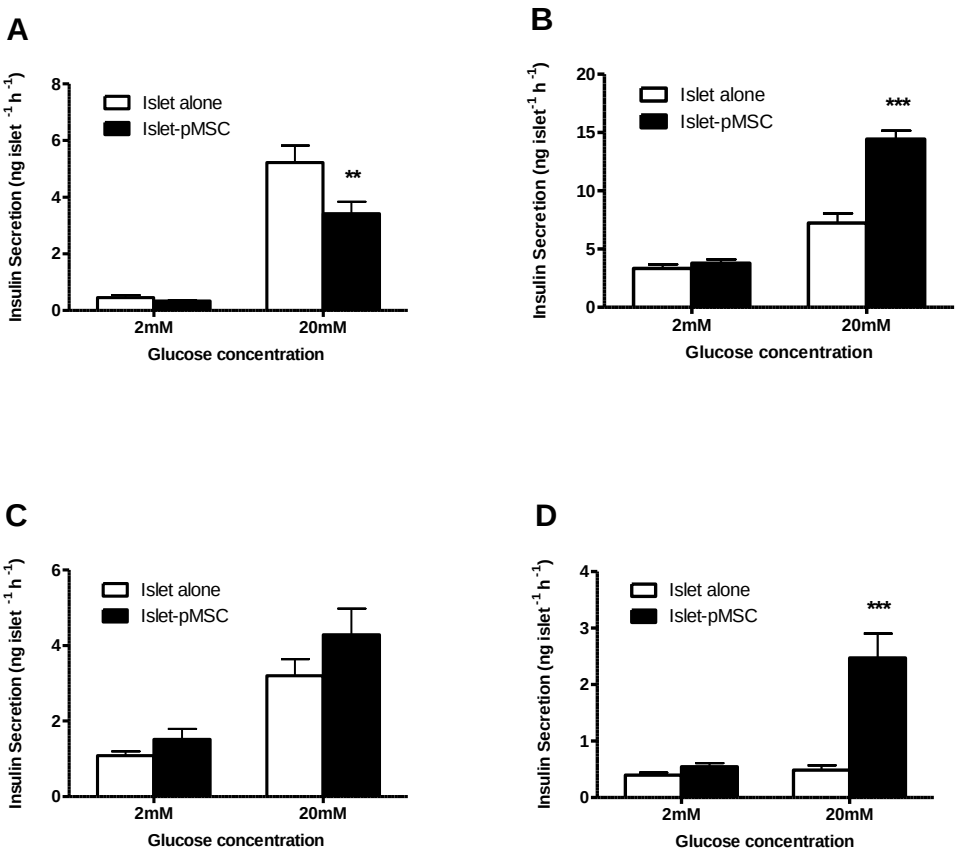

Figure 6.3: Glucose-stimulated insulin secretion *in vitro* of human islets cultured alone vs. human islets cultured directly upon a monolayer of pMSCs. In four independent experiments (A-D) insulin release at 2mM glucose (basal) and 20mM glucose (stimulated) of groups of five islets previously cultured upon a monolayer of pMSCs (black bars) or without MSCs (white bars) for 4 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, **p<0.01, ***p<0.001 vs. islet alone at the same glucose concentration. Across four independent batches of human islets pMSCs had no effect upon the basal insulin secretion of human islets, and a variable effect upon glucose stimulated secretion. The latter ranged from a detrimental effect upon insulin secretory function (A) to a trend towards increasing insulin secretion (C) and finally significantly enhancing insulin secretion (B&D) compared to islets cultured alone.

6.3.2.2 *Effects of pMSC monolayer co-culture on insulin content*

When maintained on a monolayer of pMSCs for a 4 day co-culture period, the insulin content of human islets was significantly elevated, compared to islets cultured alone, as observed in four independent islet populations (Fig. 6.4A-D). The result was considered significantly reproducible across islet populations by paired statistical analysis ($p<0.05$, n=4 repeats).

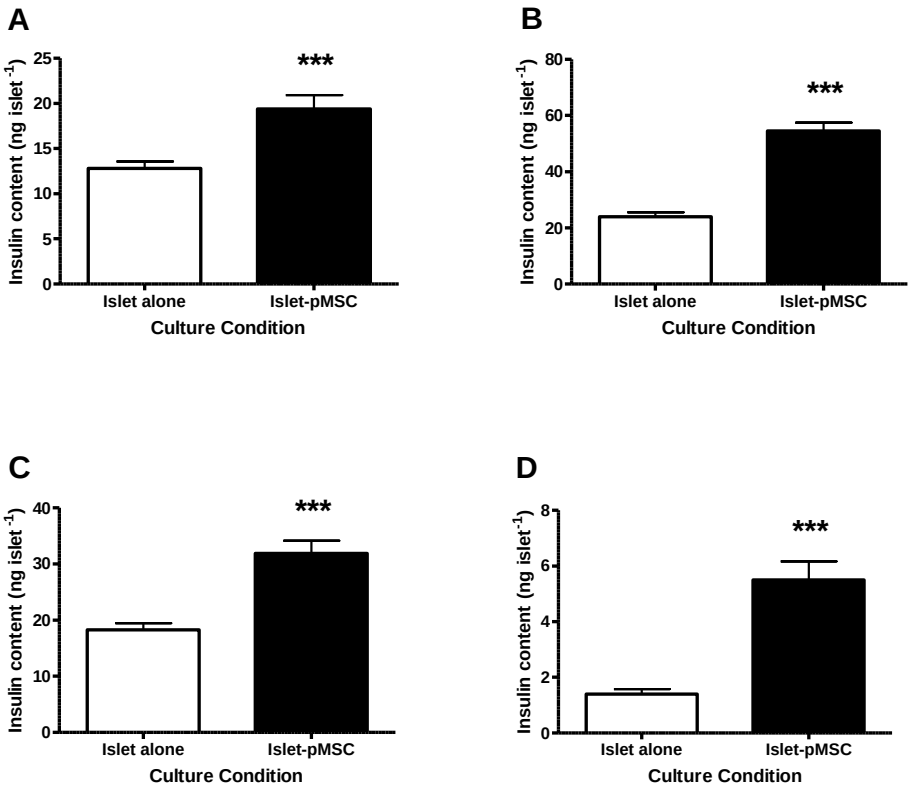

Figure 6.4: Insulin content *in vitro* of human islets cultured alone vs. human islets cultured directly upon a monolayer of pMSCs. In four independent experiments (A-D), insulin content of groups of 10 islets previously cultured upon a monolayer of pMSCs (black bars) or without MSCs (white bars) for 4 days was measured. For each experiment data are presented as mean ± SEM, n=20 observations, ***$p<0.001$ vs. islets alone. N.b. these experiments use islets from the same preparations of isolated islets used in the insulin secretory functional experiments A-C of Fig. 6.3. pMSCs consistently elevated the insulin content of co-cultured islets compared to islets cultured alone, across four independent batches of human islets.

6.3.3 Effects of bmMSC monolayer co-culture on human islet function

6.3.3.1 *Effects of bmMSC monolayer co-culture on glucose-stimulated insulin secretion*

Human bmMSCs were also trialled for co-culture, since they remain the most prevalent clinically relevant MSCs to date. In three independent islet populations bmMSCs had little effect upon basal insulin secretion (Fig. 6.5A-C). At a high glucose concentration bmMSCs also had no significant effect upon the glucose-stimulated insulin secretion in two independent islet populations (Fig. 6.5A&C), but did significantly potentiate the insulin secretory function of one islet population 1.6-fold over islets cultured alone (Fig. 6.5B). No significant effects of bmMSCs upon glucose-stimulated insulin secretion were detected across islet populations using repeated measures statistical analysis ($p>0.05$, $n=3$ repeats).

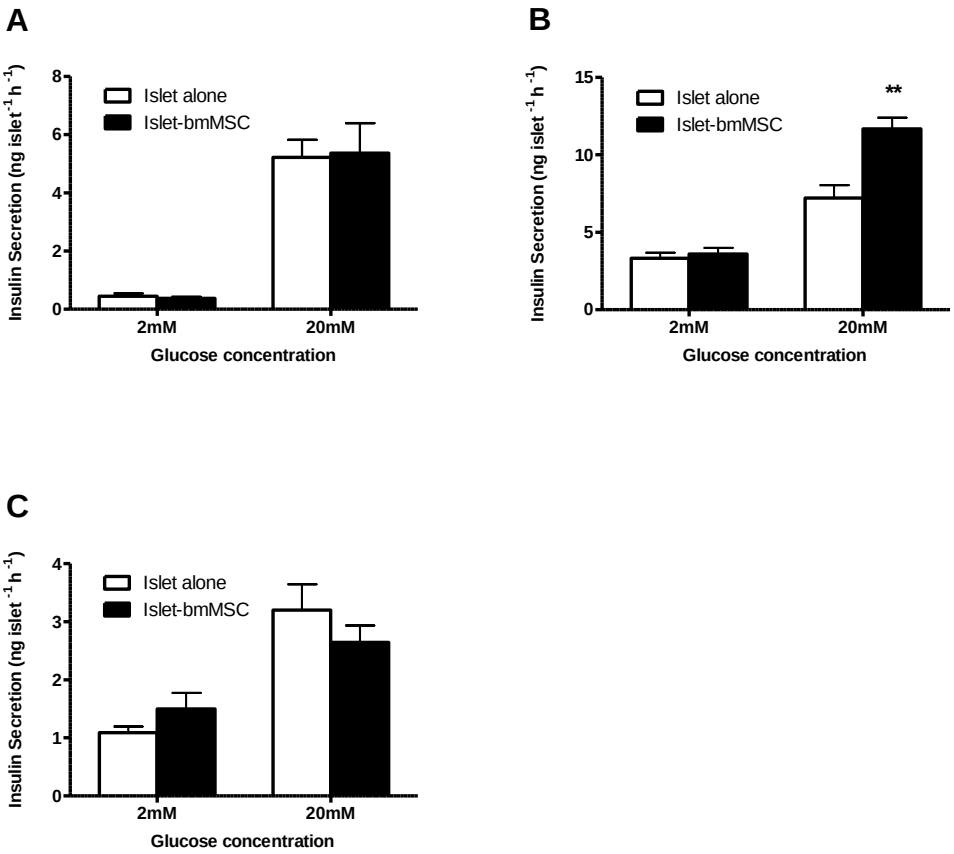

Figure 6.5: Glucose-stimulated insulin secretion *in vitro* of human islets cultured alone vs. human islets cultured directly upon a monolayer of bmMSCs. In three independent experiments (A-C) insulin release at 2mM glucose (basal) and 20mM glucose (stimulated) of groups of five islets previously cultured upon a monolayer of bmMSCs (black bars) or without MSCs (white bars) for 4 days was measured. For each experiment data are presented as mean ± SEM, n=10 observations, **p<0.01 vs. islet alone at the same glucose concentration. Across three independent batches of human islets bmMSCs had no effect upon the basal insulin secretion of human islets, and a variable effect upon glucose stimulated secretion. The latter ranged from no effect upon insulin secretory function (A&C) to significantly enhancing insulin secretion (B) compared to islets cultured alone.

6.3.3.2 *Effects of bmMSC monolayer co-culture on insulin content*

When maintained on a monolayer of bmMSCs for a 4 day co-culture period, the insulin content of human islets was significantly elevated, compared to islets cultured alone, as observed in three independent islet populations (Fig 6.6A-C). The result was considered significantly reproducible across islet populations by paired statistical analysis ($p<0.05$, n=3 repeats).

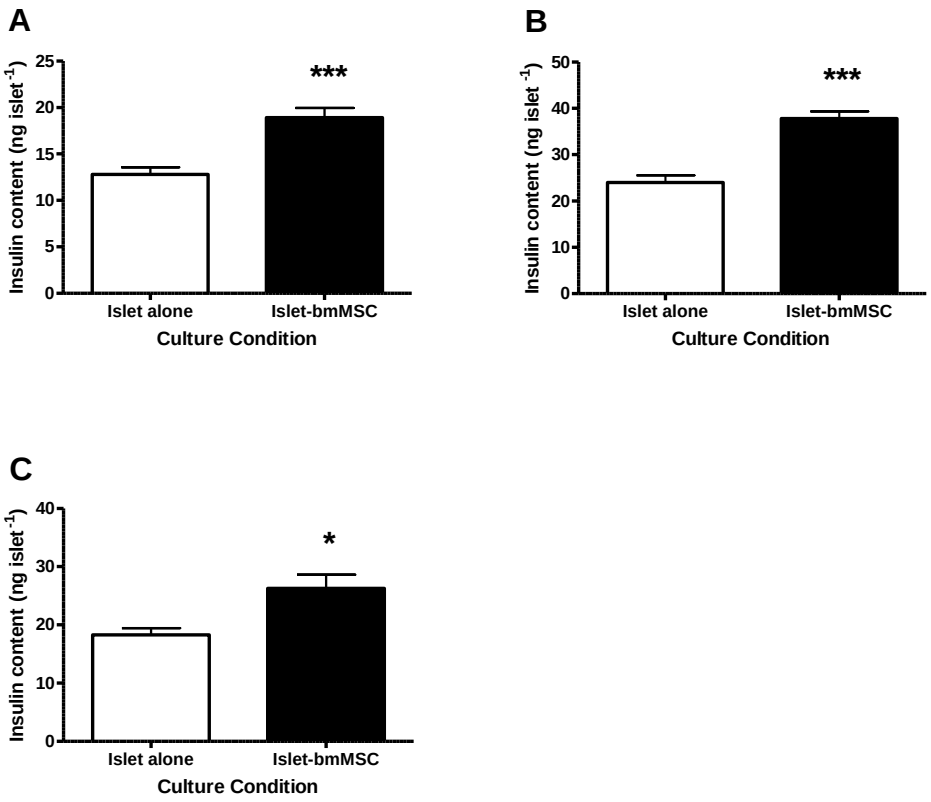

Figure 6.6: Insulin content *in vitro* of human islets cultured alone vs. human islets cultured directly upon a monolayer of bmMSCs. In three independent experiments (A-C), insulin content of groups of 10 islets previously cultured upon a monolayer of bmMSCs (black bars) or without MSCs (white bars) for 4 days was measured. For each experiment data are presented as mean ± SEM, n=20 observations, *p<0.05, ***p<0.001 vs. islets alone. N.b. these experiments use islets from the same preparations of isolated islets used in the insulin secretory functional experiments A-C of Fig. 6.5. bmMSCs consistently elevated the insulin content of co-cultured islets compared to islets cultured alone, across three independent batches of human islets.

6.3.4 Effects of MSCs on the gradual insulin content loss of cultured islets

The insulin content of human islet preparations prior to co-culture (islet alone d0) was analysed to assess whether MSCs were acting to prevent/reduce a loss of islet insulin content during culture, or whether they had the capacity to improve islet insulin levels above that of pre-cultured islets, an issue raised during mouse islet-MSC direct contact co-culture studies (Chapter 4). The culture of human islets resulted in a marked loss of insulin content, as observed in two independent batches of human islets (Table 6.1). The insulin content levels of islets cultured alone for 4 days (islet alone d4) or islets co-cultured with all three MSC types for 4 days (islet-adipMSC d4; islet-pMSC d4; islet-bmMSC d4) was significantly lower than pre-cultured islets ($p<0.001$, n=20 observations per culture condition). However, MSC co-culture was able to reduce the extent of insulin content loss during culture (Table 6.1), with all MSC co-cultured islets exhibiting significantly higher insulin content levels than islets cultured alone in both independent islet preparations ($p<0.05$, n=20 observations per culture condition).

	Islet alone d0 (ng islet^{-1})	**Islet alone d4 (ng islet^{-1})**	**Islet-adipMSC d4 (ng islet^{-1})**	**Islet-pMSC d4 (ng islet^{-1})**	**Islet-bmMSC d4 (ng islet^{-1})**
Batch 1					
Mean	135.3	24.1$^{\#}$	37.2$^{\#*}$	54.5$^{\#*}$	37.8$^{\#\Phi}$
SEM	*6.3*	*1.6*	*3.5*	*2.9*	*1.6*
Batch 2					
Mean	97.9	18.3$^{\#}$	29.5$^{\#*}$	26.3$^{\#*}$	31.9$^{\#*}$
SEM	*7.9*	*1.1*	*2.2*	*2.3*	*2.2*

Table 6.1: Insulin content *in vitro* of human islets pre-culture vs. islets cultured alone or directly upon a monolayer of adipMSC, pMSCs or bmMSCs. In two independent experiments (batch 1-2), insulin content of groups of 5 islets either prior to culture, previously cultured upon a monolayer of adipMSCs/pMSCs/bmMSCs for 4 days or previously cultured alone for 4 days was measured. For each experiment data are presented as mean ± SEM, n=20 observations, $^{\#}p < 0.001$ vs. islet alone d0, $^{\Phi}p < 0.01$ vs. islet alone d4, $^{*}p < 0.05$ vs. islet alone d4. Islet culture resulted in a loss of insulin content, regardless of culture configuration, however MSC co-culture was able to reduce the extent of this loss compared to islets cultured alone.

6.3.5 Human islet and human adipMSC-derived ECM co-culture

Human islets were co-cultured with human adipMSC-derived ECM over a 4 day time. Prior to co-culture adipMSC-derived ECM was detected throughout the tissue culture well deposited in a web-like matrix as confirmed by van Gieson staining (Fig. 6.7A). After 24 hours of co-culture, human islets attached firmly to the surface of the well and cell outgrowths from the islet interior were observed (Fig. 6.7B). Following the 4 day co-culture period islet architecture was completely lost and islets had fragmented into a monolayer of cells (Fig 6.7C). This fragmentation process was observed in two independent islet preparations, and prevented the functional analysis of islets following the 4 day co-culture period. However, islet attachment and fragmentation appeared to be independent of ECM presence, since control islets cultured alone in Nunclon tissue culture treated dishes also firmly attached to the growth surface and experienced a loss of architecture (Fig. 6.7D). Control islets maintained in suspension were the only culture group to retain typical islet morphology.

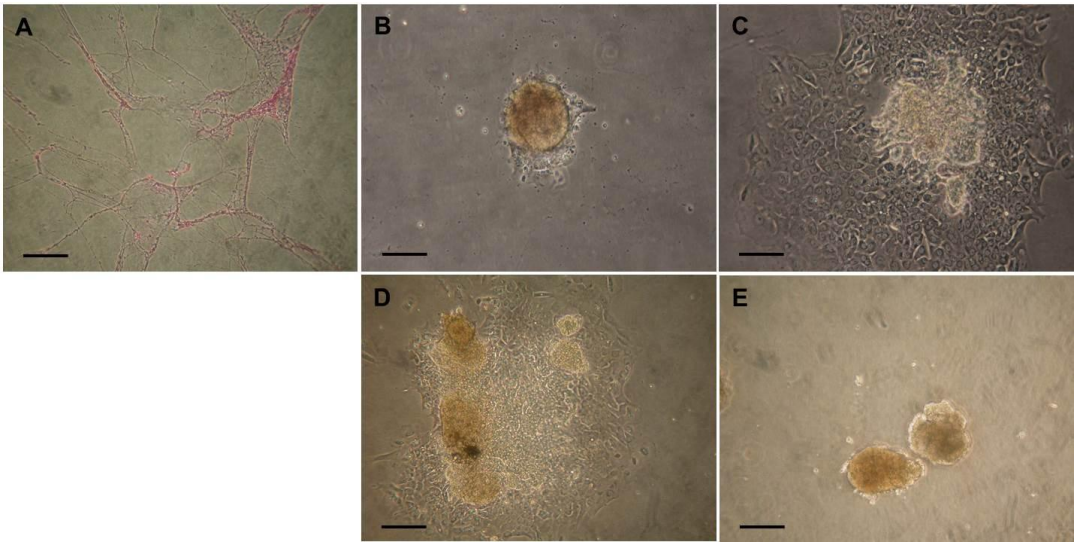

Figure 6.7: Co-culture of human adipMSC-derived ECM and human islets over a 4 day time period. Human adipMSCs deposited ECM throughout the tissue culture well prior to co-culture, as visualised by van Gieson staining (A). After initiation of co-culture, human islets adhered to the tissue culture plastic, and cell outgrowths from the islets were observed within 24 hours (B). By day 4 islets had completely fragmented and formed a cell monolayer (C). Control islets maintained on Nunclon tissue culture treated dishes also firmly attached to the growth surface after 4 days and experienced a loss of architecture (D). Only control islets cultured in suspension were able to retain typical islet morphology (E). Each image representative of at least 3 wells. Scale bar A-E; 100µm.

6.4 Discussion

Studies presented earlier in this thesis have identified a role for MSCs in the support of islet function via direct contact co-culture mechanisms using mouse tissue (Chapter 4). Although rodent islets share many features with human islets there are interspecies differences, and it is important that observations made in rodents are verified using human tissue. Therefore it was important to test the capacity of human MSCs to improve β-cell function *in vitro* using direct contact co-culture. The beneficial effects of MSC co-culture could prove beneficial for human islets during the post isolation-pre transplant stage. Since the early successes of the Edmonton protocol, which used freshly isolated islets for transplantation (Shapiro et al., 2000) there has been a return to the use of cultured islets for clinical transplantation (Hering et al., 2004), largely for logistical purposes. The culture of freshly isolated islets prior to transplantation offers a window of opportunity for islet screening and quality control (Street et al., 2004), the shipment of islets from central isolation centres to geographically distant transplant centres (Goss et al., 2002) and the initiation of immunosuppressive regimes for intended graft recipients. However islet culture is known to result in the substantial loss of functional β-cell mass (Rackham et al., 2013, Kin et al., 2008) and other islet cells such as endothelial cells (Olsson et al., 2006), therefore optimisation of culture conditions is required.

Human MSC co-culture studies were conducted with four independent human islet preparations. Unlike mouse islets, which are isolated from inbred strains of laboratory mice, human islet preparations are subject to a large degree of variability, owing to factors such as heterogeneity amongst pancreas donors and process-related variability during organ procurement. Key donor and organ procurement factors such as age, body mass index and duration of organ cold storage have been found to impact upon islet isolation successes and *in vitro* islet function (Lakey et al., 1996, Street et al., 2004). When comparing the functionality of the 4 day cultured control islets utilised in each of the four experiments of the MSC co-culture study, a large degree of variability was observed between the four

islets preparations, which was not correlated with the original purity levels of the freshly isolated islets (Table 6.2).

Islet preparation	Insulin secretion 2mM (ng/islet/hr)	Insulin secretion 20mM (ng/islet/hr)	Insulin Content (ng)	Purity (%)
1	0.45	5.22	12.8	85
2	3.32	7.22	24.0	70
3	1.09	3.20	18.3	70
4	0.39	0.49	1.4	70

Table 6.2: Functional variability between the four independent human islet preparations used in MSC co-culture studies. For each preparation the mean insulin secretion at 2mM and 20mM glucose and insulin content of 4 day cultured control islets and purity levels of the freshly isolated islet preparations are given. Mean data are presented.

Despite this variability, the *in vitro* analysis conducted in the human MSC co-culture study demonstrated that all three MSC types tested reproducibly supported human islet function across multiple islet preparations in terms of insulin content levels, whilst adipMSCs delivered the most consistent beneficial effects upon glucose-stimulated insulin secretion out of the three MSC types, as determined by repeated measures statistical analysis. This outcome raises the question of why the adipogenic-based environment generated by the adipMSCs was more beneficial to islet secretory function than the other MSC types. It is known that glucose-stimulated insulin secretion is enhanced during obesity (Perley and Kipnis, 1966, Polonsky et al., 1988) which acts to maintain fuel homeostasis. Adipocytes are a major source of peptide hormones called adipokines, which are capable of regulating a wide variety of processes that influence metabolic homeostasis. Many adipokines have been shown to directly influence beta-cell function by enhancing insulin release (reviewed by Cantley, 2014 (Cantley, 2014)). During the direct contact co-culture process the adipMSCs, which are known to be a heterogenous mix of stem an progenitor stem cells, may not only contain small subsets of adipogenic-lineage committed cells capable of

secreting adipokines, but also be exposed to insulin secreted by the beta-cells which were maintained under stimulatory glucose conditions (the glucose concentration of the RPMI-based co-culture medium is 11mM). Insulin is a key differentiation supplement used during the controlled differentiation of MSCs towards the adipogenic lineage (see Chapter 3 section 3.2.1). This exposure to insulin may promote the maturation of any adipogenic progenitors towards an adipocyte fate and further increase the release of insulinotrophic adipokines. The influence of the potentially elevated levels of adipokines in adipMSC-based co-cultures and islets may therefore have a role to play in the more consistent potentiation of glucose-stimulated insulin secretion by adipMSCs. Future studies could investigate whether adipMSC/islet co-culture promotes MSC differentiation towards an adipocyte phenotype and whether the cultures generate significant levels of adipokines.

AdipMSCs are regarded as an attractive therapeutic tool in the field of adult stem/stromal cells, due largely to their availability and accessibility. The most prevalent MSCs in clinical practise at present are bmMSCs and adipMSCs. In this study bmMSCs offered the most limited support to islet insulin secretory function out of the three MSCs types, and in addition bone marrow harvesting can be invasive, painful, associated with potential donor-site morbidity and result in low MSC yields. On the other hand, adipose tissue harvesting for adipMSC derivation is minimally invasive and can result in 500-fold greater yield of MSCs compared to their bmMSC counterparts (Hass et al., 2011, Fraser et al., 2006). If harvested from a sufficiently large volume of lipoaspirate (typically hundreds of millilitres), adipose MSCs can be obtained in their millions without expansion (Yoshimura et al., 2009). When this is coupled with methods currently being developed for the enrichment of adipMSCs from liposuction aspirates e.g. the use of adherent columns (Doi et al., 2013) and closed-system concentration devices (Cytori Celutions® system), there is the potential to generate large numbers of 'minimally manipulated' adipose MSCs without the need for culture expansion. This may have important implications for the regulatory status of the MSCs, which will be explored further in Chapter 7 - General Discussion.

The pMSCs used in the MSC co-culture study elicited the most variable effects upon islet insulin secretory function. For certain individual populations of islets pMSCs were able to enhance the glucose-stimulated insulin secretion of co-cultured islets, in comparison to control islets, to a greater degree than adipMSCs. However pMSCs were also the only MSC type tested to detrimentally impact upon the glucose-stimulated insulin secretion of a given population of human islets (Fig. 6.3A), which raises questions about their suitability as islet helper cells. The concept of stromal cells derived from the pancreas or directly from islets being detrimental towards islet cells has previously been reported. Co-culture of human MSCs derived directly from human islets has been shown to impair islet endothelial cell angiogenic behaviour and viability (Clarkin et al., 2013), whereas pancreatic stellate cells, a pro-fibrogenic cell type of the pancreas, have been shown to reduce insulin expression and induce beta-cell apoptosis following co-culture (Kikuta et al., 2013, Zha et al., 2014). The reasons as to why pMSCs would negatively impact upon islet function are not known. Only a single batch of pMSCs was utilised in this study to minimise batch-batch variability (this was also the case for adipMSCs and bmMSCs), hence the main variable between each co-culture experiment was the human islet preparation itself. Therefore the receptiveness of individual islet preparations to a given batch of MSCs may play a role in the successful outcome of co-culture. However if the effects of all three MSC types upon co-cultured islets, compared to control islets, within each islet preparation are also taken into account (Table 6.3), it can be observed that within a preparation of islets, co-culture with each MSC type can result in entirely different outcomes, which suggests there may be a role for tissue-specific MSC effects also.

MSC type	Islet preparation			
	1	**2**	**3**	**4**
adipMSC	-	/	**↑	***↑
pMSC	**↓	***↑	/	***↑
bmMSC	-	**↑	-	
	SI of 4d cultured control islets			
	11.6	2.2	2.9	1.3

Table 6.3: The effect of three different human MSC types upon the glucose-stimulated insulin secretory function of 4 day co-cultured islets from four independent islet populations. Results are reported as:
(-) no difference vs. control islets
(/) trend towards increase vs. control islets
(**↑) significant increase ($p < 0.01$) vs. control islets
(***↑) significant increase ($p < 0.001$) vs. control islets
(**↓) significant decrease ($p < 0.01$) vs. control islets
The stimulation index (SI) of cultured control islets from each islet preparation is also presented and calculated as the ratio of insulin release at 20mM glucose versus 2mM glucose.

The MSC co-culture data presented in this chapter suggests that it is not a straightforward case of one type of MSC 'fits all' for islet co-culture, which is not to be unexpected when working with human cells. In addition to increasing the number of experimental repeats to further this study, distinguishing the functional phenotypes of both the islets and the MSCs in successful and ineffective co-cultures would be useful. It is already known that MSCs are dynamic and respond to their environment. Therefore identification of any varying transcriptional activity in MSCs and islets, which may arise as a result of cross-talk between the two cell populations during co-culture, using gene expression microarray analysis could help to elucidate any key MSC and/or islet characteristics necessary for successful co-culture outcomes.

In Chapter 4, it was suggested that MSCs may be enhancing the function of islets which had become compromised during the isolation process and/or culture, whilst only maintaining the function of those islets which were relatively competent in culture. Interestingly, a similar observation was made in the human

study. As well as showing the effects of each MSC type upon co-cultured islets from each independent population of islets, Table 6.3 also shows the stimulation index (SI) of the cultured control islets used in each study. Stimulation indices were calculated by dividing the amount of insulin released at 20mM glucose by that released at 2mM glucose, and can be considered as an indicator of the functional health of the cultured islets in each experiment. Other groups have previously reported SI's of approximately 3-5 of freshly isolated human islets (Street et al., 2004, Ricordi et al., 1989) and 2-6 of cultured (1-3 days) human islets (Yeung et al., 2012, Grant et al., 1980). It can be seen in Table 6.3 that islet preparation 1 has a considerably high above average SI, and none of the 3 MSC types were able to beneficially modulate the insulin secretory function of the co-cultured islets above that of the control islets. However the remaining three islet preparations all had considerably lower SIs, closer to the lower limit of those previously reported for cultured human islets, and MSC co-culture resulted in significant enhancement of glucose-stimulated insulin secretion compared to control islets by at least one MSC type in all three islet preparations. In future studies, it would be interesting to observe whether this trend continued, especially concerning the effects of MSCs upon co-cultured islets where control islets from the same preparation have high functional quality after culture in the absence of any treatment. This may help identify potential reasons as to why MSCs do not always necessarily 'enhance' the insulin secretory function of co-cultured islets compared to control islets since sometimes they may act to maintain islets which already have good functionality and to prevent/reduce any further functional compromise. This could be investigated further by testing the *in vitro* functionality of both control islets and islets retrieved from co-culture at several different time points after the original MSC co-culture period. An assessment could then be made of the longevity of any MSC-derived beneficial effects and a comparison of the level of functional deterioration between control and co-cultured islets.

In contrast to insulin secretory function, the insulin content levels of MSC co-cultured islets were reproducibly enhanced over islets cultured alone in all islet

preparations, by all three MSC types tested. This supports the observations made in Chapter 4 that islet insulin secretory function and islet insulin content are not necessarily causally linked. The ability of human MSCs to consistently improve the insulin content levels of human islets, a finding not observed in mouse tissue, may be linked to the complex, drawn-out nature of the human islet isolation procedure. Compared to the mouse islet isolation procedure, human islet isolation is a much lengthier process, and islets are exposed to numerous stresses over a multi-hour period. Through islet handling experience, isolated human islets are also often more fragile in culture than their more robust rodent counterparts. Human MSCs may therefore have more scope to assist human islets, which are more likely to have been compromised to a greater degree during the isolation procedure than mouse islets. Table 6.1 compares the insulin content levels of cultured human islets to islets pre-culture in two independent islet preparations. The four day culture period resulted in a significant deterioration of islet insulin content for both islets cultured alone and all types of co-cultured islets. However, the extent of insulin content loss was reduced in co-cultured islets, which may be due to factors including reduced islet cell apoptosis and maintenance of islet morphology to prevent beta-cell leakage, findings which have previously been reported for both mouse and human islets co-cultured in direct contact with MSCs (Jung et al., 2011, Luo et al., 2007, Yeung et al., 2012).

MSC-deposited ECM was identified in Chapter 5 as a potential mechanism through which mouse MSCs benefitted mouse islet function during direct contact co-culture. Attempts were therefore made to co-culture human islets and human adipMSC-derived ECM in order to investigate whether this finding extended to human tissue. In addition to examining the mechanisms through which MSCs support islets, pinpointing a role for ECM could assist in the development of cell-free co-culture options, which regulatory bodies may deem less risky. Unfortunately human islet-ECM co-culture attempts in this study were unsuccessful, as in the presence of MSC-derived ECM alone human islet architecture was lost. However, islet fragmentation was observed to be independent of ECM presence since the islet architecture of control islets cultured

in Nunclon tissue-culture treated dishes was also lost, this is consistent with observations made by Luo et al. (2007). The loss of three-dimensional human islet morphology of islets cultured on ECM proteins alone has been reported previously by others (Daoud et al., 2010). The maintenance of islet architecture facilitates normal islet insulin secretion patterns (Kelly et al., 2011), however human islets fragmented and assumed monolayer cell growth in the absence of MSCs. The role of ECM support in human islet function therefore could not be confirmed utilising the experimental design employed in this study, and human islet disintegration may have be down to the fragility of human islets. This finding suggests that if 2D monolayer co-culture strategies incorporating MSCs are to be developed for the maintenance of human islets pre-transplantation, MSCs should be present for the benefit of β-cell function. Alternative 3D ECM-islet co-culture strategies e.g. scaffolds populated with ECM components (Zhang et al., 2012) would need to be considered if cell-free ECM options are to be pursued further for human islets.

In summary, the results presented within this chapter demonstrate that human MSCs have the capacity to support the function of human islets in culture. Of the three MSC types tested, adipMSCs delivered the most consistent and statistically reproducible beneficial effects. AdipMSCs represent an easily accessible and abundant source of MSCs, and have attracted attention from both scientific and clinical communities for their potential in clinical applications. Although the mechanism of functional support remains unclear, the beneficial effects of MSCs may have important implications in the clinical islet transplantation setting if co-culture with MSCs can prevent/reduce the gradual loss of insulin content and/or enhance the insulin secretory function of isolated islets. Future studies investigating the efficacy of adipMSC co-cultured islets *in vivo* will help to determine the longevity of the MSC-derived beneficial effects and provide insight into whether MSC co-culture is a suitable strategy for the maintenance of human islets in culture prior to transplantation.

Chapter 7 - General Discussion

Islet transplantation is emerging as a robust treatment option for selected patients with Type 1 diabetes. Since the introduction of the Edmonton protocol in 2000, islet transplantation has developed into a therapy that significantly reduces glycaemic variability and eliminates severe hypoglycaemia in a subset of patients with Type 1 diabetes (Shapiro et al., 2000, Shapiro et al., 2006, Ryan et al., 2005). Recent advances, including the administration of potent immunotherapy regimes, have also improved the potential of islet grafts to offer long-term insulin independence for patients (Bellin et al., 2012), with multiple global centres for transplantation now reporting five-year insulin independence rates ≥50% (Shapiro, 2012, Bellin et al., 2012, Emamaullee et al., 2010, Berney et al., 2009). Graft function and longevity are critical for successful clinical islet transplantation outcomes but, despite advances, they remain hampered by obstacles including immunological rejection, inadequate engraftment, suboptimal function of the transplanted islets and extensive cell death during the post transplantation period. Improving the function and survival of transplanted islet grafts are key considerations when developing clinical islet transplantation practices. However, the quality of human islet preparations available for transplantation impacts greatly upon the outcome of islet transplantation and should also be considered. Disengagement of islets from their 3D microenvironments during isolation and their subsequent culture prior to transplantation has been shown to leave islets susceptible to devascularisation, apoptosis, hypoxia and loss of functional cell mass (Wang and Rosenberg, 1999, Rosenberg et al., 1999, Paraskevas et al., 2000, Rackham et al., 2013). Therefore devising strategies to maintain functionally competent isolated human islets *in vitro* prior to transplantation could play an important role in enhancing clinical islet graft outcome.

The studies in this thesis were designed to investigate strategies utilising mesenchymal stromal cells to improve islet transplantation outcomes. Various populations of MSCs were isolated and characterised from clinically relevant human and mouse tissue. Phenotypic heterogeneity was observed between MSC

populations of different origin, which may correspond to heterogeneity introduced by cell culturing and/or the use of different tissue sources and species differences. In contrast different batches of MSCs isolated from the same species and tissue showed very similar phenotypes when characterised *in vitro*.

MSCs have been shown previously to enhance the outcome of islet transplantation following co-transplantation in diabetic mice *in vivo*, which has been attributed to the ability of MSCs to remodel the *in vivo* environment via their pro- angiogenic and immunomodulatory activities (Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Ito et al., 2010, Rackham et al., 2011, Sordi et al., 2010, Figliuzzi et al., 2010). However, i*n vitro* analysis conducted within this thesis showed that mouse MSCs have the capacity to directly enhance islet secretory function following a period of direct contact co-culture, independently of the *in vivo* environment. The ability of MSCs to modulate islet function, rather than revascularisation or survival, may be one of the factors contributing to the enhanced performance of MSC-islet co-transplants commonly observed in diabetic animal models *in vivo*. Since co-culture studies revealed MSCs could directly influence insulin secretory behaviour independently of key *in vivo* environmental modulators, one of the aims of this thesis was to investigate alternative mechanisms through which MSCs could be acting.

Both mouse kMSCs and adipMSCS were able to reproducibly potentiate the glucose-stimulated insulin secretory function of *in vitro* co-cultured mouse islets when a direct contact monolayer co-culture configuration was employed. Later studies showed that MSC-deposited ECM seemed likely to play a role in the support of isolated islet function. Although this has not been previously reported, numerous studies have reported the benefits of post-isolation islet-ECM restoration (Jalili et al., 2011, Zhang et al., 2012, Hammar et al., 2004, Bosco et al., 2000, Parnaud et al., 2009). In contrast, our indirect contact transwell co-culture studies were not able to support the previously reported finding of the role of MSC-secreted soluble factors in the enhancement of isolated islet function *in vitro* (Park et al., 2009, Park et al., 2010). Discrepancies

between my findings and published studies may have arisen due to experimental design differences or the dilution below effective concentration of soluble factors in the transwell configuration compared to the direct contact co-culture configuration. Jung et al (2011) reported similar co-culture findings to those presented in this thesis, and demonstrated the superior effects of direct contact co-culture over the paracrine effects of soluble factors in transwell co-culture upon islet insulin secretory function (Jung et al., 2011). In all, the literature to date and studies in this thesis suggest that MSCs might be beneficial for improving the function of isolated rodent islets both during culture and after transplantation, and a broad spectrum of MSC properties are likely to be responsible for the beneficial effects exerted over islets ranging from immunomodulation to the provision of a supportive extracellular tissue microenvironment.

Rodent-based studies are useful tools in the development of new therapeutic strategies, nevertheless key findings must always be verified in human tissue. To date, although a multitude of pre-clinical animal-based studies have been conducted regarding MSCs for islet transplantation, far fewer studies utilising human islets and MSCs have been reported. This is not surprising since human islets, which are isolated from heart-beating, brain-dead cadaver donors, are scarce with only a very limited supply available for research purposes. Fortunately, close links with King's College Hospital clinical islet isolation unit facilitated the intermittent supply of human islets for this thesis and enabled preliminary investigations into human islet-MSC co-culture. Monolayers of human bmMSCs, pMSCs and adipMSCs consistently reduced the loss of insulin content in cultured human islets, whilst adipMSCs also delivered the most reproducible beneficial effects on glucose-stimulated insulin secretion, suggesting translational potential for the mouse tissue findings. Other groups have reported the success of various human MSC types for co-culture, including bone marrow-, cord blood- and pancreatic-MSCs, although the studies employed different co-culture configurations (Park et al., 2009, Yeung et al., 2012) and co-

culture time periods (Luo et al., 2007) to those presented in this thesis and none of the studies used adipMSCs.

The data presented in this thesis, along with earlier studies from the group (Rackham et al., 2011, Rackham et al 2013) has generated a portfolio of pre-clinical *in vitro* and *in vivo* data supporting the incorporation of MSCs into islet transplantation practices, which warrants further investigation. The studies of this thesis have culminated in supporting the use of human MSCs for the maintenance of cultured human islets *in vitro* prior to transplantation. Future studies would endeavour to investigate whether these MSC-treated human islets are also beneficial for transplantation outcome. At this stage a combinatory dual strategy could be envisioned for MSCs, whereby co-culture would be used to provide a favourable microenvironment for isolated islets *in vitro* to maintain their secretory function prior to transplantation, whilst co-transplantation would enable MSCs to work locally and promote islet graft function in the early stages after transplantation. If the use of such a dual strategy were to be adopted in the clinical setting, there are a number of MSC-related factors that need to be considered. Some of these factors will be discussed below, focusing on adipMSCs which were the most consistently beneficial MSC of this thesis, unless otherwise stated.

7.1 Tumourigenicity of transplanted MSCs

Due to their therapeutic potential, MSCs have not only attracted significant attention from the scientific community, but also made noteworthy clinical progression. A search performed with 'adipose mesenchymal stromal cells' on clinicaltrials.gov in August 2014 revealed 13 clinical trials, which extended to 76 trials for a large variety of indications e.g. Parkinson's Disease, Multiple Sclerosis, Stroke, Acute Myocardial Infarction and Liver Cirrhosis, when 'adipose' was dropped from the search field. One of the major concerns with the administration of any cellular therapy, which contains subsets of stem cells, into patients is their tumour forming potential and/or promotion. Stem cells, by definition, have the capacity for self-renewal and potency which can potentially

be linked to tumourigenesis, and the greater the potency and self-renewal properties a stem cell possesses the greater the likelihood of tumour formation. The potency of stem cell subsets found in MSC preparations is more restricted than pluripotent stem cells, e.g. embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), as suggested by the greater DNA methylation at the loci of pluripotency factors OCT4 and NANOG in MSCs (Sorensen et al., 2010). This mechanism may lower the risk of tumour forming potential of MSCs compared with ESCs or iPSCs. On the other hand, although the pro-angiogenic and anti-apoptotic properties of MSCs are attractive therapeutic aspects of MSCs, they have raised concern since they also have the ability to promote tumour growth of host tissues. A number of experimental studies have tried to investigate the pro-tumourigenic nature of MSCs but delivered contradictory results with reports of MSCs both promoting (Prantl et al., 2010, Yu et al., 2008, Muehlberg et al., 2009) and inhibiting (Cousin et al., 2009, Zhu et al., 2009) tumour growth in the cancer environment. Klopp et al (2011) proposed that factors such as MSC heterogeneity, *in vitro* cell propagation, variation between *in vivo* tumour models, MSC dose and patient-patient variability in MSC isolates may account for the discrepancies between findings (Klopp et al., 2011). No conclusive statements can be made on the safety of MSCs until analysis of large-scale clinical trials in humans with extensive follow-up periods have been conducted. To date the safety evidence of MSC therapy is limited to a degree to anecdotal studies or early phase I/II clinical trials. Nevertheless, no evidence of adverse tumour formation events have been reported in clinical trial participants to date, and the world's first approved stem drug Prochymal, a bone marrow MSC based formulation, received market authorisation in Canada for the treatment of graft-vs-host disease in children in 2012 (Osiris, 2012), suggesting MSC administration in patients can be safely received.

7.2 Which MSCs have the most clinical potential?

Much discussion has taken place in this thesis about the consideration of MSC tissue-source. However, MSC 'type' extends beyond the tissue of origin. Other

factors such as autologous or allogeneic cells and cultured or non-cultured cells are also important.

7.2.1 Autologous vs. allogeneic MSCs

Adipose MSCs can be isolated both autologously (from self) or allogeneically (from genetically distinct others) both of which carry logistical or clinical advantages for the transplantation setting. One of the key advantages of allogeneic cells is the possibility of their use as an 'off the shelf' agent easily accessible from good manufacturing practice (GMP) compliant tissue banks, making them more akin to a ready to use drug and removing the need for, and potential delays of, patient tissue aspiration and MSC preparation. On the other hand autologous cells are a patient's own and in theory should be well tolerated by the patient following transplantation. The accelerated rejection of MSC allografts has been documented in diverse animal models of allo-MSC administration (Huang et al., 2010, Seifert et al., 2012, Schu et al., 2012). As an example, Huang et al (2010) reported greater clearance of allogeneic MSC preparations injected into the myocardium for treatment of myocardial infarction compared to autologous preparations, due to the induction of anti-donor immune responses following MSC differentiation (Huang et al., 2010). Autologous MSCs may therefore be able to offer longer-term persistence *in vivo* than their allogeneic counterparts, which could be an important consideration if MSCs are to be used as inducers of immune tolerance to replace immunosuppressive pharmacological agents, as well as helper cells for functional support, in islet transplantation.

The function of autologous cells is likely to be influenced by the health of the donor. Although not investigated in this study, there is some evidence that the function of MSCs isolated from a patient with diabetes may be impaired. The altered phenotype of adipMSCs derived from both rats and humans with diabetes has been reported previously, with the hyperglycaemic environment in rats being suggested as the cause of the impaired proangiogenic function of rat adipMSCs (Kim et al., 2008). Similarly transplanted autologous human adipMSCs

displayed reduced fibrinolytic activity in Type 2 diabetic patients (Acosta et al., 2013). Therefore in order to make an informed decision on the use of autologous vs. allogeneic adipMSCs for islet co-culture and/or co-transplantation, future studies must initially look towards the capacity of MSCs isolated from people with diabetes to improve islet function both *in vitro* and *in vivo*.

7.2.2 Cultured vs. non-cultured MSCs

All MSCs utilised in this thesis were manually expanded in tissue culture *in vitro*. A typical manual isolation procedure of adipMSCs involves enzymatic digestion of adipose tissue, followed by centrifugation to separate mature adipocytes from the MSC containing stromal vascular fraction (SVF) of the adipose tissue. The SVF is then cultured for the purification and expansion of MSCs. The 2D tissue-culture plastic approach to MSC culturing was sufficient to generate research-grade cell numbers appropriate for the pre-clinical small scale studies in this thesis. However, sophisticated bioreactor-based systems will need to be employed to achieve the production of clinical-grade cells at the billion-trillion dose numbers required for clinical use, whilst avoiding the inherent contamination risk and intra/inter-operator variability associated with the traditional manual techniques. A bioreactor-based device utilising microbead carriers in suspension for MSC adherence could be an attractive tool to provide an automated, high-throughput system that facilitates the clinical-scale expansion of MSCs. Such approaches are currently being investigated in industry (Lonza Inc.) for the large-scale manufacturing of cell therapies (LRMN, 2014).

As discussed in Chapter 3, MSC culture for clinical uses does however have a number of issues. The *in vitro* culture of cells intended for therapy is regarded as ‘substantial manipulation’ by regulatory bodies, since the impact of the artificial conditions imposed on cells can alter their characteristics. This matter was highlighted earlier this year when a court in the United States upheld the FDA’s ruling that cultured therapeutic stem cells should be regulated as drugs in a case against Regenerative Sciences, who were using cultured MSCs in its product

Regenexx to treat orthopaedic problems (www.the-scientist.com). Integrated systems are being developed in an attempt to generate 'minimally manipulated' adipMSCs directly from the SVF, which could benefit from less stringent regulations. An example of such an integrated system is the Celution® system developed by Cytori Therapeutic Inc (San Diego, CA, USA), which digests adipose tissue and concentrates the SVF in a closed system device designed for point-of-care operation with a processing time of 1-2 hours. The cells isolated from the system have been studied and characterised to be equivalent to manually isolated adipMSCs (Lin et al., 2008). Last year the system was approved in Australia for commercial use for 'autologous re-implantation or re-infusion of a patient's adipose-derived regenerative cells' (www.cytori.com). Future studies for this project may be directed towards functionally validating the use of concentrated SVF for islet co-culture and/or co-transplantation. The impact of incorporating cultured MSCs or minimally manipulated MSCs upon the regulatory status of islet transplantation is as of yet unknown and must be considered in the development of the project.

7.3 Regulatory implications

Somatic cell therapies, which encompass islet transplantation, can either be regulated as transplants or advanced therapeutic medicinal products (ATMPs). Part of the definition of a somatic cell therapy ATMP is offered in the European Parliament's Directive 2001/83/EC (Directive2001/83/EC, 2001):

"*…contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor…"*

Current islet isolation, purification and culture processes are regarded as minimal manipulation that do not alter the essential clinical properties of islets (EMA, 2012); as such islets isolated for transplantation are currently regulated as

transplants, not medicinal products, under the European Union (EU) Tissues and Cells directive (Directive2004/23/EC, 2004) by the UK national regulatory body Human Tissue Authority (HTA). The incorporation of MSCs into either the existing islet culture or islet transplantation protocols has significant potential to shift the regulatory status of islets towards that of medicinal product, which is a significant, costly task since such products fulfil criteria requiring clinical trials and market authorisation. Based upon data presented in this thesis, it could be argued that the use of MSCs for co-culture prior to transplantation acts to maintain the functional and structural properties of isolated human islets, rather than alter them, whilst the use of minimally manipulated adipMSCs could also be considered for co-transplantation strategies in an attempt to prevent the transition to ATMP regulatory status for islet transplantation. Nonetheless, whilst the project remains in its early pre-clinical stages, it will be valuable to get advice from the appropriate regulatory bodies and take into consideration the implications of any proposed strategies for improving islet transplantation outcome, in order to proceed with minimal deviation from existing regulations if possible and accelerate their clinical translation.

Although there are a number of issues surrounding the use of MSCs in islet transplantation, for each of the key issues discussed above, there appears to be scientific/industrial advancements on the horizon offering potential solutions. In my opinion keys steps moving forward with this project translationally would investigate the use of integrated systems such as Celution® for the generation of minimally manipulated adipMSCs to use in co-culture and co-transplantation strategies. The use of autologous adipMSCs in conjunction with the short processing times of Celution® would provide an ideal solution to the autologous vs. allogeneic debate, however in the context of patients with Type 1 diabetes autologous adipMSC functionality is uncertain, so allogeneic adipMSCs may be the more suitable option. Practically speaking, once sufficient evidence supporting the best MSC ‘type’ has been generated studies would need to focus on the large-scale operation of human MSC-islet co-culture and the retrieval of human islets from MSC monolayers. In the current studies presented in Chapter

6, human islets were retrieved by gentle pipetting/'flicking' from the monolayer bed, which is not a scalable option. Faster, large-scale islet retrieval may be achieved by the use of gentle enzymatic dissociation. Also, if dual co-culture, co-transplantation strategies are to be considered, the 'MSC-free' retrieval of islets may not be too much of a concern.

The use of MSCs in islet transplantation offers great hope for the functional support of islets both *in vitro* and *in vivo*, and although not investigated in this thesis, the immune tolerance of islet grafts *in vivo*. MSCs will make the biggest impact clinically if definitive evidence can be produced to support the efficacy of MSCs for immunomodulation in human studies and eliminate the need for immunosuppressive drugs. One of the major limiting factors of islet transplantation is the risk carried by recipient immunosuppression and is one of the reasons why islet replacement therapy is restricted to only a small subset of T1D patients, in addition to pancreas donor scarcity. Alternatively, MSCs may prove useful if they are integrated as helper cells into implantable devices (Valdes-Gonzalez et al., 2010) or co-encapsulated with cross-species islets (Zhi et al., 2012, Krol et al., 2006) for immuno-isolated xeno-islet transplantation. Porcine islets are a promising β-cell source to combat the shortage of cadaver organ donors (Hering et al., 2006, Cardona et al., 2006, van der Windt et al., 2009). Islet transplantation without immunosuppression risks and an extensive islet supply has the potential to become a more realistic alternative to insulin administration for a broader spectrum of T1DM patients and achieve mainstream clinical application.

7.4 Future scientific directions

Much of the discussion of future perspectives for this project has focussed on its clinical translation. Fundamental i*n vivo* and *in vitro* studies were required from the start of the project to ascertain the utility of MSCs in islet transplantation, prior to latter studies dissecting the mechanisms of interaction between islets and MSCs. With key data supporting the beneficial effects of MSCs in islet transplantation, specifically in the direct potentiation of glucose-stimulated

insulin secretion in islets, it is now important to take a step back and investigate the mechanisms through which MSCs may be influencing islets. Firstly, since the MSCs have had a notable effect upon β-cell insulin secretion, it would be interesting to assess how MSCs influence the β-cells ability to recognise and respond to glucose. This could be investigated by studying the influence of MSCs on the shape of the glucose-stimulated insulin secretion dose–response curve. MSCs could either be causing a left shift in the curve (i.e. beta cells become more sensitive to glucose, which may be linked to the modulation of glucose kinase activity during β-cell glucose metabolism) and/or by extending the curve (i.e. increasing the maximal amount of insulin secreted at higher glucose concentrations). Amplification of glucose-stimulated insulin secretion is likely to be linked to the latter stages of the β-cell insulin secretion cascade, which influences the exocytosis of insulin from secretory granules. Therefore, investigating changes in intracellular Ca^{2+} concentrations/influx rates or in β-cell mitochondrial respiration and ATP generation would also be useful, to assess whether these mechanisms are likely to have played a role in enhanced secretory function.

Many other fuels besides glucose influence the amount of insulin a β-cell secretes. The effect of MSCs upon the overall secretory response of β-cells, as opposed to the glucose-induced response only, could be tested by evaluating the effects of MSCs upon islet insulin secretion in response to other nutrient stimulators such as fatty acids or non-nutrient potentiators such as GLP-1 and GIP. Finally, analysis of gene expression by MSCs and islets in co-culture, to examine factors being released by the MSCs and the receptors islets are equipped with to respond to these factors may help to elucidate whether these factors can be used in the absence of MSCs for cell-free islet support *in vitro*. The identification of MSC-secreted factors beneficial to islet secretory function *in vitro* could be used for the development of islet culture medium supplemented with defined growth factors, hormones and cytokines etc. If treatment of the islets with such a medium was deemed not to have substantially manipulated them, it could be better suited to clinical islet culture protocols than the

incorporation of MSC co-culture as it would avoid the potential regulatory implications associated with the use of MSCs.

7.5 Conclusion

Overall, the studies described in this thesis suggest an important therapeutic potential for MSCs in improving the outcome of islet transplantation as a therapy for Type 1 diabetes, both by maintaining islet function *in vitro* and supporting islet engraftment *in vivo*. The beneficial effects of MSCs have been extended to clinically relevant human tissue, and at least part of the effect can be attributed to ECM laid down by the MSCs. Further scientific investigations into additional mechanisms through which MSCs affect islet function at both the cellular and molecular level, and translational studies considering MSCs with the most clinical potential for development strategies may be needed before translating these observations into improvements for clinical islet transplantation protocols.

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List of abstracts and publications from this thesis

Studies from this thesis have been included in the following peer-reviewed publications:

RACKHAM, C. L., **DHADDA, P. K**., CHAGASTELLES, P. C., SIMPSON, S. J. S., DATTANI, A. A., BOWE, J. E., JONES, P. M. & KING, A. J. F. 2013. Pre-culturing islets with mesenchymal stromal cells using a direct contact configuration is beneficial for transplantation outcome in diabetic mice. *Cytotherapy,* 15**,** 449-459.

RACKHAM, C. L., **DHADDA, P. K**., LE LAY, A. M., KING, A. J. F. & JONES, P. M. 2014. Pre-culturing islets with adipose derived mesenchymal stromal cells is an effective strategy for improving transplantation efficiency at the clinically-preffered intraportal site. *Cell Medicine,* 24 March 2014. DOI: 10.3727/215517914X680047

Studies from this thesis have been presented at the following international and national conferences:

DHADDA, P. K., RACKHAM, C. L., SIMPSON, S. J. S. & JONES, P. M. 2012. Co-culture of islets with mesenchymal stem cells in direct contact configurations improves islet function in vitro. *Diab tologia,* 55**,** S183-S183. Poster presentation at EASD conference 2012

DHADDA, P. K., RACKHAM, C. L., LE LAY, A. M., KERBY, A., HUANG, G. C., & JONES, P. M. 2013. Preculture of human islets with mesenchymal stromal cells in a direct contact configuration enhances islet function in vitro. *Transplantation,* 96**,** S1-S155. Oral presentation at IPITA conference 2013

DHADDA, P. K., RACKHAM, C. L., LE LAY, A. M., KERBY, A., HUANG, G. C., & JONES, P. M. 2013. The effects of three human mesenchymal stromal cell populations upon human islet function in vitro. *Diabetic Medicine,* 3**,** S1. Oral and poster presentation at DUK conference 2014